Full length article

Comparative immunostimulatory effect of probiotics and prebiotics in *Channa punctatus* against *Aphanomyces invadans*

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ABSTRACT

In aquaculture and human health care probiotics and prebiotics have been widely used due to their important role in enhancing beneficial gut microbiota, promoting growth, increasing disease resistance, and positively modulating the host immune system. This study reports for the first time a comparative analysis on the effect of the probiotics and prebiotics on growth, digestive enzymes activity, antioxidant activity, and immune response in *Channa punctatus* against *Aphanomyces invadans*. Among the diets enriched with *Saccharomyces cerevisiae* (*S. cerevisiae*) and Galactooligosaccharide (GOS) in *C. punctatus,* feeding $2.5 \, \mathrm{g \, kg^{-1}}$ diet did not significantly influence the mean weight gain (MWG) between weeks 2 and 4 in both the infected and control groups; however the increase in MWG became significant from weeks 6–8. Similarly, during this period the protein efficiency ratio (PER), feed conversion ratio (FCR) and protein intake (PI) did not increase significantly. The intestinal protease, lipase, and amylase enzyme activities also did not increase significantly between weeks 2 and 4, whereas the values increased significantly after 6 weeks in both groups when fed with dietary supplementation of *S. cerevisiae* and GOS. The total *S. cerevisiae* count significantly increased in the gut of infected and non-infected fish fed with *S. cerevisiae* and GOS diets while the total bacterial (TB) count decreased between weeks 6 and 8. The total superoxide dismutase (t-SOD) activity and the malonaldehyde (MDA) concentration increased significantly in the non-infected fish fed with *S. cerevisiae* and GOS supplementation diets between weeks 6 and 8 whereas the catalase (CAT) and glutathione peroxidase (GPx) activities increased significantly only on week 8. The innate immune parameters such as plasma lysozyme, acid phosphatase (ACP), and myeloperoxidase (MPO) activities increased significantly in the infected and non-infected fish fed with *S. cerevisiae* and GOS containing diets after 6 weeks. Similarly, the plasma nitric oxide (NO) level and total protein (TP) content significantly increased in the non-infected fish fed with *S. cerevisiae* and GOS containing diets between weeks 6 and 8. In the control and the non-infected fish fed with *S. cerevisiae* and GOS enriched diets caused no mortality whereas 15% and 10% mortality was observed in the infected fish fed with *S. cerevisiae* and GOS diets, respectively. This study indicates that the infected and non-infected *C. punctatus* fed with dietary supplementation of GOS diet at $2.5 \, \mathrm{g \, kg^{-1}}$ had exhibited better growth performance, digestive enzyme activities, gut microbiota composition, and immune response than that of *S. cerevisiae* diet.

1. Introduction

In India the spotted snakehead, *Channa punctatus* belonging to the Channidae family is the most important culture species in inland fisheries because it breeds during south-west and north-east monsoons period frequently; it is a natural inhabitant in sluggish muddy pond waters, beels canals, rice-fields, reservoirs rivers, and weed rich swamps. Being an air breathing fish and a carnivore it not only is amenable for controlled reproduction but also a sturdy species. In India is marching ahead in blue revolution by the improvement of inland fisheries. *C. punctatus* is a luxury aquaprotein of highly nutritional food with an insatiable demand. This fish is considered as a delicacy due to

the delicious taste, very good commercial value, low intramuscular spines, highly medicinal value as well as due to its tolerance to extreme environmental and crowded conditions; therefore, this species is a choice candidate species for intensive aquaculture has been practice in Indian subcontinent. The availability of *C. punctatus* in the wild has declined due to over exploitation, habitat degradation, environmental stress [1], and their susceptibility to Epizootic Ulcerative Syndrome (EUS) disease, which is mainly due to a fungus-like oomycete, *Aphanomyces invadans*. EUS has caused severe economic losses in culture and capture fisheries in Asia and Australia in the last few decades [2,3].

The overuse use of antibiotics in aquaculture practice to control fish and shellfish diseases has led to the development of drug-resistant or multiple antibiotic-resistant (MAR) bacteria strain [4,5] which has been known to affect terrestrial animals as well as human beings [6]. Further, the antibiotics potentially alter the normal gut microflora that increases their susceptibility and emergence of antibiotic-resistant bacteria in human [7] and aquaculture species such as fish and shellfish [8,9]. In addition, residues of antibiotics in food can produce a big problem of allergy and toxicity [10].

In this regard, probiotics and prebiotics are an efficient alternative tool to circumvent antibiotics; they also play an important role in improving gut microbiota composition, the digestibility and feed utilization increasing the weight gain, enhance disease resistance, modulate the immune system, besides promoting competitive exclusion of pathogenic microorganisms [11–13]. The efficacy of the probiotics and prebiotics on growth performance, digestive enzymes activity, antioxidant activity, and immune response in fish are very limited [14–21]. Therefore, the present study has been undertaken to make a comparative investigation on the effect of the probiotics and prebiotics on growth performance, digestive enzymes activity, antioxidant activity, and immune response in *C. punctatus* against *A. invadans*.

2. Material and methods

2.1. Diet

The formulated diet comprise groundnut oilcake and soybean flour as protein source; rice bran used as carbohydrate source; sunflower oil as lipid source; wheat flour and tapioca meal as carbohydrate source as well as binders along with mineral and vitamin premix (Table 1). All ingredients except Galactooligosaccharide (GOS) and *Saccharomyces cerevisiae* were mixed evenly in an electric blender. These ingredients comprised the basal control diet from which three enriched diets were prepared as: (1) GOS diet (2.5 g kg^{-1}), (2) *S. cerevisiae* diet (2.5 g kg^{-1}), and (3) the control diet without containing GOS or *S. cerevisiae*. The ingredients were made into dough by adding required volume of sterile distilled water and pelletized by passing through an extruder (Mesh diameter: 1 mm). The prepared feeds were air dried and stored at −20 °C until used. The proximate composition of crude protein, carbohydrate, lipid, ash, and fiber of these diets were analysed by using AOAC [22].

2.2. Pathogen

A. invadans strain was kindly provided by Prof. J.H. Lilley and maintained Prof. Chellam Balasundaram, Department of Animal Science, Bharathidasan University, Tiruchirapalli, India. The motile secondary *A. invadans* zoospores suspension was prepared by following Lilley et al. [23]. *A. invadans* was grown in glucose peptone yeast (GPY) broth by incubated at 20 °C for 4 days; afterward, it was washed out by several time in Petri dishes with autoclaved pond water (APW) and the *A. invadans* mats were keep overnight at 20 °C. Than the motile secondary zoospores were isolated and the required number of zoospores adjusted to 5×10^4 spores ml^{-1} using a Neubarr hemocytometer.

