Growth promoting and immunomodulatory effects of free and encapsulated dietary probiotics on *Labeo rohita*

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By

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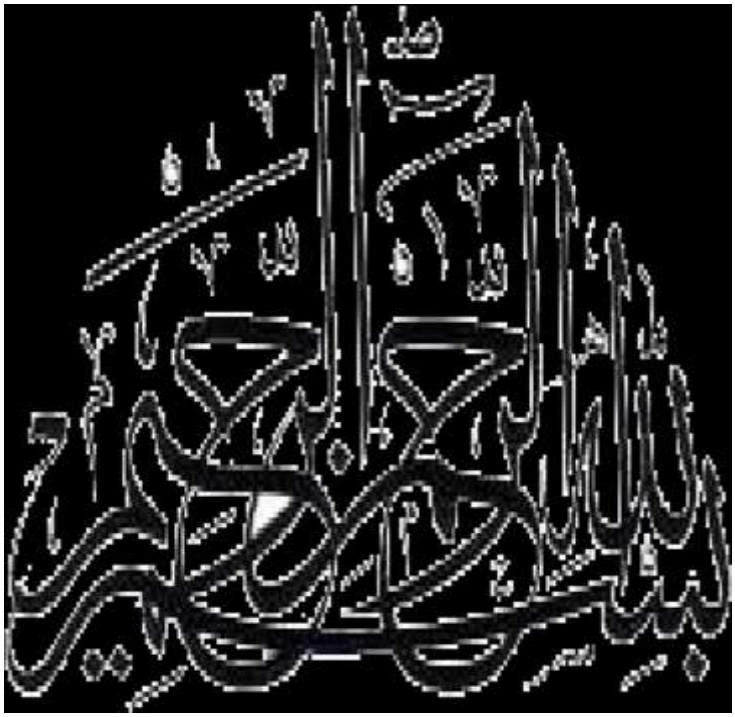

IN THE NAME OF ALLAH, THE MOST MERCIFUL
THE MOST BENEFICENT
AND
THE MOST COMPASSIONATE

CERTIFICATE

This dissertation ‘‘**Growth promoting and immunomodulatory effects of free and encapsulated dietary probiotics on *Labeo rohita*** submitted by **IMRANA AMIR** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological sciences, Quaid-I-Azam University, Islamabad, as satisfying the thesis requirement for the degree of Doctor of Philosophy in Fisheries and Aquaculture.

Supervisor: _____

(Dr. Amina Zuberi)

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_____

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Chairman

Date: _____

Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

IMRANA AMIR

Dedicated To:

“To my parents who enlightened my darkest hours by the beautiful combination of their encouraging words and invigorating tone.

To my Baba who taught me that its just time of renewal like spring, whenever it seemed like an endless summer”.

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List of Abbreviations

Abbreviations	Full Names
µl	Microliter
µM	Micromole
Alg	Alginate
ALP	alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
C	Chitosan
CFU	Colony forming unit
DO	Dissolved oxygen
EDTA	Ethylenediaminetetra acetic acid
g/dL	Gram per deciliter
$HCLO_4$	Perchloric acid
HDL	High density lipoprotein
Hrs	Hours
LDL	Low density lipoprotein
LSD	Least significant difference
M	Molar
mg/L	Milligram per liter
Min	Minutes
mM	Milli molar
MS222	Tricaine methanesulphonate
Nmol	Nanomole
OD	Optical density
PBS	Phosphate buffer saline
ppm	Parts per million
RPM	Rounds per minute
S	Starch
Sec	Seconds
SGR	Specific growth rate
X	Xanthan

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ACKNOWLEDGMENTS

Praise is to Allah, Lord of the worlds. The most beneficent, the most merciful, Who is the entire source of knowledge and wisdom, endowed to mankind; Who gave me courage and potential to pursue this goal, Whom I believe that He never spoils any efforts of good deed. Blessings of Allah be upon to his Prophet (PBUH)", the city of knowledge and blessings for entire creature, who has guided his Ummah to seek knowledge from Cradle to Grave, and enabled me to win the honor of life.

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GENERAL ABSTRACT

Several microorganisms used as probiotic for one aquatic species could be harmful to other species. For maximal benefit, selection of specific probiotic strain /species for the target fish species in a particular culture condition/environment is prerequisite. The aim of the study was to evaluate the comparative efficiency of two potential probiotics, yeast *Geotrichum candidum* QAUGC-01 and bacteria *Bacillus cereus* locally isolated from yogurt, a dairy product obtained from local store and gastrointestinal tract of adult rohu (*Labeo rohita*) respectively, and select the one for introducing into the practical culture system of the extensively culturable indigenous fish species *L. rohita.* The study was conducted in four phases. In the first phase, two sets of 11 weeks feeding experiments on two life stages (i.e. swim up fry and advanced fry of *L. rohita*) were conducted for evaluating the comparative effects of dietary *B. cereus* and *G. candidum* (at similar rate 1×10^9 CFU/g feed) on survival, growth rate, intestinal bacterial load, activities of intestinal enzymes (cellulase, amylase, protease), muscle composition, aspartate aminotransferase (AST) activity, hemato-immunological indices (RBCs, Hb, HCT, WBCs, MCHC, respiratory bursts and phagocytic activity, total protein, lysozyme, IgM) and resistance to the pathogen *Aeromonas hydrophila*. On the basis of its most significant positive impact on all studied parameters, *G. candidum* in contrast to *B. cereus* was selected for evaluating its practical application in commercial culture of *L. rohita*. In the second phase, *G. candidum* was encapsulated by using alginate alone (Alg) and in combination with starch (Alg-S) and xanthan (Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and nano-chitosan (Alg-CN, Alg-S-CN, Alg-X-CN) for improving the stability, viability and target release of probiotic. The comparative structural and *in vitro* functional characteristics of prepared microcapsules revealed that microcapsules of *G. candidum* prepared with Ag-CN as compared to other formulations

were more spherical in shape with smooth surface, and had highest viable count after exposure to three tested pH (2, 4 and 8), higher temperature (60ºC) for an hour and 60 days storage at ambient temperature and 4ºC. In the third phase of the study, compared the *in vivo* efficiency of the *G. candidum* encapsulated with alginate alone (Alg) or in combination with starch (Alg-S) and xanthan(Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and nano-chitosan (Alg-CN, Alg-S-CN, Alg-X-CN) by feeding as a dietary supplement to the fingerlings of *L. rohita* for 11weeks under semi-control conditions (reared in fiberglass tanks in the outdoor facility on prepared diet). Results indicated the most significant positive effect of Alg-CN encapsulated *G. candidum* supplemented diet on growth, feed conversion ratio, intestinal enzyme activities, hematology, AST, ALT, ALP, hemato-immunological indices, lipid profile; high density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol and triglyceride content of fish as compared to basal diet supplemented with *G. candidum* encapsulated with other encapsulating formulations. In the fourth phase, commercial application of free /un-encapsulated and Alginate-chitosan encapsulated *G. candidum* was evaluated by feeding dietary probiotic supplemented diet to fingerlings of *L. rohita* reared in earthen ponds under semi-intensive culture condition. Results indicated significantly improved growth rate, intestinal enzymes activities and hemato-immunological indices. Additionally, up regulation of heat shock protein HSP 70 gene in muscle, intestine and liver tissues, increased muscle PUFA (especially Eicosatrienoic acid, linolenic, Eicosapentaenoic acid, Docosapentaenoic acid , Docosahexaenoic acid), ratio of ω3 to ω6 fatty acids, essential amino acids (particularly phenylalanine, valine and arginine) and the reduction in serum AST and ALT activities, total cholesterol and triglycerides were also observed in a group of fingerlings fed diet supplemented with Alginate-chitosan encapsulated *G. candidum* followed by un-encapsulated probiotic. Overall results indicated the

more pronounced effect of *G. candidum* QAU-001 especially Alg-CN encapsulated *G. candidum* on survival, growth, health status, immunity, muscle proximate composition /nutritive value of *L. rohita* reared in semi control (reared in fiberglass tank on prepared feed) and semi intensive culture system (reared in earthen pond on live feed plus formulated feed). Thus, suggests its application as feed additive for early rearing of swim up fry and practical/commercial production of *L. rohita.*

General Introduction

Aquaculture refers to the rearing of aquatic organisms in fresh, brackish and marine water. It is a fastest growing sector in terms of agriculture production worldwide, and currently contributes almost above 42 percent in global fish production (FAO, 2018). Thus, it plays a vital role in reducing food deprivation and economic insecurities in underdeveloped countries. Food security prevails when people have both the social and economic access to the basic, adequate and nutritious food to meet their dietary requirements. Nowadays a major portion of animal protein is supplied by aquaculture worldwide to overcome food insecurity (Belton and Thilsted, 2014; Belton et al., 2018).

Fish is widely considered as the best replacement of red meat, due to its low carbohydrate content and highly unsaturated fatty acids, especially ω-3 long chain polyunsaturated fatty acids (ω-3 PUFA) (Yildrim et al., 2009), a well-balanced amino acid composition and lower caloric density in contrast to land animals (Tacon and Metian, 2013). Furthermore, it also contains higher content of vitamins A, B_{12} and D3, minerals, especially phosphorus, calcium, selenium, iodine, and zinc as well as rare nutrients like iron, choline and taurine (Khalili-Tilami and Sampels, 2018). Worldwide, over the past three decades, aquaculture has been rising at an average rate of 8.2% / annum (Belton and Thilsted, 2014; Belton et al., 2018). Moreover, due to the nutritional value of aquatic organism, rising population and an efficient distribution system, now aquaculture contributes over 50% of the fish required for direct human consumption (FAO, 2016).

Furthermore, due to greater demand of food supply, there is a need for increased yield from aquaculture production. To overcome this increased pressure, the semi-intensive and intensive farming systems, i.e., rearing of finfish and shellfish under controlled or semi controlled conditions at high density is gaining importance throughout the world. However, some risk factors are associated with these practices, mainly due to overstocking of fish. The major risk factor is higher mortality rate due to increased level of stress, which affects the fish immune system (Alishahi et al., 2012) and ultimately increases susceptibility of infectious and non-infectious diseases. The outbreak of diseases induced by various viruses, parasites and epizootics is impeding the economic growth of many countries (Sumathi et al., 2014) and consequently appear as the major obstacles in the sustainable growth of the aquaculture industry. The economic loss due to the outburst of diseases has reached up to billions of dollars annually (Abumorad et al., 2014).

For sustainable development of aquaculture, it is essential to overcome the spread of infectious diseases and improve the survival of cultured animals. Different chemicals, antibiotics and vaccines have been used previously as a normal practice to treat the disease outbreaks in aquaculture systems (Welker et al., 2011). Nevertheless, antibiotics and chemotherapeutic that are supposed to cure / mitigate disease outbreaks, provided limited success (Gram et al., 2001). The drawbacks associated with antibiotic treatment include development of antibiotic resistance bacteria (Osman et al., 2010), inhibition of beneficial gut microflora, immune response suppression (Maynard et al., 2012; Banerjee and Ray, 2017), deposition of residues in fish

muscle that consequently lead to potential risk to the environment, aquatic organisms and consumer i.e., human beings (Agnew and Barnes, 2007).

Beside antibiotics and chemicals, vaccination is also sometimes employed in aquaculture as a normal practice to induce strong humoral and cell mediated immune responses in fish (Zhao et al., 2017). However, they are not frequently used because of the inherent problems associated with the application like difficulty in administration, inability to provide protection throughout the culture period as well as cost of application (Lin et al., 2009; Pinpimaiet al., 2015). Moreover, vaccines could not be used alone for disease control due to specification in action, i.e. specific vaccine cures certain specific disease (Muktar et al., 2016).

There is growing need to search for alternatives to antibiotics and vaccine which should be ecofriendly, sustainable and cost effective. Nowadays probiotics appear as a safe and best alternative of antibiotic and other chemicals for the health management of fish and shellfish (Panigrahi et al., 2010; Zorriehzahra et al., 2016, Sharifuzzaman and Austin, 2017). Probiotics are feed supplements containing live or dead microbial organisms (bacteria, yeast and fungi) or their components, having a positive effect on the nutritional, intestinal and microbial balance of the host or its environment (Villamil et al., 2002; Hill et al., 2014; Zorriehzahra et al., 2016). Previously, the most extensive use of probiotics was documented in pigs, although valuable information related to the use of some bacteria in poultry as well as in human has also been reported (Floch, 2014; Saint-Cyr et al., 2016). Application of the probiotics for land-farmed animals remained an unresolved issue for many years and brought out in late 1970s.

The origin of application of probiotics in aquaculture sector is unclear, yet literature reveals an extended use of different microbes as probiotics in finfish and shellfish culture mainly in Asia (China and India) and South America (Ecuador) (Kumar et al., 2006; Nayak et al., 2007; Austin and Austin, 2012; Ibrahem, 2015; Newaj-Fyzul and Austin, 2015). It seems that the concept of probiotic in aquaculture is very recent (Cruz et al., 2012). It was considered as an effective alternative approach which might lessen the dependence on chemotherapeutics, antibiotics and vaccines (Nayak et al., 2007; Lazado and Caipang, 2014b; Akhter et al., 2015). FAO recommends the use of probiotic for improvement of fish health and quality of aquatic environment (Subasinghe et al., 2003). Their application and beneficial effects are although well documented in aquatic animals (Hai, 2015; Hoseinifar et al., 2016; Reyes-Becerril et al., 2017) but, for ever increasing demand of developing sustainable and ecofriendly therapies as well as species specific effects of probiotic, day by day, research on probiotic application in aquaculture is gaining momentum (Standen et al., 2013; Lazado et al., 2015).

Nowadays, wide varieties of microbes are in use as probiotics for improving aquaculture production (Nayak, 2010). Although, these microbes differ noticeably in their mode of action, but have some common mechanisms. Generally, probiotics improve feed conversion efficiency (FCE %), growth performance, immunity and health of host organism by competitive exclusion of pathogenic microbes and colonization of favorable gut microbiota (Burr et al., 2005; Chabrillon et al., 2005; Nayak, 2010; Ghori et al., 2018), secretion of enzymes like cellulase, amylase, protease, lipase etc, (Ibrahem, 2015; Ibrar et al., 2017; Ullah et al., 2018), production of essential vitamins, short chain fatty acids (SCFA), organic acids (formic acid, lactic acid, acetic acid), inhibitory compounds (lysozyme, bacteriocin, hydrogen peroxide , proteases) and many

other compounds like siderophores, differentiation and activation of T-cell and modulation of physiological as well as immunological responses in fish (See reviews Balcazar et al., 2007; Nayak, 2010; Ibrahem, 2015; Zorriehzahra, 2016; Sharifuzzaman and Austin, 2017).

The behavior of microbes used as probiotics vary according to their interactions with the gastrointestinal tract of the host (Ibrahem, 2015). Generally, the natural antagonism mechanism is involved in the competitive exclusion of potentially pathogenic microbes and colonization of successful probiotic microorganisms. Balcazar et al. (2007) summarized the establishment of probiotic activity in three steps, i.e., attraction, connection and attachment to host tissues and suggested the involvement of host-related (redox potential levels, body temperature, status of enzymes, genetic tolerance) and microbe-related (potential to produce lysozymes, proteases, bacteriocins, hydrogen peroxide, carbon dioxide, siderophores and organic acids which can change gut pH) factors. Furthermore, Gatesoupe (2008) suggested long time administration for successful colonization of probiotic in the intestinal tract of the host. On the basis of *in vitro* and *in vivo* tests, Lara-Flores and Guzman (2009) suggested that the adhesion and organization of beneficial microbes in the host tissues and reduction /or elimination of pathogenic bacteria is closely associated with attachment of successful probiotic to the intestinal mucus and completion for binding sites, space, essential nutrient and energy.

In aquaculture, one of the most important beneficial aspects of probiotic is the growth enhancement of the cultivated species. It is well documented that successful probiotic colonize the gut of the host and bring about a change in microbial composition and structure of the intestine (increase in the height of the villi), thus improve the growth and health of host

(Balcazar et al., 2006; Ibrahem, 2015). Probiotic supplementation improves growth rate of fish by improving appetite, feed utilization, digestibility and absorption and assimilation of nutrients (Bombaet al., 2002; Cruz et al., 2012). Several studies on both freshwater and marine animals, reported the ability of the probiotics to release exogenous proteases, amylases, trypsin, lipases, cellulases, vitamins (Vitamin K, B_{12}, folic acid etc.), fatty acids and amino acids which aid in digestive process and positively influence the growth performance, feed conversion ratio (FCR), protein efficiency ratio (PER), survival and proximate composition of fish (Irianto and Austin, 2002; Lara-Flores et al., 2003; Al-Dohail et al., 2009; Nayak, 2010; Ibrahem, 2015).

Among others, the immunostimulatory effect of various probiotics in different fish species is well documented (Nayak, 2010; Austin and Austin, 2012; Newaj-Fyzul et al., 2014; Sharifuzzaman and Austin, 2017). Many investigators observed the stimulation of both systemic as well as local gut immunity, low cholesterol level and improved lactose tolerance of fish, in response to a range of probiotics (Nayak, 2010; Kumar et al., 2012; Newaj-Fyzul et al., 2014). Generally probiotics activate the immune system of fish by effecting erythrocyte, leucocyte, granulocyte, monocytes, lymphocytes, neutrophil, macrophage, macrophage phagocytosis, respiratory burst, peroxidase content, myeloperoxidase, superoxide dismutase, complement activity, lysozyme activity, serum/plasma bactericidal activity, antiprotease activity, total protein and immunoglobulin, cytokines, levels of antibodies and acid phosphatase (Brunt and Austin, 2005; El-Rhman et al., 2009; Nayak, 2010; Hai, 2015; Sharifuzzaman and Austin, 2017).

Probiotics produce several substances with bactericidal or bacteriostatic effects on the other microbial populations such as hydrogen peroxide, bacteriocins, proteases, lysozymes and antibiotic compounds (Panigrahi and Azad, 2007; Zorriehzahra et al., 2016). In addition

probiotic also produce volatile fatty acids, e.g. butyric acid, propionic acid, lactic acid) and organic acid in the gastrointestinal tract that cause a pH reduction, thus helping to prevent the growth of pathogenic microorganisms (El-rehman et al., 2009; Zorriehzahra et al., 2016). In literature, the antibacterial (Ramesh et al., 2015), antiviral (Kamei et al., 1988; Balcazar, 2003; Harikrishnan et al., 2010; Lakshmi et al., 2013) and antifungal activity (Lategan et al., 2004; Atira et al., 2012) of several probiotics are also documented.

Moreover, in practical aquaculture practices, probiotics can also show their beneficial effects by improving the water quality parameters (see review Hai, 2015; Zorriehzahra et al., 2016). Generally, they accelerated the decomposition of organic matter, lower the concentration of phosphorus and nitrogen and controlled the concentration of hydrogen sulfide (H_2S), ammonia (NH3) and nitrite (NO_2^-) in an aquatic environment (Boyd and Massaut 1999; El-rehman et al., 2009; Cha et al., 2013). Additionally, probiotics also reduced the accumulation of organic matter (Verschuere et al., 2000; Rengpipat et al., 2003), control pathogens, deposition of nitrogen and phosphate and mitigated sediment pollution (Wang and He, 2009) and improved environmental conditions for finfish and shellfish (Park et al., 2000; Dalmin et al., 2001; Hai, 2015).

In the last thirty years, many micro-organisms (yeast and bacteria) have been recognized as probiotics and utilized in the practical semi-intensive and intensive aquaculture systems. The beneficial microorganisms vary in origin, i.e., either isolated from the host or from other sources (soil, water, dairy products, etc.) (Lazado and Caipang, 2014b; Lazado et al., 2015). It is a general concept that microorganism isolated from the similar host offer a great advantages as a probiotic (Lazado et al., 2015). However, several microbes as probiotics from the terrestrial

environment have been documented conferring several benefits to the finfish and shellfish (see review Ibrahem, 2015). Mostly microbes obtained from gut of both terrestrial and aquatic organisms use as probiotics in aquaculture (Hai and Fotedar, 2010). The selection of potential probiotic depends on several factors like adhesion to the intestinal mucosa of host, tolerance to the GI tract environment, and antagonistic activity against pathogens (Nayak, 2010; Ibrahem, 2015). However, the efficacy of selected probiotic related to source, species/strain, dose, duration and mode of the application as well as to the age, size, state and culture conditions of the fish species (Ibrahem, 2015; Ridha and Azad, 2016).

A great number of microbes (bacteria and yeast) as probiotics are now being utilized in practical aquaculture to improve survival, growth and overall production of both finfish and shellfish (see reviews Nayak, 2010; Hai, 2015; Ibrahem, 2015; Dawood and Koshio, 2016; Sharifuzzaman and Austin, 2017) like *Lactobacillus, Lactococcus, Carnobacterium, Shewanella, Bacillus, Pseudomonas, Aeromonas, Enterobacter, Bifidobacteria and Saccharomyces*. Among bacteria, Bacillus is the most widely used species in finish and shellfish culture.

Bacillus is the rod shaped spore-forming Gram-positive aerobic as well as facultative anaerobic bacterium. It can be isolated from different sources like soil, water, air, vegetables, food as well as from a gut of animal and human (Alou et al., 2015; Kotb, 2015). Bacillus species are variable in genotypic and phenotypic characters. For instance some species appears as toxin producer and opportunistic pathogen in the host (animals as well as human) (Elshaghabee et al., 2017), while others showed beneficial effects on host and appeared as potential probiotics. Bacillus spp. offers better stability during low temperature storage and heat processing of

product and higher tolerance to acidic pH (Spinosa et al., 2000; Bader et al., 2012). Additionally, low production cost, anti-oxidant, immuno-modulatory, antimicrobial activities and food fermentation ability making *Bacillus* spp. valuable for use in aquaculture production and food industry (Terlabie et al., 2006; Wang et al., 2008; Lefevre et al., 2015; Shobharani et al., 2015; Ripert et al., 2016).

Among Bacillus spp. *B. subtilis* and *B. cereus* appeared harmless to vertebrates including human (Olmos-Soto and Contreras-Flores, 2003). They demonstrated their ability to produce secondary metabolites like vaccines, antibiotics, heterologous proteins, antigens, certain chemicals (Olmos-Soto and Contreras-Flores, 2003; Westers et al., 2004; Valdez et al., 2014) which slow down the growth of many pathogenic bacteria like *V. alginolyticus* and *A. hydrophila*. Moreover, their enzymatic potential like protease, amylase, esterase, lipase, leucine arylamidase and acid phosphatase showed positive effect on the digestion of lipids and other nutrients in the gut (Kim et al., 2007).

Different species and strains of yeast are also used effectively as probiotic in aquaculture (Abraham et al., 2007 and 2008) for improving the survival rate (Villamil et al., 2002), growth (Kumar et al., 2012), resistance to stress (Rollo et al., 2006), immunocompetence (Gatesoupe, 1999; Fakruddin et al., 2017) of aquatic animals. Yeast has an ability to withstand the harsh conditions of fish GI tract like presence of enzymes, organic acids, bile salts, variation in pH and temperature and colonizes the gut (Mishra et al., 2001), support the growth of lactic acid bacteria and competitively exclude the pathogenic bacteria and their products (Onifade et al., 1999),

assimilate the cholesterol, produce glutathione, vitamin B_{12} killer toxin, and various enzymes like cellulase, protease, lipase and amylase (Boutrou and Gueguen, 2005; Fakruddin et al., 2017).

Currently, use of probiotics is gaining importance for the ecofriendly sustainable development of aquaculture. Nevertheless, there are certain challenges associated with the intake of probiotics which need to be considered to obtain beneficial effect. One of them is the minimum suggested concentration of viable cells of probiotic require on the target site of the host. It has been recommended that diet containing probiotic should contain at least 10^7 to 10^8 of alive microorganisms per gram when consumed in order to produce beneficial effects (Kim et al., 2008; Albertini et al., 2010; Russell et al., 2011; Zhang et al., 2013; Ramos et al., 2018). However, during the feed processing, long term storage and transition through the gastrointestinal tract, most of probiotics lost their viability (Mattila-Sandholm et al., 2002; Cordero et al., 2015), this show limited efficacy due to poor bioavailability of viable microorganisms on desired sites (Graff et al., 2008). Hence, it is of prime importance, that applied probiotic retain their activity and survival in the physiochemical conditions during the feed preparation process and their passage through the gastrointestinal tract (Kumar et al., 2012).

The maintenance of viability and the functionality of probiotics until they reach the gastrointestinal tract is one of the key requirements to confer the beneficial effects to the host (Ross et al., 2005; Heidebach et al., 2009; Zhang et al., 2013). Another problem associated with the probiotics is their ability to adhere to the intestinal wall, however, if unable to do so, then beneficial effects may be compromised. For the improved viability of probiotics throughout processing, storage of feed and delivery to the gastrointestinal tract, different techniques have

been developed. One of the most promising of these techniques is the microencapsulation or immobilization.

Microencapsulation is defined as a process in which microorganisms / micronutrients / material are surrounded by a material called coating material or embedded in a homogeneous or a heterogeneous matrix (Gharsallaoui et al., 2007; Huq et al., 2013; Riaz and Masud, 2013). This technology is widely being used in the pharmaceutical, food and cosmetics industry (Garg et al. 2006; Cook et al., 2013). It gives a physical barrier between the entrapped/ microencapsulated compounds and the surrounding environment, reduce the contact and reactivity with environmental oxidizing promoters like metal ions, heat and light (Ye et al., 2009). It protects probiotics from conditions like low pH, high acidity, enzymatic action, bile salt, temperature shocks and antimicrobial agents (Sultana et al., 2000; Lee et al., 2000; Cook et al., 2013). Beside protection from unfavorable conditions, microencapsulation also makes possible the controlled release and targeted delivery of an encapsulated probiotic in the gastrointestinal tract (Nualkaekula et al., 2012).

Nowadays, several microencapsulation processes are in practice to encapsulate the materials like spray drying, emulsion, extrusion, coacervation-phase, ionotropic gelation and entrapment in liposome (Benita, 2005; Gouin, 2004; Munin and Edwards-Lévy, 2011; Jyothi et al., 2010; Soukoulis and Bohn, 2018). The choice of a particular process for microencapsulation depends on the physiochemical properties of selected material/micronutrient/probiotic, coating material, size of microcapsules and on the process costs (Nesterenko et al., 2013). However, emulsion/two phase system and extrusion/droplet methods, are commonly used for encapsulation

of probiotics. By adopting these techniques, the storage stability and viability of probiotic microorganisms in fish feed and GI tract of fish could be improved (Huq et al., 2013).

Efficiency and characteristics of microcapsules depend on many factors, including, quantity of probiotic time of bio-encapsulation, encapsulating materials, hardening solution (calcium chloride), concentration of capsular matrix and hardening time (Krasaekoopt et al., 2003; Riaz and Masud, 2013). Generally, the functional groups present on the cell wall of probiotic microorganism and their cross linking with the encapsulating material influence the survival of probiotic within the microcapsule (Hernandez et al., 2013). The encapsulating or wall materials interact structurally in different ways with the probiotics, thus also affect their delivery at the target site (Riaz and Masud, 2013). The capsule forming ability and strength determines the effectiveness of any encapsulating material (Reid et al., 2007). It is recommended that wall/ encapsulating material should be cheap, biologically safe and biocompatible.

Microencapsulation is usually done by proteins, lipids and gelling polysaccharides including chitosan, alginate and xanthan (Hernandez et al., 2013). Among them alginate is one of the most commonly use encapsulating material (Joye et al., 2014). It is nontoxic, inexpensive (Lee et al., 2000), biocompatible, water soluble, anionic natural heteropolysaccharide consist of linear copolymers of β-D mannuronate and α-L-guluronate, residues. It has a powerful ability to swell in aqueous solution and reduce in molecular weight at low pH, which helps the faster degradation and release of materials from microcapsule (Gombotz et al., 1998; Krasaekoopt et al., 2006). Alginate is also hemocompatible and does not accumulate in any major organs (Rajaonarivony et al., 1993). Generally; sodium alginate is made by the addition of mineral acid

in alginate and then used for encapsulation. It is biocompatible and biodegradable, bioadhesive, pH responsive in nature and forms gels with multivalent cations (Lam et al., 2014).

Though sodium alginate is important for encapsulation but several studies confirmed their propensity towards the low pH (acidic conditions) of the stomach, loss of mechanical stability while passage through the GI tract. It is well established that alginate hydrogel is not stable in acidic medium, therefore alginate particles disintegrate quickly before reaching the target site (Joye et al., 2014). However, this degradability can easily be modified (Gombotz et al., 1998) by mixing of the alginate with other wall materials (like starch, xanthan) or coating of the alginate microcapsules with other polymers (like chitosan) and modifying the structure of the alginate (Sultana et al., 2000; Yasmin et al., 2018). The alginate in combination with starch is commonly used for encapsulating the probiotics (Riaz and Masud, 2013).

Chitosan and alginate are widely used for probiotic encapsulation (Allan-Wojtas et al., 2008). Chitosan is a high molecular weight cationic polysaccharide produced by the N-deacetylation of chitin and consist of D-glucosamine and N-acetyl-glucosamine linked through β 1-4 glycosidic bond. It is produced through varying degree of deacetylation resulting in varying amount of two monosaccharides. Thus coating of an alginate matrix using oppositely charged polymer is an effective way of modulating the physical characteristics of the wall material (Gaserod et al., 1998; Helander et al., 2001; Matricardi et al., 2008). In the chitosan coated alginate microcapsules, carboxyl group (-COOH) of the alginate and amine group (-NH2) of the chitosan interact with each other and form alginate-chitosan polyionic complex. The complex provides increased protection, biocompatibility and biodegradability and control release of the

encapsulated materials like probiotics, micronutrients more efficiently, than either one (alginate or chitosan) were used alone (Gazori et al., 2009; Yan et al., 2001; Cook et al., 2013). Alginate-chitosan (Alg-C) formulation have been generally utilized for the encapsulation of enzymes, drugs, oligonucleotides and proteins with promising results (Coppi, 2002; Gonzalez-Rodriguez, et al., 2002; Mi et al., 2002).

The efficiency of an encapsulated probiotic depends on many factors like chemical nature and concentration of the material used for encapsulation, concentration of hardening solution, process of encapsulation, size and shape of prepared microcapsule, physiology of host organism and species/ strain and dose/concentration of probiotic (Gbassi et al., 2009; Jyothi et al., 2010). Therefore, before practical application of encapsulated probiotic, literature emphasize, *in vitro* and *in vivo* simple tests for evaluating the temperature and pH tolerance, shelf life , resistance to bile and enzymes of GI tract of host and their impact on growth, immune response, metabolic function of an organism etc. (Papadimitrious et al., 2015). Although *in vitro* study provide the baseline for selection of probiotic for future *in vivo* applications (Srinu et al., 2013), however, the results based on *in vitro* tests might not comply precisely with *in vivo* conditions. Therefore, for practical /commercial application, *in vivo* studies need to be carried out. Moreover, *in vivo* efficiency of probiotics under control conditions, do not always provide the same results under practical semi intensive and intensive pond culture systems. According to Lazado et al. (2015), 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. Thus, before suggesting practical application of any microorganism as potential probiotic, effectiveness under practical pond culture systems need to be evaluated.

Keeping in view species specific or even strain specific effect of probiotic on target species in a particular culture condition/environment, the present study was designed with the aim to assess the efficiency of two local strains of potential probiotics, yeast, *G. candidum* QAUGC-01 (KTC280407) and *B. cereus* (KT021872) isolated from a dairy product (yogurt), purchased from local store and gastrointestinal (GI) tract of adult *L. rohita*, respectively, and select the best one for early rearing and commercial farming of extensively culturable indigenous species *L. rohita*. We hypothesized that with the selection of suitable probiotic, survival and growth during early rearing and production of *L. rohita* in the commercial farming system could be improved.

For achieving the target, we conducted the study in four phases. In the first phase comparative efficiency of locally isolated *G. candidum*, and *B. cereus* was evaluated by feeding as a dietary supplement to two life stages of *L. rohita*, swim up fry and advanced fry and observed their effect on growth, survival, activities of intestinal enzymes, hematological indices, the activity of aspartate aminotransferase (AST) immune response, muscle composition and resistance to pathogen *Aeromonas hydrophila*. On the basis of results *G. candidum* in contrast to *B. cereus* was selected for evaluating its practical application in commercial culture of *L. rohita*.

In the second phase of the study, *G. candidum* was encapsulated by using alginate alone (Alg) and in combination with starch (Alg-S) and xanthan (Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and nano-chitosan Alg-CN, Alg-S-CN, Alg-X-CN. To determine the best encapsulating formulation, comparative structural (shape, size and surface morphology)

and *in vitro* functional characteristics (encapsulation efficiency, thermal tolerance, pH tolerance and shelf life at different temperature) were examined.

In the third phase of the study, comparative *in vivo* efficiency of the *G. candidum* encapsulated with alginate alone and in combination with starch and xanthan and coated with chitosan and nano-chitosan was evaluated by feeding as a dietary supplement to the fingerlings of *L. rohita* and observed their impact on growth, feed conversion ratio, intestinal enzyme activities, hematology, AST, ALT, ALP, immunological indices, lipid profile (total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride content).

In the fourth phase, commercial application of free /un-encapsulated and Alginate-nano-chitosan (Alg-CN) encapsulated *G. candidum* was evaluated by feeding dietary probiotic supplemented diet to fingerlings of *L. rohita* reared in earthen ponds under semi-intensive culture condition and observing their effect on growth rate, intestinal enzyme activities, hemato-immunological indices, serum AST and ALT activities, total cholesterol and triglycerides level, expression of heat shock protein (HSP 70) gene in muscle, intestine and liver tissues as well as nutritive value of fish (muscle fatty acids and amino acids profile).

B. cereus is a Gram-positive, rod-shaped, spore-forming, aerobic as well as facultative anaerobic bacteria (size 1 × 3-4 μm). It is commonly found in food and soil. Its cell structure consists of an inner membrane and a thick peptidoglycan which functions to maintain cell shape (Ticknor et al., 2001). The cell wall consists of 50% percent polysaccharide especially neutral polysaccharide composed of N-acetylmannosamine (ManNac), N-acetylglucosamine, N-

acetylgalactosamine and glucose in a molar ratio of 1: 4: 1: 1(Amano et al., 1977). The linkage between the polysaccharide and peptidoglycan is a muramic acid 6-phosphate. The peptidoglycan of some *B. cereus* strains are unique with only a few oligomers present, the cross-linked muropeptides are dimmers, and many of the muropeptide lack the N-acetly group (Severin et al., 2004). These distinguishing characteristics affect charge on the cell surface which contributes to the attachment of an outer capsule or an S-layer in pathogenic strains. Literature revealed that some strains of *B. cereus* are opportunistic pathogen and occasionally associated with food borne illness in human and other vertebrates (Granum and Lund, 1997; Kotiranta et al., 2000). Furthermore, ability of *B. cereus* to produce moderate level proteases and poor level of amylases was also reported by Ghosh et al. (2002). In aquaculture, a few investigators reported the positive effect of this probiotic on growth and survival of fish (*Dentexdentex* L., Hidalgo et al., 2006; *Trichogaster trichopterus*, Subharanjani et al., 2015; *L. rohita*, Amir et al., 2018), while others did not observe any significant effect on growth performance and proximate composition of juvenile Nile tilapia (Garcia-Marengoni et al., 2015).

G. candidum is eukaryotic filamentous yeast. It can be isolated from soil, water, baker's dough, husks of fermentation, bread, milk, milk products and various plant substrates (Arnau et al., 1994; Gente et al., 2002). It is composed of 26.8% carbohydrate, 48% protein, 7.2% lipid, 0.9% DNA, 5.68% RNA and contains about 50 ppm copper (Quinn and Marchant, 1979), while its cell wall contains 7% protein, 8% lipid, 12% galactose, 14% mannose, 14% hexosamine and 28% glucose. Moreover, the enzymatic and chemical analysis of cell wall of *G. candidum* indicated the presence three types of polysaccharides i.e. a galactomannan, β-1, 3-glucan and chitin. Enzymatic potential of *G. candidum* indicated, higher level of α amylases, cellulases, β-

glucanases, xylanases and moderate level of proteases and lipases (Piegza et al., 2014). The use of *G. candidum* has been reported earlier in a few studies. For instance, Dabrowski et al. (1980) reported its effect on rainbow trout while using as a feed additive, while Ibrar et al. (2017) and Amir et al. (2018) used via water and as dietary supplement respectively and reported significant positive effect on early rearing of *L. rohita*. Moreover, Ghouri et al. (2018) used *G. candidum* in combination with *Enterococcus faecium* as a dietary supplement and reported positive effect on fingerlings of *L. rohita.*

Rohu, *Labeo rohita* is among seven freshwater cultureable fish species, predominantly in South Asia. It has high commercial value, good growth rate, consumer preferences and relatively easier weaning on prepared diets (Khan et al., 2008; Hussain et al., 2011). Food selection and feeding affinity of *L. rohita* showed that it is a zooplankton feeder at postlarval stage and a phytoplankton feeder at adult stage (Rahman et al., 2006). The morphology and physiology of the gastrointestinal tract of *L. rohita* change as fish grow. In early life stage, fish has limited digestive capability but with age and size, relative length of gut (RLG), slowly increases from fry to fingerlings to the juvenile stage, which reflect the change in feeding habits and digestibility of nutrient (Ramesh et al., 1999; Azim et al., 2001).

In Pakistan and most of Asian countries, aquaculture system, *L. rohita* is induced bred and the postlarvae are reared in nursery earthen ponds on live feed, while fry and fingerlings are reared in larger earthen ponds under semi-intensive culture system, i.e., on both live feed and prepared diet (Mohsin et al., 2017; Rahman et al., 2006). The higher rate of mortality (70–80%), and poor growth performance during early developmental stages of major carps are the

major constrains in the supply of good quality seed (Mohapatra et al., 2012) and increase in production of these species (Jha et al., 2015).

During early development stage, *L. rohita* like other carps do not possess well differentiated digestive and immune systems (Zapata et al., 2006; Ibrar et al., 2017) , thus lack ability to digest most of nutrient efficiently (Kolkovski, 2001) and protect themselves from pathogen. Moreover, in semi-intensive culture of *L. rohita* and other carps, the application of animal manure to maintain the fertility of earthen ponds and to stimulate plankton (live food organisms) is a common strategy (Hossain et al., 2003; Jha et al., 2004; Rahman et al., 2006; Ullah et al., 2018). However, beside many advantages, this practice involves the proliferation of pathogenic bacteria in the water bodies (Sugita et al., 1985; Jiayi et al., 1987; Quines, 1988). Thus fish is under stress both due to biological and environmental factors, which effect it growth and overall the production.

Chapter 1

Evaluation of locally isolated potential probiotics *Geotrichum candidum and Bacillus cereus* for *Labeo rohita* (Hamilton, 1822)

Abstract

Due to variation in mode of action and efficacy, "one size fits all" model can't be applied to probiotic. Several microorganisms used as probiotic for one aquatic species could be harmful to other species. For acquiring maximal benefits, selection of specific probiotic strain /species for the selected fish species in a particular culture condition/environment is prerequisite. Here local strain of bacteria *Bacillus cereus* and yeast *Geotrichum candidum*-QAUGC-01 were isolated from gastrointestinal (GI) tract of adult rohu *L. rohita* free from any disease and a dairy by-product, yogurt respectively, were selected as potential probiotics for *L. rohita*. On the basis of gut morphology and gut microbiota, two life stages i.e. swim up fry and advanced fry were used to evaluate their comparative effect on the survival, growth rate, intestinal bacterial load, intestinal enzymes activity, muscle composition, hematological indices, aspartate aminotransferase (AST) activity and immunological indices. Two sets of 11 weeks feeding experiments in triplicate were conducted in four groups each (Swim up fry: $Control_{PL}$, Y_{PL}, B_{PL}, BY_{PL}; Advanced fry: $Control_A$, Y_A, B_A, BY_A) and the study was carried out in a completely randomized manner. Control groups were given only basal feed devoid of probiotic, Y_A and B_A groups were fed basal diet supplemented with *G. candidum* and *B. cereus* respectively at similar rate i.e., 1×10^9 CFU/g feed, while BY_A group of each was fed a diet supplemented with consortium of both probiotics, each at the rate of 1×10^9 CFU/g feed. Results indicated the most significant effect ($p<0.001$) on *G. candidum* in terms of final weight, specific growth rate (SGR), biomass, survival (%), gut microbial count and muscle composition. Variation was also found in the activities of intestinal enzymes, as amylase activity in first set of experiment was considerably higher ($P<0.001$) in Y_A, whereas BY_A group showed higher activity of cellulase and protease, whereas in second set of experiment, Y_A group showed the highest activity of all

studied intestinal enzymes. Moreover, significant increase ($P<0.05$) in erythrocytes (RBCs), leukocytes (WBCs), hemoglobin (Hb) and hematocrit (HCT %) was observed in Y_A group followed by BY_A group. The immunological indices like plasma protein level, immunoglobulin (IgM), serum lysozyme activity, respiratory burst and phagocytic activity also showed a similar trend, i.e., highest in Y_A followed by BY_A and lowest values were observed in B_A group. The aspartate aminotransferase (AST) level showed opposite trend as it was highest in B_A group while lowest level was observed in Y_A group. Furthermore, after performing challenge test with *Aeromonas hydrophila*, enhanced survival ($P<0.001$) was found in Y_A group. This investigation reveals the higher efficiency of *G. candidum* as compared to *B. cereus* and suggests its application for improving *L. rohita* production.

Introduction

For the last few decades, freshwater aquaculture is showing a continuous increasing trend. It contributes 15% of the world' freshwater and more than fifty percent to the total aquaculture production in Asia. In South Asia, *L. rohita* is one of the major cultivated species due to high market value, consumer demands and appropriate growth rate (Khan et al., 2008; Hussain et al., 2011). In Pakistan and most of Asian countries, post larvae of *L. rohita* are reared in nursery earthen ponds on live feed, while fry and fingerlings are reared in larger earthen ponds under semi-intensive culture system, i.e., both on live feed and prepared diet (Rahman et al., 2006; Mohsin et al., 2017).

Hatcheries play a key role in providing fish seeds including rohu in support of freshwater aquaculture production worldwide. However, they are facing many challenges in supply of good quality seed and an increase in production of these species, like low survival (70–80% mortality) during transit from endogenous to exogenous feeding and poor growth (Mohapatra et al., 2012; Jha et al., 2015). It is well accepted that *L. rohita* like other culturable fish species, do not possess well developed/differentiated digestive and immune system during early life stages (Zapata et al., 2006; Ibrar et al., 2017), therefore they cannot efficiently digest majority of nutrients (Kolkovski, 2001) and fails to protect themselves from pathogen. As the development of immune-competence and lymphoid organs take few weeks post hatching (Zapata et al., 2006), therefore they mainly depend upon the non-specific defense systems like physical barriers, cellular defenses, protein defenses , inflammation etc. (Secombes and Ellis, 2012).

In aquaculture, feed formulation is based on the age, body size and developmental stage of the fish. During early life, fish have very small sized body and cannot consume feed particle

greater than 50-150µm in size. Normally, fish feed available locally has larger ingredient particle size. Hence, freshwater fish shows higher mortality during early life stages (Rao, 2003) and greatly affect the economy each year.

In most of South Asian countries including Pakistan, unavailability of low cost prepared/formulated feed for larvae and advanced fry on a commercial scale is a major obstacle in the expansion of major carp culture. During early rearing of culturable species including *L. rohita*, farmers rely on live food organisms like zooplankton and phytoplankton (Treece and Davis, 2000; Kolkovski, 2001). However, many inherent challenges are associated to the rearing of aquatic organism on live feed, having direct or indirect impact on the growth and survival of juveniles. There are some problems related to the utilization of live organisms as feed, including composition and availability of their nutrients because zooplanktons may have high or low level of some essential nutrients (Ibrahem, 2015). As some nutrients have high conservative nature therefore their amount and ratios cannot be manipulated to a desired level (Rainuzzo et el., 1994). Besides, the introduction of potential pathogens into the culture system (Edwards, 2013a), while producing live feed with the application of organic fertilizer (Hamre, 2016) also contribute significantly in great loss.

Live feed as well as their associated constrains can be eliminated through the development of prepared functional microdiet (Rønnestad et al., 2013). Functional diet may have the ability to enhance nutrient digestibility and improve the immunity of early life stages of fish, thus could replace the live feed required for early rearing (Ringo and Birkbeck, 1999). Since last few years, further works has been started to prepare highly digestible feed, for early growth of various fish species either with the use of partially digested protein sources like protein

hydrolysate or by supplementing enzymes and probiotics that enhance nutrients digestibility (See review Hamre et al., 2013).

For sustainable and eco-friendly development of aquaculture practice, probiotics as feed additive are gaining more attention worldwide. Generally, probiotics are alive or dead microorganisms (bacteria, yeast and fungi) or their components showing beneficial effect on the nutritional, intestinal and microbial balance of the host or its environment (Villamil et al., 2002; Hill et al., 2014; Zorriehzahra et al., 2016). Mostly, probiotics outcompete the pathogen, colonize the gastrointestinal tract of host and favor the establishment of beneficial gut microbiota (Burr et al., 2005; Chabrillon et al., 2005; Nayak, 2010; Ghori et al., 2017), improve digestibility of nutrient, FCR, growth, immunity, disease resistance and overall health status of fish by releasing enzymes like cellulase, amylase, protease, lipase etc. (Ibrar et al., 2017; Ullah et al., 2018), production of essential vitamins, short chain fatty acids (SCFA), organic acids (lactic acid, formic acid and acetic acid), inhibitory compounds (lysozyme, bacteriocin, hydrogen peroxide , proteases) and many other compounds like siderophores (Gatesoupe, 1999; see reviews Hardy et al., 2013; Ibrahem, 2015), differentiation and activation of T-cell and modulation of physiological as well as immunological responses in fish (Balcazar et al., 2006; Nayak, 2010).

The composition of the microbiota associated with the GI tract of fish is highly uneven and depends on various factors including species and life stage of organism (Ringø and Birkbeck, 1999; see review Borch et al., 2015). Generally, exposure during early life stages shows major impact on microbiota composition of adult fish (Ridha and Azad, 2016). The GI tract of most aquatic fish species during early life stages is histologically, morphologically and physiologically less elaborated, somewhat sterile in contrast to adult (Rønnestad et al., 2013). In the sterile gut of fish larvae, the establishment of microbiota is influenced on many factors like

live feed, microbes present in the rearing environment and in eggs if any (Ringo and Birkbeck, 1999). Most microbes in the gut are transient because of continuous intrusion of bacteria/microorganisms coming from food and aquatic environment (Gatesoupe, 1999). Thus, two types of microbiota are associated to fish gut, i.e., allochthonous (which incidentally visit the GI tract and after some time rejected by fish), and autochthonous (which are indigenous and have the ability to colonize the epithelial surface of host.

Many microorganisms (bacteria, yeast, fungi) with varying degree of success are now being in use as probiotics for promoting growth rate, improving survival and disease resistance and stimulating immune response of fish in aquaculture (Nayak, 2010; Navarrete and Tovar-Ramírez, 2014; see review Ibrahem, 2015; Zorriehzahra et al., 2016; Sharifuzzaman and QAUGC-01 Austin, 2017) like *Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Shewanella, Bacillus, Pseudomonas, Aeromonas, Vibrio, Enterobacter, Bifidobacteria and Saccharomyces* (see review Allameh et al., 2017). Among bacteria, Bacillus is the widely used species in aquaculture. For instance, being as probiotics, *B. cereus* and *B. subtilis* are harmless (non-toxic and non-pathogenic) to vertebrates including human (Olmos-Soto and Contreras-Flores, 2003; Farzanfar, 2006) has the ability to produce secondary metabolites like vaccines, vitamins, carotenoids, antibiotics, antimicrobial compounds, heterologous proteins, antigens, certain chemicals (Elshaghabee et al., 2017) that inhibit the growth of *V. harveyi*, *V. alginolyticus* and *A. hydrophila* and provide resistance against disease (Bagheri et al., 2008; Merrifield et al., 2010a; Amir et al., 2018; Dias et al., 2018), whereas their enzymatic performance of esterase, lipase, leucine arylamidase, and acid phosphatase show positive impact on the lipids digestion in the gut (see review Allameh et al., 2017). Moreover they also enhance

community of autochthonous bacteria, simulate multiplication of beneficial bacteria in gut, and improve immune status and antioxidant ability of fish (Wang et al., 2017).

Among yeast, *Saccharomyces cerevisiae* and *G. candidum* being resistant to many antibiotics such as sulfatides and other antibacterial agents are also used effectively as probiotic in aquaculture (Abraham et al., 2007 and 2008) for improving survival rate (Villamil et al., 2002), resistance to stress (Rollo et al., 2006), growth rate, muscle composition (Ibrar et al., 2018; Aamir et al., 2018) and immune system (Gatesoupe, 1999; Fakruddin et al., 2017) of aquatic animals. These probiotics have the ability to withstand the harsh conditions of intestinal tract like presence of enzymes, bile salts, organic acids, variation in pH and temperature, colonizes the fish intestine (Mishra et al., 2001), support the growth of lactic acid bacteria and competitively exclude the pathogenic bacteria as well as their products (Onifade et al., 1999), assimilate cholesterol, produce vitamin B_{12} killer toxin, glutathione and various enzymes like cellulase, protease, lipase and amylase (Boutrou and Guéguen, 2005; Fakruddin et al., 2017).

Tolerance to gastrointestinal environment, adhesion to the intestinal mucosa of host, antagonistic activity against pathogens are some of the important properties through which the potential probiotics were selected. Moreover, efficiency of the concerned probiotics also depends on various factors like dose/concentration and mode of the application, duration, age/size of target fish species and cultured condition (Jha et al., 2015; Ridha and Azad, 2016). Furthermore, environmental factors, temperature, oxygen, mechanical friction, osmotic pressure, hydrogen-ion concentration, water activity as well as biological factors like species, strain biotype that influence the stability of probiotics (Wang et al., 2008). Thus, one probiotic could not be used for all aquatic organisms (Sharifuzzaman and Austin, 2017). For example some microbes as probiotic are beneficial for one fish species or aquatic animals and it could be harmful for other

species (see review Hai, 2015). For obtaining maximal benefits, there is great need of specific species/strain of probiotic for selected fish species in particular culture environment.

By viewing the beneficial effects of probiotics as feed additive as well as their species specific or even strain specific effect on the fish, the present part of study was designed to evaluate the comparative efficiency of two local strains of probiotics i.e., *B. cereus* and *G. candidum*- QAUGC-01 isolated from gastrointestinal (GI) tract of an adult healthy *L. rohita* and commercial dairy product (Yogurt) purchased from local store respectively and to select the best one for early rearing of extensively culturable indigenous species *L. rohita*. For achieving the target, two life stages of target fish; swim up and advanced fry were selected, on the basis of digestive capability and gut microbiota and the effect of both probiotics as dietary supplement on the survival, growth rate, intestinal bacterial load, activities of intestinal enzymes (cellulase, amylase, protease), muscle composition, hematological indices, aspartate aminotransferase (AST) activity, hemato-immunological indices (RBCs, Hb, HCT, WBCs, MCHC, respiratory bursts and phagocytic activity, total protein, lysozyme, IgM and resistance to pathogen *Aeromonas hydrophila* was studied.

The information on the present strain of yeast and bacteria, *G. candidum*-QAUGC-01 (KTC280407) and *B. cereus* (KT021872) as potential probiotics is limited. Thus results of present part of study contributed in the already present data on various probiotics and helped in selection of probiotic for *L. rohita.*

Comparative effect of dietary *Geotrichum candidum* and *Bacillus cereus* supplementation on early rearing of *L. rohita*

Material and Methods

Potential probiotics

Yeast *G. candidum*-QAUGC-01 (KTC280407) isolated from commercial dairy product yogurt purchased from local store and gram-positive bacteria, *B. cereus* (KT021872) isolated from GI tract of wild caught adult rohu, *L. rohita* free from disease. Both probiotics having similar concentration i.e. 1×10^9 CFU g^{-1} were obtained from Department of Microbiology. Plate count was used for studying the viability of both bacterial and fungal strains before and after preparation of feed as well as during feed storage. *B. cereus* and *G. candidum* were grown on Tryptic soya agar (TSA) and Oxytetracyclin glucose agar (OGA) respectively for 24-48 h. Following incubation, plates were examined for growth and the number of colonies were counted and expressed in number of colony forming units (CFU) / ml of sample (Mandal and Ghosh, 2013). The quantified yeast and bacterial cells were centrifuged and suspensions were made with phosphate buffered saline having pH 7.2. The suspended active/alive yeast and bacterial cultures were used for top dressing.

Diet Preparation

For swim up and advanced fry of *L. rohita*, 40% and 35 % CP basal microdiet respectively were formulated and prepared by adopting already published method (Amir et al., 2018) (Table 1.1). Briefly, all dried ingredients were finely ground, weighed and mixed together in fixed ratio. For making dough, small amount of water with oil was added to the mixture. The dough/paste was passed through a small meat grinder to make spaghetti which were immediately spread on aluminum foils sheet. The feed was dried in an oven, pre- set at 60°C. Hand held pestle was used

for grinding of dried spaghetti to make fine powder. The fine powder was equally divided into four groups i.e., $control_A$, Y_A, B_A and BY_A. Each group was supplemented with their respective probiotic, i.e., control group ($control_A$) devoid of probiotic, Y_A and B_A with *G. candidum* QAUGC-01- and *B. cereus* (alive) respectively in a similar dose i.e., 1×10^9 CFU/g feed, while BY_A was supplemented with blend of both strains of probiotics in equal concentration i.e. 1×10^9 CFU/g each. The respective probiotic was added in each group by adopting similar method mentioned in our publication (Amir et al., 2018). Each group of feed dried by using freeze drier for 48 hrs and kept separately in zip lock bags and stored in a refrigerator for further use. During feeding trial, to provide feed to respective group according to particle size, feed was passed through a sieve of particular size (50-600µm). Feed particle size and feeding frequency was adjusted fortnightly with respect to the size of fry (Table 1.2). Regular preparation of the diets was done after two weeks and survivability of microbes in stored diets was examined using a common method reported by Nikoskelainen et al. (2003).

Experimental design

Broodstock of *L. rohita* was prepared in earthen ponds under semi-intensive culture conditions and induced bred by using commercially available induced spawning agent ovaprim (SGnRHa + domperidont, Syndel, USA). Female and male broodstock were injected a dose of 0.4-0.5 mL kg^1 and 0.2 mL kg^{-1} body weight, respectively. Dry method of fertilization was used to fertilize the eggs with sperm. Fertilized eggs were incubated in circular tank under flow through system. Hatchlings were maintained in tank until the absorption of yolk sac. To start first set of trial, about, 2400 swim up fry were randomly selected and equally distributed in 4 groups (200 fry per tank or 600 fry/treatment). The remaining swim up fry were stocked in earthen nursery pond. A completely randomized feeding experiment was designed and conducted under semi static

conditions in outdoor research facility. Initially, automatic feeders were used for continuous feeding of each group with their respective diet. Then, feed particle size, feeding rate and frequency was adjusted fortnightly (Table 1.2). The accurate estimation of feed consumption was not possible, because uneaten powdered feed dissolved in water within a short time, thus it was difficult to collect, dry and weigh.

During rearing, water temperature (°C), dissolved oxygen (DO, mgL^{-1}) and pH were noted twice a day (09:00 and 16:00) by using Multiparameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA), while total ammonia was checked weekly by using test kit of ammonia for freshwater (HI3824, ROMANIA). Initial temperature of water in all the tanks was 24.5±0.5°C. During experiment, it was increased gradually and at the end of experiment it reached to 26.0±0.3°C. In addition, DO level varied a little due to nonstop aeration in each tank. Furthermore, total ammonia remained $\leq$ 0.25 mg L^{-1} while pH did not show any significant difference. The water quality parameters showed no noticeable differences because the experimental set up was in in same vicinity, and trial was conducted under similar environmental conditions, at same stocking density.

Growth performance and Survival (%)

Postlarvae were weighed and counted at the time of stocking. After feeding trial and before sampling, fry were starved for about 24 hr. On the day of sampling, fry were collected separately from each tank, weighed and counted. For evaluating growth performance and survival (%), following standard formulas were adopted.

Average weight of individual fry = Total weight of all fry / Total number of fry

Weight gain WG (g) = FBW of advanced fry– IBW of postlarvae

Weight gain (%) = (FBW of the advanced fry – IBW of the postlarvae) ×100

(IBW of postlarvae)

SGR (%) = *ln* (FBW of the advanced fry) **-** ln **(**IBW of postlarvae) ×100

Experiment days

FBW: - Final body weight

IBW: -Initial body weight

SGR: - Specific growth rate

Muscle composition

For proximate composition of muscle (moisture, crude fats, crude protein, carbohydrate and ash content), 30 advanced fry were collected randomly from each tank (n= 90/treatment). Carcass of ten fry were pooled and analyzed by using standard procedure (AOAC, 2000).

Dry matter and Moisture Content

In order to find out moisture contents, washed and pre weighed china dish was taken and kept in oven at 65^0C for 10 min. Subsequently, china dish was cool in desiccator and weighed on top loading electrical balance (SHIMADZU-ELB3000, Japan). Then 5g sample was weighed and put on the pre-weighed china dish and placed in oven, set at 65^0C. The sample was dried until a constant weight is achieved. After heating, china dish was placed again in desiccator, cooled and then again weighed near 0.01g on digital balance. Percentage (%) of dry matter and moisture (%) was calculated by adopting standard formula (AOAC, 2000):

Dry matter = Weight of sample after drying / Weight of sample before drying×100

Moisture content = 100 – dry matter (%)

Moisture (%) = (Weight loses/original weight of sample) × 100

Crude protein

Crude protein in sample was determined by micro Kjeldahl's method (Cheng et al., 2017). Briefly, 2 g of sample was mixed with 5 g of digestion mixture (w/w 1g of Na_2SO_4 in 10 g of $CuSO_4$) and 30 ml conc. H_2SO_4 to digest the samples. Whole mixture was heated for 2-3 hr at 250°C until the appearance of light green color. After that mixture was cooled and distilled water was added to make the final volume of 250 ml in volumetric flask. Subsequently, 10 ml of mixture was taken and mixed with 10 ml of 40 % NaOH in Kjeldahl's apparatus. Funnel was plugged firmly, heated for 2-3 min, and then 10 ml of 2% boric acid solution was added. The liberated ammonia was collected and titrated against H_2SO_4 (0.1 N) by using 5 drops of methyl red as indicator until the appearance of golden yellow color. Total protein was calculated by multiplying the amount of nitrogen with appropriate factor (6.25). First, Nitrogen (%) was calculated by using following formula:

$$N\ (\%) = \frac{\text{Normality of } H_2SO_4 \times \text{Vol. of } H_2SO_4 \text{ used} \times 250 \times 0.014}{\text{Wt. of sample} \times 10} \times 100$$

Where,

N =, Nitrogen

Vol. = Volume

Wt. = Weight

250 = Dilution of digested mixture

0.014= Standard Vol. of H_2SO_4 to neutralize 1ml of NH_3

10= used Vol. of diluted mixture

100= percentage of nitrogen

Crude protein (%) = Nitrogen (%) × 6.25

Where,

6.25 = Assumed factor to calculate crude protein from N (%)

Crude Protein (%) = N (%) × 6.25

Fats content

Soxhlet apparatus was used to determine total fat content of sample (Folch et al., 1958). Approximately, 1 g moisture free sample was taken in pre-cleaned Soxhlet apparatus thimble, positioned in an extractor and placed correctly under the condenser of extraction apparatus. Then 150 mL of ether was added into the weighed, cleaned and dried receiving flask and connected the flask to the apparatus. Subsequently, Soxhlet apparatus temperature was increased. Whole extraction process was done at 100 ˚C for 10 hr at rate of 3-4 drops/ seconds. After extraction, thimble was dried and weighed when removed from the extractor. The percent crude fat was calculated by adopting formula given below

$$\textbf{Crude Fats (\%)} = \frac{\text{Wt. of thimble after evaporation} - \text{Wt. of empty thimble}}{\text{Wt. of sample}} \times 100$$

Crude ash

To determine crude ash, crucible was cleaned and placed in a muffle furnace oven at 100^0C for 1 hr (Folch et al., 1958). After heating, it was cooled in desiccator. Weight of empty crucible was noted. Then 5g sample was taken in pre-weighed crucible dish and then crucible was placed again in furnace and heated at 550-600 ^{0}C for 24 hr. Then crucible was cooled in desiccator at room temperature. Ash in crucible was quickly weighed as early as possible to prevent moisture absorption.

$$\text{Crude ash (\%)} = \frac{\text{Wt. of ash}}{\text{Wt. of sample}} \times 100$$

Intestinal Enzyme analysis

On the day of sampling, 18 fry from each tank were caught at random and anesthetized with MS-222 ($0.01 gL^{-1}$). They were dissected on ice bag and GI-tract of each fry was collected. To get enough sample for analysis, gut of 6 fry of same tank were pooled (3 samples / tank or 9 samples / treatment). Samples were snap frozen in liquid nitrogen and then stored at -20˚C for intestinal enzymes (amylase, protease, cellulase) analysis.

For quantitative analysis, 0.5 g gut was weighed and homogenized in 5 mL chilled PBS buffered saline (pH 7.5) by using electrical homogenizer (Model, VWR POWER 200). The resultant homogenate was centrifuged at 10, 000 × g, for 10 min at 4ºC and clear supernatant (enzyme solution) was collected in a test tube and stored at - 4ºC until analysis.

All intestinal enzyme activity assays were conducted at 25°C, while pH values of solutions/buffer were measured at room temperature (22-23°C). All reagents used for assays were of analytical grade (Sigma-Aldrich, Germany) and purchased from local supplier.

Cellulase activity

Rajoka and Malik (1997) method reported in our recent publications (Amir et al., 2018; Ullah et al., 2018) was followed for the analysis of Cellulase enzyme activity. The assay generally based on the use of carboxymethyl cellulose (CMC) as substrate and estimation of released reducing sugar i.e., glucose. Before start, solutions needed for assay were prepared. Briefly, 1 % carboxymethyl cellulose solution was prepared by weighing 1g CMC and dissolving in 100 mL distilled water with the help of magnetic stirrer set at 40ºC while for preparation of 0.1 M citrate buffer (pH 5.0) , 0.307g of citric acid monohydrate and 2.932g of sodium phosphate were dissolved in 100 mL distilled water by using magnetic stirrer. Furthermore, DNS

reagent was prepared by dissolving 1g of 3, 5 dinitrosallicylic acid in 20mL 2M NaOH. Subsequently, slowly added 30g sodium potassium tartrate and made final volume of 100mL by adding distilled water. After preparation of solutions, 1 mL of CMC solution (1%) was taken in 10 mL glass tube and mixed with 1 mL prepared citrate buffer. The resultant solution was incubated for 30 min at 50°C. Afterward, 3.0 ml DNS reagent was added and again incubated at boiling point for 15 min. Subsequently, I mL Na K tartrate (40%) was mixed and test tube having solution was cooled at room temperature. UV-Visible spectrophotometer (Agilent, 8453, USA) was set at λ 540 nm and reducing sugar i.e., glucose was measured for estimating the cellulolytic activity. One unit cellulase activity is defined as amount of the enzyme per mL filtrate that release one mg reducing sugar as glucose per min.

Amylase activity

For determination of amylase activity, 3,5-dinitrosalicylic acid (DNS) method reported previously (Ibrar et al., 2017; Amir et al., 2018) was followed. Glucose estimation (reducing sugar) at 560nm is the basis for this method. Briefly enzyme solution of 0.5 mL was taken in glass test tube, mixed with 500 µL of starch solution (1%) and kept at room temperature for 3-5 min. Subsequently, DNS reagent (1 mL) was slowly added and incubated on a preheated water bath for at least 5 min. The mixture was cooled and mixed with 10 mL reagent graded water. UV-Visible spectrophotometer (Agilent, 8453, USA) was set at λ 450 nm and absorption of resultant solution was noted for estimating amylase activity. One amylase unit was defined as the amount of enzyme in one mL filtrate that released one microgram reducing sugar as glucose min^{-1}.

Protease Activity

Methodology used by Cupp-Enyard, (2008) and reported previously in our publications (Ibrar et al., 2017; Amir et al., 2018) was followed for the protease analysis. Initially 0.65% casein solution in 50 Mm KH_2PO_4 buffer was prepared. The solution was incubated at 37ºC for 5 min. Subsequently, casein solution of 5 mL and enzyme solution 1 mL were mixed. The reaction was terminated by addition of 5 mL trichloroacetic acid reagent which was prepared by mixing 110Mm trichloroacetic in distilled water at 1:55 ratio. Solution was incubated again, after reagent addition, for 30 min at 37ºC. Subsequently, the solution was cooled at room temperature and filtered by using Whatman filter paper. From resultant filtrate, 2.0 mL was taken in separate clean 10 mL glass test tube and mixed with 5 mL Na_2CO_4 (500mM) and 1mL folin-Ciocalteau reagent (0.5mM), and heated for 30 min at 37ºC and then cooled down at room temperature. UV-Visible spectrophotometer (Agilent, 8453, USA) was set at λ 660 nm and absorption of resultant solution was noted for calculating protease activity.

