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SINGULAR EFFECTS OF *BACILLUS SUBTILIS* C-3102 OR *SACCHAROMYCES CEREVISIAE* TYPE 1 ON THE GROWTH, GUT MORPHOLOGY, IMMUNITY, AND STRESS RESISTANCE OF RED SEA BREAM (*PAGRUS MAJOR*)

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Abstract

The beneficial effects of *Bacillus subtilis* C-3102 and *Saccharomyces cerevisiae* type 1 were tested in red sea bream (*Pagrus major*) feeds. A basal diet (control) and two other diets were prepared by supplementation with *B. subtilis* C-3102 (PB) or *S. cerevisiae* type 1 (PY). After 60 days, both probiotic-supplemented groups exhibited significant enhancement in growth performance, the protein efficiency ratio (PER), and digestive enzyme secretion (protease and amylase) compared to the control group (P<0.05). The anterior, middle, and posterior parts of the intestines exhibited significantly increased values of intestinal fold height (hF), enterocyte height (hE), and microvillus height (hMV) in fish fed PB- or PY-supplemented diets (P<0.05). Serum peroxidase, anti-protease, and bactericidal activities were enhanced significantly in both probiotic-treated groups compared to the control group (P<0.05). Serum and mucus lysozyme activities improved significantly in the PB group compared to the control group (P<0.05). Catalase activity was also significantly decreased in both probiotic groups, with relatively lower activity observed in the PY group (P<0.05). Both probiotic groups showed considerably increased tolerance to freshwater exposure (P<0.05). In conclusion, *B. subtilis* C-3102 and *S. cerevisiae* type 1 can be used as functional probiotics to enhance the growth performance, digestion capacity, gut morphology, immune response, and stress resistance of the red sea bream with relatively higher efficiency by *B. subtilis* C-3102.

Key words: *Bacillus subtilis, Saccharomyces cerevisiae,* growth performance, immunity, red sea bream

The application of antibiotics in aquaculture has resulted in direct and indirect adverse effects, including environmental bioaccumulation and decreasing resistance against pathogens (Akhter et al., 2015; Zaineldin et al., 2018). Thus, the testing of several natural alternatives that can safely replace antibiotics and exhibit beneficial roles in aquatic animals is urgently needed (Dawood and Koshio, 2016). Probiotics, prebiotics, synbiotics, medicinal plants, immunostimulants, and many other feed additives have been applied and have resulted in healthy and productive aquatic organisms (Van Doan et al., 2019).

Probiotics can be considered as alive, dead or component of a microbial cell which when administered through the feed or to the rearing water, benefit the host by improving disease resistance, health status, growth efficiency, feed utilization, stress response, or overall vigor, which is achieved at least in part via enhancing the host's microbial balance or the microbial balance of the ambient environment (Merrifield et al., 2010; Ringø et al., 2020). Different forms of probiotics have been introduced for aquaculture, including bacterial cells (*Bacillus* sp., *Enterococcus* sp. and *Lactobacillus* sp.) and yeasts (*Debaryomyces* and *Saccharomyces*) (Dawood et al., 2019 b; Gatesoupe, 1999). Many reports have confirmed that the application of probiotics is highly encouraged due to the roles played by probiotics in modulating growth performance, food digestion, immune response, and disease resistance in aquatic animals (Adel et al., 2017; Dossou et al., 2018 a, b).

Many *Bacillus subtilis* strains have been used intensively via oral application in aquatic animals (Elsabagh et al., 2018; Guardiola et al., 2016; Liu et al., 2017). *B. subtilis* C-3102 provided a probiotic effect leading to improved growth performance and feed efficiency due to the manipulation of intestinal microflora (Hooge et al., 2004; Jeong and Kim, 2014). The primary mode of action of *B. subtilis* C-3102 spores seems to be in their ability to form an anaerobic environment among the intestine after germination, that has been hypothesized to favor growth and proliferation of native microfloral lactobacilli, which may result in competitive colonization exclusion of pathogenic microorganisms, and production of lactic acid to regulate and limit pathogenic microorganism within the intestine (Jeong and Kim, 2014).

Some strains of *Saccharomyces cerevisiae* are among the well-known beneficial microorganisms. The efficacy of these strains is dependent on functional components such as β-glucans, nucleic acids, and mannan oligosaccharides, and chitin (Dimitroglou et al., 2009; Ortuño et al., 2002). Yeast additionally produces different metabolites, like enzymes, oligosaccharides, amino acids, peptides, organic acids, vitamins, and alternative soluble factors (Peppler, 1982). The beneficial strains of *S. cerevisiae* can positively affect the growth performance, immune response, and disease resistance of different cultured fish species (El-Boshy et al., 2010; Li et al., 2004; Ortuño et al., 2002). *S. cerevisiae* type 1 is slowly dried during its production to prevent enzyme degradation, making it more stable and efficient than other yeast strains (Kurtzman et al., 2011). Studies have not been previously performed to the best of our information to reveal the efficaciousness of feeding aquatic animals with *S. cerevisiae* type 1 yeast.

The most applicable forms of probiotics are beneficial bacterial cells and yeast. Each of them has a specific mode of action associated with the active compounds

that can be secreted and formed in the targeted organism (Gatesoupe, 1999; Soltani et al., 2019). Supplementation of *B. subtilis* C-3102 improved the growth, feed utilization, health conditions, and immune responses in red sea bream (*Pagrus major*) (Zaineldin et al., 2018). Based on that, this research was performed to examine the beneficial effects of *B. subtilis* C-3102 or *S. cerevisiae* type 1 on the growth performance, gut morphology, digestive enzyme activity, immune response, oxidative status, and resistance against environmental stressors of red sea bream. The obtained results will be of benefit for the development of the red sea bream aquaculture industry.