Table 1
Ingredients and proximate composition of experimental diets for *C. punctatus*.

Ingredients (%)	Control	GOS	*S. cerevisiae*
Groundnut oilcake	35.0	35.0	35.0
Soybean flour	25.0	25.0	25.0
Wheat flour	10.0	10.0	10.0
Tapioca meal	10.0	10.0	10.0
Rice bran	13.0	13.0	13.0
Sunflower oil	5.0	2.5	2.5
Chromic oxide (Cr2O3)	1.0	1.0	1.0
Mineral and vitamin premix[a,b]	1.0	1.0	1.0
Galactooligosaccharide (GOS)[c]	0.0	2.5	0.0
Saccharomyces cerevisiae (*S. cerevisiae*)	0.0	0.0	2.5
Proximate composition (%)			
Crude protein	38.6 ± 1.6	37.8 ± 1.8	38.1 ± 1.5
Crude fat	11.4 ± 0.8	10.6 ± 0.7	10.2 ± 0.6
Crude fibre	4.8 ± 0.06	5.1 ± 0.08	4.5 ± 0.10
Ash	3.3 ± 0.04	3.6 ± 0.06	3.4 ± 0.05

[a] Vitamin mixture contains vitamin A 5000 IU, vitamin D 400 IU, vitamin E 20 mg, vitamin B1 (thiamin mononitrate) 4 mg; vitamin B2 (riboflavin) 5 mg; vitamin B12 (cyanocobalamine) 2 mg, vitamin C (ascorbic acid) 100 mg, nicotinamide 50 mg, pyridoxine hydrochloride 3 mg, calcium pentohenate 10 mg, and biotin 0.1 mg.
[b] Mineral mixture contains copper 300 mg; cobalt 40 mg; magnesium 2.0 g; iron 1 mg, zinc 2 g, iodine 150 mg, DL-methionine 2 g; L-lysine mono hydrochloride 4 g; calcium 25%, and phosphorous 8%.
[c] Friesland Foods Domo Co., the Netherlands.

2.3. Fish and experimental condition

Healthy *C. punctatus* (45.8 ± 1.7 g) were purchased from a local fish farm and transported into the laboratory in polythene bags. The health status of the fish was examined immediately. Then the experimental fish were properly washed with tap water, followed by 0.02% KMNO$_4$ solution, and 0.004% formalin solution to remove any microbes. The fishes were acclimatized to standard laboratory condition in 1000 l concrete tanks containing dechlorinated tube well water for one week and provided with proper aeration. During the acclimation period no mortality was observed. Afterwards, the fishes were divided into six groups of 30 fish each ($6 \times 30 = 180$ fish). Among, two groups were fed with GOS supplementation diet and another two groups fed with *S. cerevisiae* supplementation diet, while the remaining two groups were fed with the control diet. After 30 days of respective feeding, among one of control diet (C; Group I), *S. cerevisiae* diet (*S. cerevisiae*; Group II), and GOS diet (GOS; Group III) groups were injected intramuscularly (i.m.) with APW alone into the left side of fish just below the mid dorsal fin region. Another, control diet (I; Group IV), *S. cerevisiae* diet (C-*S. cerevisiae*; Group V), and GOS diet (C-GOS, Group VI) were challenged (i.m) with 0.1 ml of *A. invadans* spore (5×10^4 spores ml^{-1}) containing suspension as mentioned above according to Chinabut et al. [24]. All groups were maintained in triplicate ($3 \times 180 = 540$ fish). The respective feed was provided to each group throughout the experimental period twice a day at 09:00 and 15:00 h. The unfed diet was siphoned out after one hour of each feeding trial regularly and the water was exchanged once in a week. During the experimental period, the water quality parameters such as dissolved oxygen (6.9–8.1 mg l^{-1}), free carbon dioxide (6.5–7.9 mg l^{-1}), pH (7.3–8.0), total alkalinity (70–82 mg l^{-1}), ammonia content (0.02 ppm), and temperature (27.5–28 °C) were observed following standard methods [25]. The photoperiod was maintained 12 h light: 12 h dark in natural light and the average light intensity was 550 klx.

2.4. Growth

At the end of weeks 2, 4, 6, and 8 of post-challenge with *A. invadans*, the fishes were collected in each group after fasting for 24 h and moderately anaesthetized with MS 222 to quantify the growth

performance according to Jawahar et al. [26].

Mean weight gain (MWG) = [initial body weight] – [final body weight]

Specific growth rate (SGR) = [(in final body weight - in initial body weight)/number of days x 100]

Protein efficiency ratio (PER) = [(% protein in diet x weight of diet consumed)/100]

Feed conversion ratio (FCR) = [feed consumed]/[weight gain]

Protein intake (PI) = [(fish wet weight gain)/(fish consumed protein) x 100]

Survival rates (SR %) = [(final number of fish)/(initial number of fish) x 100]

2.5. Sampling of blood and tissues

Six fish in each experiment tank were randomly collected at the end of weeks 2, 4, 6, and 8 for blood sampling through caudal vein puncture using heparinized syringes coated with lithium heparin as anticoagulant and transfered into sterile tubes, after anaesthetizing the fish with MS-222 (NaHCO$_3$ and tricaine methane sulphonate; Sigma Chemicals, USA) 1:4000 in dechlorinated water for 2 min. After centrifugation (3000 g for 10 min at 4 °C) of blood samples, the plasma was collected and stored at −80 °C for immunological assays. Subsequently, the whole intestinal tract (except the stomach) and liver were dissected out of these fish on ice in aseptically. A half of whole intestinal tract was used for gut microbiota analysis and the remaining half was washed gently with cold distilled water, weighed, and transferred into the sterile tubes containing phosphate buffer solution (1 g intestinal tract/ 10 ml buffer) pH at 7.5 for homogenization [27]. The intestinal enzyme extracted by centrifugation at $15,000 \times g$ for 15 min at 4 °C and stored at −80 °C until used for digestive enzyme analysis [28]. The liver was homogenized in ten volumes (v/w) of ice-cold physiological saline, than centrifugation at 3000g for 10 min at 4 °C and separated supernatants for antioxidant enzymes activity.