Isolation of intestinal bacterial flora

A standard protocol was used for the isolation of gut bacterial flora. Briefly, the intestine of 5 fry per tank were dissected out, pooled and homogenized with Glass Teflon Homogenizer (PK-01200S, Thomas Scientific, USA). The homogenate was mixed with PBS in 1:10 ratio and used as inoculums for culturing of aerobic and anaerobic gut microbes and for counting using standard plate count method was adopted (Nikoskelainen et al., 2003). Briefly, 6 tryptic soya Agar (TSA) plates were dried and aseptically inoculate the agar surface with 0.1mL of diluted inoculums. Subsequently, by using sterile spreader, the inoculum was evenly distributed over the agar surface. Afterwards, 3 plates were kept in incubator set at 37°C for enumeration of aerobic

bacteria while other 3 plates were placed in anaerobic chamber for anaerobic bacteria count. After 48 hr, plates were examined for growth and morphology of colony. The bacterial colonies were counted and reported as Log CFU/g.

Challenge test

At the end of study, 45 fry per treatment (15 fry from same aquarium) were equally distributed in 12 glass aquaria well-fitted with heaters and aerators. Temperature and DO level was maintained at 26.7 ˚C and 5.7 mg L^{-1} respectively. After three day acclimatization, fry were exposed to freshly cultured pathogenic strain of *A. hydrophila* (ATCC49140) taken from an infected *L. rohita* at National Veterinary Laboratory Islamabad (Ali et al., 2016). Bacterial suspension in PBS was administered to the aquarium water at a concentration of 1.4 $\times 10^8$ CFU mL^{-1} (Das et al., 2014). However, one aquarium considered as control was not administered any pathogen. Fry of all groups were fed *ad libitum* their respective diets thrice a day and kept for 20 days. During exposure, disease symptoms and mortality was closely observed in each group to confirm that the fish death was due to introduced pathogenic strain, autopsy of freshly dead fry was done and *A. hydrophila*, was re-isolated from their infected kidney and skin.

$$\text{Mortality (\%)} = \frac{\text{Number of death in a given time}}{\text{Total number of fish used during that time}} \times 100$$

$$\text{Relative Percent Survival} = 1 - \left(\frac{\text{Percent of treatment group mortality}}{\text{Percent of control mortality}}\right) \times 100$$

Statistical analysis

All results are shown as mean ±SD. Variance of homogeneity and data normality was performed by using Bartlett and Shapiro-Wilk's tests before running any statistical analysis. Significant differences in growth performance, survival, muscle composition, enzymatic activity and

bacterial counts among experimental treatments was evaluated by using one–way ANOVA followed by LSD test at the 5 % level of significance using SPSS version 20. Log transformation was performed for bacterial count data. GraphPad Prism 5 was used for graphical representation of the data.

Experiment 1

Results

Growth and survival

The probiotic supplementation showed positive effects on *L. rohita*. ANOVA revealed considerable differences in final weight ($F_{3, 8}$= 357.67, p< 0.001), final biomass ($F_{3, 8}$= 37.844, p< 0.001) and survival % ($F_{3, 8}$= 17.68, p < 0.001) of control and probiotic fed groups. In addition, higher final weight and survival (80%) were recorded for Y_{PL} group fed *G. candidum* ($T1_{PL}$) alone followed by $T3_{PL}$ and $T2_{PL}$ respectively (Table 1.3).

Proximate analysis

Probiotic supplementation revealed significant positive effects on muscle composition of fish. Proximate composition of muscle of swim up fry reared on dietary *G. candidum* (Y_{PL}) alone resulted considerably (p=0.001) higher crude protein, crude fat and carbohydrate contents followed by combination containing *G. candidum* and *Bacillus* (BY_{PL}) and *B. cereus* alone ($T2_{PL}$ $_{PL}$) respectively (Table 1.3). However, the control group showed higher moisture (%) content while *G. candidum* ($T1_{PL}$) fed fry showed lowest value.

Analysis of intestinal enzyme

Probiotic supplementation during early rearing showed significant effect on the specific activity of digestive enzymes of *L. rohita* as well as non-fish microbial produced enzyme cellulase (Table 1.4). Highest cellulase and protease activities were found in fry reared on combination of *G. candidum* and *B. cereus* (BY_{PL}) followed by Y_{PL} and B_{PL} group respectively

(BY_{PL}> Y_{PL}> B_{PL}>Control $_{PL}$). Whilst higher amylase activity was observed in Y_{PL} group (reared on *G. candidum* alone) followed by BY_{PL} and B_{PL} groups (Y_{PL}> BY_{PL}> B_{PL}>Control $_{PL}$).

Gastrointestinal microbial load

Probiotic supplementation showed significant impact on the gut microbial load i.e., aerobic and anaerobic bacterial counts (log CFU g^{-1}). Highest aerobic bacterial count ($F_{3, 8}$ = 51.88, p< 0.001) were found in Y_{PL} group of fry followed by B_{PL} group (Y_{PL}> B_{PL}>BY_{PL}>Control $_{PL}$). Moreover, the anaerobic bacterial count was statistically comparable and significantly higher ($F_{3, 8}$ = 18.69, p< 0.001) in all probiotic fed groups as compared to control (Fig. 1.1).

Challenge test

Significantly higher survival (%) was found in probiotic fed groups as compared to control group (one way ANOVA, $F_{3, 8}$=43.58, p<0.001) after challenged with carp pathogen *A. hydrophila*. Lowest mortality was found in Y_{PL} followed by BY_{PL} and B_{PL} groups (Y_{PL}< BY_{PL}<B_{PL}<Control $_{PL}$, Fig 1.2).

Table 1.1. Formulation and proximate composition of the diet

Ingredients	**Inclusion level (g /100g)**	
	40%	**35%**
Rice bran	41	--
Mustard oil cake	39	--
Fish protein hydrolysate	8	--
Wheat flour	7	--
Fish oil	2	1
Vitamin and mineral mix	2	2
CMC[1]	1	2
Soybean meal	--	36
Gluten 60	--	37
Wheat bran	--	10
Fish meal	--	12
Total	**100**	**100**
Proximate composition (%)		
Crude Protein	39.76	35.34
Crude Lipid	9.63	8.95
Ash	5.42	7.86

[1]Carboxymethyl cellulose, used as binder.

Table 1.2. Mesh size, Feeding rate and frequency used for early rearing of *L. rohita*

Size of the mesh(µm)	Feeding rate (%)	Feeding frequency
50-100	10	Continuous feeding for a week
210	8	6 times a day (a week)
250	6	5 times a day (14 days)
420	6	3-4 times a day (14 days)
600	6	3-4 times a day (34 days)

Table 1.3. Comparative effect of *G. candidum* and *B. cereus* supplemented microdiet on growth performance (mean ±SD, n=3) and muscle composition (n=9) of *L. rohita* fry after 11 weeks feeding trial

	Groups			
	Control $_{PL}$	**Y_{PL}**	**B_{PL}**	**BY_{PL}**
IBW (mg)	0.33±0.02^{a}	0.36±0.02^{a}	0.36±0.01^{a}	0.34±0.02^{a}
FBW (g)	1.14 ±0.09^{d}	2.74±0.11^{a}	1.57±0.18^{c}	2±0.37^{b}
^{1}SGR (% body weight/day)	10.6±0.06^{c}	11.7±0.02^{a}	10.9±0.11^{c}	11.4±1.66^{b}
Initial biomass (mg)	67.8±4^{a}	72.5±1.47^{a}	73.4±2.23^{a}	68.5±4.1^{a}
Final biomass (g)	146±12.9^{d}	440±39.5^{a}	240±22.7^{c}	317±64.2^{b}
Survival (%)	64.0±2.2^{d}	80.0±3.7^{a}	76.0±3.1^{c}	78.0±2.7^{b}
Muscle composition				
Moisture (%)	74.3±0.05^{a}	71.3±0.07^{d}	73.4±0.07^{ab}	71.6±0.06^{cd}
Crude protein (%)	50.4±0.06^{d}	56.3±0.08^{ab}	52.3±0.02^{c}	55.4±0.09^{b}
Fat (%)	18.6±0.02^{d}	22.5±0.03^{a}	19±0.09^{cd}	20.3±0.07^{b}
Ash (%)	12.6±0.02^{a}	10.7±0.09^{bc}	10.4±0.06^{c}	11.5±0.08^{b}
Carbohydrate (%)	3.35±0.04^{d}	5.76±0.04^{a}	4.42±0.1^{c}	5.23±0.01^{ab}

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same row indicate significant difference ($p<0.05$). Means with different lowercase superscripts in same rows indicate significant difference at $p < 0.05$. Control$_{PL}$= Basal diet (BD) devoid of probiotic, Y_{PL}= BD +*G. candidum*, B_{PL}= BD+ *B. cereus*, BY_{PL}=BD +consortia of both probiotics.

Table 1.4. Comparative effect of *G. candidum* and *B. cereus* supplemented microdiet on intestinal enzyme activities (U mg^{-1}) of *L. rohita* fry after 11 weeks feeding trial (mean ±SE, n=9)

	Groups			
	Control $_{PL}$	**Y_{PL}**	**B_{PL}**	**BY_{PL}**
Cellulase	0.38 ± 0^{d}	0.51 ± 0.01^{b}	0.45 ± 0.03^{c}	0.65 ± 0.02^{a}
Protease	0.08 ± 0.03^{d}	0.48 ± 0.06^{ab}	0.37 ± 0.02^{c}	0.58 ± 0.04^{a}
Amylase	0.46 ± 0.03^{c}	0.72 ± 0.05^{a}	0.51 ± 0.02^{bc}	0.66 ± 0.02^{ab}

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Average with different lowercase superscripts in same row show significant difference at $p < 0.05$. $Control_{PL}$= Basal diet (BD) devoid of probiotic, Y_{PL}= BD +*G. candidum*, B_{PL}= BD+ *B. cereus*, BY_{PL}=BD +consortia of both probiotics.

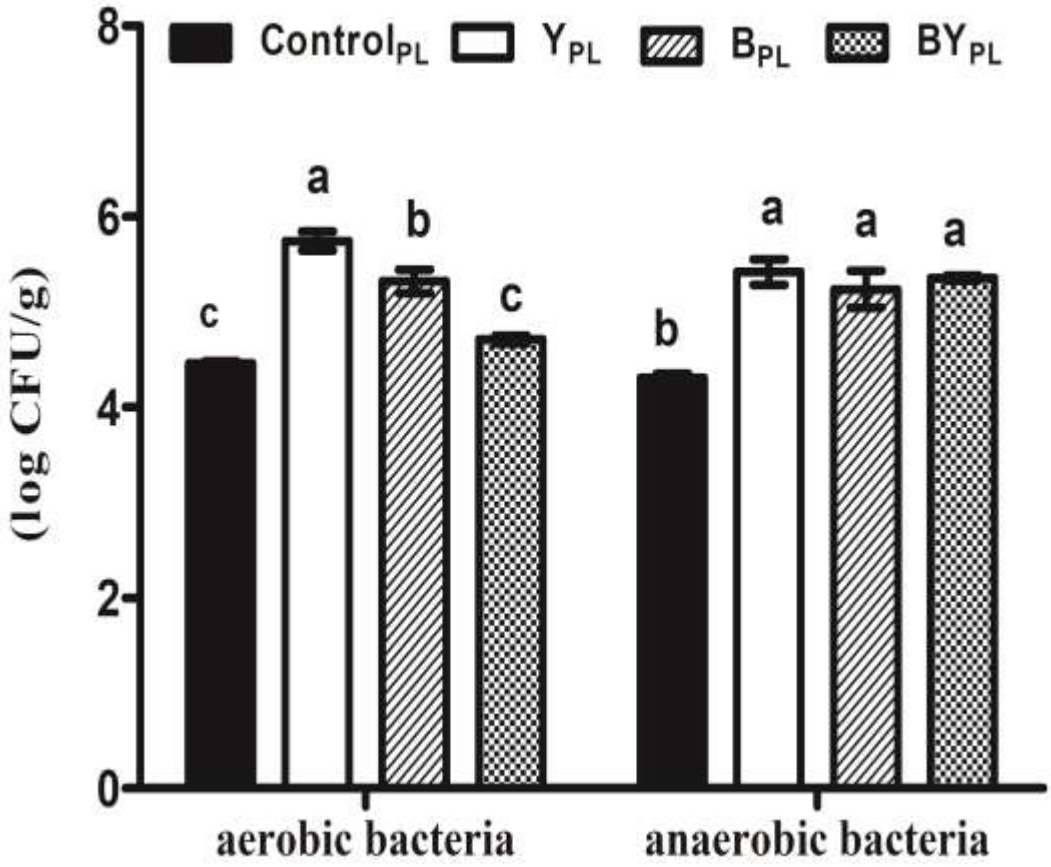

Fig. 1.1 Viable log count (CFU g^{-1}) isolated form GI tract of *L. rohita* after 11 weeks feeding of *G. candidum* and *B. cereus* supplemented microdiet. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between different groups. Means with different lowercase superscripts in same rows show significant difference at $p < 0.05$. Control $_{PL}$= Basal diet (BD) devoid of probiotic, Y_{PL}= BD +*G. candidum*, B_{PL}= BD+ *B. cereus*, BY_{PL}=BD +consortia of both probiotics.

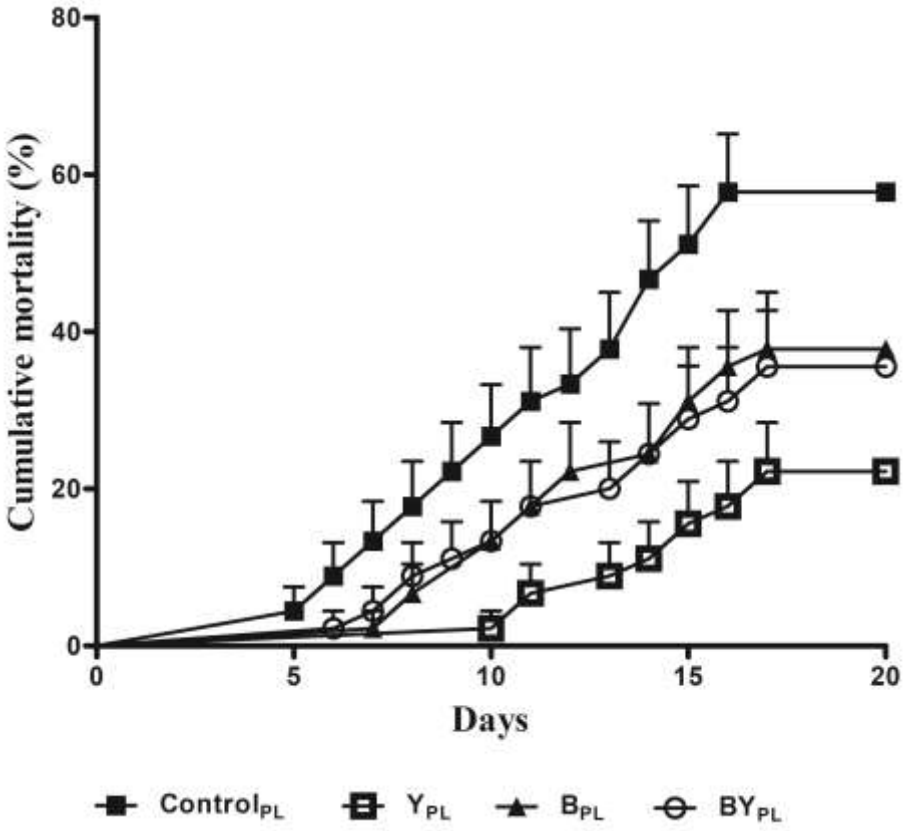

Fig. 1.2. Cumulative mortality (%) of *L. rohita* after challenged with *A. hydrophila* after 11 weeks feeding of *G. candidum* and *B. cereus* supplemented microdiet. Control $_{PL}$= Basal diet (BD) devoid of probiotic, Y_{PL}= BD +*G. candidum*, B_{PL}= BD+ *B. cereus*, BY_{PL}=BD +consortia of both probiotics (n=15).

Experiment 2

Comparative effects of dietary *Geotrichum candidum* and *Bacillus cereus* supplementation on *L. rohita* advanced fry

Experimental design and fish

About 2000 advanced fry of *L. rohita* (average body weight 2.4±0.17 g) were netted from rearing earthen pond and shifted in circular fiberglass tank (500 L capacity) having flow through system. Fish were acclimated for about 4 days and during this period they were provided with 35% crude protein diet. A completely randomize experiment was designed and conducted in replicate of three in outdoor facility during the months of July to September. For initiation of experiment, advanced fry average body weight 2.4±0.17 g were stocked in 12 separate fiberglass tanks (capacity; 150L; water level 100L), well equipped with aeration system at 1.5 g L^{-1} stocking density (about 150 fry/tank; Biomass; 356±20.9g). After shifting, they were again acclimated in their respective tanks for about two days. Afterwards, 12 tanks were divided into four groups (i.e. Control $_A$, Y_A, B_A and BY_A) and feeding trial was started by providing each group with their respective diet i.e., basal diet devoid of probiotic to control$_A$ group, while *G. candidum*, *B. cereus* and blend of both probiotics supplemented diets to Y_A, B_A and BY_A groups respectively.

Initially, respective diet was given to the experimental group four times a day (at 8:00 and 12.00 , 14:00 and 16:00) at the rate of 6% body weight, then fortnightly, feeding rate and ration as well as feed particle size was adjusted according to the size of fry/fingerling and end with twice a day (Table 1.1). Furthermore, 2 hr after feeding, unconsumed feed was collected for determination of feed conversion ratio (FCR).

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed intake (g)}}{\text{wet weight gain (g)}}$$

During feeding trial, water temperature (°C), DO (mg L^{-1}) and pH was noted twice a day (09:00 and 16:00) with multi-parameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA), while total ammonia was checked weekly using ammonia test kit (HI3824, ROMANIA) for freshwater. Water temperature of all tanks during the study period remained within range for *L. rohita* (25.8-27.6°C). Experiment was conducted in outdoor facility, therefore, initially it was 24.5±0.5°C, increased gradually and reached 26.0±0.3°C at the end of experiment. Furthermore, due to continuous aeration in each tank, DO level (5.7-6.4 mg L^{-1}) showed a small variation, while total NH_3 remained <0.25 mg L^{-1} and pH showed small fluctuation which was statistically insignificant. The experimental setup was in the same vicinity, under similar environmental conditions, thus no noticeable differences with respect to temperature, DO, pH and ammonia was observed within and between groups.

Growth performance

At the end of 11 weeks feeding trial, the fingerlings were starved for 24 hr before sampling. From each tank fish were carefully removed separately, weighed and counted in reckoning the average weight of individual fingerling. The percent weight gain (% WG), Specific growth rate (SGR), Average daily gain (ADG %), biomass and survival (%) of each group was assessed by using standard formulas as mentioned above.

Intestinal enzyme analysis

For intestinal enzyme analysis, 9 fish per tank (27 fish /treatment) were aseptically dissected by placing on ice bag and their GIT were carefully removed. The gut of 3 fish of same tank were pooled (3sample/tank or 9sample/treatment), snap-frozen in liquid nitrogen and

immediately stored at -20˚C for determination of digestive enzyme activities. For quantitative enzyme assay, gut sample (1g) was homogenized in 10 mL chilled buffered saline as described in previous experiment. The obtained mixture was centrifuged at 4500 rpm for 15min, 4ºC (Kokusan, H-103RS, Ogawa Seiki Co., LTD, Tokyo, Japan) and clear supernatant of enzyme solution was collected and stored at -4ºC until further analysis.

The intestinal protease, amylase and cellulose activities, in the control and probiotic fed groups were determined by adopting similar methods as described in above section.

Isolation of gastrointestinal bacterial flora

For the isolation of bacterial flora from gut, 9 fingerlings per tank (27 fingerlings / treatment) were aseptically dissected using standard protocol. To get enough sample, the GI tract of 3 fingerlings from each tank were pooled and similar method as mentioned above was used for isolation and counting of bacterial flora .

Muscle composition

For proximate composition of muscle (crude protein, crude fats, ash content and moisture) in the control and probiotic fed groups, 30 fingerlings per tank (n=90/ treatment) were collected at random. To acquire more sample, carcass of ten fingerlings were pooled and analyzed while following standard procedure (AOAC, 2000) as mentioned in previous section.

Evaluation of hematological indices

For complete blood count (CBC), about 9 fingerlings per tank were captured, anesthetized with MS-222 (0.10 g L^{-1} buffered with sodium bicarbonate) and blood was drawn from caudal vein by using 1 mL syringe and collected in purple top K2 EDTA tubes (LiuyangSanli Medical Technology Development Co. Ltd). In order to collect enough sample for

evaluation, blood of 3 fish from each tank was collected in the same EDTA tubes (3 samples/tank, or 9 samples/ group). CBC leukocytes (WBCs), erythrocytes (RBCs), hematocrit Value (Hct %) and hemoglobin (Hb) was determined by using hematology analyzer (Japan, SysmaxXS 800i), while RBC, Hb and Hct values were used to estimate the Mean corpuscular hemoglobin $(MCH) = [(10 \times \mathrm{Hb}) \div \mathrm{RBC}]$. Mean corpuscular hemoglobin concentration (MCHC) = [Hb / HCT (%)] and Mean corpuscular Volume (MCV) = [(HCT (%) ×10)/RBC].

Immunological parameters

For the analysis of immunological indices, 20 fingerlings from each tank (60 fingerling / group) were anesthetized with MS-222 (0.10 gL^{-1} buffered with sodium bicarbonate) and blood of each fish was taken from caudal vein through 01 mL heparinized syringe. For obtaining enough sample, blood of 5 fingerlings from same tank was collected in the same blood collecting tube. Blood samples were centrifuged (Kokusan, H-103RS, Ogawa Seiki Co., LTD, Tokyo, Japan) at 3000 rpm for 5 min. and blood serum was decanted in separate Eppendorf and saved at 4˚C for further analysis of Aspartate Aminotransferase (AST), Total Serum Proteins, Immunoglobulins (IgM) and Lysozyme enzymes activity, while fresh heparinized blood was used for phagocytic activity and respiratory burst activity.

AST activity

AST activity was estimated by assaying the rate of NADH oxidation, which is proportional to decrease in absorbance at 340 nm over time. AST / GOT kit (AMEDA Laborodiagnostik GmbH Graz Austria) was used for conducting AST assay. Briefly, 100 µL serum was taken in 2 mL Eppendorf tube and mixed with reagent A (910 µl) and reagent B (91 µl). The mixture was mixed gently and incubated for 1 min at 30˚C. UV-Visible

spectrophotometer (Agilent, 8453, USA) was adjusted at λ 340 nm and the absorbance was noted thrice at 1 min interval. The AST activity is expressed as U L^{-1}.

Total protein content

Lowry et al. (1951) method adopted by Ullah et al.(2018) used for estimation of total serum protein. For preparing standard curve, bovine serum albumin (BSA) stock solution (w/v 5 mg BSA and 5mL distilled) was prepared and used as standard protein, i.e., From stock solution, different concentration of protein (BSA) was prepared i.e., 0.05 to 1 mg/ ml. Each dilution was well mixed after adding of 1 mL alkaline copper sulfate reagent and tubes were incubated at room temperature for 10 min. Afterword 200 µl Folin-Ciocalteau solution was added in each tube and again incubated at room temperature for 30 min. Finally, absorbance was measured by using UV- visible spectrophotometer set at λ 660 nm. A standard calibration curve was plotted by taking absorption (OD) at y-axis and BSA concentrations at X-axis. The absorbance of unknown samples was noted and their concentrations were determined with the help of standard curve.

Immunoglobulin (IgM) analysis

For serum IgM, analysis, Anderson and Siwicki (1995) method, described in our publication (Ullah et al., 2018) was adopted. Briefly, through precipitation IgM was separated from serum, by mixing 100 µL serum with 100 µL, 12% polyethylene glycol. The solution was mixed well and kept in shaker incubator (Model ISS Innova 43) and incubated at room temperature with continuous shaking for 120 min. Subsequently, the solution was centrifuged for 10 min at 7000 × g. The supernatant was collected and absorbance of supernatant containing protein content as well as the serum was checked separately by using UV-visible

spectrophotometer set at λ 660 nm. Total IgM was calculated by subtracting the protein content of supernatant from total protein content in serum.

Lysozyme activity

Serum Lysozyme activity of different groups of fingerlings was estimated by adopting Anderson and Siwicki (1995) method reported by Ullah et al. (2018). Briefly, *Micrococcus lysodeikticus,* 750 µg mL^{-1} (Sigma, St Louis, MO, USA) suspended in PBS (pH 6.2) was purchased from local supplier. For conducting assay, 900 µL was taken in fresh glass test tube and mixed with in 100µL serum. The tubes were shaken well to mix the *M. lysodeikticus* and serum. The rate of change of absorbance after 1 min intervals for 10 min was checked with the help of UV- visible spectrophotometer (Agilent, 8453, USA) set at 450 nm. For calculating the lysozyme activity, lysozyme from chicken egg white (lyophilized powder, ≥40,000 units /mg protein, protein ≥90 %, Sigma-Aldrich) was used as standard.

Phagocytic activity

Nitroblue-tetrazolium dye (NBT) method, described by Anderson and Siwicki (1995) was used for the determination of free oxygen radicals produced from the phagocytes present in blood. Briefly, for the determination of Phagocytic activity, *S. aureus* (Sigma, St Louis, MO, USA) was purchased from local supplier. The assay was conducted by pipetting 100µL heparinized blood in a microwell plate, subsequently added 100µL of killed *S. aureus* 1×10^7 cells along with 100µL PBS (pH 7.2) in each well. After well mixing, the solution was left for 30 min at room temperature. Afterward, 5µL solution of each well was used for making smear on a clean glass slide. The smear was air dried, and fixed in 95% ethanol for 5 min and again air dried. Two smears per samples were prepared and stained with 7% Giemsa stain. The prepared

slides were observed under microscope with oil-immersion and enumerated phagocytes, phagocytic cells with ingested bacteria. For determination of Phagocytic activation (PA) and Phagocytic index (PI), 100 phagocytes per slide were observed and following standard formulas were adopted

$$PA = \frac{\text{Total number of phagocytes with engulfed bacteria}}{\text{Total number of phagocytes observed}} \times 100$$

$$PI = \frac{\text{No. of engulfed bacteria}}{\text{Total umber of counted phagocytes}}$$

Respiratory burst activity

Nitrobluetetrazolium (NBT) dye method explained by Anderson and Siwicki (1995), was adopted to determine the free oxygen radicals in the blood from the phagocytes. Briefly, l00 µL of blood was taken 2 mL Eppendorf tube, mixed with 0.2% 0.1 mL of NBT solution and left for 30 min at room temperature. Afterward, 50 µL of NBT-blood cell suspension and I mL N, N dimethyl formamide were mixed and centrifuged at 3000g for 5 min. The supernatant was collected in and absorbance was checked by using through UV-visible spectrophotometer set at 540 nm. For calculation, the absorbance of blank, which contained same components except blood that was replaced with distilled water, was also noted.

Challenge test

At the end of study, 30 fish per treatment (10 fish from each aquarium) were equally distributed in 12 glass aquaria well-fitted with heaters and aerators. Temperature and DO level was maintained at 26.7°C and 5.7 mg L^{-1} respectively. After three day acclimatization, freshly cultured pathogenic strain of *A. hydrophila* (ATCC49140) taken from an infected *L. rohita* at

National Veterinary Laboratory Islamabad (Ali et al., 2016) was intraperitoneally injected with 100µL suspension @1.7 $\times 10^4$ CFU/g fish as reported by Sahu et al. (2011). One aquarium was kept as control and fingerlings were injected with sterile (100µL) PBS only. Fish were fed basal diet twice a day and kept for twenty days. Fish was observed closely for disease symptoms and mortality was recorded. To verify that the mortality was due to introduced pathogenic bacteria, autopsy of the dead or moribund fish was carried out and pathogenic bacteria *A. hydrophila,* was re-isolated from the kidney and skin infected. The mortality and survival rate after challenged period was assessed according to following formula mentioned before.

Statistical analysis

All results are shown as mean ±SD. Variance of homogeneity and data normality was performed by using Bartlett and Shapiro-Wilk's tests before running any statistical analysis. Significant differences in growth performance, survival, enzymatic activity, bacterial count, hematology and immunological data among experimental treatments was evaluated by using one–way ANOVA followed by LSD test at the percent level of significance using SPSS version 20. Log transformation was performed for bacterial count data. GraphPad Prism 5 was used for graphical representation of the data.

Experiment 2

Results

Growth and survival

Yeast and bacterial based probiotics *G. candidum* QAUGC-01 and *B. cereus* ,showed significant positive effect on growth of advanced fry of *L. rohita* (Table 1.1.1). One way ANOVA indicated significant difference in final weight (n=3, ANOVA, $F_{3,12}$=84.1, p<0.001), weight gain percent (n=3, ANOVA, $F_{3,12}$=59.3, p<0.001), average daily gain (n=3, ANOVA, $F_{3,12}$=89, p<0.001), final biomass (n=3, ANOVA, $F_{3,12}$=105, p<0.001) and SGR (n=3, ANOVA, $F_{3,12}$=55.5, p<0.001) among groups. Post hoc LSD test revealed better growth performance of probiotic fed groups (Y_A, B_A and BY_A) as compared to the control group (control $_A$). Furthermore all possible pair-wise comparisons indicated highest weight gain, final biomass, ADG and SGR of Y_A group fed *G. candidum* followed by BY_A (fed consortia of both probiotics) and B_A group (fed B. *cereus* alone), Similarly, the highest survival (98%) was observed in a Y_A group followed by BY_A (95%), and B_A group (91%) while control $_A$ showed lowest survival (86%). FCR showed significant differences (n=3, ANOVA, $F_{3,\ 12}$=151, p<0.001) among all treatment groups and lowest ratio was observed in $Y_A < BY_A < B_A <$control$_A$.

Proximate analysis

Yeast and bacterial based probiotics also showed significant effect on the proximate composition of *L. rohita* (Table 1.1.1). One way ANOVA revealed significant difference in moisture (n=9, ANOVA, $F_{9,\ 36}$=198,p=0.001), crude protein (n=9, ANOVA, $F_{9,\ 36}$=495,p=0.001), fat (n=9, ANOVA, $F_{9,\ 36}$=196,p=0.001), ash (n=9, ANOVA, $F_{9,\ 36}$=55.5,p=0.001) and

carbohydrate (n=9, ANOVA, $F_{9, 36}$=30.5, p=0.001) content of all treatment groups ($control_A$, Y_A B_A and BY_A). The pair-wise comparison among groups indicated higher contents of crude protein, fat and carbohydrate in groups fed diet supplemented with probiotic as compared to control group. Furthermore, Y_A group showed the highest crude protein, fat and carbohydrate contents followed by BY_A and B_A groups (Y_A> BY_A>B_A). However higher moisture and ash was found in $control_A$ group followed by B_A group.

Intestinal enzymes

Yeast and bacterial based probiotics also showed considerable effect on the activities of intestinal enzymes of *L. rohita* (Table 1.1.2). One way ANOVA revealed significant difference in protease (n=9, ANOVA, $F_{9, 36}$=210, P< 0.001), amylase (n=9, ANOVA, $F_{9, 36}$=88.6, P< 0.001) and cellulase (n=9, ANOVA, $F_{9, 36}$=217, P< 0.001) in all treatment groups ($control_A$, Y_A, B_A and BY_A). The pair-wise comparison among groups indicated higher specific activity of all studied enzymes in probiotic fed groups as compared to control group. Furthermore, Y_A group showed the highest activities of all enzymes followed by BY_A and B_A groups.

Moreover, comparison of protease, amylase and cellulase activity in each group indicated that all three enzymes had statistically comparable activities in control group (n=9, ANOVA, $F_{2,27}$=1.29, P<0.294, Fig 1.1.1a) while probiotic fed groups showed significant difference i.e., Y_A (n=9, ANOVA, $F_{2,27}$=77.6, P< 0.001, Fig 1.1.1b), B_A (n=9, ANOVA, $F_{2,27}$= 204, P< 0.001, Fig 1.1.1c) and BY_A (n=9, ANOVA, $F_{2,27}$=111, P< 0.001, Fig 1.1.1d). Furthermore in all groups, activities of proteases and amylases were statistically comparable and significantly higher than the activity of cellulase.

Gastrointestinal microflora

Yeast and bacterial based probiotic supplementation also influenced the aerobic and anaerobic bacteria count (log CFU/g) found in GI tract of *L. rohita*. One way ANOVA revealed that aerobic (n=3, ANOVA, $F_{3, 12}$=103, P< 0.001, Fig. 1.1.2) and anaerobic bacterial counts (n=3, ANOVA, $F_{3, 12}$=73.8, P< 0.001, Fig. 1.1.2) were considerably different in all treatment groups (control $_A$, Y_A, B_A and BY_A). The post hoc LSD pairwise comparison among all groups indicated an increase in total number of aerobic and anaerobic bacterial count in all probiotic fed groups i.e. Y_A, B_A and BY_A as compared to the control (Control $_A$) group. Furthermore, among probiotic groups, higher bacterial count (both aerobic and anaerobic) was found in GI tract of Y_A followed by BY_A and then B_A group of fish (Y_A> BY_A> B_A).

Hematological indices

Hematological parameters also showed variation in response to probiotic supplemented diets (Table 1.1.3). One way ANOVA indicated significant differences (P<0.05) in the hematological values i.e. erythrocytes (n=9, ANOVA, $F_{9, 36}$=116, P< 0.001), leukocytes (n=9, ANOVA, $F_{9, 36}$=377, P< 0.001), hemoglobin (n=9, ANOVA, $F_{9, 36}$=177, P< 0.001), hematocrit (n=9, ANOVA, $F_{9, 36}$=1553, P< 0.001), MCHC (n=9, ANOVA, $F_{9, 36}$=3.50, P< 0.001), MCV (n=9, ANOVA, $F_{9, 36}$=12.5, P< 0.001) and MCH (n=9, ANOVA, $F_{9, 36}$=9.85, P< 0.001) among groups. Post hoc LSD test revealed improved hematological status of probiotic supplemented groups (Y_A, B_A and BY_A) as compared to control $_A$. Furthermore all possible pair-wise comparisons indicated higher values of erythrocytes, leukocytes, hemoglobin and hematocrit in Y_A group followed by BY_A and then B_A group (Y_A> BY_A> B_A, Table 1.1.3). However the values of MCV and MCH were found higher in control (Control $_A$) group as compared to probiotic

supplemented groups (BY_A, B_A and Y_A). Moreover, B_A and BY_A showed statistically similar values for MCV while MCH values were almost similar in Y_A and B_A group.

Aspartate aminotransferase activity (AST)

AST was affected in response to the dietary treatment. One way ANOVA showed significant difference (n=9, ANOVA, $F_{9, 36}$=131, P< 0.001, Fig 1.1.3) in all treatment groups. Post hoc LSD test showed a decreasing trend in probiotic supplemented groups as compared to the $control_A$ group. The lowest values was found in Y_A followed by BY_A and then in B_A group (Y_A< BY_A< B_A).

Immunological indices

Results showed significant positive effects of yeast and bacterial based probiotic supplementation on the innate immune status of *L. rohita* i.e. plasma protein, immunoglobulins, lysozyme, phagocytic activity and respiratory bursts activity. One way ANOVA showed significant difference in plasma protein level (n=9, ANOVA, $F_{9, 36}$=347, P< 0.001, Table 1.1.4), immunoglobulins (n=9, ANOVA, $F_{9, 36}$=133, P< 0.001, Table 1.1.4), serum lysozyme activity (n=9, ANOVA, $F_{9, 36}$=135, P< 0.001, Table 1.1.4), phagocytic activity (n=3, ANOVA, $F_{3, 12}$=30.9, P< 0.001, Fig 1.1.4 a), phagocytic index (n=3, ANOVA, $F_{3, 12}$=44.3, P< 0.001, Fig 1.1.4b) and respiratory burst activity (n=9, ANOVA, $F_{9, 36}$=171, P< 0.001, Fig 1.1.5) among all treatment groups. Post hoc LSD test revealed that plasma protein, immunoglobulins, serum lysozyme activity, phagocytic activity, phagocytic index and respiratory burst activity was higher in probiotic fed groups as compared to $control_A$ group. Furthermore all possible pairwise comparison showed the improved immune status i.e. plasma protein, immunoglobulins, serum lysozyme activity, phagocytic activity, phagocytic index and respiratory burst activity in Y_A followed by BY_A and then B_A group of fish (Table 1.1.4 and Fig 1.1.4 and 1.1.5).

Challenge test

At the end of feeding trial, fish exposed to *A. hydrophila* showed improved survival in probiotic fed groups (Fig. 1.1.6). One way ANOVA revealed significant difference in mortality (n=3, ANOVA, $F_{3, 12}$=62.2, p<0.001, Fig. 1.1.6) and relative percent survival (n=3, ANOVA, $F_{3, 12}$=41.3, p<0.001, Fig. 1.1.6) of *L. rohita*. Post hoc LSD test showed the higher mortality in $control_A$ group as compared to the probiotic fed groups (Y_A, B_A and BY_A). However among probiotic fed groups, Y_A (16%) showed lowest mortality followed by BY_A (26%) and then B_A (43%) group of fish. Conversely, for relative percent survival, lowest value was recorded in $control_A$ group as compared to the probiotic supplemented fish. Among probiotics treatments Y_A showed highest RPS (78%) followed by BY_A (65%) and then B_A (43%) group of fish.

Table 1.1.1. Growth performance (n=3) and muscle composition (n=9) of advanced fry of *L. rohita* fed *G. candidum* and *B. cereus* supplemented diet after 11 weeks feeding trial

	Groups				Pooled SEM	F value	p-value
	Control $_A$	Y_A	B_A	BY_A			
IBW (g)	2.29	2.56	2.28	2.35	0.05	6.76	0.014
FBW (g)	10.1^{d}	17.2^{a}	12.7^{c}	14.6^{b}	0.33	84.1	0.001
WG (g)	7.84^{d}	14.7^{a}	10.4^{c}	12.2^{b}	0.30	89.0	0.001
WG (%)	342^{d}	573^{a}	456^{c}	520^{b}	12.9	59.3	0.001
^{1}ADG (%)	10.3^{d}	19.3^{a}	13.7^{c}	16.1^{b}	0.41	89.1	0.001
^{2}SGR (% body weight/day)	1.95^{d}	2.51^{a}	2.25^{c}	2.40^{b}	0.04	55.5	0.001
Initial biomass (g)	344	384	342	353	7.54	6.76	0.014
Final biomass (g)	1318^{d}	2539^{a}	1737^{c}	2088^{b}	50.4	105	0.001
Survival (%)	86.6^{d}	98.0^{a}	91.1^{bc}	95.1^{ab}	0.65	56.1	0.001
^{3}FCR	3.19^{a}	1.67^{a}	2.68^{c}	2.33^{b}	0.05	151	0.001
Muscle composition							
Moisture (%)	78.4^{a}	74.01^{d}	76.5^{b}	76.4^{c}	0.130	198	0.001
Protein (%)	51.1^{d}	58.1^{a}	53.8^{c}	56.1^{b}	0.133	495	0.001
Fat (%)	9.02^{d}	11.6^{a}	10.2^{c}	11.1^{ab}	0.176	196	0.001
Ash (%)	9.08^{a}	6.53^{d}	7.72^{b}	7.02^{c}	0.147	55.5	0.001
Carbohydrate (%)	3.03^{d}	6.11^{a}	4.42^{c}	5.21^{b}	0.23	30.5	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same rows indicate significant difference at $p < 0.05$. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A =BD +consortia of both probiotics.^{1}ADG=average daily gain, ^{2}SGR= specific growth rate, ^{3}FCR= feed conversion ratio.

Table 1.1.2. Intestinal enzyme activities (U mg^{-1}) of advanced fry of *L. rohita* fed *G. candidum* and *B. cereus* supplemented diets after 11 weeks feeding trial (n=9)

Intestinal enzymes (U mg^{-1})	**Groups**				**Pooled SEM**	**F value**	**p-value**
	Control $_A$	**Y_A**	**B_A**	**BY_A**			
Protease	0.32^d	2.03^a	1.36^c	1.69^b	0.06	210	0.001
Amylase	0.31^d	2.16^a	1.34^c	1.74^b	0.09	88.6	0.001
Cellulase	0.17^d	1.18^a	0.26^c	0.74^b	0.03	217	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same rows indicate significant difference at $p < 0.05$. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A =BD +consortia of both probiotics.

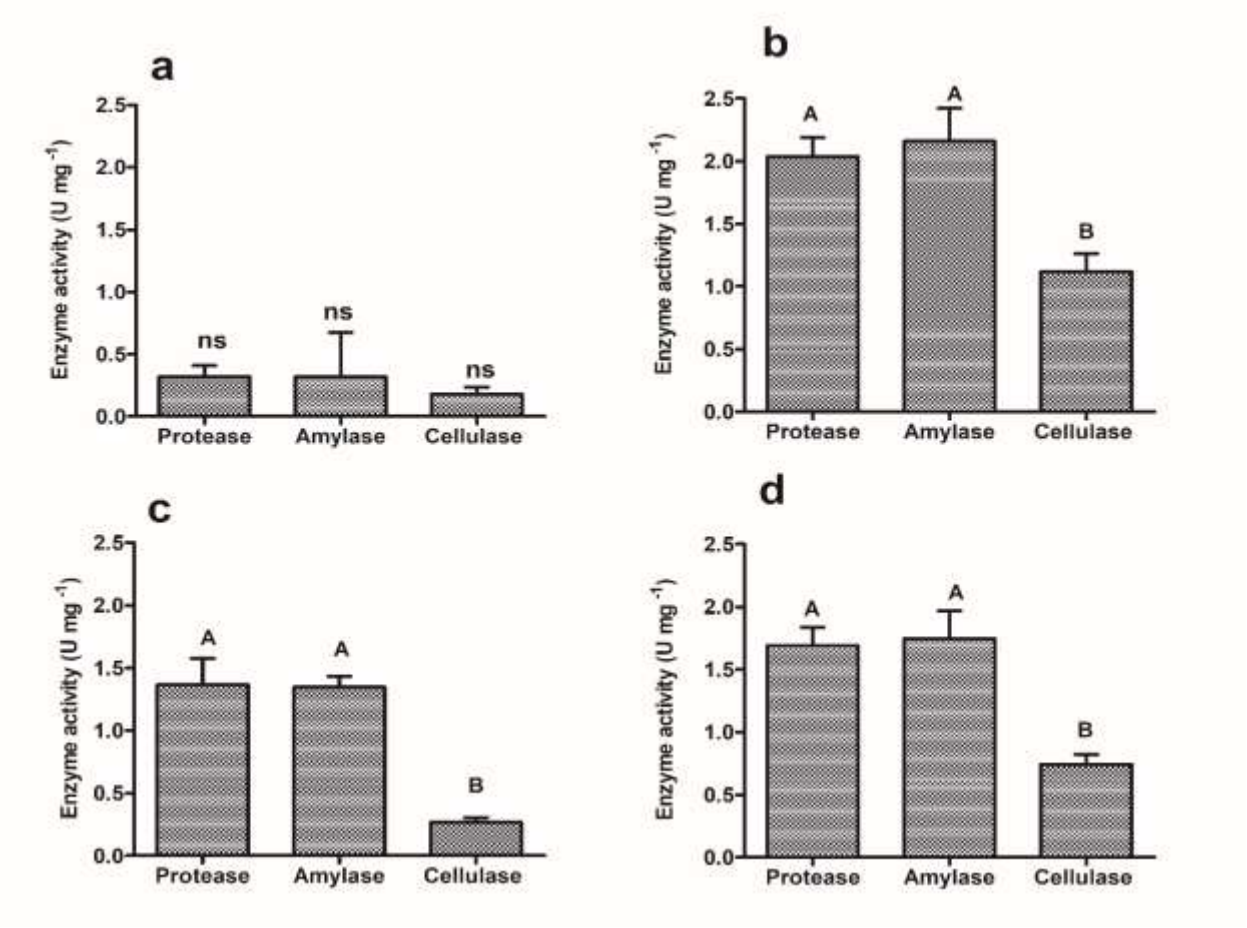

Fig. 1.1.1 Intestinal enzymes activities (U mg^{-1} protein) of advanced fry of *L. rohita* after 11 weeks feeding *G. candidum* and *B. cereus* supplemented diet. (a) group of fingerlings fed basal diet (Control $_A$) devoid of any proiotic supplement. (b) group of fish fed *G. candidum* supplemented diet (Y_A), (c) group of fish fed *B. cereus* supplemented diet (B_A) (d) group of fish fedconsortia of both prbiotic (BY_A). The above bars shows the values as mean±SD (n=9), ANOVA followed by LSD post hoc test showed pairwise comparsion between groups with respect to intestinal enzymes. Bar with different uppercase letters are considerably different at $p < 0.05$, ns=non-sigificant. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A =BD +consortia of both probiotics.

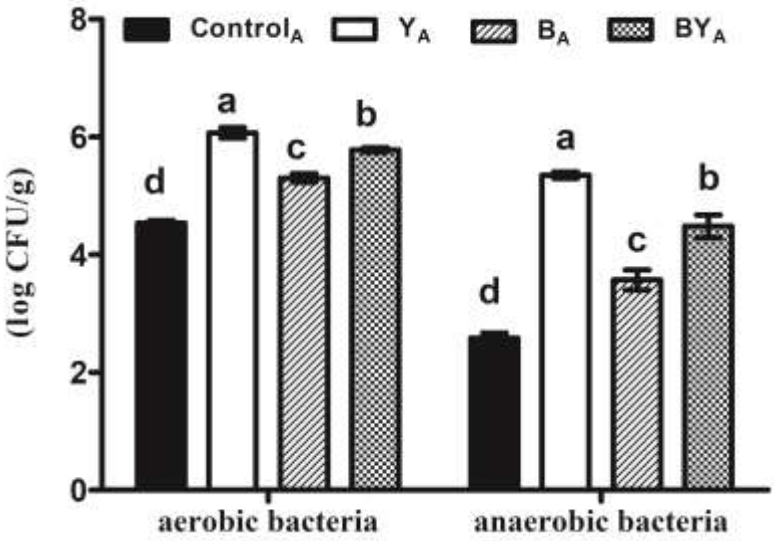

Fig. 1.1.2 Viable log counts (CFU g^{-1}) of aerobic and anaerobic bacteria separated from the GI tract of advanced fry of *L. rohita* after 11 weeks feeding *G. candidum* and *B. cereus* supplemented diet. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between different groups. Bars with different lowercase letters show significant difference ($p < 0.05$). Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD + *G. candidum*, B_A= BD+ *B. cereus*, BY_A=BD +consortia of both probiotics.

Table 1.1.3. Hematological indices of advanced fry of *L. rohita* fed *G. candidum* and *B. cereus* supplemented diet after 11 weeks feeding trial (n=9)

	Groups				Pooled SEM	F value	p-value
	Control $_A$	Y_A	B_A	BY_A			
RBC (10^6 µL)	0.79^d	2.39^a	1.34^c	1.65^b	0.07	116	0.001
Hb (g dL^{-1})	3.45^d	7.75^a	4.47^c	6.30^b	0.14	117	0.001
WBC (10^3 µL)	84.1^d	108^a	93.2^c	101^b	0.53	377	0.001
HCT (%)	15.1^d	31.9^a	20.5^c	26.1^b	0.18	1553	0.001
MCV (fL or 10^{-15})	192^a	134^d	156^{bc}	159^b	6.80	12.5	0.001
MCH (pg)	43.5^a	32.6^{cd}	33.9^c	38.4^b	1.57	9.85	0.001
MCHC (g/dL)	22.7^b	24.2^a	21.7^c	24.1^a	0.63	3.50	0.03

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same rows indicate significant difference at $p < 0.05$. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A=BD +consortia of both probiotics.

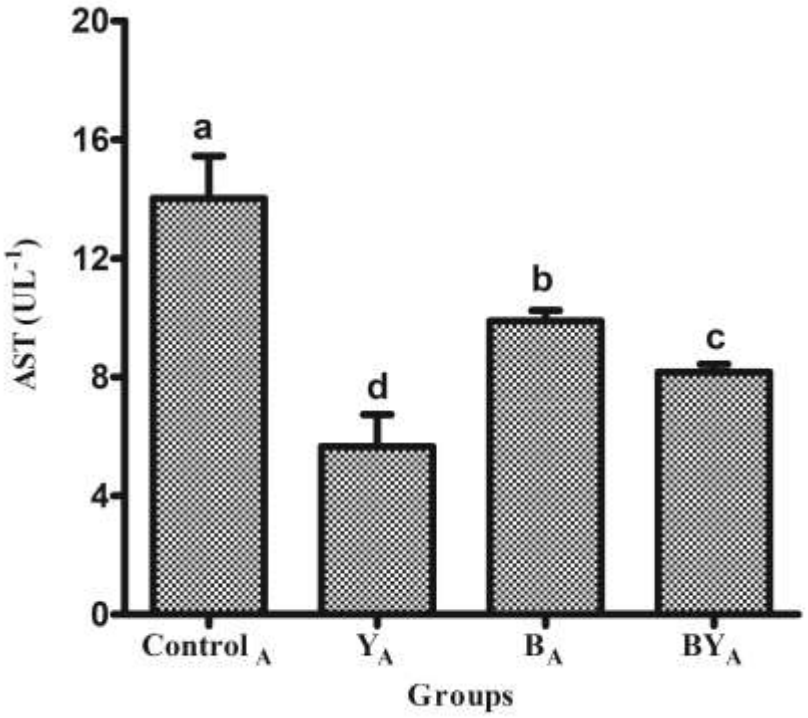

Fig. 1.1.3 Serum aspartate aminotransferase (AST) activity of advanced fry of *L. rohita* after feeding *G. candidum* and *B. cereus* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between different groups. Bars with different lowercase letters are considerably different at $p < 0.05$. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A=BD +consortia of both probiotics.

Table 1.1.4. Immunological indices of advanced fry of *L. rohita* fed *G. candidum* and *B. cereus* supplemented diet after 11 weeks feeding trial (n=9)

	Groups				Pooled SEM	F value	p-value
	Control $_A$	Y_A	B_A	BY_A			
Total serum protein (mg mL^{-1})	16.6^d	21.4^a	18.4^c	19.5^b	0.11	347	0.001
Immunoglobulin (mg mL^{-1})	4.06^d	10.3^a	6.06^c	8.26^b	0.24	133	0.001
Lysozyme activity (µg mL^{-1})	3.77^d	8.93^a	5.92^c	7.10^b	0.19	135	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same rows show significant difference at $p < 0.05$. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A =BD +consortia of both probiotics.

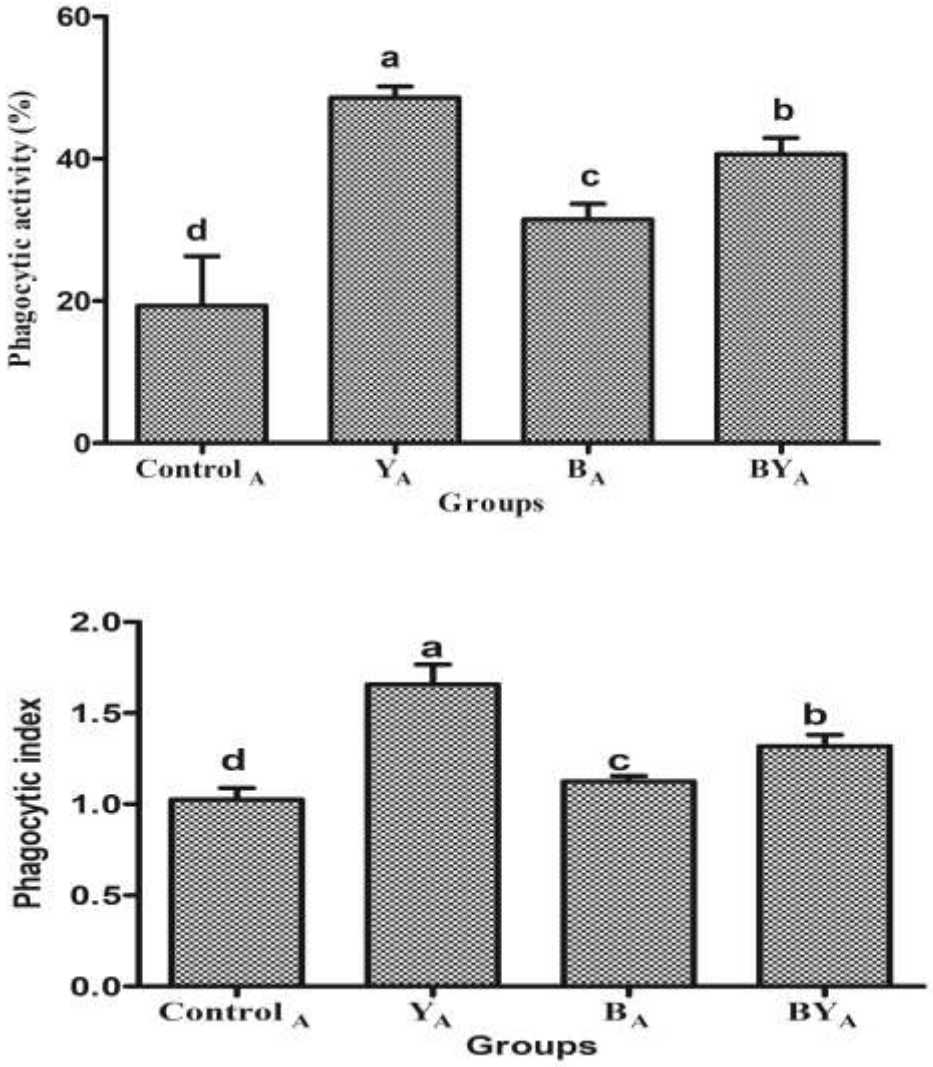

Fig. 1.1.4 (a) Phagocytic activity and (b) phagocytic index of advanced fry of *L. rohita* when fed G. *candidum* and *B. cereus* as dietary supplement for 11 weeks. Values expressed as mean (n=3). ANOVA followed by LSD post hoc test represent comparison between different groups. Bars with different lowercase letters show significant difference ($P < 0.05$). Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A=BD +consortia of both probiotics.

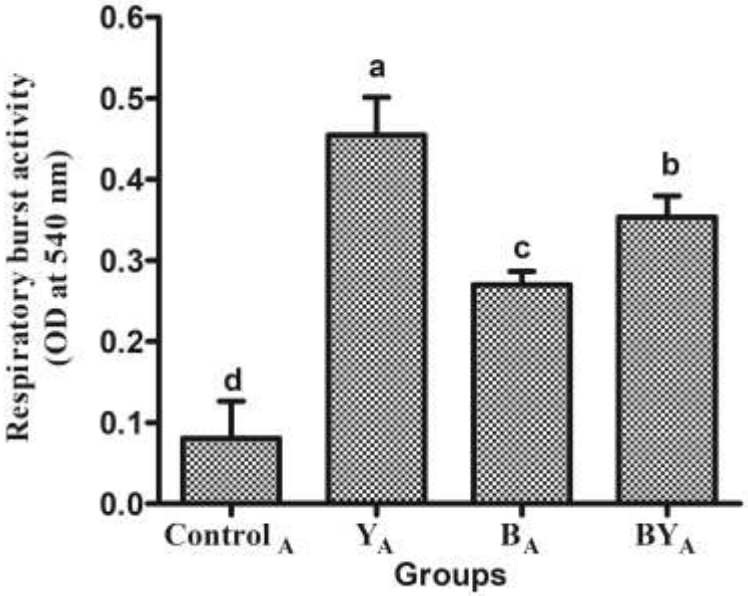

Fig. 1.1.5 Respiratory burst activity of *L. rohita* fed *G. candidum* and *B. cereus* supplemented diet for 11 weeks. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between different groups. Bars with different lowercase letters show considerable difference ($P < 0.05$). Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A=BD +consortia of both probiotics.

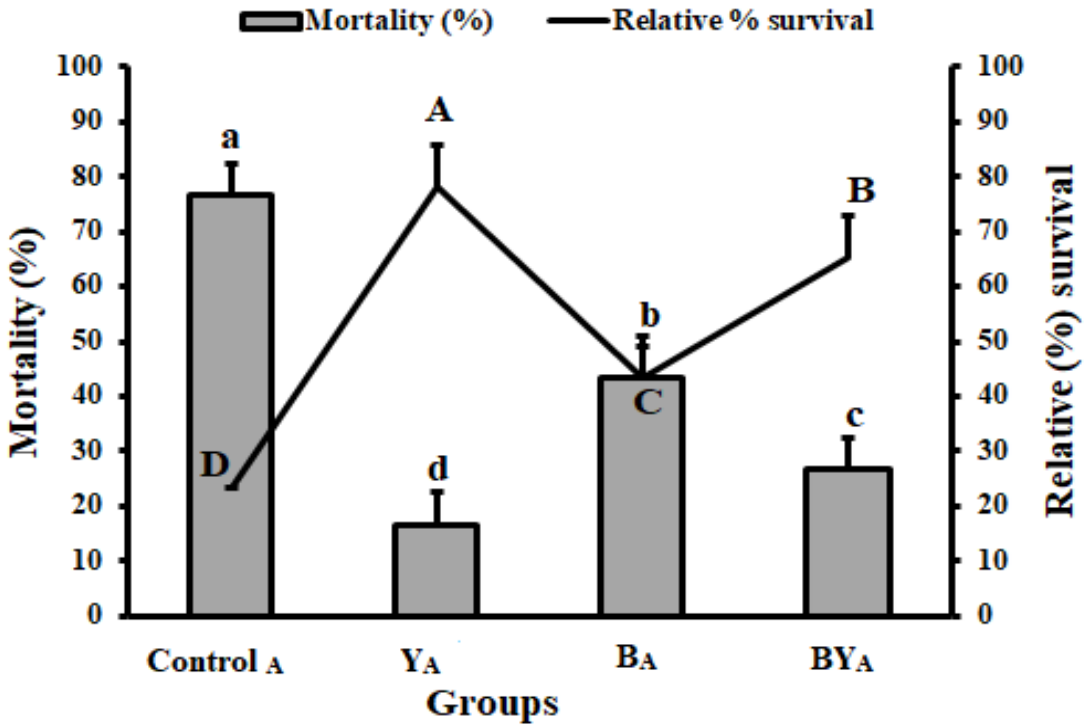

Fig. 1.1.6 Mortality and relative percent survival of advanced fry of *L. rohita* after challenged with *A. hydrophila*. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between different groups. Averages with different lowercase and uppercase letters show significant difference ($p < 0.05$) for mortality and relative percent survival respectively. Control $_A$= Basal diet (BD) devoid of probiotic, Y_A= BD +*G. candidum*, B_A= BD+ *B. cereus*, BY_A=BD +consortia of both probiotics.

Discussion

The efficacy of probiotics is diverse, as several microbes harmful for one, could be beneficial for other aquatic species as probiotic (Hai, 2015). Hence, candidate strain of microorganism as a probiotic require to be wisely chosen for acquiring maximum beneficial effects. In current part of study, we selected locally isolated strain of bacteria (*B. cereus*) and yeast (*G. candidum* QAUGC-01) prokaryotic and eukaryotic in nature, and used as probiotics for rearing of two life stages i.e., postlarvae and advanced fry of *L. rohita*. These two life stages were selected on the basis of their digestive capability and gut microbiota. Larvae of most of fish including *L. rohita* are small in size with somewhat sterile gut and inept ability to digest most of nutrient efficiently (Kolkovski, 2001), while fry and fingerlings have autochthonous and allochthonous gut microbiota originated from the microflora of live feed and rearing water, which may have inefficient digestion and nutrient assimilation and increased incidence of infectious and autoimmune related diseases due to exposure of naturally occurring pathogens in aquatic environment (Ringø and Birkbeck, 1999; Borch et al., 2015).

Here we observed probiotic specific positive effect on the survival, growth performance, muscle composition, gut microbial load and immunity of both life stages of *L. rohita* after eleven weeks rearing of on *G. candidum* and *B. cereus* enriched microdiet. All groups of same set of experiment were maintained under similar conditions and on same feeding strategy (feeding frequency, ration and particle size) except addition of probiotic in experimental diets. It seems that beneficial effects observed in experimental groups are attributed by the probiotics.

Our results were comparable with investigations and reports of other researchers about the beneficial effect of probiotic supplemented diet on various aspect of different life stages (including swim up fry, fry /fingerlings of fish) of different finfish and shellfish species e.g.,

common carp (Gupta et al., 2014), Giant freshwater prawn *Macrobrachium rosenbergii* (Gupta and Dhawan, 2012; Gupta et al., 2014), Gilthead sea bream, *Sparus aurata* (Suzer et al., 2008), juvenile Nile tilapia *Oreochromis niloticus* (Garcia-Marengoni et al., 2015; Wang et al., 2017), *Cirrhinus mrigala* (Ullah et al., 2018), and suggested the possible ecofriendly way to overwhelm constrain of high mortality and reduced growth rate especially during early life stages fish.

The efficacy of applied probiotic depends on many factors related to host (species, size, body temperature, enzymatic potential, redox potentials etc.) and microbes (strain, species, source, dose /concentration and way of application, mode of action) (Ibrahem, 2015; Ridha and Azad, 2016). Here, both potential probiotics were used for the same species of similar size, in similar concentration, i.e., 1×10^9 CFU/g feed under similar culture conditions, but *G. candidum* isolated from yogurt resulted the most pronounced positive effect on the survival (%) and growth rate of swim up fry and advanced fry in contrast to *B. cereus* (bacterial strain) isolated from the GI tract of an adult *L. rohita* free from disease (Experiment 1, Table 1.3; Experiment 2, Table 1.1.1). It seems that yeast based probiotic has a better ability to adhere and colonize in the sterile gut of postlarvae as well as has a capacity to compete the autochthonous and allochthonous gut microbiota of advanced fry of *L. rohita*. Limited literature is available on both of these probiotic strains for comparing our results.

B. cereus is also being used as probiotic in livestock but limited literature reveals its importance in aquaculture. A few investigators reported the positive impact of this probiotic on the survival and growth of fish (*Dentexdentex,* Hidalgo et al., 2006; *Trichogaster trichopterus*, Subharanjani et al., 2015) while others did not observe any significant (Tilapia: Garcia-Marengoni et al., 2015; postlarvae of shrimp, Vidal et al., 2018).

Though beneficial effects of some species /strain of yeast on survival, growth, immune response, disease resistance of fish is well documented and extensive literature address the importance *S. cerevisiae* and *Debaryomyces hansenii* (Navarrete and Tovar-Ramírez, 2014) in the nutrition and health of different fish species but limited literature is available on locally isolated *G. candidum* QAUGC-01. Previously, Ibrar et al.(2017) reported the positive effect of similar strain of *G. candidum,* on early rearing of similar fish species while supplemented in rearing water. However, here as feed additive, the observed effect on growth rate i.e., weight gain was somewhat higher than observed in earlier study. It could be due to feeding strategy and way of administration of probiotic. Here we used probiotic as a dietary supplement. Beside, quantity and quality, performance of fish species also depend on the feed particle size and feeding practices (De La, 2001). In the present study, the higher growth and survival of fish (experiment 1 and 2) even in the control group, in comparison to other reports (Jha et al., 2015) might be due to present feeding strategy. In experiment 1, Swim up fry of both control and probiotic enriched microdiet fed groups were reared with change in feeding frequency, ration and size of feed particles, initially after a weeks and then fortnightly (Experiment 1; Table 1.2), while Jha et al. (2015) used mixture of probiotics supplemented particulate feed and reared swim up of similar fish species i.e., *L. rohita* but in contrast to our strategy, did not change feed particles size, ration and feeding frequency.

Successful probiotic after colonization on the epithelium lining of host intestine, competitively exclude the pathogens and regulate the gut microbiota and enhance the nutrients digestibility by releasing various exogenous enzymes like cellulase , protease, amylase, lipase , β-glucanases, xylanases etc.((Piegza et al., 2014) . Here, the increased in survival rate and growth performance (weight gain, SGR etc.) of *L. rohita* postlarvae and fry raised on dietary

probiotics supplement, mainly *G. candidum* -QAUGC-01 could be attributed to improved levels of gut microbial load (Experiment 1, Fig. 1.1: Experiment 2, Fig. 1.1.2) and increased in secretion of amylases, proteases and cellulases (Experiment 1 & 2; Table 1.4, Table 1.1.2). The colonization of *G. candidum* in GI tract of *L. rohita* may be related to cell surface hydrophobicity gut (Vázquez-Juárez et al., 1998) and the capacity of probiotic strain to attach and proliferate (Andlid et al., 1998; Vázquez-Juárez et al., 1998). Furthermore, it could also be explain by the presence of mannose rich proteins of yeast which have a tendency to occupy receptors required by pathogenic organisms (Pillay and Kutty, 2005)

In accord to our results, Ghori et al. (2018) also observed the increased in gut microbial load of juvenile *L. rohita* after administration of similar strain of *G. candidum.* Whilst other two investigators reported the improved intestinal enzymes (amylase, protease and cellulase) activities of post larvae of similar species reared on similar strain of probiotic provided via water and as dietary supplement (Ibrar et al., 2017; Amir et al., 2018). Additionally, strong *in vitro* enzymatic potential of *G. candidum* i.e., high level of cellulases, α amylases, xylanases, β-glucanases, and moderate level of lipases and proteases (Piegza et al., 2014) in comparison to *B. cereus* which release adequate amount of proteases and reduced level of amylases (Ghosh et al., 2002) also in agreement to our observations. Nevertheless, here, the inconsistent result of the consortia of both *B. cereus* and *G. candidum* i.e., slightly enhanced enzymatic activities, but comparatively lower growth as compared to *G. candidum* alone could be explained by the level of intestinal colonization of microbes that may be overloaded, and probably negated the synergistic effect. Several scientists also observed similar low or insignificant effect of higher dose of probiotics and suggested that higher dose did not always show positive effect on the fish (Ghosh et al., 2008; Merrifield et al., 2010b).

