EFFECT OF VARIOUS FUNCTIONAL FEED ADDITIVES ON THE PERFORMANCE OF AQUATIC ANIMALS

水産動物における各種機能性飼料添加物の効果

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ENGLISH ABSTRACT

Aquaculture industry is currently recognised as one of the main supplier for food protein in human diets, thus, increased intensive farming systems have caused frequent problems including slow growth and disease occurrences in aquatic animals. The need for enhancing disease resistance and improve growth and feed utilization has become a major concern in the industry. Although antibiotics have contributed to enhance disease resistance in aquatic animals, the problems associated with spread of drug resistance pathogens, food safety problem, suppression of animal's immune systems are a major concern. Recent approaches to manage animal health by improving gut microbiota using functional feed additives have gained major recognition in aquaculture industry. The present study revealed potential functional feed additives for aqua feed development. Probiotics is a common functional feed additives used to improve performance and immune response in fish. In Asia, particularly in Japan, red sea bream *Pagros major* and amberjack *Seriola dumerili* are important cultured species due to high market values and desirable taste, however, increased demand in domestic market has been one of the major challenge. In this research, red sea bream and amberjack were used to evaluate the benefits of functional feed additives in aquatic animals. The study was divided into two parts. In the first part of the study, functional benefits of spent oleaginous yeast *Lipomyces starkeyi* in diets of *P. major* was investigated in two experiments. First, the dry yeast cells were supplemented at 0.05, 0.1, 0.5 and 1% of the dietary proportion. After 45 days of feeding period, results showed significant improvement in final body weight (FBW) and blood health (low total cholesterol, total bilarum and aspartate aminotransferase), significant improvement of immune responses (lysozyme, Immunoglobulin, Peroxidase) and antioxidant, and improved stress tolerance ability of fish. The second experiment involved the supplement of *L. starkeyi* dry cell

at 1, 1.5, 2 and 2.5 % of the dietary proportion in diets of *P. major*. After 50 days of feeding trial, significant linear relationship was established for feed intake, Specific growth rate, weight gain (%), immune responses, antioxidant and stress tolerance ability with increasing supplement ration of *L. starkeyi*. Both of the experiment indicated that spent oleaginous yeast *L. starkeyi* is a potential probiotic additive and can be supplemented up to 2.5 % of the dietary proportion for red sea bream. The second part involved two experiment, the first experiment evaluated the functional benefits of mix probiotic bacteria strain (*Streptococcus faecalis* T-110 $5x10^6$ cfu/g diet, *Bacillis Amyloliquefaciens* TOA5001 $5x10^5$ cfu/g diet) at 0.2% or mix strain *Streptococcus faecalis* T-110 ($5x10^6$ cfu/g diet), *Lactobacillus plantarum* TO-A ($4x10^5$ cfu/g diet), *Bacillus mesentericus* TO-A ($1x10^2$ cfu/g diet) and *Bacillus amyloliquefaciens* ($5x10^5$ cfu/g diet) at 1% inclusion in diets of *S. dumerili* whereby after 60 days of animal keeping, results showed significant improvement on blood profile, antioxidant activity and immune responses compared to the control diets wile reports of 70 day keeping revealed improved physiological condition, whole body fatty acids and weight gain. The second experiment evaluates mix of strain *Streptococcus faecalis* T-110 ($5x10^6$ cfu/g diet) , *Bacillis Amyloliquefaciens* TOA5001 ($5x10^5$ cfu/g diet), or mix of strain *Streptococcus faecalis* T-110 ($5x10^6$ cfu/g diet), *Lactobacillus plantarum* TO-A $4x10^5$ cfu/g diet, *Bacillus mesentericus* TO-A $1x10^2$ cfu/g diet, *Bacillus amyloliquefaciens (*$5x10^5$ cfu/g diet), or mix of strain *Streptococcus faecalis* T-110 ($5x10^6$ cfu/g diet), *Lactobacillus plantarum* TO-A ($4x10^5$ cfu/g diet), *Bacillus mesentericus* TO-A ($1x10^2$ cfu/g diet) and single strain (*Bacillus amyloliquefaciens* $5x10^5$ cfu/g diet) bacteria supplement at 0.2%, 1%, 0.5% and 0.5% respectively, in diets of red sea bream whereby after 50 days of animal keeping, results showed significant improvement in growth and immune related gene expression, immune responses, antioxidant and stress responses compared to the control. In both fish species, mix probiotic strain *Streptococcus faecalis* T-110 ($5x10^6$ cfu/g diet) and *Bacillis Amyloliquefaciens* TOA5001 ($5x10^5$ cfu/g diet) or mix of strain

Streptococcus faecalis T-110 ($5x10^6$ cfu/g diet), *Lactobacillus plantarum* TO-A ($4x10^5$ cfu/g diet), *Bacillus mesentericus* TO-A ($1x10^2$ cfu/g diet) and *Bacillus amyloliquefaciens* ($5x10^5$ cfu/g diet) showed a significant improvement in fish performance and health status of both *S. dumerili* and *P. major*, suggest a potential supplement for aquatic animal feed development.

The overall findings of this research indicated that these functional feed additives have potential to induce beneficial effects on growth and health status of *S. dumerili* and *P. major*.

List of publications in peer reviewed journal

Shadrack, R.S., Manabu, I., Koshio, S. and Waqalevu, V., 2021. Physiological condition, digestive enzyme, blood haemato-biochemistry, antioxidant, immune and stress response of juvenile red sea bream (*Pagrus major*) fed diets containing spent oleaginous yeast. *Aquaculture Reports*, 21, p.100913.

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DEDICATION

I would like to dedicate this work to my parents Mr. Spenly Shadrack Salemomo and Mrs Belinda Toa Shadrack, my siblings and those who have supported me throughout this journey. Tankio Tumas and どうもありがとうございました。

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ABREVIATIONS

ADC	Apparent digestibility coefficient	LAB	Lactic acid bacteria
ALD	Alanine aminotransferase	LB	*Lactobacillus plantarum*
AST	Aspartate aminotransferase	LS	*Lipomyces starkeyi*
BA	*Bacillus amenities*	MDA	Malondialdehyde
BAP	Biological anti-oxidant potential	MUFA	Monounsaturated fatty acid
BG	ß-glucan	MCH	Mean capsular hemoglobin
BM	*Bacillus mesentericus*	MCHC	Mean capsular hemoglobin concentration
BUN	Blood urea nitrogen	MCV	Mean capsular volume
CA	Crude ash	NBT	Nitroblue tetrazolium
CAT	Catalase enzyme activity	OD	Optical density
CFU	Colony forming unit	PCA	Principle component analy -sis
CL	Crude lipid	PBS	Phosphate buffered saline
CP	Crude protein	PER	Protein efficiency ratio
DM	Dry matter	PG	Protein gain
DHA	Docosahexaenoic acid	PR	Protein retention
d-ROM	Reactive oxygen metabolites	PUFA	Polyunsaturated fatty acid
EPA	Eicosapentaenoic acid	RBC	Red blood cells
FBW	Final body weight	S.D.	Standard deviation
FCR	Feed conversion ratio	S.E.M.	Standard error of mean
FER	Feed efficiency ratio	SF	*Streptococcus faecalis*
FI	Feed intake	SFA	Saturated fatty acid
FM	Fish meal	SGR	Specific growth rate
Glu	Glucose	SOD	Sodium oxide dismutase
HB	Hemoglobin	Sur	Survival rate
HBSS	Hank's buffered salt solution	T-Bil	Total bilirubin
Hrt	Haematocrit level	T-Cho	Total cholesterol
HPLC	High performance liquid chromatogr -aphy	TG	Triglyceride
HSI	Hepatosomatic index	T-Pro	Total protein
IgM	Immunoglobulin	T-antirotease	Total antiprotease
VSI	Viscerosomatic index	α-antiprotease	Alpha antiprotease
WG	Weight gain		
LA	Lysozyme activity		

Chapter 1: General introduction

1. General introduction

1.1 Functional feed in aquaculture

Aquaculture industry is currently one of the most promising option for sustainable animal food protein supply for human in the world. Due to technological advances, aquaculture systems have evolved into mass production using intensive farming practices resulting in frequent disease occurrences. Because of the problems associated with intensive farming, increased need for improving disease resistance, feed efficiency and growth performance of cultured animals is paramount importance in the industry (Burr *et al.*, 2005; Aguirre-Guzmán). Further, the application of chemotherapeutics or antibiotics in aquaculture may cause other problems such as evolution of disease resistance pathogen, suppressed aquatic animal's immune system, environmental hazards and food safety problem (Dawood *et al.*, 2015a).

1.2 Alternative approaches for boosting animal welfare

The increased demand for environmental friendly aquaculture has been an ongoing concerned which reflect the need to find alternative of antibiotic (Romero *et al.*, 2012; Manage *et al.*, 2018). The need for environmental friendly practices was recommended by World Health Organisation as the use of functional feed additives could replace the use of chemicals, thus, reducing environment and health impacts. Increased research on the application of functional feed additives such as probiotic, prebiotics and other sources of additives have been successfully trailed in aquatic animals (Dawood *et al.*, 2015a). Thus, functional feed additives were currently the friendliest approach in controlling diseases in aquaculture and promoting animal gut microbiota and health status. Functional feed additives are known as either nutritive or non-meditative ingredients that are added in small amount to the diets to influence the biochemical response in fish, preserving the physical or chemical qualities of the diet or to improve the quality of the culture environment. The growing interest to improve animal health

and safety of food is a global concerned which currently driving the research advances in functional feed additives. Therefore, probiotics and other functional feed additives were currently being some of the best available options to boost animal health and performances.

1.1.2 Probiotics

Probiotics are indigestible substances or ingredients that cause changes in the microbial composition or function which can positively influence nutrient absorption and improve health status of the host (Song *et al*., 2014). Yeast is one of the functional feed additives used as probiotics in different fish species. Yeast is often used as probiotic and prebiotic due to their unique mode of action in improving aquatic animal growth performance and health (Dawood *et al*., 2015; Meena *et al*., 2013; Zhang *et al*., 2020; Van Doan *et al*., 2020). Yeast produces oligosaccharides, amino acids, peptides, organic acids, vitamins and other soluble molecules (Peppler, 1982; Zaineldin *et al*., 2021). Yeast cell wall also contains polysaccharide, ß-D-glucan, chitin and mannooligosaccarides which are the important molecules in probiotics (Bowman and Free, 2006). 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). The supplementation of Baker's yeast cell walls in diets of Japanese seabass revealed an optimum range of 1-2 g/kg (Yu *et al*., 2014). Whilst it's supplementation in diets tilapia (*Oreochromis niloticus*) revealed that 0.2% is the optimal ratio with beneficial effect on immune responses and the expression of related genes (Abu-Elala *et al*., 2018). Besides, 0.4% supplementation of yeast cell wall in diets of gibel carb (*Carassius auratus gibelio*) has also improved growth and immune responses of fish (Zhang *et al*., 2020). The spent oleaginous yeast *Lipomyces starkeyi* was also reported to be a useful livestock feed (Takaku *et al*., 2020).