2.6. Digestive enzyme assays

The protease activity of the intestinal tract was analysis by casein digestion method [29]. Amylase activity was determined by the procedure of Worthington [30] and the lipase activity was determined by Natalia et al. [31]. All enzyme preparations for digestive enzyme assays were carried out on ice.

2.7. Antioxidant assays

Total superoxide dismutase (t-SOD) activity was carried out by SOD detection kit protocols (Nanjing Jiancheng Bioengineering Institute, China) according to Wang and Chen [32]. The catalase (CAT) and glutathione peroxidase (GPx) activities and reduced glutathione (GSH) concentration was determined according to Lygren et al. [33]. The malonaldehyde (MDA) concentration was examined by the thiobarbituric acid method [34]. All antioxidant enzyme preparations for antioxidant activity were carried out on ice.

2.8. Analysis of gut microbiota

The intestinal tracts were washed thoroughly with 0.85% sterile saline (SS) solution (NaCl). The excess moisture removed by sterile filter paper, then weighed and transferred into sterile tubes containing SS solution (1 g intestinal tract/9 ml SS solution) for homogenization. The homogenate solution was serially diluted to 10^{-7} with the SS solution. One hundred microlitres of the diluted homogenate solution was spread onto de man rogosa sharpe (MRS) agar (HiMedia, India) or tryptic soy (TSA) agar (HiMedia, India) in triplicate plates for determination of total viable *S. cerevisiae* and total bacteria counts, respectively after incubating 2 days at 37 °C.

2.9. Immune assays

The plasma lysozyme activity was measured by using the turbidimetric method [35] and the plasma acid phosphatase (ACP) activity was determined by disodium phenyl phosphate method [36]. The phagocytic activity was analysis by Zhang et al. [37]. The plasma myeloperoxidase (MPO) activity was carried out by Alcorn et al. [38] and the plasma nitric oxide (NO) level was determined nitrate reductase assay using a commercial kit protocols (Jiancheng Bioengineering Institute, Nanjing, China). The plasma total protein (TP) content was examined by the Biuret method [39].

2.10. Disease resistance

A duplicate group was maintained in each experiment as mention above for disease resistance study. The cumulative mortality (CM) in each group were calculated over a period of 30 days as follows: CM (%) = 100 - [(mortality of treatment group/mortality of control group)] x 100.

2.11. Statistical analysis

The obtained results are expressed as standard error of mean (mean ± SE) and the data subjected to ANOVA to find out the significant differences among the means were calculated using Duncan's multiple range test. The P value < 0.05 is considered for significant.

3. Results

3.1. Growth performance

Dietary *S. cerevisiae* and GOS did not significantly ($P > 0.05$) impact MWG in *C. punctatus* on second week. It moderately increased on week 4 and significantly increased ($P < 0.05$) on weeks 6 and 8. The SGR was not significantly influenced ($P > 0.05$) in the control, infected, and non-infected fish fed with *S. cerevisiae* between weeks 2–6, but it was varied significantly on week 8. The SGR significantly increased in infected and non-infected fish fed with GOS diet between weeks 6 and 8. The PER, FCR, and PI did not significantly vary ($P > 0.05$) among the groups during the experimental period. There were 100% SR found in the control and non-infected fish fed with *S. cerevisiae* and GOS diets. However, a high SR was found in infected fish fed with *S. cerevisiae* and GOS diets, but the difference did not vary ($P > 0.05$) among period (Table 2).

3.2. Digestive enzyme activity

The intestinal protease enzyme activity slightly increased ($P > 0.05$) among the period in the control, infected, and non-infected fish fed with *S. cerevisiae* and GOS diets. The intestinal amylase enzyme activity was similar ($P > 0.05$) in all groups; however, it significantly increased ($P < 0.05$) in the infected and non-infected fish fed with *S. cerevisiae* and GOS diets only on week 8. The intestinal lipase enzymes did not significantly increase in any group. However, in the infected and non-infected fish fed with GOS diet it significantly increased ($P < 0.05$) after 4th week (Table 3).

3.3. Intestinal gut microbiota composition

The total *S. cerevisiae* counts in the infected and non-infected fish fed with *S. cerevisiae* and GOS diets did not vary ($P > 0.05$) between weeks

Table 2

Growth performance, feed utilization, and survival rate of *C. punctatus* fed diet containing *S. cerevisiae* and GOS for 8 weeks against *A. invadans*.