The change in body composition like modification fat and protein contents is linked to their synthesis, deposition in muscle and in turn increases in growth rate. In the present study, at the end of the feeding trial, the muscle composition of fry and fingerlings reared on probiotic supplemented feed showed a considerable increase in protein and fat content as compared to fry provided basal diet. These contents were more significantly higher in those fry, which were fed diet supplemented with *G. candidum* (Table 1.3 and 1.1.1). The result of the study is in agreement with Ibrar et al. (2017) findings. It seems that *G. candidum* due to their higher enzymatic potential, improves the digestibility and absorption of nutrients more efficiently (Piegza et al., 2014), thus increasing the status of fat and protein contents in the muscle.

In literature, effect of bacillus species on body composition of fish is inconsistent. For instance, Bagheri et al. (2008) observed increase in protein contents and a decrease of crude fat in muscle of rainbow trout fry *(O. mykiss Walbaum*) after feeding *B. licheniformis* and *B. subtilis* supplemented diets for 9 weeks while Merrifield et al. (2009) did not observed any noticeable effect of similar probiotic on similar fish species. Moreover, Hassaan et al., (2018) reported non-significant effect of dietary *B. subtilis* supplementation on protein, lipid and ash content of Nile tilapia (*O. niloticus)* after 84 days feeding trial.

The effects of probiotics on health status and immunity of aquatic organism are well documented. Here the decreased serum AST level in groups fed probiotic supplementary diet as compared to a control group on basal diet also indicated the improved health status of fingerlings. Serum AST indicate the liver function and the health condition of fish (Benedeczky et al., 1984; Fakruddin et al., 2017), as it increased level indicate the liver cell damage (Kumar et al., 2011). In the present study, lowest serum AST level in a group provided dietary supplement of *G. candidum* as compared to *B. cereus* (Experiment 2, Fig. 1.1.3) may indicate the

higher efficiency and suitability of yeast base probiotic for *L. rohita*. Like present results Marzouk et al. (2008) also reported a significant decrease in AST level of *O. niloticus* fed *S. cerevisae* supplemented diet. Similarly, Solatan and El-Laithy, (2008) reported lower AST level in *O. niloticus* reared on *B. subtilis* supplementation, while according to Hassaan et al. (2014), *B. licheniformis* decrease AST level in Nile tilapia (*Oreochromis niloticus*) when its concentration increased up to 0.48×10^6 cfu g^{-1} however,beyond this level it did not show any change.

The results of complete blood count (CBC) also indicated the improved health status of advanced fry of *L. rohita* fed *G. candidum* alone followed by consortia of both probiotics supplemented diet. Here *B. cereus* also improved the RBC, Hb, Hct, WBC and MCV levels, but the impact was comparatively less as compared to *G. candidum*. In aquaculture, hematological indices are considered as a vital tool for determining the physiological change and health status of fish (Atamanalp and Yanik, 2003; Ullah et al., 2018). The influence of diet/nutrition, disease and stress on CBC of fish is well described (Rajikkannu et al., 2015; Roohi et al., 2017; Gora et al., 2018). In the present study, because of difficulty in collecting blood samples from small size fish, fingerlings from second set of experiment were used for hematological and immunity tests. No comparable study indicates the impact of *G. candidum* and *B. Cereus* on the hematology and immune response of fish. However, like our results, Goran et al. (2017) reported the positive impact of *S. cerevisiae* on hematological indices of *C. carpio*, while Hassaan et al. (2014) observed significant increase in HB, Hct, RBCs and WBCs of *O. niloticus* in response to low level (0.48×10^6 $cfug^{-1}$) of *B. licheniformis* as compared to 0.96×10^6 cfu g^{-1} dietary supplement which showed negative impact (decreased levels were noted). Moreover, some investigator did not observe any significant effect of *B. subtilis* supplemented diet on the hematological

parameters especially RBCs, Hb and Hct (*O. niloticus*: Soltan and El-ferguson, 2008; *Brycon amazonicus* breeders: Dias et al., 2012; *O. mykiss*: Kamgar and Ghane, 2014).

The mucosal surface of aquatic animal, including fish provide first-line defense i.e., t as barrier against several pathogens (bacteria and virus). It is well established that probiotic after binding on mucosal surface, improved the immunity of an organism (both innate and adaptive) (Gallo and Nakatsuji, 2011; Otte and Vordenbaumen, 2011; see review, Ibrahem, 2015). At present, information on the effects of present strains of probiotics, *B. cereus* (KT021872) and *G. candidum*-QAUGC-01 (KTC280407) on immunity of aquatic animals is limited. Here both probiotics showed a considerable increment in total serum protein, IgM level, phagocytic, respiratory burst and lysozyme activity indicating their role in improving the immune response of *L. rohita*. However, *G. candidum* showed most significant positive effects as compared to *B. cereus* (Experiment 2, Table 1.1.4).

The increased level of total serum protein in the current study may be attributed to the stimulation of defense molecules like lysozyme, IgM, agglutinins etc. In agreement to our results, many investigators reported an increase in serum protein in response to various species of *Bacillus* such as *B. amyloliquefaciens* in *Catla catla* (Das et al., 2013), *B. subtilis* in *L. rohita* (Nayak et al., 2007) and *B. licheniformis* in triangular bream, *Megalobrama terminalis* (Zhang et al., 2013). However, Hassaan et al.(2014) 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* (0.48×10^6cfu g^{-1}), while increased level (0.96×10^6cfu g^{-1}) did not show any significant effect

In fish, IgM is the key components of the humoral immune system (Watts et al., 2001). It increased level in response to probiotic indicate the stimulation of defense mechanism of fish i.e., production of antibodies against foreign invaders (see review Ibrahem, 2015). It is well

documented that successful probiotics have tendency to stimulate the production of antibodies in fish (Nikoskelainen et al., 2003; Panigrahi et al., 2004). Here, IgM level was increased in response to both probiotics, however highest levels was observed in a group of fish fed with *G. candidum* supplemented diet followed by consortia of both probiotics. It seems that in *L. rohita*, yeast in present concentration enhanced antibody production more effectively as compared to *B. cereus.* Like our results, Panigarhi et al. (2005) also reported increase IgM level in sea bream (*S. aurata*) and rainbow trout in response to the yeast cells, while Mohapatra et al. (2014) used *D. hansenii* as a dietary supplement and observed higher levels of IgM as compared to control in leopard grouper, *Mycteroperca rosacea*. Similarly, many investigators reported the elevated levels of serum IgM level in different fish species in response to *Bacillus subtilis* like *O. niloticus* (Magda et al., 2011), *O. mykiss* (Kamgar and Ghane, 2014). However, Sun et al., (2011) found that *Bacillus* supplemented diets stimulated serum IgM level in *E. coioides* up to 30 days, and thereafter, the IgM levels decreased. Conversely, Balczar et al. (2007) did not observe a significant effect of LAB groups of probiotic on IgM level of *Salmo trutta*.

Like other immunity parameter, lysozyme activity in the present study also showed the highest level in *G. candidum* supplemented diet fed group followed by fish fed diet fortified with consortia of both probiotic. The present elevated level of lysozyme indicates the stimulation of innate immune system. It is well noted that lysozyme plays a crucial role against bacterial infection (Saurabh and Sahoo, 2008; Sanchooli et al., 2012) by attacking peptidoglycan in their cell wall, thus causing lysis and stimulation of phagocytosis of bacteria by phagocytic cells (Ellis, 2001). Here, the lysozyme activity also decreased in a group of fish fed *B. cereus* supplemented diet, but its level was several fold lower than observed in response to *G. candidum,* thus also support the higher efficiency *G. candidum*. Like our results Güven and

Yalçin (2017) observed several fold increase in lysozyme activity in rainbow trout after 8 weeks feeding of *S. cerevisiae* while Jha et al. (2007) reported similar increase, in *Catla catla* after 60 days feeding dietary yeast nucleotides. Moreover, many investigators reported enhanced lysozyme activity in response to *B. subtilis* supplementation e.g. grouper, *Epinephelus coioides* (Liu et al. (2012), *O. mykiss* (Mahmoudzadeh et al., 2016). In contrary, other studies did not observe activation of lysozyme activity after feeding dietary *B. subtili*s supplementation (Zhu et al., 2010).

Here, the enhanced respiratory burst and phagocytic activity in response to probiotic supplemented diet also indicated the strengthening of bactericidal mechanisms/innate defense of fish (Ellis, 2001; Son et al., 2009). Respiratory burst generates reactive oxygen species (ROS) like superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) which are toxic to many microorganisms (Hardie et al., 1996). Generally, this process occurs in phagocytes which recognize any foreign material /potential pathogens, and by phagocytosis degrade them (Secombes, 1990; Steinhagen and Jendrysek, 1994). Several investigators reported the considerable increase in respiratory burst and phagocytic activity in response to various probiotics in different fish species. For instance, *B. subtilis* enhanced respiratory burst activity in *L. rohita* (Kumar et al., 2008), *Apostichopus japonicus* (Zhao et al., 2012). Similarly *S. cerevisiae* improved respiratory burst activity of sea cucumbers; *Sparus aurata* (Ortuno et al., 2002) Moreover Son et al. (2009) reported enhanced respiratory bursts and phagocytic activity (PA) and the phagocytic index (PI) in grouper (*E. coioides*) fed *L. plantarum* for 4 weeks, however beyond this period, no significant effect was observed. Present significant increased phagocytic activity with increased phagocytic index and respiratory bursts, indicating the correlation between respiratory burst activity and phagocytosis.

Besides supporting growth, probiotics could improve the immunity and disease resistance. Here, *L. rohita* swim up fry reared on probiotic supplemented microdiet for 11 weeks, and then exposed to *L. rohita* pathogen A. *hydrophila* showed significantly higher survival rate *G. candidum* as compared to a control group fed diet devoid of probiotic. It was also observed that group of postlarvae and fry reared on *G. candidum* as compared to *B. cereus* supplemented diet showed lower mortality rate. It seems that *G. candidum* supplement provided maximum disease resistance (Fig 1.2 and 1.1.6). Generally, successful probiotic has a better ability to colonize the gut, prevent the attachment of pathogens and regulate the gut microbiota (see review Ibrahem, 2015). Here, considerably improved survival rate (Fig. 1.1.6) could be due to the stimulation/ activation of innate immunity and the decrease in the permeability of host epithelium for toxins and macromolecules (Puddu et al., 2014). It seems, G. *candidum* has an ability to competitively exclude the opportunistic pathogens, colonize the GI tract and leave no space for pathogen attachment. In aquaculture, no comparable study is available which indicated the antagonist activity of *G. candidum* against pathogenic bacteria *A. hydrophila*. However, *in vivo* antagonist activity against other pathogens like *S. aureus* is reported by Ibrar et al. (2017), while *in vitro* antimicrobial activity of *G. candidum* alone or in combination with other probiotics against many pathogenic bacteria is reported by many investigators (Naz et al., 2013; Samuel et al., 2017; Ghori et al., 2018). Additionally, like present observation, NavinChandran et al. (2014) also noted the lower antagonistic activity of *B. cereus* against *A. hydrophila*.

Furthermore, it seems that β-glucan in the cell wall of *G. candidum* contributed in the stimulation of immune system more efficiently. β-glucan is a potent immune system stimulator (Ortuno et al., 2002; Saikai, 2005), have “receptor molecule” on the surface of phagocytes. Thus

stimulate the process of phagocytosis and also secrete cytokines (signal molecules) for stimulating the formation of new white blood cells.

In conclusion, *G. candidum*-QAUGC-01 alone had shown the most significant effects on the survival, growth, intestinal enzymes activities, muscle composition, immune response and resistance to pathogen of *L. rohita.* The better efficiency of *G. candidum* in contrast *to B. cereus* could be attributed to the constituent cell wall, i.e., β-glucans and chitin, (Sietsma and Wouter, 1971). These component could provide protection from gastric shock, better chance to withstand unfavorable conditions and adhere to the mucosa of the host (Keily and Olson, 2000; Syal and Vohra, 2014; see review Ibrahem, 2015) and activate the immune and antioxidant systems of the host (see review Ibrahem, 2015). It is well documented that β-glucan is a potent immune system stimulator (Ortuno et al., 2002; Saikai, 2005), have "receptor molecule" on the surface of phagocytes. Thus stimulate the process of phagocytosis and also secrete cytokines (signal molecules) for stimulating the formation of new leukocytes. Moreover, their larger cell volume as compared to bacteria (Gatesoupe, 2007) and the ability to release various enzymes i.e., α amylases, cellulases, β-glucanases, xylanases, proteases and lipases (Ghosh et al., 2002) may also signify their improved efficiency. On the basis of these results, *G. candidum*-QAUGC-01 supplemented microdiet could be recommended as an eco-friendly viable way to improve the survival and growth of postlarvae and advanced fry of *L. rohita*.

Chapter 2

Comparative efficiency of *Geotrichum candidum* microcapsules prepared with alginate alone and in combination with other polymers: *in vitro* evaluation

Abstract

Nowadays, microencapsulation appears as a processing technique to protect and maintain the survival, stability and target release of probiotics. However, encapsulation efficiency depends on the type, concentration and formulation of encapsulating polymers, concentration of hardening solution and process of encapsulation. Here in order to select the best encapsulating polymer/formulation, *G. candidum* was encapsulated with alginate alone (Alg) and in combination with starch (Alg-S) and xanthan (Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and chitosan nanoparticles (Alg-CN, Alg-S-CN, Alg-X-CN) by adopting simple , easy and cheap extrusion technique with similar concentration of hardening solution. The structural characteristic and surface morphology of prepared microcapsules were accomplished by fourier transform infrared spectroscopy (FT-IR) and scanning electron microscope (SEM). Moreover, *in vitro* assays were performed to evaluate the encapsulation efficiency (EE), survival, pH and temperature tolerance and viability under two different storage conditions. The SEM images of microcapsules of *G. candidum* prepared from different formulation indicated the difference in shape and morphology. The Alg-C and Alg-CN microcapsules of *G. candidum* especially Alg-CN were more spherical in shape with smooth surface and least cavities/cracks in contrast to uncoated microcapsules (Alg, Alg-S, Alg—X), which were irregular in shape with rough/ uneven surface. The encapsulation efficiency (EE) of all formulation was above 80%. The Alg-CN microcapsules revealed highest value for EE (99.3 %) followed by Alg-C (96.6%) and Alg-X-CN (96.03%) microcapsules. However, lowest EE value (83.5%) was observed when Alg alone was used as encapsulating agent. Furthermore, the chitosan coated especially Alg-CN microcapsules showed the highest viable count of *G. candidum* at three tested pH (2, 4 and 8), after exposure to higher temperature, 60ºC for an hour and after storage for 60 days at ambient

temperature and 4ºC. It seems that *G. candidum* encapsulated with Alg-CN and Alg-C had highest EE, more tolerant to pH and temperature with highest shelf life. On the basis of *in vitro* results, combination of 2% alginate 0.4 % chitosan could be recommended as encapsulating material for maintaining the % survival of this potential probiotic (*G. candidum*) in feed and GI tract of *L. rohita.*

Introduction

For the last two decades, probiotics are gaining importance for improving culture condition and health status of fish. Nowadays, probiotic application appears as an effective, ecofriendly and sustainable strategy to prevent fish diseases and improve survival and growth of cultureable fish species (See reviews Nayak, 2010; Ibrahem, 2015; Cordero et al., 2015). The probiotics are live/dead microbes, that when present in adequate amount in the GI tract of host may confer several health benefits to the organism (FAO/WHO, 2016; Shori, 2017). They prevent gastrointestinal infections, enhance immunity and decrease the serum lipids, owing to anti-carcinogenic, antimutagenic and antibacterial effects (Tannis, 2010; Cordero et al., 2015).

For probiotics to confer beneficial effects to host, their viable count must be in sufficient amount, when reached to site of action i.e.GI tract (Pinpimai et al., 2015; Shori, 2017). They must be resistant to the extreme conditions of the GI tract such as low pH, gastric enzymes and bile salts (Marteau et al., 2001). It is well documented that probiotic microorganisms in transit to target site, reduce in number of viable cells. Though, this characteristic is strongly dependent on species and strain of probiotics, but with an average only 10-25% of the ingested probiotic cells can survive and reach the target site of GI tract of fish (Del Piano et al., 2011). Beside this, environmental conditions during fish feed storage and the way of incorporation in fish feed also influenced the viability of microbes used as probiotics (Rosas-Ledesma et al., 2012). In aquaculture, probiotics are mostly provided through feed, thus microbes as probiotics should be stable and have capability to tolerate environmental stress. It is suggested that to obtain health benefits, the cell count of probiotic in diet should be in the range of 10^8 to 10^9 $CFUg^{-1}$ before ingestion, so that adequate cell at least 10^6 to 10^7 CFU could reach the GI tract of host (Shori, 2017).

To provide protection and improve the viability of probiotic microorganisms in GI tract of host and in fish feed, different techniques have been developed. One of the most promising of these techniques is the encapsulation or immobilization. A technique previously used to stabilize and control release of drug and active gradients is now gaining importance in aquaculture for improving target release of nutrients and the viability of probiotics microorganisms (Huq et al., 2013). Encapsulation is a process of packing one substance or mixture of substances in another material called coating, wall, or carrier material, to form microbeads /microcapsules (Bansodeet al., 2010; Pham-Hoang et al., 2013; Riaz and Masud, 2013). The entrapped / coated material also called payload or core material. The coating material or surrounding wall acts as a physical barrier and protect the core/ active materials from environmental insult like oxygen, gastric enzymes, bile salts etc. (Desai and Park, 2005), thus facilitate to pass through the anterior region of GI tract and reach the target site (Hassan et al., 1996; Dave and Shah, 1997; Godward and Kailasapathy, 2003). Microcapsules /microbeads have an ability not just to protect /preserve the payload/core substance but also to release it to the target site of host when required (Sultana et al., 2000; Normand et al., 2005).

Generally microencapsulation is a process to produce small capsules or beads by coating the tiny bioactive materials (core materials) with other protective materials or their mixtures. The resultant beads/microcapsules are generally spherical in shape and may be as small as few micrometers (μm) or as large as 3 mm in diameter depending upon the technique used for microencapsulation. The inner side of microcapsule holds a liquid or solid core with microorganism, whereas outside surface comprise of very thin but strong semi-permeable membrane which allow the two way selective passage of contents (Anal and Singh, 2007). Microencapsulation technique has diverse applications in different field of life like

pharmaceutical, food, biomedical and cosmetic industries (Nesterenko et al., 2013). Nowadays, several microencapsulation processes are in practice to encapsulate the ingredients like spray drying, emulsion, extrusion, coacervation-phase, ionotropic gelation and entrapment in liposome (Gouin, 2004; Benita, 2005; Reid et al., 2007; Jyothi et al., 2010; Munin and Edwards-Lévy, 2011; Soukoulis and Bohn, 2018). The choice of particular process for microencapsulation depend on the physio-chemical characteristics of core/ payload material and the wall/ coating material, size of microcapsules and on the process costs (Nesterenko et al., 2013). However, emulsion/two phase system and extrusion/droplet methods, are commonly used for encapsulation of probiotics. By adopting these techniques, the storage stability and viability of probiotics microorganisms in fish feed and GI tract of fish could be improved (Huq et al., 2013).

Efficiency and characteristics of microcapsules depend on many factors including encapsulating materials, hardening solution (calcium chloride), concentration of capsular matrix and hardening time (Krasaekoopt et al., 2003; Riaz and Masud, 2013). Generally, the functional groups present on cell wall of microorganisms used as probiotics and their cross linking with the encapsulating material influence the survival of probiotic within the microcapsule (Hernandez et al., 2013). The encapsulating or wall materials interact structurally in different ways with the probiotics, thus also affect their delivery at the target site (Riaz and Masud, 2013). The capsule forming ability and strength determine the effectiveness of any encapsulating material (Reid et al., 2007). It is recommended that wall/ encapsulating material should be cheap, biologically safe and biocompatible.

Mostly, the wall/encapsulating material affects the stability of microcapsules and the degree of protection of the payload or core ingredient (Nesterenko et al., 2013). Materials commonly used for microencapsulation are synthetic polymers and bio based materials i.e.,

lipids, animal and plant derived proteins and gelling polysaccharides like chitosan, alginate, xanthan alone or in combination (Hernandez et al., 2013; Fathi et al., 2014; Joye and McClements, 2016).

Animal source of protein (whey proteins, gelatin, casein, milk etc.) and plant sources (pea oat, wheat, barley and corn, sunflower, cereal etc.) are mostly accepted as the suitable materials for encapsulation of active materials in the cosmetic, pharmaceutical and food industries (Riaz and Masud, 2013). These materials have a benefit to form matrix of controlled sizes, without any untoward effect on sensory properties/attributes of the prepared food/core material (Chen et al., 2006; Jenkins et al., 2007). Additionally, protein as an encapsulating material also have the ability to protect, interact and reverse the binding via their functional groups with a wide range of active compounds (Chen et al., 2006). In food industry, plant source of proteins are considered to be less allergic in contrast to proteins derived from animal sources (Li et al., 2012).

Beside protein surface active lipids are also used as an encapsulating agent in various industries including pharmaceutical, food and cosmetic. Surface active lipids like phospholipids, glycolipids and acylglycerols are amphipathic in nature, have ability to self-assemble into a variety of unique micro to nanoscales supramolecular structures. These properties enhanced the gelation capacity, core ingredients/material loading capacity and *in vivo* target delivery of core material (Hernandez et al., 2013). However, compared with the protein and lipid, polysaccharides appeared as more suitable wall materials, under high temperature conditions, as protein /lipids may melt or denature (Li et al., 2012; Fathi et al., 2014). Moreover, polysaccharides also maintain their integrity, withstand the acidic condition of stomach and degraded by intestinal microbiota and releases entrapped probiotics at target site. The release rate

of encapsulated probiotics on the target site (stomach/small intestine) is related to pH variation in the gastrointestinal tract of host (Riaz and Masud, 2013).

Polysaccharides commonly used as a matrix for encapsulating the materials are pectin (Gharsallaoui et al., 2010), starches (Jeon et al., 2003; Murúa-Pagola et al., 2009), arabic gum (Shaikh et al., 2006), maltodextrin (Semyonov et al., 2010), alginates (Wikstrom et al., 2008; Huang et al., 2010) and chitosan (Pedro et al., 2009). In comparison to protein, these polymers are more soluble in the water and less viscous at higher concentrations (Nesterenko et al., 2013). Among polysaccharides, alginate and chitosan are the widely used for providing protection, stability and control release of drug and nutrient to the target site of host (Hernandez et al., 2013). Mostly encapsulation is done with alginate while chitosan is used as a coating material for improving the control release of core materials (Joye and McClements, 2014).

One of the most commonly used polysaccharide for encapsulation is Alginate. It is inexpensive, nontoxic, biocompatible, water soluble, anionic natural heteropolysaccharide consist of linear copolymers of α-L-guluronate and β-D mannuronate residues. Alginate is derived from cell wall of the brown algae (Lee et al., 2000). It has a powerful ability to swell in aqueous solution. Moreover, at low pH, reduce in molecular weight, which helps the faster degradation and release of materials from microcapsule (Gombotz et al., 1998; Sultana et al., 2000; Krasaekoopt et al., 2006). Most of the *in vitro* studies used alginate as an encapsulating material and showed satisfactory results. Moreover, *in vivo* investigations did not show the accumulation of alginate in any major organs and indicated the hamocompatible and biodegradable characteristics (Rajaonarivony et al., 1993).

Alginate as encapsulating material has a capacity to give an adequate protection to the entrapped /core material, from harsh conditions like heat /cold, acidic pH, bile salts and oxygen.

Microencapsulation with Alginate can be done by adopting simple extrusion method i.e., mixing of alginate alone or in combination with other wall materials to the $CaCl_2$ a hardening solution. In this way, the Ca^{+2} interacts with the –COOH group of alginate biopolymer and form an insoluble gel (Nazzaro et al., 2009). The Calcium–alginate gel microcapsules appears as the extensively used carrier for controlled release of many materials. It is widely used in food industries and for improving the viable count of probiotic organisms up to 80 to 95% (Sultana et al., 2000; Yan et al., 2001; Mandal et al., 2006). Yet, limited literature revealed the use of the alginate-encapsulated probiotics in aquaculture (Pinpimai et al., 2015).

Though sodium alginate is important for encapsulation but several studies indicated the mechanical instability and susceptibility of alginate microcapsules to the low pH (acidic) of the stomach while passage through the GI tract of host (Huq et al., 2017). It is well documented that the integrity of the calcium alginate microcapsule is under stress, in the presence of chelating agents (lactate, citrate and phosphates) and monovalent ions like Na^+ (Riaz and Masud, 2013). Scientists solved this problem by mixing of the alginate with other wall materials or coating of the alginate microcapsules with other polymers and modifying the structure of the alginate (Sultana et al., 2000; Yasmin et al., 2018).

Among many polymers, starch and chitosan in combination of alginate, is commonly used for encapsulating the probiotics (Riaz and Masud, 2013). Starch is semi-crystalline in nature and composed of two polymers, amylose and amylopectin. Starch granules show variation in size like wheat starch granules are the smallest one (>20 μm), while maize (10 to 20 μm) and potato starches (20 to 100 μm) being larger (Bertoft, 2017). Accordingly, maize and wheat starches are more easily digested, probably because of the increased granule surface area and enzymatic availability, thus are more appropriate for aquaculture feeds. Many investigators

suggested the alginate in combination with starch to improve the effectiveness of encapsulation (Sultana et al., 2000; Yasmin et al., 2018).

Among different polysaccharides, xanthan gum is also being used to encapsulate the ingredients. It is released as an extracellular polysaccharide from pathogenic bacteria of brassica *Xanthomonas campestris* (Becker et al., 1998). The low temperature and increase in salt concentration change the conformation of xanthan gum and form rigid coil like structure (Morris et al., 1977). Xanthan gum has been tested as a binding agent in pharmaceutical (Eyo-okon and Hilton, 2003; Riaz and Masud, 2013), aquaculture feeds (O`Mahoney et al., 2011) and for encapsulating probiotic (Chaveri et al., 2012). Xanthan gum is water soluble even at low temperature and has tolerance to wide range of pH (2-12), salt, enzymes and heat (Soma et al., 2009; Riaz and Masud, 2013). A mixture of xanthan and gellan gum was previously reported to be used for encapsulation of probiotic bacteria (Ding and Shah, 2009).

In addition to encapsulation, formation of the cover/coatings around the microcapsules also improves the physiochemical properties of the microcapsules (Krasaekoopt et al., 2003). It is advisable that coating material used for encapsulation should be biologically safe, cheap, and biocompatible. Effectiveness of coating material depends on capsule forming ability and strength (Reid et al., 2007). The cross linking of functional groups of encapsulating and coating materials determine the stability and strength of microcapsules, while interaction between functional groups of coating materials and epithelial lining of intestine determine the stability and control release of core material at the target sites. Furthermore, the interaction between microbial cell wall and the encapsulating material is important for the survival of probiotic within the microcapsule (Hernandez et al., 2013).

Many scientists observed improve physiochemical attributes of alginate microcapsules when coated with chitosan. Chitosan coated microcapsules/ microbeads are strong, stable and less susceptible to the deteriorative effects acidic pH, bile salts etc of GI tract (Krasaekoopt et al., 2003; Riaz and Masud, 2013). In the chitosan coated alginate microcapsules, -COOH group of the alginate and -NH2 of the chitosan interact with each other and form alginate–chitosan polyionic complex. The complex provides increased protection, biocompatibility, biodegradability, and control release of the encapsulated /core material more efficiently in contrast, to simple use of alginate or chitosan alone (Gazori et al., 2009). Alginate–chitosan (Alg-C) microcapsules have been extensively used for the target delivery of enzymes, drugs, micronutrients, oligonucleotides and peptides /proteins with promising results (Coppi, 2002; Gonzalez-Rodriguez, et al., 2002; Mi, et al., 2002).

Chitosan, is a high molecular-weight (mol. wt, 310 kDa) heteropolymer, containing acetylated unit N-acetyl-glucosamine and deacetylated unit D-glucosamine linked through β, 1-4 glycoside linkage. Chitosan is also biodegradable, biocompatible and non-toxic and its characteristics depend on the molecular weight and degree of acetylation (Joye and McClements, 2014). Its ability to form film, make it suitable to preserve food and to manage psychotrophic pathogens in processed meat / fish or even in fresh products (Malhotra et al., 2015). Many investigators reported the uniform and smooth surface of chitosan encapsulated microcapsules under optical and the transmission electron microscope (Tamura et al., 2002). Chitosan is being widely studied for its control release properties in drug delivery systems (Lam et al., 2014). However, there are some drawbacks while using chitosan alone, i.e., fast dissolution in the GI tract and less effective control release (Risbud et al., 2000). Moreover, antibacterial activity of chitosan limits its use for encapsulation of probiotics. To overcome these drawbacks, several

authors reported the use of chitosan as a coating agent, in the area of encapsulation and food preservation (see review Shori, 2017) and suggest an increase in mechanical strength of the microcapsules (Ribeiro et al., 1999; Serp et al., 2000). The chitosan coated, xanthan gum (Argin et al., 2009), sodium alginate (Tapia et al., 2004), carrageenan and many other compounds (Riaz and Masud, 2013), microcapsules are well documented. As a coating material, mucoadhesive nature of chitosan, increase the contact time of microcapsule with the absorption site, thus enhanced the absorption and bioavailability of core materials (Lam et al., 2014). The chitosan coated alginate encapsulated microcapsules formation is extensively mentioned in the literature (Anal et al., 2005). The positively (+ve) charged amino group of chitosan interacts with negatively (-ve) charged carboxyl group of sodium alginate (Na-Alg) and form polyelectrolyte complex (PEC). This complex improves the survival of encapsulated probiotics during the storage and also in the GI tract. Encapsulation with PEC is a much better option for delivering viable probiotics to the gut rather than encapsulating with chitosan alone (Chaveri et al., 2012).

Nowadays, nanotechnology is getting importance in every field of life. Nanoparticles of biopolymers because of their versatile characteristics like biocompatibility, low immunogenicity and biodegradability appears as suitable materials for drug and gene delivery (Nitta and Numata, 2013). It is well documented that with the change in particle size, the physical and chemical properties of material also change (Riaz and Masud, 2013). The improved stability of nanoencapsulated bioactive compounds during processing, storage and passage through the GI tract was reported by Chen et al. (2006). The surface morphology and the particle size of microcapsule effect the intercellular delivery of the core materials (Petros and DeSimone, 2010; Mizrahy and Peer, 2012; Nitta and Numata, 2013). Generally, materials at nanoscale have large surface area, rapid and higher absorption ability as well as release behavior thus appears suitable

for the delivery of bioactive substances through nanoencapsulation. However, polymeric nanoparticles show wide size distribution and low drug-loading capacity (Yang et al., 2006).

Recently, chitosan and chitosan NPs has gaining more importance as a coating material in the drug and bioactive compound delivery system. Riaz and Masud (2013) reported unsatisfactory result while using chitosan as encapsulating agent for probiotic and suggest that stability, strength and target release of probiotic can be enhanced by using chitosan in combination with other encapsulating polymers. Many investigators reported the higher viability of chitosan coated encapsulated probiotic microbes in simulated intestinal /gastric juice and during storage at different temperature (Krasaekoopt et al., 2003; Lee et al., 2004). Chitosan has special characteristics like ability to increase the permeability of cell membrane and to open the tight junctions of cell membranes, enhance the absorption of material across intestinal epithelia by increasing the residence time of microcapsule/microbead at target sites (Roldo et al., 2004). Chitosan NPs can be prepared with various biological, chemical and physical methods , but in recent years , the ionic gelation method with the use of tripolyphosphate (TPP) is gaining more attention and commonly used to prepare the nanocarriers for low molecular weight drugs, (Czerucka et al., 2007; Papadimitriou et al., 2008).

Yeast is widely used in agriculture, food and aquaculture industry. Some species and strains of yeast like *Saccharomyces cerevisiae, Saccharomyces boulardii* etc, have probiotic properties, hence are in use to to sustain and re-establish the natural gut microflora of host (Lee et al., 2004; Billoo et al., 2006; Pham-Hoang et al., 2013). In the microencapsulation domain, many investigators reported the higher efficiency of encapsulated Yeast, *S. boulardii*, (Joshi and Thorat, 2011; Duongthingoc et al., 2013), and *S. cerevisiae* (Graff et al., 2008; Hebrard et al., 2010; Pinpimaiet al., 2015) in comparison to free probiotic. The attractive properties of yeast like

relatively homogeneous and constant size (between 5-10 μm length), higher encapsulation efficiency, resistant to higher temperature and light conditions (Normand et al., 2005; Shi et al., 2007; Pham-Hoang et al., 2013) and their ability to adhere to the epithelial cells and prompt the permeability of cell membrane (Fuller et al., 2005) increase their importance in many industries. Yeast encapsulation is relatively simple and in expensive against many well-known microencapsulation technologies (Nelson et al., 2006).

Keeping in view the higher efficiency of *G. candidum* and advantages of microencapsulation technique, the present part of study was designed to encapsulate the selected probiotic *G. candidum* with alginate alone or in combination with starch and xanthan and coated the prepared microcapsules with chitosan (bulk) and nanoparticles of chitosan (nano-chitosan) and examined surface morphology and size by conducting SEM and FT-IR spectroscopy. Moreover, *in vitro* efficacy of free, uncoated and chitosan coated encapsulated *G. candidum* (Alg, Alg-C, Alg-CN, Alg-S, Alg-S-C, Alg-S-CN, Alg-X, Alg-X-C, Alg-X-CN) was determined by evaluating encapsulation efficiency (EE), tolerance to pH and temperature and shelf life by observing viable cell count after exposing three pH (2, 4 and 8), higher temperature (60ºC for an hour) and storage for 60 days at ambient temperature and 4 ºC.

Materials and Methods

Probiotic microorganism

After viewing higher efficiency in previous *in vivo* experiments, *G. candidum* as compared to *B. cereus* was selected for further study. To enhance the viability, bio-availability and shelf life, *G. candidum*, was encapsulated with alginate alone or in combination with other polymers (starch and xanthan) and coated with chitosan. For encapsulation, extrusion method was adopted which is simple, cheap and makes minimum harm and maximum viable count of probiotic (Martinsen et al., 1989; Hug et al., 2013). *G. candidum* QAU*GC*-01 was cultured as described previously in chapter 1. The cell pellet obtained with a final viable count of 10^9 CFU ml^{-1} was kept at 4°C for further use.

Solution preparation

1. Sodium alginate (2% w/v)

By using a top loading electrical balance (TX323L-SCIMADZU, Japan), 2.0 g sodium alginate was weighed and subsequently dissolved in distilled water (100 ml) by using a magnetic stirrer (200 rpm) set at 27 °C.

2. Calcium chloride (0.1M)

It was prepared by weighing 1.42 g $CaCl_2$. $2H_2O$ and dissolving in 100 mL distilled water at room temperature.

3. Starch (2% w/v)

By using an electrical balance, 2.0 g corn starch (Sigma-Aldrich Chemical Co, USA) was weighed and added in beaker having 100 mL distilled water. It was boiled for 30 min on a hot plate until it formed a gel. Subsequently, it was cooled at ambient room temperature.

4. Xanthan (1.2%)

1.2 g Xanthan (Sigma-Aldrich Chemical Co, USA) was dissolved in distilled water (100 mL) with constant stirring (200 rpm) at room temperature.

5. Chitosan (0.4% w/v)

Krasaekoopt et al. (2004) method was adopted for the preparation of a 0.4 % aqueous solution of chitosan. Briefly, 0.4 g 85% deacetylated crab shell chitosan (Sigma-Aldrich Chemical Co, USA) was dissolved in distilled water (90 mL). The solution was acidified with 0.5 mL glacial acetic acid and finally adjusted the volume to 100 mL and obtained 0.4% (w/v) concentration of chitosan. The pH 5.6 of chitosan solution was adjusted with 0.1 M) NaOH. Before use, the mixture was filtered by using Whatman NO. 41 (Thomas Scientific, USA) and autoclaved for 15 min.

6. **Sodium tripolyphosphate (TPP) (0.5% w/v)**

0.5 g of sodium tripolyphosphate (Sigma-Aldrich Chemical Co, USA) was dissolved in 100 mL distilled water and stored at 4°C until further use.

Encapsulation with various polymers

1. **Encapsulation with alginate (Alg)**

First, alginate was used to encapsulate *G. candidum* QAU*GC*-01. An extrusion method previously reported by Azarnia et al. (2008) with a few modifications was adopted. Briefly, all glassware required for encapsulation was autoclaved for 15 min. After sterilization, glassware was cooled in a fume hood. Subsequently, *G. candidum* QAU*GC*-01 culture suspension and sodium alginate solution 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 and suspension was taken in a sterile syringe (10mL) with 20 G needle and drop-wise suspension was poured in a cold 0.1M solution of $CaCl_2.2H_2O$ continuously shaken at constant speed (70 rpm). The mini orbital shaker (Thomas Scientific, USA*)* was used for gentle shaking. Precautionary measure was taken and placed the syringe 50 cm away from the solution. Microcapsules were formed at once. For hardening, the resultant capsules were allowed to remain in calcium chloride solution for further 30 min, under continuous shaking. Subsequently, the solution was filtered to harvest the capsules. To remove excess calcium chloride, capsules were washed twice with deionized water, dried and stored in sterilized petridish at 4°C.

2. **Encapsulation with alginate-starch (Alg-S)**

A slightly modified method of Sheuand and Marshall (1993), reported by Donthidi et al. (2010) was adopted to prepare alginate-starch microcapsules of *G. candidum*. Briefly, equal amount of sodium alginate and starch solutions (2% each, w/v) were mixed with gentle stirring. Afterwards

0.1% (v/v) *G. candidum* cell suspension with 10^9 CFU mL^{-1} was added to this alginate-starch suspension under sterile conditions. It was hand shaken gently (2-3 min) to ensure uniform distribution of the cells.

0.1M solution of $CaCl_2.2H_2O$ was taken in a beaker and placed on a mini orbital shaker (Thomas Scientific, USA) set at uniform speed (70 rpm). Subsequently, suspension of alginate-starch-yeast was taken in a sterile syringe (10mL) with 20G needle and drop-wise poured into a sterile solution of 0.1M $CaCl_2.2H_2O$. Precautionary measure was taken and placed the syringe 50 cm away from the solution. Microcapsules were formed at once. For hardening, the resultant capsules were allowed to remain in solution, for further 30 min under gentle shaking. Afterward, the solution was filtered to harvest the capsules. To remove excess calcium chloride, capsules were washed with deionized water, dried in a sterilized petri dish and stored at 4°C.

Encapsulation with alginate-xanthan (Alg-X)

Equal amount of prepared 1.2% xanthan and 2% sodium alginate solutions were mixed thoroughly. After mixing cell suspension of *G. candidum* was added to alginate-xanthan mixture in a ratio of 1:3 to get the final concentration of 10^9 CFU mL^{-1}. It was mixed gently for 2-3 min to obtain a homogeneous cell suspension.

About 40 mL$CaCl_2.2H_2O$ solution was taken in a 100 mL beaker and placed on a mini orbital shaker (Thomas Scientific, USA) set at uniform speed (70 rpm). Subsequently, alginate-xanthan-yeast cells suspension was taken in a sterile syringe (10mL) with 20G needle and drop-wise poured into a $CaCl_2$ solution. Precautionary measure was taken and placed the syringe 50 cm away from the solution. Microcapsules were formed at once. For hardening, the resultant capsules were allowed to remain in solution, for further 30 min under gentle shaking. Afterward,

the solution was filtered to harvest the capsules. To remove excess calcium chloride, microcapsules were washed twice with deionized water, dried in a sterilized petri dish and stored at 4°C.

A. Coating with chitosan

To control and improve the release rate of *G. candidum* in the GI tract of fish, encapsulated microcapsules prepared by alginate alone or in combination with starch and xanthan were coated with bulk and nanoform of chitosan.

1. Coating with bulk form of chitosan

Encapsulated *G. candidum* was coated with chitosan. Chitosan solution (0.4% w/v) was prepared as mentioned above. Then, 40 mL of $CaCl_2.\ 2H_2O$ solution (0.1M) was mixed with 60 mL chitosan solution, to yield cationic solutions (gelling solution) of chitosan in $CaCl_2$ (Zhou et al. 1998; Azarnia et al., 2008). 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 (Thomas Scientific, USA) set at uniform speed (90 rpm). Afterward, the mixture of *G. candidum* with polymer was taken in a sterile syringe (10mL) with 20G needle and drop-wise poured into a cationic gelling solution containing chitosan in $CaCl_2.\ H_2O$. Precautionary measure was taken and placed the syringe 50 cm away from the solution. For hardening, the resultant chitosan coated capsules were allowed to remain in solution, for further 30 min under gentle shaking. Subsequently, microcapsules coated by chitosan were filtered and washed with deionized water in order to remove excess of chitosan. The microcapsules were dried in petri dish and stored at 4°C until further use. Similar method was adopted for chitosan coating of alginate-starch (Alg-S) and alginate-xanthan (Alg-X) encapsulated *G. candidum.*

1. Coating with Nanoform of chitosan

Ionotropic gelation method reported by many investigators with a few modifications (Tsai et al., 2008; Fan et al., 2012) was used to prepare chitosan nanoparticles (NPs). Chitosan solution 0.4% (w/v) was passed through a syringe filter (Millipore, USA) to remove insoluble particles. Then, 200 mL of chitosan solution was heated in water bath for 10 min at 60°C. Afterwards, it was vigorously stirred at room temperature on a hot plate with magnetic stirrer (PC-420D, Corning®, Mexico) set at 700 rpm. Subsequently, under continuous shaking, 60 ml of (0.5% w/v) TPP aqueous solution, previously kept at 2-4°C was dropwise added (flow rate, 1 mL min^{-1}) ,into 200 mL of chitosan solution. The mixture remained on stirring for further 30 min and then sonicated for about 30 min. Resultant suspension was centrifuged at 12,000 rpm for 20 min. The supernatant was discarded and precipitate was washed with deionized water and centrifuged again twice at the same speed. The washed precipitate was suspended in ethanol and resultant suspension of chitosan NPs was used for coating of the *G. candidum* microcapsules prepared by various polymers. For coating same methodology was adopted as mentioned above.

Fourier transform infrared Spectroscopy (FT-IR)

Structural characterization of the uncoated microcapsules of g. candidum formed with alginate alone (Alg) and in combination with starch (Alg-S) and xanthan (Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and nano-chitosan (Alg-CN, Alg-S-CN, Alg-X-CN) was accomplished by Fourier transform infrared (FT-IR) spectroscopy using PerkinElmer® spectrum 65FT IR spectrometer.

Morphology of the uncoated and Chitosan coated *G. candidum* microcapsules

Shape, size and surface morphology of un-coated and chitosan coated microcapsules of *G. candidum* prepared by using natural polymers like alginate alone or in combination with starch, xanthan and chitosan was determined by using high performance scanning electron microscope (SEM) MIRA-3 (TescanFE, Brno, Czech Republic). The microcapsules were vacuum dried, put on an aluminum stub, sealed with a double-sided carbon conductive tape and then covered with a 10 nm thick layer of gold by using Sputter coater (Q150T Es- Quorum). Afterwards, SEM was conducted and images were taken.

Encapsulation efficiency (%)

To determine the encapsulation efficiency of chitosan coated/un-coated microcapsules of *G. candidum* prepared with alginate alone and in combination with starch and xanthan, a method reported by other investigators (Azarnia et al., 2008; Yasmin et al., 2018) was adopted. Briefly 0.1 g microcapsules (Alg, Alg-C, Alg-CN, Alg-S, Alg-S-C, Alg-S-CN, Alg-X, Alg-X-C, Alg-X-CN) with known concentration i.e., 10^9 $CFUg^{-1}$ were re-suspended separately in 10 mL of sterile sodium citrate (0.1 M, pH 7.0) and disintegrated by 30 min vortex at room temperature The released *G. candidum* cells were serially diluted with deionized water. Subsequently, an aliquot (100 µL) from each dilution was plated on Oxytetracycline glucose agar (OGA) and incubated at 30° for 48 hrs. After incubation, the probiotic yeast population was enumerated (log CFU g^{-1}) by using the colony counter (BC-50A Biobase, China). The encapsulation efficiency (%) of different microcapsules was calculated as:

$$EE\ (\%) = (N \div N0) \times 100$$

Where

N = No. of viable *G. candidum* cells released from the respective microcapsules,

N0 = No. of *G. candidum* cells (free) added in the respective polymer matrix during the encapsulation process.

All trials were carried out in triplicate, and results were the means of three observations (n=3)

Temperature tolerance

For determining the temperature tolerance of free/un-encapsulated *G. candidum* as well as chitosan coated and un-coated microcapsules of probiotic prepared by using different natural polymers like alginate alone or in combination with starch and xanthan, Dianawati et al. (2017) method was used. Briefly, 0.1 g microcapsules was added in 5mL of phosphate-buffered saline (PBS; pH 7.0) and incubated at 60°C for 60 min. in a preheated shaking water bath (ThermoPrecision™ SWB, Thermo Scientific)**.** Afterwards, tubes containing free and encapsulated *G. candidum* were removed from the water bath and placed at room temperature for 15 min. The released probiotic (*G. candidum*) was 10 times serially diluted and an aliquot of 100 µL of each dilution was plated on Oxytetracycline glucose agar (OGA) and incubated at 30° for 48 hrs. After incubation, viable cell count of free and encapsulated probiotics were determined by using standard plate count method mentioned above. All trials were carried out in triplicate, and results were the means of three observations (n=3)

pH Tolerance

A stock solution of phosphate buffered saline (PBS) was prepared and used for preparation of PBS of different pH i.e., 2, 4, 8 with the help of I M HCl and 0.1 M NaOH. After adjusting pH, PBS was filtered and autoclaved at 121°C. By following Castro-Cislaghi et al.(2012) method, free and encapsulated *G. candidum* with known initial counts were suspended in 5 mL PBS of different pH (2, 4 and 8). The suspended cells of free and encapsulated *G. candidum* were incubated at 27°Cin a preheated shaking water bath (Thermo Precision™ SWB, Thermo Scientific)**.** Aliquots of known volume from free and encapsulated *G. candidum* cells were taken twice at 60 min and 120 min intervals from different pH buffer and serially diluted to an appropriate concentration for plating. The viable cell counts from free and encapsulated probiotics were determined by adopting the procedure as mentioned above. All trials were carried out in triplicate, and results were the means of three observations (n=3)

Storage stability

Rosas-Ledesma et al. (2012) method was adopted for determining the effect of storage environment on the viable cell count of free/un-encapsulated and chitosan coated and un-coated microcapsules of *G. candidum* prepared by using different natural polymers like alginate alone or in combination with starch and xanthan. Briefly, coated and un-coated microcapsules were stored at controlled temperature, i.e., in a refrigerator (4°C) and at ambient room temperature (22-25°C) for 60 days. Samples for enumerating the probiotic viable count (CFU g^{-1}) were collected at two intervals i.e., after 30 days and 60 days. The collected sample (0.1 g) were re-suspended in 10 mL of sterile sodium citrate (0.1 M, pH 7.0) and disintegrated by 30 min vortex at room temperature. The released probiotic (*G. candidum*) was 10 times serially diluted and an

aliquot of 100 µL of each dilution was plated on Oxytetracycline glucose agar (OGA) and incubated at 30° for 48 hrs. After incubation, viable cell count (log CFU g^{-1}) of free and encapsulated probiotics were determined by using standard plate count method mentioned above.

Statistical analysis

All the results were summarized as mean± SD. Before using any statistical analysis, data was checked for frequency distribution by using Shapiro-Wilk and Bartlett's tests. One-way or two way ANOVA (on the basis of treatments/data) were used for comparison of different treatment groups at the significance level of 5 percent. Once difference was found, data was subjected to pairwise comparison using post hoc test such as Fisher's least significant differences test (LSD). Statistical software, SPSS version 21 (Chicago, Illinois, USA), GraphPad Prism 7 (San Diego, California, USA) and R Studio (Boston, Massachusetts, USA) were used for the statistical analysis and graphical explanation.

Results

Fourier transform infrared (FT-IR) Spectroscopy

Structural characterization of un-coated and chitosan coated microcapsules of *G. candidum* prepared by using natural polymers like alginate alone or in combination with starch and xanthan are presented in Fig. 2.1 –2.9.

In FT-IR spectra, the functional group regions, ranging between 3500 -1500 cm^{-1}. However, the disappearance of any peaks in FTIR spectra of the uncoated and chitosan coated microcapsules of *G. candidum* prepared with alginate alone and in combination with starch and xanthan indicated the interaction of polymer with core material (probiotic).

The FT-IR spectra of alginate encapsulated *G. candidum* (Alg) (Fig. 2.1) , peak around 1700 cm^{-1} indicated the presence of carboxylic group (–COOH) of alginate. Further, absorption peak of alginate at 3431 cm^{-1} was due to the OH– stretching. This also confirmed the presence of alginate.

Presence of peaks around 3000 cm^{-1} —3100 cm^{-1} in alginate microcapsules of *G. candidum* coated with chitosan (Alg-C) indicated the stretching vibrations due to overlapping of the O–H (of alginate) and N–H of chitosan. An additional peak at 3400 cm^{-1} indicated the presence of N-H group of chitosan (Fig. 2.2). Few peaks at 3200-3300 also indicated the overlapping interaction between hydrogen bonded (O-H) and amine (NH_2) group which are characteristic functional group of alginate and chitosan respectively.

A broad and intense peak at about 3400 cm^{-1}, indicated the formation of new hydrogen bonds, between –OH and –NH_2 groups in chitosan and carboxylic (–COOH) group of sodium alginate. It was present in alginate microcapsules of *G. candidum* with nanoform of chitosan

(Alg-CN) indicated the presence of N-H derivative which confirmed the complex formation between alginate and nano-chitosan (Fig 2.3). Furthermore peak at 1700 $cm^{-1}$was detected due to the presence of carboxylic group (-COOH).

Moreover the presence of peak at 1600 cm^{-1}in spectra of both Alg-C and Alg-CN microcapsules, represented the presence of amidic group between chitosan and alginate which confirmed the synthesis of alginate- chitosan complex. Presence of peak at 1384 cm^{-1} is due to NH_3^+-COO^- interaction.

Fig. 2.4 shows the appearance of peak at 2900 cm^{-1} indicated the presence of alkyl group (-R) which confirms the starch presence in Alg-S microcapsules of *G. candidum*. Less peaks with broader area were observed in alginate- starch microcapsules. Peaks around 1700 cm^{-1} was due to the presence of carboxylic group (-COOH) of alginate present in alginate-starch microcapsules.

FT-IR spectrum shown in Fig. 2.5 illustrated the alginate-starch microcapsules of *G. candidum* with chitosan coat (Alg-S-C), a broad peak at around 3200 cm^{-1} to 3500 $cm^{-1}$was clearly shown which is due to -OH group, while a sharp peak at 3400 cm^{-1} is due to NH— derivative of chitosan. Smaller peaks were also observed which showed the presence of alginate-starch complex formation with chitosan coat. Fewer peaks were present around 1300 cm^{-1} which was due to NH^+_3—COO^- which confirmed the presence of alginate, starch and chitosan complex.

FT-IR spectra of alginate-starch-chitosan nano-chitosan (Alg-S-CN) microcapsules of *G. candidum* illustrated very sharp and many additional peaks due to strong interaction between alginate-starch and nano-chitosan (Fig 2.6). A number of peaks were observed between 1100 to

1300 cm $^{-1}$ while presence of peak at 1109 cm $^{-1}$ was indicative of three linkage C-O-C whereas peak at 1700 cm^{-1} corresponds to carboxylic group (-COOH) and around 1600 cm^{-1} was the indicative of amidic group. Peak at 1383 cm $^{-1}$ is due to NH^{+}_{3}—COO^{-} which confirmed the presence of strong interaction between alginate-starch and nanoform of chitosan. An additional peak at 3378 cm^{-1} (Fig 2.6) was due to the presence of —NH derivative which corresponds to complex formation between alginate-starch core and nanoform of chitosan coating.

FT-IR spectrum of alginate-xanthan (Alg-X) microcapsules of *G. candidum* is shown in Fig. 2.7. one peak around 3300 cm^{-1} to 3500 cm^{-1} which was due to the presence of –OH group of alginate while an additional peak showed the presence of Carboxylic group (-COOH) around 1700 cm^{-1} confirms the alginate presence in alginate-xanthan microcapsules. Additionally xanthan gum presence was evident through ketone group (R-C=O-R) appearance on FT-IR spectra around 1600 cm^{-1} which established due to the synthesis of complex formation between alginate and xanthan.

In Fig. 2.8 alginate-xanthan microcapsules of *G. candidum* coated with chitosan (Alg-XC) showed the amidic (O=C-NH_2) group presence, while chitosan was confirmed with peak at 1728 cm^{-1} whereas xanthan presence was recognized by kenotic group (R-C=O-R) around 1690 cm^{-1}. This confirms the synthesis of chitosan complex with alginate and xanthan which was mandatory for the successful coating of alginate-xanthan microcapsules with chitosan coat.

Alginate xanthan microcapsules of *G. candidum* with nano-chitsoan (Alg-X-CN) depicted the strong complex formtion due to the presence of nanoform of chitsoan with additional absorption spectrum of various functional groups such as peaks at 2200-2100 cm^{-1} due to C≡C stretching and few bands at 1650-1700 cm^{-1} due to presnece of acidic group (Fig. 2.9). Xanthan presence was evident from the peak obtained near 1690 cm^{-1} which indicated the

ketonic group presences while peaks around 1700- 1728 cm^{-1} confirmed the presence of amidic group of chitosan. A sharp peak at 3378 showed the presence of (OH— group) of alginate. Additionaly ketonic group presence also confirm the strong interaction between alginate-xanthan and nanoform of chitosan.

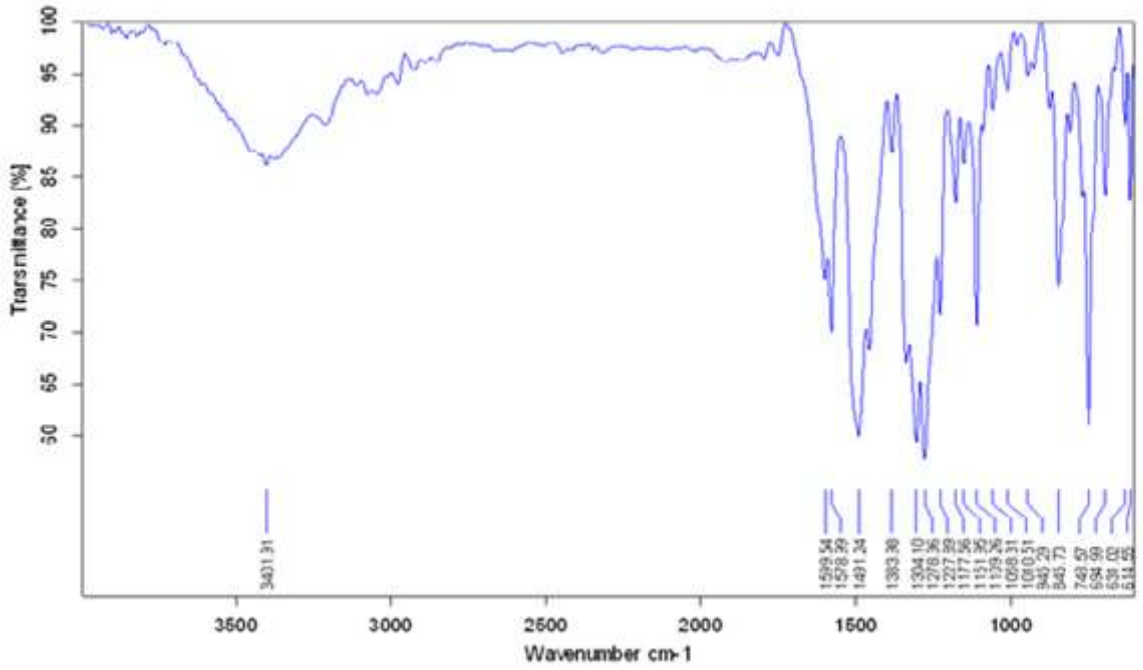

Fig. 2.1. FT-IR spectra of alginate (Alg) microcapsules of *G. candidum* showing absorption spectrum of different functional groups

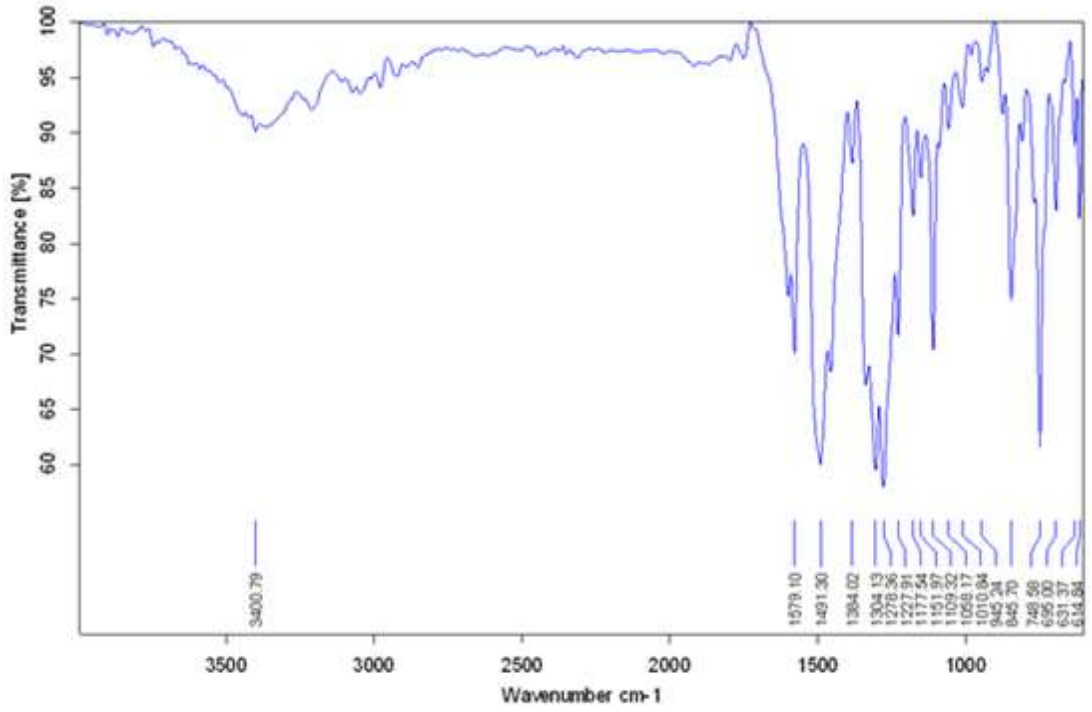

Fig. 2.2. FT-IR spectra of alginate encapsulated and coated with chitosan (Alg-C) microcapsules of *G. candidum* showing absorption spectrum of different functional groups

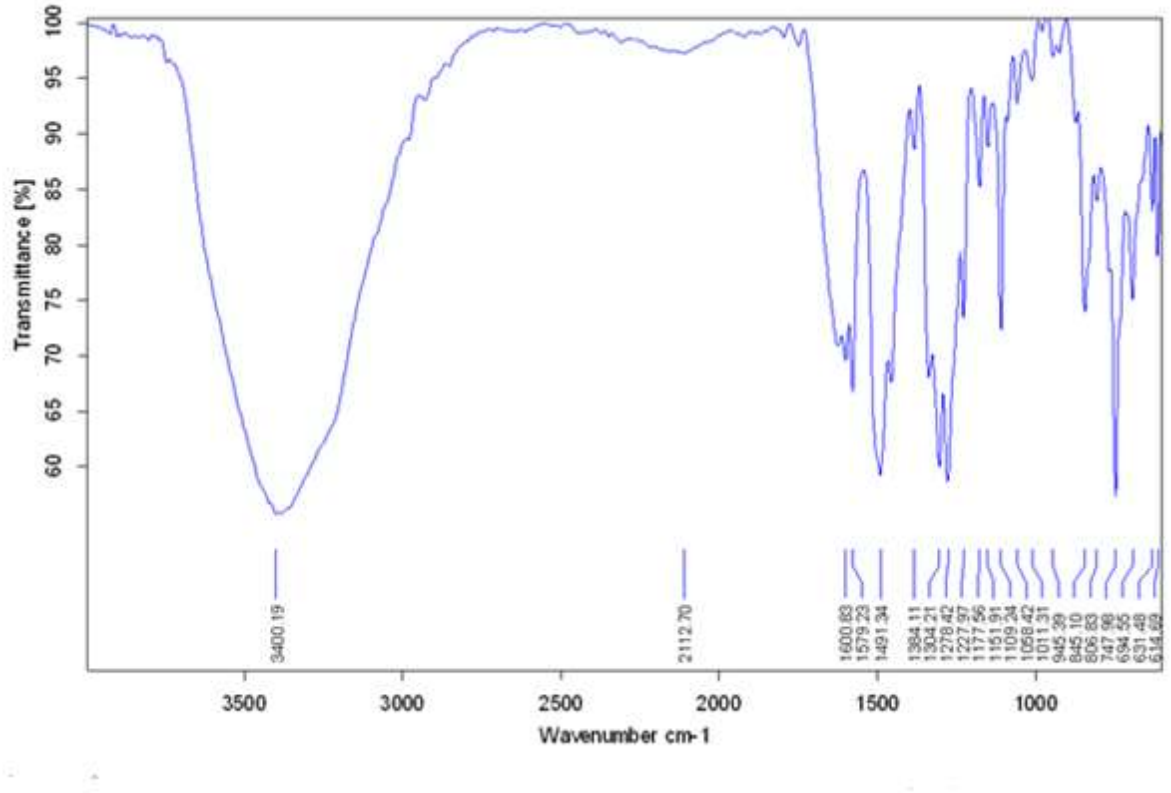

Fig. 2.3. FT-IR spectra of alginate encapsulated and coated with nano-chitosan (Alg-CN) microcapsule of *G. candidum* showing absorption spectrum of different functional groups

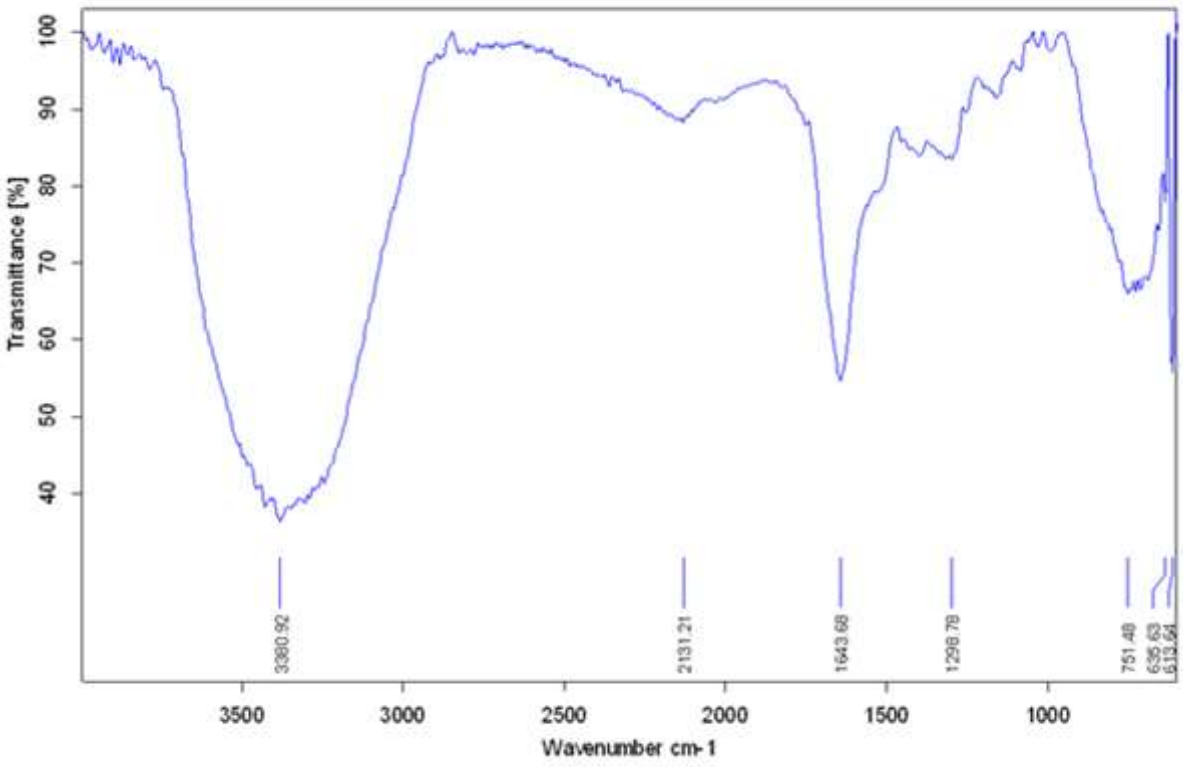

Fig. 2.4. FT-IR spectra of alginate-starch encapsulated (Alg-S) microcapsule of *G. candidum* showing absorption spectrum of different functional groups