Probiotic bacteria were recently proven to be useful feed supplements for boosting growth, immune function, and resistance to pathogens (Ringø *et al.*, 2018). Probiotics as friendly additives include lactic acid bacteria, *Bacillus*, and *Saccharomyces* (Niu *et al.*, 2019). It was reported that the combination of probiotic bacteria strains may complement or improve the health of an individual strain (Niu *et al.*, 2019, Giri *et al.*, 2014) because the use of a single probiotic can never be suitable for all host species due to dissimilarity in the physiological and physio-chemical status of a host of the surrounding environment (Lazado *et al.*, 2015). The inclusion of multi-strain probiotic bacteria in diets of olive flounder reduces the potential adverse effects of low fishmeal diets (Niu *et al.*, 2019). The individual strain of probiotic bacteria such as *Lactobacillus plantarum* (LP) was reported to improved feed intake (FI), specific growth rate (SGR), weight percent (WG%), antioxidants, and immune enzyme in koi carp (Zhang *et al.*, 2020), while mix probiotic bacteria (*Streptococcus faecalis* (SF), *Lactobacillus plantarum* (LP) and *Bacillus mesentericus* (BM)) numerically improves WG%), SGR and relative condition factor in red sea bream (Shadrack *et al.*, 2021)

Red sea bream (*Pagros major*) is one of the most common warm water cultured species in Japan due to its economic value. Until now, the use of yeast supplement as probiotic in red sea bream is limited. On the same note, amberjack *Seriola dumerili* is one of the famous cultured species in Japan due to its economic value and delicacy. There has been very limited study on the efficacy of single or mix probiotic bacteria strain in diet of amberjack and red sea bream. Thus, the following are the objectives of the present study.

1. Although many studies have been conducted to evaluate the commercially available additives on performance of Japanese cultured species, there is still available additive that require further investigation.
2. To investigate effects of two or more probiotic bacteria strains on performance, feed utilization, immune responses, antioxidant and stress tolerance ability in fish.

3. To determine the effect of spent oleaginous yeast supplementation on performance, survival, immune response, antioxidant activity and stress résistance in juvenile red sea bream.
4. To investigate effects of spent *Lipomyces Starkeyi* yeast strain supplementation on performance, blood chemistry, digestive enzyme activity, immune response and stress tolerance ability in fish.
5. To investigate the supplementation of probiotic bacteria *Streptococcus faecalis, Bacillis Amyloliquefaciens, Streptococcus faecalis, Lactobacillus plantarum, Bacillus mesentericus, Bacillus amyloliquefaciens* as single or mix of strain on growth, survival, immune response, antioxidant activity and stress resistance in juvenile amberjack and red sea bream.

Chapter II: General materials and methods

2. General material and methods

2.1 Diet preparation

The main ingredient for the experiments was brown fish meal containing 67% crude protein and 8% crude lipid. The main lipid sources were Pollack liver oil and Soybean lecithin. The ingredient was binder with activated gluten to produce pellets. Wheat flour was the main carbohydrate source in the diet and cellular powder was used to adjust the weight to 100 % total proportion. The functional feed supplement was added to the diet following the percentage proportion of the experimental diets. The feed additives were first mixed with the lipid source (Pollack liver oil and soybean lecithin) and stir by hand for 5 minutes, then with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan) for 15 minutes prior to adding other ingredients. The ingredients were stirred with a spatula for 5 minutes and then with a food mixture (DK 400, Yamato Scientific, Tokyo, Japan) for 15 minutes. Then, based on the weight of the ingredient, 30-40 percent water was added and stirred for the next 10 minutes. Before pelleting, the pH of the ingredients was adjusted to neutral (pH 7.2) using 4N sodium hydroxide. Pelleting was made using a meat grinder with a 1.2 mm diameter opening and then dried in a convection oven at 45 degrees to less than 10% of the moisture content. Finally, the dry pellets were packed in polypropylene bags and stored in a freezer at -28 ℃ until use.

2.2 Animal keeping and experimental condition

The experiment was conducted at the Kamoike Marine Research facility, Faculty of Fisheries, Kagoshima University, Japan. The juvenile fish were purchased from a commercial hatchery (Miyazaki prefecture, Japan) and were stocked in 100 L polyethylene tanks filled with 80 L of seawater in a flow-through seawater system at 1.51 L per minute, with continuous aeration. The juvenile fish were fed with commercial feed for 7 days' acclimation period. The rearing water

condition was maintained as follows; (26.1 ± 1.2 ℃), pH (8.1 ± 0.5), salinity (33.1 ± 0.5 PSU), and dissolved oxygen (6.1 ± 0.5 mg/L).

After the acclimation period, fish were weigh randomly to minimise average initial body among experimental treatments and stocked into the rearing tank (triplicate tank per treatment). Feeding was conducted twice daily at 8 am and 4 pm over the days of trial period. Uneaten feed was siphoned after one hour of feeding, then dried using a freeze drier. The weight was used later for the calculation of feed intake (FI).

2.3 Sampling

At the end of the 50 day of the feeding trial, fish were starved for 24 hours prior to sample collection. During sampling, Eugenol (4- Allylomethoxyphenol, 50 ml/L) was used to anesthetize the fish for weight and length data collection. Five juvenile fish were collected per tank and stored at -20 ℃ for the final whole body analysis.

Blood was collected using heparinized (n=5) and non-zheparinized (n=3) syringes. A small fraction of the heparinized blood was used to determining haematocrit by following the micro-hematocrit technique. The heparinized blood was centrifuged at 3000 x g for 15 minutes using a high-speed refrigerated microcentrifuge and the plasma obtained was kept in a freezer at -80 ℃ until used. The non-heparinized blood was kept at room temperature for 2 hours and then centrifuged at 3000 x g for 15 minutes to collect serum which was then stored at -80 ℃ until used.

2.4 Biochemical procedures

2.4.1 Proximate composition

The diet and whole-body proximate composition were determined following standard procedures outline in Chemists (1990).

2.4.1.1 Moisture

A mechanical convection oven (Dk400, Yamato Scientific CO., Tokyo, Japan) was used to dry the diet samples at 135 ℃ to constant and the weight loss represents moisture content. Fish whole body was dried to constant weight using a freeze dryer (Eyela freeze dryer FD-1, Tokyo Rikakikai Co. Ltd., Japan).

Moisture content was determined based on the following equation:

Moisture (%) = {(weight before drying -weight after drying/weight before drying)} × 100

2.4.1.2 Crude protein

Crude protein was quantified following the Kjeldahl nitrogen method (Kjeltec System 1002 tecator, Sweden) and evaluating the protein content. Briefly, 0.2g of sample and 2 g of catalase (K2SO4:CuSO4) at a ratio of 9:1 were digested in concentrated H_2SO_4 (10 ml) and 30% H_2O_2 (5 ml) for 90 min at 420 ℃. The digested samples were distilled in 50 ml of 30-40% of NaOH from the Kjeldhal distilling apparatus (Kjeltec System 1007, Tecator, Sweden). 150 ml of the distillate in H_3BO_3 solution mixed with methylene blue and methyl red indicators in ethanol and titrated with 0.1N H_2SO_4 to neutral pH. The percentage of nitrogen was used to calculate the crude protein (%) according to the following equation:

Nitrogen (N %) = {14.008 × (volume of sample titrant- volume of blank titrant) × 0.1 × F}/ {Sample weight (g) × 10}

F = Factor of 0.1 N sulfuric acid solution

Crude protein (%) = % N × 6.25

2.4.1.3 Ash

Ash content was quantified by burning at 550 ˚C in a muffle furnace for 4 hours and the final product represents ash content. The ash content was calculated as follows:

Ash (%) = (weight of ash/ sample weight) × 100

2.4.1.4 Crude lipid

Crude lipid content was determined following the Soxhlet extraction method. Briefly, 2g of was placed in a thimble and covered with cotton. The thimble was placed in soxlhet extraction tube and 125 ml diethyl ether was added into the extraction flask and placed onto the soxhlet extraction apparatus for 5-6 hour. The flask with the fats was dried using a rotary in rotary vacuum evaporator (Eyela SB 1100, CCA 1111), then dried in an oven at 110 ℃ to constant weight. Crude lipid was calculated using the following equation:

Crude lipid= [weight of fat (g)/ dry sample weight (g)] × 100

2.4.2 Fatty acid

The total lipid of test diets and fish whole body were determined according to Bligh and Dyer (1959) method. Briefly, total lipid was extracted using chloroform: methanol (1:1, v/v), then the derivative was converted to methyl esters using a method described by Kavanagh (1981). The lipid class of the extract was separated using SEP-PAK silica column chromatography cartridges (waters corporation) and eluted with chloroform or methanol for polar and neutron lipid respectively. The analysis of polar and neutron lipid of the whole body were determined following the method described by Juaneda and Rocquelin (1985). The methyl esters (23:0) was incorporated into samples and esterified to quantify fatty acid content (Joseph and Ackman 1992). A mixture of 23:0 methyl ester (1mg C23 in 1 ml n-hexane) as internal standard was measured and added to each sample base on this equation: (mg TL x 0.8/20; mg PL x 0.5/20)

(Oswald et al. 2019). The content of the mixture was evaporated to dryness, then boron trifluoride (BF3-MeOH 14%, 1 ml) and dichloromethane (0.5 ml) were added and mixed thoroughly with a touch mixer. The reactive vial with the content was heated at 95-100°C for 1 hour and then cooled immediately in an ice bath to cease the reaction. A volume of 0.5 ml NaCl and 1 ml hexane were added forming a sample (hexane) layer. This layer was transferred to sample vials and evaporated under nitrogen gas to dryness. The fatty acid concentration in the vial was adjusted with n-hexane at a ratio of 20 mg:1ml. The Shimadzu AO - 20I GC 2010 gas chromatograph (Supelco, Inc.) was used for separating and quantifying the fatty acid content of the sample. The quantification of relative fatty acid was determined by measuring the area under the peak of the chromatograph corresponding to each fatty acid.

