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Effects of yeast (*Saccharomyces cerevisiae*) on the intestinal microbiota of GIFT Tilapia (*Oreochromis mossambicus*)

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Abstract

This study was conducted to assess the impact of yeast (*Saccharomyces cerevisiae*) as potential probiotic for improving the intestinal microbiota of GIFT Tilapia (*Oreochromis mossambicus*) as improved intestinal microbiota can enhance the resistance and improve the gut morphology. The study was carried out for 60 days to investigate the influence of different levels (0.15%, 0.30%, 0.45%, 0.60%, 0.75% and 1%) of *S.cerevisiae* (named as SC1, SC2, SC3, SC4, SC5 and SC6). Isolation and identification of intestinal microbiota was conducted as describe in methods. The results showed significantly better effects on intestinal microbiota ($P<0.05$) as compared to control. The maximum number of intestinal microbiota was observed in SC4 ($5.5x10^8$ CFU/ ml) treatment. In this study, various strains of intestinal bacteria were isolated and identified. A total of 384 bacterial strains were isolated from the fish and classified into six taxonomic groups; *Acinetobacter, Bacillus,* Enterobacteriaceae*, Vibrio, Pseudomonas and* Vibrionaceae (*Aeromonas*). The significant difference was observed in Vibrionaceae ($P<0.05$), while highly significant ($P<0.01$) differences were observed in Acinetobacter, Enterobactericeae, Pseudomonas, other gram negative and gram-positive bacteria counts in treatments fed with dietary probiotics as compared to control. It can be concluded that the addition of 0.60% *S. cerevisiae* in the diet can enhance the intestinal microbiota of GIFT Tilapia.

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Introduction

Microbes exist in the aquatic environment and enter in the digestive tract of fish and make commensal intestinal microbiota inside intestine which provide a suitable environment for them (Mondal *et al.,* 2008). The indigenous gut microbiota defends against the colonization of orally introduced microorganisms by mechanisms including competition at mucosal surfaces for substrates and receptors (Plant *et al.*, 2003; Galindo *et al.,* 2009). The intestinal microbiota is very important for the host (Sugita *et al.,* 1991; Sarkar and Ghosh, 2014) because it plays vital role in growth, digestion, disease control and can influence the health of host (Sivasubramanian *et al.,* 2012). Various researchers have studied intestinal microbiota in vertebrates over the past decades (Ferguson *et al.,* 2010). The addition of feed additives like probiotics in aqua feeds (Merrifield *et al.,* 2010; Dimitroglou *et al.,* 2011) can develop microbial balance and improve gut morphology (Lara-Flores *et al.,* 2003; El-Haroun *et al.,* 2006; Aly *et al.,* 2008; Wang *et al.,* 2008; Standen *et al.,* 2013). Bacterial profile in the intestine is usually a replication of microbes existing in the environment. The bacterial load in the gut of fish depends upon the quantum and food type ingested recently (Gibson *et al.,* 2004; Dimitroglou *et al.,* 2010; Thillaimaharani *et al.,* 2012). Probiotics play an important role in various species of fish such as pollock (Gatesoupe, 2008), rainbow trout (Aubin *et al.,* 2005), channel catfish (Shelby *et al.,* 2007) and Nile tilapia (Shelby *et al.,* 2006; Merrifield *et al.,* 2011). Different bacteria flourished in the gut of fish (Ringo *et al.,* 2007) like rod-shaped bacteria in *Labeorohita* (Ghosh *et al.,* 2010); coccoid and rod-shaped bacteria in *Salvelinus alpinus* adherent bacteria and yeast (rod shaped bacilli or round shaped cocci) in *Oreochromis* spp. which was confirmed by various techniques like SEM and 16SrDNA sequence analysis (Saha *et al.,* 2006; Ray *et al.,* 2007; Sarkar and Ghosh, 2014). Several researchers used live and dead probiotic in aquaculture to observe benefit of their supplementation on intestinal microbiota (Irianto and Austin, 2003; Panigrahi *et al.,* 2005; Ringo *et al.,* 2006ab; Taoka *et al.,* 2006; Gatesoupe, 2008; Merrifield *et al.,* 2011).

