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SPECIFIC IMPORTANCE OF LOW LEVEL DIETARY SUPPLEMENTATION OF *LYPOMYCES STARKEYI CB1807* YEAST STRAIN IN RED SEA BREAM (*PAGRUS MAJOR*)

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Abstract

Most probiotic yeast supplements in fish exhibit beneficial effect at ≤1% of the dietary proportion. This study aimed at evaluating the specific effects of *Lypomyces starkeyi CB1807* yeast strain supplemented at ≤1% of dietary proportion on the performance of juvenile red sea bream (*Pagrus major*, 1.9 ± 0.04 g). Five diets were supplemented with yeast at graded levels of 0% (control diet 'CD1'), 0.05% (D2), 0.1% (D3), 0.5% (D4), and 1.0% (D5). After 45 days of feeding trial, significant (P<0.05) improvement was detected on final body weight (FBW) and body weight gain (BWG) in fish fed D3 and D5 compared to control. Low values of total cholesterol (T-Cho) and aspartate aminotransferase (AST) were recorded in fish groups fed on D2, D4, and D5, respectively. Fish fed on D3, D4 and D5 diets showed high (*P*<0.05) values of serum, mucus and liver lysozyme compared to control. Fish fed on D5 showed high values of total immunoglobulin (Ig) compared to control. Fish fed on D2 showed strong correlation with biological antioxidant activity (BAP), superoxide dismutase (SOD) and catalase activity (CAT). The biological antioxidant potential (BAP) activity in fish fed on D2 was significantly higher compared to control (P<0.05). The reactive oxygen metabolites (d-ROM) were significantly lower in fish fed on D2 and D3 compared to CD1 (P<0.05). Peroxidase activity was improved significantly (P<0.05) in fish fed on D3, D4 and D5 compared to control. The tolerance ability (LT_{50}) of fish fed on D5 against low salinity stress was significantly higher compared to control (P<0.05). It was concluded that dietary benefits of spent *L. starkeyi* yeast at ≤1% showed considerable improvement in antioxidant capacity in red sea bream, *P. major*.

Key words: red sea bream, growth performance, oxidative status, blood health, immune response

Red sea bream (*Pagrus major*) is one of the important cultivated fish species in eastern Asia (Sugama, 2002; Dawood et al., 2016 a; El Basuini et al., 2016) due to its high survival rate and low feed conversion ratio (Kim et al., 2012). The rapid development of intensive red sea bream aquaculture has increased occurrences of diseases which lead to significant economic losses (Cerezuela et al., 2012; Hoseinifar et al., 2020). Antibiotics application is a traditional way to better the health of farmed animals; however, it has consequential negative effect on environment causing problem with bioaccumulation and reduce organism's ability to build resistance against pathogens (Akhter et al., 2015; Zaineldin et al., 2018). This indicates the need to assess the efficacy of natural alternatives which can enhance growth response and improve overall condition of aquatic animals (Zaineldin et al., 2021). Functional feed supplement including probiotics have been certified to be useful for improving organism health status through immunomodulation (Dawood and Koshio, 2016; Van Doan et al., 2020).

Functional feeds include probiotic such as microbial cells which are supplied to animal through feed or the rearing water. Probiotics induce beneficial effect on the host organism by enhancing disease resistance, improve growth and improve tolerance to stress as a result of improved microbial balance in the host or improved environmental condition (Merrifield et al., 2010; Ringø et al., 2020; Zaineldin et al., 2021; Lieke et al., 2020). To date, many forms of probiotics were introduced in aquaculture as feed supplements, including bacteria cells of *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Carnobacterium* genera, and yeast (Dawood et al., 2019 b; Chauhan and Singh, 2019; Van Doan et al., 2020). Several reports have confirmed and encourage the role of yeast as both probiotic and prebiotic due to their beneficial effects on aquatic animals (Navarrete and Tovar-Ramirez, 2014; Islam et al., 2021; Jahan et al., 2021; Zhang et al., 2020; Van Doan et al., 2020; Lieke et al., 2020).

Naturally yeast generate biomolecules (Peppler, 1982), and the cell wall comprises specific compounds (Bowman and Free, 2006), useful for animal feed sup-

plement and these compounds are responsible for the differential dietary effect of each yeast strain in animal health (Dimitroglou et al., 2009; Ortuño et al., 2002). β-D-glucans have capacity to regulate living organism's immune system especially in fish and humans (Meena et al., 2013; Taylor et al., 2007; Zhang et al., 2021). The optimum dietary supplementation of baker's yeast cell walls was 0.1–0.2% in Japanese seabass (*Lateolabrax japonicus*) (Yu et al., 2014) and 0.2% in tilapia (*Oreochromis niloticus*) (Abu-Elala et al., 2018). Similarly, inclusion of yeast cell walls at 0.4% in diets of gibel carb (*Carassius auratus gibelio*) showed considerable improvement in growth and immune responses (Zhang et al., 2020). Several studies have reported the potential use of yeast cells as protein source for aquaculture and livestock feeds (Sahlmann et al., 2019; Øverland and Skrede, 2017). However, oleaginous yeast was previously reported to be a potential feed supplement for animals (Overland and Skrede, 2017; Blomqvist et al., 2018) while dietary substitution of fish oil by oleaginous yeast oil has no adverse effect on fish (Blomqvist et al., 2018). The procedure involving the oleaginous yeast fermentation process was described in Takaku et al. (2020), while the general procedure on yeast production from lignocellulose biomass was described in Overland and Skrede (2017). Supplementation of oleaginous yeast improved growth performance and immune response in juvenile red sea bream *Pagrus major* at 1–2.5% of the dietary proportion (Shadrack et al., 2021). In contrast most previous studies on the utilization of probiotic yeast reported beneficial effects at ≤1% of dietary proportion (Yu et al., 2014; Sutthi and Thaimuangphol, 2020; Abu-Elala et al., 2018; Zhang et al., 2020; Islam et al., 2021; Jahan et al., 2021). Thus, it is anticipated that supplementation of spent *L. starkeyi* yeast at low proportion (≤1%) in diet of fish could still induce specific beneficial effects comparable to reports of other related probiotic yeast supplements.