2.4.3 Amino acid

The total amino acid content of the diet was determined according to the method describe in Teshima et al. (1986) and Kader et al. (2010). The concentration of the total amino acid (TAA) was quantified using high-performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan). Briefly, 2 mg of sample was spiked with a known concentration of norleucine as internal standard, then 4 N methanesulfonic acid was added and hydrolysed at 110 °C for 22 hr. Finally, the pH of the hydrolysate was adjusted to 2.2 ± 0.05, then filtered and stored at 4 °C. The separation and chromatographic analysis of the amino acid content was conducted using an HPLC ion exchanging resin column.

2.4.4 Bacteria count

The viability of the bacteria cells incorporated in feed were assessed by spreading onto 3M™ petrifilm aerobic count plates (Thomas Scientific, USA) in triplicate per treatment. The lactic acid bacteria content in feed were determine by spreading onto 3MTM petrifilm anaerobic

count plate (Thomas Scientific, USA) in triplicate per treatment. Briefly, after the diets were prepared, 1 g of the test diet was homogenized in 10 ml PBS (0.05 m, pH 7.4). then 1 ml of the solution was serially diluted in 10 ml PBS buffer to 4th dilution. Finally, 1 ml of each dilution was spread over the 3MTM petrifilm plate and incubated at 26 ˚C for 3-5 days. The bacteria colony forming unit (CFU g-1) were counted using a colony counter (ACK-3 AS ONE, Japan) as describe in Ren et al. (2007). In similar manner, the total bacteria count and lactic acid bacteria count were determining from fish intestinal content.

2.4.5 Blood parameters

Blood was collected using heparinized (n=5) and non-zheparinized (n=3) syringes. A small fraction of the heparinized blood was used to determining haematocrit by following the micro-hematocrit technique. The heparinized blood was centrifuged at 3000 x g for 15 min using a high-speed refrigerated microcentrifuge and the plasma obtained was kept in a freezer at -80 ˚C until used. The non-heparinized blood was kept at room temperature for 2 hr and then centrifuged at 3000 x g for 15 min to collect serum which was then stored at -80 ˚C until used.

Blood plasma chemical parameters were measured using dry chemical kits with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Array, Inc. Kyoto, Japan). The biological antioxidant potential (BAP) and derivative of reactive oxygen metabolites (d-ROM) from blood serum were determined with an automated analyzer using a chemical kit (FRAS4, Diacron international s.r.l., Grosseto, Italy), according to the manufactures instruction. The red blood cells (RBCs) were counted with a haemocytometer (Houston, 1990), immediately after dilution with Natt and Herrick's solution. The blood haemoglobin concentration was determined using a dry chemical kit with an automated analyzer (Lygren et al., 2001).

2.4.6 Immune response

2.4.6.1 Immunoglobulin

Immunoglobulin from serum and mucus was determine following the method of Siwicki and Anderson 1993. Briefly, 100 µl was blended with 12% polyethelyne glycol (Sigma) and incubated at room temperature for 120 min and then centrifuged at 5000 x g for 15 min at 4 °C. The supertant was diluted with 0.85% NaCl and the protein content was determined using Bradford (1976) method. The differences between the polyethelyne glycol treated sample and the protein content of untreated sample represent the IgM value.

2.4.6.2 Protease, total-antiprotease, α-protease

Protease activity of serum and mucus was determined using azocasein hydrolysis assay (Cordero et al., 2016). Briefly, equal volume of serum or skin mucus was incubated with 100mM ammonium bicarbonate buffer containing 0.7 azocasein (Sigma-Aldrich) for 19 h at 25 °C in triplicate. 4.6% trichloroacetic acid (TCA, Sigma–Aldrich) was added to stop the reaction and the mixture centrifuged at 13,000×g, for 5 min. Supernatants were collected and transferred to a 96-well plate containing 100 µl well−1 of 0.5 N NaOH. The optical density was read at 450 nm using a plate reader. Skin mucus was replaced by trypsin solution (5 mg ml^{-1}) as positive control (100% of protease activity), or by buffer, as negative control (0% activity).

The antitrypsin activity of sera was measured following the methods described by Magnadóttir et al. (2005). 20 µl of serum was incubated with 20 µl of standard trypsin solution (Sigma-Aldrich, 1000-2000 BAEE, 5 mg ml^{-1}) at room temperature (~22°C) for 10 min using Eppendorf tubes. 200 µl of 0·1 mol l−1 PBS and 250 µl of 2% (w/v) azocasein solution (20 mg ml−1 PBS) were added and incubated further for 1h. 500 µl of 10% trichloro acetic acid (TCA; Fisher) was added and incubated for another 30 min. Then the tubes were centrifuged at 9,000 rev min−1 for 5 min and then 100 µl of the supernatant in each tube was placed into the wells of a microtitre flat bottom plate (Nalge Nunc, Hereford, UK) filled with 100 µl of 1N sodium

hydroxide. The optical density (OD) was read at 450 nm on a Smartspec 3000 spectrophotometer (Bio-Rad). Inhibition of trypsin activity was calculated by comparing with a 100% control sample, which contained the buffer to replace serum, and a negative control where the buffer replaced both serum and trypsin. The percentage of trypsin inhibition represent total anti-protease activity.

The α1-antiprotease was performed following the method by Ellis (1999). Briefly 10 µl of serum was incubated with 20 µg trypsin in 100 µl of Tris–HCl (50 mmol l−1; pH 8·2) (Sigma-Aldrich). Volume of each tube were made up to 200 µl with Tris–HCl and incubated at room temperature (~22°C) for 1 h. Then, 2 ml of 0.1 mmol l−1 Na benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA; Sigma-Aldrich) was added and incubated for another 15 min. 500 µl of 30% acetic acid was added ceasing the reaction and the OD read at 450 nm. The serum blank contained 100 µl of Tris instead of trypsin, and the positive control contained trypsin but no serum.

2.4.6.3 Lysozyme activity

Lysozyme activity was measured with turbidometric assays (Lygren et al., 1999). 10 µl of serum or mucus was put into microplate well and 190 µl of substrate (0.2 mg Micrococcus lysodeikticus, lyophilized cell, Sigma, USA /ml PBS, pH 7.4) was added and the OD was read at 45 0nm (ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan) after 1 min and 5 min after incubation and gentle shaking at room temperature. Each unit change of enzyme represent the amount of enzyme that causes the decrease in absorbance of 0.001/ min.

2.4.6.4 Bacteria activity

Serum and mucus bacteria activity were determining spectrophotometrically at 570 nm following the modified method describe in El Basuini *et al.*, (2020). The amount of serum or

mucus were mixed with bacteria suspension (*Escherichia coli* $1x10^8$, IAM1239 cell line, Kagoshima, Japan) at 1:1 ratio (50 µl sample: 50 µl bacteria suspension) and incubate at 25 °C for 2.5 h with gentle rotation using micro-tube rotator (Wavex-Tube Rotator E11270). Incubated mixture were placed into 96 well microplate containing 15 µl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, Egypt) (5 mg ml^{-1}) for 15 min at 25 °C with gentle shaking. The resulted formazan from the mixture was dissolved with 50 µl of dimethyl sulfoxide (DMSO). The bacteria suspension in PBS without serum or mucus was used as positive control. The optical density of the solution was read at 570 nm and the antimicrobial activity presented as percentage of E. Coli inhibition relative to the positive control as the following:

$$E.coli\ inhibition\ \% = \frac{\text{OD Control} - \text{OD Sample}}{\text{OD Control}} \text{ x } 100$$

2.4.6.5 Peroxidase activity

The peroxidase activity was measured as describe in Salinas et al., (2008) with few modifications. In brief, 15 µl of serum were diluted in 35 µl of Hanks buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates, followed by addition of 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA). The mixture was incubated for 15 minutes, and then the colour developing from the reaction was stopped by adding 50 µl of 2 M sulphuric acid. Thereafter, the OD was measured using a plate reader at 450 nm. PBS was used as blank instead of Serum.

2.4.6.5 Nitro blue tetrazolium assay

The Nitro blue tetrazolium assays was performed in whole blood sample using Anderson and Siwicki (1995). Briefly, 100 µl of blood was place in a microtilter well plate and an amount (1:1, v/v) of 0.2 % NBT solution (Sigma, USA) was added and incubated at room temperature for 30 min. About 50 µl of NBT blood cell suspension was added to a glass tube containing 1

ml l N, N-dimethylformamide (Sigma, USA). The mixture was centrifuged at 3,000 rpm for 5 min. The supernatant was measured at 540 nm. Dimethylformamide was used as the blank.

2.4.7 Antioxidant and lipid peroxidation enzymes

The superoxide dismutase (SOD) activity of blood serum or liver was measured using the SOD assay kit ((Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instruction. The percentage reaction rate of enzyme with the WST was presented as SOD activity unit. One unit of SOD is defined as the amount of enzyme that inhibits the reduction reaction of WST-1 by 50 %. The malondialdehyde (MDA) concentration as an indicator of lipid peroxidation and was measured using the Colorimetric TBARs Microplate Assay Kit (Oxford Biomedical Research, Inc., USA) following the manufacturers protocol. The OD was measured spectrophotometrically at 532 nm and the MDA level was expressed as nmol/ml serum.

Catalase (CAT) enzyme activity was performed spectrophotometrically according to Goth (1991) using the measure the stable complex formed from reaction between hydrogen peroxide with ammonium molybdate. Briefly, 50 µl of serum was added to 1 ml of substrate (65 µmol per ml H_2O_2 in 60 mmol l-1 phosphate buffer, pH 7.4) and incubated at 37 °C for 60 s. The yellow complex formation between ammonium molybdate and hydrogen peroxide was measured spectrophotometrically at 405 nm against a blank (serum was replaced with distilled water). Each unit of CAT decomposes 1 µmol of hydrogen peroxide per min in assay condition and the CAT is expressed as kilo unit per litre.

2.4.8 Mucus amount

Nine fish (3 fish per tank) were collected and the skin was washed with PBS and distilled water, followed by gentle rubbing with a sterilized piece of cotton over 200 mm^2 of the body surface

according to the protocol described in (Dawood et al., 2020b). The cotton containing the mucus was transferred into a 1.5 mL tube and suspend in 1ml PBS (pH=7.4). Then the samples were centrifuged at 2000 x g, 4 °C for 10 min. The supernatant was collected and transferred into new 1.5 ml tubes and stored at -80 °C. Total protein from the mucus sample was quantified following the method of Bradford (1976). Amount of secreted mucus was expressed as mean relative value in the basis of total protein according to Yokoyama et al., (2006).