Material and methods

Fish and the experimental program

The experiment was done at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Juveniles of red sea bream were collected from a local private fish hatchery (Ogata Suisan Co., Kumamoto, Japan). For two weeks, the fish were raised under laboratory conditions in a 500-L tank for acclimatization. During the acclimatization period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. Each tank was fitted with an inlet and outlet; the tanks were continuously aerated and maintained under a natural light/dark regime. Twenty fish with an average initial weight of 16 g were assigned randomly to nine (3 tanks × 3 treatments) 100-L polycarbonate tanks previously prepared. The fish were fed the test diets to visual satiation for 60 days by hand twice a day at 9:00 and 16:00 h. All fish were weighed in bulk every two weeks to monitor the growth, and therefore the health condition of the fish was visually checked. The water quality parameters throughout the feeding trial were reported as follows: water temperature, 20.8 ± 1.9°C; pH 8.1 ± 0.7; and salinity, 33.5 ± 0.5 PSU (practical salinity unit).

Ingredient and experimental diets

Basal diet had no probiotic additives (control), and two other test diets were formulated to contain 1 g per kg diet *B. subtilis* C-3102, and *S. cerevisiae* type 1 (PB and PY, respectively) were formulated to compare the beneficial effects of *B. subtilis* (Calsporin, *Bacillus subtilis* C-3102, live spore = 1×10^{10} colony forming units (CFU) per g) obtained from Calpis Co., Ltd. (Tokyo, Japan) and *S. cerevisiae* type 1 obtained from Sigma Aldrich (Table 1). Dietary components were adequately mixed in a food mixer for 10 min. The mixture was then passed through a meat grinder (Royal, Tokyo, Japan, type 22VR–1500) with an appropriate diameter (1.6–2.1 mm) to prepare pellets under room temperature. New test diets were made every two weeks to maintain the actual bacterial and yeast numbers incorporated in the experimental diets. The pellets were air-dried in a dry air mechanical oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50°C for 3 hours then stored in a freezer at –20°C until use. *B. subtilis*-selective medium (polypeptone yeast agar medium) was used to detect the actual counts of *B. subtilis* within the feed. The medium contained 1% polypeptone, 0.5% dry yeast, 2% agar, and 1% NaCl (Salma Zohora et al., 2011). In 1 ml of phosphate-buffered saline (PBS, pH=7.4), about 0.1 g of feed was homogenized. Se-

rial dilutions were prepared and distributed onto plates of *B. subtilis*-selective media, which were then incubated at 30°C for 2–3 days. The levels of *S. cerevisiae* type 1 in the diets were confirmed by culturing the dilutions on YPD agar plates. The medium contained 2% peptone, 1% yeast extract, 2% glucose, and 1.5% agar (Ausubel et al., 1998). The actual counts of *B. subtilis* in the diet were 4.88×10^{9} CFU g^{-1}, and the actual counts of *S. cerevisiae* type 1 were 5.19×10^{5} CFU g^{-1}.

Table 1. Dietary formulation and proximate composition of the basal diet

Ingredients	(%)	Proximate composition (% dry matter basis)	
Fishmeal (67%)[1]	30.5	Protein	52.7
Soybean meal (47%)[2]	45.15	Lipid	10.7
Soybean lecithin[3]	3	Ash	11.5
Pollack liver oil[4]	5.5	GE (KJg^{-1})[12]	19.1
Vitamin mixture[5]	3		
Mineral mixture[6]	3		
Stay-C[7]	0.3		
Activated gluten[8]	5		
Amino acid premix[9]	3.05		
α-Cellulose+ PB[10] or PY[11]	1.5		

[1-9] According to Zaineldin et al. (2018); [10]Calsoporin: (*Bacillus subtilis* C-3102, live spore = 1×10^{10} CFU g^{-1}) was supplied by Calpis Co., Ltd (Tokyo, Japan). [11]YSC1 (yeast *Saccharomyces cerevisiae* type 1) was supplied by Sigma Aldrich Co., St. Louis, MO, USA. [12] Growth energy was calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5, and 17.2 kJ/g, respectively and carbohydrate was calculated by the difference: 100- (protein + lipid + ash).

Sample collection

Fish sampling was performed 24 h after the last ration. The body weights of the fish were measured individually in each tank at the beginning, after one month, and at the end of the trial. The growth and feed utilization parameters [weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion efficiency (FCE), and protein efficiency ratio (PER)] were calculated using the following formulae:

$$WG\ (\%) = (FBU - IBU) \times 100/IBW$$
$$SGR\ (\%\ per\ day) = \{Ln\ (FBW) - Ln\ (IBW)/duration\} \times 100$$
$$Survival\ (\%) = 100 \times (final\ no.\ of\ fish/initial\ number\ of\ fish)$$
$$FI\ (g\ fish/60\ days) = (dry\ diet\ given - dry\ recovered)/number\ of\ fish$$
$$FCE = live\ WG\ (g)/dry\ feed\ intake\ (g)$$
$$PER = live\ WG\ (g)/dry\ protein\ intake\ (g)$$

where: *IBW* is the initial body weight (g), and *FBW* is the final body weight (g).

Three fish were randomly sampled from each treatment replicate tank, and blood was collected from these fish by puncture of the caudal vein using heparinized (1600 IU per ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled.

Also, non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit, while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min at 4°C and then stored at –80°C until analysis.

