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RESEARCH PAPER

Geotrichum candidum Enhanced the *Enterococcus faecium* Impact in Improving Physiology, and Health of *Labeo rohita* (Hamilton, 1822) by Modulating Gut Microbiome Under Mimic Aquaculture Conditions

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Abstract

The present study is designed to evaluate the impact of potential probiotics *Enterococcus faecium* QAUEF01 in single and its mix-culture with *Geotrichum candidum* QAUGC01 on the *Labeo rohita* (Hamilton, 1822). In the mix-culture, both bacteria and yeast survived comparatively better under mimic gut conditions and showed higher hydrophobicity. Moreover, mix-culture showed comparatively more antipathogenic activity. A feeding trial of 90 days for *L. rohita* fingerlings comprising of three treatments, control group fed on basal diet, second group fed on *E. faecium* supplemented diet and third group was fed on mix-culture probiotics supplemented diet. Mix-culture probiotics fed group showed significantly higher ($P<0.05$) growth as compared to control. Better specific growth rate (SGR) was significantly correlated with the feed conversion ratio (FCR) and feed conversion efficiency (FCE), protease and cellulase activity in probiotic fed fishes. The applied probiotics established well in fish gut and shown no harmful impact on fish physiology. Probiotic application distinctly modulated fish gut microbiome evident by increased level of friendly microbiota and exclusion of potential fish pathogens. The results suggested an effective eco-friendly strategy to boost the fish productivity.

Keywords: Probiotics, fish physiology, etagenomics and gut microbial diversity.

Introduction

Aquaculture is the mainstream industry that provides safe and nutritious protein rich food for mankind across the globe (Mohapatra *et al.*, 2012). High feed costs, low productivity, and disease outbreaks are the drawbacks slowing its growth. Sustainability, economy generation and healthy food are the focal areas of aquaculture. Vaccines, antibiotics and immune-stimulators though customary for infectious control, but their misuse is debilitating the environmental quality and elevating the antibiotic resistance multifold through the food chain (Cabello, 2006; Sapkota *et al.*, 2008; Marshall and Levy, 2011; Aly and Albutti, 2014). *Labeo rohita* is one of the economically important fish in Pakistan owing to its taste, meat quality and consumer demand. Low productivity and feed conversion efficiency are key issues barring *L. rohita* productivity, which might be enhanced by the appropriate administration of probiotics in fish feed. Lack of well-established immune system and digestive systems in the early life cycle of fishes makes them highly vulnerable to pathogens, thus causing heavy mortality losses and economic downsides (Ghosh *et al.*, 2004; Wang *et al.*, 2008). The scientific community should culminate the mismanagement of antibiotics and efforts should be made to adopt preventive measure with the use of probiotics (Brugère *et al.*, 2010). Production of antimicrobial compounds by probiotics make them potent against pathogens thus saving the host (Dahiya *et al.*, 2012). The significant advantages associated with probiotic use are host defense, improved digestibility, stress tolerance, pathogen inhibition, increased reproductive capacity and immune stimulation (Nour and El-Ghiet, 2011; Martínez Cruz *et al.*, 2012; Giri *et al.*, 2013). *Enterococcus faecium* is a putative probiotic especially used in farmed aquatic species (Sun *et al.*, 2010). Bacteriocin production is reported for many *Enterococcus* species and antibacterial potential of *G. candidum* along with its enzymatic activity and safe status prompted to design the present study. The objective was to assess their probiotics potential in single and mixed form followed by their impact on the physiology and the intestinal community composition of *L. rohita* fingerlings. According to our information it was the first time to check the impact of both microorganisms mix-culture in aquaculture.

Materials and Methods

Ninety fingerlings of *L. rohita* with an average weight of 5.90 ± 0.02g were collected from Faisalabad Fish Hatchery (FFH) before transferring them to fisheries and aquaculture research station, Quaid-i-Azam University Islamabad, where they were shifted to glass aquaria of volume (60 x 35 x 35 cm^3) and a stocking density of 1.5 g/L was maintained. Two microbial strains *Enterococcus faecium* QAUEF01 (NCBI accession: KP256006) and *Geotrichum candidum* QAUGC01 (NCBI accession: KT280407) were previously isolated from the local fermented product "Dahi".

Assessment of Probiotics Potential of Selected Microbial Strains in Mimic Gut Conditions

In the present study, *E. faecium* QAUEF01 combined with *G. candidum* QAUGC01 was used. They were screened for probiotics potential by evaluating them for cell surface hydrophobicity, bile tolerance, antibiotic susceptibility and antimicrobial activity.

The ability of the strains to tolerate bile salt was determined by method of Walker and Gilliland (Walker and Gilliland, 1993) with some modifications. In this assay, the 100 µL of bacterial strains at their log phase were inoculated in 10 mL of sterilized tryptic soy broth (TSB; Oxoid, UK) present in test tubes while the 100 µL of yeast strains at their log phase were inoculated in 10 mL of sterilized oxy-tetracycline glucose broth (OGB) present in the test tubes. The bile tolerance of mix-culture was calculated out by adding 50 µL from both yeast and bacteria at their log phases. Test tube containing bile salts (1g/l0mL), inoculum, lysozyme (0.01g/10mL) solution and pH 3 served as experimental while test tubes containing only inoculum with pH 7 were considered as control. Cultures were incubated for 24 hours at 150 revolutions per minute (rpm) for 37° C and 30° C respectively. After 0 hours, 2 hours, 6 hours and 24 hours interval, samples were successively taken out and the comparative survival of the strains was measured by using spectrophotometer at 600nm. Experiment was conducted in triplicate.

$$\% \text{ Survival} = [\text{OD of bile media} / \text{OD of control media}] \times 100$$