2.4.9 Low salinity stress

The fresh water from city water was dechlorinatemd by strong aeration for 24 hr and mix with sea water as low salinity water. A refractor meter was used to confirm the salinity of the test water. A minimum of 4 juvenile fish per tank were randomly selected and were placed in a 20 L transparent glass aquaria containing 18 L of dechlorinated water (0.2 %). The test was conducted in triplicate for each experimental treatment. The initial percentage of the fish was 100% survival and converted to log value ([log10 (100) = 2]. Calculation was based on the data collected every 10 min. The log survival values were plotted against time of death to determine the duration of 50% mortality according to the equation from Moe et al. (2004).

$Y = aX + b$ (8)

where Y = log10 (survival), X = time to individual death of fish (min). LT50 (X)

obtained when Y = 1.7 as log10 (50) = 1.7.

2.4.10 Digestibility assessment

The digestibility of crude protein, crude lipid, and dry matter was measured indirectly using chromium oxide as an inert marker. Fish were fed with a diet containing chromium oxide for 5

days to be accustomed to the feed. Thereafter, feeding to satiation was conducted twice per day and faeces were collected 3 hours after each feeding session using a siphon and a fine-mesh nylon net. Faeces were freeze-dried and milled to powder form. The quantification of chromium oxide in diet and faeces were made following the method of Furukawa (1966). The following formula was used to determine the apparent digestibility coefficients.

ADC nutrient (%) = 100 - (% Cr_2O_3 diet/ %Cr_2O_3 faeces ×% nutrient faeces /% nutrient diet

ADC dry matter (%) = 100 – (100 - (% Cr_2O_3 diet/ % Cr_2O_3 faeces)

2.4.11 Real-time PCR analysis

Livers were obtained from dissecting the fish, weighed, and placed in fivefold of RNAlater (Invitrogen; Thermo Fisher Scientific K. K., Tokyo, Japan) solution and stored at -80 ℃ until analysis. The RNA from liver samples was extracted using the RNeasy Mini Kit 50 (Qiagen; Hilden, Ger- 321 many). Briefly, 30mg liver was placed in a sterilized tube (1.5 ml), homogenized, and centrifuged at 1,2000 rpm for 15 s. The supernatant was collected and mixed with 70% ethanol. After completing the RNA extraction, the Prime 324 ScriptTM RT Master Mix Kit (Takara Bio Inc. Shiga, Japan) was used to obtain the cDNA following the manufacturer protocol. Finally, the PCR analysis was performed using the SYBR Master Mix kit (Thermo Fisher Scientific K. K., Tokyo, Japan) using the primers presented in table (2). The elongation factor (β-Actin) was tested for stability and used as a housed-keeping gene (Hossain et al., 2016). Amplification of genes was made with CFD-3120 Mini Opticon Real-Time PCR System (BIO-RAD, Singapore) according to the following steps: 2 min denaturation at 95 °C, 40 cycles at 95 °C for 15 s, and 65 °C for 30 s. Each assay was performed in triplicate 0 °C for 30 s.

2.4.12 Statistical analysis

The Kolmogorov-Smirnov test and Shapiro-Wilk test were performed to verify the normality of the data and homogeneity of variance was confirmed using Levene test. One-way analysis of variance ANOVA was performed using Palaeontology Statistical software version 3.21 (Hammer et al., 2001). The probability of $P < 0.05$ was considered significant. The significant differences were further evaluated using Tukey- Kramer post hoc test. The multivariate analysis such as the Principle Component Analysis (PCA) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical algorithm, hierarchical single linkage clustering, Non-metric Multi-Dimensional Scaling (NMDS) plot, Analysis of Similarities (ANOSIM) and Permutational Multivariate Analysis of Variance (PERMANOVA) were conducted only on standardized data.

Chapter III: Efficiency of functional material as feed additives for cultured fish species

Experiment I: Specific effects of low level dietary supplementation of *Lipomyces starkeyi* CB1807 yeast strain in red sea bream (*Pagrus major*)

Specific importance of low level dietary supplementation of *Lypomyces starkeyi CB1807* yeast strain in red sea bream (*Pagrus major*)

3.1.1 Abstract

Most probiotic yeast supplement 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 (LT50) of fish fed on D5 against low salinity stress were 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.

Keywords: red sea bream (*Pagrus major*); growth performance; oxidative status; blood health; immune response.

3.1.2 Introduction

Red sea bream (*Pagrus major*) is one of the important cultivated fish species in eastern Asia (Sugama 2002; Dawood *et al.*, 2016a; 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 increase occurrences of diseases which lead to significant economic losses (Cerezuela *et al.*, 2012; Hosseinifar *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 pathogen (Akhter *et al.*, 2015; Zaineldin *et al.*, 2018). This indicate 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; Ringo *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.*, 2019b; Chauhan and Singh 2018; 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 of specific compounds (Bowman and Free 2006), useful for animal feed supplement and these compounds are responsible for the differential dietary effect of each yeast strain in animal health (Dimitoglou 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 in Japanese seabass (Lateolabrax japonicus) ranged from 0.1-0.2% (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 (Blomqyist *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 Pagros 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.

3.1.3 Material and Method

3.1.3.1 *Ethical statement and dietary yeast product*

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 spent oleaginous yeast (*Limpocey Skarkeyi*) used in this present study was kindly provided by Fuji Oil Holdings Inc., Japan and Toray Industries, Inc., Japan. The oleaginous spend yeast dry cells contains 69.26% lipid, 6.80% protein and 3.4 % ß-glucan of the sample weight. The dry cells were obtained after the fractional separation of oil, fats, sugar, alcohol and other residuals. The dry yeast cells were stored at -18 degree until use.

3.1.3.2 *Test Diet*

The formulation and chemical composition of the experimental diets are shown in Table1. The spent oleaginous yeast dry cells were incorporated in the percentage proportion of the experimental diet 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 yeast supplement was thoroughly mixed with lipids that were combined ingredients such as Pollack liver oil, soybean lecithin and n-3 HUFA before adding to the dry ingredients. The mixture of all the ingredients was stirred with a spatula for 5 min, then thoroughly mixed with

a food mixer for 15 min. The mixture was added with water (30 – 40% of the dry ingredients, and then mixed for another 15 min). The pH of the mixture was adjusted to the range of 7.0 – 7.5 with 4 N sodium hydroxide whenever necessary. The mixture was then passed through a meat grinder of 1.2 mm diameter openings and cut into pellets, and then oven-dried in a mechanical convection oven at 50 ℃ to less than 10% moisture content. The test diets were packed into polypropylene bags and stored in a freezer at -28 ℃ until use.

3.1.1 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 [4]	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 [14]	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 $NaHSO_3 \cdot 3H_2O$ (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 (Nicotic 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.

Table 3.1.2 Fatty acids (mg/ 1000 mg diet) and total amino acid (AA g/ 100 g diet) contents of experimental diets

Parameters	Experimental diets				
	CD1	D2	D3	D4	D5
Fatty acids					
SFA [1]	57.47±1.6	61.02±3.16	60.76±0.89	58.83±1.41	59.96±0.46
MUFA [2]	52.85±1.6	46.1±3.16	46.45±0.89	48.86±1.41	50.74±0.46
16:2n-6+16:2n-4	2.04±0.13	2.01±0	2.05±0.05	2.01±0.02	1.94±0
16:4n-3	2.43±0.1	2.42±0.08	2.42±0.19	2.41±0.03	2.37±0.19
18:2n-6	25.86±0.34	25.94±0.7	25.7±1.23	24.93±0.26	23.94±0.24
18:3n-6	0.83±0.02	0.88±0.02	0.87±0.08	0.87±0.07	0.82±0.04
18:3A	0.3±0.03	0.29±0.05	0.33±0.09	0.26±0.01	0.26±0
18:3n-3	3.34±0.02	3.32±0.2	3.31±0.28	3.1±0.01	2.97±0.02
18:4n-3	2.85±0.04	2.78±0.09	2.77±0.15	2.76±0.02	2.57±0.04
18:4n-1	0.33±0.02	0.31±0.01	0.3±0.04	0.3±0.02	0.28±0
20:2n-6	0.26±0.01	0.18±0.09	0.18±0.03	0.09±0.13	0.22±0.01
20:3n-6	0.12±0	0±0	0.14±0.19	0.11±0.15	0.37 ± 0.38
20:4n-6	1.42±0.02	1.36±0.08	1.37±0.05	1.34±0.02	1.25±0.02
20:3n-3	0.09±0	0.05±0.07	0.11±0	0.05±0.07	0.05±0.07
20:4n-3	1±0.01	1.03 ± 0.01	0.99 ± 0.02	0.91±0.02	0.86±0.01
20:5n-3	20.91±0.34	20.24±0.82	20.46±1.14	19.92±0.41	18.97±0.23
22:3n-6	0.68±0.02	0.62±0.12	0.68±0.06	0.71 ± 0.08	0.64±0.02

22:5n-3	2.65±0.03	2.48±0.12	2.63±0.16	2.4±0.19	2.35±0.05
22:5n-6	0.29±0.03	0.25±0.03	0.28±0.04	0.13±0.13	0.13±0.13
22:6n-3	16.36±0.17	15.78±0.6	16.25±1.32	15.66±1.14	14.58±0.15
PUFA [3]	79.11±1.04	85.11±2.59	82.11±0.64	79.13±3.41	79.48±0.62
n-3 PUFA	47.21±0.55	53.45±1.7	50.46±2.51	44.8±1.83	42.35±0.01
n-6 PUFA	29.47±0.39	29.23±0.82	29.22±1.69	31.92±5.22	34.76±0.45
n-3/n-6 [4]	1.79±0	2.02±0	1.93±0.18	1.59±0.3	1.75±0.02
22:6n-3/20:5n-3 [5]	0.78±0	0.78±0	0.79±0.02	0.79±0.04	0.77±0.02
Amino acids					
Arginine	4.68 ± 0.17	5.71 ± 1.53	4.45 ± 0.08	4.61 ± 0.2	4.54 ± 0.07
Histidine	3.39 ± 0.12	3.02 ± 0.49	3.31 ± 0.04	3.16 ± 0.16	3.16 ± 0.13
Isoleucine	3.09 ± 0.08	2.66 ± 0.44	2.72 ± 0.06	2.62 ± 0.2	2.6 ± 0.08
Leucine	0.62 ± 0.14	0.55 ± 0.19	0.67 ± 0.06	0.57 ± 0.04	0.58 ± 0.04
Lysine	2.75 ± 0.05	2.55 ± 0.21	2.44 ± 0.1	2.39 ± 0.11	2.39 ± 0.08
Methionine	5.77 ± 0.24	5.41 ± 0.53	5.58 ± 0.02	5.45 ± 0.19	5.48 ± 0.1
Phenylalanine	3.16 ± 0.31	3 ± 0.36	3.19 ± 0.03	2.96 ± 0.26	3.01 ± 0.17
Threonine	2.07 ± 0.1	4.21 ± 2.87	2.07 ± 0.01	2.02 ± 0.12	2.05 ± 0.09
Tryptophan	0.76 ± 0.02	0.78 ± 3	0.79 ± 0.02	0.79 ± 0.12	0.86 ± 0.17
Valine	4.78 ± 0.04	6.06 ± 1.94	4.76 ± 0.24	4.74 ± 0.29	5.05 ± 0.27
Taurine	0.78 ± 0.05	0.71 ± 0.1	0.76 ± 0.02	0.72 ± 0.01	0.74 ± 0.01

CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast

Values are the means of duplicate groups represented as mean ± S.E. Different superscript letters indicate a significant difference ($p < 0.05$) of means. The absence of superscript letters indicates no significant difference in means between groups.