The parameters to be assessed in this investigation in *P. major* includes growth performance, blood chemistry, stress resistance, antioxidant, and immune response.

Material and methods

Ethical statement and yeast product detail

Ethical statement

The rules of Animal Experiment in Kagoshima University does not apply to fish. However, the animal care protocol (number of fish, fish handling, safety of feed ingredients etc.) was considered in this study.

The *Lypomyces starkeyi CB1807* spent yeast dry cells was sourced from Fuji Oil Holdings Inc., Japan. The yeast strain was bred and separated by joint research conducted by Nigata University of Pharmacy and Applied Life Sciences and Fuji Oil Holdings Inc. The spent *L. starkeyi* cells contains 69.26% lipid, 6.80% protein measured with standard AOAC method (AOAC, 1999). The β-glucan content from dry yeast cells was 3.4% of the sample weight, measured using β-glucan commercial kit (Neogen Megazyme Ltd.) in accordance with the guidelines set out by the manufacturer. The dry cells were obtained after the fractional separation of oil, fats, sugar, alcohol, and other residuals. The yeast product was stored in a fridge at temperature below –18°C for further use.

Test diets

Table 1 presents the diet formulation and proximate composition of experimental diets. The dry cells of *L. starkeyi* were incorporated in the diet based on percentage proportion at graded levels of 0% (control diet CD1), 0.05% (D2), 0.1% (D3), 0.5% (D4), and 1.0% (D5). The total proportion was adjusted to 100% by adding α-Cellulose powder. The supplements were thoroughly mixed in lipid ingredients including pollack liver oil, soybean lecithin and n-3 HUFA prior to combining with the main ingredients. All ingredients were mixed with a spatula in an aluminium bowl, followed by another round of mixing for 15 minutes using a food mixer. A 30–40% water was added to the ingredient mixer based on the dry weight, and mixed for another 15 minutes. The mix ingredient was passed through 1.2 mm diameter openings of a meat grinder and cut into pellets. The pellets were oven-dried at 50°C using a mechanical convection oven to 10% moisture content. The test diets were filled into polypropylene bags and kept in a freezer at –28°C for further use.

Experimental trial

The feeding trial was conducted at Kamoike Marine Research facility, Faculty of Fisheries, Kagoshima University, Japan. *P. major* juveniles were collected from a commercial hatchery in Miyazaki prefecture, Japan. Firstly, fish were acclimated to the laboratory conditions for one week while feeding with commercial fish meal diet. Feed trial was done in 100-L polyethylene tanks containing 80 L of seawater connected to a flow-through seawater system. The tanks were equipped with an inlet and outlet valve, allowing for continuous aeration while sweater flow rate supplied to each tank was 1.5 L per minute. The water quality monitored during experiment was as follows: temperature 26.1 ± 1.2°C, pH 8.1 ± 0.5, salinity 33.1 ± 0.5 psu and dissolved oxygen 6.1 ± 0.5 mg/L.

Red sea bream juveniles (n = 300, 1.9 ± 0.03 g) were randomly weighed and placed (20 fish per tank) into 15 experimental tanks of 5 treatment group. Apparent satiation feeding regime was applied twice a day for 45 days. Uneaten feed was collected by one hour after feeding by siphoning and dried in a freeze drier.

Sample collection

The final sampling was conducted at the end of the trial (45 days) after a 24-hour fasting period. 50 ml/L Eugenol (4-allylmethoxyphenol) solution was used to anesthetize the fish before and body weight and length measurements were taken. A total of 5 fish were randomly collected from each tank and stored in a freezer at –20°C for further whole body proximate analysis.

Table 1. Experimental diets ingredients and proximate composition

Ingredients	Experimental diets				
	CD1	D2	D3	D4	D5
Brown fish meal[1]	57	57	57	57	57
Soybean meal[2]	18	18	18	18	18
Pollack liver oil[3]	2	2	2	2	2
Soybean lecithin	2	2	2	2	2
n-3 HUFA[5]	0.5	0.5	0.5	0.5	0.5
Methionine[6]	0.14	0.14	0.14	0.14	0.14
Lysine[7]	0.5	0.5	0.5	0.5	0.5
Taurine[8]	0.09	0.09	0.09	0.09	0.09
Vitamin mix[9]	4	4	4	4	4
Mineral mix[10]	4	4	4	4	4
Vitamin C ester[11]	0.3	0.3	0.3	0.3	0.3
Activated gluten[12]	6	6	6	6	6
α-cellulose[13]	5.47	5.42	5.37	4.97	4.47
Yeast[1]	0	0.05	0.1	0.5	1
Total	100	100	100	100	100
Proximate composition					
Crude protein	51.0 ± 0.6	51.7 ± 0.3	51.9 ± 0.0	51.9 ± 0.2	51.9 ± 0.7
Crude lipid	11.1 ± 1.0	11.2± 0.1	11.1 ± 0.3	12.0 ± 0.2	12.5 ± 0.2
Crude ash	14.0 ± 0.1	14.1 ± 0.1	14.1 ± 0.0	14.1 ± 0.1	14.1 ± 0.1
Carbohydrate[15]	17.4 ± 2.3	18.0 ± 1.4	18.5 ± 0.6	18.0 ± 0.6	15.9 ± 1.1
Gross energy (KJ/g)[16]	19.4± 0.16	19.7 ± 0.12	19.8 ± 0.04	20.1 ± 0.13	19.9 ± 0.09