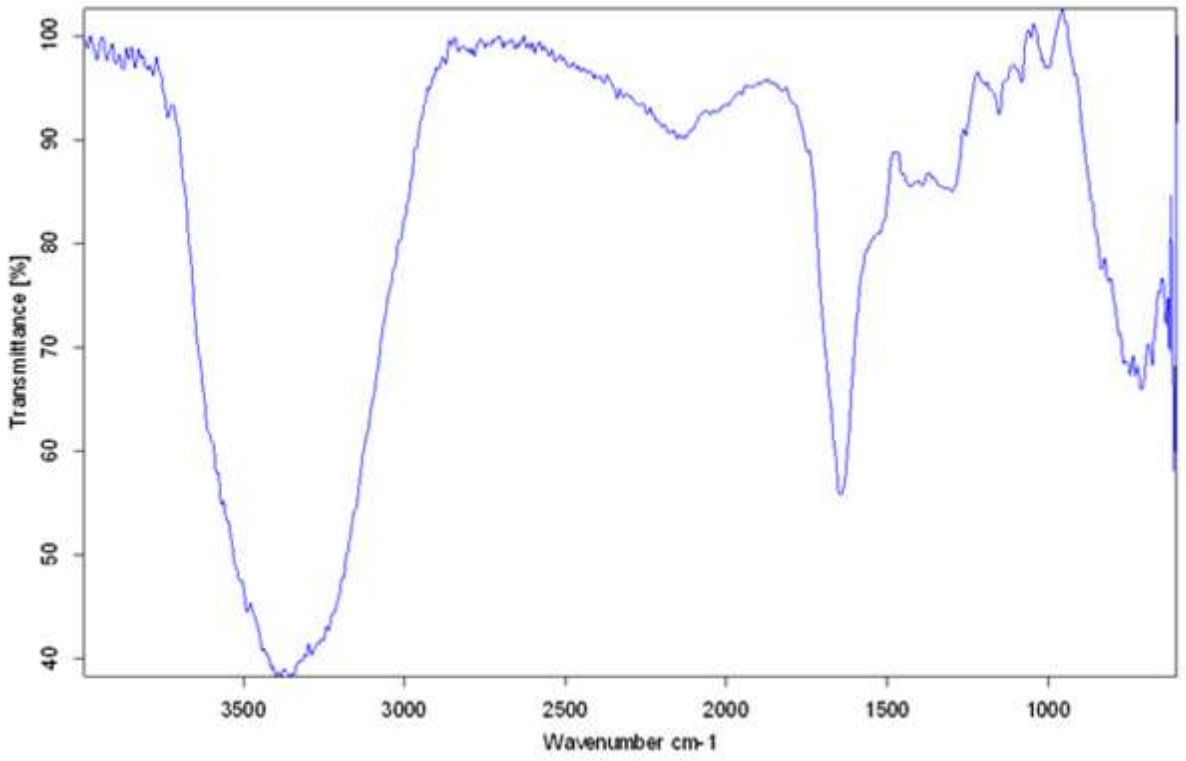

Fig. 2.5. FT-IR spectra of alginate-starch encapsulated and coated with chitosan (Alg-SC) microcapsule of *G. candidum* showing absorption spectrum of different functional groups

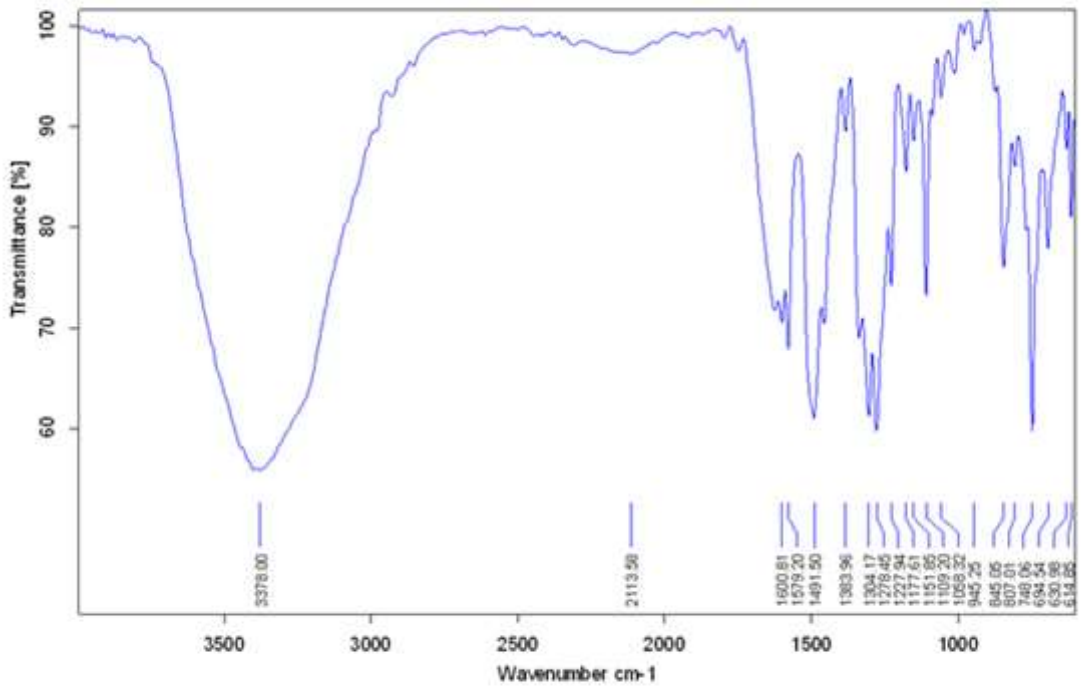

Fig. 2.6. FT-IR spectra of alginate-starch- encapsulated and coated with nano-chitosan (Alg-S-CN) microcapsule of *G. candidum* showing absorption spectrum of different functional groups

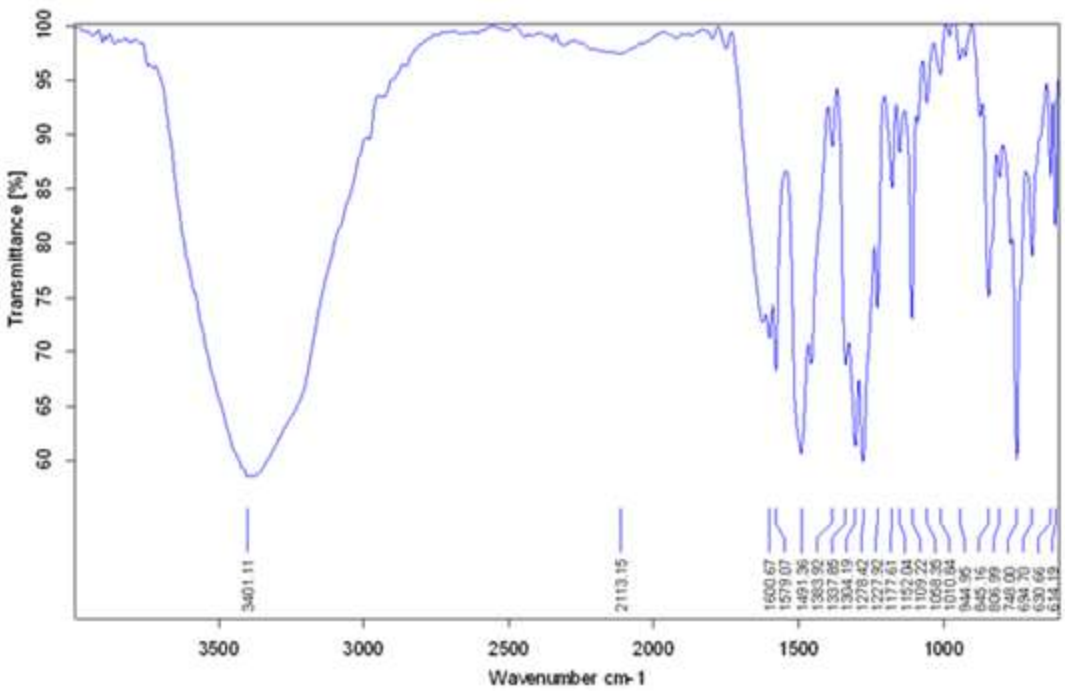

Fig. 2.7. FT-IR spectra of alginate-xanthan encapsulated (Alg-X) microcapsule of *G. candidum* showing absorption spectrum of different functional groups

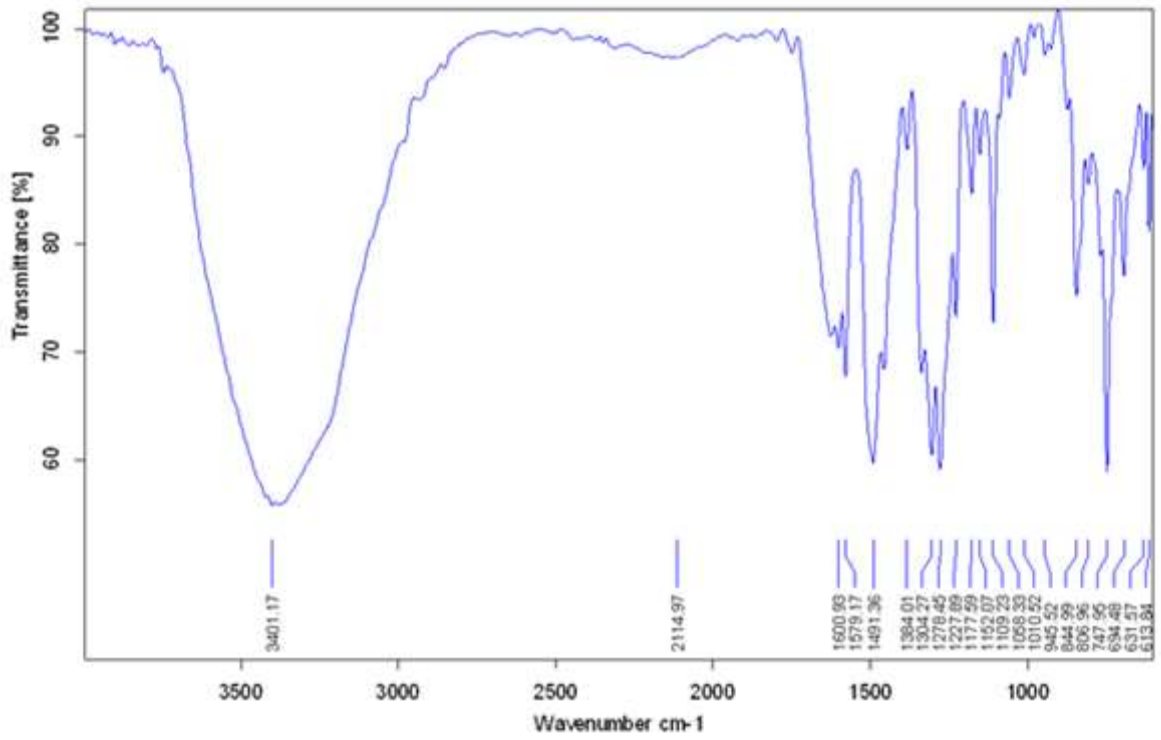

Fig. 2.8. FT-IR spectra of alginate-xanthan encapsulated and coated with chitosan (Alg-XC) microcapsules of *G. candidum* showing absorption spectrum of different functional groups

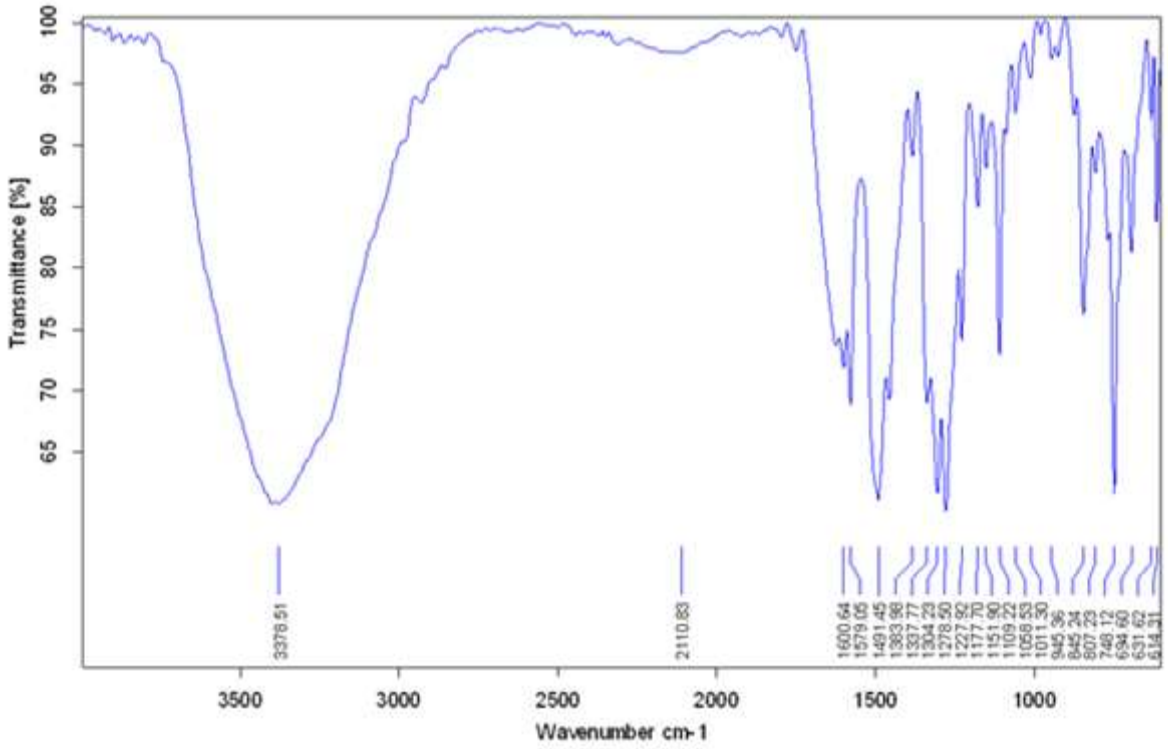

Fig. 2.9. FT-IR spectra of alginate-xanthan encapsulated and coated with nano-chitosan. (Alg-X-CN) microcapsules of *G. candidum* showing absorption spectrum of different functional groups

Surface morphology

The scanning electron microscopic, (SEM), images showing the surface morphology of un-coated and chitosan coated microcapsules of *G. candidum* prepared by alginate alone or in combination with starch and xanthan are presented in Fig. 2.10-2.12. The shape and size of the uncoated, coated microcapsules was variable with a mean diameter of about 125-165µm (Table 2.1).

The alginate (Alg) microcapsules appeared irregular in shape with rough uneven, swollen surface (Fig. 2.10 a), while chitosan coated microcapsules (Alg-C and Alg-CN) were spherical in shape with smooth surface without any cavities and cracks. The closer observation revealed that Alg-CN (encapsulated with alginate and coated with nano-chitosan) are more spherical in shape with smooth surface (Fig. 2.10b and 2.10c).

The Alginate-starch microcapsule (Alg-S) of *G. candidum* was also irregular in shape with wrinkles, pores and large cavities on the surface (Fig. 2.11 d). However, chitosan and chitosan NPs coated Alginate-starch microcapsules (Alg-SC and Alg-S-CN) were somewhat spherical in shape with smooth surface (Fig. 2.11 e and f). Again nano-chitosan coated microcapsules appeared more regular in shape (spherical) with smooth and shiny surface.

Scanning electron photomicrographs of Alginate- Xanthan microcapsules of *G. candidum* are shown in Fig. 2.12. The Alginate-xanthan (Alg-X) microcapsules were irregular in shape with rough/ bulging surface (Fig. 2.12 g), while chitosan coated Alginate-Xanthan, microcapsules (Alg-XC) showed an improved surface morphology (Fig. 2.12 h). However microcapsule's surface was not smooth although they were opaque in color as compared to other forms of microcapsules. Furthermore alginate-xanthan microbeads with nano-form chitosan

coating (Alg-X-CN) resulted in an improvement in texture of the microcapsules along with smooth, non-porous and opaque *G. candidum* microcapsules (Fig. 2.12 i).

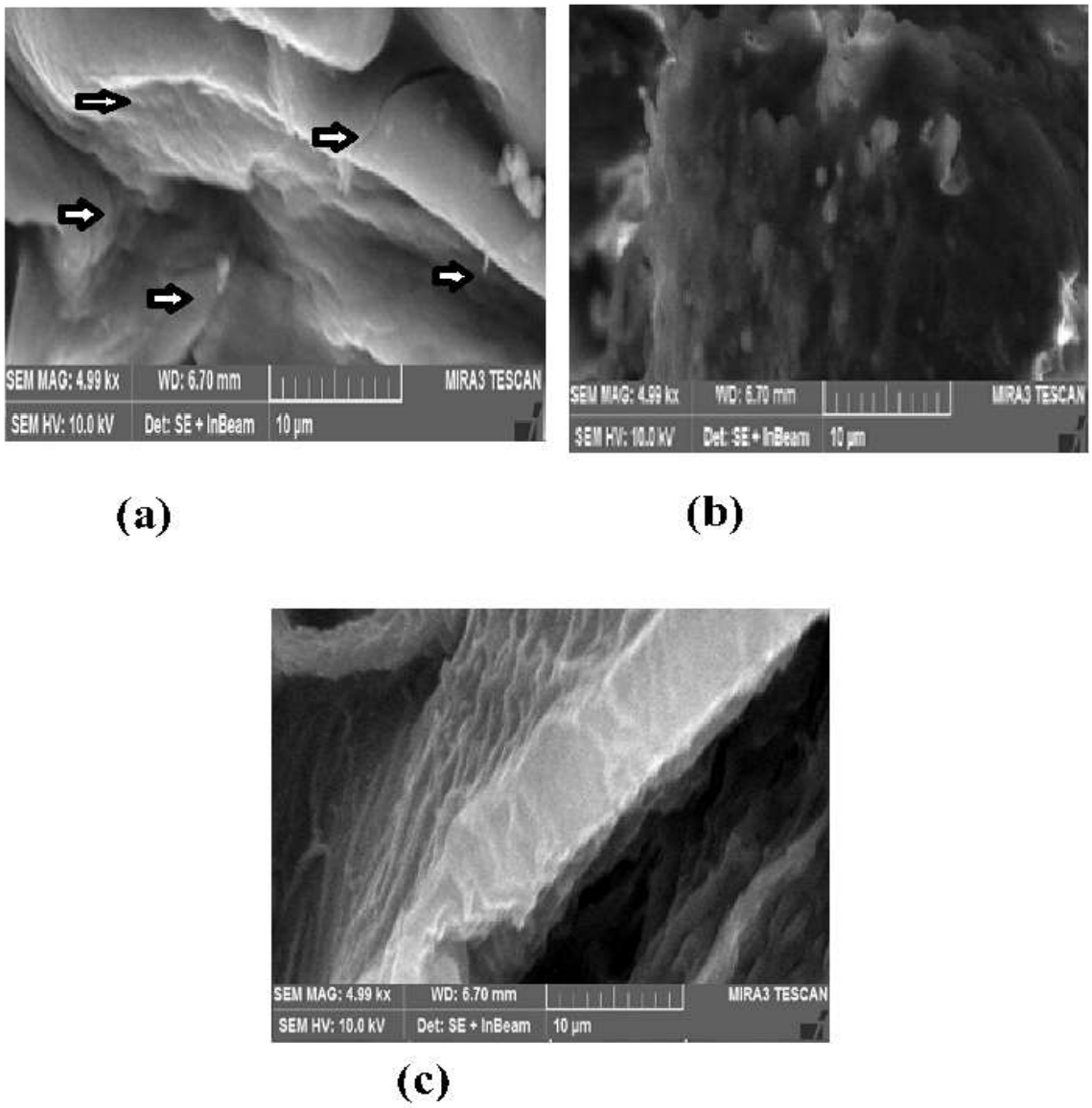

Fig. 2.10. Scanning electron photomicrograph of (a) alginate encapsulated (b) alginate encapsulated and coated with chitosan(c) alginate encapsulated and coated with nano-chitosan microcapsules of *G. candidum.*

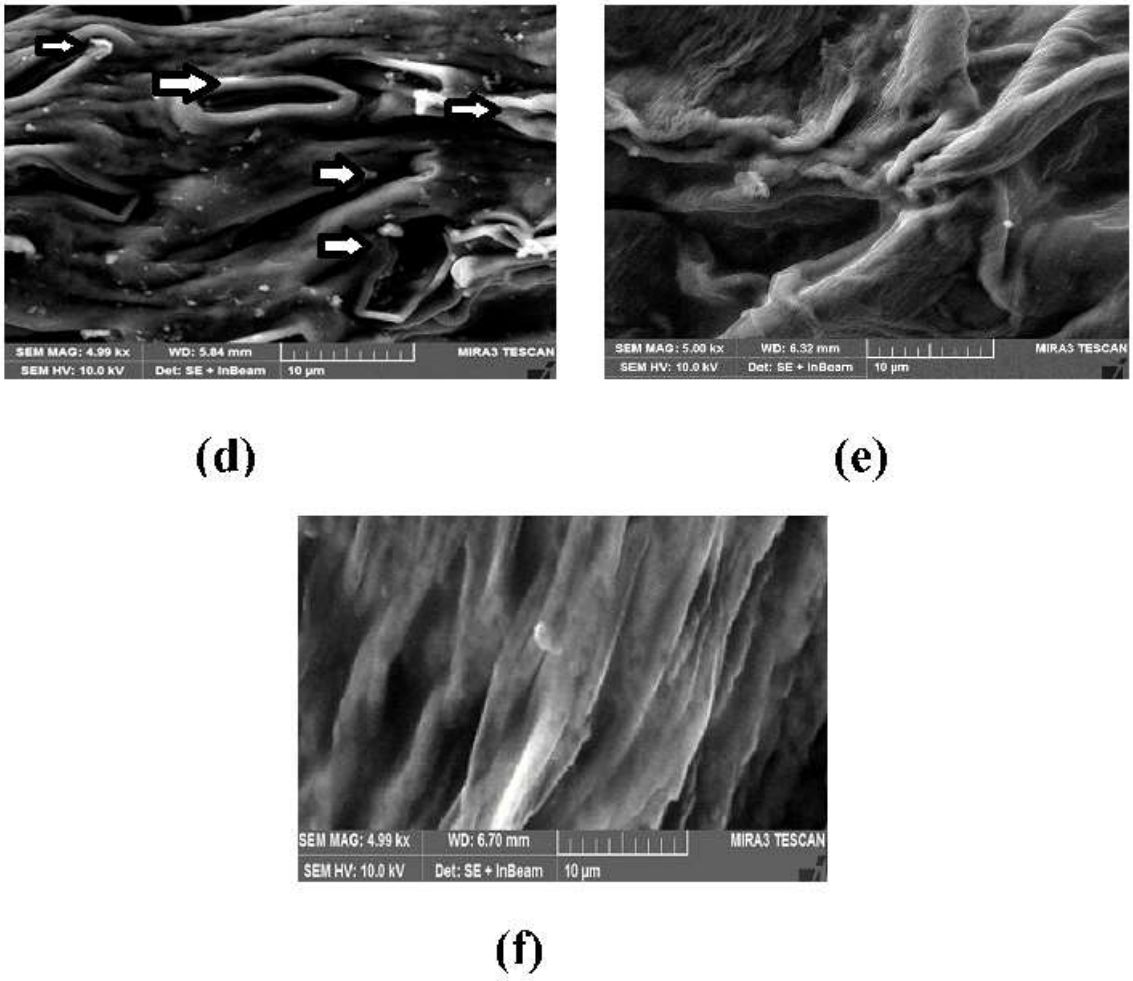

Fig. 2.11. Scanning electron photomicrograph of (d) alginate-starch encapsulated (e) alginate-starch encapsulated and coated with chitosan (f) alginate-starch- encapsulated and coated with nano-chitosan microcapsules of *G. candidum.*

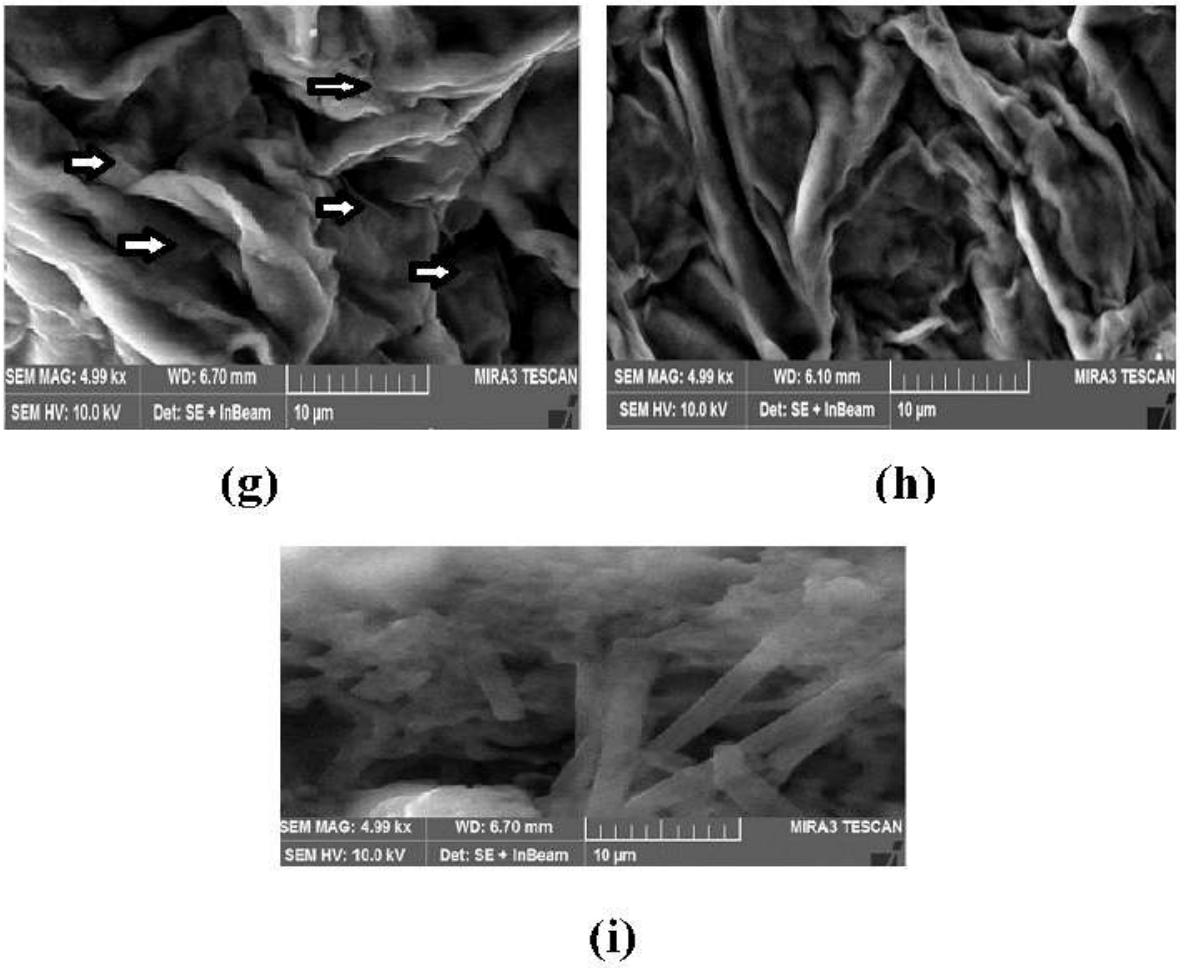

Fig. 2.12. Scanning electron photomicrograph of (g) alginate-xanthan encapsulated (h) alginate-xanthan encapsulated and coated with chitosan (i) alginate-xanthan encapsulated and coated with nano-chitosan microcapsules of *G. candidum.*

Encapsulation/coating efficiency (EE) (%)

The EE (%) of un-coated and chitosan coated microcapsules of *G. candidum* prepared by alginate alone or in combination with starch and xanthan is presented in Table 2.2 and Fig. 2.13. One way ANOVA revealed a significant difference in encapsulation efficiency (n=3, ANOVA, $F_{8, 27}$= 14, p= 0.001, Table 2, Fig.13). The Post hoc LSD test showed higher efficiency of microcapsules of *G. candidum* where alginate was used in combination with other polymer in contrast to alone for encapsulation. Furthermore, pairwise comparison indicated the significantly higher EE (%) of chitosan coated as compared to un-coated microcapsules. Additionally, EE (%) further improved when nano-chitosan was used as coating material (Table 2.2). The Alg-CN microcapsules showed the highest EE followed by Alg-C and Alg-X –CN microcapsules.

Temperature tolerance

The viable cell count of *G. candidum* before incubation and after 60 min incubation at 60ºC is presented in Table 2.3.2. Temperature treatment showed significant variable effect on the % survival of free, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone and in combination with starch and xanthan (Table 2.3.2). Two way ANOVA showed significant effect of time (ANOVA, $F_{1, 60}$=1430, p<0.001, table 2.3.1) and encapsulating/coating formulation (ANOVA, $F_{9, 60}$=65.4, p<0.001, Table 2.3.1). Moreover significant interaction between time × encapsulating/coating formulation at fixed temperature i.e., 60ºC indicated the combined effects of both variable on % survival of *G. candidum* (ANOVA, $F_{9, 60}$=58.4, p<0.001, Table 2.3.1). Overall results indicated the highest % survival of *G. candidum* in alginate-nano-chitosan (Alg-CN) while free *G. candidum* showed the lowest value after 60 min incubation at 60ºC

Table 2.1. Size of chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan

Microcapsule formulation	Size (µm)
Alg	125±25
Alg-C	136±15
Alg-CN	131±12
Alg-S	142±19
Alg-S-C	153±22
Alg-S-CN	149±20
Alg-X	148±24
Alg-X-C	165±32
Alg-X-CN	155±10

Values are represented as mean± SD (n=12). Alg, alginate encapsulated; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

Table 2.2. Encapsulation efficiency (%) of chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan

Microcapsule formulation	*Encapsulation efficiency (%)
Alg	83.5 ± 2.75^{e}
Alg-C	96.6 ± 1.57^{ab}
Alg-CN	99.3 ± 0.45^{a}
Alg-S	90.4 ± 2.91^{d}
Alg-S-C	$93.8^{d}\pm1.32^{bc}$
Alg-S-CN	94.9 ± 1.57^{bc}
Alg-X	91.7 ± 2.28^{cd}
Alg-X-C	94.6 ± 2.86^{bc}
Alg-X-CN	96.03 ± 1.88^{ab}

* viable cell count

Values are represented as mean± SD (n=3).ANOVA followed by LSD showed comparisons among various microcapsules of *G. candidum*. Means with different lowercase superscript letters (abc) differs significantly ($p<0.05$) in columns.; Alg, alginate encapsulated ;Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ;Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ;Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

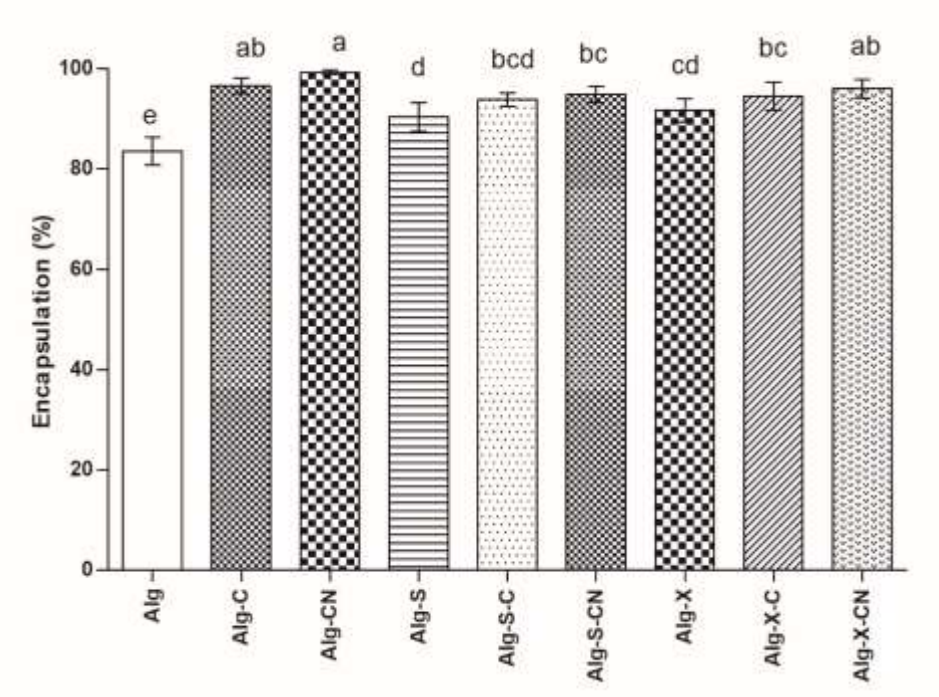

Fig. 2.13. Encapsulation efficiency (%) of chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan

The above bars shows the values as mean± SD (n=3). ANOVA followed by LSD showed comparisons among various microcapsules of *G. candidum*. Means with different lowercase superscript letters (abc) differs significantly ($p<0.05$); Alg, alginate encapsulated ;Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ;Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ;Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

Table 2.3.1. Summary of ANOVA comparing the survival (%) of chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan exposed to higher temperature (60°C) for 60 min

Source of variation	Df	SS	MS	F	P
Time	1	144.02	144.02	1430	<0.001
*Treatment	9	59.28	6.58	65.43	<0.001
Time× Treatment	9	52.93	5.88	58.42	<0.001

*Encapsulating/coating formulation

Table 2.3.2 Survival (%) of free and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at 60°C

Encapsulating /coating formulation	Viable *G. candidum* cells (log CFU g^{-1})		
	0 min	60 min	Survival (%)
Free-Geo	9.73 ± 0.23^{A}	$1.79\pm0.43^{j,B}$	18.5 ± 4.98^{g}
Alg	9.2 ± 0.26^{A}	$4.93\pm0.21^{I,B}$	53.6 ± 0.8^{f}
Alg-C	9.71 ± 0.09^{A}	$8.33\pm0.17^{b,B}$	85.7 ± 1.93^{a}
Alg-CN	9.88 ± 0.1^{A}	$9.05\pm0.55^{a,B}$	91.6 ± 6.26^{a}
Alg-S	9.26 ± 0.27^{A}	$5.64\pm0.53^{gh,B}$	60.8 ± 4.37^{ef}
Alg-S-C	9.38 ± 0.13^{A}	$6.43\pm0.47^{ef,B}$	68.5 ± 4.99^{cd}
Alg-S-CN	9.41 ± 0.10^{A}	$6.91\pm0.23^{de,B}$	73.4 ± 2.71^{bc}
Alg-X	9.28 ± 0.25^{A}	$6.11\pm0.51^{fg,B}$	65.9 ± 6.92^{de}
Alg-X-C	9.49 ± 0.19^{A}	$7.26\pm0.26^{ced,B}$	76.5 ± 4.21^{b}
Alg-X-CN	9.56 ± 0.16^{A}	$7.47\pm0.39^{c,B}$	78.1 ± 2.96^{b}

Values are presented as mean ± SD (n=3). ANOVA followed by LSD show comparisons between groups (within the column) and within the group (within the row). Means sharing different lowercase superscript letters (abc) within the column and uppercase letter (ABC) in row indicate significant differences ($P < 0.05$). Free Geo, un-encapsulated, Alg, alginate encapsulated ; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

Effect of different pH

Tolerance to harsh environmental conditions is a very critical requirement for the encapsulated microorganisms. The efficacy of free and encapsulated *G. candidum* was observed at three different pH (2, 4 and 8).

Viability of free and encapsulated *G. candidum* at pH 2

Free/unencapsulated and encapsulated *G. candidum* exposed to acidic pH 2 for duration of 120 min caused significant change in the number of viable cells (Table 2.4.2). Two way ANOVA revealed that at pH 2, viable counts were significantly affected by exposure time (n=3, ANOVA, $F_{2, 18}$= 510, $p<0.001$, Table 2.4.1, Fig 2.14) and encapsulating/coating formulation (n=3, ANOVA, $F_{9, 18}$= 125, $p<0.001$, Table 2.4.1). Furthermore a significant interaction between exposure time× encapsulating/coating formulation (n=3, ANOVA, $F_{2, 18}$=33.6, $p<0.001$, Table 2.4.1) indicated the combined effects of both factors on viable cells counts of *G. candidum*.

The Post hoc LSD test revealed the decrease in viable cell counts of free and encapsulated *G. candidum* with an increase in time. Moreover, higher viable counts were obtained from alginate -nano-chitosan coated (Alg-CN, Alg-C and Alg-X-CN respectively) as compared to uncoated microcapsules (Table 2.4.2, Fig. 2.14). Furthermore, un-encapsulated (free) *G. candidum* showed the lowest viable cell counts at both duration (60 and 120 min).

Viability of free and encapsulated *G. candidum* at pH 4

Free/unencapsulated and encapsulated *G. candidum* exposed to pH 4 resulted in a significant change in number of viable cells of *G. candidum* (Table 2.5.2). Two way ANOVA revealed that at pH 4, viable cell counts were significant affected by exposure time (n=3, ANOVA, $F_{2, 18}$=28.4, $p<0.001$, Table 2.5.1, Fig. 2.15) and encapsulating/coating formulation (n=3, ANOVA, $F_{9, 18}$=25.1, $p<0.001$, Table 2.5.1). Furthermore significant interaction between exposure time×

treatment (ANOVA, $F_{2, 18}$=7.37, p<0.001, Table 2.5.1) revealed the manner in which viable cell counts were influenced by both factors. Post hoc test LSD showed that cell counts were significantly reduced in free form as compared to encapsulated forms.

Furthermore after 60 min duration Alg-CN showed higher number of viable cells while Alg-C, Alg-XCN, Alg-X-C and Alg-SCN showed statistically comparable (p>0.01) and lower number of viable *G. candidum* cells. Moreover, after 120 min, *G. candidum* microcapsules viz. Alg-CN, Alg-C, Alg-XCN and Alg-XC remained stable in terms of total cell counts but Alg-S-CN, Alg-X, Alg-SC, Alg-S and Alg respectively showed a slight decline in viable cell counts. However free cells of *G. candidum* showed a sharp decline (about 4 log reduction) after 120 min at pH 4 (Table 2.5.2, Fig. 2.15).

Viability of free and encapsulated *G. candidum* at pH 8

Free/unencapsulated and encapsulated *G. candidum* kept for 30 and 60 min pH 8 showed a significant change in viable number of cells (Table 2.6.2). Two way ANOVA showed that at pH 8, the viable number of cells were affected significantly by the exposure time (n=3, ANOVA, $F_{2, 18}$=73.3, p<0.001, Table 2.6.1, Fig. 2.16) and encapsulating/coating formulation (n=3, ANOVA, $F_{9, 18}$=79.3, p<0.001, Table 2.6.1, Fig. 2.16). Furthermore, significant interaction between exposure duration×encapsulating/coating formulation (n=3, ANOVA, $F_{2,18}$ =27.4, p<0.001, Table 2.6.1) revealed the manner in which the viable cell counts were affected by the combined effect of both variables. The Post hoc LSD test showed that both factors (duration and encapsulating/coating formulation) caused a significant reduction in the total number of alive yeast cells. After 60 min exposure at pH 8, all microcapsules except alginate encapsulated, showed statistically comparable viable cell counts. However, free/unencapsulated *G. candidum* showed a sharp reduction in the total number of viable cells. Moreover, after 120 min, Alg-CN

microcapsules of *G. candidum* showed highest number of viable cells, while free/unencapsulated probiotic showed lowest viable counts (Table 2.6.2).

Comparison of pH conditions, exposure duration and treatments

Comparative effect of independent variables, pH (Fig. 2.17) and exposure time (Fig. 2.18) indicated that extreme condition like pH 2 and prolong exposure, i.e., 120 min showed the most significant effect on viable counts of chitosan coated and uncoated microcapsules of *G. candidum* prepared with alginate alone and in combination of starch and xanthan (Fig. 2.20). Exposure of pH 4 and 8 for short, duration, i.e., 30 min showed a relatively stable number of viable cells of *G. candidum* (Fig. 2.20). Moreover, at all pH and exposure periods, higher (above 8 log CFU/g) number of viable cells were observed in encapsulated as compared to un-encapsulated (free) *G. candidum,* and in chitosan coated in contrast to uncoated microcapsules (Fig. 2.19). The maximum viable counts were observed in nano-chitosan coated in contrast to chitosan coated microcapsules.

Furthermore, two way ANOVA with significant interaction between encapsulating/coating formulation × exposure time at three pH (ANOVA, $F_{18, 238}$= 8.65, $p<0.001$, Table 2.7, Fig. 2.22) as well as between encapsulating/coating formulation × pH at all exposure periods (ANOVA, $F_{20, 238}$= 5.03, $p<0.001$, Table 2.8, Fig. 2.21) also indicated the combined effect of all factors on the viable cell counts of *G. candidum*.

Table 2.4.1 Summary of ANOVA comparing the viable cell counts (log CFU g^{-1}) of free, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at pH 2

Source of variation	Df	SS	MS	F	P
Time	2	143.03	71.51	510.857	<0.001
*Treatment	9	157.63	17.51	125.112	<0.001
Time: Treatment	18	84.87	4.71	33.68	<0.001

*encapsulating/coating formulation

Table 2.4.2. Effect of pH 2 on viable cell count (log CFU g^{-1})of free and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at two interval

Encapsulating/ Coating formulation	Viable *G. candidum* cells (log CFU g^{-1})		
	0 min	60 min	120 min
Free-Geo	$9.52\pm0.28^{abc,A}$	$3.48\pm0.42^{f,B}$	$2.56\pm0.53^{c,C}$
Alg	$9.07\pm0.15^{bc,A}$	$6.95\pm0.20^{e,B}$	$3.5\pm0.65^{bc,C}$
Alg-C	$9.66\pm0.15^{ab,A}$	$9.36\pm0.21^{ab,A}$	$8.68\pm0.75^{a,A}$
Alg-CN	$9.94\pm0.07^{a,A}$	$9.76\pm0.11^{a,B}$	$8.97\pm0.57^{a,A}$
Alg-S	$9.04\pm0.29^{bc,A}$	$7.59\pm0.26^{de,B}$	$3.76\pm0.39^{bc,C}$
Alg-S-C	$9.38\pm0.13^{abc,A}$	$8.34\pm0.26^{cd,B}$	$7.61\pm0.40^{a,C}$
Alg-S-CN	$9.49\pm0.15^{abc,A}$	$8.63\pm0.38^{bc,B}$	$7.73\pm0.56^{a,C}$
Alg-X	$9.17\pm0.22^{bc,A}$	$7.65\pm0.25^{de,B}$	$4.5\pm0.45^{b,C}$
Alg-X-C	$9.45\pm0.28^{abc,A}$	$8.81\pm0.34^{bc,B}$	$7.84\pm0.73^{a,C}$
Alg-X-CN	$9.60\pm0.18^{abc,A}$	$9.20\pm0.25^{ab,B}$	$8.42\pm0.52^{a,C}$

Values are presented as mean ± SD (n=3). ANOVA followed by LSD show comparisons between groups (within the column) and within the group (within the row). Means sharing different lowercase superscript letters (abc) within the column and uppercase letter (ABC) in row indicate significant differences ($P < 0.05$). Free Geo, un-encapsulated, Alg, alginate encapsulated ; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

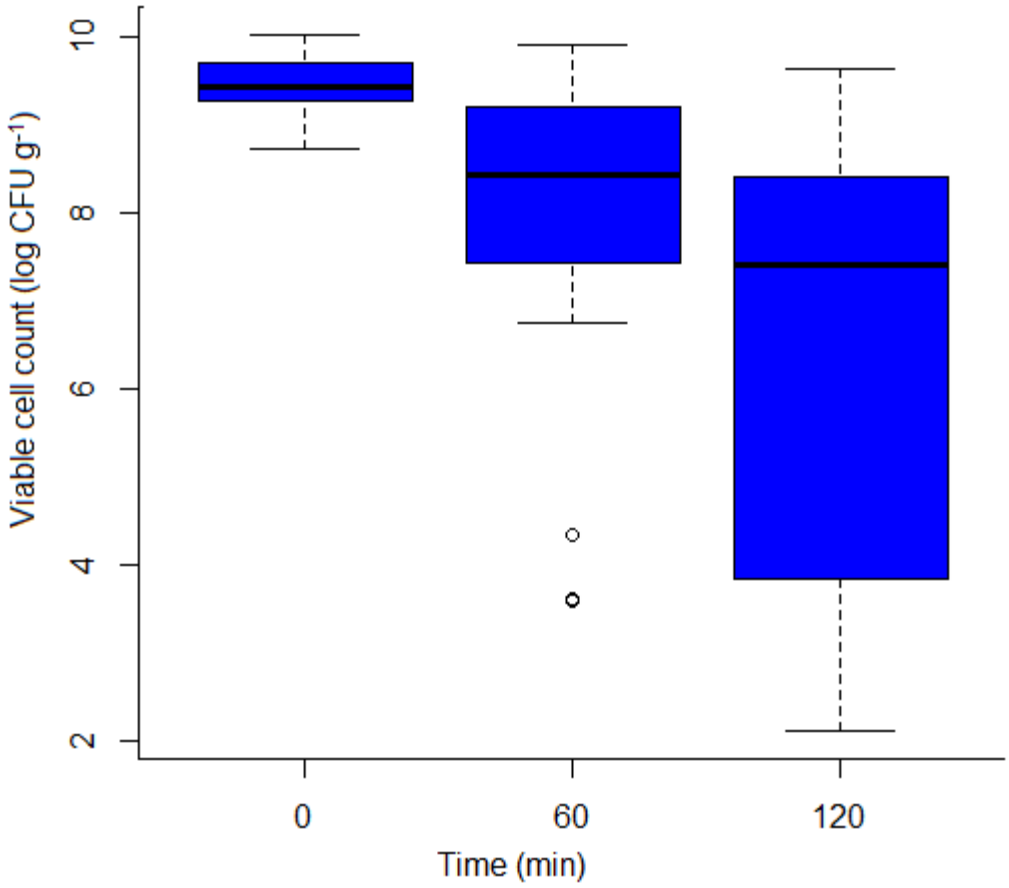

Fig. 2.14. Effect of exposure period on viable cell counts (log CFU g^{-1}) of *G. candidum* at pH 2

Table 2.5.1 Summary of ANOVA comparing the viable cell count (log CFU g^{-1}) of free, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at pH 4

Source of variation	Df	SS	MS	F	P
Time	2	8.391	4.195	28.42	<0.001
*Treatment	9	33.429	3.71	25.16	<0.001
Time× Treatment	18	19.58	1.08	7.37	<0.001

*Encapsulating/coating formulation

Table 2.5.2 Effect of pH 4 on viable cell count (log CFU g^{-1}) of free/unencapsulated, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at two interval

Encapsulating/ Coating formulation	Viable *G. candidum* cells (log CFU g^{-1})		
	0 min	60 min	120 min
Free-Geo	$9.52\pm0.28^{ab,A}$	$7.38\pm0.59^{e,B}$	$5.68\pm1.08^{c,C}$
Alg	$8.45\pm0.49^{c,A}$	$7.98\pm0.76^{de,B}$	$7.45\pm0.35^{b,C}$
Alg-C	$9.61\pm0.16^{ab,A}$	$9.49\pm0.08^{ab,B}$	$9.31\pm0.1^{a,C}$
Alg-CN	$9.94\pm0.06^{a,A}$	$9.8\pm0.10^{a,B}$	$9.48\pm0.52^{a,C}$
Alg-S	$9.38\pm0.13^{ab,A}$	$8.4\pm0.51^{cde,B}$	$7.81\pm0.53^{b,C}$
Alg-S-C	$9.04\pm0.29^{bc,A}$	$8.34\pm0.11^{cde,B}$	$8.20\pm0.42^{b,C}$
Alg-S-CN	$9.49\pm0.15^{ab,A}$	$8.79\pm0.12^{abcd,B}$	$8.69\pm0.35^{b,C}$
Alg-X	$9.17\pm0.22^{bc,A}$	$8.58\pm0.2^{bcd,B}$	$8.45\pm0.35^{b,C}$
Alg-X-C	$9.45\pm0.28^{ab,A}$	$9.06\pm0.11^{abc,B}$	$9.02\pm0.3^{a,C}$
Alg-X-CN	$9.60\pm0.18^{ab,A}$	$9.25\pm0.1^{abc,B}$	$9.17\pm0.27^{a,C}$

Values are presented as Mean ± SD (n=3). ANOVA followed by LSD show comparisons between groups (within the column) and within the group (within the row). Means sharing different lowercase superscript letters (abc) within the column and uppercase letter (ABC) in row indicate significant differences ($P < 0.05$). Free Geo, un-encapsulated, Alg, alginate encapsulated ; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

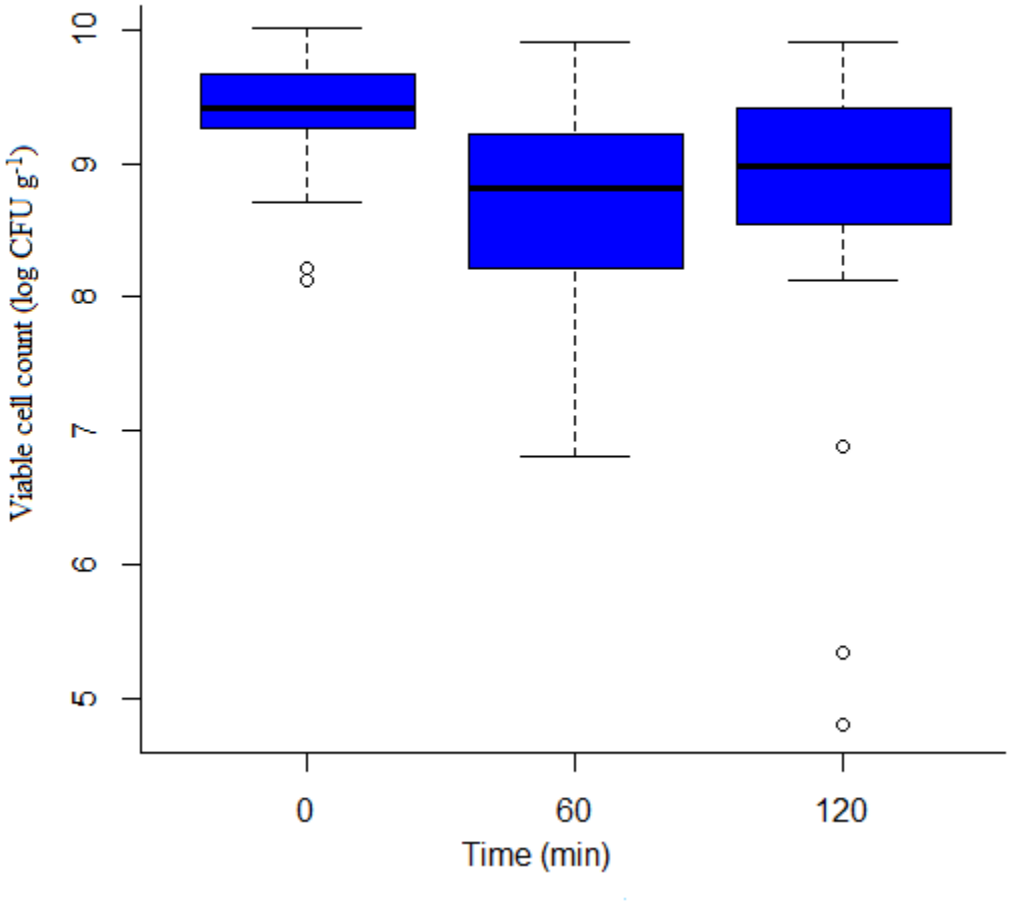

Fig. 2.15. Effect of exposure time (min) on viable cell counts (log $CFUg^{-1}$) of *G. candidum* at pH 4

Table 2.6.1 Summary of ANOVA comparing the viable cell counts (log CFU g^{-1}) of free, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at pH 8

Source of variation	Df	SS	MS	F	P
Time	2	13.610	6.805	73.341	<0.001
*Treatment	9	66.226	7.358	79.303	<0.001
Time×Treatment	18	42.793	2.544	27.41	<0.001

*Encapsulating/coating formulation

Table 2.6.2. Effect of pH 8 on viable cell count (log CFU g^{-1}) of free/unencapsulated, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan at two intervals

Encapsulating/ Coating Formulation	Viable *G. candidum* cells (log CFU g^{-1})at pH 8		
	Time		
	0 min	60 min	120 min
Free-Geo	$9.52\pm0.28^{ab,A}$	$6.48\pm0.36^{c,B}$	$3.64\pm0.74^{c,C}$
Alg	$8.45\pm0.49^{c,A}$	$8.32\pm0.3^{b,B}$	$8.26\pm0.15^{b,C}$
Alg-C	$9.66\pm0.15^{ab,A}$	$9.71\pm0.02^{a,B}$	$9.31\pm0.1^{ab,C}$
Alg-CN	$9.94\pm0.17^{a,A}$	$9.88\pm0.07^{a,B}$	$9.71\pm0.08^{a,C}$
Alg-S	$9.04\pm0.29^{bc,A}$	$9.26\pm0.33^{a,B}$	$8.62\pm0.4^{b,C}$
Alg-S-C	$9.38\pm0.13^{ab,A}$	$9.31\pm0.2^{a,B}$	$9.01\pm0.5^{ab,C}$
Alg-S-CN	$9.49\pm0.15^{ab,A}$	$9.37\pm0.39^{a,B}$	$9.07\pm0.39^{ab,C}$
Alg-X	$9.17\pm0.22^{bc,A}$	$9.15\pm0.3^{a,B}$	$8.26\pm0.15^{b,C}$
Alg-X-C	$9.45\pm0.28^{ab,A}$	$9.44\pm0.22^{a,B}$	$9.11\pm0.29^{b,C}$
Alg-X-CN	$9.6\pm0.18^{ab,A}$	$9.64\pm0.31^{a,B}$	$9.26\pm0.27^{ab,C}$

Values are presented as Mean ± SD (n=3). ANOVA followed by LSD show comparisons between groups (within the column) and within the group (within the row). Means sharing different lowercase superscript letters (abc) within the column and uppercase letter (ABC) in row indicate significant differences ($P < 0.05$). Free Geo, un-encapsulated, Alg, alginate encapsulated ; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

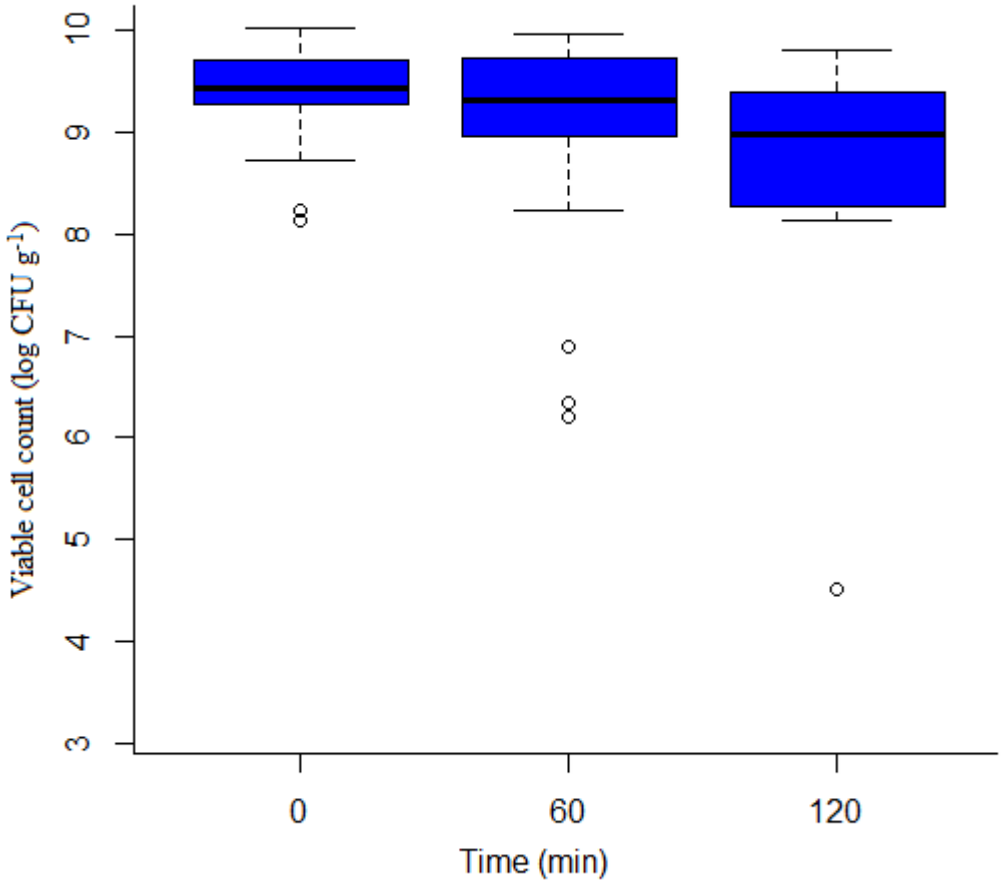

Fig. 2.16. Effect of exposure time (min) on viable counts ($logCFUg^{-1}$) of *G. candidum* exposed at pH 8

Table 2.7. Summary of ANOVA with interaction between treatment (free and encapsulated *G. candidum*) and exposure time (0, 60 and 120 min)

Source of variation	Df	SS	MS	F	P
*Treatment	9	231.81	25.76	39.09	<0.001
Time	2	108.6	54.32	82.43	<0.001
*Treatment× time	18	102.6	5.7	8.65	<0.001
Residuals	238	225	0.95		

*Encapsulating/coating formulation

Table 2.8. Summary of ANOVA with interaction between treatment (free and encapsulated *G. candidum*) and pH (2,4 and 8)

Source of variation	Df	SS	MS	F	P
*Treatment	9	231.81	25.76	39.09	<0.001
pH	2	61.02	30.51	46.3	<0.001
*Treatment×pH	20	95.31	4.77	5.038	<0.001
Residuals	238	225	0.95		

*Encapsulating/coating formulation

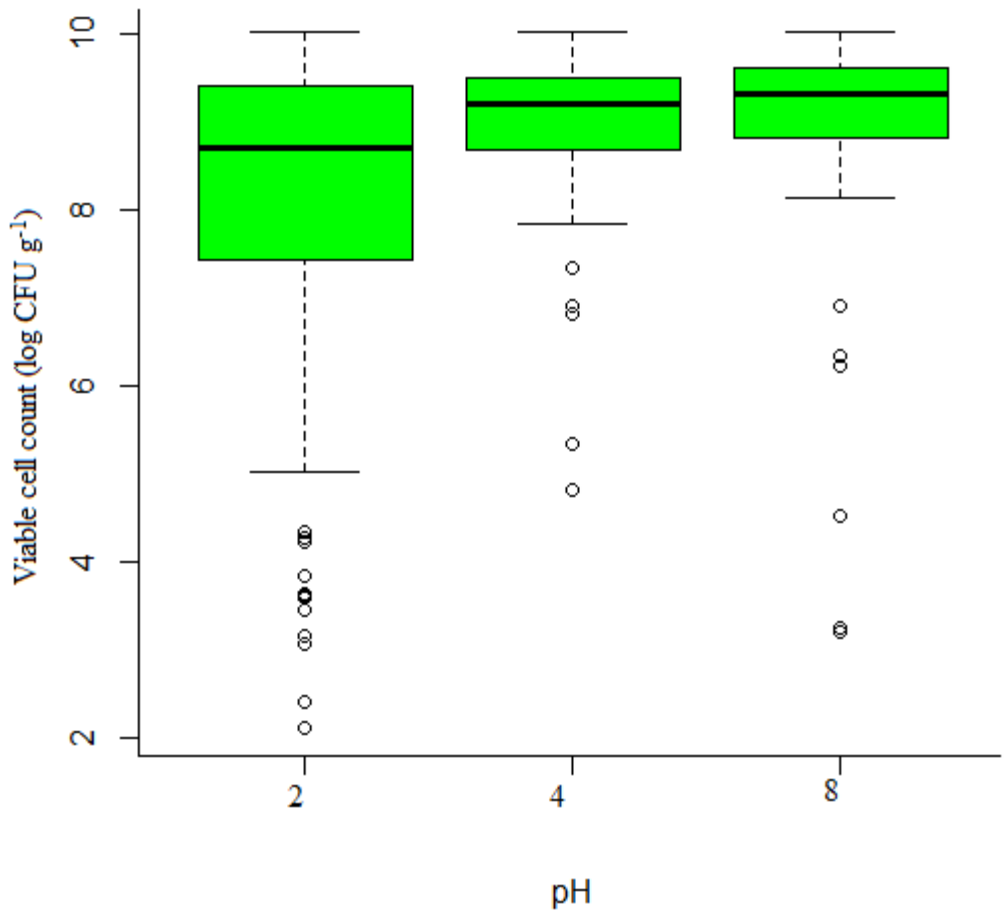

Fig. 2.17. Effect of three different pH (2, 4 and 8) on viable counts(logCFUg^{-1})of free /unencapsulated and encapsulated *G. candidum*

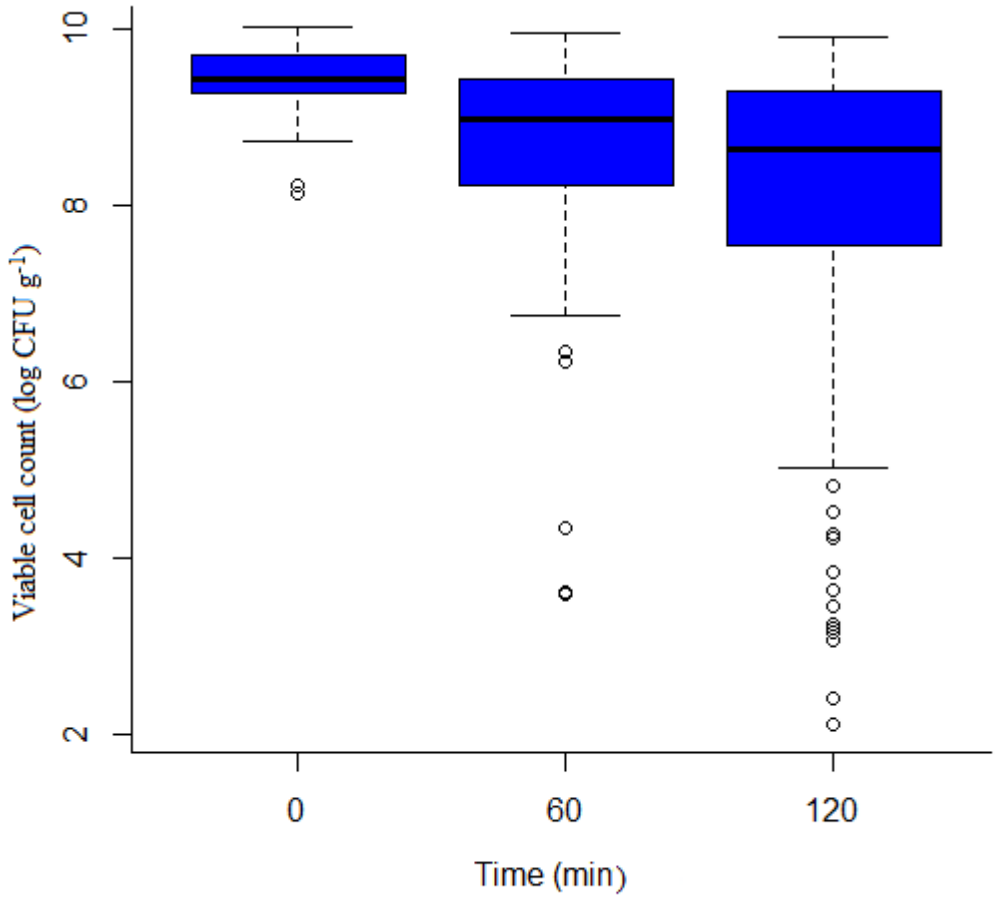

Fig. 2.18. Effect of exposure time 0, 60 and 120 min on viable counts(log CFU g^{-1}) of free /unencapsulated and encapsulated *G. candidum*

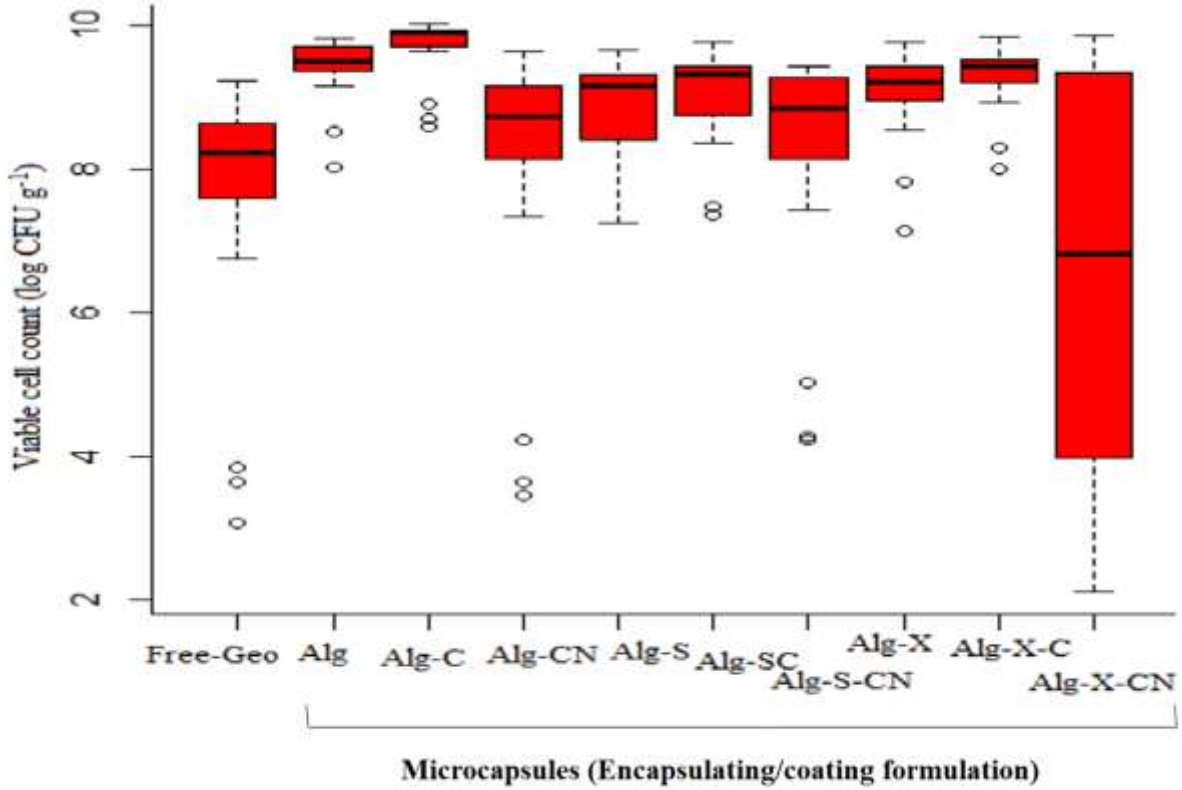

Fig. 2.19. Effect of encapsulating/coating formulation on viable counts (log $CFUg^{-1}$) of *G. candidum*

Values are represented as mean± SD (n=3). Free Geo=non-encapsulated, Alg, alginate encapsulated ;Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ;Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ;Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

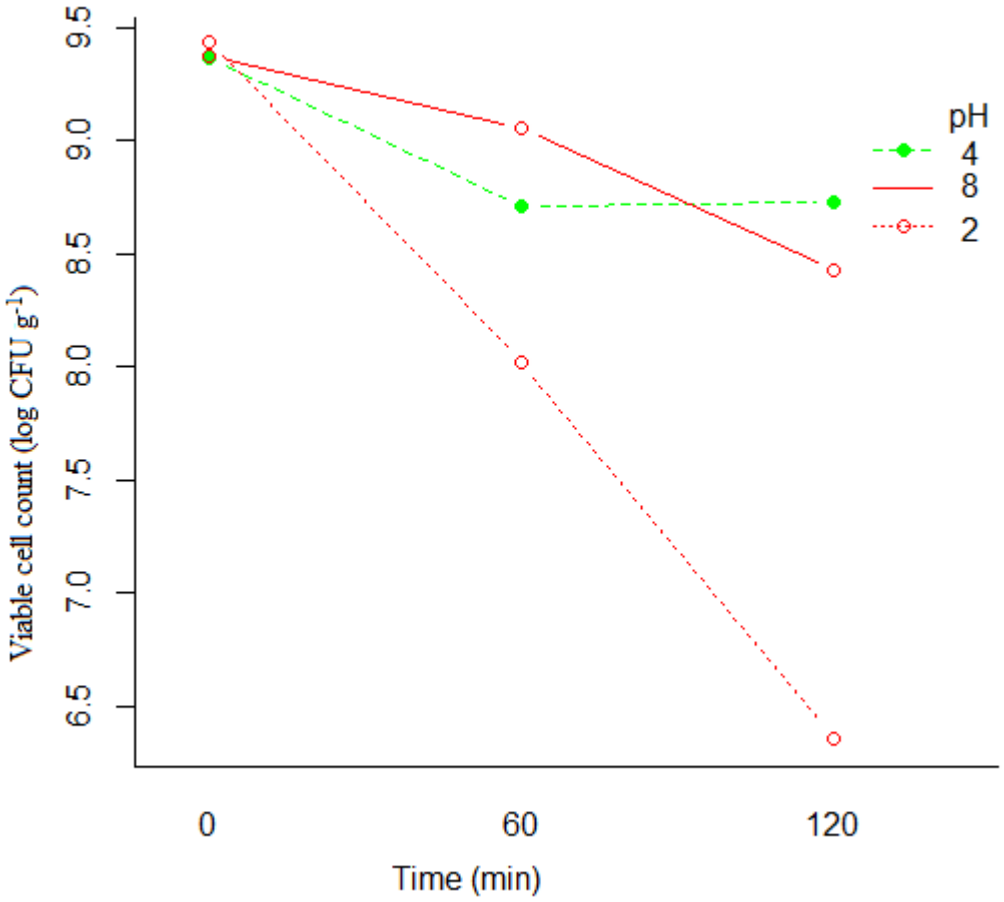

Fig. 2.20. Combine effect of exposure time (min) and pH (2,4 and 8) on viable cell count (log $CFUg^{-1}$)

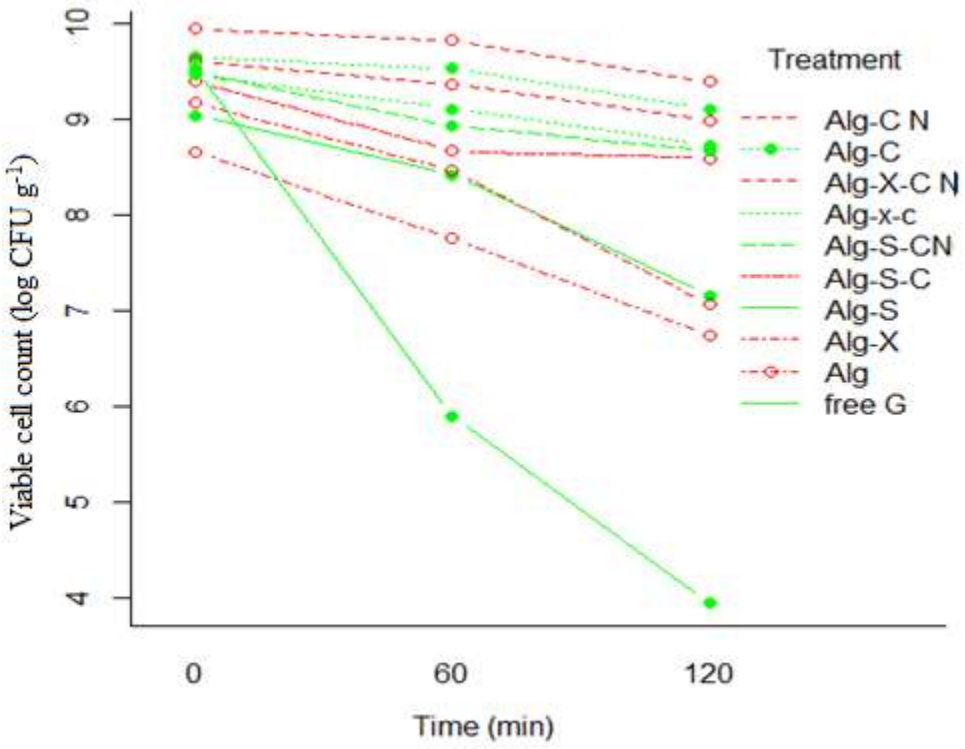

Fig. 2.21. Combined effect of encapsulating/coating formulation and exposure time (0, 60 and 120 min) on viable cell count (log $CFUg^{-1}$).