[1] SFA (saturated fatty acids): 12:0; 13:0; iso-14:0; 14:0; anteiso-15:0; iso-16:0; anteiso-16:0; 15:0; 16:0; iso-17:0; anteiso-17:0; 17:0; iso-18:0; 18:0; 19:0; iso-20:0; 20:0 and 22:0.

[2] MUFA (monounsaturated fatty acids): 14:1n-7; 14:1n-5; 16:1n-9; 16:1n-7; 17:1; 18:1n-11 + 18:1n-9; 18:1n-5; 19:1; 20:1n-11; 20:1n-9; 20:1n-7; 22:1n-9 and 22:1n-7.

[3] PUFA (polyunsaturated fatty acids).

[4] n-3/n-6: ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids.

[5] 22:6n-3/20:5n-3: ratio of docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA).

3.1.3.3 *Fish and experimental condition*

The feeding trial was conducted in Kamoike Marine Research facility, Faculty of Fisheries, Kagoshima University, Japan. Juvenile red sea breams Pagros major were collected from a commercial hatchery in Miyazaki prefecture, Japan. The fish were at first acclimated to the laboratory conditions for a period of one week prior to the experimental trial. A commercial fish meal diet was fed to the fish during this acclimation period. The feeding trial was conducted in 100-l polyethylene tanks filled with 80 L of seawater in a flow-through seawater system, and each tank was equipped with an inlet and outlet valve, with continuous aeration. The flow rate of the seawater was maintained at 1.5 L per minute throughout the experimental period. The water quality parameters (Mean and S.E) values monitored during experiment being considered optimum for red sea bream juvenile were in terms of water temperature 26.1 ± 1.2 ℃, pH 8.1 ± 0.5, Salinity 33.1 ± 0.5 psu and Dissolve Oxygen 6.1 ± 0.5 mg/L.

Red sea bream juveniles (n = 300 fish) with an initial weight of 1.9 ± 0.03 g were randomly assigned into 5 experimental groups at 20 fish per tank. Apparent satiation feeding regime was applied twice a day for 45 days. Uneaten feed was collected by siphoning after one hour of feeding and then dried using a freeze drier.

3.1.3.4 *Sampling*

The final sampling was conducted at the end of the trail (45 days) after a 24 h fasting period. Eugenol (4-Allylmethoxyphenol), 50 ml/L solution was used to anesthetize the fish before and body weight and length measurements were taken. A pooled sample of 5 individual fish from each of the replicate tanks was collected randomly and stored in a freezer at -20 ℃ for final whole-body analysis.

The blood from the caudal vein of fish from each replicated tank was extracted 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 min using a high-speed refrigerated microcentrifuge and keep at -80 °C. The non-heparinised blood samples were kept at room temperature for 2 h, and then centrifuged at 3,000 x g for 15 min to collect serum.

A sum of three fish from each replicate tank were dissected for viscera and liver weight measurements to determine viscerasomatic index (VSI) and hepatosomatic indices (HSI) (El Basuini et al., 2021). The collected liver was pooled together and stored at – 80 °C for further analysis. Calculation of VSI and HSI is shown following the equations below.

VSI = (Viscera weight / fish body weight) × 100

HSI = (Liver weight / fish body weight) × 100

Nine fish from each treatment (3 fish /tank) were randomly assigned for skin mucus sampling. Briefly, the skin of fish was washed with distilled water individually, then skin mucus was collected from 200 mm^2 of its body surface by rubbing it with a sterilized piece of cotton and immediately suspending it in 1 ml phosphate buffered saline (PBS, pH = 7.4) and centrifuged at 2000 x g, 4 °C for 10 min and the supernatant was transferred into a 1.5 ml centrifuge tube and stored at -80 °C for further analysis (Dawood et al., 2015b).

3.1.3.5 *Growth performance and feed utilization*

The following calculations were used to evaluate growth performance and feed utilization for the juvenile red sea bream feeding trial as described by Kader et al. (2012).

Body weight gain (BWG%) = [(final weight – initial weight) /initial weight] x 100

Specific growth rate (SGR%) = [(Ln (Final weight) - Ln (initial weight))/duration (45 days)] x 100

Survival (%) = (Fish no. at 45 days/ Fish no. at the beginning of the experiment) x 100

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

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

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

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

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

Condition factor (CF) = (Fish weight, g / Fish length, cm^3) x 100

3.1.3.6 *Biochemical and antioxidant activity*

The moisture, crude protein, total lipids and ash from the ingredient, diets and fish whole body were analysed in triplicate using standard methods (AOAC 1990), while gross energy was calculated using the combustion value for carbohydrate, protein and lipid. The total amino acid (TAA) of the diet and fish whole body was determined using high-performance liquid chromatography (HPLC) with the method of Kader et al. (2010). Gas chromatography (GC) was used to separate and quantify fatty acid (FA) following the procedure described by Teshima et al. (1986) and Oswald et al. (2019).

The plasma chemical parameters were measured spectrometrically using an automated analyser (SPOTCHEM™ EZ model SP-4430, Array, Inc. Kyoto, Japan). The biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) from blood plasma were measured with an automated analyser (FRAS4, Diacron international s.r.l., Grosseto, Italy), following the procedure describe by Morganti et al. (2002) and Dawood et al. (2015a). Plasma catalase activity was determined according to previous method (Cecchini et al.,2000; Goth 1991).

3.1.3.7 *Non-specific immune responses*

The lysozyme activity of serum and mucus was determined following the turbidometric assay describe 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 lysodeikticus, lyophilized cell, Sigma, USA) /ml PBS (pH 7.4) was added and incubated at room temperature with gentle shaking. The absorbance was read with Multiskan FC (Thermo Fisher Scientific K.K., Japan) at 450 nm after 1 and 5 minute. A unit of enzyme was determined as the amount that causes a decrease in absorbance at 0.001/min. The total serum immunoglobulin (IgM) was determined following the method of Siwicki et al. (1994), briefly describe in Yeganeh et al. (2021).

The total peroxide from plasma was measured as described by Salinas et al. (2008) with a few modifications. 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. The colour developing from the reaction was stopped by adding 50 µl of 2 M sulphuric acid. The optical density of the reaction was measured at 450 nm with a plate reader. PBS was used as a blank instead of serum. For tissue analysis, 25 mg of liver tissue was homogenized in 250 µl RIPA buffer containing protease inhibiter (0.1%, v/v) and centrifuged at 3000 x g for 10 min at 4 °C. The supernatant was used for the analysis.

The plasma superoxide dismutase (SOD) activity was measured with the SOD assay Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) following the manufacturers instruction, briefly describe in Dawood et al. (2016c). Plasma malondialdehyde (MDA) was measured using Colorimetric TBARS microplate assay Kit (Oxford Biomedical Research, Inc., USA) following the manufactures instructions, briefly describe in Dawood et al. (2017c).

3.1.3.8 *Low salinity stress test*

At the end of the feeding trial, 3 fish per tank were subjected to low salinity water to examine the tolerance against stress. The fish were randomly selected and transferred into a 20L stress glass tank containing dechlorinated (strong aeration for 24 hrs) low salinity water (0.2%). The stress tank containing the fish was placed inside a 100L black tank containing 50% water volume less than the height of stress tank (Dawood *et al.* 2017a; Dawood *et al.* 2017b). Continuous water flow was supplied through the tank to keep the stress tank to keep it under ambient temperature. The stress tank was equipped with continuous aeration. The test was terminated when all fish died in the test tank. The number of dead fish in each tank was recorded every 10 min. The amount of time taken to reach 50% death was calculated according to Ren *et al.* (2007).

3.1.3.9 *Statistical analysis*

The statistical analysis was performed using a Paleontological statistical software package for education and data analysis version 3.21 (Hammer *et al.* 2001). The normality of the data was confirmed by the Kolmogorov-Smirnov test and homogeneity of variances by Levene's test before analysis of variances (ANOVA) test. Probabilities of $p < 0.05$ were considered significant, and significant differences between means were evaluated using Tukey-Kramer post hoc test. The principle component analysis was conducted using XLSTAT (2019) on standardized data of the important variables assessed in this investigation (Table 8).

3.1.4 Results

3.1.4.1 *Growth parameters and nutrient 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 3. The fish group fed on D3 and D5 showed significantly high ($p<0.05$) final body weight (FBW) and body weight gain (BWG%) compared to fish fed on the control diet (CD1). Feed intake (FI) and protein gain (PG) were numerically higher ($p>0.05$) in fish group fed on D5. Low feed conversion ratio (FCR) were observed in fish group fed yeast supplemented diets (D2; D4; D5) compared to fish fed the control CD1 ($p>0.05$, Table 3). The survival rate was not significantly different among all fish groups ($p>0.05$, Table 3).