The tendency of adhesion of microorganism to hydrocarbons is an index for their cell surface hydrophobicity. The method used by (Rosenberg *et al.*, 1980) was used with some modifications. Briefly, bacterial culture and combination of *G. candidum* QAUGC01 and *E. faecium* QAUEF01 were grown overnight in TSB at 37° C and 30°C. 2 mL culture was transferred to microtubes and was subjected to centrifugation (Eppendorf centrifuge 5417R) at 6000rpm for 5 minutes. Supernatant was discarded and pellets were washed twice with phosphate buffer (pH 7.4), and were then added to 3mL autoclaved distilled water. Subsequently, optical density (O.D) was taken at 600nm. 0.6mL of xylene was added to these samples vortexed vigorously and was incubated for 30minutes. Formation of two phases occurred. Water phase was carefully pipetted out and growth was again measured at 600nm. The decrease in the O.D of aqueous phase was taken as measurement of hydrophobicity which is calculated by the following formula.

$$\text{Hydrophobicity Percentage (\%)} = [(A0 - A1) / A0] \times 100$$

Where,

A0 =Optical density before mixing the xylene

A1 = Optical density of the aqueous layer

The antibiotic susceptibility was checked by using antibiotic disc diffusion method on TSA plates. 24 hour cultures grown in TSB were adjusted to 0.5 McFarland's standards. Sterile cotton swab dipped in inoculum was spread evenly on the TSA plates. The antibiotic discs were placed on agar surface and incubated at 37°C for 24 hours. Sensitivity pattern was assessed using vancomycin, cefpirome, ampicillin, ceftriaxone, ciprofloxacin, chloramphenicol, ceftazidime, piperacillin, and moxifloxacin.

The inhibitory activity of *E. faecium* QAUEF01 and consortium of *E. faecium* QAUEF01 with G. *candidum* QAUGC01 was checked by well diffusion method against test pathogens (*Staphylococcus aureus* ATCC 2593, *Listeria monocytogenes* ATCC 1393, *Salmonella enterica* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922). The supernatant was diluted according to 0.5 McFarland's standard (Khunajakr, 2008) followed by lawn formation with the help of sterile swabs on TSA plates. 6mm diameter well was cut into agar plates and 50µL of supernatant fluid (probably having antibacterial activity) was added to each well. Zone of inhibition was checked after incubation of 24 hours at 37°C.

Preparation of Fish Feed

The probiotics strains of *E. faecium* QAUEF01 with G. *candidum* QAUGC01 were inoculated in TSB at 37°C for 24 hours and at 30°C for 48 hours respectively. After centrifugation at 14000 rpm for 10 minutes at 4°C, the bacterial and yeast pellet was washed twice with 0.9% normal saline and was added to feed to a CFU of 10^9 cells/g of feed. These cell suspensions were then sprayed on 35% basal diet (Basal diet composition: soybean meal, 212 g/Kg, sun flower meal 212 g/Kg, white fish meal, 105 g/Kg,

gluten 30%, canola meal 212 g/Kg, rice polish 52 g/Kg, Dicalcium phosphate 10 g/Kg, carboxymethyl cellulose 10 g/Kg, vitamin premix 20 g/Kg, vegetable oil 10 g/Kg and wheat bran 52 g/Kg) in biosafety hood, while control diet was sprayed by 0.9% saline and dried at room temperature. Feed preparation was repeated after every two weeks till 90 days and stored at 4°C to maintain cell viable count.

Experimental Design and Feeding Trial

Fish fed with 35% basal diet at 3% body weight twice a day under controlled conditions were acclimatized for two weeks before the commencement of experiment. Water quality was assured by monitoring pH, temperature, dissolved oxygen and NH_3 concentration at daily basis by using by Multi-parameter Hanna (Hanna, HI 9147). The water was changed daily and was maintained 25±1°C, dissolved oxygen from 5.5 to 6.4 mg L^{-1}, pH from 7.5 to 7.9, while total ammonia concentration was less than 0.25 mg L^{-1}. Fecal material of fish contains ammonia, so it was drained out along the undigested food and water to avoid NH_3 toxicity and alkalinity issue, because it causes damage to the fish outer surfaces like gills, eyes, and skin. Samplings for the determination physiological factors were done at 45 days and 90th day of experiment.

Determination of Fish Growth Parameters

Growth performance in terms of percentage weight gain (%WG), specific growth rate (SGR), feed conversion ratio (FCR), feed conversion efficiency (FCE) was measured by following the protocols used by (Firouzbakhsh *et al.*, 2011).

Detection of Digestive Enzyme from Fish Gut

The analysis of protease (Tsuchida *et al.*, 1986), amylase (Bernfeld, 1955) and cellulase (Denison and Koehn, 1977) were performed .

Fish Nutritional Value

Proximate analysis of the dried flesh samples was done in triplicate (AOAC., 2000). Crude fats and crude protein were determined using Soxhlet apparatus and micro Kjeldhl method respectively (Sutharshiny and Sivashanthini, 2011). Total ash content was determined by incinerating the sample for 24 hours at 600°C in muffle furnace.