Free Geo=non-encapsulated, Alg, alginate encapsulated ;Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan;Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

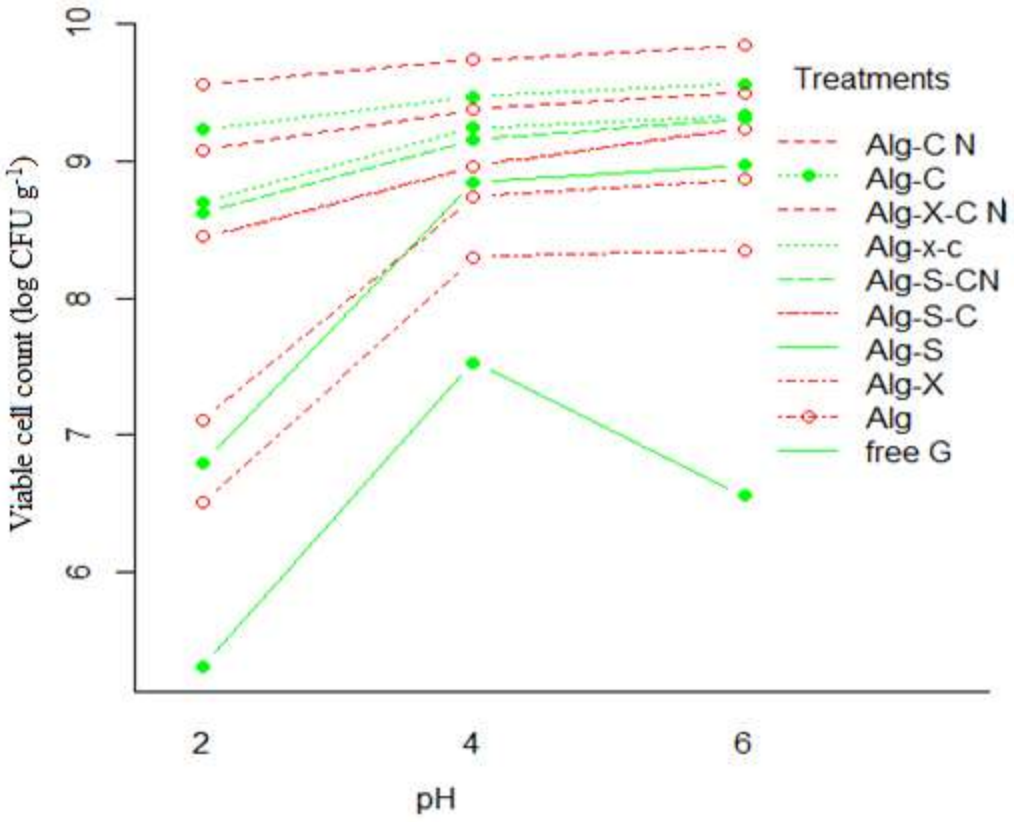

Fig. 2.22. Combined effect of encapsulating/coating formulation and pH (2, 4 and 8) on viable cell count (log $CFUg^{-1}$).

Free Geo=non-encapsulated, Alg, alginate encapsulated ; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

Effect of storage conditions

The effect of storage at 4ºC and ambient temperature for 30 and 60 on viable cell count (log CFU g-1) of free (un-encapsulate), chitosan coated and uncoated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan is shown in Table 2.9.2 and 2.9.3. ANOVA revealed that storage condition (ANOVA: $F_{1, 180}$=223, p<0.001, Table 2.9.1, Fig 2.23), storage duration (days) (ANOVA: $F_{2, 180}$=1220, p<0.001, Table 2.9.1) and encapsulating/coating formulation of microcapsules (ANOVA: $F_{9, 180}$=19.6, p<0.001, Table 2.9.1) significantly affect the viability (viable cell count) of *G. candidum.* The post hoc test revealed significant difference in viable cells of free and chitosan coated and uncoated microcapsules of *G. candidum* after 30 days storage at 4ºC (Fig. 2.25). With increase in storage time, the viable cell count decrease and difference between free/unencapsulated, and encapsulated *G. candidum* as well as between chitosan coated and uncoated microcapsules of probiotic become more pronounced. At 4ºC storage, Alg-CN microcapsule showed highest stability in term of maximum viable cell count followed by Alg-C. However, free/unencapsulated *G. candidum* showed the lowest number of viable cells (Table 2.9.3 and Fig. 2.24).

The viable cell count of free/unencapsulated and, chitosan coated and uncoated microcapsules of probiotic *G. candidum* prepared with alginate alone and in combination with starch and xanthan after 30 and 60 days storage at ambient temperature followed the similar trend as observed when stored at ambient temperature i.e., viable number of cells decrease with an increase storage duration and maximum viable count in Alg-CN (Table 2.9.3). However, the numbers of viable *G. candidum* cells were less at ambient temperature as compared to storage at 4ºC (Fig. 2.24).

Furthermore, ANOVA with significant interaction between encapsulating/coating formulation× storage days (ANOVA: $F_{18, 180}$= 88, p<0.001, Table 2.9.1, Fig 2.25), storage temperature× encapsulating/coating formulation (ANOVA: $F_{9, 180}$= 9.35, p<0.001, Table 2.9.1, Fig 2.24), storage temperature × storage days (ANOVA: $F_{2, 180}$= 76.9, p<0.001, Table 2.9.1) as well as storage temperature× days× encapsulating/coating formulation (ANOVA: $F_{18, 180}$= 3.99, p<0.001, Table 2.9.1), also indicated the combined effect of all factors on the viable cell counts of *G. candidum*.

Table 2.9.1. Summary of ANOVA showing the effect of storage temperature (4°C and ambient), storage days (0, 30 and 60 days) and encapsulating/coating formulation on viability of *G. candidum*

Source of variation	Df	SS	MS	F	P
Temperature	1	5.61	5.61	223.3	<0.001
Days	2	61.3	30.6	1220	<0.001
*Treatment	9	94.4	10.4	417	<0.001
Temperature× days	2	3.87	1.93	76.9	<0.001
Temperature×*treatment	9	2.11	0.23	9.35	<0.001
Days ×treatment	18	39.8	2.21	88.5	<0.001
Temperature× days× *treatment	18	1.80	0.10	3.99	<0.001

*Encapsulating/coating formulation

Table 2.9.2. Viable cell count (log CFU g^{-1}) in the chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan, stored for 60 days at 4ºC

Treatment	Initial count	Stored at (4°C)	
		30 Days	60 Days
Free-Geo	$9.52\pm0.28^{abc,A}$	$7.84\pm0.11^{i,B}$	$5.6\pm0.23^{h,C}$
Alg	$9.07\pm0.15^{c,A}$	$8.14\pm0.06^{h,B}$	$7.32\pm0.1^{g,C}$
Alg-C	$9.68\pm0.16^{ab,A}$	$9.55\pm0.1^{ab,B}$	$9.36\pm0.10^{ab,C}$
Alg-CN	$9.94\pm0.06^{a,A}$	$9.83\pm0.09^{a,B}$	$9.63\pm0.13^{a,C}$
Alg-S	$9.04\pm0.29^{c,A}$	$8.42\pm0.04^{gh,B}$	$7.65\pm0.08^{fg,C}$
Alg-S-C	$9.38\pm0.13^{abc,A}$	$8.79\pm0.19^{ef,B}$	$8.29\pm0.04^{e,C}$
Alg-S-CN	$9.49\pm0.15^{abc,A}$	$8.95\pm0.12^{de,B}$	$8.53\pm0.05^{de,C}$
Alg-X	$9.17\pm0.22^{bc,A}$	$8.54\pm0.08^{fg,B}$	$7.90\pm0.09^{f,C}$
Alg-X-C	$9.45\pm0.28^{abc,A}$	$9.11\pm0.02^{cd,B}$	$8.71\pm0.15^{cd,C}$
Alg-X-CN	$9.60\pm0.18^{abc,A}$	$9.29\pm0.06^{bc,B}$	$9.01\pm0.13^{bc,C}$

Values are presented as Mean ± SD (n=3). ANOVA followed by LSD show comparisons between groups (within the column) and within the group (within the row). Means sharing different lowercase superscript letters (abc) within the column and uppercase letter (ABC) in row indicate significant differences ($P < 0.05$). Free Geo, un-encapsulated, Alg, alginate encapsulated ; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

Table 2.9.3. Viable cell count (log CFU g^{-1}) in chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan and stored for 60 days at ambient temperature

Treatment	Initial count	Stored at ambient	
		30 Days	60 Days
Free-Geo	$9.52\pm0.28^{abc,A}$	$6.50\pm0.16^{g,B}$	$4.47\pm0.29^{i,C}$
Alg	$9.07\pm0.15^{c,A}$	$7.02\pm0.14^{f,B}$	$7.06\pm0.13^{h,C}$
Alg-C	$9.68\pm0.16^{ab,A}$	$9.35\pm0.10^{b,B}$	$9.19\pm0.05^{ab,C}$
Alg-CN	$9.94\pm0.06^{a,A}$	$9.74\pm0.07^{a,B}$	$9.56\pm0.09^{a,C}$
Alg-S	$9.04\pm0.29^{c,A}$	$7.39\pm0.12^{e,B}$	$7.37\pm0.13^{gh,C}$
Alg-S-C	$9.38\pm0.13^{abc,A}$	$7.89\pm0.08^{d,B}$	$7.9\pm0.17^{ef,C}$
Alg-S-CN	$9.49\pm0.15^{abc,A}$	$8.26\pm0.11^{d,B}$	$8.16\pm0.07^{de,C}$
Alg-X	$9.17\pm0.22^{bc,A}$	$7.42\pm0.13^{e,B}$	$7.56\pm0.06^{fg,C}$
Alg-X-C	$9.45\pm0.28^{abc,A}$	$8.63\pm0.15^{c,B}$	$8.57\pm0.19^{cd,C}$
Alg-X-CN	$9.6\pm0.18^{abc,A}$	$9.06\pm0.12^{b,B}$	$8.78\pm0.12^{bc,C}$

Values are presented as Mean ± SD (n=3). ANOVA followed by LSD show comparisons between groups (within the column) and within the group (within the row). Means sharing different lowercase superscript letters (abc) within the column and uppercase letter (ABC) in row indicate significant differences ($P < 0.05$). Free Geo, un-encapsulated; Alg, alginate encapsulated; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan ; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan ; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

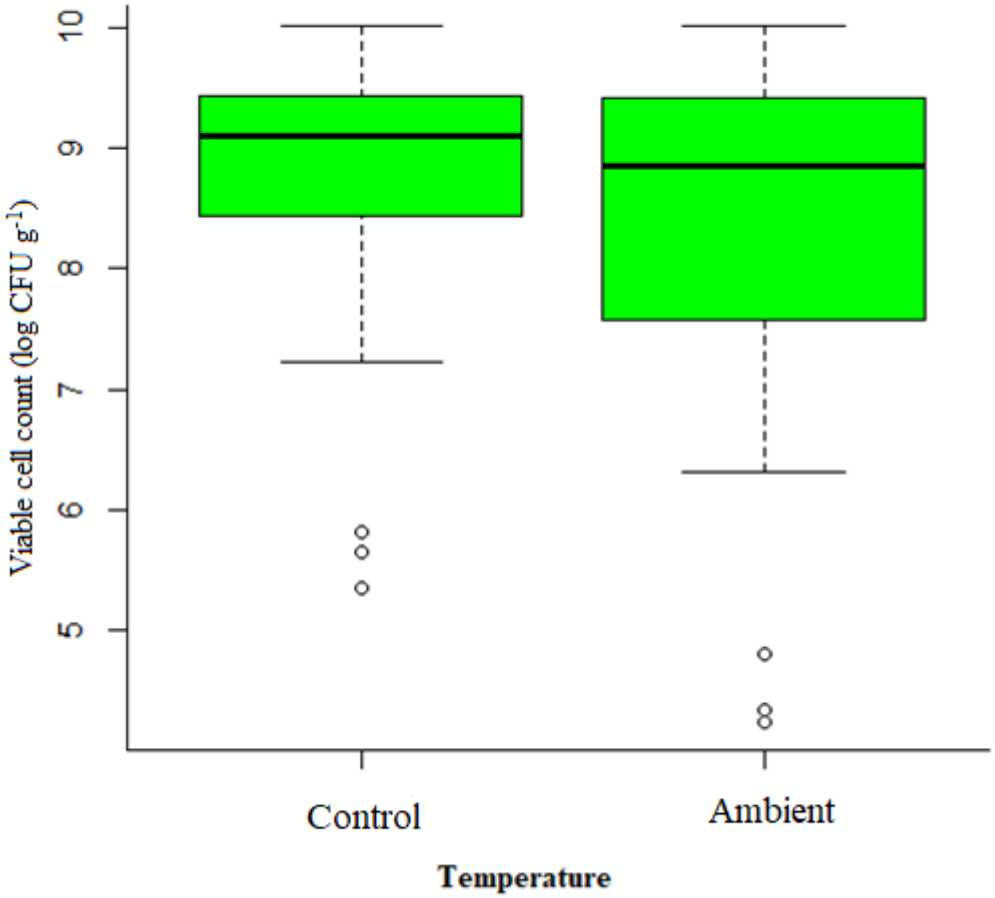

Fig. 2.23. Effect of storage temperature (4°C and ambient) on viable cell count (log $CFUg^{-1}$) of free and encapsulated *G. candidum* stored for 60 days

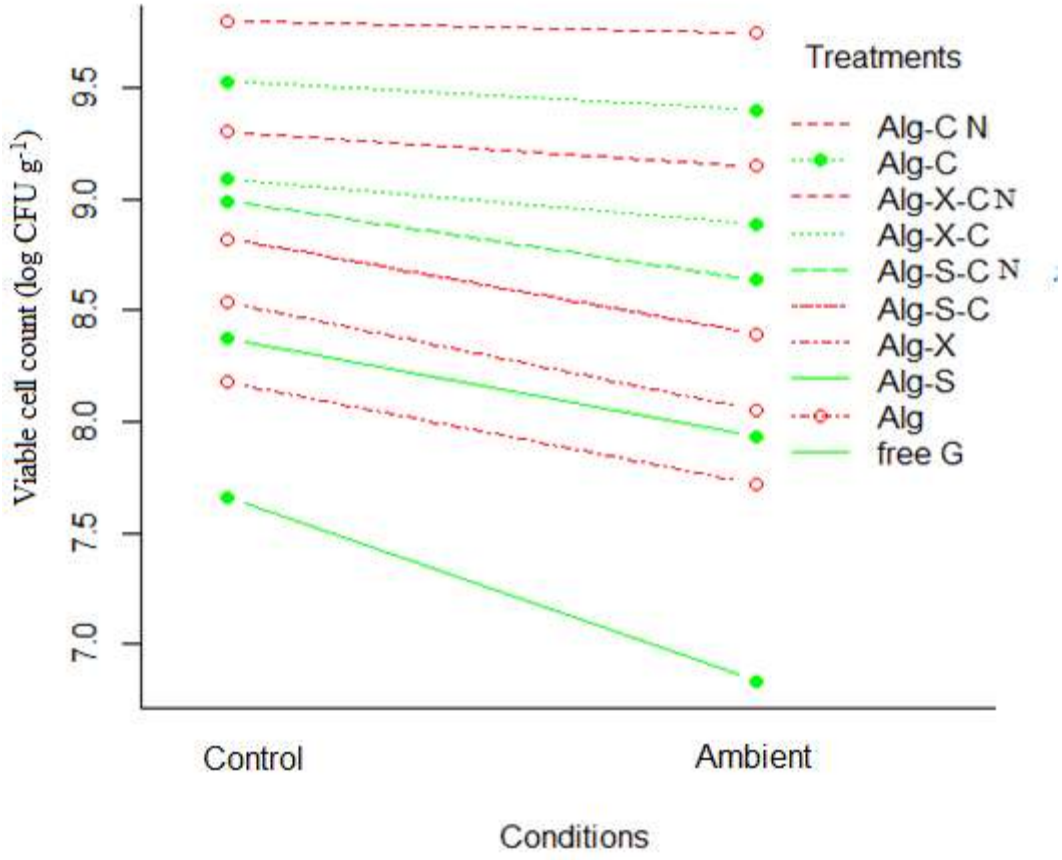

Fig. 2.24. Combined effect of temperature (4°C and ambient) and encapsulating/coating formulation on viable cell count (log $CFUg^{-1}$) of *G. candidum*.

Free G, non-encapsulated; Alg, alginate encapsulated; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

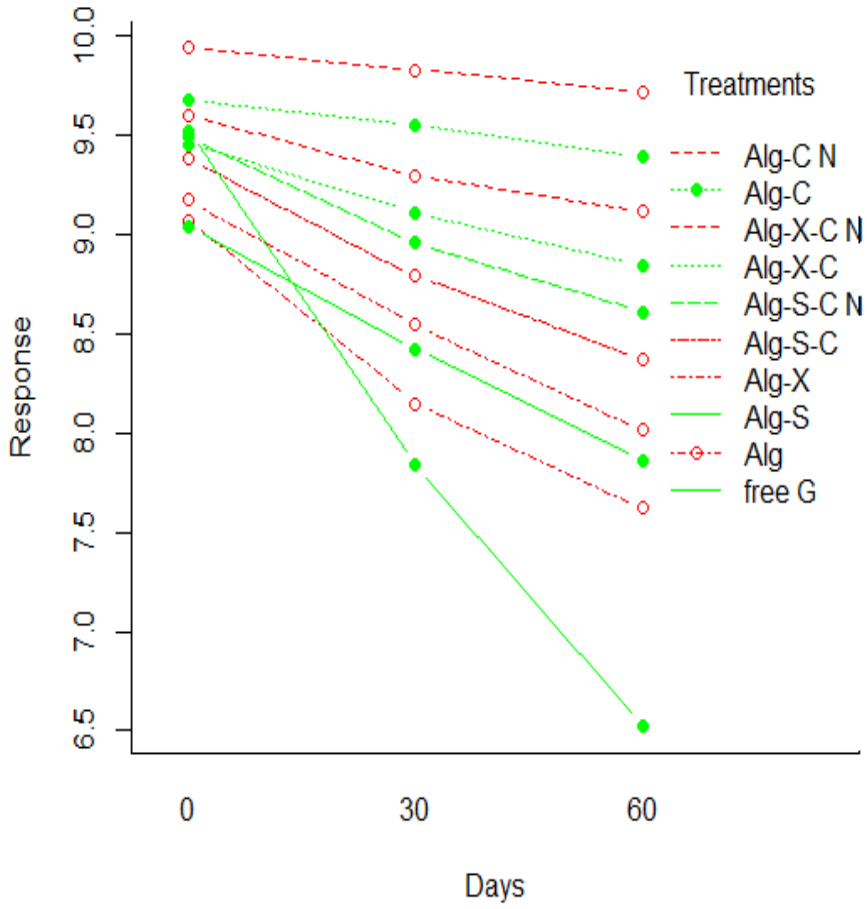

Fig. 2.25. Combined effect of treatments (free and encapsulated) and storage days (0, 30,60 days) on viable count (log CFU g^{-1}) of free and encapsulated *G. candidum* stored for 60 days

Free G, non-encapsulated; Alg, alginate encapsulated; Alg-C, alginate encapsulated and coated with chitosan; Alg-CN, alginate encapsulated and coated with nano-chitosan; Alg-S, alginate-starch encapsulated; Alg-S-C, alginate-starch encapsulated and coated with chitosan; Alg-S-CN, alginate-starch- encapsulated and coated with nano-chitosan; Alg-X, alginate-xanthan encapsulated; Alg-X-C, alginate-xanthan encapsulated and coated with chitosan; Alg-X-CN, alginate-xanthan encapsulated and coated with nano-chitosan.

Discussion

Probiotics provide health benefits to the host, when sufficient viable count present in the digestive tract of host (Pinpimai et al., 2015; Shori, 2017). The tolerance of probiotic organisms to harsh environmental conditions, gastrointestinal secretions and ease of storage are the major constrains in the practical application of probiotics in aquaculture. It is well documented that microorganisms use as probiotics in transit to target site, reduce in number of viable cells. Nowadays, microencapsulation appears as a promising technique to provide protection and improve the viability of probiotic microorganisms in feed and GI tract of host (Huq et al., 2013). However, efficiency of microcapsule depends on the encapsulating materials (biopolymers), process of encapsulation and size of microcapsules (Nesterenko et al., 2013).

In this study, selected probiotic *G. candidum* was encapsulated with alginate alone or in combination with different polymers (starch and xanthan) by adopting extrusion method which is simple, easy and cost effective (Hug et al., 2013). Moreover, to further improve the stability and target release, the prepared microcapsules were coated with chitosan (bulk) and chitosan NPs. This all was done in order to improve viability of *G. candidum* in feed and at the target site of host (*L. rohita*) and select the encapsulating agent/formulation for this probiotic on the basis of *in vitro* results. Pinpimai et al. (2015) reported the enhanced viability of encapsulated *S. cerevisiae* during storage and simulated gastric conditions in contrast to free probiotic.

The size of microcapsule is a very important factor which determines the encapsulation efficiency (Yasmin et al., 2018). In the present study, microcapsules prepared from different formulation were somewhat variable in size. Among all, microcapsules prepared with alginate alone showed the lowest diameter while Alg-X-C was highest in size. Many factors like type and

concentration of the polymer, diameter of syringe (needle), concentration of hardening solution ($CaCl_2$), stirring speed and the distance between syringe needle and hardening solution effect the size and shape of microcapsule (Valero-Cases and Frutos, 2015; Mokhtari et al., 2017). Here except type and concentration of polymers, all factors were constant. The present slight variation in size may be due to interaction between different polymers like alginate with chitosan, alginate with starch, alginate with xanthan etc.

The SEM images of microcapsules prepared from different formulation indicated the difference in shape and morphology. The microspheres prepared with chitosan coating were more spherical in shape with smooth surface and least cavities/cracks in contrast to uncoated microspheres, which were irregular in shape with rough/ uneven surface. This variation could be due to cross linking reaction between polymers and calcium as well as within polymers (in case of combination of polymers) used for encapsulation and coating (Yasmin et al., 2018). It is well documented that coating of alginate microcapsule with chitosan enhance the cross linking between alginate and chitosan molecules thus form complex structure with smaller pores and cavities on the surface of the microcapsule (Lee et al., 2004). Zanjani et al. (2014) also reported the change in shape and morphology of alginate capsule when coated with chitosan.

In the present study SEM images were observed after drying the microcapsules. In the process of drying water was removed from the microcapsules and resulted wrinkle and crack on the surface. However, in case of the chitosan coated microcapsules, the structural collapse were not prominent, may be due to alginate –chitosan matrix which allow the microcapsules to remain in shape , when water was removed (Yasmin et al., 2018). The topography of Alg, Alg-S and Alg-X indicated that microcapsules were not smooth and had wrinkles and cavities /channels

throughout the surface (Fig 2.10a, 2.11d and 2.12g: Arrows indicated cavities), may be due to fast release of water in the process of drying (Shi et al., 2006; Belščak-Cvitanović et al., 2015).

The encapsulation efficiency depends upon various factors including process of encapsulation, formulation and concentration of the encapsulating polymers, hardening solution (Lee et al., 2004; Jyothi et al., 2010). In the present study, extrusion technique was adopted and more than 90% encapsulation efficiency of all microcapsule formulations except Alginate (Alg) alone (EE, 83%) was observed which is the indicated of the effectiveness of encapsulating polymers and process of encapsulation. Like our results many other investigators also reported the lower encapsulation efficiency (EE) of chitosan free alginate (Azarnia et al., 2008) and suggested that encapsulation effectiveness could be improved either by mixing alginate with other polymers or coating the Alg-microcapsule with chitosan (Krasaekoopt et al., 2003). Our results of higher EE of other microcapsule formulations support this view (Table 2.2). Beside chitosan, we also used other biopolymers, starch and xanthan in combination with alginate (Alg-S, Alg-X) to encapsulate the *G. candidum* and observed higher encapsulation efficiency, above 90% and 91 % respectively, while this EE was further improved with chitosan coating. It seems that alginate–chitosan complex decreases the rate of diffusion of water across the alginate matrix (Cook et al., 2013). Although the concentration of hardening solution (CaCl2) also affects the EE but here we used similar concentration of $CaCl_2$ in all formulation.

The shape of the microcapsule also determines the EE. The spherical microcapsule with smooth and compact wall provides better protection to the probiotic from external environment in contrast to irregular with rough surface (Yasmin et al., 2018). Here we also observed highest EE of Alg-CN followed by Alg-C. It seems that chitosan –coated alginate microcapsule system favor the entrapment, provide protection and maintain the survival and stability of *G. candidum*.

It is well documented that chitosan increases the release time, control permeability/absorption and also enhances the mucoadhesive nature of the microcapsule (Cook et al., 2013). Previous studies also observed significant effect of chitosan coating on the stability of alginate microcapsule and on the viable count of encapsulated probiotic microbes (Krasaekoopt et al*.,* 2003; Cook et al., 2013). For instance Chavarri et al. (2010), reported the decrease in porosity of the alginate microcapsule coated with -chitosan can reduce leakage of entrapped probiotic.

Although, microencapsulation technology is beneficial for the stability and control release of probiotic but in the present study *G. candidum* microencapsulation showed further improved results in the presence of nano-chitosan coating. It may be due to increase surface area that directly related to release and diffusion of probiotic. According to Jia (2003) nano encapsulation of probiotic, increased the surface area of microcapsule and improve the attachment of microcapsule to the gut lining. The improved efficiency nano encapsulation i.e., more stability and enhanced bioavailability of probiotic throughout the GI tract has also been reported by Kumari et al. (2013). The future importance of nano encapsulation for the improved transfer of probiotics to the specified receptor cells of the GI tract has been predicted by Vidhyalakshmi et al., 2009). Nowadays, the nanoscale delivery system is gaining more importance because it is more effective and delivers the contents more efficiently than microscale delivery systems (Agrawal et al., 2014).

For probiotic, more than 10^6 CFU g^{-1} viable cell count at the target site is require to provide possible health benefits to the host (Nayak, 2010). It is essential that microorganisms have to remain sufficiently available at the target site in the GI tract of fish. The tolerance of the probiotic microorganisms to the intestinal enzymes, pH and bile of fish and the ease of storage are the major constrains for the practical use of the probiotics in aquaculture sector. Here we

evaluated the potential practical application of locally isolated, microorganism *G. candidum* (yeast) as probiotic in aquaculture. Although, it is reported that yeast has ability to withstand under many unfavorable conditions (Nayak, 2011), however it is not sufficiently stable during feed processing/storage and in the GI tract of host (Arslan et al., 2015). Several yeast strains for example *S. boulardii* is genetically resistant to the gastric acidic pH (Buts and Bernasconi, 2005). However, Fietto et al., (2004) documented that an exposure to acidic condition (pH 2) for a 60 min period is lethal to the viability of the *S. boulardii* and its probiotic potential. Here the most significant decreases (many fold) in viable cell count of free/unencapsulated *G. candidum* at all tested pH especially at pH 2 (Table 2.4.2, Fig. 2.14) are in agreement to literature.

The *in vitro* results of this study and recent report of Pinpimai et al. (2015) indicated that the viability of yeast cells at different pH can be improved by encapsulation. In the present study, chitosan coated especially Alg-CN microcapsules showed the highest viable count of *G. candidum* at all tested pH (2, 4 and 8). The *in vitro* study is a useful tool to select and introduce successful probiotic in practical / commercial application (Nayak, 2011; Srinu et al., 2013). The guidelines of the evaluation of probiotic organisms indicated the importance of *in vitro* acid tolerance test for assessment of probiotics (Mirlohi et al., 2009; Yasmin et al., 2018). Literature also emphasize on the consideration of pH of GI tract of host before designing the probiotic encapsulation system, as pH sensitive hydrogels swells only at suitable pH leading to the release of the entrapped probiotic (Lin et al., 2009).

The present *in vitro* results showed the effect of polymer formulation used for encapsulation of *G. candidum* on the viability of yeast cells at different pH. Here again chitosan coated microcapsules (Alg-CN, Alg-C, Alg-X-CN, Alg-XC, Alg-S-CN and Alg-S-C) showed higher number of viable yeast cell while microcapsules without chitosan coating (Alg-X, Alg-S

and Alg) showed almost similar but lower number of viable of cells at all pH values. This may be due to smooth surface, least cavities/channels of microcapsules, decreases porosity which reduces the leakage of the encapsulated probiotic or prevent the entrance of surrounding solution in the capsule (Chavarri et al., 2010). According to Song et al. (2013) spherical shape microcapsule is important to maintain the texture and provide better protection to the encapsulated probiotics. The decreased in number of viable cell count of *G. candidum* microcapsule devoid of chitosan may be due to direct contact of probiotic cells to the external medium as irregular shape microcapsules with channels on surface allow the quick and easy diffusion of external medium or leakage of microbial cells out of the matrix (Rathore et al., 2013).

Here, the highest and somewhat similar viable cell counts of Alg-C and Alg-CN microcapsule at all pH in contrast to other chitosan coated microcapsule indicating the promising wall and coating material for yeast encapsulation. The release of encapsulated probiotic strain i.e. *G. candidum*-01 from alginate chitosan microcapsule appears as pH dependent. For instance, at pH 2, both Alg-C and Alg-CN showed significant decrease (but less than as compared to other microcapsules) in viable cells of *G. candidum* as compared to pH 4 and 8, where no significant (Fig. 2.17) effect on the viable cell count was observed. It seems that even at acidic pH, chitosan-alginate complex provide protection to the core material (*G. candidum*). Previous studies indicated the low pH sensitivity of the alginate- chitosan microcapsules (Anal, et al., 2003; Anal and Stevens, 2005). In acidic solutions due to the ionic bond between the alginate and chitosan microcapsules keep maintain their integrity and effectively prevent the direct exposure to acidic pH (Daly and Knorr, 1988; Zhang et al., 2011; Ren et al., 2017). Furthermore, a study dealing with comparative evaluation of *S. cerevisiae* and *S. boulardii*, reported the higher stability of *S.*

boulardii at different pH, even at pH below 2, while viability of *S. cerevisiae* showed significant reduction at all studied pH (6, 3 and 2). However, both strains were not able to survival at pH 1 (Edwards-Ingram et al., 2007). Here, we were designing the encapsulating system for *L. rohita*, which GI tract never attains pH 1, therefore we did not test the tolerance of microcapsules at this pH.

The maintenance of the viable cell count of probiotic microorganism before consumption is also an imperative aspect which needs to be considered, in order to achieve desired probiotic count in the intestinal tract of host (Rosas-Ledesma et al., 2012). For aquaculture applications, the dose of probiotic usually varies from 10^6-10^{10} CFU g^{-1} feed (Nayak, 2010). The higher dose may have some deleterious effects and fail to provide the adequate protection against pathogen/disease while, a lowest dose may not be suitable for colonization of adequate number of microbial cells in the GI tract of fish, thus fail to provide the required level of immune responses (Nikoskelainen et al., 2001; Zhang et al., 2011). Additionally, during processing and storage of feed, a further decrease in the viable count of microbes (below efficacy level) has also been reported (Tripathi and Giri, 2014).

Here Alg-CN and Alg-C microcapsules containing *G. candidum* showed highest tolerance to temperature. After exposure to 60ºC for an hour, the % survival of *G. candidum* encapsulated with Alg-CN was significantly highest (91%) followed by ~85% when encapsulated with Alg-C. Although *G. candidum* in other encapsulating formulation also showed improved temperature tolerance, as compared to free probiotic but the % survival was considerably lower. It is well documented that alginate use in encapsulation provide physical barrier and protects the microorganism used as probiotic from unfavorable surrounding environment (Mandal et al., 2006b). Nevertheless, some investigators reported the insufficient

protection and leakage of entrapped probiotic/core material from alginate microcapsule (Sultana et al., 2000; Mandal et al., 2006b). Here we also observed the lowest % survival of *G. candidum* when capsulated with Alg alone as compared to other formulation. It seems that Alg alone unable to provide sufficient protection against thermal stress. Furthermore un-encapsulated *G. candidum* was not able to withstand the exposure of high temperature (60 °C) and showed only 18% survival after one hour exposure. It seems that the encapsulation and coating protect the *G. candidum* against higher temperature.

During storage at 4ºC and under ambient condition, the viable count of *G. candidum* showed the similar trend, i.e., the highest number of viable cells when encapsulated with Alg-CN followed by Alg-C. However, *G. candidum* in all chitosan un-coated microcapsules showed lower viable count in contrast to chitosan coated microcapsules, after 60 days storage at 4ºC and ambient temperature. For probiotic products, culture viability during storage is a reasonable measure of their activity (Tripathi and Giri, 2014). Here, the viable count of *G. candidum* encapsulated in different formulation decreased with the passage of time (30 and 60 days) during storage at both conditions. However, encapsulated yeast (*G. candidum*) cells showed less variation in terms of total number of viable cells, stored at 4°C in refrigerated conditions (Fig 2.25). The observed greater fluctuation in total count of viable yeast cell, when stored at room temperature might be due to the fluctuations in surrounding temperature. The viable cell count of encapsulated *G. candidum* (with all encapsulating polymers) was above the recommended therapeutic level of probiotic (10^7 log CFU g^{-1}) as compared to free/un-encapsulated *G. candidum* which showed about 40% decreased in viable cell count after 60 days storage even at 4ºC.

Like our results, other investigators also reported the significant positive effect of encapsulation on viable cell count of different probiotics. Pinpimai et al. (2015) did not observe any viable cell of unencapsulated *S. cerevisiae* during 14 days storage at ambient temperature, while viability of encapsulated probiotic especially with skim milk alginate in contrast to alginate alone was significantly higher. Similarly, free /unencapsulated *L. plantarum* showed significant loss of viable cells during 38 days storage at room temperature or under refrigerated conditions, while encapsulated probiotic showed limited decrease in viable cell count (Brinques and Ayub, 2011). Çabuk and Harsa. (2015) also observed lowest viability of free *L. acidophilus* after 4 weeks of storage at 4 °C while whey protein and pullulan encapsulated cells had higher viable cell count under same conditions. Like our results many investigators reported the improved viability of the encapsulated probiotic microorganisms during storage, when alginate was used in combination with chitosan (Aly et al., 2008b; Chávarri et al., 2010; Utiswannakul et al., 2011).

Overall results of this part of study indicated the higher encapsulation efficiency and improved tolerance to different temperature, pH and storage conditions of encapsulated *G. candidum* in contrast to free/unencapsulated probiotic. Moreover, among all formulations, alginate with chitosan or nano-chitosan appeared as most suitable wall material for the *G. candidum* as with this formulation, the prepared microcapsules were more spherical in shape with least dent/crack on surface and showing highest encapsulation efficiency, shelf life and viable cell count of entrapped probiotic after exposure to higher temperature (60°C) and various pH (2, 4 and 8). On the basis of *in vitro* results, combination of 2% alginate with 0.4 % chitosan could be recommended as encapsulating material for improving the % survival of this potential

probiotic (*G. candidum*) in feed and GI tract of *L. rohita* during feed preparation, feed storage and exposure to GI tract conditions.

Although, size, shape, morphology and *in vitro* tests provide information regarding the general characteristics, functionality and viable cell count of potential probiotic under different conditions, however postulation based on *in vitro* results might not follow precisely with *in vivo* conditions. Hence, before practical application, *in vivo* evaluation of selected probiotic needs to be carried out.

Chapter 3:

Comparative efficiency of *Geotrichum candidum* microcapsules prepared with alginate alone and in combination with other polymers: *in vivo evaluation*

Abstract

Nowadays microencapsulation technique is getting importance to improve the viability and target release of probiotics. However, interaction /crosslinking of encapsulating material with the functional groups present on wall of probiotic microorganism and to the epithelial lining of host GI tract influence the survival of probiotic within the microcapsule and their target release. Here, an 11 weeks feeding experiment was conducted, for evaluating the *in vivo* comparative efficacy of *G. candidum* encapsulated with , alginate alone (Alg) or in combination with starch (Alg-S) and xanthan (Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and nano-chitosan (Alg-CN, Alg-S-CN, Alg-X-CN). Fingerlings of *L. rohita* (average weight, 15.63±0.35g) were evenly distributed in eleven groups, each with three replicate i.e., Control (control $_{FL}$) was fed diet devoid of probiotic while others were fortified with probiotic *G. candidum* QAUGC-01 at the rate of 1×10^{9}CFU g^{-1}. $Y1_{FL}$ was provided free or un-encapsulated *G. candidum*, while other groups were given diet fortified with microcapsules of *G. candidum* prepared with Alg , Alg-C and Alg-CN to $T2_{FL}$, $T3_{FL}$ and $T4_{FL}$ groups respectively, Alg-S, Alg-S-C and Alg-S-CN to $T5_{FL}$, $T6_{FL}$ and $T7_{FL}$ groups respectively while Alg-X, Alg-X-C and Alg-X-CN to $T8_{FL,}$ $T9_{FL}$ and $T10_{FL}$ groups respectively. Results indicated significant effect of free/encapsulated probiotic supplemented diet on growth, feed conversion ratio, intestinal enzyme activities, hematology, metabolic enzymes (AST, ALT and ALP), immunological indices (Total serum protein contents, IgM level, lysozyme activity, phagocytic activity, respiratory burst activity), lipid profile: total cholesterol, high and low density lipoprotein (HDL, LDL) and triglyceride content of fish as compared to basal diet devoid of probiotic. However, $T4_{FL}$, $T3_{FL}$, $T7_{FL}$, $T6_{FL}$, $T10_{FL}$ and $T9_{FL}$ groups provided chitosan coated microcapsules of *G. candidum* supplemented diets showed comparatively improved performance as compared to $T2_{FL}$, $T5_{FL}$ and $T8_{FL}$ groups of fish fed

diet supplemented with uncoated microcapsules. Furthermore, among chitosan coated microcapsules, Alg-CN microcapsules of *G. candidum* showed the highest efficiency i.e., most significant positive effect on all the studied parameters (growth performance, FCR, activity of intestinal enzymes, hemato-immunological indices, AST, ALP, ALT and lipid profile) followed by Alg-C microcapsules of *G. candidum*. It appears that Alginate-nano chitosan encapsulating formulation is the best to improve the viability and target release of locally isolated *G. candidum* QAU001 in the gastrointestinal tract of *L. rohita*. Thus, suggests a possible way of improving the growth and health status of *L. rohita* by feeding diet supplemented with Alg-CN microcapsules of *G. candidum*.

Introduction

Most probiotics are supplied as live supplements in feed to benefit the host. Generally, probiotic inhibit the pathogenic microbes, improve immune response, survival and growth rates, enhance digestion and increase feed utilization, promote anti-mutagenic and anti-carcinogenic activity and improve water quality in the culture systems (Harikrishnan et al., 2010; Andani et al., 2012).

Nowadays, probiotics are extensively being used for the development of sustainable aquaculture practices, however there are certain challenges associated with the intake of probiotics. Despite the aforementioned advantages, the viability of live probiotic cells during the food processing, long term storage and transition through the gastrointestinal tract of host are not reliable to confer the desired beneficial effects (Cordero et al., 2015). The least suggested viable cell count ranges from 10^8 to 10^9 CFU per day dose^{-1} (Albertini et al., 2010; Ramos et al., 2018). Therefore, currently, there is great interest in getting better or improved viability of probiotics to be administered in the diet.

Among all the methods developed to improve probiotic viable count, encapsulation is one of the most relevant to improve survival during processing, storage and release in the gastrointestinal tract when administered orally (Cordero et al., 2015). The chief goal to encapsulate probiotics is to protect them from the unfavorable conditions during food processing (Tripathi and Giri, 2014), storage (Sousa et al., 2013) and after consumption, transit to GI tract (Sun and Griffiths, 2000) as well as from high oxygen levels (Sunohara et al., 1995). Microencapsulation protect probiotics from the conditions like low pH, high acidity, digestive enzymes (Sousa et al., 2013), bile salt (Lee et al., 2000), temperature shocks and antimicrobial agents (Sultana et al., 2000). It is suggested that encapsulation protect the probiotics from interfacial inactivation (Nazzaro et al., 2012).

Many investigators reported the most pronounced effect of encapsulated as compared to free/unencapsulated probiotics. For instance dietary supplementation of alginate-skim milk microcapsules of *S. cerevisiae* and *Lactobacillus rhamnosus* showed most pronounced effect on weight gain (%), SGR and, FCR of Nile tilapia (*O. niloticus*) as compared to un-encapsulated probiotics (Pinpimai et al.,2015; Pirarat et al., 2015). Similarly, Alginate microcapsules of *Schewanella putrefacien* showed higher viability/survival in the GI tract of Senegalese sole (Rosas-Ledesma et al., 2012). Moreover, Cordero et al. (2015) used alginate encapsulated probiotic strain Pdp 11 and observed significant increase in IgM level in gilthead seabream, *Sparusaurata* L. as compared to free/unencapsulated probiotic.

Several types of biopolymers and coating materials have been tested such as alginate, pectin, chitin and chitosan (Cordero et al., 2015; Tee et al., 2014) alone or in combination like alginate-chitosan, alginate-gelatin and alginate-skim milk microcapsules for encapsulation of probiotics (Zhao et al., 2012; Jiang et al., 2013). It is well documented that most of encapsulating agents when used alone form loose network, which cause leakage of core material, thus limits the beneficial effects (Zhang et al., 2011). However, this problem can be solved either by using combination of polymers or by coating encapsulating probiotic (microcapsule) with another material/natural polysaccharide like chitosan.

Alginate, is a natural-polysaccharide, has an ability to form gel in the presence of multivalent cations under mild conditions, thus permit the preservation and viability of the probiotic microorganisms (Trabelsi et al., 2013). Several investigators used alginate for encapsulating the probiotics (bacteria and yeast) (Sultana et al., 2000; Chandramouli et al., 2004; Gouin, 2004; Riaz and Masud, 2013). However, alginate microcapsule have some disadvantages like chemically not stable and disintegrate in the presence of low pH and excess amount of monovalent ions and other conditions (Krasaekoopt et al., 2003; Lee et al., 2004). Therefore other scientists used alginate in combination with other compounds like

starch, glycerol, 10 % skim milk, xanthan, chitosan etc (Krasaekoopt et al., 2003; Lee et al., 2004; Anal and Stevens, 2005; Huq et al., 2013; Pinpimai et al., 2015) and suggested that other supporting material/compound/polymer is required to improve the physiochemical characteristics of microcapsules (shape, morphology, stability, resistance to pH and temperature and target release etc).

Nowadays Alginate with chitosan formulation is getting attention, because the electrostatic-interaction of -COOH group of alginate with the -NH2 group of chitosan form a complex matrix that reduces their porosity (Ren et al., 2017), limit the leakage of core materials (Chavarri et al.,2010), provide more stability against GI tract harsh conditions (pH, bile, enzymes etc) of host (Zhou et al., 2000; Krasaekoopt et al., 2003) and improved bioavailability of probiotics at target site (Kumari et al., 2013). Moreover, chitosan has excellent properties for aquaculture applications, because it is a growth promoter and immuno-stimulator, thus also provide protection against the actions of certain microorganisms (Bautista-Baños et al., 2006; Jose et al., 2012).

The efficiency of microencapsulation depends on many factors like chemical nature and concentration of the material used for encapsulation, concentration of hardening solution process of encapsulation, size and shape of microcapsule, physiology of host organism and species/strain and amount of probiotic (Gbassi et al., 2009; Jyothi et al., 2010). Therefore, before practical application of encapsulated probiotics, literature emphasizes *in vitro* and *in vivo* simple tests for evaluating the temperature and pH tolerance, shelf life, resistance to bile and enzymes of GI tract of host and their impact on growth, immune response, metabolic function of an organism etc. (Papadimitriou et al., 2015). Although *in vitro* study provides the baseline information for selection of successful probiotics for future practical/commercial applications (Srinu et al., 2013), however, the results depends on the *in vitro* experiments

might not follow exactly the *in vivo* conditions. Hence, *in vivo* evaluation needs to be conducted.

The present part of study was designed to compare the *in vivo* efficacy of *G. candidum* encapsulated with alginate alone or in combination with xanthan and starch and coated with chitosan (bulk) and nano-chitosan, by observing their effect on survival, growth, FCR, intestinal enzyme activity, metabolic enzymes (AST, ALT, ALP), hemato-immunological indices and serum lipid profile of *L. rohita* fingerlings.

Materials and Methods

An 11 weeks feeding experiment was conducted, for evaluating the *in vivo* efficacy of *G. candidum* QAUGC-01 encapsulated with alginate alone or in combination with starch and xanthan and coated with chitosan (bulk) or nano-chitosan in comparison to free (un-encapsulated) *G. candidum*.

Probiotic organism

G. candidum QAUGC-01 was cultured as described in chapter 1 and encapsulated by various encapsulating agents i.e., alginate encapsulated (Alg), alginate encapsulated and coated with chitosan (Alg-C), alginate encapsulated and coated with nano-chitosan (Alg-CN), alginate-starch encapsulated (Alg-S), alginate-starch encapsulated and coated with chitosan (Alg-S-C), alginate-starch- encapsulated and coated with nano-chitosan (Alg-S-CN), alginate-xanthan encapsulated (Alg-X), alginate-xanthan encapsulated and coated with chitosan (Alg-X-C), alginate-xanthan encapsulated and coated with nano-chitosan (Alg-X-CN), as previously reported in chapter 2 in materials and methods sections. Moreover, probiotic cell density was measured by standard method reported by Nikoskelainen et al. (2003).

Feed preparation and probiotic supplementation

The 35% CP basal diet was formulated and prepared as described in chapter 1 (Table 1a). The prepared diet was divided in 11 groups i.e. Control (Control $_{FL}$) devoid of probiotic while others were fortified with probiotic *G. candidum* QAUGC-01 at the rate of 1×10^9 CFU/g, $Y1_{FL}$ group contained free or un-encapsulated *G. candidum*, while other treatment groups were supplemented with *G. candidum* encapsulated with Alg ($T2_{FL}$), Alg-C ($T3_{FL}$), Alg-CN ($T4_{FL}$), Alg –S ($T5_{FL}$), Alg-S-C ($T6_{FL}$), Alg-S-CN ($T7_{FL}$), Alg-X ($T8_{FL}$), Alg-X-C ($T9_{FL}$), Alg-X-CN ($T10_{FL}$). Diet was fortified with probiotic by top dressing as described previously.

The pelleted (1mm) feed was dried by placing in freeze dryer for 48 hrs. After drying the feed was stored in air-tight jars and placed in refrigerator. Viability of free and encapsulated forms of *G. candidum* QAUGC-01 in feed was checked using standard method (Nikoskelainen et al. 2003; Amir et al., 2018). The feed was freshly prepared fortnightly to avoid any extreme variation in probiotic cell count.

Fish collection and maintenance

About fourteen hundred fingerlings of *L. rohita* were procured from a Government Fish Hatchery Manawan, Lahore, Pakistan. Fish was transported in well aerated oxygen filled plastic bags to the Fisheries and Aquaculture Research Centre, Quaid-i-Azam University Islamabad, Pakistan, and after tempering stocked in the five circular fiberglass tanks (capacity: 350 Gallon) having flow through system. Fish were acclimated for 4 days and during that period, provided 35% crude protein basal diet.

Experimental design

A completely randomize experiment was designed and conducted in replicate of three in an outdoor facility during the months of June to August. For initiation of experiment, active fingerlings (average weight, 15.63±0.35g) apparently with no infection or disease symptoms were randomly stocked in 33 separate small fiberglass tanks (capacity; 500L) at a stocking density of 2.0 g L^{-1} (about 40 fingerlings/ tank). Tanks were supplied with the aerators in orders to maintain the dissolved oxygen level. After shifting, they were again acclimated in their respective tanks for about two days. Afterwards, 33 tanks were randomly divided into eleven groups i.e. Control $_{FL}$, $Y1_{FL}$, $Y2_{FL}$, $Y3_{FL}$, $Y4_{FL}$, $Y5_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y8_{FL}$, $Y9_{FL}$ and $Y10_{FL}$. Feeding trial was started by providing each group to their respective diet i.e. basal diet to $Control_{FL}$ group and free or un-encapsulated *G. candidum* to $Y1_{FL}$, while all other groups were given their respective diet supplemented with microcapsules of *G. candidum* prepared

with Alg, Alg-C, Alg-CN to $Y2_{FL}$, $Y3_{FL}$ and $Y4_{FL}$ groups respectively, Alg-S, Alg-S-C and Alg-S-CN to ($Y5_{FL}$, $Y6_{FL}$ and $Y7_{FL}$ respectively while Alg-X, Alg-X-C and Alg-X-CN to $Y8_{FL}$ $Y9_{FL,}$ $Y10_{FL}$ groups respectively. Initially, they were fed three times daily (8:00, 12:00 and 16:00 hrs), with 4% body weight and end with twice a day (9:00 and 16:00 hrs) at the rate of 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 15-20% exchanged with freshwater.

During feeding trial, water temperature (°C), DO level (mg L^{-1}) and pH of water were noted twice a day (09:00 and 16:00 hrs) with multi-parameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA), while total ammonia was checked weekly using ammonia test-kit. At the start of experiment, water temperature of all the groups was 24.3±0.3°C. Gradually, it was increased with time and peak (28.2±0.2°C) during the month of August. Furthermore, as each tank nonstop aerated, therefore, DO level (5.8-6.7 mg L^{-1}) showed a small variation with respect to each group, while total ammonia in each tank was < 0.25 mg L^{-1}. The pH value of each tank showed small fluctuation which was statistically insignificant. The experimental setup was in same vicinity, under similar environmental conditions, thus no noticeable differences among all experimental groups, were observed in water quality parameters.

Growth study and sample collection

After completion of 11 weeks experiment, fingerlings of each group were starved for about 24 hrs before sampling. They were carefully removed separately from each tank with scoop net, weighed by using top loading balance (SHIMADZU-ELB3000, Japan) and counted for determining the average weight of individual fingerlings. The survival (%), weight gain (g),

(% WG), SGR and final biomass was estimated as described in chapter 1. For hepatosomatic index (HSI) following formula was used:

$$\text{HIS (\%)} = \frac{\text{Wet wt. of liver}}{\text{Wet body wt. of fish}} \times 100$$

Intestinal enzyme analysis

For intestinal enzyme analysis, 12 fish per tank (36 fish/group) were anesthetized with MS-222 (0.10 g L^{-1} buffered with sodium bicarbonate), dissected on ice pad by adopting standard aseptic methods for intestinal enzyme estimation described previously in chapter 1. GI tract of 4 fish of the same tank were pooled (3 samples/tank or 9 samples/group), snap-frozen in liquid nitrogen and immediately stored at -20˚C for determination of intestinal enzyme activities as reported previously (chapter 1).

Hematological analysis

For complete blood count, about 12 juvenile fish, from each fiberglass tank (36 juvenile fish/group) were captured through scoop net and blood was drawn from the caudal vein by using 2mL sterile heparinized syringes (24G, B-D®Discardit™ Becton Dickinson, Spain). Blood samples were collected in lavender top K2 VACUETTE®-EDTA tubes (Liuyang Sanli Medical Technology Development Co., LTD). In order to get enough amount, blood of 3 fish from each tank was pooled in same EDTA tubes (3 samples/tank or 9 samples/group). The heparinized blood was used for CBC determination i.e. red blood cells ($\times 10^6 \mu L^{-1}$), hemoglobin (gdL^{-1}), hematocrit (%), white blood cells ($x10^3 \mu L^{-1}$), MCH (pg), (gdL^{-1}) and MCV (fL) as described previously in chapter 1.

Immunological analysis

Blood sample from 12 fish per tank (36 fingerlings/group) was also collected with 2 ml heparinized syringe (24G, B-D®Discardit™ Becton Dickinson, Spain) in red top EDTA

VACUETTE®tubes (LiuyangSanli Medical Technology Development Co., LTD). For taking enough sample, blood drawn from 4 fish of same tank was collected in the same blood collecting tube (9 sample/group). Serum was separated by centrifuging whole blood for 5 min at 3000 rpm and saved at 4˚C for further analysis of AST, total Serum proteins, IgM and lysozyme enzyme activity, while fresh heparinized blood was used for phagocytic activity and respiratory burst activity by following procedure already mentioned in chapter 1. Furthermore, total cholesterol, triglycerides, HDL, LDL, alanine aminotransferase (ALT) activity and alkaline phosphatase (ALP) activity were measured using following methods.

Alanine aminotransferase (ALT)

ALT activity was measured by using alanine aminotransferase Activity Assay KIT MAK-052 (SIGMA-ALDRICH). Assay was performed by adopting standard procedure. Briefly, before initiating analysis, all reagents allowed to come to room temperature. Subsequently, Master reaction mix (1 mL) was prepared by mixing 860 µL ALT Assay buffer with 100µL ALT substrate and 20 µL each of fluorescent peroxidase substrate and ALT enzyme mix. Afterword, serum sample (10 µL) was added to 96 well microplate and equal volume of ALT buffer was added in it, to make a final volume of 20 µL. For positive control, 5 µL of the ALT positive control was added to wells, and volume was adjusted to 20 µL with ALT buffer. Subsequently, Master reaction mix (100µL) was added in each of standard, positive control and samples wells. The mixture in each well was mixed well for 3 min by placing plate on horizontal shaker (Model ISS Innova 43). After that, initial measurement was taken and absorbance was read at 570 nm (Agilent, 8453, USA). Then microplate was incubated at 30 °C by taking measurement after every 5 min until 20 min. The change in absorbance from initial and final reading for samples and positive control was measured. ALT activity was calculated and expressed as UL^{-1}. One unit of ALT is defined as amount of enzyme that generates 1.0 nmole of pyruvate $min^{-1.}$

Alkaline phosphatase (ALP)

ALP activity was measured using ALP kit (Thermo Scientific reagent kit, Cat # TR11320). Assay was performed by following standard procedure. Briefly, 200 µL of reagent 1 (consisting of 46 $mmolL^{-1}$ AMP, 2.55 $mmolL^{-1}$ Mg acetate, 1.27 $mmolL^{-1}$ $ZnSO_4$ and 2.55 $mmolL^{-1}$ EDTA) and 50 µL of reagent 2 (containing 81.6 $mmolL^{-1}$ 4-NPP, pH 10.75±0.10 at 20 °C) were mixed with 5 µL serum sample. The automatic chemistry analyzer (MICRO-LAB 200, Germany) was set at 405 nm and absorbance was noted.

Total cholesterol

Greiner Diagnostic GmbH kit (Unter Gereuth, Bahlingen-Germany) was used for determination of total cholesterol. Briefly, 1 mL Reagent R was mixed with 10µl serum sample and incubated at ambient temperature for about 5 min and absorbance was noted at 500 nm by using chemistry analyzer (MICRO- LAB 200, Germany). Total cholesterol was calculated as mg dL^{-1}.

Triglycerides

Serum triglyceride level was measured by using AMP diagnostic kit (AMEDA Labor diagnostik GmbH, Germany). Serum sample (1µL) was mixed with Reagent R (1 mL) and incubated for 15 min at 25°C. Then, absorbance at 500 nm was noted by using chemistry analyzer (MICRO- LAB 200, Germany). Triglyceride level was measured as mg dL^{-1}.

HDL and LDL

HDL and LDL levels in control and experimental groups of fish were determined by using Quantitation kit MAK045 (Sigma-Aldrich). Serum sample (100µL) was mixed with 2× LDL precipitation buffer (MAK045B-Sigma Aldrich) and incubated for 10 min at ambient temperature. Subsequently, centrifuged (Kokusan, H-103RS, Ogawa Seiki Co., LTD, Tokyo,

Japan) the sample at 2000g for 10 min. Supernatant fraction containing HDL was transferred to a separate eppendorf tube while precipitate containing LDL was centrifuged again to remove any residual HDL. Then the resultant precipitate was re-suspended in 200 µL of PBS (pH 7.3). Afterwards 50 µL of reaction mixture, containing 44 µL cholesterol essay buffer, 2 µL each of cholesterol probe and cholesterol esterase and 1 µL cholesterol enzyme mix and was incubated at 25°C for 60 min then absorbance was measured at 570 nm (MICRO-LAB 200, Germany).

Statistical analysis

Before running any statistical analysis, data was assessed for variance homogeneity and normality distribution by using Bartlett and Shapiro-Wilk's tests. All data was represented as mean ± SD. Significant differences in growth performance, survival, enzymatic activity, hematology and immunological indices among experimental treatment groups were identified by using one–way ANOVA. Once significant differences were found comparison among the means were made using post hoc test i.e. LSD test using similar Software as described previously. All the results were statistically evaluated at the significance level of 0.05. For graphical representation of the data GraphPad Prism 5 was used.