Indices	Weeks	C	I	*S. cerevisiae*	GOS	C-*S. cerevisiae*	C-GOS
MWG(g)	2	12.8 ± 1.7[a]	10.2 ± 1.4[a]	20.1 ± 1.8[a]	23.3 ± 1.3[a]	18.7 ± 1.6[a]	23.3 ± 1.3[a]
	4	18.2 ± 1.9[b]	16.8 ± 1.8[b]	28.4 ± 1.5[b]	31.1 ± 1.6[b]	25.2 ± 1.8[b]	29.8 ± 1.8[b]
	6	26.1 ± 2.2[c]	22.3 ± 1.9[c]	37.4 ± 1.9[c]	40.2 ± 2.8[c]	34.6 ± 2.0[c]	38.7 ± 2.4[c]
	8	37.5 ± 2.8[d]	29.1 ± 1.6[d]	49.5 ± 2.0[d]	54.2 ± 3.2[d]	45.3 ± 2.4[d]	52.5 ± 2.8[d]
	4	18.2 ± 1.9[b]	16.8 ± 1.8[b]	28.4 ± 1.5[b]	31.1 ± 1.6[b]	25.2 ± 1.8[b]	29.8 ± 1.8[b]
SGR (%)	2	1.07 ± 0.02[a]	1.01 ± 0.03[a]	1.86 ± 0.03[a]	1.95 ± 0.04[a]	1.62 ± 0.02[a]	1.82 ± 0.03[a]
	4	1.19 ± 0.03[a]	1.08 ± 0.02[a]	2.24 ± 0.02[a]	2.57 ± 0.03[a]	2.05 ± 0.04[a]	2.44 ± 0.05[a]
	6	1.31 ± 0.02[a]	1.12 ± 0.04[a]	2.61 ± 0.04[a]	2.88 ± 0.06[b]	2.23 ± 0.03[a]	2.75 ± 0.05[b]
	8	1.43 ± 0.02[a]	1.18 ± 0.04[a]	2.93 ± 0.05[b]	3.23 ± 0.05[c]	2.38 ± 0.04[b]	3.11 ± 0.04[c]
PER (%)	2	1.01 ± 0.02[a]	0.83 ± 0.03[a]	1.21 ± 0.03[a]	1.34 ± 0.03[a]	1.14 ± 0.04[a]	1.25 ± 0.03[a]
	4	1.09 ± 0.03[a]	0.88 ± 0.02[a]	1.33 ± 0.02[a]	1.49 ± 0.04[a]	1.25 ± 0.03[a]	1.41 ± 0.03[a]
	6	1.11 ± 0.02[a]	0.99 ± 0.03[a]	1.45 ± 0.02[a]	1.56 ± 0.02[a]	1.33 ± 0.03[a]	1.50 ± 0.04[a]
	8	1.14 ± 0.03[a]	1.03 ± 0.02[a]	1.51 ± 0.03[a]	1.78 ± 0.02[b]	1.42 ± 0.04[a]	1.60 ± 0.03[a]
FCR	2	0.83 ± 0.01[a]	0.81 ± 0.02[a]	1.21 ± 0.02[a]	1.48 ± 0.03[a]	1.15 ± 0.02[a]	1.37 ± 0.03[a]
	4	0.87 ± 0.01[a]	0.84 ± 0.03[a]	1.38 ± 0.03[a]	1.53 ± 0.04[a]	1.20 ± 0.03[a]	1.45 ± 0.03[a]
	6	0.90 ± 0.02[a]	0.86 ± 0.03[a]	1.41 ± 0.04[a]	1.62 ± 0.03[a]	1.32 ± 0.03[a]	1.49 ± 0.04[a]
	8	0.94 ± 0.02[a]	0.88 ± 0.02[a]	1.44 ± 0.04[a]	1.67 ± 0.04[a]	1.37 ± 0.02[a]	1.52 ± 0.05[a]
PI (%)	2	0.54 ± 0.02[a]	0.51 ± 0.01[a]	0.58 ± 0.02[a]	0.61 ± 0.03[a]	0.54 ± 0.02[a]	0.56 ± 0.03[a]
	4	0.56 ± 0.02[a]	0.52 ± 0.02[a]	0.60 ± 0.03[a]	0.66 ± 0.02[a]	0.57 ± 0.03[a]	0.58 ± 0.04[a]
	6	0.57 ± 0.03[a]	0.53 ± 0.02[a]	0.62 ± 0.03[a]	0.69 ± 0.03[a]	0.59 ± 0.04[a]	0.63 ± 0.03[a]
	8	0.59 ± 0.03[a]	0.55 ± 0.01[a]	0.64 ± 0.02[a]	0.72 ± 0.04[a]	0.60 ± 0.03[a]	0.66 ± 0.04[a]
SR (%)	2	100.0 ± 0.0	23.4 ± 1.12[a]	100.0 ± 0.0	100.0 ± 0.0	89.4 ± 1.35[a]	94.9 ± 1.02[a]
	4	100.0 ± 0.0	23.4 ± 1.12[a]	100.0 ± 0.0	100.0 ± 0.0	89.4 ± 1.35[a]	94.9 ± 1.02[a]
	6	100.0 ± 0.0	25.2 ± 1.21[a]	100.0 ± 0.0	100.0 ± 0.0	92.8 ± 1.50[a]	96.4 ± 1.26[a]
	8	100.0 ± 0.0	27.6 ± 1.34[b]	100.0 ± 0.0	100.0 ± 0.0	92.8 ± 1.50[a]	96.4 ± 1.26[a]

MWG: mean weight gain, SGR: specific growth rate, PER: protein efficiency ratio, FCR: feed conversion ratio, PI: protein intake, SR: survival rates, C: control, I: infected. The data expressed as mean ± SD, n = 6 and the difference in the same row as superscripts indicate significant differences ($P < 0.05$).

2 and 4, but it significantly increased ($P < 0.05$) after 6th week. The total bacterial (TB) count was high in control and infected groups fed with control diet. The TB count though decreased did not vary in the infected and non-infected fish fed with *S. cerevisiae* and GOS diets, but the difference became significant only the non-infected fish fed with GOS after 6th week (Table 4).

3.4. Antioxidant activity

The t-SOD activity and MDA concentration did not vary significantly ($P > 0.05$) among the groups on weeks 2 and 4. But these values significantly increased ($P < 0.05$) the non-infected fish fed with *S. cerevisiae* and GOS diets as well as in infected fish fed with GOS diet between weeks 6 and 8 (Figs. 1 and 2). The CAT and GPx activities were not significantly influenced ($P > 0.05$) in the infected and non-infected fish fed with *S. cerevisiae* and GOS diets during the experiment, but in the non-infected fish fed with GOS diet it varied significantly ($P < 0.05$) on 6th and 8th week (Figs. 3 and 4). The GSH activity did

not significantly vary among groups on weeks 2 and 4. However, it was significantly high in the non-infected fish fed with *S. cerevisiae* and GOS diets on weeks 6 and 8, but not in the infected fish fed with these diets during these periods (Fig. 5).

3.5. Immunological activity

The immunological activity of plasma lysozyme activity and plasma ACP activity did not vary significantly among groups on weeks 2 and 4. The difference became significant in non-infected fish fed with *S. cerevisiae* and GOS diets on week 6 as well as in infected and non-infected fish fed with *S. cerevisiae* and GOS diets on week 8 (Figs. 6 and 8). The phagocytic activity did increase significantly on 4th week. It varied significantly in the non-infected fish fed with *S. cerevisiae* and GOS diets on week 4; it was also significant in both infected and non-infected fish fed with *S. cerevisiae* and GOS diets on weeks 6 and 8 (Fig. 7). The MPO activity did not vary ($P > 0.05$) among groups between weeks 2 and 4. The MPO activity was significant in the non-infected fish with *S.*

Table 3

Effect of digestive enzyme in *C. punctatus* fed diet containing *S. cerevisiae* and GOS for 8 weeks against *A. invadans*.