Using sterilized cotton, skin mucus was collected from the body surface (200 mm^2) and suspended immediately in 1 ml of PBS (pH=7.4) and centrifuged (2000×g, 10 min, 4°C) (MX-160, Tomy Seiko Co., Ltd., Tokyo, Japan). The tube supernatant was moved into another centrifuge tube (510-GRD, QSP, San Diego, CA, USA) and held at a temperature of –80°C until further analysis was carried out.

Digestive enzyme analysis

Digestive tracts were washed and separated. Midguts and hindguts were cut into small pieces, pooled together, and stored at –80°C for the digestive enzyme measurement. Amylase activity was determined according to Worthington (2011). The following formula was used to identify specific enzyme activity: specific enzyme activity (U) = µmol maltose released/mg enzyme in reaction mixture × 3 min. The protease enzyme activity was determined using a non-specific protease activity assay from Sigma (Cupp-Enyard, 2008). In this method, proteases split peptide bonds, and casein acts as a substrate. Lipase activity was assayed according to Mustafa et al. (2016). Experiments were carried out in triplicate, and the standard deviations from the mean values were calculated.

Intestinal microbial count

At the end of the experiment, after 24 h cessation of feeding, three fish from each tank were sampled randomly to enumerate the total intestinal microbiota and determine the survival of *B. subtilis* and *S. cerevisiae* in the red sea bream gastrointestinal tract (GIT). The fish GITs were sampled according to He et al. (2009). Samples were diluted serially ten times with PBS, and 100 µl of the solution was distributed over triplicate TSA plates (Trypto-Soya agar, Nissui Pharmaceutical Co., Ltd., Japan) to assess total bacterial counts. *B. subtilis*-selective medium containing 1% polypeptone, 0.5% dry yeast, 2% agar, and 1% NaCl were used to detect viable *B. subtilis*. Also, YPD agar plates were used to determine viable *S. cerevisiae* in the GITs. After the inoculation of each dilution in the agar plates, they were incubated for 3 to 5 days at 25°C. CFU ml^{-1} values were detected for viable bacterial counts (Moe et al., 2004).

Intestinal morphology analysis

Three fish from each replicate tank were used to study the intestinal morphology. For the intestinal sample preparation, the whole gastrointestinal tract (GIT) was removed, divided into the esophagus, stomach, pyloric caeca, and intestine. After that, the intestinal part was collected and divided into three regions, the proximal, middle, and distal regions. All tissues were washed twice with PBS (pH=7.4) and fixed immediately in Davidson's solution (agitated for 5 min) for 8 h.

Fixed tissues were then gradually dehydrated in ethanol (70 to 100%), cleaned twice with xylene (1 and 2 h), and embedded in paraffin. Sections of 5-µm thickness

were collected and stained with hematoxylin and eosin (H&E). Two cross-sectional slices were prepared from each tissue. The tissue slices were stained with H&E and then examined under a light microscope (Eclipse 50i; Nikon, Tokyo, Japan) and camera (Digital Sight DS2MV with a DS-L2 control unit, Nikon) by using Sigma Scan Pro 5 software. Intestinal fold height (hF), enterocyte height (hE) and microvillus height (hMV) were measured using ImageJ analysis software with magnifications of 100×, 200× and 400×, respectively. For each tissue, 10 measurements were obtained, according to the method described by De Los Santos et al. (2007).

Biochemical and blood analyses

The experimental diets were analyzed for moisture, crude protein, total lipid, and ash content in triplicate using standard methods (AOAC, 2007). The hematocrit was determined using the micro-hematocrit technique. Using commercial reagent kits (Arkray, Inc., Kyoto, Japan), the chemical parameters of plasma, including glucose, blood urea nitrogen, total bilirubin, glutamyloxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), triglyceride, total cholesterol, and total protein levels, were measured by an automated analyser (SPOTCHEM™ EZ model SP-4430, Arkray, Inc., Kyoto, Japan) following the manufacturer's protocol (Tatsumi et al., 2000). Blood plasma was also used to measure the biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) levels spectrophotometrically with an automated analyser (FRAS4; Diacron International s.r.l., Grosseto, Italy) according to the method described by Morganti et al. (2002).

Evaluation of immunological parameters

Lysozyme activity in serum and mucus was measured with turbidimetric assays (Lygren et al., 1999). An enzyme activity unit was defined as the amount of enzyme that produced a decrease in absorbance of 0.001/min. The activity of total peroxidase in the serum and mucus was measured by following Salinas et al. (2008). Bactericidal activities in serum and mucus were measured as described by Iida et al. (1989). The bactericidal activity definition was as follows: (CFU of blank group – CFU of each group)/CFU of blank group × 100.

The activity of anti-protease was measured by following Hanif et al. (2004). The trypsin inhibition percentage was calculated as follows: anti-protease activity (%) = (A1-A2 /A1) × 100. A1 is the control trypsin activity (without serum), and A2 is the residual trypsin activity after serum addition. The catalase (CAT) enzyme activity was determined by using a spectrophotometric method, according to Goth (1991).

Freshwater stress test

Five fish were randomly selected from each rearing tank and transferred to 9 blue-colored 20-L rectangular glass aquariums (three replicates) filled with 15 L tap water, which was aerated one day before. The tanks were fitted with continuous aeration and maintained during the stress test under ambient temperature. The duration for 50% mortality (LT_{50}) of red sea bream was determined, according to Dawood et al. (2015). Higher values indicate greater tolerance in the freshwater test.

Statistical analysis

All data were statistically checked using one-way ANOVA (Super ANOVA 1.11, Abacus Concepts, Berkeley, CA, USA). P<0.05 probabilities were considered significant. Significant differences between means were analysed using the Tukey-Kramer test.