Results

Growth performance and survival of *L. rohita* fingerlings

The dietary intake of encapsulated *G. candidum* indicated the most significant positive effect on the growth rate indices of *L. rohita* fingerlings in contrast to the free/un-encapsulated probiotic, while the efficiency of different groups of encapsulated *G. candidum* showed variation on the basis of used encapsulating agent and coating with chitosan (Table 3.1). One way ANOVA showed significant variation in final weight (n=3, ANOVA, $F_{10, 33}$=196, p<0.001), weight gain percent (n=3, ANOVA, $F_{10, 33}$=126, p<0.001), SGR (n=3, ANOVA, $F_{10, 33}$=135, p<0.001), final biomass (n=3, ANOVA, $F_{10, 33}$=208, p<0.001), HSI (n=3, ANOVA, $F_{10, 33}$=21, p<0.001), FCR (n=3, ANOVA, $F_{10, 33}$=39.8 , p<0.001) and survival (n=3, ANOVA, $F_{10, 33}$=4, p<0.001) between different groups. The LSD post hoc test indicated improved growth performance in all probiotic fed groups ($Y1_{FL}$, $Y2_{FL}$, $Y3_{FL}$, $Y4_{FL}$, $Y5_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y8_{FL}$, $Y9_{FL}$ and $Y10_{FL}$) as compared to control $_F$ (Table 3.1). Furthermore, fish fed encapsulated *G. candidum* ($Y2_{FL}$, $Y3_{FL}$, $Y4_{FL}$, $Y5_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y8_{FL}$, $Y9_{FL}$, and $Y10_{FL}$) showed a significantly higher growth rate in terms of weight gain, final biomass, SGR value as compared to un-capsulated/free form of *G. candidum* ($Y1_{FL}$).

Furthermore, groups provided encapsulated *G. candidum* with chitosan coating supplemented diets ($Y4_{FL}$, $Y3_{FL}$, $Y7_{FL}$, $Y6_{FL}$, $Y10_{FL}$ and $Y9_{FL}$) showed comparatively higher growth rate as compared to other groups of fish fed similar encapsulated *G. candidum* but without chitosan coating ($Y2_{FL}$, $Y5_{FL}$ and $Y8_{FL}$). Similarly, nano-chitosan coated *G. candidum* microcapsules as compared to chitosan (bulk) coated one showed more pronounced effect. Overall, $Y4_{FL}$ group showed the highest weight gain, SGR, final biomass

and lowest FCR followed by $Y3_{FL}$ group, while among probiotic fed groups, $Y1_{FL}$ showed lowest values of growth performances indices and the highest value of FCR.

During experimental period, except control group all other groups (probiotic fed) showed 100% survival.

Table 3.1. Growth performance of *L. rohita* fingerlings, after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum*, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan (n=3)

	Groups											Pooled SEM	F ratio	p-value
	Control $_{FL}$	Y1$_{FL}$	Y2$_{FL}$	Y3$_{FL}$	Y4$_{FL}$	Y5$_{FL}$	Y6$_{FL}$	Y7$_{FL}$	Y8$_{FL}$	Y9$_{FL}$	Y10$_{FL}$			
IBW (g)	15.1	15.4	15.2	15.7	15.4	15.7	15.9	16.1	15.6	15.5	15.8	0.15	3.48	0.613
FBW (g)	26.6^{j}	30.5^{i}	32.3^{gh}	49.6^{b}	56.4^{a}	34.3^{g}	41.3^{d}	45.1^{c}	34.1^{g}	36.2^{f}	38.4^{e}	0.63	196	0.001
Weight gain(g)	11.5^{j}	15.1^{i}	17.1^{gh}	33.8^{b}	40.9^{a}	18.6^{g}	25.3^{d}	29.0^{c}	18.4^{g}	20.7^{f}	22.6^{e}	0.65	176	0.001
Weight gain (%)	75.9^{j}	97.6^{i}	112^{gh}	214^{b}	265^{a}	118^{g}	159^{d}	180^{c}	117^{g}	133^{f}	142^{e}	4.90	126	0.001
^{1}SGR	0.74^{h}	0.89^{g}	0.98^{f}	1.5^{b}	1.7^{a}	1.02^{e}	1.25^{d}	1.35^{c}	1.02^{e}	1.11^{d}	1.16^{d}	0.24	135	0.001
Initial biomass (g)	1212^{b}	1238^{ab}	1220^{b}	1262^{ab}	1235^{ab}	1258^{ab}	1273^{ab}	1288^{a}	1254^{ab}	1243^{ab}	1269^{ab}	12.2	3.48	0.007
Final biomass (g)	2115^{j}	2446^{i}	2589^{hi}	3970^{b}	4514^{a}	2749^{g}	3304^{d}	3612^{c}	2728^{gh}	2901^{f}	3077^{e}	49.9	208	0.001
^{2}FCR	3.93^{a}	3.11^{b}	2.88^{bc}	1.58^{ef}	1.41^{f}	2.76^{bcd}	1.89^{e}	1.63^{ef}	2.83^{bcd}	2.68^{cd}	2.49^{d}	0.12	39.8	0.001
Survival (%)	96.0^{b}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	0.55	4.00	0.003
^{3}HSI	0.65^{h}	0.92^{g}	1.04^{fg}	1.53^{ab}	1.72^{a}	1.11^{efg}	1.34^{cd}	1.44^{bc}	1.08^{efg}	1.21^{def}	1.26^{cde}	0.07	21.0	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Averages with different lowercase superscripts in same rows shows significant difference at $p < 0.05$. Control$_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*,Y2$_{FL}$= Alginate encapsulated, Y3$_{FL}$=Alginate encapsulated and coated with chitosan, Y4$_{FL}$=Alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$= alginate-Starch encapsulated, Y6$_{FL}$=Alginate-Starch encapsulated and coated with chitosan, Y7$_{FL}$=Alginate-Starch- encapsulated and coated with nano-chitosan, Y8$_{FL}$=Alginate-Xanthan encapsulated, T9$_{FL}$=Alginate-Xanthan encapsulated and coated with chitosan, Y10$_{FL}$=Alginate-Xanthan encapsulated and coated with nano-chitosan.[1]Specific growth rate (% body weight day^{-1}), [2]feed conversion ratio, [3]hepatosomatic index.

Intestinal enzyme activities

Yeast based encapsulated *G. candidum* dietary supplement also showed most significant positive effect in contrast to free/un-encapsulated probiotic on the activities of intestinal enzymes of *L. rohita* (Table 3.2). One way ANOVA revealed significant differences in protease (n=9, ANOVA, $F_{10, 99}$ = 670, p=0.001), cellulase (n=9, ANOVA, $F_{10, 99}$ = 435, p=0.001) and amylase (n=9, ANOVA, $F_{10, 99}$= 284, p=0.001) activity in all experimental groups. The pairwise comparison among groups showed a significant increase in overall intestinal enzymatic activity in all groups fingerlings fed *G. candidum* supplemented diet in comparison to the control$_F$ group. Furthermore, all groups ($Y2_{FL}$, $Y3_{FL}$, $Y4_{FL}$, $Y5_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y8_{FL}$, $Y9_{FL}$, and $Y10_{FL}$) fed encapsulated *G. candidum* supplemented diet in contrast to free/un-encapsulated *G. candidum* ($Y1_{FL}$) showed significantly higher intestinal enzymatic activities.

The close observations of data revealed that those groups ($Y4_{FL}$, $Y3_{FL}$, $Y7_{FL}$, $Y6_{FL}$, $Y10_{FL}$ and $Y9_{FL}$) which fed encapsulated *G. candidum* with a coating of chitosan showed most pronounced effect on intestinal enzymatic activities as compared to groups of fingerlings ($Y2_{FL}$, $Y5_{FL}$ and $Y8_{FL}$) fed diet supplemented with encapsulated *G. candidum* but without chitosan coating. Furthermore, intestinal protease, amylase and cellulase activity in different groups of fingerlings follow the trend $Y4_{FL}$>$Y3_{FL}$>$Y6_{FL}$> $Y7_{FL}$>$Y10_{FL}$>$Y9_{FL}$>$Y5_{FL}$>$Y8_{FL}$>$Y2_{FL}$>$Y1_{FL}$>Control $_{FL}$, while activity of observed three enzymes in different groups followed the similar trend i.e., amylase > protease and cellulase.

Table 3.2. Intestinal enzyme activities (U mg^{-1}) in gastrointestinal tract of *L. rohita* fingerlings, after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum*, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan (n=9)

Intestinal enzymes (U mg^{-1})	Groups											Pooled SEM	F ratio	p-value
	Control $_{FL}$	Y1$_{FL}$	Y2$_{FL}$	Y3$_{FL}$	Y4$_{FL}$	Y5$_{FL}$	Y6$_{FL}$	Y7$_{FL}$	Y8$_{FL}$	Y9$_{FL}$	Y10$_{FL}$			
Protease	$0.36^{i,A}$	$0.65^{h,B}$	$0.82^{gh,B}$	$3.12^{b,A}$	$3.69^{a,A}$	$1.18^{f,C}$	$2.64^{c,A}$	$2.79^{c,A}$	$1.01^{fg,C}$	$1.69^{e,C}$	$2.38^{d,A}$	0.04	670	0.001
Cellulase	$0.26^{j,A}$	$0.74^{i,C}$	$0.95^{h,B}$	$2.69^{b,C}$	$2.96^{a,C}$	$1.69^{f,B}$	$2.20^{cd,B}$	$2.37^{c,B}$	$1.34^{g,B}$	$1.94^{e,B}$	$2.07^{de,B}$	0.04	435	0.001
Amylase	$0.28^{i,A}$	$1.38^{h,A}$	$1.50^{gh,A}$	$2.92^{b,B}$	$3.25^{a,B}$	$1.95^{f,A}$	$2.58^{cd,A}$	$2.80^{bc,A}$	$1.7^{g,A}$	$2.13^{ef,A}$	$2.35^{de,A}$	0.05	284	0.001
Pooled SEM	0.06	0.03	0.52	0.04	0.06	0.03	0.03	0.03	0.04	0.04	0.05	---	---	---
F ratio	0.63	174	46.6	34.7	34.2	162	61.1	59.3	94.2	25.9	9.40	---	---	---
P value	0.53	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	---	---	---

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between and within groups. Means with different lowercase superscripts in same rows are significantly different at $p < 0.05$ and show comparison between groups, however uppercase superscript letters in same column shows difference within group. Control $_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*,Y2$_{FL}$= alginate encapsulated, Y3$_{FL}$=alginate encapsulated and coated with chitosan, Y4$_{FL}$=alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$= alginate-Starch encapsulated, Y6$_{FL}$= alginate-starch encapsulated and coated with chitosan, Y7$_{FL}$=alginate-starch- encapsulated and coated with nano-chitosan, Y8$_{FL}$=alginate-xanthan encapsulated, Y9$_{FL}$=alginate-xanthan encapsulated and coated with chitosan, Y10$_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

Hematological indices of *L. rohita* fingerlings

The evaluation of hematological indices also confirmed the most significant positive effect of encapsulated as compared to free/un-capsulated *G. candidum* dietary supplementation on *L. rohita* fingerlings (Table 3.3). One way ANOVA indicated significant differences in RBC (n=9, ANOVA, $F_{10, 99}$= 852, p=0.001), WBC (n=9, ANOVA, $F_{10, 99}$= 462, p=0.001), Hb (n=9, ANOVA, $F_{10, 99}$= 2324, p=0.001), Hct (n=9, ANOVA, $F_{10, 99}$= 1634, p=0.001), MCV (n=9, ANOVA, $F_{10, 99}$= 71.1, p=0.001), MCH (n=9, ANOVA, $F_{10, 99}$= 79, p=0.001) and MCHC (n=9, ANOVA, $F_{10, 99}$= 146, p=0.001) between groups. The Post hoc LSD test showed improved status of hematological indices of all probiotic supplemented diet fed groups in comparison to a $control_F$ group taken diet devoid of any supplement. Furthermore, results also indicated that encapsulated *G. candidum* as compared to free/un-encapsulated *G. candidum* showed most significant effect on erythrocytes, leukocytes, hemoglobin and hematocrit (Table 3.3).

The close observation of data also revealed the most significant effect of encapsulated *G. candidum* having chitosan coating on RBCs, WBCs, Hb and Hct values of $Y4_{FL}$, $Y3_{FL}$, $Y7_{FL}$, $Y6_{FL}$, $Y10_{FL}$ and $Y9_{FL}$ groups of fingerlings as compared to encapsulated *G. candidum* without chitosan coating ($Y2_{FL}$, $Y5_{FL}$ and $Y8_{FL}$). Overall results indicated the most significant increase in RBCs, WBCs, Hb, Hct and decreased in MCV, MCH and MCHC in $Y4_F$ group of fingerlings followed by $Y3_{FL}$ (Table 3.3).

Table 3.3. Hematological indices of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum*, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan (n=9)

Hematological indices	Groups											Pooled SEM	F ratio	p-value
	Control $_{FL}$	Y1$_{FL}$	Y2$_{FL}$	Y3$_{FL}$	Y4$_{FL}$	Y5$_{FL}$	Y6$_{FL}$	Y7$_{FL}$	Y8$_{FL}$	Y9$_{FL}$	Y10$_{FL}$			
RBC (10^6 µL)	0.82^{j}	1.09^{i}	1.24^{h}	2.46^{b}	2.73^{a}	1.39^{g}	2.10^{d}	2.26^{c}	1.27^{h}	1.60^{f}	1.87^{e}	0.021	852	0.001
WBC (10^3 µL)	113^{j}	138^{i}	157^{h}	249^{b}	266^{a}	172^{g}	223^{d}	237^{c}	167^{g}	184^{f}	203^{e}	2.24	462	0.001
Hb (g dL^{-1})	4.76^{j}	6.09^{i}	7.14^{h}	10.4^{b}	10.7^{a}	7.69^{g}	9.08^{d}	9.75^{c}	7.18^{h}	8.20^{f}	8.95^{e}	0.04	2324	0.001
HCT (%)	10.1^{k}	17.4^{j}	20.2^{i}	34.6^{b}	37.4^{a}	24.7^{g}	30.2^{d}	32.5^{c}	21.4^{h}	26.9^{f}	28.8^{e}	0.20	1634	0.001
MCV (fL or 10^{-15})	125.2^{g}	159.8^{c}	161.9^{c}	140.5^{ef}	137^{f}	177.4^{a}	143.9^{e}	143.9^{e}	168^{b}	168.1^{b}	154^{d}	1.88	71.1	0.001
MCH (pg)	58.5^{a}	55.8^{b}	57.2^{ab}	42.2^{e}	39.1^{f}	55.1^{b}	43.2^{e}	43.1^{e}	56.3^{ab}	51.2^{c}	47.8^{d}	0.79	79	0.001
MCHC(g dL^{-1})	46.9^{a}	34.9^{b}	35.3^{b}	30.05^{d}	28.5^{e}	31.06^{d}	30.02^{d}	29.9^{d}	33.5^{c}	30.4^{d}	31.02^{d}	0.42	146	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Averages with different lowercase superscripts in same rows shows significant difference at $p < 0.05$. Control $_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*,Y2$_{FL}$= alginate encapsulated, Y3$_{FL}$=alginate encapsulated and coated with chitosan, Y4$_{FL}$=alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$= alginate-Starch encapsulated, Y6$_{FL}$= alginate-starch encapsulated and coated with chitosan, Y7$_{FL}$=alginate-starch-encapsulated and coated with nano-chitosan, Y8$_{FL}$=alginate-xanthan encapsulated, Y9$_{FL}$=alginate-xanthan encapsulated and coated with chitosan, Y10$_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

Aspartate aminotransferase (AST) activity

After 11 weeks feeding trial, serum AST activity also showed significant differences between different groups of *L. rohita* fingerlings provided free/un-encapsulated probiotic and encapsulated *G. candidum* supplemented diets (Fig. 3.1). One way ANOVA depicted significant difference in serum AST activity among different groups (n=9, ANOVA, $F_{10, 99}$= 312, p=0.001), while Post hoc LSD test indicated significantly lower AST activity in probiotic fed groups vs control$_F$ group and encapsulated *G. candidum* fed groups in contrast to un-encapsulated/free probiotic fed group. Moreover, $Y3_{FL}$, $Y4_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y9_{FL}$ and $Y10_{FL}$ groups of fish fed diet fortified with encapsulated *G. candidum* with chitosan coating showed most significant decrease in serum AST level as compared to $Y5_{FL}$, $Y8_{FL}$ and $Y2_{FL}$ groups of *L. rohita* fed diet supplemented with encapsulated without chitosan coating *G. candidum*. Overall, $Y4_{FL}$ and $Y3_{FL}$ groups showed the lowest and statistically comparable serum AST activity while control $_F$ showed the highest value (Fig. 3.1).

Alanine aminotransferase (ALT) activity

The encapsulated *G. candidum* supplementation showed the most pronounced effect on serum ALT activity of *L. rohita* as compared to free/un-encapsulated probiotic. Moreover, *G. candidum* encapsulated with different encapsulating agents and coating with chitosan showed significant variation in the efficiency of probiotic. One way ANOVA showed significantly different serum ALT activity among different groups (n=9, ANOVA, $F_{10, 99}$= 109, p=0.001, Fig 3.2), while Post hoc LSD test showed significantly lower ALT activity in probiotic fed groups as compared to control $_{FL}$ group as well as in encapsulated *G. candidum* fed groups in contrast to free/un-encapsulated probiotic fed group ($Y1_{FL}$).

Furthermore, the comparative efficiency of encapsulated *G.candidum,* indicated lower level of ALT activity in groups of fingerlings ($Y3_{FL}$, $Y4_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y9_{FL}$ and $Y10_{FL}$), where the

supplemented encapsulated probiotic had chitosan coating. Overall, the lowest AST activity was observed in $Y4_{FL}$ group (diet supplemented with nano-chitosan coated encapsulated *G. candidum*) followed by $Y3_{FL}$ group (diet supplemented with chitosan (bulk) coated encapsulated *G. candidum*). However, Control $_{F}$ group (fed basal diet) showed the highest ALT activity followed by $Y1_{FL}$ group (fed diet supplemented with un-encapsulated probiotic) (Fig. 3.2).

Alkaline phosphatase (ALP) activity

The encapsulated dietary *G. candidum* supplementation showed the most significant positive effect on the serum ALP activity of *L. rohita* as compared to free/un-encapsulated probiotic. Moreover coating materials used for encapsulation also showed significant effect on the efficiency of G. *candidum*. One way ANOVA showed significant differences in serum ALP activity among different groups (n=9, ANOVA, $F_{10, 99}$= 400, p=0.001, Fig. 3.3), while Post hoc LSD test showed significantly higher activity of ALP in probiotic fed groups as compared to a control $_{FL}$ group as well as in encapsulated *G. candidum* fed groups, in contrast to un-encapsulated probiotic fed group ($Y1_{FL}$). Moreover, groups of fish fed diet supplemented with chitosan coated encapsulated *G. candidum* ($Y3_{FL}$ and $Y4_{FL}$ respectively) showed the highest ALP activity as compared to other groups where supplemented encapsulated *G. candidum* was devoid of chitosan coating. Overall, the highest ALT activity was observed in $Y4_{FL}$ followed by $Y3_{FL}$> $Y7_{FL}$> $Y6_{FL}$> $Y10_{FL}$> $Y9_{FL}$> $Y5_{FL}$>$Y2_{FL}$>$Y1_{FL}$ while control$_{F}$ group showed the lowest activity (Fig. 3.3).

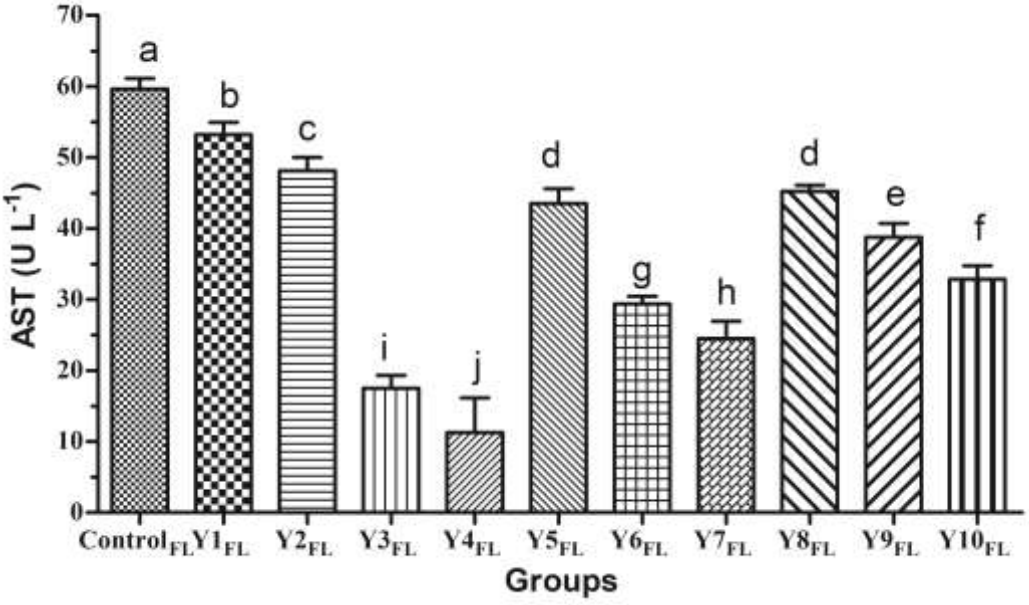

Fig. 3.1. Serum aspartate aminotransferase (AST) activity of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum,* chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between different groups. Means followed by different lowercase superscripts letters on bars are considerably different at $P < 0.05$. Control $_{FL}$= without any probiotic supplement, $Y1_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*, $Y2_{FL}$=alginate encapsulated, $Y3_{FL}$=alginate encapsulated and coated with chitosan, $Y4_{FL}$=alginate encapsulated and coated with nano-chitosan, $Y5_{FL}$= alginate-Starch encapsulated, $Y6_{FL}$= alginate-starch encapsulated and coated with chitosan, $Y7_{FL}$=alginate-starch- encapsulated and coated with nano-chitosan, $Y8_{FL}$=alginate-xanthan encapsulated, $Y9_{FL}$=alginate-xanthan encapsulated and coated with chitosan, $Y10_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

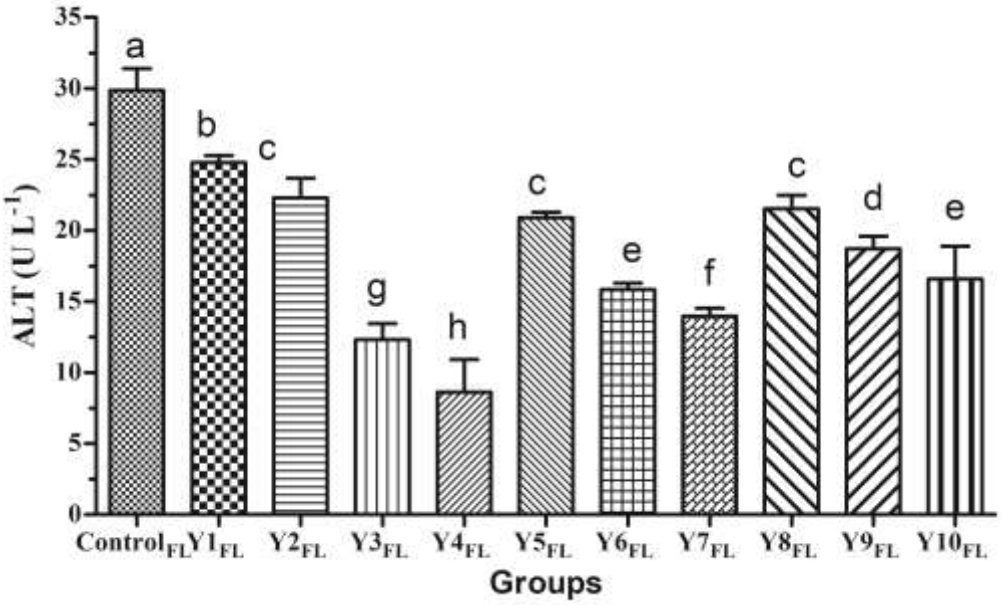

Fig. 3.2. Serum alanine aminotransferase (ALT) activity of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum* and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between different groups. Means with different lowercase letters on bars are considerably different at $P < 0.05$. $Control_{FL}$= diet devoid of probiotic supplement, $Y1_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*, $Y2_{FL}$=alginate encapsulated, $Y3_{FL}$=alginate encapsulated and coated with chitosan, $Y4_{FL}$=alginate encapsulated and coated with nano-chitosan, $Y5_{FL}$= alginate-starch encapsulated, $T6_{FL}$= alginate-starch encapsulated and coated with chitosan, $Y7_{FL}$=alginate-starch- encapsulated and coated with nano-chitosan, $Y8_{FL}$=alginate-xanthan encapsulated, $Y9_{FL}$=alginate-xanthan encapsulated and coated with chitosan, $Y10_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

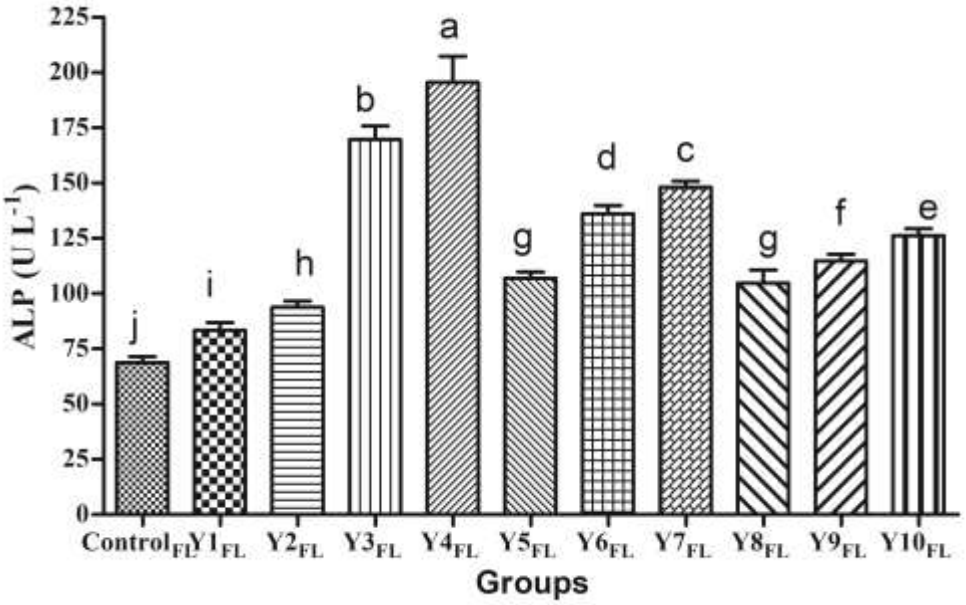

Fig. 3.3. Alkaline phosphatase (ALP) activity of *L. rohita* after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum* and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between different groups. Averages with dissimilar lowercase letters on bars shows significant difference at $P < 0.05$. $Control_{FL}$ = without any probiotic supplement, $Y1_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*, $Y2_{FL}$= alginate encapsulated, $Y3_{FL}$=alginate encapsulated and coated with chitosan, $Y4_{FL}$=alginate encapsulated and coated with nano-chitosan, $Y5_{FL}$=alginate-starch encapsulated, $T6_{FL}$= alginate-starch encapsulated and coated with chitosan, $Y7_{FL}$=alginate-starch- encapsulated and coated with nano-chitosan, $Y8_{FL}$=alginate-xanthan encapsulated, $Y9_{FL}$=alginate-xanthan encapsulated and coated with chitosan, $Y10_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

Immunological indices of *L. rohita* fingerlings

Results indicated the most significant positive effect of encapsulated *G. candidum* on immunological indices of *L. rohita* fingerlings as compared to un-encapsulated/free probiotic. One way ANOVA revealed significant difference among groups with respect to total serum protein content (n=9, ANOVA, $F_{10, 99}$=651, p=0.001, Table 3.4), immunoglobulin (n=9, ANOVA, $F_{10, 99}$=779, p=0.001, Table 3.4), serum lysozyme activity (n=9, ANOVA, $F_{10, 99}$= 299, p=0.001, Table 4), Phagocytic activity (n=3, ANOVA, $F_{10, 33}$= 35.9, p=0.001, Fig.3.4), phagocytic index (n=3, ANOVA, $F_{10, 33}$= 30.1, p=0.001, Fig. 3.5) and respiratory burst activity (n=9, ANOVA, $F_{10, 99}$= 113, p=0.001, Fig. 3.6). The Post hoc LSD test revealed that total protein, immunoglobulin, lysozyme level, respiratory burst activity, phagocytic activity and phagocytic index were significantly higher in probiotic fed groups in comparison to a $control_F$ group. Furthermore, among probiotic fed groups, improved immune status was observed in response to encapsulated *G. candidum* supplemented diet as compared to un-encapsulated/free *G. candidum* ($T1_{FL}$).

Moreover, encapsulated *G. candidum* with chitosan coating ($Y3_{FL}$, $Y4_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y9_{FL}$ and $Y10_{FL}$) showed a more pronounced effect on immunological indices as compared to the encapsulated *G. candidum* devoid of chitosan coating ($Y5_{FL}$, $Y8_{FL}$ and $Y2_{FL}$). The comparative evaluation of groups where encapsulated *G. candidum* had chitosan coating, indicated significantly higher total protein, IgM, lysozyme, phagocyte and respiratory burst activity in $Y4_{FL}$ group followed by $Y3_{FL}$ group while the lowest levels in $Y10_{FL}$ group of fish. However, $Y2_{FL}$, $Y5_{FL}$ and $Y8_{FL}$ groups, supplemented with encapsulated G. *candidum* devoid of chitosan coating showed statistically comparable impact on total protein, IgM, serum lysozyme and respiratory bursts activity of *L. rohita* (Table 3.4, Fig. 3.6).

Table 3.4. Immunological indices of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum*, chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan (n=9)

	Groups													
	Control$_{FL}$	**Y1$_{FL}$**	**Y2$_{FL}$**	**Y3$_{FL}$**	**Y4$_{FL}$**	**Y5$_{FL}$**	**Y6$_{FL}$**	**Y7$_{FL}$**	**Y8$_{FL}$**	**Y9$_{FL}$**	**Y10$_{FL}$**	**Pooled SEM**	**F-ratio**	**p-value**
Total serum protein (mg mL^{-1})	10.2^{j}	12.1^{i}	15.0^{h}	25.9^{b}	29.9^{a}	16.1^{g}	20.7^{d}	23.2^{c}	15.6^{gh}	17.6^{f}	19.0^{e}	0.23	651	0.001
IgM (mg mL^{-1})	1.35^{i}	1.93^{h}	2.31^{gh}	10.8^{b}	12.6^{a}	2.52^{g}	7.23^{d}	8.94^{c}	2.50^{g}	3.59^{f}	5.12^{e}	0.14	779	0.001
Lysozyme activity (µg mL^{-1})	3.77^{i}	5.11^{h}	5.95^{g}	10.7^{b}	12.2^{a}	6.32^{fg}	8.13^{d}	8.96^{c}	5.95^{g}	6.43^{f}	7.45^{e}	0.15	299	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Averages with different lowercase superscripts in same rows shows significant difference at $p < 0.05$. Control $_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*,Y2$_{FL}$= alginate encapsulated, Y3$_{FL}$=alginate encapsulated and coated with chitosan, Y4$_{FL}$=alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$= alginate-Starch encapsulated, Y6$_{FL}$= alginate-starch encapsulated and coated with chitosan, Y7$_{FL}$=alginate-starch- encapsulated and coated with nano-chitosan, Y8$_{FL}$=alginate-xanthan encapsulated, Y9$_{FL}$=alginate-xanthan encapsulated and coated with chitosan, Y10$_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

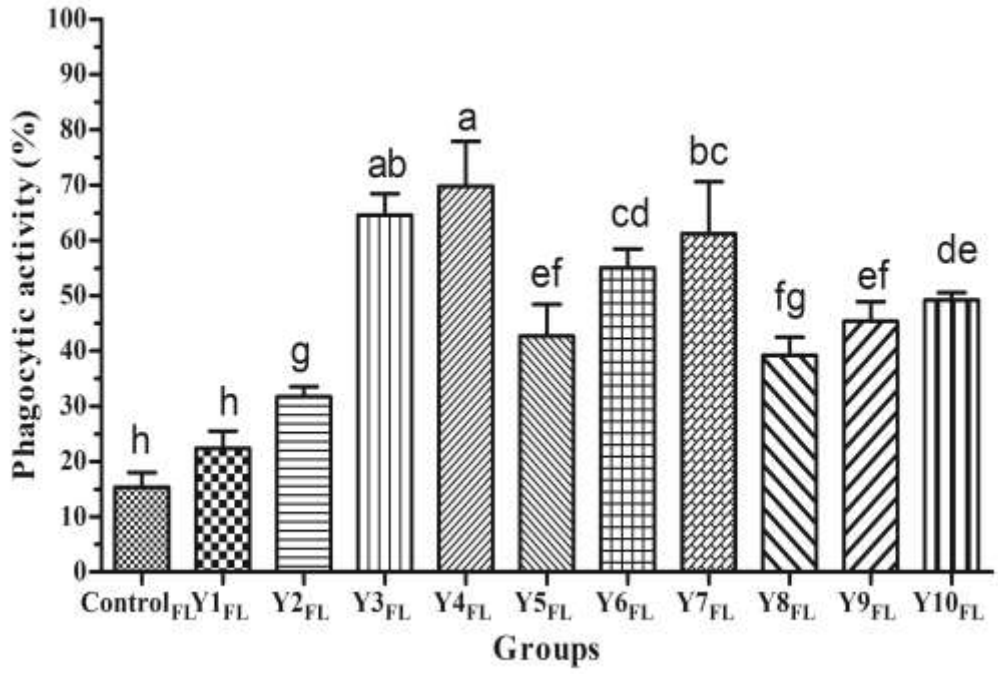

Fig. 3.4. Phagocytic activity (%) of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum* and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between different groups. Averages with different lowercase alphabets on bars show significant difference at $P < 0.05$. Control $_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*, Y2$_{FL}$=alginate encapsulated, Y3$_{FL}$=alginate encapsulated and coated with chitosan, Y4$_{FL}$=alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$=alginate-starch encapsulated, Y6$_{FL}$=alginate-starch encapsulated and coated with chitosan, Y7$_{FL}$=alginate-starch- encapsulated and coated with nano-chitosan, Y8$_{FL}$=alginate-xanthan encapsulated, Y9$_{FL}$=alginate-xanthan encapsulated and coated with chitosan, Y10$_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

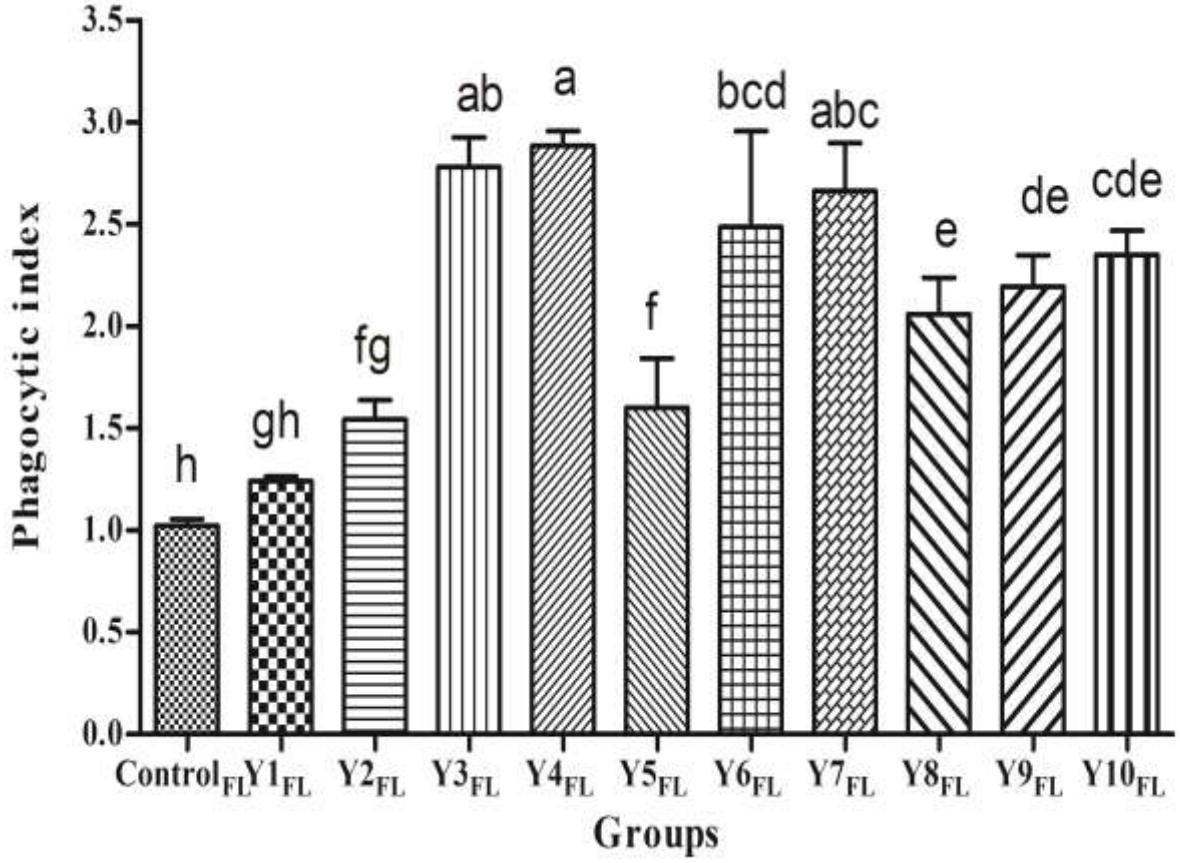

Fig. 3.5. Phagocytic index of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum* and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between different groups. Averages with different lowercase alphabets show significant difference at $P < 0.05$. Control $_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*, Y2$_{FL}$= alginate encapsulated, Y3$_{FL}$=alginate encapsulated and coated with chitosan, Y4$_{FL}$=alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$= alginate-Starch encapsulated, Y6$_{FL}$=alginate-starch encapsulated and coated with chitosan, Y7$_{FL}$=alginate-starch encapsulated and coated with nano-chitosan, Y8$_{FL}$=alginate-xanthan encapsulated, Y9$_{FL}$=alginate-xanthan encapsulated and coated with chitosan, Y10$_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

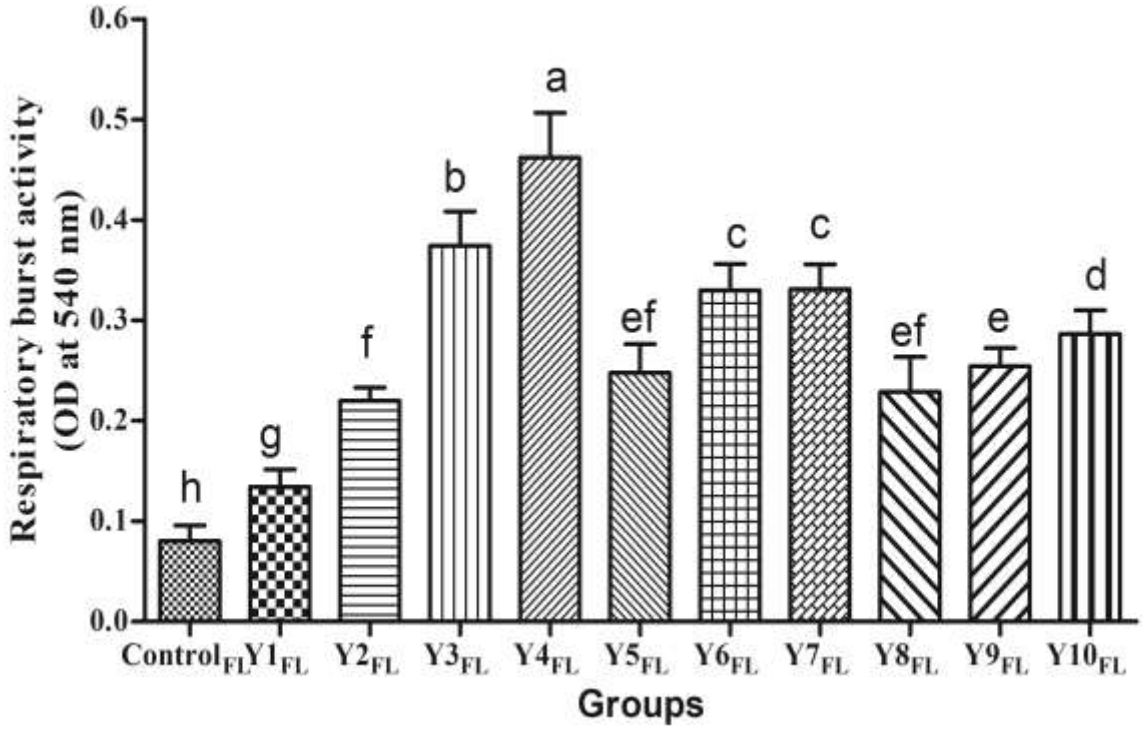

Fig. 3.6. Respiratory burst activity of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum* and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between different groups. Averages with different lowercase alphabets show significant difference at $P < 0.05$. Control $_{FL}$= without any probiotic supplement, $Y1_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*, $Y2_{FL}$= alginate encapsulated, $Y3_{FL}$=alginate encapsulated and coated with chitosan, $Y4_{FL}$=alginate encapsulated and coated with nano-chitosan, $Y5_{FL}$=alginate-starch encapsulated, $Y6_{FL}$= alginate-starch encapsulated and coated with chitosan, $Y7_{FL}$=alginate-starch encapsulated and coated with nano-chitosan, $Y8_{FL}$=alginate-xanthan encapsulated, $Y9_{FL}$=alginate-xanthan encapsulated and coated with chitosan, $Y10_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

Lipid profile

The encapsulated *G. candidum* supplementation also showed considerable effect on serum lipid profile of *L. rohita* fingerlings in comparison to un-encapsulated/free probiotic (Table 3.5), while *G. candidum* encapsulated with various encapsulating agents and with and without coating of chitosan showed variable results. One way ANOVA revealed significant difference in cholesterol (n=9, ANOVA, $F_{10, 99}$= 97.3, p=0.001), LDL (n=9, ANOVA, $F_{10, 99}$= 5.65, p=0.001), HDL (n=9, ANOVA, $F_{10,99}$=54.2, p=0.001) and Triglycerides (n=9, ANOVA, $F_{10, 99}$= 352, p=0.001) levels among different groups. The post hoc LSD test showed comparatively decrease levels of total cholesterol, LDL and triglyceride in probiotic fed groups as compared to the control$_{FL}$ group. Similarly encapsulated *G. candidum* supplementation more significantly lowers the total cholesterol, LDL and triglyceride levels as compared to the free/un-encapsulated *G. candidum* ($Y1_F$). The comparative evaluation of encapsulated *G. candidum* revealed the most pronounced decreasing trend in total cholesterol and triglycerides in response to encapsulated probiotic having chitosan coating ($Y3_{FL}$, $Y4_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y9_{FL}$ and $Y10_{FL}$) as compared to devoid of chitosan coating ($T5_{FL}$, $T8_{FL}$ and $T2_{FL}$). Additionally, comparative efficiency of chitosan coated *G. candidum* indicated the lowest total cholesterol and triglyceride values in $Y4_{FL}$ group followed by $Y3_{FL}$ group, while the highest levels were observed in $Y9_{FL}$ group of fish (Table 3.5).

The encapsulated *G. candidum* supplemented group showed a pronounced decline in LDL concentration and the lowest concentration was found in $Y4_F$ followed by $Y3_F$ group while all other groups supplemented with encapsulated dietary probiotic i.e. $Y2_{FL}$, $Y5_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y8_{FL}$, $Y9_{FL}$ and $Y10_{FL}$ showed statistically similar values (Table 3.5). Conversely the highest LDL concentration was observed in control$_{FL}$ followed by $Y1_{FL}$ group of fish.

Furthermore, *G. candidum* supplementation showed significant positive effect on HDL concentration and an increasing trend was observed in *G. candidum* fed groups of fish as compared

to control $_{FL}$. It was evident that more obvious increase in HDL concentration was found in response to encapsulated *G. candidum* as compared to free/un-encapsulated probiotic ($Y1_{FL}$). Furthermore, comparison among encapsulated *G. candidum* fed groups revealed that encapsulated probiotic with chitosan coating ($Y3_{FL}$, $Y4_{FL}$, $Y6_{FL}$, $Y7_{FL}$, $Y9_{FL}$ and $Y10_{FL}$) showed highest HDL concentration as compared to other groups ($Y5_{FL}$, $Y8_{FL}$ and $Y2_{FL}$). Overall, highest HDL value was observed in $Y4_{FL}$ followed by $Y3_{FL}$ while all groups supplemented with encapsulated *G. candidum* without chitosan coating i.e. $Y8_{FL}$, $Y5_{FL}$ and $Y2_{FL}$ showed statistically comparable HDL concentration (Table 3.5).

Table 3.5. Lipid profile of *L. rohita* fingerlings after 11 weeks feeding diet supplemented with free/un-encapsulated *G. candidum* and chitosan coated and un-coated microcapsules of *G. candidum* prepared with alginate alone or in combination with starch and xanthan (n=9)

	Groups													
	Control$_{FL}$	**Y1$_{FL}$**	**Y2$_{FL}$**	**Y3$_{FL}$**	**Y4$_{FL}$**	**Y5$_{FL}$**	**Y6$_{FL}$**	**Y7$_{FL}$**	**Y8$_{FL}$**	**Y9$_{FL}$**	**Y10$_{FL}$**	**Pooled SEM**	**F ratio**	**P value**
Total Cholesterol (mg dL^{-1})	290^{a}	276^{ab}	272^{ab}	102^{f}	49.6^{g}	255^{b}	158^{e}	150^{e}	230^{c}	203^{d}	165^{e}	7.88	97.3	0.001
LDL (mg dL^{-1})	88.1^{a}	74.9^{ab}	67.1^{bc}	47.6^{c}	21.8^{d}	63.4^{bc}	59.9^{bc}	58.2^{bc}	65.2^{bc}	61.0^{bc}	60.1^{bc}	6.93	5.65	0.001
HDL (mg dL^{-1})	39.9^{h}	66.2^{g}	78.8^{f}	177^{b}	189^{a}	89.6^{f}	143^{c}	151^{c}	80.3^{f}	107^{e}	132^{d}	6.52	54.2	0.001
Triglycerides (mg dL^{-1})	379^{a}	350^{b}	287^{c}	89.6^{i}	54.6^{j}	218^{e}	131^{gh}	123^{h}	255^{d}	180^{f}	144^{g}	5.67	352	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Averages with different lowercase superscripts in same rows shows considerable difference at $p < 0.05$.Control $_{FL}$= without any probiotic supplement, Y1$_{FL}$= supplemented with free or un-encapsulated *G. candidum*, while other treatment groups are supplemented with encapsulated *G. candidum*,Y2$_{FL}$= alginate encapsulated, Y3$_{FL}$=alginate encapsulated and coated with chitosan, Y4$_{FL}$=alginate encapsulated and coated with nano-chitosan, Y5$_{FL}$= alginate-starch encapsulated, Y6$_{FL}$= alginate-starch encapsulated and coated with chitosan, Y7$_{FL}$=alginate-starch encapsulated and coated with nano-chitosan, Y8$_{FL}$=alginate-xanthan encapsulated, Y9$_{FL}$=alginate-xanthan encapsulated and coated with chitosan, Y10$_{FL}$=alginate-xanthan encapsulated and coated with nano-chitosan.

Discussion

In aquaculture, probiotics administration has been shown to improve growth, the nutritional and health status as well as immunity of many cyprinid fish (Allameh et al., 2016). The probiotic application in finfish culture has certain limitations such as tolerance of probiotic organisms to gastrointestinal conditions and the ease of storage which restrict the practical use of probiotics in aquaculture. Microencapsulation appears as an alternative promising technique for improving the use of probiotic microorganisms for sustainable development of aquaculture. However, efficiency of microencapsulation depends on types and concentration of microencapsulating agents, process of encapsulation, concentration of hardening solution, size of microcapsule as well as on the physiology of host organism (Jyothi et al., 2010). Before practical application of encapsulated probiotics, literature emphasize, *in vitro* and *in vivo* simple tests for evaluating the temperature and pH tolerance, shelf life, resistance to bile and enzymes of GI tract of host and their impact on growth, immune response, metabolic function etc. of an organism (Papadimitriou et al., 2015) .

We have already evaluated the positive impact of free/unencapsulated *G. candidum* on growth, survival, digestive enzyme activity, proximate composition, hematological indices and immune response of *L. rohita* (Chapter 3). For improving stability, shelf life and viable cell count at the target site, *G. candidum* was encapsulated with Alginate alone and in combination with starch and xanthan and coated with chitosan and nano-chitosan, and conducted various *in vitro* tests like encapsulation efficiency, temperature and pH tolerance and shelf life (Chapter 2) and observed best results when *G. candidum* was encapsulated with Alginate and coated with chitosan (bulk) or nano-chitosan. However, it is well documented that hypothesis based on the results of *in vitro* assays may not follow exactly with *in vivo* physiological conditions, thus *in vivo* evaluation of selected probiotic is required to check the comparative effects of *G. candidum* encapsulated/coated with Alg, Alg-C, Alg-CN, Alg-S, Alg-S-C, Alg-S-CN, Alg-X, Alg-X-C, Alg-X-CN, on growth performance,

metabolic enzyme and intestinal enzymes activities, innate immunity and serum lipid profile of *L. rohita*.

In the present part of the study, no mortality was found in *L. rohita* fed both free and encapsulated *G. candidum* supplemented diet throughout the feeding trial. It seems rearing condition was suitable for *L. rohita*. Like previous results i.e. the positive effect of free *G. candidum* on survival (%), growth performance, intestinal enzymes activities, hematological indices, immunological parameters (Total protein content, IgM level, Lysozyme activity, Phagocytic activity ,Respiratory burst activity of fry (chapter 1), the present results also indicated the significant positive effect of dietary free *G. candidum* supplementation in contrast to basal diet (devoid of probiotic) on the survival (%), growth performance (increase in body weight, SGR), intestinal enzymes activities (Protease, cellulose and amylase), hematological indices (increased RBCs, WBCs, HB, HCT and MCV while decreased in MCH and MCHC) , immune responses (enhanced levels of total serum protein, IgM, Lysozyme activity, phagocytic activity, respiratory burst activity) on the fingerlings of *L. rohita*.

Our finding are in accord to the observations of many investigators, where dietary probiotics supplementation showed beneficial effect on growth rate (Rahiman et al., 2010; Mohapatra et al., 2012; Giri et al., 2014; Pinpimai et al., 2015; Subharanjani et al., 2015; Ullah et al., 2018), immune response (Sun et al., 2009; Gupta et al., 2014; Won et al., 2013; Ullah et al., 2018) proximate composition (Mohapatra et al., 2012; Dawood and Koshio, 2016; Amir et al., 2018; Ullah et al., 2018) intestinal enzymes activities (Yanbo and Zirong, 2006; Amir et al., 2018; Ullah et al., 2018) and resistance against pathogens (Amir et al., 2018; Ullah et al., 2018) of finfish and shellfish. This could be due to competitive exclusion of pathogenic microbes, colonization of *G. candidum* in the GI tract of *L. rohita*, secretion of exogenous enzymes, stimulation of immune response, reduction in permeability of the host epithelium to toxins/macromolecules and release of inhibitory factors and improved disease (Aly et al., 2008a; Puddu et al., 2014, Ibrahem, 2015). Furthermore, it seems that

β-glucan and chitin in the cell wall of *G. candidum* contribute in improving the health status and immunity of fish. It is well documented that β-glucan, chitin and its derivatives alter the immune responses in fish and mammals (Ortuno et al., 2002; Cuesta et al., 2003).

Here, we also observed positive effect of *G. candidum* on the activity of AST, ALT and ALP. AST is considered as a good indicator of reduced functioning of liver and spleen (Fakruddin et al., 2017) which may be due to nutritionally imbalanced feed and feeding practice (Xu et al., 2003), while ALT more specifically indicate liver cell damage and higher serum cholesterol level (Fakruddin et al., 2017). Here, decrease in AST level in all probiotic supplemented diet groups showed an improved liver functioning as compared to control group. It might be due to reduced cortisol induced protein catabolism (Freeman and Idler, 1973) resulting in a reduction of stress level in probiotic fed groups. It is well documented that ALT and ALP catalyse the synthesis and deamination of amino acids, therefore during stress, in order to meet the high energy demand of the organisms, involve in the conversion of protein to carbohydrate (Waarde and Henegouwen, 1982). In accord to our results, El-Rhman et al. (2009) reported significant decreased in activities of plasma AST and ALT in *Oreochromis niloticus* in response to dietary supplement of two strain of probiotic *Pseudomonas spp.* and *Micrococcus luteus*. Many other investigators also reported decrease in activities of liver enzymes (ALT, ALP, AST, LDH) in response to dietary probiotic *B. licheniformis* and *B subtilis* in *L. calcarifer* (Adorian et al., 2018), *Bacillus licheniformis* and dietary yeast extract (0.5%) in *O. niloticus* (Hassaan et al., 2014), *S. cerevisiae* in *O. niloticus* (Marzouk et al., 2008) and *S. cerevisiae* in *Sarotherodon galilaeus* L. (Abdel-Tawwab et al., 2006).

Alkaline phosphatase (ALP) is also an important polyfunctional enzyme, play significant role in liver function and bone development. In alkaline medium, it catalyses the trans-phosphorylases reaction and responsible for hydrolysis of many compound containing phosphate. It also plays a significant role in maintaining the intestinal homeostasis, while its activity could be controlled through diet (Lalles, 2010). Here, increased alkaline phosphatase (ALP) activity in response to

dietary *G. candidum* may be due to stimulation of phosphorus-metabolism or increased availability retention of certain minerals. It is well documented that low serum ALP is related to deficiency of mineral in fish (Sugiura et al., 2004). Like our results, rainbow trout, (*Oncorhynchus mykiss*) also showed an increased alkaline phosphate activity after administration *Lactobacillus rhamnosus* (Panigrahi et al., 2010). Similarly, in another study Ma et al. (2013) observed significantly increased ALP activity in *A. japonicus* when fed dietary yeast *Hanseniaspora opuntiae* C21 supplemented diet.

Beside above mentioned parameters, we also observed a decreasing trend in LDL, total cholesterol and triglycerides levels in groups of *L. rohita* fed *G. candidum* supplemented diet as compared fish fed diet devoid of probiotic. 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* (Øverland et al., 2013). Conversely, Piccolo et al. (2015) reported slight rise in total cholesterol and triglycerides level in European sea bass (*Dicentrarchus labrax*) following 90 days giving *Lactobacillus plantarum* supplemented diet. The observed lipid lowering effect might be attributed to the digestion and incorporation of the cholesterol into the cellular membranes the yeast or may be due to binding of the cholesterol to the cellular surfaces of the microbes and de-conjugation of intestinal bile acids (Kumar et al., 2012; Fakruddin et al., 2017). Many investigators conducted *in vitro* assays and found decrease in cholesterol from the culture medium in response to several strains of *Lactococcus* and *Lactobacillus* (Kimoto et al., 2002: Lye et al., 2010). Similar to the bacterial strains, lower in cholesterol from laboratory culture medium was also reported in response to some yeast species including *S. boulardii* and *S. cerevisiae* (Psomas et al., 2003). 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 (Sindhu and Khetarpaul, 2003; Fakruddin et al., 2017) and suggested the involvement of cell wall component of

yeast such as β-glucan in lowering plasma cholesterol level (Waszkiewicz-Robak and Bartnikowska, 2009).

Here we also observed that fingerlings reared on experimental diets supplemented with encapsulated *G. candidum* as compared to un-encapsulated/free probiotic, showed significantly higher growth rate, intestinal enzyme activity, hematology, ALP activity, immunological indices, a significant decrease in FCR, AST, ALT activities and most prominent changes in serum lipid profile. Furthermore the most significant positive effect was observed when diet supplemented with Alg-CN microcapsules ($Y4_{FL}$ group) followed by Alg-C microcapsules ($Y3_{FL}$ group).

Like our results, several studies reported the positive effects of bio-encapsulated probiotics on growth performance of different fish species like Touraki et al. (2012) reported the increased weight gain in spiny headed sturgeon fed bio encapsulated *Artemia* species, while, Adineh et al. (2011) observed similar enhanced growth rate of *L. rohita* fed diet containing bio-encapsulated *Artemia* with *bacillus* and *Saccharomyces*. Similar to our results, other scientists also reported significantly improved weight gain (%), SGR and low FCR of Nile tilapia (*O. niloticus*) when fed diet supplemented with alginate-skim milk microcapsules of *Saccharomyces cerevisiae* and *Lactobacillus rhamnosus* compared to un-encapsulated probiotics (Pinpimai et al., 2015; Pirarat et al., 2015). In another study, Cordero et al. (2015) observed an increase in IgM level in gilthead seabream, *Sparusaurata* L., after two weeks administration of probiotic strain Pdp 11 encapsulated in alginate. It seems that the improved efficiency of encapsulated in contrast to free probiotic observed here and reported by many investigators is due to physical barrier (capsule) which provides protection/resistance against temperature, bile salts, enzymes and pH of the GI tract of host, thus delivers the probiotic to the target site without damage (Rosas-Ledesma et al., 2012).

However, low efficiency of dietary free/unencapsulated *G. candidum* in contrast to capsulated one may be due to decrease in viable cell count of probiotic while passing through the

host gastrointestinal tract (Kailasapathy and Rybka, 1997). Many scientists reported the effect of bile salts/acids, intestinal enzymes and pH of GI tract on the viability of probiotic microorganisms and suggested the need of physical barrier for the probiotics, during their passage through the gastrointestinal tract (Kailasapathy, 2002). The use of encapsulated probiotic in aquaculture is somewhat new, therefore limited literature is available which revealed its impotence.

The efficiency of microcapsule depends on the encapsulating materials (biopolymers), process of encapsulation and size of microcapsules (Nesterenko et al., 2013). The capsule forming ability and strength determine the effectiveness of any encapsulating material (Reid et al., 2007). Generally, the functional groups present on cell wall of probiotics microorganisms and their cross linking with the encapsulating material influence the survival of probiotic within the microcapsule (Hernandez et al., 2018). while interaction between functional groups of coating materials and epithelial lining of host GI tract determine the stability and control release of core material (probiotic) at the target sites. The encapsulating or wall materials interact structurally in different ways with the probiotics, thus also affect their delivery at the target site (Riaz and Masud, 2013).

Here, *G. candidum* encapsulated with alginate and coated with nano-chitosan (Alg-CN) showed the most significant effect on all studied parameters followed by Alg-C. These results are consistent with the *in vitro* results where both these microcapsules showed higher resistance to temperature and pH as well as had greater encapsulating efficiency and shelf life. Many investigators used alginate for encapsulating the probiotics (bacteria and yeast) and reported many advantages over other encapsulating materials like cheap, easy to prepare and handle (Chandramouli et al., 2004; Gouin, 2004; Riaz and Masud, 2013). However, other scientists used alginate in combination with other compounds like starch (Sultana et al., 2000; Kailasapathy, 2002; Krasaekooptet al., 2003), glycerol (Hansen et al., 2002), 10% skim milk (Pinpimai et al., 2015), chitosan (Krasaekoopt et al., 2003; Lee et al., 2004; Anal and Stevens, 2005; Huq et al., 2013) and suggested that other supporting

material/compound is required to improve the physiochemical characteristics of microcapsules (shape, morphology, stability, resistance to pH and temperature and target release etc.).

The higher efficiency of Alg-CN and Alg-C microcapsules of *G. candidum* may be due to shape and morphology of capsules. In the previous chapter, we observed that Alg-CN and Alg-C microcapsules were more spherical in shape with smooth surface and least cavities/cracks in contrast to uncoated microcapsules, which were irregular in shape with rough/uneven surface. This variation could be due to cross linking reaction between polymers used for encapsulation and coating (Yasmin et al., 2018). It is well documented that coating of alginate microcapsule with chitosan enhance the cross linking between alginate and chitosan molecules thus form complex structure with smaller pores and least cavities on the surface of the microcapsule (Lee et al., 2004; Huq et al., 2013). It seems that smooth, complex alginate and chitosan interaction prevent the leakage and deterioration of probiotic (*G. candidum*) during passage to GI tract and release it at target site. Chavarri et al. (2010) also reported the decreased porosity of alginate microcapsule and reduced leakage of the encapsulated probiotic when coated with chitosan. It is well documented that chitosan coated alginate capsule are stronger (Mortazavian et al., 2007) and less effected to the GI tract conditions (pH, bile, enzymes etc) of host (Zhou et al., 2000; Krasaekoopt et al., 2003).

Moreover, the highest efficiency of Alg-CN as compared to all encapsulating formulation including Alg-C may be due to increase surface area of nano-chitosan coating that positively improved the release rate and diffusion of *G. candidum* at the target site. The very thin layer (nano-chitosan coating) may improve the resistance of microcapsules without enlarging their size (Pandey et al., 2016b; Ansari et al., 2017). However, efficiency of encapsulation decreased due to reduction in the surface area, (Yoksan et al., 2010) as in case of chitosan (bulk) coating. The particle size of the coating material is also playing significant role in adhesion, interaction and absorption of microcapsule with GI tract of host (Agrawal et al., 2014). Thus, the higher efficiency of Alg-CN

microcapsules of *G. candidum* may be due to their more stability and enhanced bioavailability throughout the mucosa of the GI tract (Kumari et al., 2013).

The increased biological activity of encapsulated especially Ag-CN microcapsules of *G. candidum* may also be due to pH dependent release of probiotic at target site. The enhanced release of probiotic from microcapsules has been reported in alkaline condition (Alishahi et al., 2011). It is well documented that, microcapsules release their contents upon reaching the intestinal pH or neutral to higher pH (Champagne and Fustier, 2007), where calcium is progressively displace and microcapsules starts to dissolve and the contents are released (Ansari et al., 2017). This pH dependent release in the fish was previously explained for rainbow trout by Wang et al. (2009). In the rainbow trout stomach, the release rate was very low due to the enhanced electrostatic interaction, whereas in the intestine due to weak electrostatic interaction microcapsules disintegrate and increase the release rate of the encapsulated material (Yasmin et al., 2018).

Generally, fish intestinal tract consist of different segments with acidic pH value in stomach portion and neutral to basic values in intestinal segment (Rosas-Ledesma et al., 2012). According to literature, at intestinal pH, microcapsules starts swelling and release rate is quite high, however, the best release rate from alginate-chitosan microcapsules can be achieved at pH 7.4 (Ansari et al., 2017). The present study was conducted to select and encapsulate probiotic for *L. rohita*, where stomach is absent and the pH of intestinal fluid ranging from 6.2-7.1. It seems pH of the intestinal fluid favor the release of *G. candidum* from the Alg-CN and Alg-C microcapsule throughout the GI tract of target species. Similar increased survival of alginate microcapsules containing fish probiotic bacteria (*Schewanella putrefaciens*) was obtained within the GI tract of Senegalese sole (Rosas-Ledesma et al., 2012).

Our *in vivo* results are consistent with *in vitro* results and it appears that alginate in combination with nano-chitosan (Alg-CN) and chitosan (bulk) Alg-C are the best encapsulating materials for improving the target release of *G. candidum* in the GI tract of L. *rohita*. Keeping in

view the results i.e., best growth performance, lowest FCR, most significant improvement in the status of hemato-immunological indices, status of intestinal enzymes activities, reduction in AST, ALT and serum lipid profile in groups of fingerlings fed Alg-CN and Alg-C microcapsules supplemented diet in comparison to other groups, Alg-CN and Alg-C microcapsules of *G. candidum* could be recommended for improving the production of *L. rohita*.

Chapter 4:

Effect of dietary Alginate-chitosan encapsulated *Geotrichum candidum* on growth, immunity and nutritive value of rohu (*L. rohita*) in semi-intensive culture system

Abstract

A number of microorganisms have been evaluated as probiotics in aquaculture to enhance growth, improve digestibility of nutrients and the host immune system of several fish species in controlled conditions. Concurrently, search is going on to select suitable microbes which could be used as probiotics for practical, semi-intensive /semi control culture system. Here commercial application of Alginate-chitosan encapsulated *G. candidum* was evaluated by feeding dietary probiotic supplemented diet to fingerlings of *L. rohita* reared in earthen ponds under semi-intensive culture conditions. About 720 fingerlings with average body weight 20 ± 2.34 g were evenly distributed in three groups, and were stocked in 120 m^2 earthen ponds located at the Fisheries and aquaculture facility, Quaid-i-Azam University using three ponds per group. The control group (Y0) was fed 35% protein diet without any supplement while the two treated groups (Y1 and Y2) were fed basal diet supplemented with un-encapsulated (free) and nano-chitosan coated alginate microcapsules (Alg-CN) of *G. candidum*, respectively, each containing 10^9 CFU g^{-1} diet for a period of 11 weeks. Results indicated significantly improved growth rate , intestinal enzyme activities (protease, amylase and cellulase), hemato-immunological indices (RBCs, Hb, HCT, WBCs, MCHC, respiratory bursts and phagocytic activity, total protein, lysozyme, IgM), expression of heat shock protein HSP 70 gene in muscle, intestine and liver tissues, status of muscle PUFA (especially Eicosatrienoic acid, linolenic, Eicosapentaenoic acid, Docosapentaenoic acid , Docosahexaenoic acid), ratio of ω3 to ω6 fatty acids, essential amino acids (especially valine , threonine, isoleucine, phenylalanine, Arginine) as well as in the reduction of serum AST and ALT activities, total cholesterol and triglycerides in Y1 and Y2 groups of fish fed *G. candidum* supplemented diets as compared to basal diet. However, most

pronounced ($p<0.001$) positive effect was observed in a Y2 group of fingerlings fed Alg-CN encapsulated *G. candidum* supplemented diet as compared to free probiotic. In conclusion, it seems that probiotic especially nano-chitosan coated alginate microcapsules of *G. candidum* showed more pronounced effect on health status, immunity and nutritive value of *L. rohita* reared in semi intensive culture system. Thus, suggests its application as feed additive in practical/commercial semi intensive earthen pond culture system.