Enzymes	Weeks	C	I	*S. cerevisiae*	GOS	C-*S. cerevisiae*	C-GOS
protease	2	0.27 ± 0.02[a]	0.25 ± 0.03[a]	0.30 ± 0.02[a]	0.36 ± 0.03[a]	0.26 ± 0.03[a]	0.36 ± 0.03[a]
	4	0.29 ± 0.03[a]	0.27 ± 0.02[a]	0.33 ± 0.03[a]	0.40 ± 0.04[a]	0.28 ± 0.02[a]	0.40 ± 0.04[a]
	6	0.30 ± 0.02[a]	0.28 ± 0.03[a]	0.36 ± 0.03[a]	0.44 ± 0.03[a]	0.31 ± 0.03[a]	0.44 ± 0.04[a]
	8	0.33 ± 0.03[a]	0.30 ± 0.02[a]	0.38 ± 0.02[a]	0.49 ± 0.04[a]	0.34 ± 0.03[a]	0.46 ± 0.03[a]
Amylase	2	2.06 ± 0.03[a]	2.01 ± 0.02[a]	2.53 ± 0.04[a]	2.86 ± 0.05[a]	2.67 ± 0.04[a]	2.74 ± 0.04[a]
	4	2.13 ± 0.04[a]	2.10 ± 0.03[a]	2.75 ± 0.05[a]	3.36 ± 0.06[a]	2.97 ± 0.05[a]	3.17 ± 0.05[a]
	6	2.21 ± 0.04[a]	2.14 ± 0.03[a]	3.36 ± 0.06[a]	4.56 ± 0.06[b]	3.34 ± 0.05[a]	4.23 ± 0.05[b]
	8	2.29 ± 0.03[a]	2.18 ± 0.02[a]	4.12 ± 0.08[b]	5.33 ± 0.08[b]	3.88 ± 0.06[b]	4.78 ± 0.06[b]
Lipase	2	2.88 ± 0.04[a]	2.72 ± 0.03[a]	2.94 ± 0.04[a]	3.11 ± 0.05[a]	2.73 ± 0.05[a]	3.06 ± 0.05[a]
	4	2.97 ± 0.05[a]	2.86 ± 0.04[a]	3.23 ± 0.06[a]	3.69 ± 0.06[a]	2.98 ± 0.06[a]	3.86 ± 0.06[a]
	6	3.06 ± 0.04[a]	2.98 ± 0.03[a]	4.27 ± 0.07[a]	5.21 ± 0.08[b]	3.43 ± 0.05[a]	5.13 ± 0.07[b]
	8	3.19 ± 0.05[a]	3.03 ± 0.04[a]	4.46 ± 0.08[b]	5.86 ± 0.08[b]	3.96 ± 0.06[b]	5.62 ± 0.08[b]

C: control, I: infected. The data expressed as mean ± SD, n = 6 and the difference in the same row as superscripts indicate significant differences ($P < 0.05$).

Table 4
Total *C. punctatus* count and total bacterial (TB) count (Log CFU g^{-1} of intestine) in *C. punctatus* fed diet containing *S. cerevisiae* and GOS for 8 weeks against *A. invadans*.

Indices	Weeks	C	I	*S. cerevisiae*	GOS	C-*S. cerevisiae*	C-GOS
S. cerevisiae	2	4.36 ± 0.42^a	4.04 ± 0.24^a	4.48 ± 0.32^a	4.63 ± 0.46^a	4.21 ± 0.26^a	4.35 ± 0.40^a
	4	4.38 ± 0.26^a	4.18 ± 0.32^a	4.75 ± 0.45^a	4.91 ± 0.38^a	4.51 ± 0.40^a	4.64 ± 0.31^a
	6	4.44 ± 0.30^a	4.27 ± 0.36^a	5.83 ± 0.40^b	6.15 ± 0.28^b	4.87 ± 0.46^a	4.98 ± 0.36^a
	8	4.53 ± 0.34^a	4.36 ± 0.20^a	5.96 ± 0.36^b	6.36 ± 0.32^b	5.57 ± 0.38^b	5.83 ± 0.30^b
TB count	2	7.33 ± 0.58^a	7.74 ± 0.40^a	7.36 ± 0.52^a	7.15 ± 0.45^a	7.44 ± 0.50^a	7.22 ± 0.42^a
	4	7.47 ± 0.50^a	7.86 ± 0.38^a	7.04 ± 0.48^a	6.56 ± 0.34^a	7.23 ± 0.44^a	6.74 ± 0.42^a
	6	7.55 ± 0.46^a	7.94 ± 0.44^a	6.57 ± 0.40^a	6.23 ± 0.46^b	6.71 ± 0.38^a	6.45 ± 0.42^a
	8	7.61 ± 0.42^a	8.12 ± 0.48^a	6.73 ± 0.47^a	6.07 ± 0.41^b	6.88 ± 0.46^a	6.28 ± 0.48^a

C: control, I: infected. C: control, I: infected. The data expressed as mean ± SD, n = 6 and the difference in the same row as superscripts indicate significant differences ($P < 0.05$).
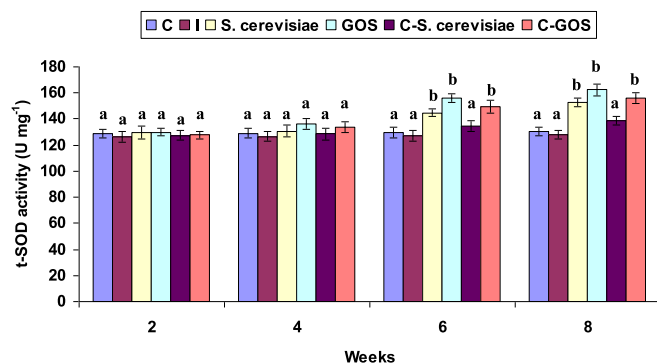
Fig. 1. Total superoxide dismutase (t-SOD) activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. Each column and bar represents mean ± SE and the different alphabets represent a statistical difference between means ($P < 0.05$).
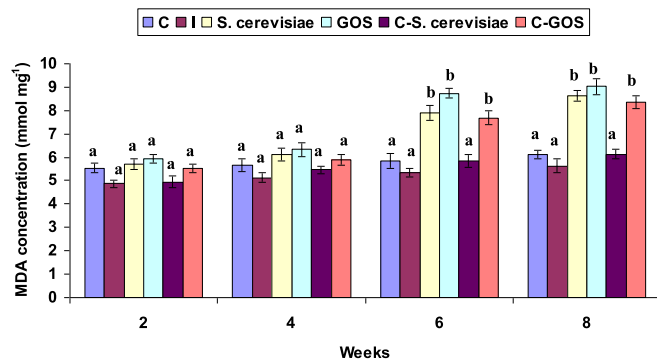

Fig. 2. The malonaldehyde (MDA) concentration of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.

Fig. 3. The catalase (CAT) activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.