[1]Nihon Suisan Co. Ltd (Tokyo, Japan); [2]J. Oil Mills, Japan; [3, 4]Riken, Tokyo, Japan; [5]Highly unsaturated fatty acid n-3: (eicosapentaenoic acid) EPA 0.25 g and (docosahexaenoic acid) DHA 0.25; [6, 7, 8]Nacalai Tesque, Inc., Kyoto, Japan; [9]Vitamin mixture, g/kg diet [*β*-carotene, 0.10; Vitamin D3, 0.01; Menadione NaHSO3·3H2O (K3), 0.05; DL-*α*-tocopherol acetate (E), 0.38; thiamine-nitrate (B1), 0.06; riboflavin (B2), 0.19; pyridoxine-HCl (B6), 0.05; cyanocobalamin (B12), 0.0001; biotin, 0.01; inositol, 3.85; niacin (nicotinic acid), 0.77; Ca pantothenate, 0.27; folic acid, 0.01; choline chloride, 7.87; *ρ*-aminobenzoic acid, 0.38; cellulose, 1.92]; [10]Mineral mixture, g/kg diet [$MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate, 12.09; $Al(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; $Ca(IO_3)_2$, 0.01; $CoSO_4$, 0.04]; [11]L-ascrobil-2 phosphates-Mg; [12]A-glu SS-Glico Nutrition Company Ltd. Osaka, Japan; [13]Nippon Paper Chemicals, Tokyo, Japan; [14]Dry yeast, Fuji Oil Holdings Inc., Toray Industries Inc., Japan; [15]Carbohydrate % = 100 – (crude protein % + crude lipid % + crude ash %); [16]Gross energy calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

Fish were randomly selected and blood was collected from the caudal vein using heparinised (fish no. =5) and non-heparinised (fish no. =3) syringes. The micro-haematocrit technique was used to determine haematocrit from heparinised blood. Blood plasma was obtained by centrifugation of heparinised blood samples at 3000 x g for 15 minutes using a high-speed refrigerated microcentrifuge and keep at –80°C. The collected non-heparinised blood samples were allowed to stand at room temperature for 2 hours prior to centrifugation at 3000 × g for 15 minutes for collection of serum.

For viscerasomatic index (VSI) and hepatosomatic indices, 3 fish per replicated tank were dissected and weighed for the calculation. Livers of fish from each tank were pooled and stored at –80°C for further analysis. VSI and HSI were calculated according to the following equation:

$$VSI = (Viscera\ weight/fish\ body\ weight) \times 100$$
$$HSI = (Liver\ weight/fish\ body\ weight) \times 100$$

Skin mucus was randomly collected from 3 fish per tank. Briefly, the skin of individual fish was washed with distilled water. Then a sterilised piece of cotton was rubbed over 200 mm^2 of its body surface. The cotton was immediately suspended in 1 mL phosphate buffered saline (PBS, pH = 7.4) in a 1.5 ml Eppendorf tube. The collection was centrifuged at 2000 x g, 4°C for 10 min and the supernatant was filled into a new Eppendorf tube and stored at –80°C for further analysis.

Growth performance and feed utilization

The calculations of growth responses and feed utilization are as follows:

$$Body\ weight\ gain\ (BWG\%) = [(final\ weight - initial\ weight)/initial\ weight] \times 100$$
$$Specific\ growth\ rate\ (SGR\%) = [(Ln\ (final\ weight) - Ln\ (initial\ weight))/duration\ (45\ days)] \times 100$$
$$Survival\ (\%) = (no.\ of\ fish\ at\ 45\ days/no.\ of\ fish\ at\ the\ beginning\ of\ the\ experiment) \times 100$$

Feed intake (FI, g/45 days) = (dry diet given – dry uneaten recovered diet)/no. of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g)/dry protein intake (g)

Protein gain (PG, g/kg weight gain) = [(final weight, g × final whole body protein content /100) – (initial weight gain, g × initial whole protein body content/100)] × 1000/weight gain (g)

Protein retention (PR, % of intake) = (protein gain, g/kg × 100)/protein intake g/kg

Condition factor (CF) = (fish weight, g/fish length, cm^3) × 100

Proximate contents

The proximate content such as moisture, crude protein, total lipids and crude ash from diets and fish whole body were analysed using standard methods (AOAC, 1990) in triplicate samples. Gross energy was calculated from the combustion value of carbohydrate, protein, and lipid.

Blood and antioxidant capacity

The chemical components of plasma samples were analysed spectrometrically with an automated analyser (Spotchem™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). The biological antioxidant potential (BAP) and by-products of reactive oxygen metabolites (d-ROMs) in plasma samples were determined using an automated analyser (FRAS4, Diacron International s.r.l., Grosseto, Italy). Plasma catalase (CAT) activity was determined according to previous method (Cecchini et al., 2000; Góth, 1991).

Immune responses

The serum and mucus lysozyme (LYZ) activity was measured using the turbidometric assay described by Lygren et al. (2001). In brief, a 10 µl of sample was placed into 96-well microplate and 190 µl of substrate (0.2 mg *Micrococcus luteus*, lyophilized cell, Sigma, USA)/ml PBS (pH 7.4) was added and incubated with gentle shaking at room temperature. The absorbance was read with Multiskan FC (Thermo Fisher Scientific K.K., Japan) at 450 nm after 1 and 5 minutes. LYS amount was calculated as the amount of enzyme causing a decrease in absorbance at 0.001/min. The total immunoglobulin (Ig) from serum was quantified according to method of Siwicki et al. (1994).

The total peroxidase (GPx) from plasma was measured as described by Salinas et al. (2008). In brief, 15 µl of serum was diluted with 35 µl of Hank's buffered salt solution (HBSS) without Ca^{2+} or MG^{2+} in a flat bottom 96 well microplate. Next, a 50 µl of peroxide substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added and incubated for 15 min and the reaction was stopped by adding 50 µl of 2 M sulphuric acid. The optical density of the reaction was taken at 450 nm using a plate reader. PBS was used as a blank instead of serum. For liver tissue, 25 mg sample was homogenized in 250 µl RIPA buffer containing protease inhibitor (0.1%, v/v) and centrifuged at 3000 x g for 10 min at 4°C. The supernatant was collected for the analysis.

The plasma superoxide dismutase (SOD) activity was measured with the SOD assay Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) following the manufacturer's instruction. Plasma malondialdehyde (MDA) was measured using Colorimetric TBARs microplate assay kit (Oxford Biomedical Research, Inc., USA) following the manufacturer's instructions.