Introduction

From the last decade, with an increase demand of ecofriendly sustainable development of aquaculture practices, research on microorganisms as potential probiotic for fish and other aquatic organism is continuously increasing (Sreedevi and Ramasubramanian, 2011; Dimitroglou et al., 2011; Iribarren et al., 2012; Ibrahem, 2015; Dawood and Koshio, 2016). Most of the probiotics used earlier for improving the health status of livestock are now being introduced in aquaculture practices. Until now, several microorganisms as probiotics have been tested to improve growth, enzymatic activity and manipulation of host immune responses of several fish species in controlled conditions (Lobo et al., 2014; Ibrahem, 2015). Concurrently, search for suitable microorganisms which could successfully be used as probiotics in semi-controlled pond farming /practical aquaculture conditions is continuously being undertaken (Nayak and Mukherjee, 2011; Lazado et al., 2015).

Generally, probiotic work depends on biological and physiochemical factors like, size and age of the culture aquatic species including fish (Jha et al., 2015; Ridha and Azad, 2016), culture condition, environmental factors (Wang et al., 2008). Thus, one probiotic could not be used for all aquatic organisms (Sharifuzzaman and Austin, 2017), under all culture conditions. For example some microbes as probiotic are beneficial for one fish species or aquatic animals, could be harmful for other species (see review Hai, 2015). Similarly a particular probiotic may be effective in controlled conditions but could be ineffective in extensive and semi-intensive practical aquaculture practices. According to Lazado et al. (2015), selected probiotic under controlled condition, may not be able to produce desired outcome in practical semi-intensive pond culture system due to physico-chemical conditions of the host and culture condition. Although probiotics are gaining increased scientific and commercial interest in finfish and

shellfish aquaculture worldwide (Dawood and Koshio, 2016), however a few studies addresses their application in the semi-intensive culture of major carps (Miscevic et al., 2012; Jha et al., 2015; Khatun and Saha, 2017; Ullah et al., 2018).

In south Asian countries especially Pakistan, Bangladesh and India, semi-intensive carp culture is very old extensively used practice for obtaining production (Miah et al., 1997; Rahman et al., 2006). The key characteristic of this system is the dependence on the combination of artificial and natural feed (Hepher and Pruginin, 1982) and it is managed by the addition of lime, organic and in organic fertilizers, supplemented feed etc. Semi-intensive culture system is somewhat labor intensive, with risk of mortalities due to poor management and outburst of diseases (Sreedevi and Ramasubramanian, 2011).

The utilization of animal manure (cow, sheep, goat and pig dung, poultry and duck dropping etc.) for pond fertilization is a common practice in semi intensive pond culture systems (Hossain et al., 2003; Jha et al., 2004). It is commonly used to stimulate plankton (live food organisms) production. However, beside many advantages, this practice involve in the proliferation of pathogenic bacteria in the water bodies like *Pseudomonas, Aeromonas* spieces (Sugita et al., 1985; Quines, 1988), which may cause various kind of diseases in aquatic organisms like skin ulcerations, furunculosis , albinoderma, erythroderma (Das, 2004; Sihag and Sharma, 2012). Normally, fish in pond remain healthy even in the presence of pathogens but any fluctuation in abiotic factors cause stress and shift the balance in favor of the disease (Godara et al., 2015).

The intestinal microbiota of fish has a continuous interaction with the surrounding aquatic environment and functions of host (Verschuere et al., 2000). Many investigators

observed this relationship and suggested that microorganism in an aquatic environment regulate the composition of the gut microbiota of host and vice versa (Cahill, 1990; Verschuere et al., 2000). Moreover, some scientists emphasize the larger influence of immediate ambient environment in aquaculture on the health status of fish than terrestrial animals or humans. The gut mucosal surface represents the sites where intestinal microbiota and environmental antigens interact with the host. According to Pérez et al. (2010) colonization of normal microbiota in the intestinal mucosal surfaces of host enhance the immune regulatory functions of the gut, while imbalanced microbiota may disturb the immune regulatory functions and contribute to the development of diseases.

However, the complex ecosystem of intestinal microbiota can be modulated with the manipulation of environmental and nutritional factors (Pérez et al., 2010). 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 probiotics have tendency to improve water quality (Banerjee et al., 2010; Aguirre-Guzman et al., 2012; Mohapatra et al., 2012), inhibit or out compete pathogenic microbes, to colonize the intestinal mucosal surface, improve survival, growth, immune response, disease resistance and nutritive values of fish by producing inhibitory substances having antibacterial (Zapta and Lara-Flores, 2013; Newaj-Fyzul and Austin, 2014), antiviral (Balcazar, 2003; Lakshmi et al., 2013) and antifungal (Lategan et al., 2004) activity, various extracellular enzymes like cellulases, lipases, proteases etc. (Wang et al., 2008), stimulation of the activity of cytokines, phagocytes, lysozyme, acid phosphatase (Pirarat et al., 2006; Lara-Flores and Aguirre-Guzman, 2009) and differentiation as well as activation of T-cell (see review

Ibrahem, 2015) and formation of short chain fatty acids and essential vitamins (See reviews, Hardy et al., 2013; Perez-Sanchez et al., 2014).

Many microorganisms (bacteria and yeast) with varying degree of success are now being extensively applied as probiotics in shellfish and finfish culture especially in Asia and South America (Kumar et al., 2006; Nayak et al., 2007;Walker, 2008; see review Ibrahem, 2015). Here present part of study was design to assess the commercial value of selected probiotic by feeding free/unencapsulated and Alg-CN encapsulated *G. candidum* supplemented diets to fingerlings of *L. rohita* reared in earthen ponds under semi-intensive culture condition, an extensively used practice in Pakistan. On the basis of previous results, we assumed that Alg-CN encapsulated *G. candidum* QAUGC01 (Accession number KTC280407) would improve the production and nutritive value of fish by improving growth, digestibility of nutrients and health status of fish.

Materials and Methods

Probiotic microorganism

The practical application of nano- chitosan coated alginate (Alg-CN) 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. Probiotic *G. candidum* was cultured, processed and encapsulated as mentioned previously in chapter 1 and 2.

Probiotic supplementation in the feed

Locally available feed ingredients were purchased from Oryza organics private limited and feed pellets (2 mm) with crude protein (35%) were prepared and stored as mentioned in Chapter 1 (part 2). The prepared pelleted diet was divided in to three groups. First control group pellets were sprayed PBS (2.5 mL of PBS g^{-1} feed) without any probiotic while pellets of others groups Y1 and Y2 were top dressed with free and encapsulated *G. candidum* respectively at the same rate 1×10^{9} CFU g^{-1} . For avoiding, extreme variation in microbial count, fortnightly, fresh diets were prepared and stored at 4°C.

Fish collection and management

About nine hundred fingerlings of *L. rohita* (average body weight 20 ± 2.24g) were purchased from Government Fish Hatchery Manawan Lahore, Pakistan. Fish was transported in well aerated oxygen filled plastic bags to the Fisheries and Aquaculture facility and shifted in tanks having flow through system. They were acclimated for about 2 days and during this period provided 35% crude protein diet.

Experimental design

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

Earthen ponds preparation

All ponds at Fisheries and aquaculture facility were rectangular in shape with an average size of 120 m^2 and depth of 1.45 m. Before initiation of experiment, all earthen ponds, adjacent to each other, were sun dried, earthwork including repair of dikes was completed. They were treated with calcium carbonate at the rate of 125 kg ha^{-1} (Ali et al., 2008) and fertilized with cow dung at the rate of 3333.33 kg ha^{-1} (Javed et al., 1992; Ullah et al., 2018) to enhance pond productivity. Animal manure was spread evenly on the pond bottom and was exposed to the sun for several days. After the application of manure, the ponds were half filled with water obtaining from nearby freshwater Rumli stream. However, when water become fertile, more water was added and maintained level up to 1.3 m. Throughout the experiment, pond productivity and water level were maintained with addition of fertilizers (animal manure and Diammonium phosphate (DAP)) and water. Occasionally, ponds productivity 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 9 earthen ponds. After shifting they were again acclimated in their respective tanks for about two days. Afterwards 9 earthen ponds, each with 80 fish were randomly assigned a treatment group (240 fish/ group), Y0, Y1 and Y2. Feeding trial was started by providing each group to their

respective diet i.e. devoid of any probiotic to Y0 group while un-encapsulated and encapsulated G. *candidum* supplemented diets to Y1 and Y2 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 11 weeks.

During feeding trial, daily water temperature (°C), DO level (mg L^{-1}) and pH value were checked with multiparameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA) while total ammonia was checked weekly. The DO level and temperature showed fluctuation during feeding trial and ranges DO, 4.5–6.2 mg L^{-1} and temperature 25.6–28.6°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.

Growth performance

After feeding trial, fish were starved for 24 hrs before harvesting. On the day of sampling, the outlet of pond was opened and water was drained in fish collecting basin. Fish of each pond were collected separately, weighed and counted the number for evaluating growth performance with formulas as described in previous chapters.

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 (on ice bag) by adopting standard aseptic method and their gastrointestinal-tracts (GI tract) were carefully removed as described in chapter 1. The GI tract of 2 fish of 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 intestinal enzymes amylase, protease, and microbial origin cellulase were determined with their respective standard methods as reported previously in chapter 1.

Hematological indices

At random nine 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 ($\times 10^6$ µ L^{-1}), Hb (g dL^{-1}), HCT (%), MCH (pg), MCHC (g dL^{-1}) and MCV (fL) determination as described previously in chapter 1.

Immunological indices

For serum collection, blood sample from 15 fish per pond (45 fingerling / group) was also collected from caudal vein by using 2 mL heparinized syringe (24G, B-D®Discardit™ Becton Dickinson, Spain) in red top EDTA VACUETTE® tubes (LiuyangSanli Medical Technology Development Co., LTD). For obtaining enough sample, blood of 5 fingerlings from same pond was collected in the same blood collecting tube (9 sample/treatment). Blood samples were centrifuged (Kokusan, H-103RS, Ogawa Seiki Co., LTD, Tokyo, Japan) for 5 min at 3000 rpm and separated serum was decanted in fresh Eppendorf and saved at 4˚C for the analysis of total Serum proteins, immunoglobulins (IgM) and lysozyme enzymes activity, while fresh heparinized

blood was used for phagocytic activity and respiratory burst activity by following procedure already mentioned in chapter 1.

Aspartate Aminotransferase (AST)

The AST / GOT kit mentioned in chapter 1 was used for measurement of serum AST activity.

Alanine aminotransferase (ALT)

Serum ALT activity was measured using alanine aminotransferase Activity Assay KIT MAK-052 (SIGMA-ALDRICH). Assay was performed by adopting standard procedure.

Total cholesterol

Total cholesterol was measured by using Greiner Diagnostic GmbH kit (UnterGereuth, Bahlingen- Germany). Assay was performed by adopting standard procedure.

Triglycerides

Serum triglycerides were measured by using AMP diagnostic kit (AMEDA Labordiagnostik GmbH, Germany) according to standard procedure described previously in chapter 3.

Gene expression

For evaluating, HSP 70 gene expression in each group, 9 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 3 fish from same pond were pooled (9 sample per treatment) and preserved in RNA later and stored at -80°C for further use.

Extraction of Total RNA and cDNA synthesis

High pure RNA tissue kit (Roche, Basel, Switzerland) was used for extraction of total RNA from each sample. To check the purity of extracted RNA NanoDrop- ND1000 (Tech. Inc., Wilmington, USA) was used. Then total RNA was converted into cDNA for qPCR using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific, Lithuania). The extracted RNA (1µg) 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 molecular studies.

PCR condition 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 a. PCR conditions and cycle number was 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 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 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, $2^{-\Delta\Delta C_T}$ comparative threshold, C_T method was used for calculating gene expression level

Table (a) Primers used for expression study

Gene	Product size (bp)	Primer sequence	Reference
HSP 70	114	Forward CACAATCACCAACGATAAGGG Reverse TTGGCAGACACCTTTTCACGC	(Maji et al., 2017)
β-actin	200	Forward AGACCACCTTCAACTCCATCATG Reverse CCGATCCAGACAGAGTATTTACGC	(Maji et al., 2017)

Amino acid analysis

For Amino acid profile from muscle of fish, high performance liquid chromatography (HPLC) based amino acid analyzer (CBM-20A, Shimadzo, Japan), available at Medicinal botanical center of Pakistan council for scientific research (PCSIR) Laboratory, complex Peshawar was used.

Extraction of amino acid

For the analysis of amino acids, 10 fish from each tank (30 fish / group) were anesthetized with MS222 (0.01 g L^{-1} buffered with sodium bicarbonate) and muscle of each fish was aseptically collected. For obtaining sufficient sample, muscle of 10 fish from same tank was

collected in the same tube (3sample/treatment) and stored at -20 °C until further use. Muscle tissue samples were thawed and about 50 g of tissue sample was dried to a constant weight in vacuum oven.

Dried muscle tissues were ground using glass pestle motor and transferred to a dried glass tube. Subsequently, 5 ml HCl (0.1 %) was added and test tube was shaken vigorously for 2-3 min and centrifuged at 3500 rpm for 15 min. Supernatant was collected and filtered through a membrane filter (0.45µm) and used for amino acids profile.

Preparation of reaction solutions:

Preparation of buffer solution

In order to prepare the borate buffer, 40.7g of Na_2CO_3 and 13.57g of H_3BO_3 were dissolved in 600 ml of sterilized double distilled water. The solution was mixed thoroughly to make clear solution.

Solution A

The solution was prepared by mixing 0.2 ml of Sodium Hypochlorite solution (7-10% Na ClO) with 500 ml of prepared buffer. The solution was filtered by passing through membrane filter (pore size, 0.45µm).

Solution B

This solution was prepared by dissolving 0.4 g Ortho-Phthalaldehyde (OPA) in 7 ml ethanol and mixed with 450 ml of the prepared borate buffer solution. In this mixture 0.5 g N. Acetyl Cysteine was added and shaken vigorously in order to make homogeneous solution. To remove impurities resultant solution was filtered through membrane filter (pore size, 0.45µm).

Preparation of mobile phase 'A'

Citrate tribasic (19.6 g) was dissolved in 600 ml of deionized water and then mixed with 70 ml of ethanol and 7.8 mL per-chloric acid. After mixing, volume was raised to 1 L by adding double deionized water and pH of the mobile phase was adjusted (pH 3.2) by adding the perchloric acid.

Preparation of mobile phase 'B'

Citrate Tribasic (58.8 g) and boric acid (12.4 g) was dissolved in 800 ml of double distilled water. After this 25 ml of NaOH (4 N) was added and volume was raised to 1L. The pH of solution was adjusted (pH 10) by using NaOH and filtered.

Preparation of mobile phase 'C'

Sodium hydroxide (0.2 N) solution was prepared by dissolving 8 g of Sodium hydroxide in double distilled water (1L) and filtered.

Afterwards, 20 µl sample, was injected in HPLC based amino acid analyzer (Shimadzu, Japan). The column used for amino acid profile was sodium based with a fluorescent detector. Fluorescence was detected at excitation and emission wavelength of 350nm and 450nm respectively. Amino acids were separated by using mobile phase A and B. After separation they were spell out from the column where reaction solution (solution A and B) was combined with mobile phase (containing amino acids) and initiated the post column derivatization. After derivatization, the complex was passed to the cuvette for detection of different amino acids.

Fatty acid analysis

Fatty acids were analyzed from muscle tissue using Gas chromatography mass spectrometry (GC-MS) at medicinal botanical center of Pakistan council for scientific research (PCSIR) Laboratory, complex Peshawar.

For the analysis of fatty acids, 10 fish from each tank (30 fish / group) were anesthetized with MS222 (0.01 g L^{-1} buffered with sodium bicarbonate) and muscle of each fish was aseptically collected. For obtaining enough sample, muscle of 10 fish from same pond was collected in the same tube (3 sample/treatment). Muscle were immediately frozen in liquid nitrogen and stored at -20 °C until further use.

Extraction of total lipid

The extraction of total lipid, preparation of fatty acids methyl esters (FAMEs) and composition of FAMEs was analyzed by adopting previously adopted procedure (Afridi et al., 2018). Briefly, 20g muscle tissues were dried in Vacuum drying oven (Lab Tech Korea) for three days at 50˚C. Subsequently dried muscle tissues were taken n conical flask of Soxhlet apparatus and added n-hexane (Fisher scientific, UK). The flask was heated for about 7 hrs and extracted oil was condensed with the help of Rotary vacuum evaporator and shifted to glass vials. Extra hexane was evaporate by keeping vials overnight at ambient temperature (Poitevin, 2016).

Preparation and analysis of FAMEs on GC- MS

Poitevin (2016) procedure previously reported (Afridi et al., 2018) was adopted and FAMEs was prepared. The prepared FAMEs solution was filtered by membrane filter paper (pore size, 0.45µm). The FAMEs composition was analyzed with gas chromatography-mass spectrometer (GC-MS-QY2010, Plus Shimadzu, Kyoto, Japan) having 100 m CP Sil 88 capillary

column (film thickness, 0.20 µm; i.d, 0.25 µm, Chrompack, Middleburg, Netherlands) and a flame ionization detector. Initially, at the time of sample injection, the column temperature for 1 min was set at 80°C, then increased at the rate of 2°C per min to 215°C and maintained this 30 min. The Inlet and detector temperature were 220°C and 230°C respectively, while mode of injection was split with a ratio of 100:1. The flow rate 1 mL/min was set for H_2 carrier gas. Most of the fatty acid peaks were identified and quantified using FAMEs standard Mix SUPELCO (CRM47885, Bellefonte, USA) containing 37 component was used as external standard (Gao et al., 2006; Wen et al., 2017).

Statistical analysis

Before running statistical analysis, data was assessed for variance homogeneity and normality distribution by using Bartlett and Shapiro-Wilk's tests. All data was represented as mean ± SD. Significant differences in growth performance, survival, intestinal enzymatic activity, hematology and immunological parameters, HSP-70 gene expression, amino acid and fatty acid profile among 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.

Results

Growth performance

A diet supplemented with Alg-CN encapsulated probiotic *G. candidum* showed a most significant positive effect on growth performance and survival of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions (Table 4.1). One way ANOVA indicated significant differences in final weight (n=3, ANOVA, $F_{2, 9}$ =40, p<0.001, Table 1), weight gain (n=3, ANOVA, $F_{2, 9}$ =42.6, p<0.001, Table 4.1), weight gain percent (n=3, ANOVA, $F_{2, 9}$ =24.6, p<0.001, Table 4.1), final biomass (n=3, ANOVA, $F_{2, 9}$= 47.2, p <0.001, Table 1), SGR (n=3, ANOVA, $F_{2, 9}$ =22.3, p<0.001, Table 4.1) and survival (n=3, ANOVA, $F_{2, 9}$ =89.6, p<0.001, Table 4.1) between Y0 (fed feed devoid of *G. candidum*), Y1 (fed un-encapsulated *G. candidum* supplemented diet) and Y2 (fed Alg-CN encapsulated *G. candidum* supplemented diet). The Post hoc LSD test indicated improved growth performance of Y1 and Y2 groups as compared to Y0. Furthermore, all possible pairwise comparisons indicated that among probiotic fed groups, higher weight gain, final biomass and SGR was observed in Y2 group followed by Y1. Similarly, the highest survival (99%) was observed in Y2 group followed by Y1 (96%) while Y0 group showed lowest survival (89%) during the experimental period.

Table 4.1. Comparative effect of free/un-encapsulated and Alg-CN encapsulated *G. candidum* supplemented diet on growth performance 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
	Y0	Y1	Y2			
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 $p < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2 , fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.[1]SGR= specific growth rate

Activity of intestinal enzymes

Digestion and absorption of material can be inferred from the activity of intestinal enzymes in the gastrointestinal tract. Here Alg-CN encapsulated *G. candidum* showed a most pronounced effect on the activities of intestinal enzymes of fingerlings *L. rohita* reared in ponds under semi intensive culture conditions (Table 4.2). One way ANOVA revealed significant differences in the activities of protease (n=9, ANOVA, $F_{2, 27}$= 207, p<0.001, Table 4.2), cellulase (n=9, ANOVA, $F_{2, 27}$= 346, p<0.001, Table 4.2) and amylase (n=9, ANOVA, $F_{2, 27}$= 135, p<0.001, Table 4.2) of Y0, Y1 and Y2 groups fed diet devoid of probiotic and supplemented with free and Alg-CN encapsulated *G. candidum*. The pairwise comparison between groups indicated higher activities of all studied enzymes in probiotic fed groups (Y1 and Y2) as compared to control group (Y0), while Y2 group fed diet supplemented with Alg-CN encapsulated G. *candidum* (Y2) showed the highest activities of all three enzymes (Table 4.2).

Moreover, comparison of protease, amylase and cellulase activity within group, showed statistically significant difference in all studied enzymes in each group of fish, Y2 (n=9, ANOVA, $F_{2, 27}$=48.8, P< 0.001), Y1 (n=9, ANOVA, $F_{2, 27}$=104, P< 0.001, Table 4.2) and control group (n=9, ANOVA, $F_{2, 27}$=14.0, p=0.001, Table 4.2). Moreover, in all groups, activity of amylase was significantly higher followed by protease and cellulase respectively.

Table 4.2. Comparative effect of free (un-encapsulated) and Alg-CN 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)

Intestinal enzymes (U mg^{-1})	**Groups**			**Pooled SEM**	**F ratio**	**p-value**
	Y0	**Y1**	**Y2**			
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 $p < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum.*

Hematological indices

The Alg-CN encapsulated *G. candidum* supplemented diet showed a most significant effect on hematology of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions. One way ANOVA revealed significant differences in erythrocytes (n=9, ANOVA, $F_{2, 27}$=104, p<0.001, Table 4.3), leukocytes (n=9, ANOVA, $F_{2, 27}$=570, p<0.001, Table 4.3), hemoglobin (n=9, ANOVA, $F_{2, 27}$=588, p<0.001, Table 4.3), hematocrit (n=9, ANOVA, $F_{2, 27}$=89.9, p<0.001, Table 4.3), mean corpuscular volume (n=9, ANOVA, $F_{2, 27}$=8.66, p<0.001, Table 4.3), mean corpuscular hemoglobin (n=9, ANOVA, $F_{2, 27}$=12.7, p<0.001, Table 4.3) and mean corpuscular hemoglobin concentration (n=9, ANOVA, $F_{2, 27}$=3.90, p=0.03, Table 4.3) of Y0, Y1 and Y2 group. The Post hoc test showed higher values of RBCs, WBCs, Hb and HCT in probiotic fed groups (Y2 and Y1) as compared to control group. Furthermore, comparison among probiotic fed groups revealed significantly higher concentration of RBCs, WBCs and Hb content in a Y2 as compared to Y1 group. Conversely, significantly (p=0.001) lower concentration of MCV observed in Y2 group, as compared to Y1 and Y0 having statistically comparable concentration (p=0.14). Similarly, Y0 showed highest MCH concentration followed by Y1 and then Y2 group.

Table 4.3. Comparative effect of free (un-encapsulated) and Alg-CN 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					
	Y0	**Y1**	**Y2**	**Pooled SEM**	**F ratio**	**p-value**
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 $p < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

Aspartate aminotransferase activity (AST)

Alg-CN encapsulated *G. candidum* supplemented diet significantly affected the serum AST activity of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions (Fig. 4.1). One way ANOVA revealed significant differences in AST activity between Y0, Y1 and Y2 groups (n=9, ANOVA, $F_{2, 27}$=118, $p<0.001$, Fig. 4.1). The Post hoc LSD test indicated significantly lower serum AST activity in probiotic supplemented diet fed groups (Y2 and Y1) as compared to the control group (Y0). Furthermore Y2 group showed significantly lowest AST activity.

Alanine aminotransferase activity (ALT)

One way ANOVA also revealed a significant difference in the activity of ALT between Y0, Y1 and Y2 groups (n=9, ANOVA, $F_{2, 27}$=83.8, $p<0.001$, Fig. 4.2). The pairwise comparison showed significantly decrease ALT activity in Y2 and Y1 groups as compared to the control group (Y0). However, among *G. candidum* fed groups (Y1 and Y2), the lowest serum ALT activity was observed in the Y2 group.

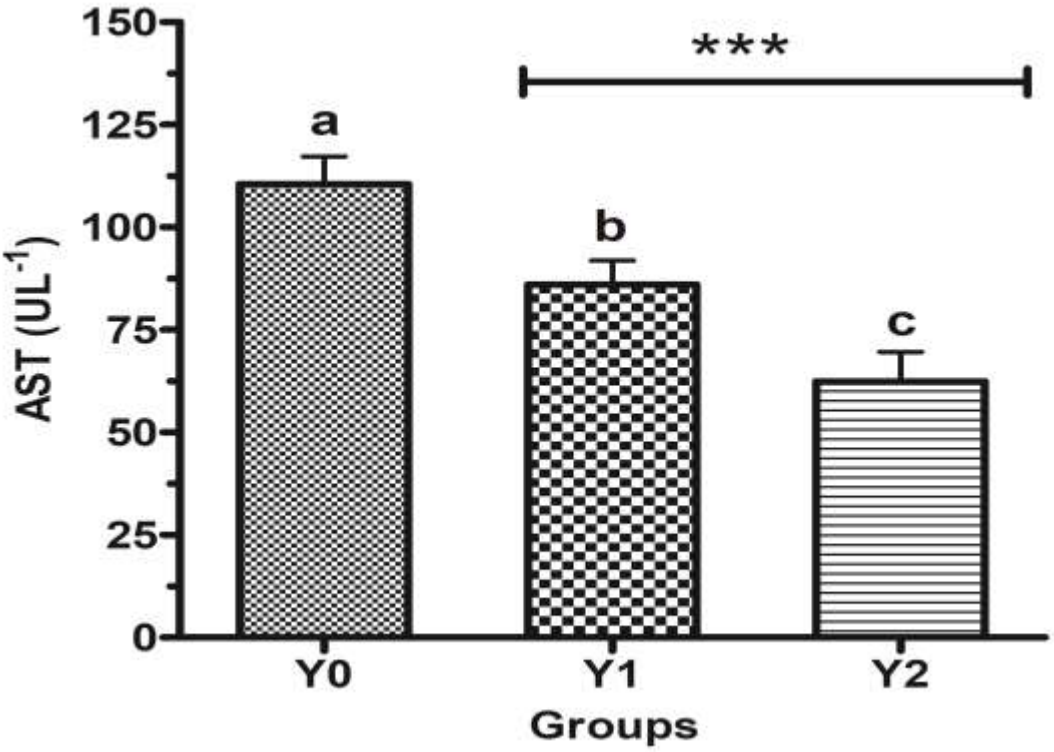

Fig. 4.1. Aspartate aminotransferase activity of *L. rohita* fingerlings after 11 weeks feeding of free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between groups. Average followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

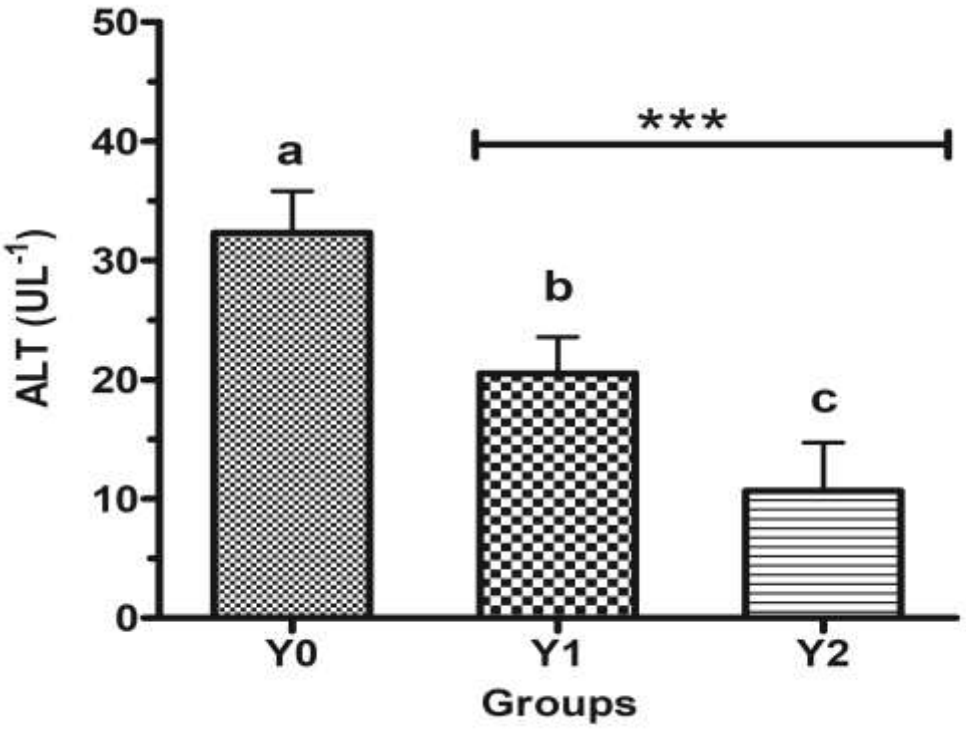

Fig. 4.2. Alanine aminotransferase activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

Immunological indices

Alg-CN encapsulated *G. candidum* dietary supplementation positively affected the immunological indices of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions. One way ANOVA revealed significant differences in serum total protein (n=9, ANOVA, $F_{2, 27}$=104, p<0.001, Table 4.4), immunoglobulin (n=9, ANOVA, $F_{2, 27}$=603, p<0.001, Table 4.4), lysozyme activity (n=9, ANOVA, $F_{2, 27}$=439, p<0.001, Table 4.4), phagocytic activity (n=3, ANOVA, $F_{2, 9}$=146, p<0.001, Fig 4.3), phagocytic index (n=3, ANOVA, $F_{2, 9}$=194, p<0.001, Fig 4.4) and respiratory burst activity (n=9, ANOVA, $F_{2, 27}$=421, p<0.001, Fig 4.5) between Y0, Y1 and Y2 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 (Y1 and Y2) as compared to control group (Y0). Furthermore all possible pairwise comparison showed the most significant positive effect of Alg-CN encapsulated *G. candidum* followed free probiotic.

Table 4.4. Comparative effects of free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet on immunological 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
	Y0	Y1	Y2			
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

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 $p < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

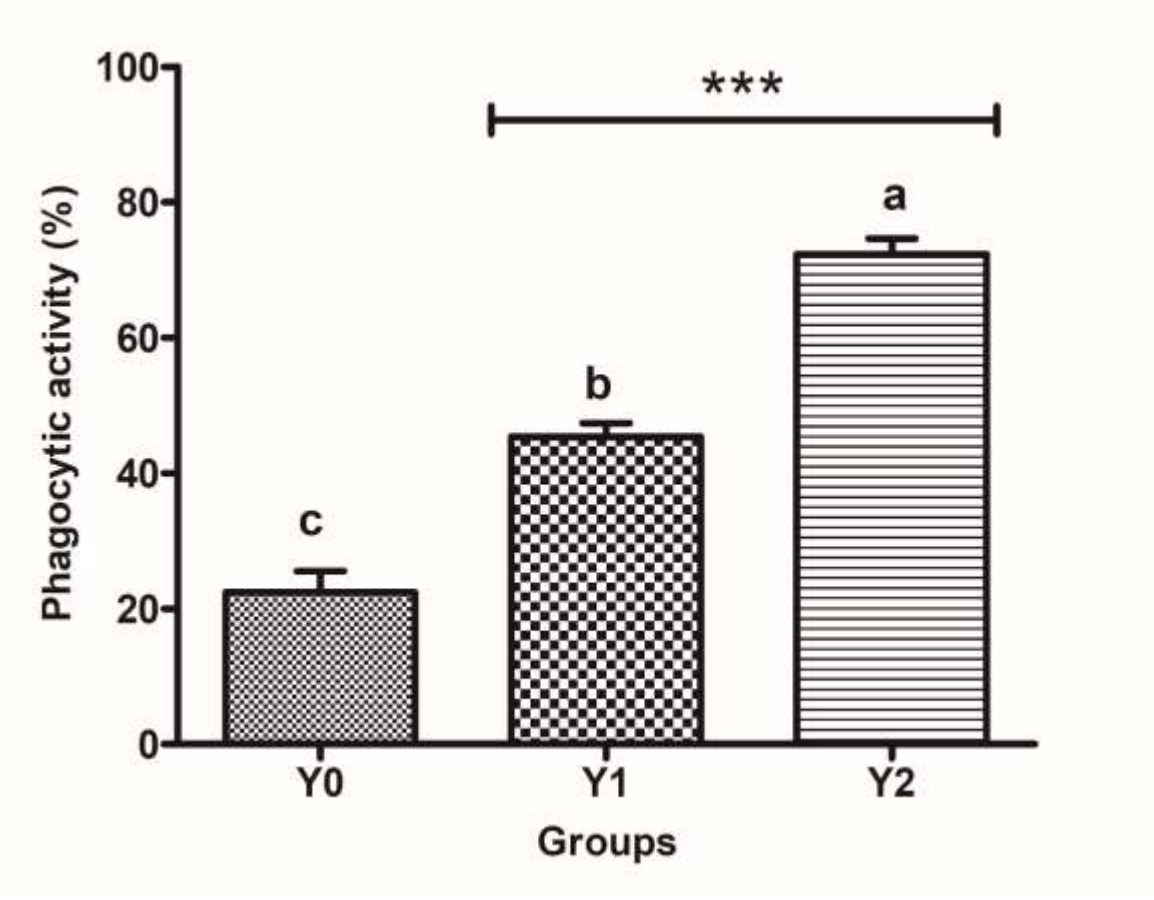

Fig. 4.3. Phagocytic activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

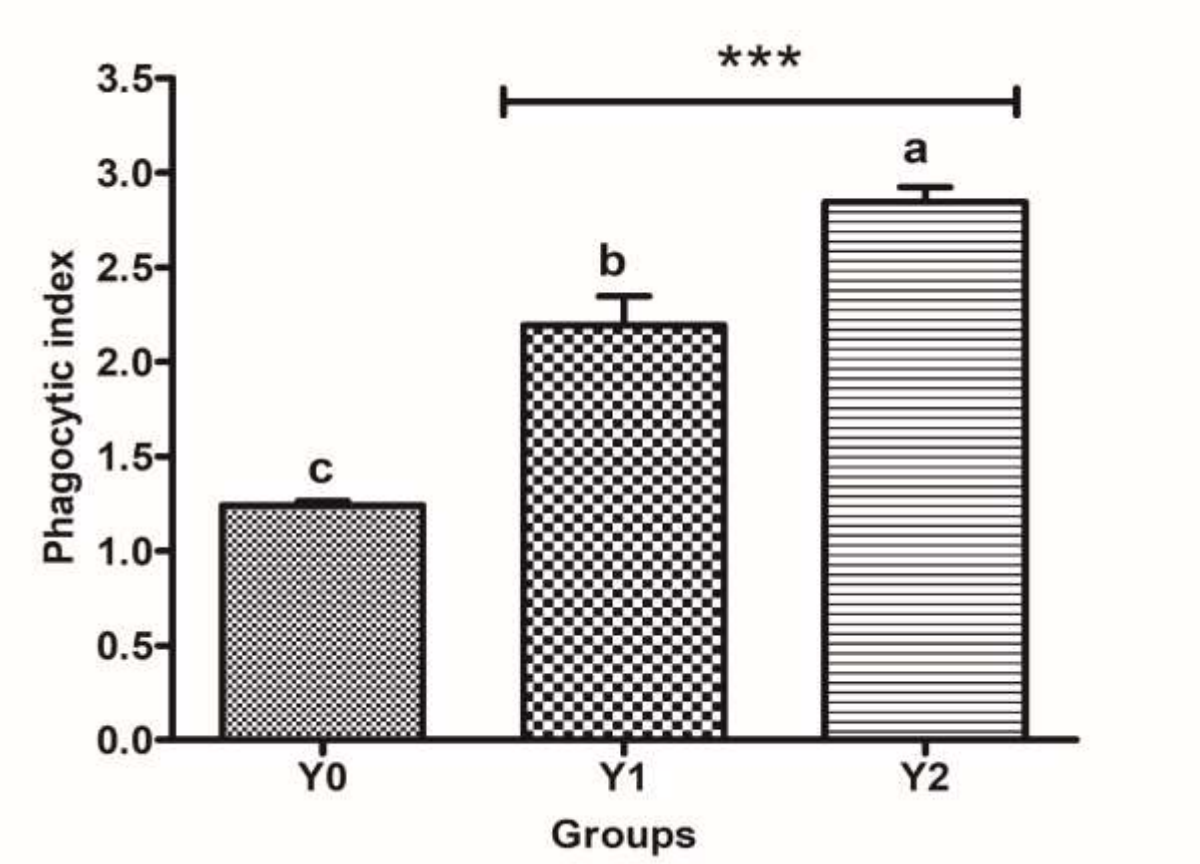

Fig. 4.4. Phagocytic index of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=3. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

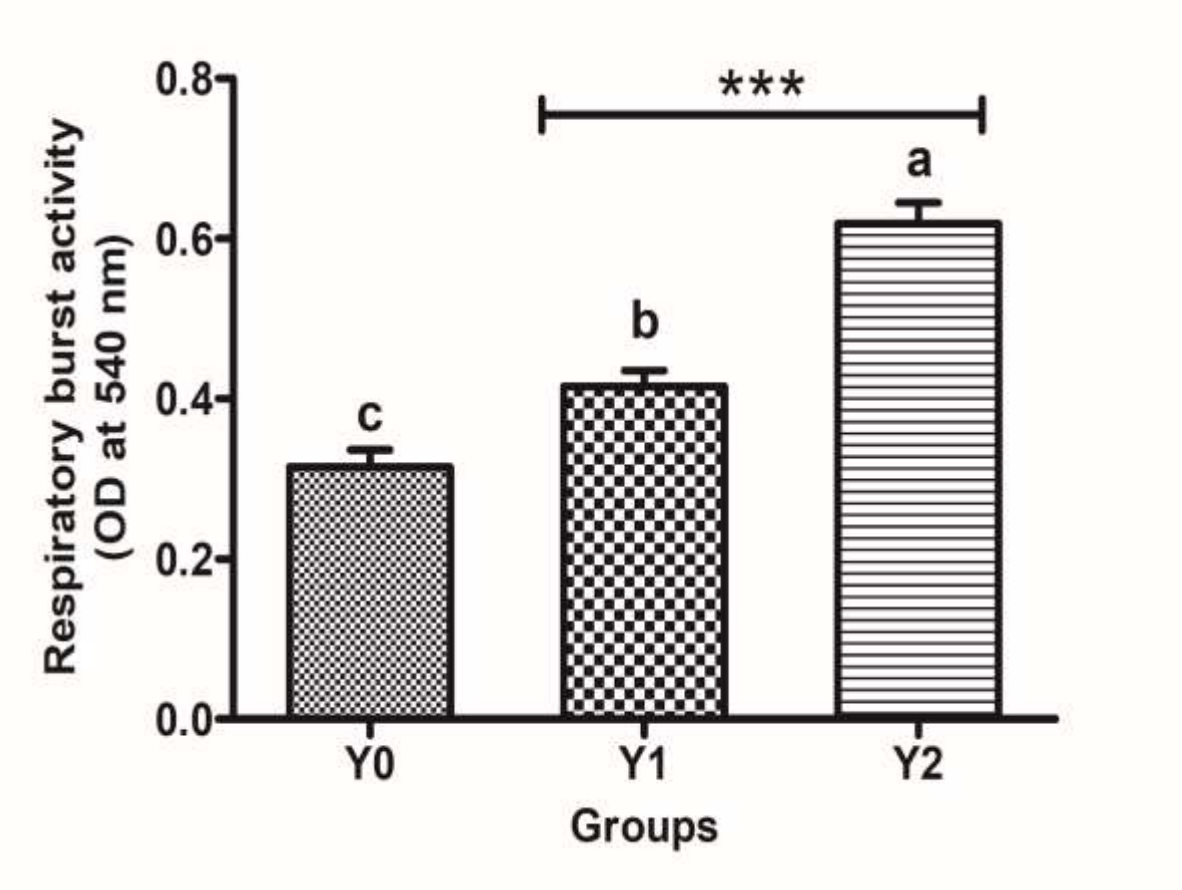

Fig. 4.5. Respiratory burst activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

Lipid profile

G. candidum in Alg-CN encapsulated form showed the most significant effect on the serum lipid profile of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions. One way ANOVA showed significant differences in total cholesterol (n=9, ANOVA, $F_{2, 27}$=68.9, p<0.001, Table 4.5) and triglyceride level (n=9, ANOVA, $F_{2, 27}$=150, p<0.001, Table 4.5) between different groups (Y0, Y1 and Y2). The Post hoc LSD test revealed lower levels of total cholesterol and triglyceride in probiotic fed groups (Y2 and Y1) as compared to control group (Y0). Furthermore, all possible pairwise comparison showed the lowest values total cholesterol and triglyceride in Y2, followed by Y1 group, while Y0 group showed the highest levels.

Table 4.5. Comparative effect of free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet on lipid profile of *L. rohita* fingerlings after 11 weeks rearing in ponds under semi intensive culture conditions (n=9)

	Groups			Pooled SEM	F ratio	p-value
	Y0	Y1	Y2			
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

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 $p < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

Gene expression

The Alg-CN encapsulated in contrast to free (un-encapsulated) *G. candidum* supplemented diet showed the most significant positive effect on HSP 70 gene expression in different tissue of *L. rohita* fingerlings. One way ANOVA revealed significant differences in HSP 70 gene expression in the muscle (n=9, ANOVA $F_{2, 27}$= 17.9, p=0.001, Fig 4.6), intestine (n=9, ANOVA $F_{2, 27}$= 51.7, p=0.001, Fig 4.7) and liver (n=9, ANOVA $F_{2, 27}$= 71.3, p=0.001, Fig 4.8) of fish from Y0, Y1 and Y2 groups. The Post hoc LSD test revealed up-regulation of HSP 70 gene in probiotic fed groups (Y1 and Y2) as compared to control group (Y0). Furthermore, all possible pairwise comparison showed significantly higher HSP 70 expression in muscle, intestine and liver tissues of Y2 group as compared to the Y1 group of fish. The muscle tissues of Y0 and Y1 groups showed statistically comparable and lower expression related to HSP 70 protein (LSD post hoc, p=0.121, Fig 4.6) as compared to Y2 group.

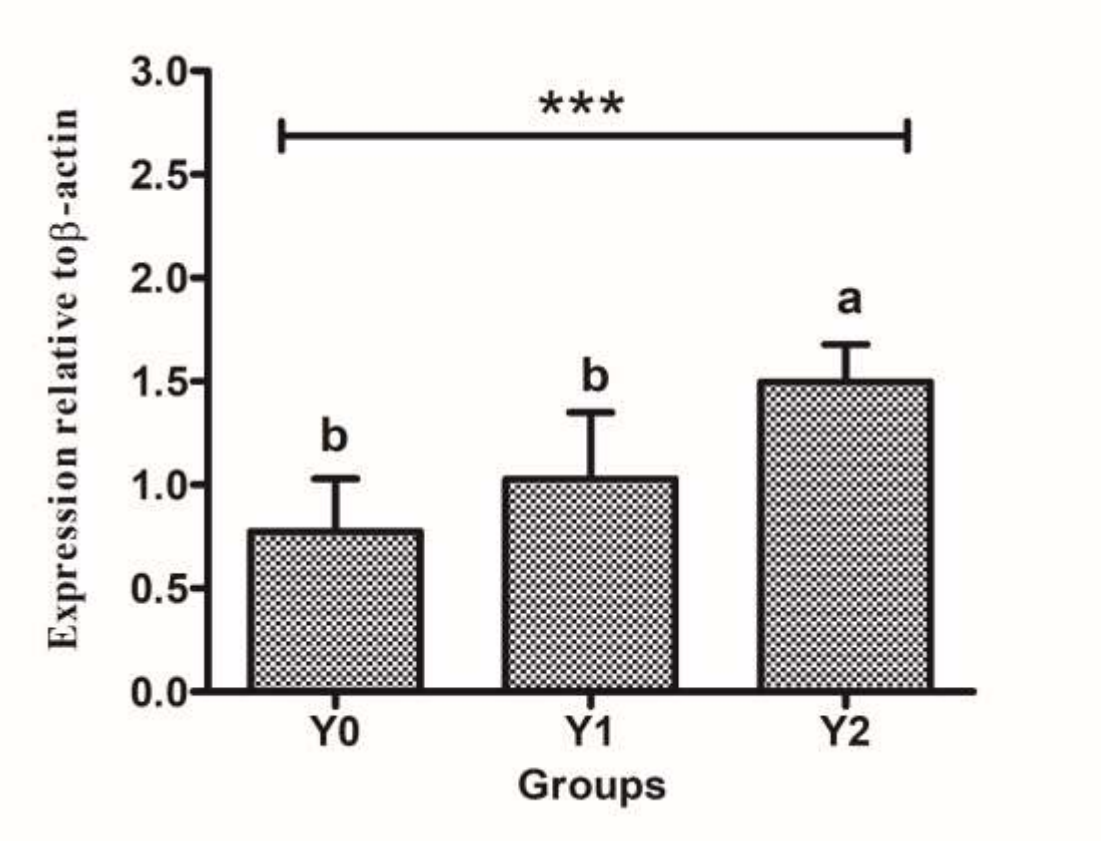

Fig 4.6. HSP 70 gene expression in the muscle of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

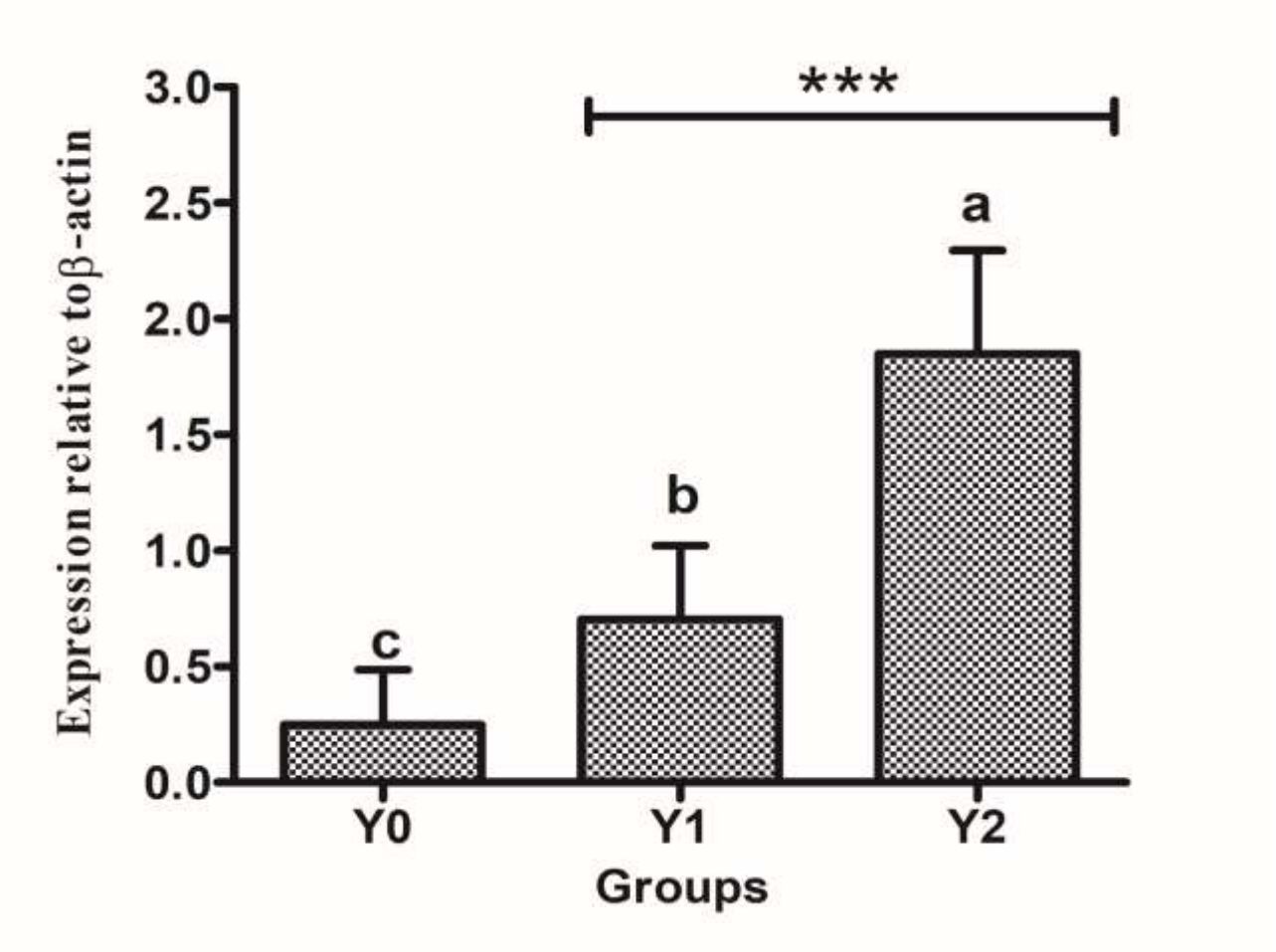

Fig 4.7. HSP 70 gene expression in the intestine of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$.Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

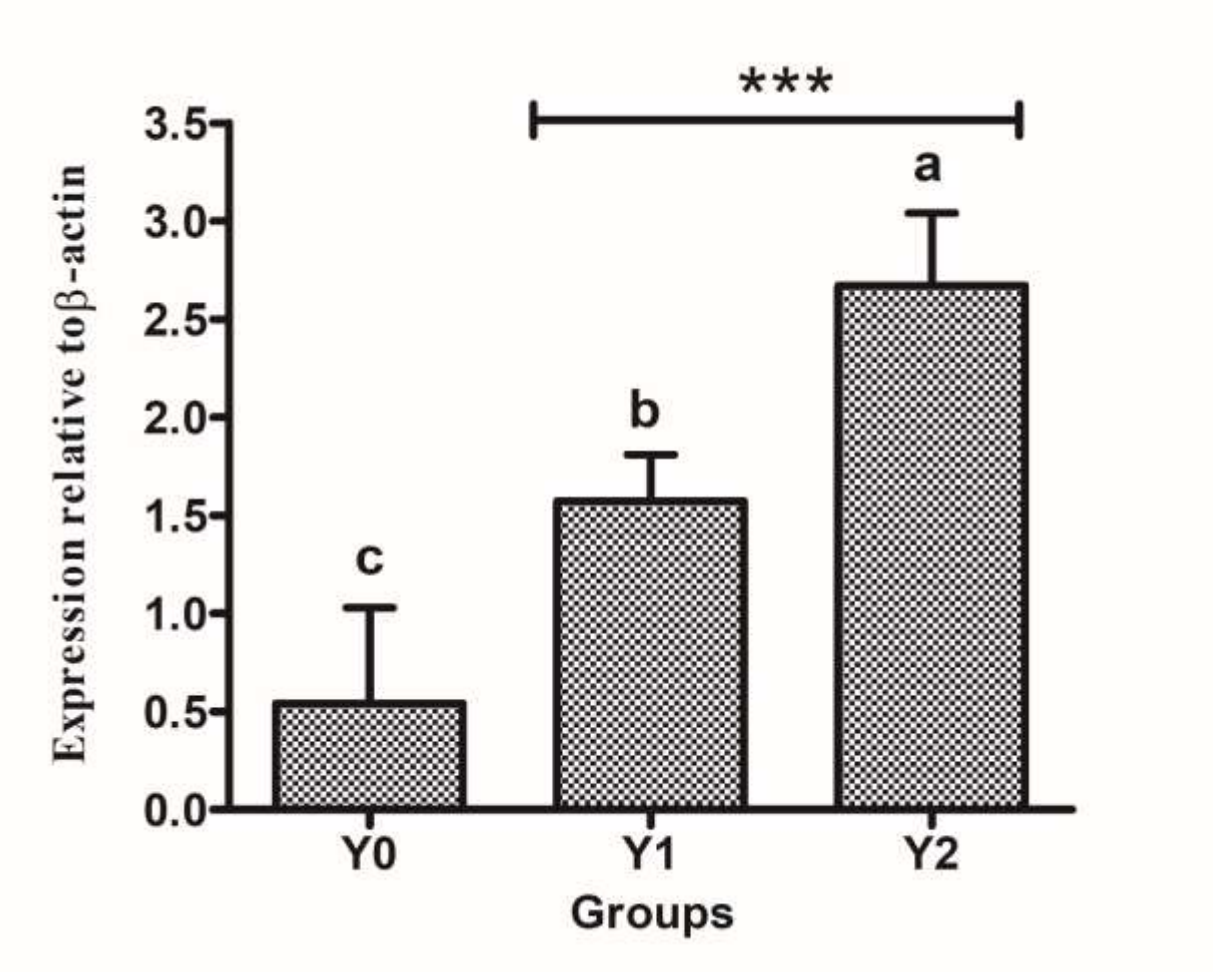

Fig 4.8. HSP 70 gene expression in the liver tissue of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and Alg-CN encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n=9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*

Muscle amino acid (AA) composition

Dietary Alg-CN encapsulated as compared to free (un-capsulated) *G. candidum* supplemented diet showed the most significant positive effect on muscle amino acid composition of *L. rohita* fingerlings reared in earthen ponds under semi intensive culture conditions (Table 4.6). One way ANOVA showed significant differences in essential amino acids (n=3, ANOVA, $F_{2, 9}$=148, p=0.001, Table 4.6) and nonessential amino acids concentration (n=3, ANOVA, $F_{2, 9}$=65.8, p=0.001, Table 4.6) between Y0, Y1 and Y2 group of fish. Post hoc LSD test revealed that both essential and nonessential amino acids were found higher in probiotic fed groups (Y1 and Y2) as compared to control group (Y0). Furthermore all possible comparisons among groups indicated that significantly higher essential and nonessential amino acids concentration was observed in Y2 group followed by Y1. Moreover, methionine, tyrosine and serine showed statistically comparable ($p > 0.05$) concentration found in the muscle of all three (Y0, Y1 and Y2) groups of fish (Table 4.6).

Table 4.6. Comparative effect of free (un-encapsulated) and Alg-CN encapsulated *G. candidum* on muscle amino acid composition (Concentration %) of *L. rohita* after 11 weeks rearing in earthen ponds under semi intensive culture conditions (n=3)

	Groups			F ratio	p value
	Y0	Y1	Y2		
Essential amino acids (EAA)					
Thr	2.84±0.49^{c}	5.47± 0.51^{b}	6.68± 0.49^{a}	46.7	0.001
Val	4.54± 0.63^{b}	5.80± 0.72^{b}	10.07± 0.57^{a}	61.0	0.001
Met	1.40± 0.52^{a}	2.63± 0.35^{a}	3.33± 0.77^{a}	8.72	0.017
IIe	ND	3.04± 0.45^{a}	3.49± 0.25^{a}	121	0.001
Leu	1.21±0.71^{c}	2.55±0.11^{b}	4.15±0.57^{a}	23.1	0.002
Phe	4.99±0.67^{c}	9.61±0.93^{b}	13.8±1.46^{a}	51.8	0.001
His	3.23±0.71^{c}	5.51±0.75^{b}	8.93±1.53^{a}	21.9	0.002
Lys	2.40± 0.83^{b}	4.51± 0.63^{b}	7.90± 1.68^{a}	17.6	0.003
Tyr	4.08± 0.82	3.04± 0.65	4.12± 0.73	2.07	0.207
Arg	0.99± 0.67^{c}	5.59± 1.10^{b}	8.25± 0.70^{a}	56.3	0.001
Conditionally essential amino acids (CEAA)					
Gly	1.81± 0.57^{c}	2.56±0.8^{b}	5.21±0.73^{a}	20.1	0.002
Glu	2.22± 1.23^{b}	4.77± 0.62^{a}	5.29± 0.84^{a}	17.0	0.003
∑EAA	32.7±4.87^{c}	55.1±2.33^{b}	81.3±2.63^{a}	148	0.001
Non-essential amino acids (NEAA)					
Ala	5.85±1.78^{bc}	6.16± 0.71^{b}	9.33± 0.54^{a}	8.44	0.018
Asp	0.39±0.27^{b}	0.77± 0.34^{a}	1.9± 0.28^{a}	20.8	0.002
Ser	2.76±1.05	3.63± 0.56	4.54± 1.22	2.43	0.168
∑NEAA	9.0±0.49^{c}	10.5±0.95^{b}	15.7±0.77^{a}	65.8	0.001

Result presented as mean± SD. One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same rows are significantly different at $p < 0.05$ Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*. Thr: threonine, Glu: glutamic acid, Gly: glycine, Val: valine, Met: methionine, lle: isoleucine, Leu: leucine, Tyr: tyrosine, Phe: phenylalanine, His: histidine, Lys: lysine, Arg: Arginine, Ala: alanine, Asp: aspartic acid, Ser: serine. ND: not detected.

Muscle Fatty acid composition

Dietary Alg-CN encapsulated as compared to free (un-capsulated) *G. candidum* most significantly modify the muscle fatty acid composition of *L. rohita* reared in earthen ponds under semi intensive culture conditions. One way ANOVA revealed significant differences in the total amount of muscle saturated fatty acids (n=3, ANOVA, $F_{2, 8}$= 47.9, p=0.001, Table 4.7), monounsaturated fatty acids (n=3, ANOVA, $F_{2, 8}$= 163, p=0.001, Table 4.7) , polyunsaturated fatty acid (n=3, ANOVA, $F_{2, 8}$= 1153, p=0.001, Table 4.7), unsaturated fatty acids (n=3, ANOVA, $F_{2, 8}$=922, p=0.001, Table 4.8), saturated to unsaturated ratio (n=3, ANOVA, $F_{2, 8}$=225, p=0.001, Table 4.8) as well as **ω**-3 to ω-6 ratio (n=3, ANOVA, $F_{2, 8}$=564, p=0.001, Table 4.8) between Y0, Y1 and Y2 groups. The muscle fatty acid composition of fish fed Alg-CN encapsulated diet (Y2) showed significantly higher levels of un-saturated fatty acids, lower saturated to un-saturated fatty acid ratio as well as the considerably higher ratio of ω -3 to ω-6 followed by Y1 group fed un-encapsulated *G. candidum*. Conversely, muscle tissues of Y0 group of fish showed significantly higher concentrations of saturated fatty acid, high ratio of saturated to un-saturated fatty acid and considerably lower ratio of ω -3 to ω-6.

Table 4.7. Comparative effect of free (un-encapsulated) and Alg-CN capsulated *G. candidum* on muscle fatty acid composition (Concentration %) after 11 weeks rearing in earthen ponds under semi intensive culture conditions (n=3)

Fatty acids	No. of Carbon atoms	Groups			F ratio	p value
		Y0	Y1	Y2		
Myristic acid	14:00	0.73± 0.07^{c}	1.54±0.07^{b}	1.88±0.07^{a}	193	0.001
Pentadecanoic acid	15:00	0.62±0.15^{c}	0.96±0.07^{b}	1.35±0.08^{a}	35.3	0.001
Palmitic acid	16:00	32.4±1.2^{a}	26.9±1.24^{b}	21.7±1.01^{c}	64.1	0.001
Margaric acid	17:00	1.53±0.05^{c}	1.86±0.04^{b}	2.12±0.01^{a}	139	0.001
Stearic acid	18:00	22.7±1.13^{a}	20.7±0.36^{b}	18.4±0.54^{c}	24.2	0.001
Docosanoic acids	22:00	0.35±0.03^{a}	0.27±0.01^{b}	0.22±0.03^{b}	18.3	0.001
∑Saturated		58.3±2.33^{a}	52.0±1.00^{b}	45.7±1.04^{c}	47.9	0.001
Heptadecenioc acid	17:01	6.09±0.11^{c}	6.58±0.10^{b}	7.64±0.17^{a}	104	0.001
Oleic acid	18:1(n-9)	0.67±0.02^{b}	0.73±0.01^{b}	0.80±0.03^{a}	20.7	0.001
Eicosenoic acid	20:01	0.58±0.02^{c}	0.64±0.03^{b}	0.76±0.04^{a}	24.6	0.001
∑Monounsaturated		7.34±0.14^{c}	7.96±0.07^{b}	9.21±0.15^{a}	163	0.001
Linoleic acid (ω6)	18:2(n-6)	9.26±0.33^{a}	8.86±0.08^{b}	5.36±0.09^{c}	329	0.001
g-linolenic acid (ω6)	18:3(n-6)	0.23±0.01^{a}	0.19±0.01^{b}	0.14±0.02^{c}	25.8	0.003
Linolenic acid (ω3)	18:3(n-3)	0.37±0.02^{a}	0.41±0.02^{a}	0.49±0.01^{a}	0.25	0.789
Eicosatrienoic acid (ω3)	20:3(n-3)	0.51±0.03^{c}	0.62±0.01^{b}	0.91±0.03^{a}	159	0.001
D-G-linolenic acid (ω6)	20:3(n-6)	ND	0.63±0.02^{b}	3.35±0.01^{a}	5705	0.001
Arachidonic acid (ω6)	20:4(n-6)	5.38±0.34^{a}	4.22±0.14^{b}	3.91±0.07^{c}	37.2	0.001
Eicosapentaenoic acid (EPA) (ω3)	20:5(n-3)	1.23±0.02^{c}	2.18±0.10^{b}	3.77±0.08^{a}	775	0.001
Docosapentaenoic acid (ω3)	20:5(n-6)	ND	ND	2.62±0.11^{a}	1960	0.001
Docosahexaenoic acid (DHA) (ω3)	22:6 (n-3)	5.39±0.04^{c}	8.91±0.22^{b}	9.61±0.17^{a}	551	0.001
∑ ω3		7.51±0.06^{c}	12.1±0.17^{b}	14.8±0.19^{a}	1538	0.001
∑ ω6		14.87±0.02^{b}	13.9±0.23^{c}	15.7±0.08^{a}	83.1	0.001
∑Polyunsaturated		22.4±0.05^{c}	26.0±0.27^{b}	30.2±0.19^{a}	1153	0.001
Ratio (ω3/ ω6)		0.50±0.00^{c}	0.87±0.02^{b}	0.93±0.01^{a}	564	0.001

Result presented as mean± SD. One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different lowercase superscripts in same rows are significantly different at $p < 0.05$ Y0, fed basal diet devoid of *G. candidum*, while Y1 and Y2, fed diet supplemented with free (un-encapsulated) and Alg-CN encapsulated *G. candidum*.

Table 4.8. Comparative effect of free (un-encapsulated) and Alg-CN capsulated *G. candidum* on muscle fatty acid composition (Concentration %) after 11 weeks rearing in earthen ponds under semi intensive culture conditions (n=3)

	Groups			F ratio	P value
	Y0	Y1	Y2		
Total fatty acids	88.1±1.27[a]	86.0±0.49[a]	88.1±1.27[a]	2.93	0.129
∑Saturated fatty acids	58.3±1.34[a]	52.0±0.57[b]	45.7±0.60[c]	47.9	0.001
∑Unsaturated fatty acids	29.7±0.07[c]	34±0.20[b]	39.3±0.17[a]	922	0.001
Ratio (Saturated\ unsaturated)	0.66±0.05[a]	0.60±0.03[b]	0.53±0.02[c]	225	0.001

Discussion

In recent years, use of probiotic in practical aquaculture has gain attention and throughout the world many indigenous microorganism have been tested as probiotics by conducting *in vitro* and *in vivo* experiments. However, *in vivo* efficiency of probiotics under control conditions, do not always provide same results under practical semi intensive pond culture systems. According to Lazado et al. (2015), 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. Thus, before suggesting practical application of any microorganism as potential probiotic, it is prime important to check its effectiveness under practical ponds culture systems.

Here for evaluating the commercial importance of *G. candidum* QAU01 both in free and Alg-CN encapsulated form, we conducted the feeding trial in earthen ponds under semi-intensive culture conditions and observed similar positive effect on the growth, survival, intestinal enzyme activities, hematology, AST and ALT activities, innate immunity of *L. rohita* as observed previously, while rearing almost similar size fish (fingerlings) on totally prepared diet in fiberglass tanks (chapter 3). In this part of study fingerlings were reliance both on live feed (planktons) and prepared diet, a common way in semi intensive culture system. During experimental period, culture conditions were managed by adopting commonly used practice (Rahman et al., 2006) i.e., with the help of organic and in organic fertilizers, lime and supplemented feed. Occasionally, we applied cow dung and DAP, for stimulating and maintaining the growth of live food organism (natural plankton).