3.1.4.2 *Whole body proximate, biometric indices, fatty acids, and amino acids profiles*

Red sea bream juvenile whole-body proximate analysis, biometric parameters, fatty acids and amino acids profiles at the end of the feeding trial (45 days) are presented in Tables 4 and 5. No significant difference were observed in the whole body proximate values among treatment groups ($P>0.05$, Table 4). Condition factor (CF) was numerically higher in fish group fed supplemented diets (D2; D4; D5) compared to the control group (CD1) ($p>0.05$, Table 4). The amino acid profile of fish whole body showed numerically high ($p>0.05$) taurine, valine and methionine in fish group fed D2 compared to control group (CD1) (Table 5). A numerically high ($p>0.05$) arginine was observed in fish group fed D5 compared to the control (CD1) and the supplemented group (D2; D3; D4). The whole body fatty acid showed a numerically low ($p>0.05$) n-3 and n-6 fatty acid ratio in fish group fed D2 compared to control group CD1 and supplemented groups (D3; D4; D5) (Table 5). Monounsaturated fats (MUFA) was numerically high ($p>0.05$) in fish group fed on yeast supplement (D2; D3; D4; D5) compared to fish group fed on control (CD1) diet. The whole body PUFAs were not significantly different ($p>0.05$) in

fish fed the control diets (CD1) compared to fish fed yeast supplemented diets (D2; D3; D4; D5). Overall, the fish whole body proximate, fatty acid and amino acid were not significantly different ($p>0.05$) among supplemented groups and the control group although numerically increases ($p>0.05$) were observed (Table 4&5).

3.1.4.3 *Blood status*

Table 6 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) 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-Bil, and AST were recorded in fish groups fed supplemented diet D2, D4, and D5, respectively ($P>0.05$).

3.1.4.4 *Non-specific immune response*

Figures 1 and 2 display the first line of defence in the fish body such as the lysozyme and total immunoglobulin activity between fish fed the dietary treatment. Fish groups fed on D5 showed significantly high values of Serum, mucus and liver lysozyme activity compared to the control and other treatment groups ($p<0.05$, Fig.1, a, b, c). The IgM activity of fish fed the yeast supplement was significantly higher in fish group fed on D5 compared to the control diet ($p<0.05$, Fig.2). Fish group fed D2, D3 and D4 should improve IgM activity compared to the fish fed the control diet D1.

3.1.4.5 *Antioxidant capacity*

At the end of the experiment, antioxidant capacity of juvenile red sea bream fed test diets for 45 days is presented in Table 7 and Figures 3 and 4. Fish group fed on D2 diet exhibited the lowest values of MDA compared to D3, highest value of SOD compared to D3 group, the lowest d-ROM compared to control CD1 group, and the highest BAP activity compared to D5 ($p<0.05$, table 7). The lowest BAP activity was observed in fish fed D5 compared to D2 and D4 ($p<0.05$, Table 7). MDA was numerically high ($P>0.05$) in D3 compared to CD1, D4 and D5 while fish group fed D2 showed lower values compared to D3 ($p<0.05$). The general pattern of the combine effect of BAP and d-ROM (Fig. 3) shows a balance between antioxidant and oxidative stress. Zone A reflect a good condition where D2 groups experience low oxidative with high tolerance ability. Zone B shows a balance between oxidative stress and tolerance ability where D4 was favoured similarly to Zone C where D3 was favoured. D5 and CD1 (control diet) were in zone D due to high oxidative stress and low tolerance ability. Figure 4 shows values of catalase and peroxidase activities of red sea bream after 45 days of experimental period. Plasma catalase activity was significantly higher in D2 treated group compared to lower values in D3 and D5 treated groups ($p<0.05$). Meanwhile, liver catalase activity was significantly lower in D5 compared to control CD1 group and other treated groups ($p<0.05$, Fig.4b). The serum peroxidase activity significantly high values fish fed D3 and D4 treated groups compared to control CD1 ($p<0.05$, Fig.4C). A higher value in liver peroxidase was observed in D4 and D5 treated group compared to control CD1, D2, D3 treated group ($p>0.05$, Fig.4c, d).

3.1.4.6 *Low salinity stress*

Time (min) to 50% mortality of juvenile red sea bream exposed to low salinity stress after 45 days of a feeding period on test diets is shown in Figure 5. The log rank 50% mortality showed fish fed D5 has a significantly ($p<0.5$) high tolerance ability compared to fish group fed the

control group (CD1), however, fish fed yeast supplement diets showed numerically high tolerance to stress conditions compared to control group ($p>0.05$, Fig. 5).

3.1.4.7 *PCA variable distribution*

The principle component analysis was conducted to show the variable correlated to the test diets and their distribution shown in Table 8 and Figure 6. The variable such FCR, WG%, SGR and FBW showed the highest loading in PC1 while AST, ALT, T-Bill and MDA showed highest positive loading in PC2. The SOD, BAP and CAT showed highest negative loading in PC1 while Glu, CP% and 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 diet D4, D2 and control D1 where associated with antioxidant activity. The diet D4 and control diet D1 were near the neutral region of the PC1 and PC2.

Table 3.1.3 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

CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0%

yeast. Values presented as means of triplicate ± S.E. Values with different superscripts indicate significant difference ($p < 0.05$). Absence of superscripts indicate no significant difference. [1] IBW (g/fish): Initial body weight; [2] FBW (g/fish/45days): 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 convention ratio; [7] PER: Protein efficiency ratio; 8 PG: Protein gain.

Table 3.1.4 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

CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast. Values are means of triplicate groups ± S.E. Absence of superscript letters indicates no significant difference between treatments. 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 3.1.5 Fatty acids (mg/ 1000 mg dry weight) and total amino acid (AA g/ 100 g^{-}dry weight) contents of red sea bream whole-body fed experimental diets for 45 days

Parameters	Experimental diets				
	CD1	D2	D3	D4	D5
Fatty acids					
SFA [1]	89.15 ± 5.05	94.19 ± 3.13	92.88 ± 0.31	92.06 ± 1.25	91.15 ± 2.93

MUFA [2]	66.26 ± 10.68	66.07 ± 1.14	74.94 ± 3.1	73.68 ± 1.87	74.32 ± 8.05
16:2n-6+16:2n-4	1.04 ± 0.25	1.18 ± 0.09	1 ± 0.1	0.88 ± 0.03	0.88 ± 0.11
16:4n-3	0.44 ± 0.63	0.28 ± 0.02	0.23 ± Tr	0.17 ± Tr	0.13 ± 0.02
18:2n-6	10.65 ± 3.96	11.21 ± 0.37	8.35 ± 2.31	6.44 ± 0.31	7.65 ± 0.56
18:3n-6	0.28 ± 0.39	0.36 ± 0.17	0.37 ± 0.2	3.52 ± 4.98	0.44 ± 0.11
18:3A	0.21 ± 0.05	0.18 ± 0.03	0.16 ± 0.02	0.25 ± 0.01	0.18 ± 0.01
18:3n-3	0.37 ± 0.19	0.64 ± 0.05	0.99 ± 0.74	0.53 ± 0.03	3.92 ± 4.81
18:4n-3	0.33 ± 0.2	0.17 ± 0.02	0.17 ± 0.1	0.48 ± 0.1	0.32 ± 0.02
18:4n-1	0.1 ± 0.15	0.11 ± 0.16	0.07 ± 0.1	0.18 ± 0.03	0.17 ± 0.01
20:2n-6	0.11 ± 0.16	0.15 ± 0.21	0.25 ± 0.1	0.17 ± 0.05	0.22 ± 0.01
20:3n-6	ND	ND	ND	0.08 ± 0.12	0.14 ± 0.1
20:4n-6	0.34 ± 0.01	0.35 ± 0.01	0.36 ± 0.11	0.08 ± 0.11	0.13 ± 0.13
20:3n-3	ND	ND	ND	0.04 ± 0.05	0.15 ± 0.11
20:4n-3	0.17 ± 0.24	0.18 ± 0.04	0.2 ± 0.06	0.99 ± 0.06	0.16 ± 0.06
20:5n-3	3.3 ± 1.48	2.37 ± 0.43	2.62 ± 0.78	1.58 ± 0.41	2.15 ± 0.7
22:3n-6	ND	ND	ND	ND	ND
22:5n-3	0.48 ± 0.68	0.43 ± 0.07	0.52 ± 0.17	0.93 ± 1.32	0.44 ± 0.24
22:5n-6	ND	ND	ND	ND	0.14 ± 0.19
22:6n-3	4.59 ± 2.55	3.28 ± 0.62	3.49 ± 1.09	2.13 ± 0.62	3 ± 1.02
PUFA [3]	22.19 ± 10.2	19.42 ± 0.9	17.54 ± 5.65	19.61 ± 1.43	19.01 ± 2.27
n-3 PUFA	9.23 ± 4.96	7.08 ± 1.24	7.98 ± 2.94	6.66 ± 3.42	10.16 ± 2.88
n-6 PUFA	11.9 ± 4.48	12.07 ± 0.32	9.32 ± 2.72	10.29 ± 5.47	8.72 ± 0.63
n-3/n-6 [4]	0.85 ± 0.17	0.64 ± 0.14	0.93 ± 0.07	0.91 ± 0.83	1.28 ± 0.49
22:6n-3/20:5n-3 [5]	1.35 ± 0.17	1.38 ± 0.01	1.33 ± 0.02	1.34 ± 0.04	1.39 ± 0.02
Amino acids					
Arginine	6.59 ± 0.2	6.15 ± 0.9	6.5 ± 0.73	6.55 ± 0.19	6.62 ± 0.07
Histidine	4.12 ± 0.18	3.88 ± 0.61	4.12 ± 0.47	4.19 ± 0.01	4.04 ± 0.04
Isoleucine	3.51 ± 0.05	3.32 ± 0.47	3.61 ± 0.46	3.59 ± 0.04	3.56 ± 0.05
Leucine	5.04 ± 0.26	3.3 ± 3.05	4.87 ± 1.5	4.93 ± 0.3	4.75 ± 0.2
Lysine	3.69 ± 0.04	3.23 ± 0.95	3.85 ± 0.6	3.86 ± tr	3.79 ± 0.05
Methionine	4.85 ± 0.14	5.09 ± 0.27	4.8 ± 0.33	4.66 ± 0.02	4.67 ± 0.09
Phenylalanine	5.49 ± 0.11	4.34 ± 1.95	5.67 ± 0.63	5.64 ± 0.03	5.57 ± 0.05
Threonine	6.02 ± 0.11	4.27 ± 2.75	5.94 ± 0.89	5.96 ± 0.09	5.92 ± 0.14
Tryptophan	0.24 ± Tr	0.13 ± 0.03	0.11 ± 0.02	0.1 ± 0.02	0.11 ± 0.02
Valine	2.61 ± 0.13	3.31 ± 0.97	2.66 ± 0.14	2.56 ± 0.03	2.61 ± 0.13
Taurine	0.34 ± 0.01	0.25 ± 0.05	0.19 ± 0.02	0.24 ± 0.03	0.24 ± 0.02

CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast. Values are the means of duplicate groups represented as mean ± S.E. Different superscript letters indicate a significant difference ($p < 0.05$) of means. The absence of superscript letters indicates no significant difference in means between groups.