Low salinity stress test

Low salinity stress test was conducted at the end of feeding trial using 3 fish per tank to examine the capacity to respond to stress condition. The fish were randomly selected and transferred into a 20L stress glass tank containing dechlorinated (strong aeration for 24 hrs), low salinity (0.2%) water. The stress tank was placed onto a 100L black tank of 50% water lower than the height of stress tank (Dawood et al., 2017 a, b). Continuous water flow was supplied through the black tank to keep the stress tank under ambient temperature. The stress tank was equipped with continuous aeration. The number of dead fish was recorded for each tank every 10 minutes. The time of 50% death was calculated according to Ren et al. (2007).

Statistical analysis

The statistical analysis was conducted using the Paleontological statistical software package for education and data analysis version 3.21 (Hammer et al., 2001). Data normality was checked by Kolmogorov-Smirnov test while homogeneity of variances by Levene's test prior to performing one-way analysis of variances (ANOVA) test. The probability <0.05 was considered significant, and the mean differences were further evaluated using Tukey-Kramer post hoc test. The principal component analysis was conducted using XLSTAT (2019) on standardized data of the important variables assessed in this investigation.

Results

Growth and feed utilization

The growth parameters, nutrient utilization, and survival rate of juvenile red sea bream fed the experimental diets for 45 days are shown in Table 2. The fish group fed on D3 and D5 showed significantly high ($P<0.05$) final body weight (FBW) and BWG% compared to fish fed CD1. FI and PG were numerically higher ($P>0.05$) in fish group fed on D5. Low FCR ($P>0.05$, Table 2) was observed in fish group fed on D2, D4, D5 compared to the control. Survival rate was observed to be the same between all fish groups ($P>0.05$, Table 2).

Body proximate composition and biometric indices

The whole body proximate composition and biometric indices for *P. major* at 45 days were presented in Table 3. No significant difference was detected in whole body proximate composition among fish groups (P>0.05). CF was numerically high in fish fed supplemented diets D2, D4, D5 compared to control (P>0.05).

Blood status

Table 4 presents modulation in blood profile of *P. major* at 45 days of experimental period. Haematocrit was numerically high in fish fed probiotic incorporated diets (D2–D5) than in fish fed the control diets (P>0.05). A significantly higher total cholesterol (T-Cho) was observed in fish fed D3 compared to D2. Similarly, a significantly high total bilirubin (T-Bill) was observed in D3 compared to D4 and D5, and significantly high AST compared to D5 (1%) (P<0.05). Blood urea nitrogen (BUN), alanine aminotransferase (ALT), total glycerides (TG), and total protein (TP) values were not different among fish groups fed the test diets (P>0.05). The lowest value of T-Cho, T-Bill, and AST were recorded in fish groups fed supplemented diet D2, D4, and D5, respectively (P>0.05).

Table 2. Growth parameters, feed efficiency and survival rate of red sea bream fed experimental diets for 45 days

Parameters	Experimental diets				
	CD1	D2	D3	D4	D5
IBW (g/fish)[1]	1.9 ± 0.0	1.9 ± 0.1	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0
FBW (g/fish)[2]	15.45 ± 0.3 a	16.20 ± 0.5 ab	17.2 ± 0.5 b	16.1 ± 0.55 ab	17.55 ± 0.35 b
BWG (%)[3]	588.0 ± 10.0 a	647.5 ± 4.5 ab	689.0 ± 16.64 b	642.1 ± 26.7 ab	730.0 ± 19.0 b
SGR[4]	4.3 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	4.2 ± 0.2	4.3 ± 0.3
FI (g/fish/45 days)[5]	11.37 ± 0.82	11.27 ± 0.52	11.61 ± 0.86	11.22 ± 0.86	12.57 ± 1.14
FCR[6]	1.1 ± 0.08	1.05 ± 0.09	1.11 ± 0.03	1.1 ± 0.12	1.0 ± 0.06
PER[7]	2.6 ± 0.1	2.4 ± 0.2	2.59 ± 0.0	2.51 ± 0.2	2.49 ± 0.1
PG[8]	247.7 ± 34.7 ab	230.8 ± 11.7 b	252.8 ± 17.7 ab	239.6 ± 15.3 ab	272.4 ± 9.0 a
Survival rate (SR%)	90 ± 7.1	95 ± 4.1	95 ± 4.1	91.7 ± 6.2	90 ± 0.0

Values are means ± S.E (n=3). Different letters indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast. [1]IBW (g/fish): Initial body weight; [2]FBW (g/fish/45 days): Final body weight; [3]BWG (%): Weight gain percentage per fish; [4]SGR(%/day): Specific growth rate; [5]FI (g/fish/45 days): Feed intake per fish per day; [6]FCR: Feed conversion ratio; [7]PER: Protein efficiency ratio; [8]PG: Protein gain.

Table 3. Whole-body proximate composition and biometric indices of juvenile red sea bream

Parameter	Experimental diets				
	CD1	D2	D3	D4	D5
Moisture	69.6 ± 1.50	71.0 ± 0.70	69.9 ± 0.80	69.2 ± 0.90	68.6 ± 0.30
Crude protein	17.2 ± 1.10	16.8 ± 0.30	16.6 ± 0.90	16.9 ± 0.30	17.3 ± 0.0
Crude lipid	4.8 ± 0.90	3.5 ± 0.30	4.7 ± 0.70	5.4 ± 0.80	5.3 ± 0.30
Crude ash	4.9 ± 0.10	4.9 ± 0.10	5.1 ± 0.10	5.0 ± 0.10	5.0 ± 0.20
CF[1]	2.03 ± 0.10	2.38 ± 0.10	1.96 ± 0.00	2.12 ± 0.10	2.39 ± 0.00
HSI[2]	1.20 ± 0.40	1.10 ± 0.30	1.10 ± 0.30	1.20 ± 0.30	1.20 ± 0.40
VSI[3]	7.0 ± 0.60	6.80 ± 0.60	7.10 ± 0.70	7.6 ± 1.2	6.7 ± 0.80

Values are means ± S.E (n=3). Different letters indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast. Crude protein, crude lipid, and ash are expressed on a wet weight basis. [1]CF: condition factor; [2]HSI: hepatosomatic index; [3]VSI: viscerasomatic index.