It is well accepted that probiotic work depends on biological and physiochemical factors like, age and size of the cultured fish species (Jha et al., 2015; Ridha and Azad, 2016), culture

condition and environmental factors (Wang et al., 2008). Here feeding trial was conducted in replicate of three under similar semi-intensive conditions, feeding rate, feeding frequency and on almost similar size of fingerlings. Thus improved health status (blood profile), growth rate, intestinal enzymes activity, immune response, HSP 70 gene expression, unsaturated fatty acids and essential amino acids concentrations and decreased levels of total cholesterol, triglycerides in Y2 group of fish followed by Y1 as compared to Y0 group indicated the beneficial effect of *G. candidum* supplementation especially encapsulated (Alg-CN) probiotic. It seems that *G. candidum* in semi intensive culture conditions also, competitively exclude the pathogens and colonize the intestinal mucosal surface of host and exert its beneficial effects on physiology of fish.

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 (Pérez et al., 2010). 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 (Godara et al., 2015). Very low mortality in the present study both in control and probiotic fed groups indicate the favorable culture conditions.

The application of animal manure to maintain fertility of earthen ponds and to stimulate plankton (live food organisms) is a common strategy in modern aquaculture systems and extensively used practice in Asian countries including Pakistan (Hossain et al., 2003; Rahman et al., 2006; Ullah et al., 2018). However, beside many advantages this practice involve in the proliferation of pathogenic bacteria in the water bodies like *Pseudomonas, Aeromonas* species (Gupta et al., 2013; Godara et al., 2015), which may cause various kind of diseases in aquatic organisms like skin ulcerations, furunculosis , albinoderma, erythroderma (Sihag and Sharma,

2012; Das and Sahoo, 2014). Many investigators reported the presence of a range of pathogenic microbs like *E. coli, Salmonella, Yersinia enterocolitica, Campylobacter, Listeria monocytogenes* etc. in animal manure in addition to the common gut microflora (Pell, 1997; Guan and Holley, 2003). Here somewhat higher survival rate and no sign of disease may be due to improved water quality of culture system (Banerjee et al., 2010; Mohapatra et al., 2012), which reduce the incidence of diseases (Irianto and Austin, 2002; Newaj-Fyzul et al., 2007; Silva et al., 2013). In the present study, we did not test ponds water for pathogens and simply checked the water quality parameter. However, many scientists reported the lower incidence of pathogens and improved water quality of ponds when yeast was provided either directly or indirectly via feed (Wang et al., 2005; Deng et al., 2013; Achupallas et al., 2016).

The relationship of the gut microbiota to the aquatic habitat or food is well established (Verschuere et al., 2000). In the present study we provided *G. candidum* as dietary supplement and it seems that this probiotic after colonization to the target site, regulated the composition of mirobiota of gut and aquatic environment (Cahill, 1990; Verschuere et al., 2000), improved digestibility of nutrients by secreting exogenous enzymes (Piegza et al., 2014; Ibrar et al., 2017; Amir et al., 2018), stimulated the immune response by improving the status of total protein (Hassaan et al., 2014), IgM (Magda et al., 2011) levels and activity of lysozymes (Guven and Yalcin, 2017) and phagocytic activity (Son et al., 2009), and provide protection against pathogens (Ibrar et al., 2017; Amir et al., 2018) either by release of inhibitory factors or decrease in the permeability of host epithelium for macromolecules and toxins and activation of innate immunity (Puddu et al., 2014).

Like previous results, the present study also indicated the most significant positive effects of Alg-CN encapsulated *G. candidum* on every studied parameters of *L. rohita* as compared to

free *G. candidum.* Our results are in agreement to the findings of many investigators, alginate encapsulated probiotic administration has been shown to improve growth, as well as immune responses in fish e.g., Senegalese sole (Rosas-Ledesma et al., 2012), Gilthead seabream (Cordero et al., 2015) and alginate-skim milk microcapsules in Nile tilapia (Pirarat et al., 2015). 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 probiotic microorganisms in transit to target site, reduce in number of viable cells (Ansari et al., 2017). Similar to this study, Pirarat et al. (2015) reported higher increase in weight gain and specific growth rate of Nile tilapia fed alginate encapsulated *Lactobacillus rhamnosus* as compared to control group.

Beside observing positive effect of G. *candidum* on all previously studied parameters while rearing fingerlings of *L. rohita* in fiberglass tanks on prepared diet, we extended the study and also observed effect on the heat shock protein HSP 70 gene expression and nutritive value of fish i.e., amino acids and fatty acids profile. The heat shock protein HSP70 gene was selected because it is involved in the protection of protein structure and also stop cell self-destruction (apoptotic) mechanisms (Rollo et al., 2006). 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 (Tovar-Ramírez et al., 2010), hence maintaining their normal function (Banerjee et al., 2010) and protein homeostasis (Yamashita et al., 2010). A higher HSP70 level indicates a greater potential to respond to the stressful conditions possibly present in fish farms. Zhang et al. (2015) observed the regulatory effect of HSP70 on pro-inflammatory cytokine production in grass carp, while others reported the implication of HSP70 gene in both

innate and adaptive immunity (Daugard et al., 2007; Zhang et al., 2015). 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 Alg-CN encapsulated *G. candidum* as compared to free form. Like our results, Liu et al. (2013) also reported an up-regulation of HSP70 gene in intestine, kidney and spleen of tilapia after administration of *Lactobacillus brevis* supplemented diet. Furthermore, other scientists also observed similar an increased HSP70 expression in response to various probiotics, in different tissues of different fish species. For instance, Bagni et al. (2005) observed an up-regulation of HSP70 in liver of sea bass after feeding yeast β-glucans fed, while Rollo et al. (2006) observed similar trend in fish species after administration of *Lactobacillus fructivorans* and *L. plantarum*. Moreover, *Lactobacillus* consortium induced HSP-70 expression in intestine of *L. rohita* (Maji et al., 2017).

Fish is the best source of protein with balance and physiologically beneficial amino acids compositions like arginine, histidine, lysine, taurine (Økland et al., 2005; Metzner et al., 2006). A characteristic fish flavor is also related to a free amino acid level found in the muscle tissue. Additionally, some amino acids like Glutamate, histidine and glycine substantially contribute to the taste, texture and quality of fish meat (Li et al., 2009). It is well established that the nutritional value of meat is characterize not only by the content of protein but also on the composition of amino acid. In the present study, the muscle amino acid composition of *L. rohita* fed Alg-CN encapsulated *G. candidum* supplemented diet showed the highest values of all essential amino acids followed by free probiotic fed group (Table 4.6). We observed the highest contents of phenylalanine, followed by valine. No comparable study is available which show the effect of probiotic on amino acid composition of fish. However, in broiler "Vetosporin Active"

consisting of different strains of *Bacillus subtilis* improved the amount lysine, leucine, isoleucine leucine and valine in meat. Here all muscle amino acids showed increasing level and all groups showed similar trend with respect to amino acid composition. One possible reason may be that probiotic especially *G. candidum* also secrete proteases to digest peptide bonds in the proteins and therefore break down proteins into their constituent monomers and the free amino acids, which can benefit the nutritional status of the fish (Mohapatra et al., 2012). *G. candidum* was reported to most efficiently convert phenylalanine, tyrosine and tryptophan into the lipid soluble antimicrobial metabolites i.e. phenyllactic acid, indoleacetic acid and hydroxyl-phenyllactic acid during *in vitro* conditions (Naz et al., 2013). Moreover, achieving AA modulation in fish muscle with probiotic supplementation hold great potential for growth and health management in aquaculture. This may help to use *G. candidum* microcapsules and its application to formulate functional and environment friendly aquafeeds.

Beside protein, fish is also considered as excellent source of polyunsaturated omega ω-3 fatty acid, eicosapentaenoic acid (EPA, 20:5 ω -3) docosahexaenoic acids (DHA, 22:6 ω -3) and as well as the alpha linolenic (22:6 ω -6), a precursor of EPA and DHA (Ugoala et al., 2009; Mohamed and Al-Sabahi, 2011). Fatty acids biosynthesis in fish depends upon nutritional and environmental factors (Mráz and Pickova, 2011; Afridi et al., 2018), while the variation in fatty acid profile of fish within species can be attributed to difference in their natural and prepared diets (Khan et al., 2015; Afridi et al., 2018).

The quantity and quality of dietary source of lipid also contribute in modifying the muscle fatty acid profile of fish (Qiu et al., 2017). However, in the present study all groups of fish were provided similar diet except the probiotic supplementation and cultured under similar semi-intensive pond culture system. Therefore , here, lower in concentration of saturated fatty

acids (SFA), while increased in monounsaturated fatty acids (MUFA), polyunsaturated acids PUFA) especially docosahexaenoic acids (DHA) and Eicosapentaenoic acid (EPA) and ω3/ω6 ratio in the muscle of *L. rohita* fed probiotic supplemented diets in contrast to diet devoid of *G. candidum* could be due to improved viability of *G. candidum* and exogenous secretion of lipases in fish gut which triggered the production and assimilation of essential fatty acids. Another possible reason may be that *G. candidum* induce fermentation of non-digestible dietary carbohydrate and produce short chain fatty acids (SCFA) such as acetate, propionate and butyrate which showed positive impact on lipid metabolism (Hardy et al., 2013).

The observed muscle fatty acid compositions of *L. rohita* was in accord to observe by Afridi et al. (2018). They also reported the similar lower concentration of saturated fatty acids (SFA), , higher contents of monounsaturated fatty acids (MUFA), polyunsaturated acids PUFA) especially docosahexaenoic acids (DHA) and Eicosapentaenoic acid (EPA) and ω3/ω6 ratio in similar size of *L. rohita*. It seems *L. rohita* have good proportion of essential fatty acids and feed additives further enhanced their concentrations. Fatty acid composition of fish muscle is related to the nutritional quality and oxidative stability of lipids. For instance, monounsaturated (MUFA) are fatty acids are generally believed to positively modulate the serum lipids (Liu et al., 2013), while polyunsaturated fatty acids (PUFA) are among the most important and dominating fatty acids with anti-arrhythmic, anti-inflammatory, antithrombotic, hypolipidemic and vasodilatory properties (Simopoulos, 2016; see review, Sokoła-Wysoczańska et al., 2018).

Like other animals, fish are also not capable of synthesizing essential fatty acids (EFAs), thus indicate the requirement to provide them via food (Kiralan et al., 2010; Afridi et al., 2018). Both alpha-linolenic acids (ALA) and linoleic acids (LA) are essential because they are involved in the synthesis of several other fatty acids like docosahexaenoic eicosapentaenoic acid and

arachidonic acid (Salim de Castro et al., 2015). We observed that *G. candidum* supplementation not only increased the concentration of EFAs but also others ω 3 and ω 6 PUFA in the muscles of *L. rohita*. Moreover increase contents of muscle DHA and EPA reflected the endogenous synthesis of these FAs from ALA. It seems that higher digestibility and availability of nutrients in the presence of *G. candidum* make this possible.

Generally, ω6 and ω3 fatty acids are not inter-convertible, metabolically and functionally distinct, and often have important opposing physiological effects, therefore their balance is important (Simopoulos, 2016). The ω-6 fatty acids increase the membrane permeability which then increases intracellular triglycerides, while omega-3 fatty acids suppress lipogenic enzymes and increase beta oxidation which reduces the fat deposition (Simopoulos, 2016). Omega-6 and omega-3 fatty acids work together to create balance, homeostasis, within the body. According to some scientists, 1:1 ratio of ω -3 to ω -6 fatty acids is good from nutritional point (Simopoulos, 2016) while other suggested 1:1 to 1:5 ratios with predominant amount of EPA and DHA, best for a healthy human diet, (Osman et al., 2001). The outcomes of the present study indicated that *G. candidum* supplement balance the ω 3/ ω 6 ratio by increasing the PUFA in favor of ω -3 in contrast to ω -6 fatty acids. Moreover 1:0.93 ω -3 / ω -6 ratio in the muscle of Y2 group as compared to 1:0.87 in Y1 group could be due to more stability, viability and target release of encapsulated *G. candidum* as compared to free probiotic. The improved status of essential amino acids, essential fatty acids and the ratio of ω -3/ ω -6 ratio in fish body indicated the additional benefits of using probiotic supplements in the feed of *L. rohita* for improving the nutritional value of fish.

The present improved survival, growth rate, intestinal enzymes activities, hematology, immune responses, HSP 70gene expression, muscle amino and fatty acid contents of *L. rohita*

reared on probiotic supplemented diets under semi intensive earthen pond culture conditions indicated practical application of selected probiotic *G. candidum*. Additionally, higher efficiency of Alg-CN encapsulated *G. candidum* QAUGC01 may be attributed to the improved stability, viability of *G. candidum* in the feed and bioavailability in the GI tract of *L. rohita*. Based on these results, Alg-CN encapsulated *G. candidum* supplemented diet could be recommended as an eco-friendly viable way to supplement the conventional feed for improving production and nutritive value of *L. rohita*.

General discussion

Nowadays, probiotics are among the most attractive dietary supplements for improving the health status and growth of fish. Currently, wide varieties of microorganisms are in use as probiotics for improving aquaculture production (Nayak, 2010; Sharifuzzaman and Austin, 2017). However, the behaviors of microbes used as probiotic vary according to their interactions with the gastrointestinal tract of the host (Ibrahem, 2015). A microorganism used as probiotic for one aquatic species could be harmful to other species (see review Hai, 2015). Thus, a specific probiotic strain /species to target fish species in a particular environment is required for obtaining maximum production.

The present study was conducted for selecting a suitable probiotic for widely culturable fish species of Pakistan, *L. rohita*, from two potential probiotics i.e., indigenous strains of yeast *G. candidum* QAUGC-01 (accession number KTC280407) and bacteria *Bacillus cereus* (accession numberKT021872) isolated from yogurt a dairy by-product and gastrointestinal tract of adult *L. rohita* respectively. The selection of potential probiotic based on many different factors, including tolerance to gastrointestinal environment, adhesion to the intestinal mucosa of host and antagonistic activity against pathogens (See review Ibrahem, 2015). The morphology and physiology of the gastrointestinal tract of *L.rohita* change as fish grow. In early life stage, fish has limited digestive capability and mainly feed zooplanktons but with age and size, relative length of gut (RLG), slowly increases from fry to fingerlings to the juvenile stage, which reflect the change in feeding habits (preferring plant materials) and digestibility of nutrient (Ramesh et al., 1999; Azim et al., 2001).

Initially, on the basis of gut morphology and digestive capability, two life stages i.e. swim up fry and advanced fry were used to evaluate the comparative effect of indigenous local strains of probiotics. Larvae of most of fish, including *L. rohita* are small in size with somewhat sterile gut and inept ability to digest most of nutrient efficiently (Kolkovski, 2001), while fry and fingerlings have autochthonous and allochthonous gut microbiota originated from the microflora of live feed and rearing water, which may have inefficient digestion and nutrient assimilation and increased incidence of infectious and autoimmune related diseases due to exposure of naturally occurring pathogens in aquatic environment (Ringø and Birkbeck, 1999; Borch et al., 2015).

We observed probiotic specific positive effect on both life stages of fish. *G. candidum* showed the most significant positive effect on survival (%), growth performance, intestinal enzymes activities, hematological indices, immunological parameters (Total protein content, IgM level, lysozyme activity, phagocytic activity, respiratory burst activity and disease resistance of fry as well as fingerlings against *A. hydrophila*as compared to *B. cereus* (chapter 1).The better efficiency of *G. candidum* as compared to *B. cereus* could be due to cell wall constituent of *G. candidum* i.e., chitin, β-glucans (Sietsma and Wouter, 1971) as well as larger cell volume as compared to bacteria (Gatesoupe, 2007), which provide protection to unfavorable conditions, facilitie to out-compete other microbes and adhere to GI tract of the host (Keily and Olson, 2000; Syal and Vohra, 2014; see review Ibrahem, 2015) and occupy more space. .

It is well documented that β-glucan is a potent immune system stimulator (Ortuño et al., 2002; Saikai, 2005), have “receptor molecule” on the surface of phagocytes. Thus stimulate the process of phagocytosis and also secrete cytokines (signal molecules) for stimulating the formation of new white blood cells. In the current study, both probiotics were used in similar

concentrations, i.e., 1×10^9 CFU/g, however, *G. candidum* is a eukaryote (size: 6-12 × 3-6 µm) in contrast to *B. cereus* which is prokaryote in nature and smaller in size (1 × 3-4 µm), thus *G. candidum* adhere more space on the mucosal surface of the host.

The higher efficiency of *G. candidum* may also be due to its strong enzymatic potential i.e., higher level of α amylases, cellulases, β-glucanases, xylanases and moderate level of proteases and lipases (Ghosh et al., 2002; Piegza et al., 2014) in contrast to *B. cereus* that produce moderate level of proteases and poor level of amylases (Ghosh et al., 2002). The need of increase level of amylase and proteases during early life stages of *L. rohita* has already been reported (Umalatha et al., 2016). Based on results, *G. candidum*-QAUGC01was selected for our target species.

In Pakistan and most of Asian countries, *L. rohita* is cultured in semi-intensive culture system i.e. on live and artificial feed (Mohsin et al., 2017; Bakhtiyar et al., 2017).Most of scientists, including Sun et al. (2011) reported the 5 to 10% decrease in the amount of the probiotic in feed after 30 days of storage. Moreover, viable cell count of probiotic microorganisms also reduce in number in transit to the target site (cook et al., 2013). However, to confer beneficial effects to the host, sufficient numbers of viable cell count of probiotic at target site is required (Rosas-Ledesma et al., 2012; Pinpimai et al., 2015)

The shelf life of probiotics could be increased by storing the prepared feed in cool and dry place (Lara-Flores, 2011) or by encapsulating the probiotic (Huq et al., 2013). Nowadays encapsulation of probiotics with different coating materials is gaining more importance. Microencapsulation protects the core material from harsh conditions such as high temperature, pH, salinity of water, harsh environment of the GI tract (acidic pH, enzymes, bile salts etc)

(Corona-Hernandez et al., 2013). However, the efficiency and characteristics of microcapsules depend on many factors, including encapsulating materials, hardening solution (calcium chloride), concentration of capsular matrix, and hardening time (Krasaekoopt et al., 2003; Riaz and Masud, 2013).For improving the survival, stability and the target release of selected probiotic as well as for selection of best encapsulating formulations, encapsulation of *G. candidum* was also conducted with alginate alone (Alg) and in combination with starch (Alg-S) and xanthan (Alg-X) and coated with chitosan (Alg-C, Alg-S-C, Alg-X-C) and chitosan nanoparticles (Alg-CN, Alg-S-CN, Alg-X-CN), by adopting simple, easy and cheap extrusion technique with similar concentrations of hardening solution. We selected Alginate as it is biodegradable, hemocompatible and did not show any accumulation in any major organs (Rajaonarivony et al., 1993). It is widely used in food industries and for obtaining the survival of probiotic organisms up to 80 to 95% (Sultana et al., 2000; Mandal et al., 2006a).

Though some investigators reported the use of alginate as encapsulating material, but several studies confirmed their susceptibility towards the acidic pH conditions of the stomach, loss of mechanical stability while passage through the GI tract (RiazandMasud, 2013). According to Huq etal, (2017), alginate matrix is porous in nature, susceptible to low pH conditions, consequently, provides a less protection. In view of these factors, we also used alginate in combination with other polymers (starch and Xanthan as an encapsulating material and chitosan as a coating material).

Before practical application of encapsulated probiotics, literature emphasizes, *in vitro* and *in vivo* simple tests for evaluating the temperature and pH tolerance, shelf life, resistance to bile and enzymes of the GI tract of host and their impact on growth, immune response, metabolic

function of an organism etc. (Papadimitriouset al., 2015). Therefore, structural characteristic, surface morphology, and *in vitro* assays were performed to evaluate the encapsulation efficiency (EE), pH and temperature tolerance and viability under two different storage conditions (ambient and 4ºC). Overall results indicated the higher encapsulation efficiency, survival and improved tolerance to different temperature, pH and storage conditions of encapsulated *G. candidum* in contrast to free /un-encapsulated probiotic (chapter 2).

Among all formulations, alginate with nano-chitosan and chitosan coating appeared, as a most suitable wall material for the *G. Candidum,* since with this formulation, the prepared microcapsules were more spherical in shape with least dent/crack on the surface and showing highest encapsulation efficiency, shelf life and viable cell count of entrapped probiotic after exposure to higher temperature (60°C) and various pH (2, 4 and 8).It may be due to cross linking between alginate and chitosan molecules and formation of complex structure with smaller pores and cavities on the surface of the microcapsule (Lee et al., 2004). Zanjani et al. (2014) also reported the change in shape and morphology of alginate capsule when coated with chitosan. Furthermore, like our results many other investigators also reported the lower encapsulation efficiency (EE) of chitosan free alginate (Azarnia et al., 2008) and suggested that encapsulation effectiveness could be improved either by mixing alginate with other polymers or coating the Alg-microcapsule with chitosan (Krasaekoopt et al., 2003).

On the basis of *in vitro* results, a combination of 2% alginate 0.4 % chitosan or nano-chitosan was selected and recommended as encapsulating material for improving the % survival of this potential probiotic (*G. candidum*) in the feed during feed preparation, feed storage and GI tract of *L. rohita*. Although, *in vitro* results of the encapsulated probiotic could indicate the

functionality and viability of potential probiotic, however, assumptions based on *in vitro* experiments might not comply exactly with *in vivo* conditions. Therefore, for *in vivo* evaluation 11 weeks experimental trial was conducted in order to check the comparative effects of *G. candidum* encapsulated / coated with Alg, Alg-C, Alg-CN, Alg-S, Alg-S-C, Alg-S-CN, Alg-X, Alg-X-C, Alg-X-CN, on *L. rohita* fingerlings reared in fiberglass tanks on prepared feed (under semi control condition). Our results were in accord to *in vitro* results. Here, we also observed that fingerlings reared on experimental diets supplemented with encapsulated *G. candidum* as compared to un-encapsulated/free probiotic, showed significantly improved growth rate, intestinal enzyme activity, hematological indices, ALP activity, immunological indices, a significant decrease in FCR, AST, ALT activities and most prominent changes in serum lipid profile (chapter 3). Furthermore the most significant positive effect was observed when diet supplemented with *G. candidum* encapsulated with alginate and coated with nano-chitosan (Alg-CN microcapsules), followed by Alg-C microcapsules.

Like our results, several studies reported the positive effects of bio-encapsulated probiotics on growth rate, FCR, innate immunity of different fish species (Adineh et al., 2011;Tourakiet al., 2012; Pinpimai et al., 2015; Pirarat et al., 2015; Cordero et al.,2015).The improved efficiency of encapsulated in contrast to free probiotic observed here and reported by many investigators may be due to physical barrier (capsule) which provide protection/resistance against temperature, bile salts, enzymes and pH of the GI tract of the host, thus deliver the probiotic to the target site without damage (Rosas-Ledesma et al., 2012).

The higher efficiency of Alg-CN and Alg-C microcapsules of *G.candidum* may be due to Alginate and chitosan interaction and the formation of the complex which prevent the leakage

and deterioration of probiotic (*G. candidum*) during passage to GI tract and release it on target site. Chávarriet al. (2010) also reported the decreases porosity of alginate microcapsule and reduces leakage of the encapsulated probiotic when coated with chitosan. It is well documented that chitosan coated alginate capsules are stronger (Mortazavian et al., 2007) and less effected to the GI tract conditions (acidic pH, bile, enzymes etc) of the host (Zhou et al., 2000; Krasaekoopt et al., 2003).

Moreover, the highest efficiency and increased biological activity of Alg-CN as compared to all encapsulating formulations including Ag-C may be due to small particle size of the coating material that increase the surface area and improve the adhesion, interaction and absorption and pH dependent release rate of probiotic in the GI tract of the host (Agrawal et al., 2014). Thus, the higher efficiency of Alg-CN microcapsules of *G. candidum* may be due to their higher stability and enhanced bioavailability throughout the mucosa of the GI tract (Kumari et al., 2013). The present study was conducted for the *L. rohita*, where the stomach is absent and the pH of intestinal fluid ranging from 6.2-7.1. It seems pH of the intestinal fluid favor the release of *G. candidum* from the Alg-CN and Alg-C microcapsules. According to literature, at intestinal pH, microcapsules starts swelling and release rate is quite high, however, the best release rate from alginate-chitosan microcapsules can be achieved at pH 7.4 (Ansari et al., 2017). Keeping in view the results Alg-CN and Alg-C microcapsules *G. candidum* could be recommended for improving the production of *L. rohita*.

We conducted all above experiments, in fiberglass tanks under semistatic and semi-control conditions in the outdoor facility. The postlarvae, fry and fingerlings were dependent on the outside source of feed i.e., prepared diet. We also changed the water frequently, thus prevent

the establishment of live feeds as well waste material for decomposition. However, for commercial production, *L. rohita* are reared in earthen ponds under semi intensive culture system.

In semi-intensive culture system, the application of animal manure to maintain the fertility of earthen ponds and to stimulate plankton (live food organisms) is a common strategy (Hossain et al., 2003; Jhaet al., 2004; Rahman et al., 2006; Ullah et al., 2018). Thus fish depends on live feed and additional prepared feed is provided to improve growth and to stimulate the immune system. However, beside many advantages, this practice involves the proliferation of pathogenic bacteria in the water bodies like *Pseudomonas, Aeromonas*species (Jiayiet al., 1987;Quines, 1988; Sugita et al., 1996), which may cause various kinds of diseases in aquatic organisms like skin ulcerations, furunculosis , albinoderma, erythroderma (Das, 2004; Sihag and Sharma, 2012). Many investigators reported the presence of a range of pathogenic microbes like *E.coli*, *Salmonella, Yersinia enterocolitica, Campylobacter, Listeria* monocytogenes etc. in animal manure in addition to the common gut microflora (Pell, 1997; Guan and Holley, 2003). Moreover, accumulation of waste materials and their decomposition also make this system different from culturing in fiberglass tanks. It seems that fish in semi-intensive culture system, are at greater risk of diseases.

Literature revealed that, the *in vivo* efficiency of probiotics under control conditions, do not always consistent with semi intensive earthen pond culture systems. 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 (Lazado et al., 2015). Thus, before suggesting practical application of selected probiotic *G. candidum* QAUGC-

01, we conducted feeding trial in earthen ponds under semi intensive culture conditions (chapter 4). Fingerlings of *L. rohita* were fed 35% CP diet supplemented with similar doses of free/un-encapsulated and Alginate-chitosan encapsulated *G. candidum*QAUGC-01 as used in a previous experiment.

Our results of semi intensive culture system were in agreement with previous results (Chapter 3). We observed similar significant positive impact of Alg-CN encapsulated *G. candidum* followed by free probiotic on growth rate, intestinal enzyme activities (protease, amylase and cellulase), hemato-immunological indices (RBCs, Hb, HCT, WBCs, MCHC, respiratory bursts and phagocytic activity, total protein, lysozyme, IgM). Beside these, we also observed up-regulation of heat shock protein HSP 70 gene in muscle, intestine and liver tissues, improved nutritive value of fish in term of improved status of muscle PUFA (especially Eicosatrienoic acid, linolenic, Eicosapentaenoic acid, Docosapentaenoic acid , Docosahexaenoic acid), ratio of ω3 to ω6 fatty acids, essential amino acids (especially arginine, isoleucine, threonine, valine and phenylalanine) and the reduction in serum AST and ALT activities, total cholesterol and triglycerides in group of fingerlings fed Alg-CN encapsulated *G. candidum* supplemented diet as compared to free probiotic.

A higher muscle, liver and intestine heat shock protein HSP70 expression in probiotic fed groups indicates a greater potential of fish to respond to the stressful conditions possibly present under semi intensive culture conditions. The involvement of HSPs gene in the folding and translocation of newly synthesized proteins, repair or bringing back the damaged proteins to their normal conformation (Tovar-Ramírez et al., 2010), maintenance of their normal function (Banerjee et al., 2017) and protein homeostasis (Yamashita et al., 2010) are well documented. It

seems that under stressful semi intensive culture system *G. candidum* especially Alg-CN encapsulated one; provide protection and keeping functions normal by stimulating HSP70 gene expression in different tissues. Like our results, many scientists reported the up regulation of HSP70 gene in different organs of various fish species after feeding probiotics. For instance Liu et al. (2012) reported an up-regulation of HSP70 gene in the intestine, kidney and spleen of tilapia after administration of *Lactobacillus brevis* supplemented diet, while Bagni et al. (2005) observed an up-regulation of HSP70 gene in the liver of sea bass after feeding yeast β-glucans.

Fish is the best source of protein with balance amino acid compositions and an excellent source of polyunsaturated omega ω-3 (EPA and DHA) and ω -6 (ALA) fatty acids (Saba and Muhammad 2000; Ugoala et al., 2009) and essential amino acids (Marshall, 1994;Metzner et al., 2001;Økland et al., 2005). The digestibility and availability of nutrient from natural and prepared diets play significant role in changing the nutritive value of fish within the species (Puustinen et al., 1985; Sargent et al., 1995; Ahigren et al., 1996).The present increased in the contents of all essential amino acids, PUFAs, ratio of ω3/ω6 fatty acids in probiotic fed groups, especially in response to Alg-CN encapsulated *G.candidum,* show the additional benefit of this product and it may be due to improve digestibility and availability of nutrient for downstream metabolism and production of other amino acids and fatty acids.

Our results indicated that even in the presence of challenging conditions (semi intensive culture system, *G. candidum* QAUGC-01showed promising results either by producing inhibitory compounds and controlling aquatic environment (Wang et al., 2005; Deng et al., 2013; Achupallas et al., 2016) or by competitive exclusion of pathogens, colonization on the target site, regulation of the composition of mirobiota of host gut (Cahill, 1990; Verschuere et al., 2000). It seems that *G. candidum* showed its positive impact by secreting exogenous enzymes, stimulating

immune response, or by decreasing the permeability of host epithelium for macromolecules and toxins (Puddu et al., 2014). However, the higher efficiency of Alg-CN encapsulated *G. candidum* QAUGC01 may be attributed to the improved stability, viability of *G. candidum* in the feed and bioavailability in the GI tract of *L. rohita*.

In conclusion, the improved gut microbial load, intestinal enzymes activity (proteases, amylases and cellulases), muscle protein and fat contents, erythrocytes (RBCs), leukocytes (WBCs), hemoglobin (Hb) and hematocrit (HCT %), plasma protein level, IgM, serum lysozyme activity, respiratory burst, phagocytic activity, expression of heat shock protein (HSP 70) gene in muscle, intestine and liver tissues muscle as well as PUFA (especially Eicosatrienoic acid, linolenic, Eicosapentaenoic acid, Docosapentaenoic acid , Docosahexaenoic acid), ratio of ω3 to ω6 fatty acids, essential amino acids particularly phenylalanine, valine and arginine), and reduction in serum AST and ALT activities, total cholesterol and triglycerides in postlarvae, advanced fry reared under semi control and semi-static conditions (on prepared diet) and on fingerlings of *L. rohita* reared under both semi control/ semi-static and semi-intensive earthen pond (on both live feed and prepared diet) conditions on *G. candidum* supplemented diet as compared to basal diet devoid of probiotic indicated the positive impact of *G. candidum* on early rearing and grow out stage of *L. rohita* .

Moreover, improved structural characteristics i.e., more spherical in shape, with smooth surface and higher encapsulation efficiency, *in vitro* thermal and pH tolerance as well improved shelf life of *G. candium* encapsulated with alginate and coated with nano-chitosan(Alg-CN microcapsules of *G. candidum*) and chitosan (bulk) (Alg-C microcapsules of *G. candidum*) as compared to other microcapsules prepared with Alginate alone or in combination with starch and

Xanthan and coated with chitosan indicated that Alginate-nano-chitosan/chitosan is best formulation for encapsulation of *G. candidum*

Furthermore, in *in vivo* evaluation, the most significant positive effect of Alg-CN encapsulated *G. candidum* supplemented diet on growth, feed conversion ratio, intestinal enzyme activities, hematology, AST, ALT, ALP, hemato-immunological indices, lipid profile; high density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol and triglyceride content of fish as compared to basal diet supplemented with *G. candidum* encapsulated with other encapsulating formulations (Alg, Alg-S, Alg-S-C, Alg-S-CN, Alg-X, Alg-X-C and Alg-X-CN) also indicated the higher efficiency of Alg-CN formulation for the encapsulation of *G. candidum.*

On the basis of results, *G. candidum* could be recommended for improving the survival and growth during early rearing and Alg-CN encapsulated *G. candidum* for practical/commercial production of *L. rohita.* Moreover, further, experiments and molecular level study is suggested for understanding the mode of action of this probiotic

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ORIGINAL ARTICLE

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Evaluation of yeast and bacterial based probiotics for early rearing of *Labeo rohita* (Hamilton, 1822)

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Abstract

Relatively poorly developed digestive and immune systems appear as key factors for low survivability and reduced growth rate of major carp during early development stages. Here, an attempt has been made to improve the digestive capability and immune response of *Labeo rohita* larvae by feeding diets supplemented with yeast and bacterial based probiotics *Geotrichum candidum* QAUGC-01 and *Bacillus cereus*, locally isolated from yogurt and gastrointestinal tract of an adult healthy *L. rohita* respectively, and evaluate their effects on the survival, growth, digestive enzyme activity, muscle composition and intestinal bacterial load. Eleven week feeding experiment in triplicate with four groups was conducted in a completely randomized design. Control group was given only basal feed devoid of probiotic, T1 and T2 groups was provided basal diet supplemented with *G. candidum* and *B. cereus* respectively at similar rate, that is, 1×10^9 CFU/g feed, while T3 group was fed a diet supplemented with consortium of both probiotics, each at the rate of 1×10^9 CFU/g feed. *G. candidum* showed the most significant effect ($p < 0.001$) on final weight, SGR, biomass, survival (%), microbial gut count and muscle composition. Intestinal enzymes activities also showed significant variation, as amylase activity was significantly ($p < 0.001$) higher in T1, while T3 group of fish showed higher activity of cellulase and protease. Likewise, after challenge with *Aeromonas hydrophila*, T1 group showed significantly ($p < 0.001$) higher survival. This study signifies the higher efficiency of *G. candidum* in comparison to *B. cereus* and suggests its inclusion in the diet for early rearing of *L. rohita*.

KEYWORDS

growth performance, intestinal bacterial load, muscle composition, probiotics, survival

1 | INTRODUCTION

Freshwater aquaculture contributes more than 50% to the total aquaculture production in Asia. *Labeo rohita* is among seven freshwater fish species cultivated predominantly in South Asia because of its high commercial value, good growth rate, consumer preferences and relatively easier weaning on prepared diets (Hussain et al., 2011; Khan, Shakir, Khan, Abid, & Mirza, 2008).

Throughout the world, hatcheries are playing significant role in the supply of fish seed of various fish species including *L. rohita* in support of freshwater aquaculture production. However, higher rate of mortality (70%–80%), and poor growth performance during early developmental stages of major carps are the major constrains in the supply of good quality seed (Mohapatra et al., 2012) and increase in production of these species (Jha, Bhujel, & Anal, 2015). Most of culturable species including *L. rohita* during early development stage do not possess well differentiated digestive and immune systems (Ibrar, Zuberi, Amir, Imran, & Noor, 2017; Zapata, Diez, Cejalvo, Gutierrez-de Frias, & Cortes, 2006), thus, lack ability to digest most of nutrient

efficiently (Kolkovski, 2001) and protect themselves from pathogen. Generally immune-competence and maturation of lymphoid organs developed after certain weeks of hatching (Zapata et al., 2006), hence, primarily they rely on non-specific defense systems (Secombes & Ellis, 2012).

In most of Asian countries, unavailability of cheap prepared larval-feed on a commercial scale, compel farmer to rear postlarvae of both finfish and shellfish including *L. rohita* on live food organisms (Kolkovski, 2001) and faces many inherent problems, which directly or indirectly affect the growth and survival of juvenile like deficiency of several essential nutrients in Rotifers and *Artemia*, appearance of unwanted microorganism and pathogens while producing a live feed through pond fertilization as well as variation in the ratio and quantity of nutrients in the live organisms (Hamre, 2016).

It is generally assumed that prepared microdiet that can improve the digestibility of nutrient and immune competency of early juvenile could replace the live feed (Cahu & Zambonino-Infante, 2001) From the last decade, research is in progress to prepare diet for early rearing of different fish species either using more digestible source of protein such as protein hydrolysates, or improving the digestibility of nutrients with enzymes or probiotic supplements (See review Hamre et al., 2013).

Nowadays, for eco-friendly and sustainable development of aquaculture, probiotics are gaining more importance. Generally, probiotic improve the nutritional and health status of host organism by out competing the pathogens and promoting the growth of favorable intestinal microbial populations (Burr, Gatlin, & Ricke, 2005; Ghori, Tabassum, Ahmad, Zuberi, & Imran, 2018). It improves mucosal defenses of the gastrointestinal tract, non-specific immune response, resistance against pathogens, tolerance against stress, survival, growth performance and feed conversion efficiency of fish either as a result of production of digestive enzymes like amylase, protease, lipase as well as cellulose degrading enzyme (cellulase), short chain fatty acids (SCFA), and essential vitamins or by differentiation and activation of T-cell (see reviews Hardy, Harris, lyon, Beal, & Foey, 2013; Ibrahem, 2015; Ullah et al., 2018).

A large number of bacteria and yeast with varying degree of success are now being predominantly used as probiotics in aquaculture for enhancing the survival and improving the growth performance of fish (see review Ibrahem, 2015). The selection of potential probiotic based on many different factors like tolerance to gastrointestinal environment, adhesion to the intestinal mucosa of host, antagonistic activity against pathogens. However, efficacy of selected probiotic depends on dose, duration and mode of the application, age and size of the cultured fish species (Jha et al., 2015; Ridha & Azad, 2016).

Currently, most of studies focus on evaluating the impact of different probiotics on fingerlings and adult fish. Yet, research on the impact of probiotics on early juvenile is limited. Therefore, major objective of this study was to investigate and compare the effects of fungal and bacterial based probiotics *Geotrichum candidum* and *Bacillus cereus*, locally isolated from commercial dairy product (Yogurt) and gastrointestinal tract of an adult healthy *L. rohita* respectively on early rearing of *L. rohita* by evaluating their impact on survival, growth, activities of intestinal enzymes and muscle composition. Our hypothesis was that successful colonization of beneficial microbes in the sterile underdeveloped gut of swim up fry would enhance the growth and survival of *L. rohita* by improving digestibility of nutrients and immune responses.

2 | MATERIALS AND METHODS

2.1 | Fungal and bacterial strain

Fungal probiotic *G. candidum*-QAUGC01 (KTC280407) and gram-positive bacterial strain, *B. cereus* (KT021872) contained similar concentration of microbes, 1×10^9 CFU/g was obtained from the Department of Microbiology. *G. candidum* and *B. cereus* were isolated from fermented milk product Dahi (yogurt) and gastrointestinal tract of an adult healthy rohu, *L. rohita* respectively. The viability of fungal and bacterial strain before and after feed preparation as well as during feed storage was investigated using plate count. Bacteria, *B. cereus* was grown on Tryptic soya agar (TSA) at 37°C while yeast, *G. candidum* was grown on Oxytetracycline glucose agar (OGA) for 24–48 hr and for estimating the colony forming unit, Mandal and Ghosh (2013) standard method was used.

2.2 | Diet Preparation

For early rearing of *L. rohita*, 40% crude protein basal microdiet was prepared by following Jha et al. (2015) method with formulation mentioned in Table 1. The prepared dried feed pellets were grounded with hand held pestle into fine powder. The powder was divided into four groups and each group was supplemented with their respective probiotic, for example, control group (C) without any supplement, T1 and T2 diets with *G. candidum* and *B. cereus* respectively at similar rate, that is, 1×10^9 CFU/g feed, while T3 group diet was supplemented with blend of both strains, that is, *G. candidum* (1×10^9 CFU/g) and *B. cereus* (1×10^9 CFU/g). To add probiotic in experimental diets, respective probiotic was dissolved in phosphate buffer and sprayed on powdered feed. All diets were dried in freeze drier for 48 hr, and stored in zip lock bags at low temperature for further use. Before providing feed to respective groups, it was initially passed through a sieve with fine mesh (50–100 μm), while feed particle size was adjusted fortnightly according to size of fry (Table 2). All diets were prepared regularly after 2 weeks and survivability of microbes in stored diets was checked by adopting commonly used method (Nikoskelainen, Ouwehand, Bylund, Salminen, & Lilius, 2003).

2.3 | Experimental design and Fish

L. rohita broodstock was maintained and induced bred using commercially available induced spawning agent, ovaprim (SGnRHa + domperidont, Syndel, USA) at the rate of 0.5 ml/kg to female and 0.2 ml/kg to male at Fisheries and Aquaculture research station, Islamabad. Fertilized eggs and hatchlings were kept in hatching tanks

TABLE 1 Formulation and proximate composition of the diet

	Inclusion level (g /100 g)
Ingredient	
Rice bran	41
Mustard oil cake	39
Fish protein hydrolysate	8
Wheat flour	7
Fish oil	2
Vitamin and mineral mix	2
CMC[a]	1
Total	100
Proximate composition (%)	
Crude protein	39.76
Crude lipid	9.63
Ash	5.42

[a]Carboxymethyl cellulose, used as binder.

TABLE 2 Mesh size, feeding rate and frequency used for early rearing of *Labeo rohita*

Size of the mesh (μm)	Feeding rate (%)	Feeding frequency
50–100	10	Continuous feeding for a week
210	8	6 times a day (a week)
250	6	5 times a day (14 days)
420	6	3–4 times a day (14 days)
600	6	3–4 times a day (34 days)

up to 4 days or until the absorption of the yolk sac. To initiate experiment, approximately, 200 swim up fry of *L. rohita* were weighed and stocked per tank in triplicate for each treatment. The experiment was performed with completely randomized design under semi static conditions in an outdoor facility. Initially, each experimental group was provided their respective diet continuously with the aid of automatic feeder and fortnightly, feed particle size, feeding rate and frequency was adjusted and end with four times a day (at 8:00, 12.00, 14:00 and 16:00 hr) at the rate of 6% body weight (Table 2). The precise determination of feed consumption was not possible as uneaten powdered feed was difficult to collect, dry and weigh. After 11 weeks, the fry from control and each probiotic fed group were weighed and counted.

During rearing, dissolved oxygen (DO, mg/L), temperature (°C) was noted twice a day (09:00 AM and 04:00 PM) using DO meter (Oxi 3205 SET 1, Germany), while pH and total ammonia were checked weekly using a pH meter (pH 3110 SET 2, Germany) and ammonia test kit for freshwater (HI3824,ROMANIA) respectively. Initially, water temperature of all tanks was 24.5 ± 0.5°C. It was increased gradually and at the end of experiment reached to 26.0 ± 0.3°C. Furthermore, DO level showed a small fluctuation due to continuous aeration in each tank However, total ammonia remained ≤0.25 mg/L while pH did not show any significant difference. The experiment was conducted in similar environmental conditions, at same stocking density and all tanks were in the same vicinity, therefore, water quality parameters of control and treatment groups did not show any noticeable differences.

2.4 | Growth and survival

The approximate number of swim up fry and biomass of each tank was noted at the time of stocking. At the end of the experiment, the fry were starved for 24 hr, collected separately from each tank, weighed and counted in reckoning the average weight of individual fry. The mean ± *SD* increase in body weight, per cent weight gain (WG %), specific growth rate (SGR), biomass and survival (%) were assessed using the standard formulas.

2.5 | Isolation of intestinal bacterial flora

To isolate gut bacterial flora, five fry from each tank (15 fry/treatment) were aseptically dissected using standard protocol. The intestine of five fry from each tank were pooled and homogenized with hand held homogenizer and resultant suspension was diluted in sterile PBS up to 10 times (10^{-10}) and used as inoculums. Standard plate count method (Nikoskelainen et al., 2003) was used to culture the aerobic and anaerobic gut microbes. The bacterial colonies formed were counted and reported as log CFU/g.

2.6 | Muscle composition

For estimation of proximate composition of muscle in the control and probiotics fed groups, 12 advanced fry from each tank (n = 36/treatment) were taken. To get enough sample, carcass of four fry were pooled and analyzed by adopting standard procedure (AOAC, 2000), while total crude protein and fat contents were analyzed using Soxthlet and Micro Kjeldahl apparatus respectively.

2.7 | Enzyme analysis

At the end of the experiment, 18 fry per tank were immediately anesthetized with MS-222 (100 mg/L), dissected by placing on ice bag and gastrointestinal tract of each fry was removed. The guts of six fry from same tank were pooled (three samples per tank or nine samples per treatment), initially kept in liquid nitrogen and subsequently stored at lower temperature (−20˚C) for determination of intestinal enzymes, protease, amylase and cellulase. For quantitative assay, 0.5 g gut was homogenized in 5 ml chilled buffered saline with hand held electrical homogenizer (Model, VWR POWER 200). The resultant homogenate was centrifuged at 4,500 rpm for 15 min at 4°C and clear supernatant (enzyme solution) was collected in a test tube and stored at 4°C until analysis.

2.8 | Cellulase activity

The activity of microbial origin cellulose degrading enzyme in the intestine of *L. rohita* was determined by adopting the method described by Denison and Koehn (1977). The assay based on the use of carboxymethyl cellulose (CMC) as a substrate and estimation of released reducing sugar at 540 nm. One unit cellulase activity was referred as the amount of the enzyme per mL filtrate that releases one mg reducing sugar as glucose per min.

2.9 | Amylase activity

To determine amylase activity, 3,5-dinitrosalicylic acid (DNS) method reported previously (Ibrar et al., 2017; Ullah et al., 2018) was adopted. The method generally based on estimation of glucose (reducing sugar) at 560 nm. One amylase unit was defined as the amount of enzyme in 1 mL filtrate that released one microgram reducing sugar as glucose min^{-1}.

2.10 | Protease activity

Standard method previously reported by Ullah et al. (2018) and Ibrar et al. (2017) was used for determination of protease activity. In the assay, casein is used as substrate and one unit protease activity is defined as the amount of tyrosine (micromoles) released from casein min^{-1}.

2.11 | Challenge test

From remaining fry, randomly 45 fry of each group were equally distributed in 12 glass aquaria (15 fry per aquarium). Each aquarium contained 30 L water, well-equipped with aeration system and a heater for maintaining temperature and DO level. The temperature of the heaters was set at 26.5°C and DO level was maintained around 5.5 mg/L. After 2 days of acclimatization, three aquaria of each group were exposed to freshly culture pathogenic bacteria, *Aeromonas hydrophila*, isolated from an infected *L. rohita* at National Veterinary Laboratory Islamabad (Ali et al., 2016). Bacterial suspension in PBS was administrated to the aquarium water at a concentration of 1.4 × 10 CFU/mL(Das et al., 2014). However, one aquarium considered as control was not administrated any pathogen. Fry of all groups were fed ad libitum their respective diets thrice a day and kept for 20 days. During that period, closely observed disease symptoms and mortality in each group. To confirm that the fish death was due to introduced pathogenic strain, autopsy of freshly dead fry was done and *A. hydrophila*, was re-isolated from their infected kidney and skin.

2.12 | Statistical analysis

Results of all selected parameters were presented as mean ± *SD*. SPSS version 20 was used for statistical analysis. Homogeneity of variance was determined by Levene's test. Comparison among different groups on the basis of growth performance, survival, muscle composition, enzymatic activity and bacterial counts were made by one–way ANOVA followed by Tukey's test at the 5% level of significance.

3 | RESULTS

3.1 | Growth and survival

Probiotic supplementation showed positive effects on *L. rohita*, ANOVA revealed significant differences in final weight ($F_{3,8}$ = 357.67, $p < 0.001$), final biomass ($F_{3,8}$ = 37.844, $p < 0.001$) and survival % ($F_{3,8}$ = 17.68, $p < 0.001$) of control and probiotic fed groups. However, higher final weight and survival (80%) were recorded for T1 group fed *G. candidum* (T1) alone followed by T3 and T2 respectively (Table 3).

3.2 | Proximate analysis

Results showed significant positive effects of probiotic supplements on muscle composition of fish. Proximate composition of muscle of swim up fry reared on dietary *G. candidum* (T1) alone showed significantly (p = 0.001) higher crude protein, crude fat and carbohydrate contents followed by combination containing *G. candidum* and *Bacillus* (T3) and *B. cereus* alone (T2) respectively (Table 3). However, the control group showed higher moisture (%) content while *G. candidum* (T1) fed fry showed lowest value.

3.3 | Enzyme analysis

Probiotic supplementation during early rearing showed significant effect on the specific activity of digestive enzymes of *L. rohita* as well as non-fish microbial produced enzyme cellulase (Table 4). Highest cellulase and protease activities were observed in fry reared on combination of *G. candidum* and *B. cereus* (T3) followed by T1 and T2 group respectively. While higher amylase activity was found in T1 group (reared on *G. candidum* alone) followed by T3 and T2 groups.

3.4 | Gastrointestinal microbial load

Probiotic supplementation increased the gut microbial load, that is, aerobic and anaerobic bacterial counts (log CFU/g). T1 group of fry reared on dietary *G. candidum* showed highest aerobic bacterial count ($F_{3,8}$ = 51.88, $p < 0.001$) followed by T2 group. Moreover, the anaerobic bacterial count was statistically comparable and significantly higher ($F_{3,8}$ = 18.69, $p < 0.001$) in all probiotic fed groups as compared to control (Figure 1).

3.5 | Challenge test

Probiotic fed groups showed significantly higher survival (%) as compared to control group (one-way ANOVA, $F_{3,8}$ = 43.58, $p < 0.001$)

TABLE 3 Comparative effect of *Geotrichum candidum* and *Bacillus cereus* supplemented microdiet on growth performance (mean ± *SD*, *n* = 3) and muscle composition (*n* = 9) of *Labeo rohita* fry

	Control	T1	T2	T3
Parameter				
Initial weight (mg)	0.33 ± 0.02^{a}	0.36 ± 0.02^{a}	0.36 ± 0.01^{a}	0.34 ± 0.02^{a}
Final weight (g)	1.14 ± 0.09^{d}	2.74 ± 0.11^{a}	1.57 ± 0.18^{c}	2 ± 0.37^{b}
Initial biomass (mg)	67.8 ± 4^{a}	72.5 ± 1.47^{a}	73.4 ± 2.23^{a}	68.5 ± 4.1^{a}
SGR (% body weight/day)	10.6 ± 0.06^{c}	11.7 ± 0.02^{a}	10.9 ± 0.11^{c}	11.4 ± 1.66^{b}
Final biomass (g)	146 ± 12.9^{d}	440 ± 39.5^{a}	240 ± 22.7^{c}	317 ± 64.2^{b}
Survival (%)	64.0 ± 2.2^{d}	80.0 ± 3.7^{a}	76.0 ± 3.1^{c}	78.0 ± 2.7^{b}
Muscle composition				
Moisture (%)	74.3 ± 0.05^{a}	71.3 ± 0.07^{d}	73.4 ± 0.07^{ab}	71.6 ± 0.06^{cd}
Crude protein (%)	50.4 ± 0.06^{d}	56.3 ± 0.08^{ab}	52.3 ± 0.02^{c}	55.4 ± 0.09^{b}
Fat (%)	18.6 ± 0.02^{d}	22.5 ± 0.03^{a}	19 ± 0.09^{cd}	20.3 ± 0.07^{b}
Ash (%)	12.6 ± 0.02^{a}	10.7 ± 0.09^{bc}	10.4 ± 0.06^{c}	11.5 ± 0.08^{b}
Carbohydrate (%)	3.35 ± 0.04^{d}	5.76 ± 0.04^{a}	4.42 ± 0.1^{c}	5.23 ± 0.01^{ab}

Note. Mean values with different letters in same row indicate significant ($p < 0.05$) differences in groups

TABLE 4 Intestinal enzymes specific activities (μ/mg) of *Labeo rohita* fry after feeding *Geotrichum candidum* and *Bacillus cereus* supplemented microdiet for 11 week (mean ± SE, *n* = 9)

Enzyme	Control	T1	T2	T3
Cellulase	0.38 ± 0^{d}	0.51 ± 0.01^{b}	0.45 ± 0.03^{c}	0.65 ± 0.02^{a}
Protease	0.08 ± 0.03^{d}	0.48 ± 0.06^{ab}	0.37 ± 0.02^{c}	0.58 ± 0.04^{a}
Amylase	0.46 ± 0.03^{c}	0.72 ± 0.05^{a}	0.51 ± 0.02^{bc}	0.66 ± 0.02^{ab}

Note. Mean values with different letters in same row indicate significant ($p < 0.05$) differences in groups

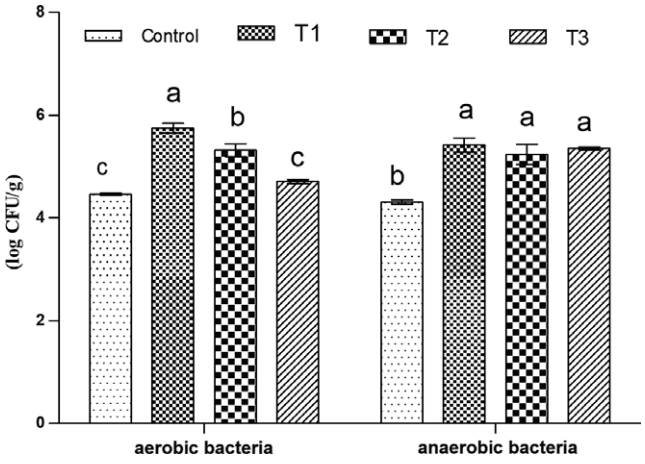

FIGURE 1 Viable log count (CFU/g) of aerobic and anaerobic bacteria isolated form GI tract of *Labeo rohita*

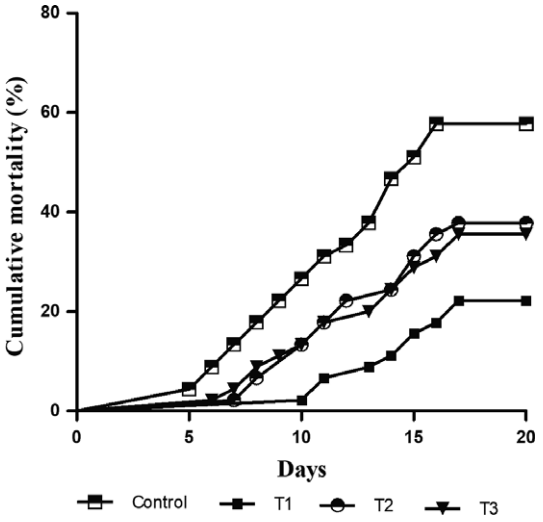

FIGURE 2 Cumulative mortality (%) of *Labeo rohita* after challenged with *Aeromonas hydrophila*

after challenged with carp pathogen *A. hydrophila*. Lowest mortality was observed in T1 followed by T3 and T2 groups (Figure 2).

4 | DISCUSSION

Feed and feeding practices are among the most important factors in the early rearing of fish which needs to be explored to attain higher survival and growth at earlier stages of rearing. Here, we reared swim up fry of *L. rohita* for 11 week on 40% CP *B. cereus* and *G.candidum*-QAUGC-01 enriched microdiets and observed positive effect on the growth, survival, muscle composition and resistance to disease. Like our results, few other investigators also observed beneficial effect of probiotics on early rearing of different fish species (Gilthead sea bream, Suzer et al., 2008; Giant freshwater prawn, Gupta & Dhawan, 2012; common carp, Gupta, Gupta, & Dhawan, 2014) and suggested the possible way to overcome constrain of high mortality during early rearing.

Type and species of probiotic, their source, dose and mode of application determine the efficiency of applied probiotic (Ridha & Azad, 2016). In this study, both probiotics were used in similar concentration, that is, 1×10^9 CFU/g feed but G. *candidum* isolated from dairy fermented product showed the most significant effect on survival and growth of swim up fry as compared to *B. cereus* isolated from the gastrointestinal tract of an adult healthy *L. rohita*. Scanty of literature is available on the comparative effect of bacteria and yeast on early rearing of fish. Nevertheless, the higher efficiency of *G. candidum* could be explained by the better adherence ability to the GI tract of host (Keily & Olson, 2000; Syal and & Vohra, 2014), their components like chitin and β-glucans (Sietsma & Wouter, 1971) which can stimulate the antioxidant and immune system of host (see review Ibrahem, 2015), larger cell volume and ability to produce diverse enzymes, that is, α amylases, cellulases, β-glucanases, xylanases, proteases and lipases (Ghosh, Sen, & Ray, 2002) as compared to *B. cereus*.

Previously, Ibrar et al. (2017) also observed beneficial effect of similar strain of *G. candidum* as water additive on early rearing of rohu. However, here the observed weight gain is several folds higher than reported in earlier study. It seems that way of administration of probiotic and feeding strategy affect the results of rearing (De La, 2001). We changed the feeding rate, frequency as well feed particle size of feed fortnightly (Table 2) and observed most significant effect on growth and survival of swim up fry of *L. rohita* even in control group as compared to other studies (Ibrar et al., 2017; Jha et al., 2015), where *L. rohita* spawn were reared on fixed feeding frequency, ration and particles size.

Successful colonization of probiotic can modulate 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. Here, the better growth and survival of *L. rohita* spawn reared on probiotics supplement, especially *G. candidum* may be attributed to elevated levels of intestinal microbial load (Figure 1) and enhanced secretion of proteases, amylases and cellulases (Table 4).Like our results, the positive impact of *G. candidum* on intestinal microbial load (Ghori et al., 2018) and intestinal enzymatic activities (Ibrar et al., 2017) of juvenile *L. rohita* has already been reported. Furthermore, in vitro studies showing strong enzymatic potential of *G. candidum*, that is, higher level of α amylases, cellulases, β-glucanases, xylanases and moderate level of proteases and lipases (Piegza, Witkowska, & Stempniewicz, 2014) in contrast to *B. cereus* that produce moderate level of proteases and poor level of amylases (Ghosh et al., 2002) also support our results. However, here, the inconsistent result of the blend of both probiotics (*G. candidum* and *B. cereus* @ 1×10^9 CFU/g each), that is, slightly higher enzymatic activities, but lower growth of fry as compared to *G. candidum* alone could be explained by the level of intestinal colonization that may be in excess and possibly negated the synergistic effect. Many other investigators also observed that higher level of probiotic did not always increase growth performance of fish (Ghosh, Sinha, & Sahu, 2008; Merrifield, Dimitroglou, Bradley, Baker, & Davies, 2010).

The change in body composition like protein and fat are linked to their synthesis, deposition in muscles and in turn increase in growth rate (Abdel-Tawwab, Khattab, Ahmad, & Shalaby, 2006). In this study, at the end of the feeding trial, the muscle composition of fry reared on probiotic supplemented feed showed a considerable increase in protein and fat content as compared to fry provided basal diet. These contents were more significantly higher in those fry, which were fed diet supplemented with G. *candidum* (Table 3). The result of the study is in agreement with Ibrar et al. (2017) findings. It seems that *G. candidum* due to their higher enzymatic potential, improves the digestibility and absorption of nutrients more efficiently (Piegza et al., 2014), thus, increasing the status of fat and protein contents in the muscle.

Besides promoting growth, probiotics have potential to protect the organism from infectious diseases. In the present study, *L. rohita* larvae reared on probiotics supplemented feed for 11 week and exposed to carp pathogenic bacteria A. *hydrophila* showed significantly reduced mortality as compared to control group fed diet devoid of probiotic. The results further revealed optimum disease resistance with *G. candidum* as compared to *B. cereus* (Figure 2). Potential probiotics are usually able to colonize the gut more successfully and prevent the attachment of pathogens (see review Ibrahem, 2015). In our study, significantly higher survival (Figure 2) may be due to the decrease in the permeability of host epithelium for macromolecules and toxins or activation of innate immunity (Puddu, Sanguineti, Montecucco, & Viviani, 2014). It seems that *G. candidum* out compete the proliferation of opportunistic pathogens and leave no space for their attachment in the GI tract of fry. No comparable study is available to reveal the antagonist activity of *G. candidum* against *A. hydrophila* in fish. However, in vivo antagonist activity against *Staphylococcus aureus* (Ibrar et al., 2017), as well as in vitro antimicrobial activity alone (Naz, Gueguen-Minerbe, Cretenet, & Vernoux, 2013; Samuel, Prince, & Prabakaran, 2017) or in mix culture with *Enterococcus faecium* (Ghori et al., 2018) against many pathogenic bacteria is well established. Moreover, like our results NavinChandran et al. (2014) reported low level antagonistic activity of *B. cereus* against *A. hydrophila*.

Overall results of the present study showed that *G. candidum*-QAUGC01 alone has great potential to improve survival, growth rate, proximate composition of muscle and resistance to pathogen during early rearing of *L. rohita*. These positive effects may be ascribed to their morphological structure, higher enzymatic potential and immune-stimulatory effect. Based on these results, *G. candidum*-QAUGC01 supplemented microdiet could be recommended as an eco-friendly viable way to replace the live feed and improve the survival and growth of *L. rohita* during early rearing.

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ETHICAL APPROVAL

Experiment on fish was conducted by following the Ethics approved by the society for prevention of cruelty to animals (SCPA), Pakistan. Moreover, the study was approved by the ethical committee of Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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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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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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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 (× $10^3 \mu L^{-1}$), RBCs (× 10^6 μ L^{-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*.

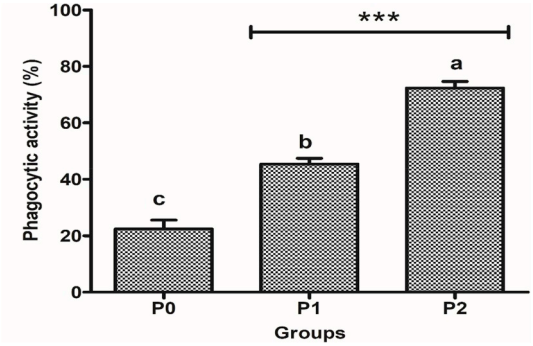

Fig. 1. Phagocytic activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 3. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different 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*.

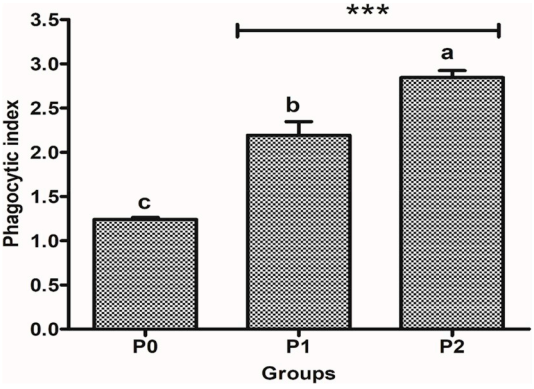

Fig. 2. Phagocytic index of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 3. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different 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*.

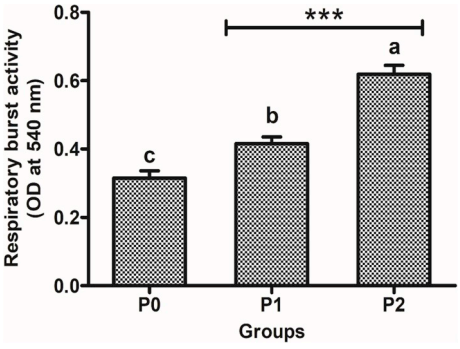

Fig. 3. Respiratory burst activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by 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*.

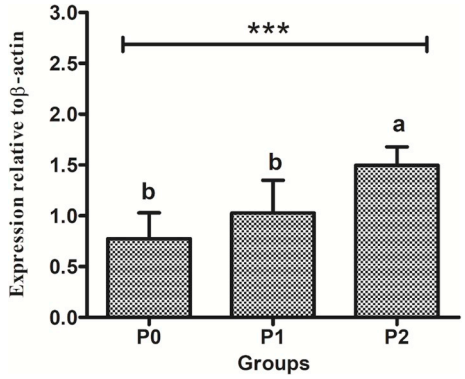

Fig. 4. HSP 70 gene expression in the muscle of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed 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.

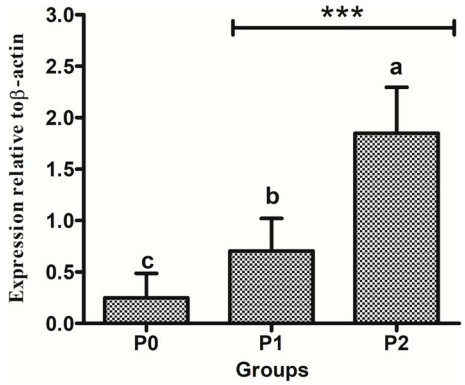

Fig. 5. HSP 70 gene expression in the intestine of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages 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*.

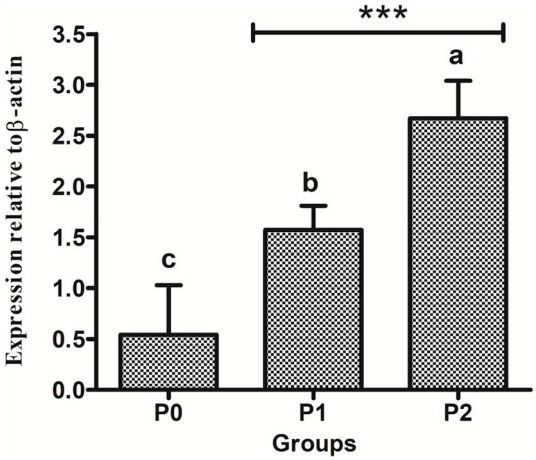

Fig. 6. HSP 70 gene expression in the liver tissue of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages 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*.

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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.

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