Results

Growth performance and feed utilization

No significant (P>0.05) differences were detected in the growth performance, feed efficiency, and survival rate among the tested groups after 30 days of the feeding trial. On the day 60, the final body weight, WG, SGR and PER were significantly (P<0.05) improved in fish fed *B. subtilis* compared to controls, with relatively higher values in fish fed diets supplemented with *S. cerevisiae* type 1 (Table 2). FI and survival rates showed no significant differences (P>0.05) among the tested groups.

Digestive enzyme activities

Protease activity was significantly enhanced (P<0.05) in the fish fed *B. subtilis* compared to the control group, but there was no difference compared to the PY group (Table 3). Additionally, amylase activity was significantly enhanced in both probiotic-treated groups (PB and PY) compared to the control (P<0.05), with no differences between the PB and PY groups. Lipase activity showed no significant (P>0.05) difference between the tested groups and the control (Table 3).

Intestinal microbial count

After 60 days, the total bacterial counts in guts of red sea bream fed dietary probiotics showed a numerical increase compared to those in the guts of fish fed the control diet, but no statistical significance was reported (P>0.05). Neither *B. subtilis* nor *S. cerevisiae* was found in fish fed the control diet. *B. subtilis* was determined in fish supplied *B. subtilis* C-3102, and *S. cerevisiae* was determined in fish fed *S. cerevisiae* type 1 (Table 4).

Intestinal morphology analysis

The anterior, middle, and posterior parts of the intestine exhibited significantly (P<0.05) increased hF values in fish fed PB or PY. Simultaneously, the PB group showed higher values than the PY group. On the other hand, the hE values in fish fed PB and PY increased significantly compared to the control group (P<0.05), and without any difference observed between the probiotic groups (Table 5).

The hMV values were significantly (P<0.05) higher in the anterior parts of fish fed PB or PY probiotics than in those of the control fish, but there was no difference between the probiotic groups. In the middle intestine, the hMV significantly (P<0.05) increased in fish fed PB compared to the control, but there was no difference compared to the PY group (Table 5). In the distal part of the intestine, no significant differences were observed in the hMV among the groups (P>0.05).

Table 2. Growth performance of red sea bream fed test diets for 30 and 60 days

Parameters	After 30 days			After 60 days		
	control	PB	PY	control	PB	PY
Initial body weight (g)	16.03±0.3	16.03±0.3	15.9±0.5	16.03±0.3	16.03±0.3	15.9±0.5
Final body weight (g)	38.1±0.44	40.35±0.24	37.05±0.8	68.05±1.14 a	74.2±1.03 b	70.7±0.7 ab
Weight gain (%)	137.97±6.8	151.8±2.9	133.57±5.3	324.7±3.1 a	363.5±13.5 b	345.6±5.5 ab
Specific growth rate (% day^{-1})	1.34±0.04	1.42±0.02	1.3±0.03	2.2±0.015 a	2.36±0.03 b	2.3±0.03 ab
Feed intake (g $fish^{-1}$ 60 $days^{-1}$)	26.3±0.19	27.76±0.9	26.9±0.22	57.01±1.15	59.85±1.8	58.08±4.3
Feed conversion efficiency	0.84±0.03	0.88±0.027	0.79±0.023	0.9±0.02	0.97±0.01	0.9±0.04
Protein efficiency ratio	–	–	–	1.68±0.04 a	1.8±0.02 b	1.78±0.02 ab
Survival rate	–	–	–	91.7±1.7	95±2.9	95±2.9

Values are means of triplicate groups' ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

Table 3. Specific activities of digestive enzymes of red sea bream fed test diets for 60 days

Parameters	Test groups		
	control	PB	PY
Protease (unit mg^{-1})	0.18±0.02 a	0.28±0.02 b	0.22±0.002 ab
Lipase (unit mg^{-1})	1.9±0.2	2.1±0.1	1.95±0.2
Amylase (unit mg^{-1})	2.5±0.002 a	3.55±0.23 b	3.5±0 .001 b

Data represent means± pooled SEM. Values with different letters are significantly different (P<0.05). Values with the same letter are not significantly different (P>0.05).

Table 4. Intestinal microbial counts (CFU /g intestine) of red sea bream fed test diets for 60 days

Parameters	control	PB	PY
Total bacterial count	(3.76±0.57) $\times 10^7$	(5.8±1.15) $\times 10^7$	(4.64±0.55) $\times 10^7$
B. subtilis	Nil[1]	(3.87±0.47) $\times 10^5$	Nil[1]
S. cerevisiae	Nil[1]	Nil[1]	(3.6±1.5) $\times 10^3$

[1]Nil means not existed.

Table 5. Micromorphology of the intestine of red sea bream fed test diets for 60 days

Item		Test groups		
		control	PB	PY
Proximal	hF (μm)[1]	1007.7±70.6 a	1366.9±35.6 b	1187.7±64.2 ab
	hE (μm)[2]	89.1±3.8 a	152.2±4.8 b	141.4±1.6 b
	hMV (μm)[3]	12.7±0.6 a	18.2±1.2 b	17.05±0.6 b
Middle	hF (μm)	697.3±2.35 a	1098.4±7.6 b	1131.3±16.4 b
	hE (μm)	91.6±1.7 a	153.4±2.9 b	148.2±4.8 b
	hMV (μm)	12.3±1.1 a	17.2±1.4 b	15.3±0.4 ab
Distal	hF (μm)	667.4±10.8 a	996.9±33.7 b	1020.1±13.9 ab
	hE (μm)	85±1.6 a	154.4±4.07 b	148±10.4 b
	hMV (μm)	13.9±1.6	15.5±0.24	16.7±1.02

Data represent means± pooled SEM. Values with different letters are significantly different (P<0.05). Values with the same letter are not significantly different (P>0.05).
[1]hF, fold height; [2]hE, enterocyte height; [3]hMV, microvillus height.