Study of Hematological Parameters

Blood was collected by using K2E (EDTA) coated micro syringe from caudal vein and was pooled together in 3 replicates of 18.0 mg/10ml of blood K2E (EDTA) coated tubes for each sample as the blood drained from each fish was very small in quantity so we had to pool the sample and analyzed by using hematological analyzer (Sysmex KX-21N^{TM}). The hematological parameters studied were red blood cells(RBCs), haemoglobin (HGB), hematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), MCV (mean corpuscular volume), WBCs (white blood cells), platelets (PLT), lymphocytes (Lym)

Fish Gut Microbiome Analysis

DNA extraction from intestinal samples was done by using Favor Prep Stool DNA isolation mini kit (Favorgen, Taiwan) as per manufacturer instructions/supplier protocol. The samples were qualitatively analyzed by gel electrophoresis followed by quantitative determination by Nano drop 1000 spectrophotometer (Thermo scientific, USA). DNA was preserved at -20°C for advance processing. The intestinal samples fed on *E. faecium* QAUEF01 and mixture of *E. faecium* QAUEEF01 with *G. candidum* QAUGC01, basal diet (control) were subjected to PCR by targeting V4 region to generate 16SrRNA library. PCR was done in triplicate. PCR reaction under the V4 region was amplified by using 515F (GTGCCAGCMGCCGCGGTAA)/806R(GGACTAC HVGGGTWTCTAAT) and ITS primer pairs (Caporaso *et al.*, 2011). PCR programed performed by Hot Star Taq Plus Master Mix Kit (Qiagen, USA) using 20µL reaction mixture, consisted of initial denaturation at 94°C for 3 minutes followed by 30 cycles denaturation at 94°C for 30 seconds, annealing at 53°C for 40 seconds. The final step of extension was performed at 72°Cfor 1 minute.

Amplification and relative intensity of bands was determined by using 2% agarose gel, after that equal proportion of all samples pooled together and purified by using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to formulate Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallow water, TX, USA) by using Illumina MiSeq platform as per manufacturer's protocol. MR DNA pipeline was used as statistical and bioinformatics tool for data analysis. Operational taxonomic units (OTUs) were defined by clustering at different similarity cut off level. Final OTUs were taxonomically classified using BLAST against a curated database derived from RDPII, RDPI and RDPI (DeSantis *et al.*, 2006) and NCBI(*www.ncbi.nlm.nih.gov*,http://rdp.cme.msu.edu).

Statistical Analysis

Comparison among the treatments was carried out by one-way analysis of variance (ANOVA) using XLSTAT followed by Duncan's multiple range test. Alpha diversity was applied for evaluation of species diversity among samples using OTUs, Shannon and Simpson indices. Comparison was made at the 5%

probability levels. The results were presented as means ±SE (Standard error).

Results

Assessment of Probiotics Potential in Mimic Gut Conditions

Growth of *G. candidum* QAUEF01 mix-culture with *E. faecium*, QAUEF01 showed better survival rate as compared to single strain probiotics, *E. faecium* QAUEF01 (Figure1). Mix-culture also shown more hydrophobic as compared to *E. faecium* QAUEF01 (Figure 2).

Antibiotic Susceptibility

Antibiotic susceptibility pattern showed that *E. faecium*QAUEF01 was sensitive to Vancomycin (30), Gentamycin (10), Chloramphenicol (30), Moxifloxacin (5), Piperacillin (100), Imipenem (10) intermediate to Ciprofloxacin (5) and resistant to Cefpirome CPO (30) and Ampicillin (25).

Antimicrobial Activity

Although single strain and mix-culture probiotics were found to possess antimicrobial activity against all tested pathogens but comparatively *E. faecium* QAUEF01 in combination with *G. candidum* QAUGC01 mostly produced larger inhibitory zones (Table 1).

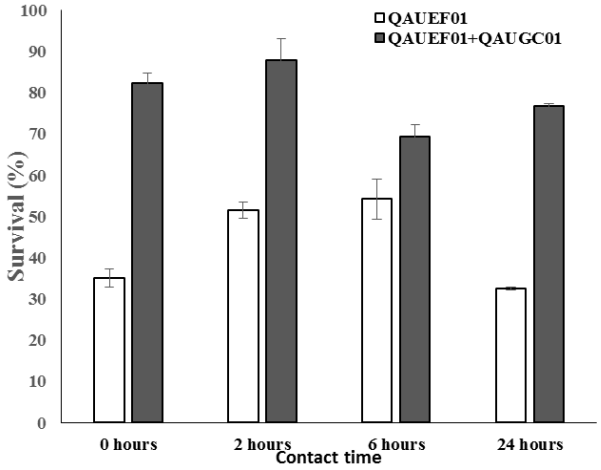

Figure 1. Survival of *Enterococcus faecium*QAUEF01and *Enterococcus faecium* QAUEF01combined with *Geotrichum candidum* QAUGC01 in mimic gut condition (data is represented as mean±SE (n=3).

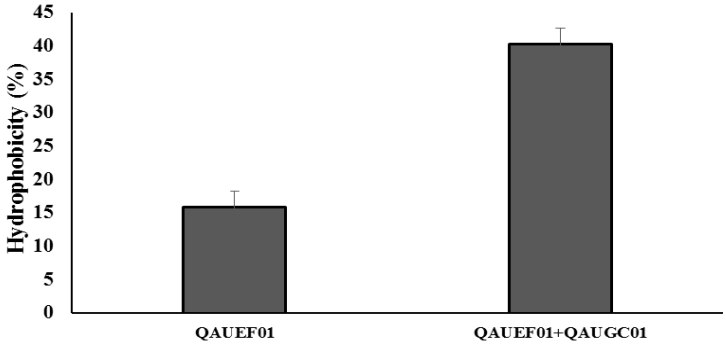

Figure 2. Hydrophobicity percentages of QAUEF01 (*Enterococcus faecium*) and QAUEF01+QAUGC01 (Mix-culture of *Enterococcus faecium and Geotrichum candidum*). Data expressed as mean ±SE (n=3).

Table 1. Antimicrobial assessment against pathogens, data presented as mean± SE

Probiotics	Zone of inhibition (mm) against Pathogens				
	P. aeruginosa	*S. enterica*	*E. coli*	*L. monocytogenes*	*S. aureus*
E. faecium QAUEF01	19± 01	24±0.8	21±1.2	21±1.4	18±1.73
E. faeciumQAUEF01 + G. candidumQAUGC01	24.5±1.25	28±1	21±0.57	23.6±1.8	28.3±0.8

*Each represented value is means of three replicates.