[1] SFA (saturated fatty acids): 12:0; 13:0; iso-14:0; 14:0; anteiso-15:0; iso-16:0; anteiso-16:0; 15:0; 16:0; iso-17:0; anteiso-17:0; 17:0; iso-18:0; 18:0; 19:0; iso-20:0; 20:0 and 22:0. [2] MUFA

(monounsaturated fatty acids): 14:1n-7; 14:1n-5; 16:1n-9; 16:1n-7; 17:1; 18:1n-11 + 18:1n-9; 18:1n-5; 19:1; 20:1n-11; 20:1n-9; 20:1n-7; 22:1n-9 and 22:1n-7.

[3] PUFA (polyunsaturated fatty acids). [4] n-3/n-6: ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids. [5] 22:6n-3/20:5n-3: ratio of docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA). ND = not detected; Tr= trace (tr < 0.01).

Table 3.1.6 Blood health parameters of juvenile red seabream 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

CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast. Values are means of triplicates represented as means ± S.E. Different superscript letters indicate significant difference ($p < 0.05$). Absence of letters indicates no significance difference between groups. T-Cho: total cholesterol; Bun: blood urea nitrogen; T-Bill: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: total glycerides; TP: total protein.

Table 3.1.7 Antioxidant capacity of juvenile red seabream 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]

CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast. The values are means of triplicate groups represented as mean ± S.E. Different superscript letters indicate significant difference ($p < 0.05$) of means. Absence of letters indicate no significance difference of means between groups.

Table 3.1.8 Table of principle component axis (PCA) loading plot for the variables observed in juvenile red sea bream fed the test diet for 45 days.

Variables	PC1	PC2	PC3	PC4
FI	0.67	-0.47	-0.53	0.22
FCR	0.94	-0.14	-0.01	-0.30
WG%	0.93	-0.36	-0.10	-0.07
SGR	0.93	-0.34	-0.11	-0.04
PG	0.87	-0.48	0.09	-0.08
FBW	0.94	-0.24	-0.15	-0.18
Hrt	0.05	0.54	-0.84	0.02
Glu	0.60	-0.75	-0.15	-0.24
T-Cho	0.40	0.67	0.50	0.37
BUN	0.65	0.65	0.33	0.22
T-Bil	0.44	0.81	-0.23	-0.31
AST	0.31	0.88	-0.17	0.32
ALT	0.57	0.80	-0.20	0.02
TG	0.04	0.77	0.43	0.47
TP	0.68	0.65	-0.05	-0.34
MDA	0.16	0.79	0.13	-0.58
BAP	-0.87	0.43	-0.22	0.12
d-ROM	0.33	-0.07	0.92	0.22
SOD	-0.91	-0.19	-0.37	-0.07
PER	0.39	0.61	-0.22	0.66
CAT	-0.82	-0.46	0.01	0.34
LYS	0.41	-0.55	-0.54	0.49
IgM	0.37	-0.27	-0.60	0.65
CP%	-0.03	-0.69	0.71	0.17
Lipid%	0.75	-0.40	0.44	0.28

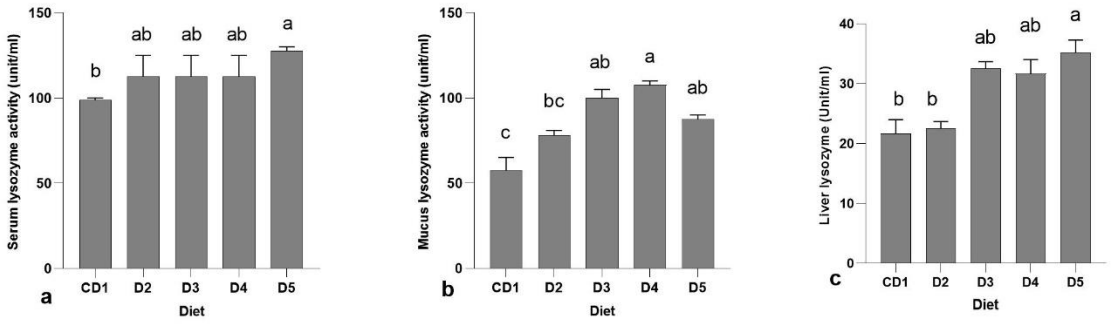


Fig. 3.1.1 Lysozyme activity of juvenile red sea bream fed test diets for 45 days. (a) Serum lysozyme activity (unit/ml); (b) Mucus lysozyme activity (unit/ml); (c) Liver lysozyme (unit/mg). Values are means (n=3) ± standard error (SE). Different superscripts indicate significant difference ($p<0.05$) between treatment means. Absence of letters indicate no significant differences CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast.

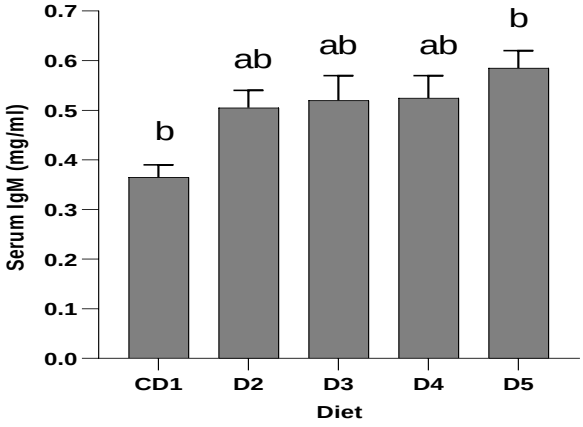


Fig. 3.1.2 Serum total Immunoglobulin (IgM) of juvenile red sea bream fed test diets for 45 days. Values (mg/ml) are means ± SE (n=3). CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0.1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast.

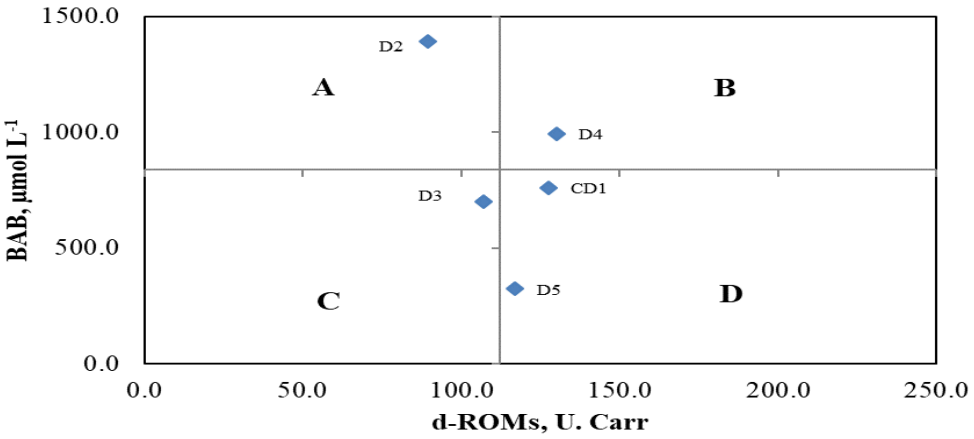


Fig. 3.1.3 Oxidative condition of juvenile red sea bream fed test diets for 45 days. Values represent the means ± SE (n=3). The central axis is the means of both d-ROM and BAP for the dietary groups. Zone A: high antioxidant capacity and low reactive oxygen metabolites (good health condition); Zone B: 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 with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast.

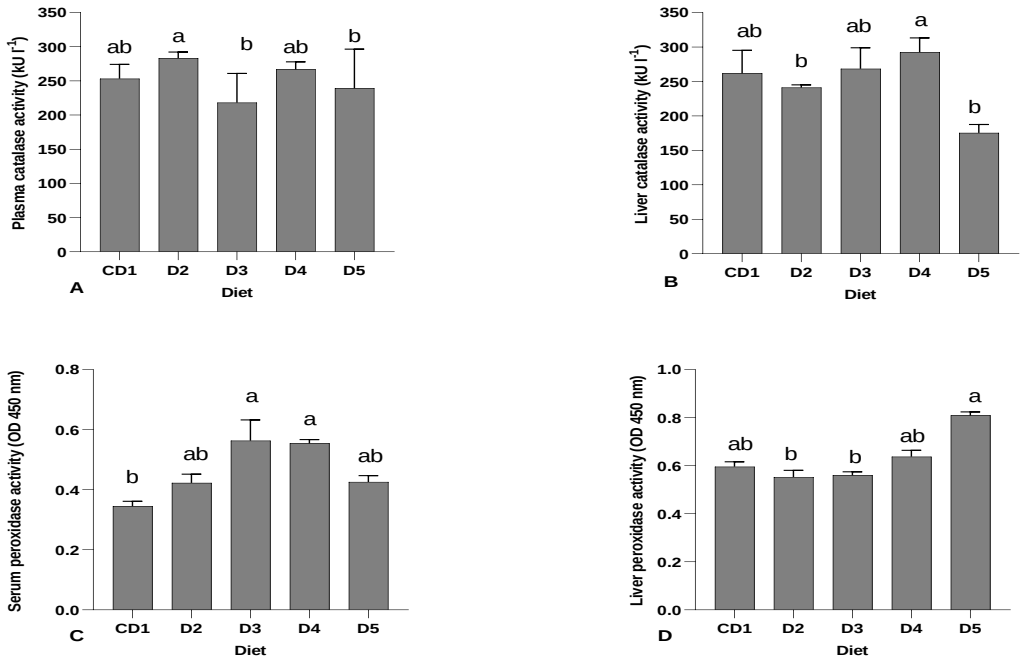


Fig. 3.1.4 Catalase (kU/l) (a&b) and peroxidase activities (c&d) (OD at 450 nm) of juvenile red sea bream after the 45-days of experimental. Different superscripts indicate significant differences ($p<0.05$) between treatment means. Absence of letters indicate no significant differences CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast.

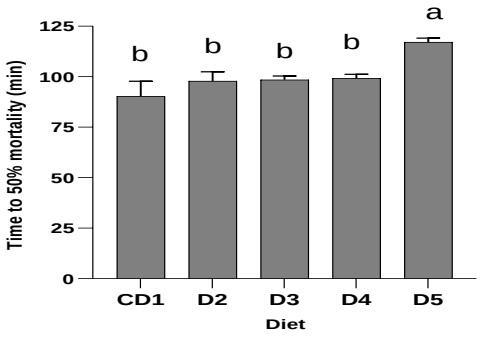


Fig. 3.1.5 Time (min) to 50% mortality of juvenile red sea bream exposed to low salinity stress after 45 days feeding test diets. The values are means ± pooled S.E. Absence of letters indicate no significant differences. CD1= the control or basal diet with no yeast supplements; D2 = the

basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast.