Blood biochemical parameters

No significant differences were observed in the blood parameters (Table 6). The total cholesterol levels, haematocrit, and total bilirubin levels were numerically higher in fish fed diets supplemented with *B. subtilis* C-3102. The GOT and GPT levels were not significantly influenced by dietary *B. subtilis* and *S. cerevisiae* (P>0.05).

Oxidative status parameters

The oxidative status and combined effect patterns of the BAP and d-ROMs of red sea bream fed the experimental diets are presented in Figure 2 and Table 6. Dietary supplementation with *B. subtilis* C-3102 significantly (P<0.05) enhanced the BAP value compared to that of the control group, with no difference compared to the PY

group (Table 6). On the other hand, no differences were detected for the d-ROMs of the fish (P>0.05). The combined pattern of BAP and d-ROMs showed that fish fed *B. subtilis* C-3102 or *S. cerevisiae* type 1 exhibited a good oxidative status and an acceptable condition, respectively, while fish fed the control were stressed (Figure 1).

Table 6. Blood biochemical parameters of red sea bream fed test diets for 60 days

Parameters	Test groups		
	control	PB	PY
Haematocrit (%)	34±1	35.3±0.3	34±0.6
T-Cho (mg/dl)[1]	239±2	244±1	239.5±0.5
T-Bill (mg/dl)[2]	0.25±0.05	0.45±0.15	0.25±0.05
BUN (mg/dl)[3]	7±0	6.5±0.5	6.5±0.5
GOT (IU/l)4	160.5±50.5	166±37	230.5±10
GPT (IU/l)5	10±0	10±0	16±6
Triglyceride (mg/dl)	252±14	256±25	249.5±32.5
Glucose (mg/dl)	58.5±3.5	55.5±2.5	53.5±2.5
Total protein (g/dl)	4.05±0.2	3.75±0.03	4±0.29
BAP[6]	2517±47.4 a	2873±106.8 b	2650.7±53.2 ab
d-ROMs[7]	84±18.5	80.3±10.9	79.3±18.6

Data represent means ±pooled SEM. Values with different letters are significantly different (P<0.05). Values with the same letter are not significantly different (P>0.05).

[1]T-Cho: total cholesterol, [2]T-Bill: total bilirubin, [3]BUN: blood urea nitrogen, [4]GOT: glutamyl oxaloacetic transaminase, [5]GPT: glutamic-pyruvate transaminase, [6]BAP: biological antioxidant potential; [7]d-ROMs: reactive oxygen metabolites.

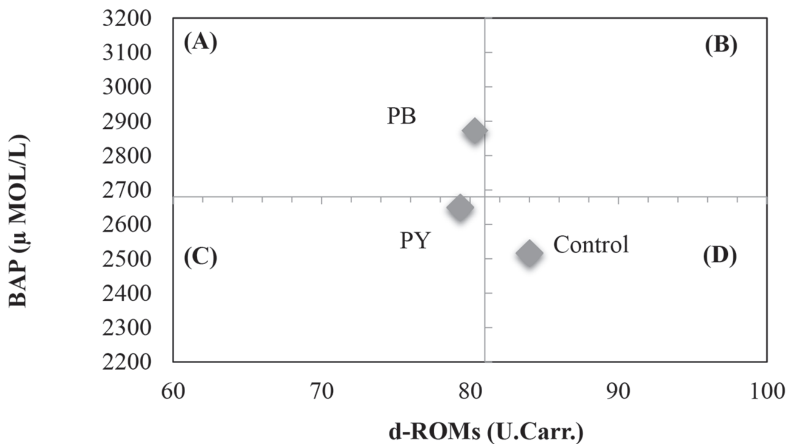

Figure 1. Oxidative status in red sea bream fed test diets for 60 days. Values are expressed as mean ± SEM. Central axis based on mean values of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) from each treatment. Zone (A): high BAP and low d-ROMs (good condition); Zone (B): high BAP and high d-ROMs (acceptable condition); Zone (C): low BAP and low d-ROMs (acceptable condition); Zone (D): low BAP and high d-ROMs (stressed condition)