Fig. 4. The glutathione peroxidase (GPx) activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.

cerevisiae and GOS diets on week 6, but not in infected fish fed with *S. cerevisiae* and GOS diets. However, in the infected and non-infected groups fed with GOS diet the MPO activity varied significantly than in the infected and non-infected fish fed with *S. cerevisiae* diet on 8th week (Fig. 9). The plasma NO level and TP content did not vary initially on weeks 2 and 4 among experimental groups. These increased significantly in the non-infected fish fed with *S. cerevisiae* and GOS diets as well as infected fish fed with GOS diets on weeks 6 and 8 (Figs. 10 and 11).

3.6. Disease resistance

There was no cumulative mortality (CM) in the control and non-infected fish fed with *S. cerevisiae* and GOS diets for 30 days. A lower
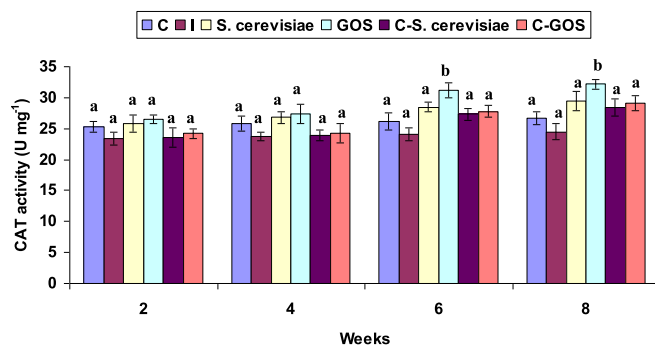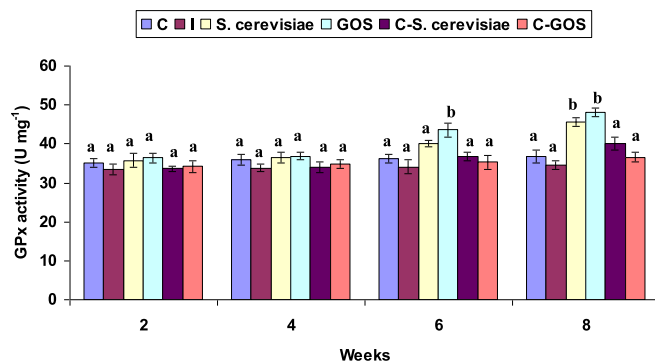
mortality of 15% and 10% was observed in the infected fish fed with *S. cerevisiae* and GOS diets. However, the mortality was 85% in the infected fish fed with control diet without *S. cerevisiae* and GOS (Fig. 12).

4. Discussion

S. cerevisiae is single cell eukaryote yeast, widely used in making wine, bread, beer, and improving human and animal health. The GOS comprise non-digestible food ingredients used for stimulating the growth of beneficial bacteria in the intestine of the host. The ability of the probiotics and prebiotics are improving digestive enzymes activity, antioxidant activity, gut microflora composition, survival rate, and its immune modulation functions [40–42] but there is a paucity of information in fish. Therefore, the present study was performed to make a comparative analysis of *S. cerevisiae* and GOS as probiotics and
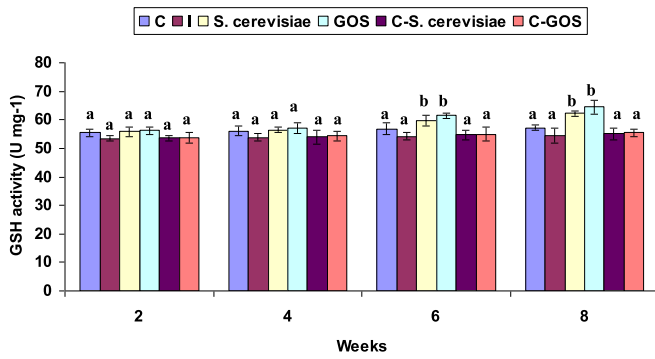
Fig. 5. The reduced glutathione (GSH) activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.
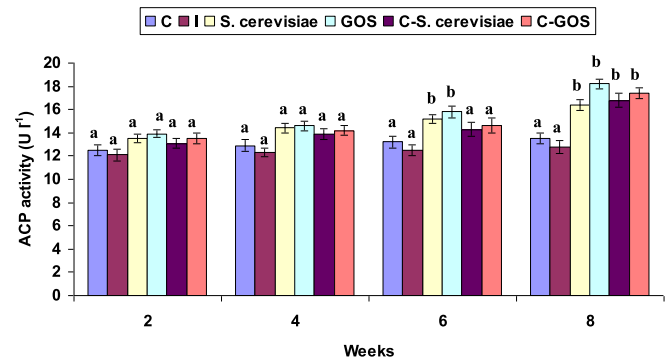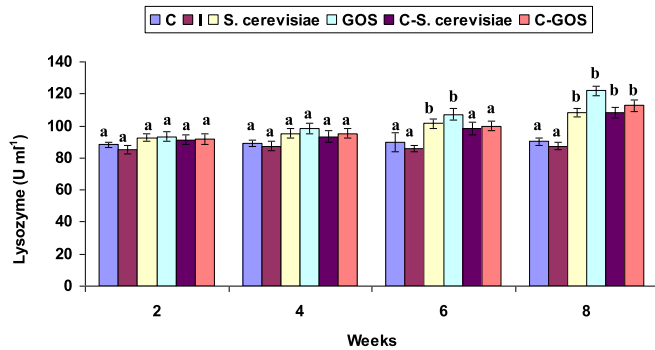

Fig. 6. The plasma lysozyme activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.
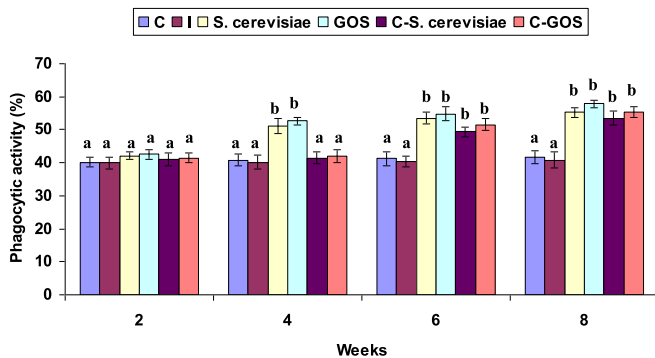

Fig. 7. The plasma phagocytic activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.

prebiotics in *C. punctatus* against *A. invadans* for the first time. In the present study, dietary *S. cerevisiae* and GOS in *C. punctatus* at $2.5 \, \text{g} \, \text{kg}^{-1}$ did not significantly impact of MWG on 2nd week, but it moderately increased on 4th week and significantly between weeks 6 and 8. The PER, FCR, and PI values were not significantly influenced by dietary *S. cerevisiae* and GOS at $2.5 \, \text{g} \, \text{kg}^{-1}$ in *C. punctatus*. It has been reported that in Atlantic salmon, carp, and gilthead sea bream fed with diet containing prebiotics, MOS did not significantly affect the FCR, SGR, and PER values [43–46]. This was also confirmed in Gulf of Mexico sturgeon, hybrid tilapia, and Atlantic salmon when fish were fed with a prebiotics such as MOS [47–49]. On the contrary, in yellow catfish, rainbow trout, European sea bass, and gilthead sea bream enriched diet of 0.2% MOS is sufficient to improve the growth performance [50–54].