Table 4. Blood health parameters of juvenile red sea bream juvenile fed experimental diets for 45 days

Parameter	Experimental diets				
	CD1	D2	D3	D4	D5
Haematocrit (%)	36.7 ± 1.2	41.0 ± 2.2	42.0 ± 3.3	39.0 ± 5.4	37.0 ± 7.0
Glucose (mg/dl)	66.3 ± 4.1	65.3 ± 4.8	66.3 ± 5.4	66.0 ± 3.6	72.5 ± 0.5
T-Cho (mg/dl)	207.7 ± 34.9 ab	184.3 ± 20.4 b	221.3 ± 7.9 a	211.0 ± 28.2 ab	201.5 ± 11.5 ab
Bun (mg/dl)	13.3 ± 3.7	12.3 ± 2.6	14.3 ± 2.6	14.7 ± 2.1	12.0 ± 2.0
T-Bill (mg/dl)	0.5 ± 0.1 ab	0.5 ± 0.1 ab	0.7 ± 0.2 a	0.4 ± 0.1 b	0.4 ± 0.0 b
AST (IU/L)	131.7 ± 78.2 ab	151.0 ± 22.6 ab	231.7 ± 73.5 a	167.0 ± 32.3 ab	98.5 ± 18.5 b
ALT (IU/L)	33.3 ± 16.0	30.7 ± 11.3	78.7 ± 44.5	36.7 ± 13.0	23.5 ± 5.5
TG (mg/dl)	170.7 ± 69.7	149.3 ± 54.1	193.3 ± 55.2	189.7 ± 41.1	126.5 ± 28.5
TP (g/dl)	3.0 ± 0.3	2.9 ± 0.2	3.3 ± 0.2	2.9 ± 0.1	3.0 ± 0.0

Values are means ± S.E (n=3). Different letters indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast. T-Cho: total cholesterol; Bun: blood urea nitrogen; T-Bill: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: total glycerides; TP: total protein.

Immune response

Figures 1 and 2 present the first line of defence such as LYS and Ig activity in *P. major* fed the test diets at 45 days. The LYS activity in serum, mucus and liver of fish fed on D5 was significantly higher compared to fish fed D2, D3, D4 and the control (P<0.05, Figure 1 A, B, C). The Ig activity was significantly improved in fish fed D5 than control diet (P<0.05, Figure 2) while numerical improvement was observed in D2, D3 and D4 fish group compared to control (P>0.05)

Antioxidant activity

The antioxidant activity of juvenile *P. major* was presented in Table 5, Figure 3 and Figure 4. The lowest values of MDA were observed in fish fed D5 compared to fish fed the rest of supplemented diets and the control, while a significant difference was detected when compared to D3 (P<0.05). The SOD values were significantly improved in D2 fish group compared to D3, however, no significant differences (P>0.05) were detected in the values of SOD between CD1, D2, D4 and D5. The d-ROM values were significantly low in fish group fed D2 and D3 compared to fish fed CD1 (P<0.05). Highest BAP activity was observed in fish group fed D2 compared to all supplement groups and the control (P<0.05). The lowest value of BAP was observed in fish fed D5 but was not significantly different in relation to CD1 (P<0.05). The combined effect of BAP and d-ROM (Figure 3) reflects the balance between antioxidant activity and oxidative stress. Zone A reflects good condition, thus, D2 groups experience low oxidative stress and a high tolerance ability. Zone B reflects the balance between oxidative stress and tolerance ability, thus, D4 exhibit a stress free state. Zone C shows a stabilized condition due to reduced antioxidant activity and oxidative stress, thus, D3 is in a stable state. Zone D reflects high oxidative stress, thus, D5 and CD1 (control) fish group experiences high oxidative stress and low tolerance ability. Figure 4 illustrates catalase (CAT) and peroxidase activities (GPx) of red sea bream after 45 days of feed trial. Elevated plasma CAT was recorded in fish fed D2 compared to D3 and D5 (P<0.05). Meanwhile, liver catalase activity was significantly lower in fish fed D5 compared to CD1, D2, D3 and D4 (P<0.05, Figure 4 B). Serum GPx activity was significantly enhanced in D3 and D4 fish group compared to control fish group (P<0.05, Figure 4 C). Liver GPx activity was not significantly different amongst fish fed all test diets (P>0.05, Figure 4 C, D).

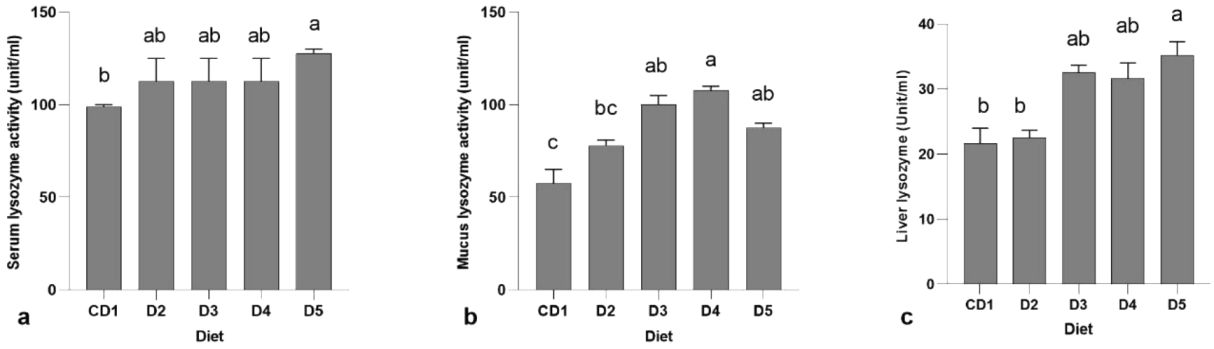


Figure 1. Lysozyme activity of red sea bream juveniles in 45 days of feeding. Lysozyme activity of serum (a), mucus (b) and liver. Values are means ± S.E. Different superscripts indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0. 1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast

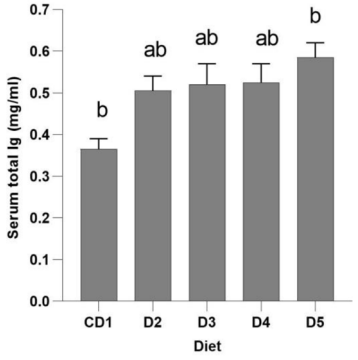


Figure 2. Serum total immunoglobulin (Ig) of red sea bream juveniles in 45 days of feeding. Values are means ± S.E. Different superscripts indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast

Table 5. Antioxidant capacity of juvenile red sea bream fed test diets for 45 days

Parameters	Experimental groupings				
	CD1	D2	D3	D4	D5
MDA (nmol/ml)	161.7±11.25 a	137.9±7.4 ab	171.72±11.15 a	135.72±5.02 ab	117.92±5.59 b
SOD (50% inhibition)	34.8 ± 0.6 ab	46.1 ± 12.2 a	29.5 ± 3.6 b	33.6 ± 0.0 ab	33.2 ± 0.9 ab
d-ROMs (µMol/L)	127.5 ± 0.7 a	89.5± 31.8 d	107 ± 4.2 c	130 ± 3.1 ab	117 ± 3.2 ac
BAP (U. Carr)	759.5±6.5 bc	1393.5±1.5 a	699±6 b	994.5±5.5 b	324.0±1.0 c

Values are means ± S.E (n=3). Different letters indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast.

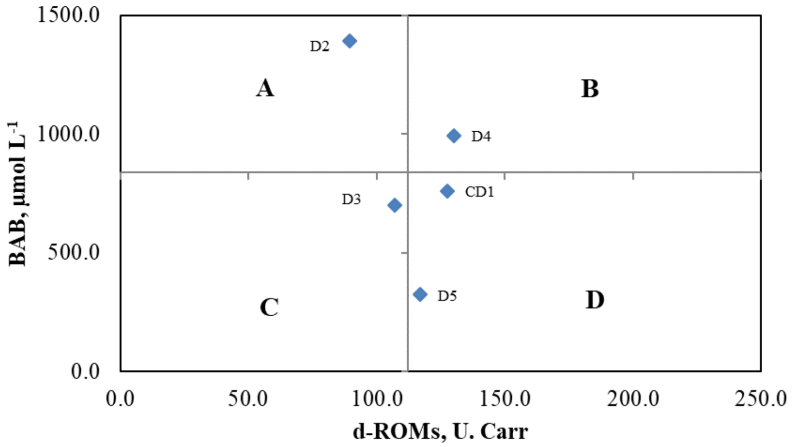


Figure 3. Oxidative condition of red sea bream juveniles in 45 days of feeding. Values represent the means ± SE (n=3). The means of d-ROM and BAP are presented on the central axis. Zone A: high antioxidant capacity and low reactive oxygen metabolites (good health condition); Zone B: high antioxidant capacity and low reactive oxygen metabolite (acceptable health condition); Zone C: low antioxidant potential and low reactive oxygen metabolite (acceptable condition); Zone D: low antioxidant potential and high reactive oxygen metabolite (poor condition). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast

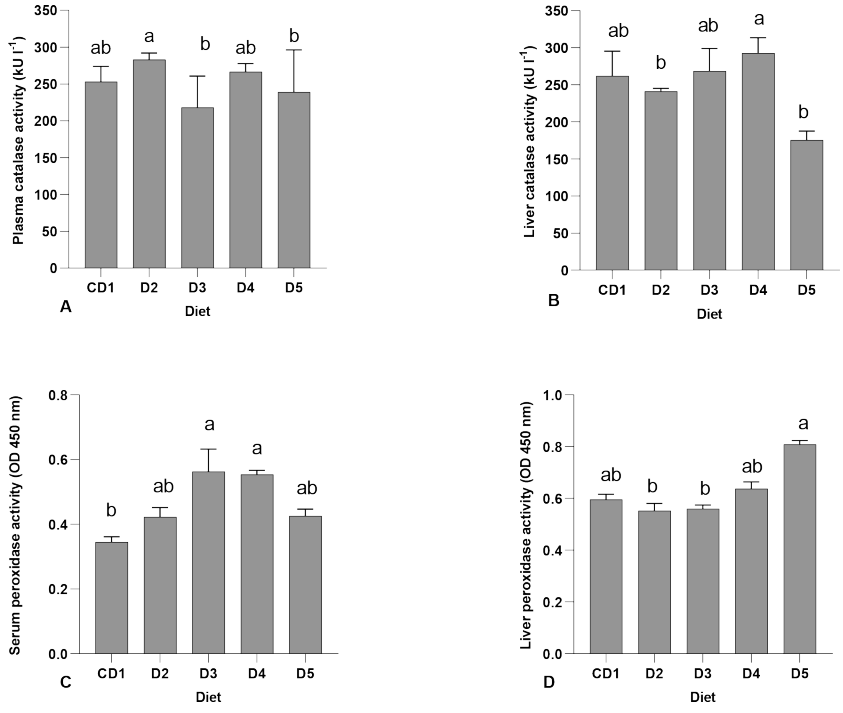


Figure 4. Catalase (kU/l) and peroxidase activities (OD at 450 nm) of red sea bream juveniles in 45 days of feeding. Different superscripts indicate significant differences (P<0.05) between treatment means. Values are means ± S.E (n=3). Different superscripts indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast

Low salinity stress

Time (min) to 50% mortality (LT_{50}) of juvenile *P. major* exposed to low salinity stress after 45 days of feeding is presented in Figure 5. Results showed significant tolerance ability to salinity stress in fish fed compared to control (P<0.05) while numerical improvement was observed in fish fed D2, D3 and D4 (P>0.05, Figure 5).