Marine and freshwater fishes contain specific indigenous intestinal microbiota due to feeding habitat which may be affected due to nutritional status, environmental conditions and fish age (Olafsen, 2001; Vine *et al.,* 2006; Dimitroglou *et al.,* 2014). Previously, Spanggaard *et al.* (2000) detected the intestinal microbiota of 48 rainbow trout by comparing direct microscopic counts with plate counts (tryptone soya agar, TSA). Sivasubramanian *et al.* (2012) isolated communal Gram-positive and Gram-negative bacteria *Aeromonas, Vibrio*, *Pseudomonas, Lactobacillus, Bacillus, Acinetobacter, Enterobacter* and *Flavobacterium* from the gut of three estuarine fishes. This intestinal microbiota with antibacterial abilities was helpful to prevent the growth of attacking bacteria in intestines of freshwater and marine fishes as one strain of *Bacillus* was used successfully to eliminate pathogenic *Vibrio* from *Centropomus undecimalis*.

Tilapia (*Oreochromis mossambicus*) is most important cultivated fish species after salmonids and carps (Fessehaye, 2006). The intestinal microbiota is commercially important in fishes due to control of fish diseases and proper handling and preparation of fish feed. Tilapia is an omnivorous fish and can be cultured in freshwater as well as seawater. Its digestive tract is 5-7 times longer as its body length and the impact of microbes on host mucosa is also poorly understood (Standen *et al.,* 2013) due to which this species was selected for studying the intestinal microbiota (Thillaimaharani *et al.,* 2012). Probiotic applications have established a range of benefits in fish including tilapia (Lara-Flores et al. 2003; El-Haroun *et al.,* 2006; Pirarat *et al.,* 2006; Shelby *et al.,* 2006; Taoka *et al.,* 2006; Aly *et al.,* 2008; Wang *et al.,* 2008). Most of these studies are limited to growth, immunity, digestive enzymes activity and haematology but fewer studies have been conducted about the gut microbiota to understand the mechanisms on endogenous microbiota (Ferguson *et al.,* 2010). The intestinal microbiota of freshwater fish

has been investigated (Austin, 2002; Ghosh *et al.,*2010; Ray *et al.,* 2012) and can be divided into autochthonous or allochthonous on the basis of ability to adhere and colonize in the gut (Ringo *et al.,* 2003). Several researchers described probiotics as the major microbial colonizers in the gut of fish (Pond *et al.,* 2006) including yeast Gatesoupe, 2007; Mandal and Ghosh, 2013; Banerjee and Ghosh, 2014; Sarkar and Ghosh, 2014). Therefore, the current study was designed to investigate the role of yeast (*S. cerevisiae*) on gut microbiota in GIFT tilapia (*O. mossambicus*).

Materials and methods

In this study, the influence of different levels of dietary supplementation of probiotic bacteria *Saccharomyces cerevisiae*on intestinal microbiota of GIFT Tilapia (*Oreochromis mossambicus*) was carried out at the Fish Research Laboratory, Department of Zoology, Government College University Faisalabad, Pakistan.

Experimental design

The experimental fish specimens were fed daily with basal fish feed (5% of their body weight at 9.00 am and 4.00 pm) before the start of the trials. The study involved control and treatment groups with three replicates for each group, and their culture period was 60 days. *Saccharomyces cerevisiae* was selected for this investigation. This 60 days trial was divided into 6 treatments, i.e., SC1 to SC6 and was fed with basal fish feed plus probiotic (*S.cerevisiae*) @ 0.15% or 0.30% or 0.45% or 0.60% or 0.75% or 1.0 %, respectively and one control (C) group, fed with only basal diet. This trial was also conducted in triplicate. *S. cerevisiae* was purchased from Sigma-Aldrich.