Evaluation of Physiological Parameters

Comparative % WG, FCR, FCE and SGR between control and probiotics fed groups are shown in (Table 2 and 3). Single strain probiotics *E. faecium* QAUEF01 and combination of *E. faecium* QAUEF01 with *G. candidum*, QAUGC01 showed significantly high (P<0.05) % WG, FCE and SGR. Significantly low FCR was observed by probiotics fed groups as compared to control, *E. faecium* QAUEF01 efficiency, improved FCR among all treatments used. Multispecies probiotics fed fishes showed highest % WG and SGR at the 45th day of trial.

Mix-culture probiotics showed significantly high protease activity (45/90 days), cellulase activity (90th day) as compared to control (Table 4). Proximate analysis showed that the fishes fed on probiotics supplemented diet exhibited significantly higher crude protein (P<0.05) as compared to control, maximum crude protein was recorded by mix-culture probiotics *E. faecium* QAUEF01 combined with *G. candidum*, QAUGC01, crude fats were also found to significantly higher as compared to control after 45 days of trial for fishes fed on probiotics, maximum fat content was exhibited by the group fed on *E. faecium* QAUEF01, while 90th day data illustrated significantly higher fat content in control as compared to experimentally treated groups. Ash content was found to be sufficiently high in control as compared to probiotics fed groups at 45th day while non-significant variation existed between control fed and probiotics fed groups at 90th day (Table 5). Hematological assessment indicated that the probiotics fed groups showed significantly high (P<0.05) RBCs, haemoglobin (90 days), hematocrit (HCT), white blood cells (WBCs) as compared to control fed fishes. Significant impact of *E. faecium* QAUEF01 was observed on mean corpuscular hemoglobin (MCHC) (90th day), WBC (45/90days), platelet (90th day), lymphocyte % (90th day), mix-culture probiotics (QAUEF01+QAUGC01) significantly improved WBCs (90th day) (Table 6 and 7).

Table 2. Growth parameters of *Labeo rohita* fed with basal diet (control) vs probiotic feed (45 days)

Parameters	Control (Tc)	*E. faecium QAUEF01*	*E. faecium QAUEF*01 + *G. candidum QAUG*C01
	After 45th day of trial		
% Growth	44.30[b] (±1.22)	78.98[a] (±3.24)	82.09[a] (±1.37)
FCR	3.39[a] (±0.05)	2.30[b] (±0.18)	2.62[b] (±0.19)
FCE	29.45[b] (±0.50)	43.70[a] (±2.45)	38.18[ab] (±1.97)
SGR	1.22[b] (±0.028)	1.93[a] (±0.060)	1.99[a] (±0.025)

*Values are represented as mean ±SE (n=3) followed by ANOVA (P<0.05), different superscripts (a, b, c) depicts significant variation according to Duncan statistical test (a>b>c).

Table 3. Growth parameters of *Labeo rohita* fed with basal diet (control) vs probiotic feed (45 days)

Parameters	Control (Tc)	*E. faecium QAUEF01*	*E. faecium QAUEF*01 + *G. candidum QAUG*C01
	After 90th day of trial		
% Growth	86.8[c] (±1.48)	118.43[a] (±1.81)	115.15[b] (±2.31)
FCR	5.28[ab] (±0.09)	5.02[b] (±0.04)	5.50[a] (±0.14)
FCE	18.91[ab] (±0.33)	19.92[a] (±0.19)	18.18[b] (±0.42)
SGR	0.69[a] (±0.008)	1.025[a] (±0.07)	1.997[a] (±0.05)

*Values are represented as mean ±SE (n=3) followed by ANOVA (P<0.05), different superscripts (a, b, c) depicts significant variation according to Duncan statistical test (a>b>c).

Table 4. Digestive enzymes of *Labeo rohita* fed with basal diet (control) vs probiotics (single and consortium form)

Tested Enzymes	Control		*E. faecium QAUEF01*		*E. faecium*QAUEF01 + *G. candidum*QAUGC01	
	45th day	90th day	45th day	90th day	45th day	90th day
Protease	0.018[b] (±6.48E-05)	0.0162[b] (±4.51E-05)	0.01517[c] (±0.00015)	0.016[b] (±0.0002)	0.026[a] (± 9.61E-05)	0.02624[a] (±3.9E-05)
Cellulase	0.1535[a] (±0.0002)	0.16297[b] (±0.0006)	0.1373[b] (±0.002)	0.145[c] (±0.00053)	0.15346[a] (±0.0001)	0.1822[a] (±0.0003)
Amylase	0.0314[a] (±0.0001)	0.0320[a] (±3.37E-05)	0.03182[a] (±0.0002)	0.0304[b] (±8.49E-05)	0.0253[b] (±0.0003)	0.0282[c] (±3.94E-05)

*Values are represented as mean ±SE (n=3) followed by ANOVA(P<0.05), different superscripts (a, b, c) depicts significant variation according to Duncan statistical test (a>b>c)

Table 5. Biochemical analysis of *Labeo rohita* supplemented with basal diet and probiotics

Parameters	Control (Tc)		*E. faecium QAUEF01*		*E. faecium*QAUEF01 + *G. candidum*QAUGC01	
	45th day	90th day	45th day	90th day	45th day	90th day
Crude proteins	65.5[b] (± 01)	70.5 [c] (±0.50)	73.48 [a] (±0.611)	78.75[b](± 0.01)	69.13 [b] ± (0.9)	85.75 [a](±0.57)
Crude fats	8.5 [b] (± 0.70)	18.3 [a] (±1.1)	13 [a] (±0.83)	14 [b] (±0.36)	10.6 [ab] (±0.43)	13.6 [b](±0.63)
Ash content	14.7 [a] (±0.3)	16 [a] (±1.40)	10.8 [b] (±0.57)	12 [a] (±0.57)	11[b](±0.57)	14.5 [a](±0.63)

*Values are represented as mean ±SE (n=3) followed by ANOVA (P<0.05), different superscripts (a, b, c) depicts significant variation according to Duncan statistical test (a>b>c).