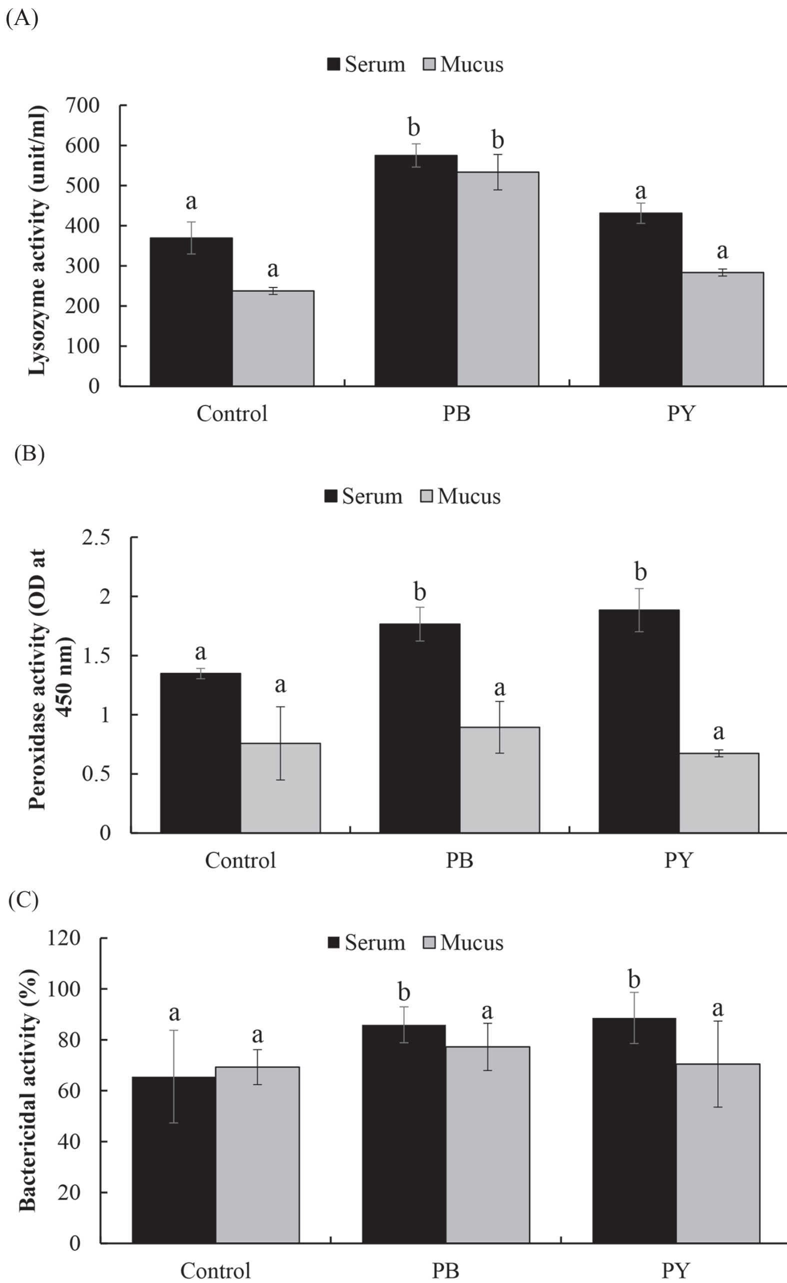

Figure 2. Lysozyme activity (unit ml^{-1}) (A) peroxidase activity (OD at 450 nm) (B) and bactericidal activity (%) (C) of serum and mucus in red sea bream fed test diets. Data represent means ± pooled SEM. Values with different letters are significantly different ($P<0.05$). Values with the same letter are not significantly different ($P>0.05$)

Immune responses

Fish fed *B. subtilis* showed significantly enhanced serum and mucus lysozyme activities compared to the control and PY groups (P<0.05; Figure 2 A). The serum peroxidase activity increased significantly (P<0.05) in fish fed *B. subtilis* C-3102 or *S. cerevisiae* type 1 compared to the control, while no significant difference on activity was observed in the mucus samples (Figure 2 B). Furthermore, the serum bactericidal activity was significantly (P<0.05) increased in the fish fed *B. subtilis* compared to the control group, with no difference compared to the PY group, while no activity was demonstrated in the mucus samples (Figure 2 C). The serum anti-protease activity was significantly (P<0.05) boosted in fish fed *B. subtilis* C-3102 compared with the control group, with no difference compared to the PY group (Figure 3). Surprisingly, catalase activity was significantly (P<0.05) decreased in fish fed *S. cerevisiae* type 1 and *B. subtilis* C-3102 compared to the control group (Figure 4).

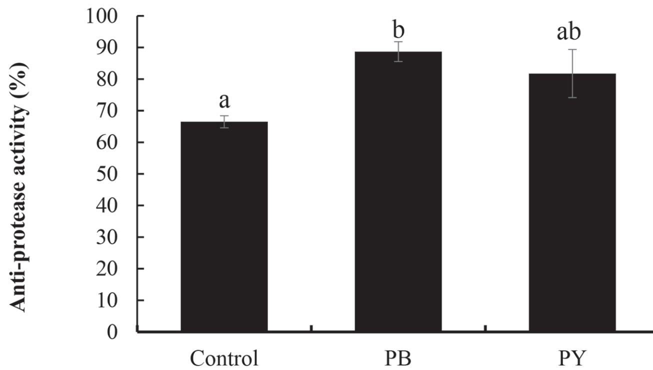

Figure 3. Serum anti-protease activity (%) of red sea bream fed on experimental diets for 60 days. Data represent means± pooled SEM. Values with different letters are significantly different (P<0.05). Values with the same letter are not significantly different (P>0.05)

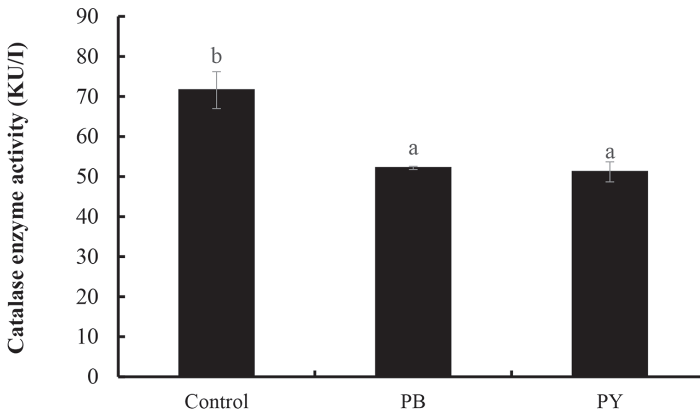

Figure 4. Serum catalase activity (KU/I) in red sea bream fed test diets. Data represent means± pooled SEM. Values with different letters are significantly different (P<0.05). Values with the same letter are not significantly different (P>0.05)

Freshwater stress tolerance

The LT_{50} value was significantly ($P<0.05$) higher in fish fed *B. subtilis* C-3102 compared to the control group, with no difference compared to the PY group (Figure 5).