Feed preparation

The basal fish feed was prepared by common ingredients which were purchased from the local market and its proximate chemical analysis was carried out according to AOAC (2000). All these ingredients were mixed with boiled water and converted into paste or into semi moist dough, which was passed through electrical mincer to make pellets and Kenwood Multi-processor was used for this purpose. These pellets were dried for a few days at the room temperature, then crushed to make fine particles. The proposed doses of Probiotic were added freshly with these fine particles at the time of feeding. Feed was given twice a day (9.00 am and 4.00 pm each day) @ 5% of body weight for the entire period of experiment.

Intestinal microbiota

At the end of 60 days trials, five fish were taken from each experimental treatment for estimating intestinal microbiota. The GIT tract was removed aseptically from its entirety. Now the microbial populations from this GIT tract were identified by adopting the method of Merrifield *et al.,* 2011. Subsequently, inter-fish differences have been stated already by Spanggaard *et al.,* 2000 and Liu *et al.,* 2008. So, 2 fishes from each tank were sacrificed to get faecal material yielding three samples per treatment. Spread plate method was used for determination of total aerobic heterotrophic bacterial populations and MRS for Lactic acid bacteria. For this purpose, serial dilution of samples was carried out by using PBS and its 100 ml was spread onto duplicate TSA plates (Oxoid, Basingstoke, UK). Incubation of MRS and TSA plates was conducted at 30 ^{o}C (comparable to tilapia culture conditions) for 48 h and then bacterial colonies were counted from statistically feasible plates for calaulation of colony forming units (CFU g^{-1}) (Standen *et al.,* 2013). Microbiota was isolated by using the method described by Sivasubramanian *et al.,* 2012 with some alterations.

Isolation and identification of bacteria

Gut homogenate (01 ml) was spread aseptically and was mixed with sterile double strength PBS (9ml) against Nutrient agar. Incubation at TSA plates for 24-48 hrs at 37 ^{o}C was carried out. Colonies of bacteria on the TSA plates were counted and described as cfu/g. Agar slants were used to purify the isolates and bacterial strains were detected up to the species (Buchanan and Gibbons, 1974). In early steps, isolates were identified by observing following activities: Motility, catalase activity, oxidation/ fermentation, glucose gas, glucose acid, gram stain,

citrate utilization, oxidase activity and pigment production. Bacterial isolates were passed through Secondary tests to identify them at the genus level which include production of amylase, lipase activity, developing ability on sodium chloride media (0%), production of protease, and gelatinase activity.

Statistical analysis

The data was analyzed by using two-way ANOVA (analysis of variance). The data was presented as treatment mean ± Standard deviation and the variation of means among different groups. P values < 0.05 was considered to be significant; using Duncan's multiple range test. Software package (SPSS, version 17) was used for statistical analysis.

Results

After 60 days trail, total counts were conducted by either DAPI staining or culturing which showed higher similarity in most of the intestinal samples. It indicated that cultured microorganisms were dominant in the intestine of tilapia fish and both direct and plate counts varied between individual fish with 3-5 log units and was effectively identified.

Table 1. Analysis of variance (Mean squares) for Bacterial counts and physiologic identification of strains isolated from TSA plates sampled from GIFT Tilapia (*O. mossambicus*) intestine fed with different doses of probiotic (*S. cerevisiae*)after 60 days.

Source of variation	Degrees of freedom	Mean squares						
		Acinetobacter	Enterobactericeae	Vibrionaceae	Pseudomonas spp	Other Gram-negative	Gram-positive	Total number
Treatment	6	3.7143**	17.429**	5.429*	19.714**	8.8571**	10.714**	83.429**
Error	14	0.4286	2.143	1.429	1.857	0.8571	0.857	7.007
Total	20							

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01).

Qualitative studies on gut microbiota

Results of qualitative analysis of gut microbiota possessing the bacterial loads with different treatments ranged was from $3.9x10^8$ to $5.5x10^8$ CFU/ ml. The maximum number of microbiota was $5.5x10^8$ CFU/ ml in SC4 group and minimum ($3.9x10^8$ CFU/ ml) in SC1 in treatments while it was observed $4.6x10^8$ CFU/ ml in control (C) group. Total count in SC2, SC3, SC5 and SC6 were $4.8x10^8$ CFU/ ml; $4.9x10^8$ CFU/ ml; $4.1x10^8$ CFU/ ml; $4.4x10^8$ CFU/ ml, respectively.