Table 6. Hematological analysis of *L. rohita* fed on control and multispecies probiotic at 45 day

Parameters	Control (Tc)	*E. faecium QAUEF01*	*E. faecium*QAUEF01 + *G. candidum*QAUGC01
RBCs($10^6\mu L^{-1}$)	1.81[c] (±0.02)*	2.53 [a] (±0.02)	2.03 [a] (±0.023)
HGB(gdl^{-1})	6.7 [a] (±1.00)	8.2 [a] (±0.12)	6.5 [a] (±0.12)
HCT (%)	24 [b](±0.8)	27.2 [a] (±0.23)	22.4 [b] (±0.23)
MCH(pg)	36.6 [a] (±1.00)	32.33 [b] (±0.52)	31.3[b] (±0.12)
MCHC(gdL^{-1})	27.9 [a](±0.90)	30.1 [a] (±0.29)	29 [a] (±0.69)
MCV(fL)	131.1 [a] (±0.50)	107.5 [b] (±0.34)	107.7 [b] (±0.57)
WBCs(10^3 uL^{-1})	178.4 [b] (±1.00)	188.7 [a] (±0.97)	159.2 [c] (±0.28)
PLT%	13 [a] (±1.00)	40 [a] (±0.57)	62 [b](±0.57)
Lym%	97.6 [a] (±1.00)	98 [a] (±0.30)	98.9 [a] (±0.05)

*Values are represented as mean ±SE (n=3) followed by ANOVA, different superscripts depicts significant variation according to Duncan test (P<0.05) a>b>c.

Table 7. Hematological analysis of *L. rohita* fed on control and multispecies probiotic at 90 day

Parameters	Control (Tc)	*E. faecium QAUEF01*	*E. faecium*QAUEF01 + *G. candidum*QAUGC01
RBCs ($10^6\mu L^{-1}$)	1.075 [c] (±0.005)	1.99 [a] (±0.052)	1.56 [b] (±0.08)
HGB (gdl^{-1})	4.3 [b](±0.3)	7.9 [a] (±0.057)	5.833 [ab] (±0.44)
HCT (%)	13.9 [b] (±0.50)	20.1 [a] (±0.75)	17.2 [a] (±0.57)
MCH (pg)	43.55 [a] (±0.95)	40.6 [a] (±0.93)	42.8[a] (±0.83)
MCHC (gdL^{-1})	35 [b] (±0.70)	39.4 [a] (±0.78)	34.83 [b] (±0.69)
MCV(fL)	123.75 [a] (±0.35)	101.76 [b](±0.75)	122.35 [a] (±0.60)
WBCs (10^3 uL^{-1})	142.3 [c] (±2.10)	239.66 [a] (±2.02)	177.03 [b] (±2.48)
PLT%	87.5 [b] (±0.50)	212.3 [a] (±0.68)	55.93 [c](±1.09)
Lym%	77.25 [b] (±0.55)	97.4 [a] (±0.95)	80.66 [b](±0.01)

Fish Gut Microbial Diversity

The control group Tc showed 6 phyla, Proteobacteria (97.74%), Actinobacteria (1.28%), Firmicutes (0.46%), Planctomycetes (0.39%), Bacteroidetes (0.07%) and Chlamydia (0.001%). Dominating species were, *Achromobacter xylosoxidans* (0.32%), *Pseudomonas psychrophila* (87.2%), *Pseudomonas fragi* (3.85%), *Rhodobacter sp.* (0.51%) (Figure 3 & 4) Control group showed five fungal phyla where Ascomycota (98.50%), Basidiomycota (1.48%), Neocallimastigomycota (0.002%), Glomeromycota (0.001%) and Cryptomycota (0.0005%). Dominating fungal species were *Debaryomyces hansenii* (88.61%), *Trichoderma longibrachiatum* (4.11%), *Knufia epidermidis* (2.09%).

Metagenomic analysis revealed that *E. faecium* QAUEF01 fed groups had seven phyla where Firmicutes were 99.04% Proteobacteria (0.87%), Actinobacteria (0.059%), Bacteroidetes (0.0080%), Cyanobacteria (0.00484%), Planctomycetes (0.003227%), Spirochaetes (0.003227%). Dominating species are *Paenibacillus lactis* (85.83838%), *Bacillus szutsauensis* (12.74%), *Bacillus licheniformis* (0.1717%), *Pseudomonas psychrophila* (0.1633%), *Achromobacter xylosoxidans* (0.190%). Metagenomic based diversity analysis of fungal community showed that three fungal phyla were present Basidiomycota (97.59%), Ascomycota (2.39%), Neocallimastigomycota (0.0019%). Dominating fungal species found were *Cryptococcus magnus* (86.65%), *Galactomyces geotrichum* (0.34%), *Debaryomyces hansenii* (1.623%), *Galactomyces candidum* (0.20%).