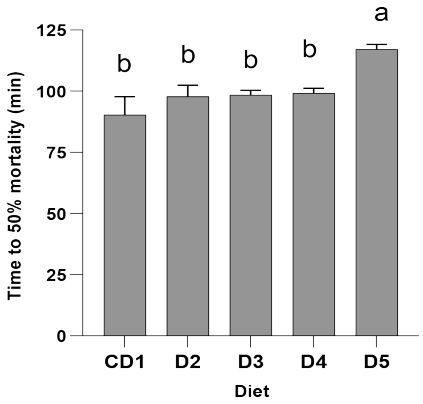


Figure 5. Time (min) to 50% mortality (LT_{50}) of red sea bream juveniles after 45 days of feeding and exposed to low salinity stress condition. Values are means ± pooled S.E. Different superscripts indicate significant differences between treatment means (P<0.05). CD1 = the control or basal diet; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0.1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast

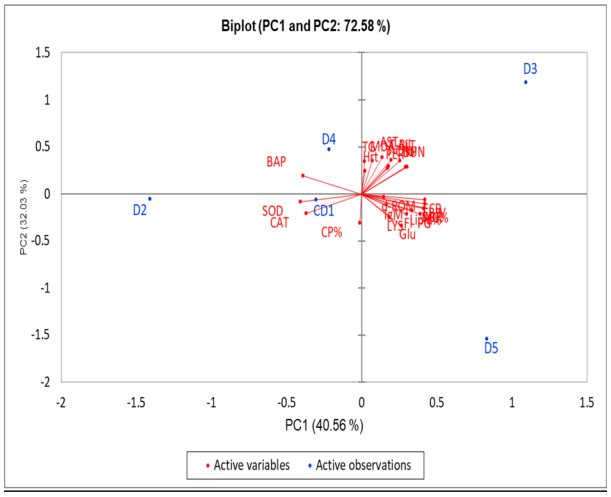


Figure 6. The principal component analysis of parameters of red sea bream juveniles in 45 days of feeding. The PC1 separates the variables horizontally and explained 40.56% of the total variances. The PC2 separates the variables vertically and explained 32.03% of the variance. The variables contributed to the separation are indicated by line and the length of the line indicates the strength of the correlation of variable to PC1 and PC2. CD1 = the control or basal; D2 = basal diet + 0.05% yeast; D3 = basal diet + 0. 1% yeast; D4 = basal diet + 0.5% yeast; D5 = basal diet + 1.0% yeast

The principal component analysis (PCA) of variable distribution

The principal component analysis was conducted to show variable correlation to the test diets and their distribution shown in Figure 6. Variables such FCR, WG%, SGR and FBW showed the highest loading in PC1 (Principal Component 1) while AST, ALT, T-Bil and MDA showed highest positive loading in PC2 (Principal Component 2). The SOD, BAP and CAT showed highest negative loading in PC1 while Glu (Glucose), CP% and Lysozyme (LYS) showed highest negative loading in PC2. The distribution of variable in PCA revealed most parameters were correlated with fish fed diet D3 and D5 while fish fed D4, D2 and CD1 were associated with antioxidant activity. The D4 and CD1 were near the neutral region of PC1 and PC2.

Discussion

Aquaculture is a major panacea to low animal protein for human consumption. Introduction of intensive farming systems has led to improvement in production per unit area on one hand, whilst contributing to high disease incidences and slow growth in farm animals, on the other hand. Recently many approaches have been used to improve growth and wellbeing of the cultured species such as improving nutritional value of feed ingredients (Dossou et al., 2018 a), modernising aquaculture systems (Liang and Chien, 2013; Mansour and Esteban, 2017), and incorporating feed additives (prebiotics, probiotics, immunostimulants, exogenous enzymes, nucleotides hormones and antioxidants) in feed formulation (Dawood et al., 2018). Dietary probiotic supplements improved growth performances and enhanced disease resistance of animals in captivity (Oliva-Teles, 2012). The oleaginous yeast has nutritional derivatives including lipid, glucans and mannooligosaccharides that can be utilized by fish (Blomqvist et al., 2018). Studies have indicated the benefits of β-glucan from yeast cell wall on improving the immune system of fish and its function as barriers to prions (Huyben et al., 2018). Although studies have determined the effects of probiotic yeast supplements in the diet of aquatic animals (Navarrete and Tovar-Ramírez, 2014; Ozório et al., 2012). Apparently there is a dearth of documented information on dietary utilization of low (≤%) levels of *L. starkeyi* in comparison to other yeast strains in aquaculture (Yu et al., 2014; Sutthi and Thaimuangphol, 2020; Abu-Elala et al., 2018; Zhang et al., 2020; Islam et al., 2021; Jahan et al., 2021). To this end, *P. major* was used to test for the specific beneficial effects of ≤1% of spent *L. starkeyi* yeast supplemented in diet of fish on growth and health condition.

The result of growth and feed utilization showed an improved FI for fish fed on D5 (1%) with a lower FCR, suggesting a better nutrient uptake and metabolism. The FBW and BWG% were improved in fish fed supplemented diet D3 and D5 (1%), demonstrating an improved feed utilization and nutrient absorption made possible by improving the gut microflora (Rimoldi et al., 2020). Similarly, gibel carps (*Carassius auratus gibelio*) diets supplemented with yeast cells showed high FBW at 0.4% of the dietary proportion (Zhang et al., 2020). Additionally, the current results are consistent with those of Huang et al. (2020) who reported significant improvement in WG

and FCR of *Lates calcarifer* fed tilapia piscidin 4-expressing yeast.

Blood indices are good reflectors of the fish condition and reflect the responses to stressors and external stimuli (El Basuini et al., 2017, 2016). The blood indices of *P. major* in the present study were within the acceptable limits (Kader et al., 2010; Takagi et al., 2001; Uyan et al., 2007). Low values of triglycerides (TG) were observed in fish fed yeast supplementation which is consistent with Tao et al. (2015) who reported improved blood profile of ruminant calves fed dietary yeast glucan. The lowest plasma AST value was found in fish group fed D5 (1%) while plasma ALT values were lower in yeast supplemented group compared to control. High AST and ALT in blood indicate presence of an enzyme located in the cytoplasm which is released into circulation after liver damage (Nagai et al., 1989; Vermeulen et al., 1992). However, the decrease in AST and ALT for fish in yeast supplemented group suggests a beneficial effect for aquatic animal feeds (Hassaan et al., 2018). Plasma total protein (TP) values were not significantly different and statistically same in fish of all treatment group, which indicates a stable fish health condition for fish. The low plasma cholesterol (T-choc) level in the yeast supplement group may be linked to the effects of the yeast glucan content in the fish diet (Robbins and Seeley, 1977; Tao et al., 2015).