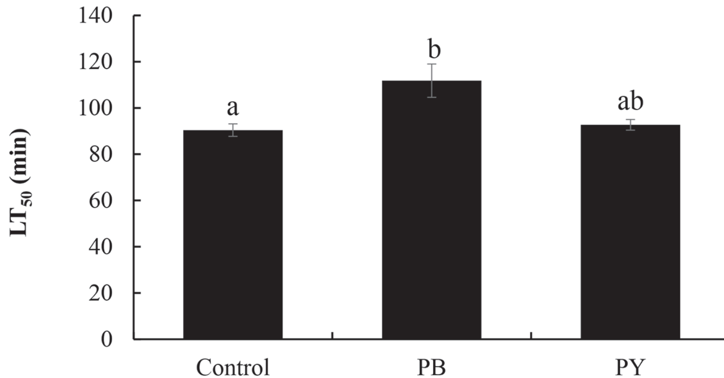

Figure 5. LT_{50} (min) calculated from the lethal time of juvenile red sea bream exposed to fresh water. Data are expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different ($P<0.05$)

Discussion

The use of probiotics and associated products is considered a practical approach to decreasing antibiotic usage in aquaculture (Carbone and Faggio, 2016; Kothari et al., 2019). *B. subtilis* has been used to improve several fish species' performance, including red sea bream (Zaineldin et al., 2018). However, there is no information regarding the effect of *S. cerevisiae* type 1 on the performance of red sea bream.

In the current study, the parameters FBW, WG, SGR, and PER were enhanced in red sea bream fed probiotic-supplemented diets for 60 days. The obtained results are consistent with previous studies on red sea bream or Nile tilapia (Pinpimai et al., 2015; Zaineldin et al., 2018). The improved growth performance observed with both types of probiotics might be ascribed to the enhanced activities of intestinal digestive enzymes and beneficial microbes, resulting in rapid digestion (Suzer et al., 2008).

Bacillus species can participate in digestive processes by producing extracellular enzymes (amylases, proteases, and lipases) (Sun et al., 2010; Zaineldin et al., 2018). In this study, *S. cerevisiae* type 1 showed a similar mechanism, increasing the secretion of extracellular enzymes, especially proteases and amylases, which might have improved nutrient digestibility, leading to improved growth performance and feed efficiency in red sea bream. Similar observations have also been documented for other fish species, which exhibited increased feed utilization with probiotic-based diets (Dawood et al., 2019 c; Talpur et al., 2014).

The absorption capability of the intestinal surface subsequently increased with increasing intestinal villus length, improving dietary nutrient utilization, and ultimately improving growth performance (Pirarat et al., 2011). The intestinal morphometric results in the present study revealed significant improvement in the groups fed a *B. subtilis* C-3102- or *S. cerevisiae* type 1-supplemented diet. The proximal and middle hE, hF and hMV values were significantly high in fish fed probiotic-based diets, whereas the distal parts showed increased hE and hF values only. This result was consistent with previous studies that demonstrated positive effects on fish's gut morphology after probiotic feeding (Cerezuela et al., 2012; Merrifield et al., 2011; Pirarat et al., 2011). The beneficial effects of yeast on intestinal morphology in the present study might be due to the presence of mannooligosaccharides (MOS) that are found in the yeast cell wall. The results showed an improvement in the morphology of villi and microvilli in red sea bream fed dietary *B. subtilis* C-3102 or *S. cerevisiae* type 1. Concurrently, MOS may prevent harmful bacteria from becoming attached to mannose residues on intestinal epithelial cells, thereby alleviating atrophy of villi and microvilli due to inflammation caused by pathogens and associated toxins (El-Boshy et al., 2010; Ran et al., 2015).

The blood parameters of fish are essential tools for the detection of abnormalities associated with nutritional strategies, environmental stressors, diseases, and immunological responses (Abdel-Daim et al., 2019; Dawood et al., 2017; Faggio et al., 2014). The blood parameters obtained in the present study are considered to be within the normal range for red sea bream based on previous findings (El Basuini et al., 2017). The glucose content, haematocrit, total cholesterol content, and total bilirubin content were not significantly affected in the fish fed experimental diets. However, the bacteria-based probiotic group showed numerically high values of haematocrit, total cholesterol content, and total bilirubin content, indicating an improvement in the health status of red sea bream. Interestingly, the levels of blood glucose displayed average values without significant differences among the groups, which indicate that the supplementation of *B. subtilis* C-3102 or *S. cerevisiae* type 1 had no stressful impact on the performances of red sea bream under the current trial conditions.

It is suggested that beneficial microorganisms act as efficient antioxidants (Geng et al., 2012). Oxidative stress is an emerging risk factor for health in aquaculture and can produce high rates of reactive oxygen species (ROS), resulting in oxidant activity exceeding the neutralizing ability of antioxidants and reduced antioxidant system efficiency (Geng et al., 2012; Merrifield et al., 2011). The antioxidant enzymes can catalyze the dismutation of two superoxide radicals into hydrogen peroxide and oxygen to eliminate the cellular environment's damaging ROS (Pasquini et al., 2008). For assessing the oxidative stress status of fish, the oxidative stress was determined using free radical analytical units by measuring d-ROMs and BAP in fish plasma (Ballerini et al., 2003). In our study, red sea bream fed diets supplemented with *B. subtilis* C-3102 or *S. cerevisiae* type 1 showed no impaired antioxidative status, indicating a good health status. In this respect, the obtained results demonstrated that continuous probiotic administration (*B. subtilis* C-3102 or *S. cerevisiae* type 1) for 60 days successfully decreased the adverse effects of oxidative stress via the synthesis of ROS in activated leukocytes. Similar findings have indicated that probiotic-

enriched diets can increase antioxidant enzymes in fish blood (Martínez Cruz et al., 2012).