Enterococcus faecium QAUEF01 combined with

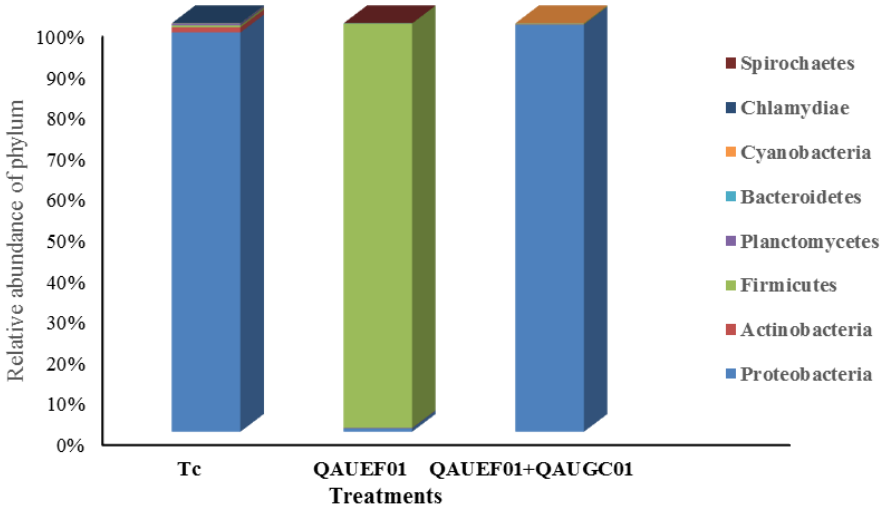

Figure 3. Taxonomic composition of bacterial communities at phylum level.

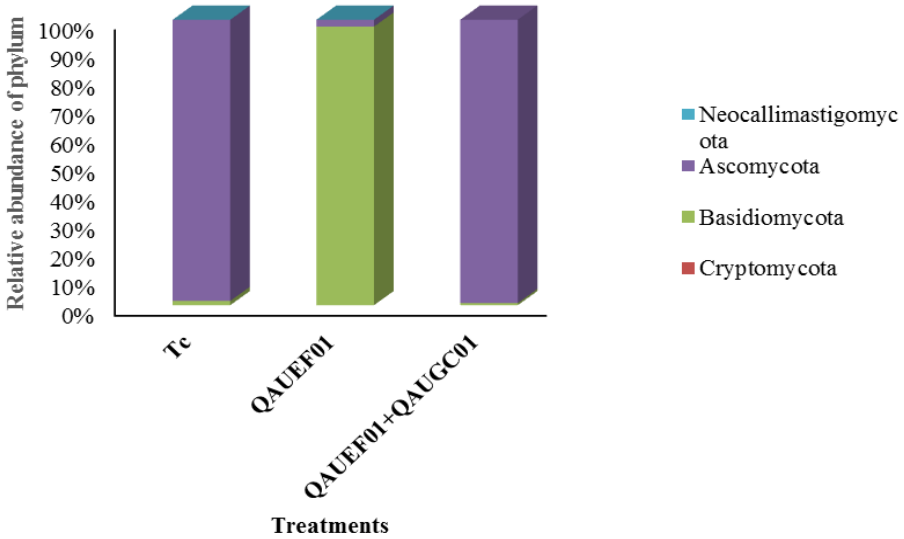

Figure 4. Taxonomic composition of fungal communities at phylum level.

G. candidum QAUGC01 fed group was represented by 6 phyla Proteobacteria (99.69%), Firmicutes (0.24%), Actinobacteria (0.047%), Planctomycetes (0.007581%), Bacteroidetes (0.007581%) and Cyanobacteria (0.00151%). Dominating bacterial species were *Achromobacter xylosoxidans* (50.8%), *Klebsiella oxytoca* (25.9%), *Serratia quinivorans* (13.7%) (Figure 3). Fungal phyla Ascomycota (99.25%), Basidiomycota (0.735%). Dominating fungal species were *Galactomyces candidum* (35.83%), *Galactomyces Geotrichum* (31.12%), *Galactomyces sp.* (22.40%), *Debaryomyces hansenii* (2.75%) (Figure 4) the gut microbial diversity at genus level is graphically represented (Figure 5 and 6). Diversity measure of metagenomic analysis is represented in Table 8.

Discussion

Gut microbiome has a pivotal impact on fish physiology that leads to better growth and resistance against stress factors. However, imbalanced gut microbiome results in poor feed response and higher mortality. Probiotics application can be an effective strategy instead of antibiotics and chemical feed additives for controlling infections and physiological disorder provided their higher viable number persists over a considerable duration in the host gut (López *et al.*, 2003). Proficient probiotic action is the interplay of their interactions with resident microbial community and ability to withstand digestive tract conditions. The better survival and adaptability of *E. faecium* QAUEF01 in the presence of *G. candidum* QAUGC01 under drastic gut conditions was might be

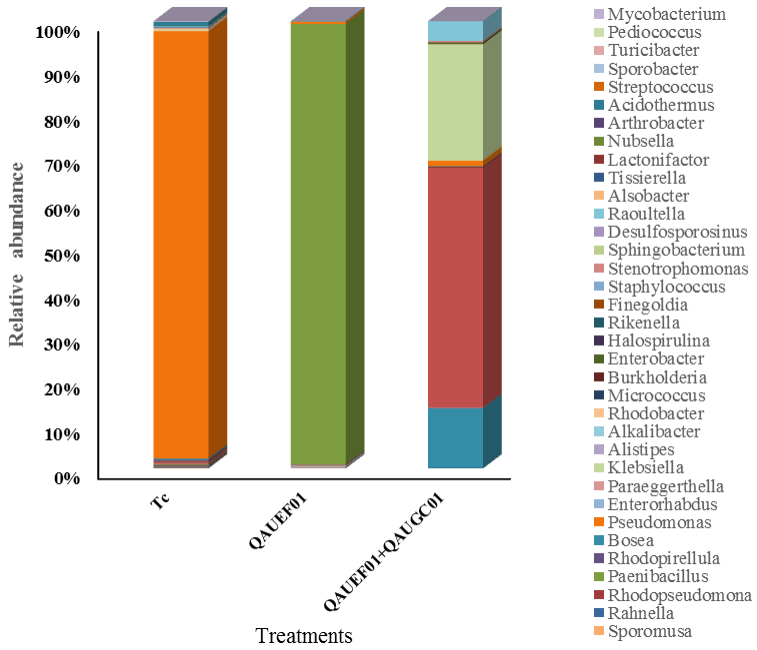

Figure 5. Bacterial composition at Genus level.