Fig. 8. The plasma acid phosphatase (ACP) activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.

Further, there were 100% SR found in the control, infected, and non-infected fish fed with *S. cerevisiae* and GOS diets. Similarly, it was reported that addition of MOS in the feed increases the survival rate in European seabass, rainbow trout, gilthead seabream, Nile tilapia, and catfish [51,54–57]. The present results suggest that feeding *S. cerevisiae* and GOS diets did not affect the growth and mortality the normal and infected fish.

The intestinal protease and amylase enzyme activities were slightly increased in the control, infected, and non-infected fish fed with dietary *S. cerevisiae* and GOS; however, it significantly increased in the infected and non-infected fish on being fed with these diets on week 8. The intestinal lipase enzymes in the infected and non-infected fish fed GOS diet significantly increased only after 4th week. The increasing level of intestinal enzyme activity suggests that ingestion of *S. cerevisiae* and GOS had an intrinsic limit in fish. In fish the digestive ability is based on the levels of digestive enzyme activities which ultimately affect growth and health [58]. The improvement of digestive enzyme activity has been reported in some fish when fed with prebiotics-supplemented diets such as MOS [37,50,59,60]. Certainly, the intestinal enzyme activity in fish fed with *S. cerevisiae* and GOS did not cause any further increase between weeks 2 and 4; however, the mechanism of the effect in fish is currently unknown.

The prebiotics such as MOS and fructooligosaccharides (FOS) prevent the attachment and colonization of pathogenic microorganisms in the digestive tract of fish [54,61,62] which induce selective colonization of the gut beneficial microbes, including bifidobacteria/LAB [50,62]; therefore, the prebiotics may be improve or a positive effect on fish health. The total *S. cerevisiae* count in the gut of infected and non-infected fish fed with *S. cerevisiae* and GOS diets did not significant increase between weeks 2 and 4, but it increased significantly after 6th week. This suggests that GOS exhibit an indirect influence on nutrient digestibility by providing a conductive environment for the proliferation of *S. cerevisiae* in the gut and by manipulating the host gut microbiota in favor of a beneficial microbial community. On the other hand, the TB count has shown low in the infected and non-infected fish fed with *S. cerevisiae* and GOS diets. The presence of beneficial microbial communities has been linked to the increased activities of digestive enzymes in the gut [63].

Normal physical conditions can create a balance between reactive oxygen species (ROS) production and the antioxidant defense system [64]. The ability of scavenging activity and the antioxidant enzymes capacity are correlated with all kinds of patho-physiological circumstances [65]. The t-SOD activity and the MDA concentration did not significantly differ among the groups on weeks 2 and 4, whereas it was significantly increased in the non-infected fish fed with *S. cerevisiae* and GOS diets as well as infected fish fed with GOS diet between weeks 6 and 8. The CAT and GPx activities did not significantly influence the infected and non-infected fish fed with *S. cerevisiae* and GOS diets
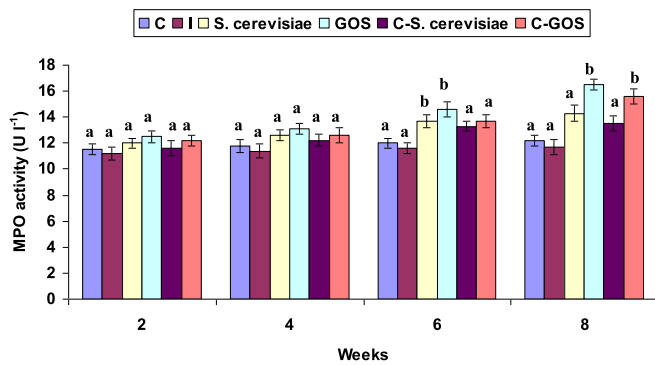
Fig. 9. The plasma myeloperoxidase (MPO) activity of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.
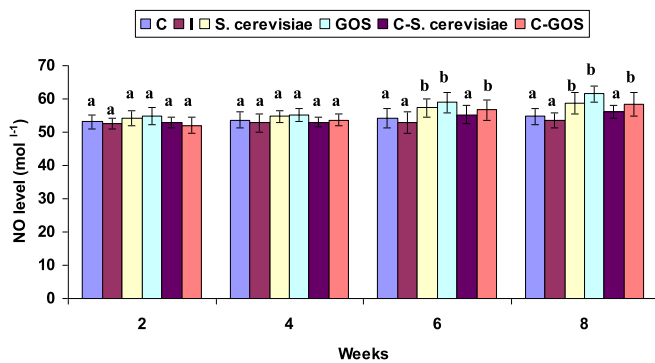

Fig. 10. The plasma nitric oxide (NO) level of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.
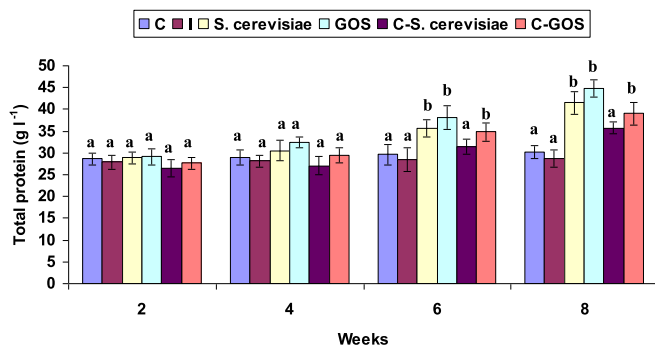

Fig. 11. The plasma total protein (TP) content of *C. punctatus* (n = 6) fed with *S. cerevisiae* and GOS diets against *A. invadans*. C: control, I: infected. The statistical information is seen in Fig. 1.