Several immune responses were measured in this study to confirm the beneficial effects of the probiotic *B. subtilis* C-3102 or *S. cerevisiae* type 1 on red sea bream immunity and, in turn, fish's ability to resist infectious diseases. Skin mucus is vital in protecting the fish body from environmental hazards, including infectious diseases. In this study, we tested bactericidal, peroxidase, and lysozyme activities in both serum and mucus samples of red sea bream. Bactericidal activity is an essential and helpful tool to assess host resistance against pathogenic bacteria. The results of the present study showed that the probiotic-treated groups exhibited the highest serum bactericidal activity. Likewise, Dawood et al. (2016) and Zaineldin et al. (2018) reported that dietary probiotics significantly increased the bactericidal activity of red sea bream. Similarly, other fish species showed enhanced bactericidal activity after *B. subtilis* administration, including Nile tilapia (Elsabagh et al., 2018), rainbow trout (Martínez Cruz et al., 2012) and grouper (*Epinephelus coioides)* (He et al., 2009). Several probiotic strains can modulate lysozyme activity in the serum and mucus of fish, as reported in rainbow trout and red sea bream (Dawood et al., 2016; Ran et al., 2015). Consistent with previous studies, in our study, the bacteria-based probiotic group showed significantly high serum and mucus lysozyme activity in red sea bream. Chiu et al. (2010) found that probiotic yeast succeeded in enhancing lysozyme activity in grouper. However, yeast-based probiotics did not affect the lysozyme activity of red sea bream in this study. These differences might be due to individual variations among fish species. Peroxidase is an important enzyme that utilizes oxidative radicals in fish (Salinas et al., 2008). In the present study, serum peroxidase activity was significantly enhanced in both probiotic supplementation groups. At the same time, no significant difference was observed in the mucus samples, similar to previous data in sea bream, indicating the immunostimulant properties of probiotic bacteria and yeast in red sea bream (Salinas et al., 2008).

Anti-protease is an important tool for innate immunity, leading to delayed infection by or inhibition of pathogens that produce toxic proteases (Ellis, 2001). In the present study, serum anti-protease activity increased significantly in the PB group, which indicated that the immune status of the tested red sea bream is strongly influenced by probiotic incorporation in the diet of red sea bream (Ellis, 2001).

Catalase activity is a diagnostic tool for acute pancreatitis, haemolytic disease, and some liver diseases (Goth et al., 1982; Kim et al., 2015). Catalase also catalyses the breakdown of hydrogen peroxide to water and molecular oxygen as an antioxidant enzyme (Goth et al., 1982). In this study, fish fed probiotic-based diets exhibited decreased catalase activity compared to the control, which indicates that the fish in the probiotic-treated groups experienced optimal liver health and physiological conditions. These findings are consistent with those of previous studies, which reported that probiotics might enhance catalase activity (Ellis, 2001; Yang et al., 2012).

Dietary probiotics can regulate the gut microbiota, selectively stimulate beneficial bacteria, and suppress some potentially harmful species (Gobi et al., 2018). Using the plate culture method, red sea bream fed diets with the probiotics *B. subtilis* C-3102 and *S. cerevisiae* type 1 exhibited increased numbers of total bacteria in

the gut. The gut count of *B. subtilis* in the second treatment was determined to be $3.87\pm0.47\times10^5$ CFU/g, and the *S. cerevisiae* count in the red sea bream gut was $3.6\pm1.5\times10^3$ CFU/g in the third treatment. Despite the presence and colonization of both probiotic strains in the gut, further studies are needed.

Currently, aquaculture environments contain various stressors, which may affect the hormonal secretion rates, metabolism, and immunity of cultured fish species (Dawood et al., 2019 a; Kurtzman et al., 2011). The stress test was used to determine the health status of red sea bream by measuring the tolerance to freshwater (LT_{50}). Dietary *B. subtilis* C-3102 or *S. cerevisiae* type 1 supplementation increased the resistance of red sea bream to freshwater stress in the present study. These probiotics encounter a variety of stress factors, including temperature, acid, and bile, increased concentration of specific ions or nutrient depletion, exposure to osmotic and oxidative stress in product matrices along with passage through the GIT transit that may detrimentally affect their viability and their functionality (Terpou et al., 2019). Probiotics need to either adapt to such a dynamic environment or be protected to survive and become available in adequate quantities and deliver their health benefits. The increased resistance to freshwater stress of red sea bream fed probiotic-supplemented diets indicates the health status of the fish.

Overall, the results are confirming that the probiotics application has a species-specific mode of action, which means that the influence of probiotics depends on the dose, feeding regimes, and organisms as well as the microorganism strain and concentration (Cao et al., 2020; Soltani et al., 2019). Regarding the comparison effects of *B. subtilis* C-3102 or *S. cerevisiae* type 1 on red sea bream performances, both exhibited beneficial effects on red sea bream when compared to the control with the superior potential impact of *B. subtilis* C-3102 *vs S. cerevisiae* type 1. However, further studies are required to confirm this hypothesis by applying multiple microorganisms with several doses in the case of red sea bream and the other aquatic animals.

Conclusion

In conclusion, dietary *B. subtilis* C-3102 or *S. cerevisiae* type 1 had beneficial effects on red sea bream in terms of growth performance, gut morphology, digestive enzyme activity, and immune response oxidative status, and resistance against environmental stressors. To be more specific, red sea bream fed *B. subtilis* C-3102 showed relatively higher performance and wellbeing than *S. cerevisiae* type 1.

Conflict of Interest

The authors declare that there is no conflict of interest associated with this work.

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