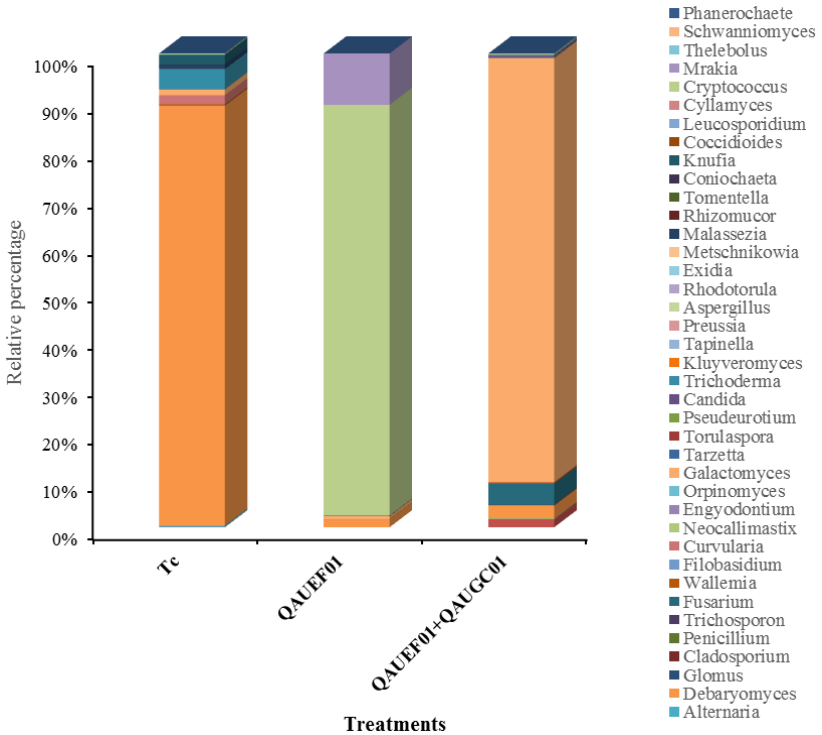

Figure 6. Fungal composition at Genus level.

Table 8. Diversity measure of metagenomic analysis

Probiotics	No. of reads		No. of OTUs		Shannon index		Simpson index		Observed species	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
E. faecium QAUEF01	71030	52595	133	70	0.13	0.49	0.03	0.23	133	70
*E.faecium QAUEF01+ G.candidum*QAUGC01	66219	15672	167	97	1.36	1.48	0.65	0.72	167	97
Control	67926	381936	158	169	0.70	0.57	0.23	0.78	158	169

*Number of analyzed sequences, Diversity richness (OTUs) and Diversity index (Shannon and Simpson) for 16S rRNA sequencing libraries of treated and control samples.

a complementary metabolic impact of both microorganisms. Better survival in acidity and bile salts involves multiple mechanisms such as proton pumps, chaperones participating in repair of damaged proteins, amino acid decarboxylation and transport, stress induced changes in cell wall, fatty acid and isoprenoid biosynthesis (Bron *et al.*, 2004). These properties are already well documented for *G. candidum* (Boutroua and Gueguen, 2005; Khoramnia *et al.*, 2013). But this is first report where *G. candidum* improve the tolerance of *E. faecium* in mimic gut conditions.

Microbial adherence to intestinal epithelium is a prerequisite for colonization in gastrointestinal tract (Collado *et al.*, 2007). More attachment of *E. faecium* to xylene surface was observed in presence of *G. candidum*. This could be either due to adherence of bacteria to yeast surface or because of some unexplainable interaction between both microorganisms. The higher hydrophobicity is an *in vitro* marker indicates a better attachment to intestinal epithelium (Kos *et al.*, 2003). The *E. faecium* QAUEF01 was found sensitive to vancomycin, chloramphenicol, gentamycin, moxifloxacin and imipenem and was resistant against cefpirome and ampicillin. Prevalence of multiple drug resistance has been documented for *Enterococcus* strains (Landman and Quale, 1997; Leclercq, 1997). Vancomycin is used to treat multiple drug resistant infections and our strain used in this experiment was found to safe as it was sensitive to vancomycin (Arthur and Courvalin, 1993). It was also demonstrated that mix-culture probiotic has higher antagonistic activity against tested pathogens. The production of antimicrobial compounds such as organic acids, hydrogen peroxide and bacteriocins are well known in *E. faecium* (González *et al.*, 2007). Moreover, antimicrobial activity of *G. candidum* is also well documented (Naz *et al.*, 2013).

The significant higher weight gain was observed in fishes fed on *E. faecium* QUEF01 and mix-culture probiotics. Each probiotic utilized nutrients differently, thus having a variable impact on growth. Therefore, the underlying mechanism for a rise in the growth rate was might be due to release in vitamins, digestive enzymes leading to better digestibility and growth. Moreover, the type of the feed supplied also stimulate digestive enzymes production and have immediate effect on fish health and growth (Shan *et al.*, 2008). Continual administration of probiotics over an extended period of time helps them to colonize in intestinal mucosa to express their multiple health effects on host.