Quantitative studies on gut microbiota

The intestinal microbiota of *O. mossambicus* was analyzed in fishes which were fed with different doses of *S. cerevisiae*. Several strains of bacteria were isolated which were six and identified as *Bacillus cereus*, *Bacillus licheniformis*, *Enterococcus faecalis, Vibrio sp.*, *Virgibacillus pantothenticus* and *Virgibacillus alginolyticus*. As per observations after 60 days trial 80% of the isolated bacteria were Gram positive in the gut of *O. mossambicus* which were identified as *B. cereus, B. licheniformis, V. pantothenticus, E. faecalis* and Gram negative were *Vibrio* sp. and *V. alginolyticus* present in gut contents. The identification of isolates was carried out by various biochemical tests and it was observed that *Pseudomonas, Vibrio* and *Bacilli* were also common bacteria found in all fishes. *Vibrio*, *Aeromonas* and *Pseudomonas* were predominant bacterial genera in tilapia intestine. *Bacillus* and *Corynebacterium* were predominant groups to tolerate the adverse effects of digestive enzymes. Composition of the intestinal microbiota determined by physiological identification, contain *Acinetobacter*, Enterobactericeae (*Citrobacter, Proteus*) Vibrionaceae (*Aeromonas, Plesiomonas*); *Pseudomonas* spp (*Pseudomonas*) and other Gram-negative (Beta-proteobacteria) and Gram-positive (*Streptococcus, Carnobacterium* and *Bacillus*). A total of 384 bacterial strains were isolated from the fish and classified into six taxonomic groups; *Acinetobacter, Bacillus,* Enterobacteriaceae, *Vibrio, Pseudomonas and* Vibrionaceae (*Aeromonas*). All

the isolates from the fish gut were tested for their biochemical characters. The result of biochemical test in this study indicated that *Acinetobacter* were 6 %, Enterobactericeae 36%, Vibrionaceae 15%, *Vibrio* 12%, *Pseudomonas* 21% and *Bacillus* were 10 %. About 70 % of the strains utilized citrate and 30% of strains showed positive in indole, 10% of strains showed positive Haemolysis and H_2S production test. Methyl red test showed positive on 15 % of the isolates. Analysis of variance for intestinal microbiota after 60 days exposed that significant difference ($P<0.05$) in Vibrionaceae. Highly significant ($P<0.01$) differences were observed in Acinetobacter, Enterobactericeae, Pseudomonas, other Gram negative and Gram positive bacteria (Table1 and 2).

Table 2. Comparison of means (±SE) for Bacterial counts and physiologic identification of strains isolated from TSA plates sampled from GIFT Tilapia (*O. mossambicus*) intestine fed with different doses of probiotic (*S. cerevisiae*) after 60 days.

Treatment	Acinetobacter	Enterobactericeae	Vibrionaceae	Pseudomonas spp.	Other Gram-negative	Gram-positive	Total number
C	2.00 ± 0.00^{C}	21.00 ± 1.00^{AB}	7.00 ± 1.00^{AB}	17.00 ± 1.00^{A}	5.00 ± 0.58^{CD}	6.00 ± 0.58^{AB}	58.00 ± 1.25^{AB}
SC1	4.00 ± 0.58^{AB}	22.00 ± 1.00^{A}	7.00 ± 0.58^{AB}	9.00 ± 0.58^{B}	4.00 ± 0.00^{D}	7.00 ± 0.00^{AB}	53.00 ± 1.49^{BC}
SC2	3.00 ± 0.00^{BC}	21.00 ± 0.58^{AB}	9.00 ± 0.58^{AB}	11.00 ± 0.00^{B}	6.00 ± 0.00^{BCD}	5.00 ± 0.00^{B}	55.00 ± 1.39^{B}
SC3	2.00 ± 0.00^{C}	16.00 ± 0.58^{C}	8.00 ± 0.00^{AB}	12.00 ± 1.00^{B}	8.00 ± 0.58^{AB}	6.00 ± 0.58^{AB}	52.00 ± 1.85^{BC}
SC4	5.00 ± 0.00^{A}	17.00 ± 0.00^{BC}	6.00 ± 0.00^{B}	11.00 ± 0.58^{B}	6.00 ± 0.00^{BCD}	2.00 ± 0.00^{C}	47.00 ± 0.84^{C}
SC5	$3.00\pm0.58B^{C}$	22.00 ± 1.15^{A}	10.00 ± 1.15^{A}	12.00 ± 1.15^{B}	9.00 ± 1.00^{A}	8.00 ± 1.00^{A}	64.00 ± 2.35^{A}
SC6	4.00 ± 0.58^{AB}	20.00 ± 1.00^{ABC}	8.00 ± 0.58^{AB}	10.00 ± 0.58^{B}	7.00 ± 0.58^{ABC}	6.00 ± 0.58^{AB}	55.00 ± 1.00^{B}