The supplementation of yeast as a functional diet alters the immune response of fish by acting on the immune cells through metabolic, neurological or endocrine pathways (Dossou et al., 2018 b). Phagocytosis activity is the first line of defence mechanism in cells and is expressed by lysozyme activity, thus forming the defence mechanism of non-specific immune responses (Song et al., 2021). Lysozymes are produced by leukocyte cells which helped in the lysis of microbe's cell wall, triggering the activation of the immunity complementary system (Cecchini et al., 2000). The significant improvement in activity of serum, mucus and liver LYS in *P. major* fed yeast supplement diets demonstrates the enhancement of the phagocytosis cells which could be related to ß-glucan content in *L. starkeyi* yeast. ß-glucan supplemented in diet of red sea bream revealed an increase in LYS activity of fish group fed the supplemented diets (Dawood et al., 2017 b). Total Ig in fish blood is a reflector of humoral immunity and is expressed well in blood serum (Yeganeh et al., 2021). The presence of a concentrated Ig in blood serum acts as an immediate protection against pathogen (Magnadottir et al., 2010). The Ig level in serum of some fish species was reported to range between 0.25 and 23.5 mg/ml, and variations could be related to fish size, age, environmental condition, and disease status (Ceusta et al., 2004). The results in the present study showed significant improvement in Ig concentration in fish fed yeast supplement diets which is consistent with previous findings on effects of probiotics supplement (Allameh et al., 2017; Sun et al., 2012) or yeast supplement (Huang et al., 2020). The improved immune response of *P. major* in the present study demonstrates immunomodulatory enhancement by spent *L, starkeyi* product.

The antioxidant defence system in animal is linked with the health status (Martínez-Álvarez et al., 2005). Lack of balance in the antioxidant defence system leads to oxidative stress, causes DNA hydroxylation, protein denaturation, lipid peroxidation, apoptosis, and ultimate cell damage (Hoseinifar et al., 2020). The balance in the antioxidant and oxidative stress is measured by quantifying the derivatives of the reactive oxygen metabolites (d-ROM) and BAP (Mzengereza et al., 2021; Shadrack et al., 2021). Probiotics have been a friendly alternative with promising results on improving antioxidant defence mechanism in fish (Hoseinifar et al., 2020). Dietary yeast glucans are well known for modulating the antioxidant defence mechanism in fish (Rodrigues et al., 2020). This study showed BAP and d-ROM values in fish fed D2 (0.05%), D3 (0.1%) and D4 (0.5%) were improved compared to those in D5 (1%) and the control. The significantly ($P<0.05$) high BAP activity in the D2 and numerically high activity in D4 groups suggest the potential effect of low level (<1%) *L. starkeyi* yeast supplementation in aquatic animals. Lipid peroxidase (MDA) and other important antioxidant enzymes, such as SOD, CAT and GPx were important indicator of fish defence system. Lipid peroxidation of the cell membrane is measured by MDA activity (Aliahmat et al., 2012). The analysis of MDA revealed the lowest value in the yeast supplemented groups except for D3 (0.1%). This result is in accordance with a previous study where it was found that the inclusion of yeast has a defensive influence that reduces the elevated level of MDA for tilapia (Xu et al., 2015). The SOD activity was improved for fish in supplemented group D2 (0.05%) compared to all test diets. A study on supplementation of yeast at 0.6% of the diet for Nile tilapia showed improved activity of SOD, which indicated improved antioxidant status in fish (Xu et al., 2015). Catalase is an antioxidant enzyme that defends cells against a wide variety of toxic substances including chemicals, metabolites and oxidative stress (Singh et al., 2002). Similarly, catalase activity was numerically enhanced in fish group fed yeast supplement compared to the control. Oxidative stress is said to be related to important mechanisms of drug abuse, which in this case correlates to the decline in CAT activity in fishes (Andriamialinirina et al., 2020). The peroxidase activity was also higher in the yeast supplemented groups, indicating the important role in removing excess ROS and stimulating the homeostasis of the cell (Dawood et al., 2019 a, 2016 b). The high CAT and GPx in liver of fish in supplemented group indicate an improved antioxidant defence system (Sohet et al., 2009; Sun et al., 2014).

The ability of fish to tolerate stress is an important nutritional aspect of a functional diet. The challenge test was conducted often to gauge the ability of fish against biological and physical stressors (Barton, 2002). Amongst the stressors, low salinity is an important stressor to assess response of fish to stress and was normally

performed after nutritional trials (Dawood et al., 2017 b; Dossou et al., 2018 a; Hossain et al., 2016). The stress results obtained in the present study indicated an improved stress tolerance ability in fish fed on D5 ($P<0.05$). Improved stress tolerance in fish could be related to efficient utilization of feed as a result of improved in microvilli alignment which allows for better use of nutrient and available energy to synthesis of adrenal steroids against the stressor (Soleimani et al., 2012).

The parameters assessed using PCA revealed two clusters correlated with fish group fed D3 and D5 suggesting that yeast supplement has boosted the growth, feed utilization and immune response of red sea bream. The fish group fed D2 showed a strong association with antioxidant activity. Moreover, this finding suggests that *L. starkeyi* supplement at ≤1% has improved the growth, antioxidant and immune response of juvenile red sea bream.

Conclusion

From the present research, the dietary supplementation (0.05, 0.1, 0.5 and 1% proportion) of spent *L. starkeyi* yeast in *P. major* has significantly improved growth performance and feed utilization with a detectable optimum at 1% proportion. Blood parameters were improved, such as lowering of TG, AST, and T-Cho. The immune responses activity of LYS and Ig were significantly enhanced in fish fed on D5. The antioxidant and stress tolerance ability of fish was also improved in fish fed 0.05% (D2) and 1% (D5) yeast supplement, respectively. Considering the graded level of *L. starkeyi* yeast used, antioxidant activity was improved even at low (0.05%) dietary proportion, suggesting a potential probiotic for improving antioxidant activity in fish.

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Conflict of interest

There is no conflict of interest for authors.

Data availability statement

The corresponding author declares that data supporting the results of the current study are available and may be furnished upon appropriate request.

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