Crude protein content and protease activity of groups fed on mix-culture probiotics was significantly enhanced. This could be associated with better establishment of combination of yeast and bacteria in fish intestinal epithelium. *Enterococcus* and *Saccharomyces* are commonly used as feed probiotics in animal nutrition (Lauková *et al.*, 2008). Survival of microorganisms in fish gut is dependent on numerous factors such as acid and bile resistance, composition of the feed given and competition of intestinal microbial community (Succi *et al.*, 2005). The provision with constant probiotics to sustain their number by exceeding multiplication then their expulsion. Our results of higher FCE and lower FCR in fishes fed on probiotics *E. faecium*, QAUEF01 are in accordance with early reports (Abdel-Tawwab *et al.*, 2008). Low FCR and high FCE percentage in tilapia fed on a yeast supplemented diet was reported (Abdel-Tawwab *et al.*, 2010). Growth promoting effects are also dependent on water quality, fish body temperature, enzyme level and genetic resistance (Balcázar *et al.*, 2006). The results of the current study and earlier observations suggest that changes by probiotic supplementation on the chemical composition of fish can be linked to the variations in the deposition rate and formation of muscles (Rumsey *et al.*, 1990), improved feed intake, enhanced digestibility of the nutrients and efficient absorption (Abdel-Tawwab *et al.*, 2008). Probiotics act by increasing the intestinal villi thereby increasing the absorptive surface area and ultimately digestion (Biloni *et al.*, 2013; Jayaraman *et al.*, 2013; Afsharmanesh and Sadaghi, 2014). Probiotic block the intestinal infection route by site specific attachment to mucosal epithelium, detoxify detrimental substances in the diet, produce vitamins, thus keeping the gut healthy (Ringø *et al.*, 2010).

The present study showed an improvement in the number RBCs, hemoglobin, HCT, platelets and lymphocytes in probiotics treated groups as compared to control indicating their hematopoietic stimulation activity. Production of antimicrobial compounds by microbiota in gut could has have a significant impact of blood profile that untimely leads to better physiology and growth (Verschuere *et al.*, 2000). Therefore, enhanced growth in our experiment is indirect associated with antagonisms against pathogen by *G. candidum* in addition to direct impact of digestive enzymes (Dieuleveux *et al.*, 1998; Eida *et al.*, 2013; Bakar, 2014). Similar mechanism behind probiotic impact was reported by researchers for certain fish species (Asadi Rad *et al.*, 2012; Hassaan *et al.*, 2014; Jha *et al.*, 2015). The fishes fed on mix-culture probiotics shown significantly high cellulase and protease contents in gut, thus facilitating nutrients availability by efficient feed conversion, rapid absorption and improved metabolism. It has already been reported earlier that probiotics application compensate the deficiency of adequate enzymes due to immature digestive tract during early life stages of many fishes (Ibrar *et al.*, 2017). Better feed digestion and metabolism due to higher enzymatic activity in fish gut improved crude protein and fat content in response to the probiotic fed group in our study. It was also reported earlier about the positive impact of probiotics on protein and fat content such as *Oreochromis niloticus* fed on multiple specie

probiotics comprising of *Streptococcus faecium*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* supplemented diet (Lara-Flores *et al.*, 2003). It is reported that *Bacillus licheniformis* and yeast extract also enhanced protein and fat in fish flesh (Hassaan *et al.*, 2014).

Modulation of gut microbiome for healthy diversity of beneficial microbiota is the most difficult task in natural environmental conditions. In our findings; *Proteobacteria* was found to be dominant phyla in control and the group fed on multispecies probiotics other most commonly found bacteria were *bacteroidetes*, *firmicutes* and *actinobacteria*. Higher *Proteobacteria* counts have also being documented by a study conducted on aquaculture sediment (Wu *et al.*, 2012). *Proteobacteria* and *firmicutes* are important intestinal microbes of carps and other fishes while *Bacteroidetes* are relatively less abundant (Xing *et al.*, 2013; Xia *et al.*, 2014). Taxonomic composition and microbial interaction with host is vital for fish health. Common fish pathogens such as *Aeromonas*, *Vibrio* and *Flavobacterium* were absent in fish gut. The *Staphylococcus saprophyticus* a potent fish pathogen was present in control, but in probiotics fed group it was in very low percentage but was eliminated by multiple specie probiotics which might be due to competitive exclusion or production of antimicrobials by probiotics. In our study several potentially cellulose degrading species such as *Clostridium* and *Ruminococcus* spp. were detected which also corresponds to the dietary habits of *L. rohita*. Previously conducted studies on *L. rohita* microbial diversity has also shown similar results (Flint *et al.*, 2012; Singh, 2017). Some of the *firmicutes* especially *Streptococcus* and *Bacillus* used as probiotics in aquaculture were also detected in our study, but in low percentage similar results were procured by (Singh, 2017). *Actinobacteria* are important phyla known for secondary metabolite production, were far less dominant in our experiment. These findings are in agreement to previous trial on *L. rohita* (Wu *et al.*, 2012; Singh, 2017). The present study showed that *G. candidum* successfully adhered and colonize in fish GIT tract. Polyamines by certain yeasts helps in adherence to mucosa epithelium might be responsible for the colonization of *G. candidum* QAUGC01 (Andlid *et al.*, 1995).

Conclusion

In our study, mix-culture probiotic shown higher sustainability in mimic gut conditions. This leads to an improved growth, FCR, FCE and SGR, tissue crude protein content of *L. rohita* fed on mix-culture probiotic for specific duration. Probiotic fed fishes shown an improved feed digestion and health strongly related to increase in enzymes activity and absence of pathogenic bacteria in gut. The overall positive impact probiotic on fishes could be related significantly to an increase in level of friendly microbiota in gut those played a pivot role in improvement of physiology and wellbeing. However, further pilot studies are required to understand molecular mechanisms involved in interaction of probiotic with resident fish gut microbiome.

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