Means sharing similar letter in a column are statistically non-significant ($P>0.05$).

Discussion

In the present study, Enterobactericeae (*Citrobacter, Proteus*) Vibrionaceae (*Aeromonas, Plesiomonas*); Pseudomonas spp. (*Pseudomonas*) other Gram-negative (Beta-proteobacteria) and Gram-positive (*Streptococcus, Carnobacterium* and *Bacillus*) were observed. A total of 384 bacterial strains were isolated from the fish and classified into six taxonomic groups; *Acinetobacter, Bacillus, Enterobacteriaceae, Vibrio, Pseudomonas and Aeromonas*. All the isolates from the fish gut were tested for their biochemical characters. The result of biochemical test in this study indicated that *Acinetobacter*6%, Enterobactericeae 36%, Vibrionaceae 15%, *Aeromonas* 12%, *Pseudomonas* 21%, and *Bacillus* were 10 %. About 70% of the strains utilize citrate and 30 % of strains showed positive in indole, 10% of strains showed positive Haemolysis and H_2S production test. Methyl red test showed positive on 15 % of the isolates. The study displayed that intestinal microbiota had protective effect against pathogenic bacteria and retard pathogens to colonize in the intestine of targeted fish. In the present study, bacterial counts were higher as compared to the control (C) groups fed with basal feed and similar observations were recorded by Bagheri *et al.* (2008) after feeding yeast, *Saccahromyces cerevisiae*. Previous studies confirmed the presence of bacilli (*Bacillus subtilis*) and cocci (*Staphylococcus* sp.) in different fish species *Mugilcaphalus* (Nagvenkar *et al.,* 2006); *Salmosalar* (Ringo *et al.,* 2008); *Labiorohita* (Ghosh *et al.,* 2010); *Labeobata* (Mondal *et al.,* 2010); freshwater teleosts (Ray *et al.,*2012) and *O. mossambicus* (Sarkar and Ghosh, 2014). Therefore, the current study agreed with previous studies and confirmed the improvement in intestinal microbiota after addition of 0.60% *S. cerevisiae* in the diet of GIFT Tilapia.

Conclusion

Qualitative analysis of gut microbiota possessing the bacterial loads with different treatments ranged from $2.8\times10^{6}$to 4.5×10^{6} CFU/g ml^{-1}. The maximum number of microbiota was 4.5×10^{6} CFU/g ml^{-1} in SC4

group. Significant difference were observed in Vibrionaceae ($P<0.05$), while highly significant differences were observed in Acinetobacter, Enterobactericeae, Pseudomonas, and gram-positive bacteria. The biochemical test in this study indicated that *Acinetobacter*6 %, Enterobactericeae 36 %, Vibrionaceae 15 %, *Aeromonas* 12 %, *Pseudomonas* 21 % and *Bacillus* were 10%.

In this study, bacterial counts were higher in treatment groups as compared to the control (C) groups fed with basal feed only which indicate beneficial effects of yeast on intestinal microbiota.

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