whereas it was significant in the non-infected fish fed with GOS diet on 8th week. In the present study, the infected fish fed without *S. cerevisiae* and GOS diets have shown low liver t-SOD and CAT activities, whereas MDA content was high, which is possibly due to the incapability of both enzymes to overcome the extremely high levels of ROS and the excessive ROS could in turn inactivate SOD and CAT activities [66]. The antioxidant enzymes activities may be saturated under a sustained compromised situation due to excessive accumulation of MDA [67]. The infected fish, liver t-SOD and CAT activities could improve the utilization of the diet containing $2.5\,g\,kg^{-1}$ of *S. cerevisiae* and GOS, which may be contribute to dietary antioxidants assimilated or that GOS plays a key role in antioxidant activity. The GSH activity did not significantly vary among groups on weeks 2 and 4, but it was
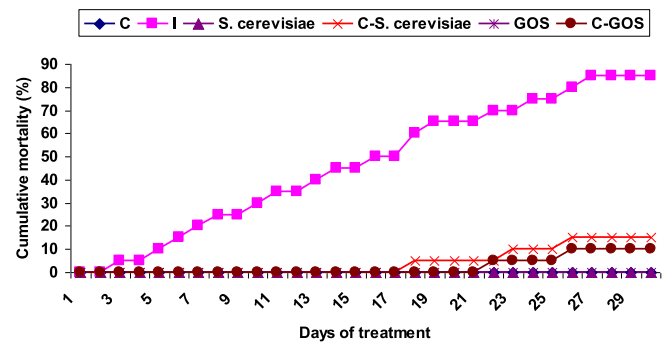

Fig. 12. The cumulative mortality (CM) of *C. punctatus* (n = 20) fed with *S. cerevisiae* and GOS diets against *A. invadans*.

significantly high in the non-infected fish fed with *S. cerevisiae* and GOS diets on weeks 6 and 8. It hypothesized that the antioxidative properties of GOS in the intestinal microbiota may serve as a defensive mechanism and therefore assists to overcome exo- and endogenous oxidative stresses. In juvenile blunt snout bream the COS might enhance the antioxidant capacity; this was supported by the fact that the antioxidant enzymes are capable of scavenging ROS and products of lipid peroxidation, thereby protecting cells and tissues from oxidative damage [68]. In fact, when the immune response is increased; animal cells produce ROS, which are highly microbicidal [69]. In order to keep an ongoing balance between antioxidants and ROS, the major antioxidant enzymes, such as SOD, CAT and GPx representing the first line of defense against oxidative stress are generated [70]. The administration of FOS was observed significantly low levels of liver MDA content indicate that COS may be inhibit the process of lipid peroxide. It was supported by this fact that MDA level is a direct evidence of the toxic processes caused by free radicals [71]. The mechanism underlying this process is quite unknown.

Prebiotics are not digested but act as an immunostimulant to enhance growth performance and phagocytosis in fish [72] and they help to maintain beneficial microbiota in the gastrointestinal tract [12]. The plasma lysozyme and ACP activities were not significant among groups on weeks 2 and 4 whereas it was significantly enhanced in the infected and non-infected fish fed with *S. cerevisiae* and GOS diets after 6th week. This indicates that GOS could improve the nonspecific immune response in *C. punctatus* and this immunostimulatory effect modulating the growth of beneficial bacteria such as *Bacillus* and LAB [73]. As a typical lysosomal enzyme, ACP activity expectedly has the similar trend with that of lysozyme [74]. The cell wall contain lipopolysaccharides of the beneficial bacteria with immunostimulatory properties [75,76]. The MPO activity exhibited no statistical difference among groups between weeks 2 and 4, but it was significantly increased in the non-infected fish with *S. cerevisiae* and GOS diets after 6th week. In infected and non-infected fish fed with GOS diet significantly higher MPO activity was exhibited than when fed with *S. cerevisiae* diet on 8th week. The higher lysozyme and MPO activities may be attributed to the relatively high leukocyte numbers [77,78]. Fish lysozyme has been identified histochemically both in monocytes and neutrophils [77], whereas MPO activity is a peculiar and specific hemeprotein released by neutrophils [79].

The plasma NO level and TP content did not significantly vary initially on weeks 2 and 4 among experimental groups. These values were exhibited significantly in the non-infected fish fed with *S. cerevisiae* and GOS diets as well as infected fish fed with GOS diets between weeks 6 and 8. The prebiotics FOS effectively enhanced the immune responses in turbot, blunt snout bream, and gilthead sea bream [19,80,81]. The application of prebiotics enhanced innate immune parameters including ACP [81], lysozyme activity [82], respiratory burst [83], SOD [84], and phagocytic [85] activities.

There was no cumulative mortality in control and the non-infected

fish fed with *S. cerevisiae* and GOS diets whereas a low mortality of 15% and 10% was observed in the infected fish fed with *S. cerevisiae* and GOS diets, indicating that *S. cerevisiae* and GOS could effectively reduce mortality against *A. invadans*. Further, the lower mortality might have been achieved through the enhanced cellular and humoral immune defenses response activated by *S. cerevisiae* and GOS. It was reported that prebiotics, like FOS can enhance cellular and humoral immune defense response which may control fish diseases [86,87]. In the infected fish fed with control diet the mortality was 85%. This may be attributed to the oxidative stress and immunosuppression caused by *A. invadans* as a result of reduced the resistance to fungal infection. Thus the oxidative stress usually makes fish more susceptible to diseases [88].

In conclusion, this preliminary study in *C. punctatus* fed with dietary supplementation of *S. cerevisiae* and GOS at $2.5\,\mathrm{g\,kg^{-1}}$ had positively improved the growth performance, digestive enzyme activities, gut microbiota composition, and their immune response. A better performance was observed in the infected and non-infected fish fed with GOS diet at $2.5\,\mathrm{g\,kg^{-1}}$ than when fed with *S. cerevisiae* diet. GOS may be provide a sufficient nutrient for gut beneficial microbes that stimulate the growth, digestive enzyme activity, antioxidant activity, innate immune response, and disease resistance in *C. punctatus* against *A. invadans*. In this regard proper feeding schedule and optimal administration doses of prebiotics are both important in improving the immunity and providing resistance to fish against microbial infection. However, the beneficial effect of *S. cerevisiae* and GOS in different concentration as a dietary supplement in other fish species against pathogen is necessary before it is recommended as feed additive for aquaculture species.

Acknowledgements

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