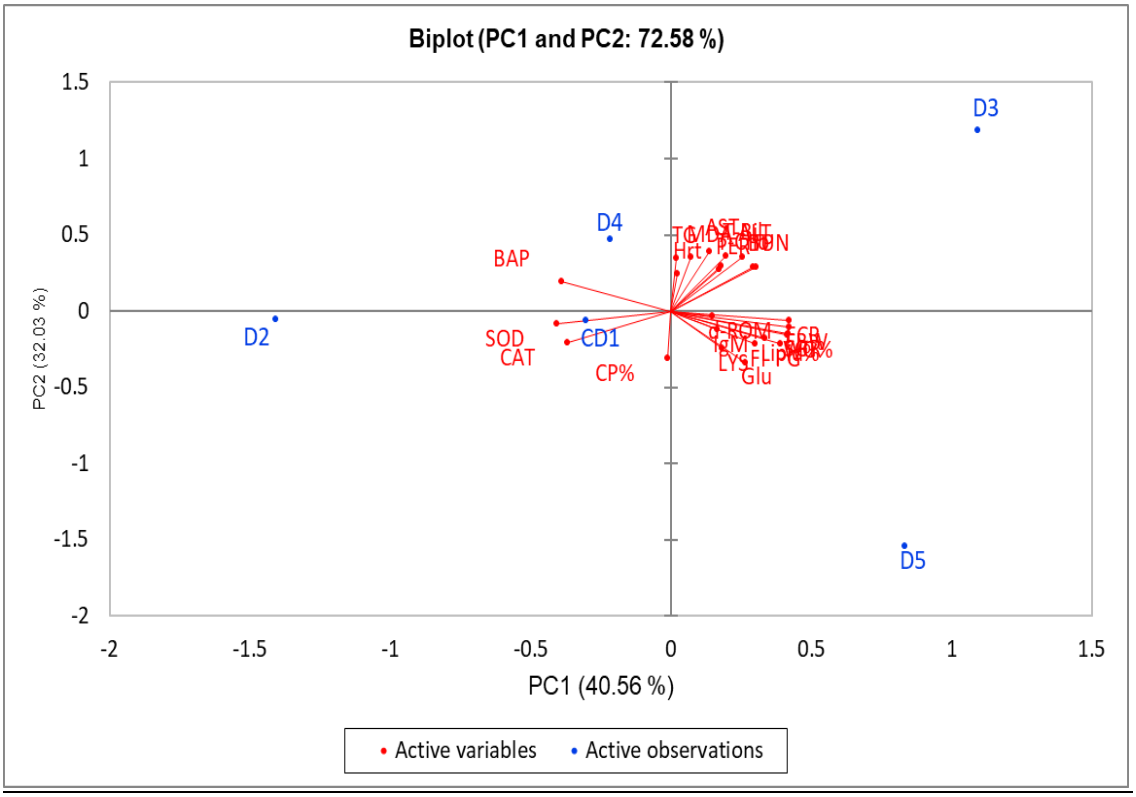


Fig. 3.1.6 The principle component analysis of parameters measured in the fish group fed the test diet for 45 days (See table 8). The PC1 separate the variables horizontally and explained 40.56% of the total variances. The PC2 separates the variables vertically and explained 32.03% of the variance. The variables contributed to the separation are indicated by line and the length of the line indicate the strength of the correlation of variable to PC1 and PC2. CD1= the control or basal diet with no yeast supplements; D2 = the basal diet + 0.05% yeast; D3 = the basal diet + 0. 1% yeast; D4 = the basal diet + 0.5% yeast; D5 = the basal diet + 1.0% yeast.

3.1.5 Discussion

Aquaculture of species at high population density has raised many challenges that need better strategies to improve quality and maximise production. 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*. 2018a), modernising aquaculture systems (Liang and Chien 2013; Mansour and Esteban 2017), and incorporating feed additives (prebiotics, probiotics, immunostimulant agents, exogenous enzymes, nucleotides hormones and antioxidants) in feed formulation (Dawood *et al*. 2018a). Dietary supplement for growth and immunostimulant are known to be useful for enhancing growth and disease resistance of cultured species (Oliva-Teles 2012). The oleaginous yeast has nutritional derivatives include lipids, glucans and mannooligosaccharides that can be utilized by fish (Blomqvist *et al*. 2018). Studies have indicated the positive effect of yeast cell wall ß-glucan on the immune system of fish and also 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: Johan *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 feed intake (FI) for yeast supplement at D5 (1%) dietary proportion with a lower FCR, suggesting a better nutrient uptake and metabolism. The final body weight (FBW) and body weight gain (BWG%) was significantly higher in fish group fed D3 and D5 (1%), demonstrating an improved feed utilization and nutrient absorption stimulated by the yeast supplement due to modulation of

intestinal microflora (Rimoldi *et al.* 2020), resulting in an improved growth performance of fish (Navarrete and Tovar-Ramírez 2014). Fish fed D4 showed no significant differences, however, the analysis of yeast cells in feed would add further information for a better explanation on this scenario. Yeast cell wall supplemented in diets of gibel carps (*Carassius auratus gibelio*) improved FBW at 0.4% of the dietary proportion (Zhang *et al.*,2020). Additionally, Huang *et al.* (2020) investigated the performance of *Lates calcarifer* fed tilapia piscidin 4-expressing yeast (*Pichia pastoris*), where results showed significant improvement in weight gain (WG) and feed conversion ratio (FCR). In our study, growth performance and feed utilization improved with increasing dietary supplementation of yeast.

The use of oleaginous yeast derived oil from glucose substrate (wheat straw) has already been investigated in fish (Blomqvist *et al.*, 2018; Hatlen *et al.*, 2012), but little is known about the dietary effect of the spent oleaginous yeast on amino acid profile in fish. Amino acids have unique properties, such as maintaining the integrity, growth and function of the intestine, normalizing inflammatory cytokine secretion, improving T-lymphocyte numbers and specific T cell functions, and increasing the section of IgA by lamina propria cells in aquatic animals (Ruth and Field 2013). In the present study, whole body amino acid profile revealed a slight increase in Taurine, Valine and Methionine for fish group fed D2 (0.05%) while a numerically high Arginine in fish group fed D5 (1%), suggesting an improved fish health condition (antioxidant and immune responses) in fish fed dietary yeast supplement.

The fatty acid profile of the diet with a low amount of n-6/n-3 ratio is important for animal disease prevention. This study revealed a decrease in the total amount of n-6 fatty acid in fish groups fed yeast supplemented diets, which is in line with the findings of Blomqvist *et al.* (2018) where yeast fed diets in sea bass decreased the n-6 fatty acid ratio in fish whole body. Conversely, a low n-3/n-6 ratio is important as high ratio in modern diets leads to a variety of diseases (Simopoulos 2006). The fish fed diet D2 (0.05%) yeast showed relatively low n-3/n-6

ration compared to the high ratio found in fish group fed D5 (1%) yeast supplement group. The whole body MUFA increases in fish group fed yeast supplement diet, suggest the functional properties of the yeast in improving the health of fish. Thus, spent oleaginous yeast from wheat straw fermentation is an important feed supplement for fish. On the other hand, it is important to note that the fatty acid composition of the yeast varies with strain and cultivation conditions (Brandenburg *et al.*, 2016; Brandenburg *et al.*, 2018; Olstorpe *et al.*, 2014). Consequently, choosing a better yeast strain and improving culture conditions can have positive influence on fatty acid composition such as the n-6/n-3 ratio that is beneficial for animal feed (Blomqvist *et al.*, 2018).

Blood parameters are good indicators of the fish health status and reflect the responses to stressors and external stimuli (El Basuini *et al.* 2017; El Basuini *et al.* 2016). The blood indices recorded in this present study were within the acceptable limits for red sea bream (Kader *et al.* 2010; Takagi *et al.* 2001; Uyan *et al.* 2007). The decreased in blood triglycerides (TG) in fish group fed yeast supplement diets suggest the effectiveness of yeast supplementation, and a similar trend was observed by Tao *et al.* (2015) on the blood profile of ruminant calves as a result of dietary yeast glucan supplementation to the diet. The lowest plasma AST values was found in fish group fed D5 (1%) while plasma ALT values were lowest in yeast supplemented groups but were not significantly different from the control group. High AST and ALT in blood are linked to damage in the liver cause by an enzyme located in the cytoplasm which is release into circulation after being damaged (Nagai *et al.*, 1989; Vermeulen *et al.*, 1992). However, the decrease in AST and ALT in fish fed yeast supplemented diets suggest a beneficial effect for aquatic animal feeds. Plasma total protein (TP) values were not significantly different between all treatment group, which indicates a stable fish health condition for fish. The low plasma cholesterol (T-choc) level in the yeast supplement group is linked to the effects of the yeast

glucan content in the fish diet, as such effect was noted in previous studies (Robbins and Seeley 1977; Tao *et al*., 2015).

The supplementation of yeast as a functional diet alters the immune response of fish directly or indirectly by impressing on their immune cells in either metabolic, neurological or endocrine pathways (Dossou *et al*., 2018b). The first line of the defence mechanism in fish is phagocytosis activity of the cells and is expressed in lysozyme activity (Cecchini *et al*., 2000; Dawood *et al.,* 2016a); thus, forming the non-specific immune defence mechanism of the immune systems (Dawood *et al*., 2015a). Lysozymes are produced by leukocytes cells which lysis the cell wall of microbes and stimulate the activation of the immunity complementary system (Cecchini *et al*., 2000). The results of the present study showed significantly high lysozyme (serum, mucus and liver) activity for yeast supplemented group which demonstrate the enhancement of the phagocytosis cells stimulated by dietary yeast ß-glucan in the supplemented diet which is consistent with the observation on yeast ß-glucan causing increase in lysozyme activity in Atlantic salmon (*Salmo salar* L.) (Paulsen *et al*., 1991: Engstad *et al.,* 1992). A study by Dawood *et al*. (2017b) on dietary supplementation of ß-glucan in red sea bream diets revealed an increase in lysozyme activity in the fish group fed supplemented diets. Immunoglobulin (IgM) in blood is an adequate indicator of humoral immunity and fish well-being which is also a significant component of blood serum (Yeganeh *et al*.,2021). The concentration of IgM provides an immediate and wide range of protection against pathogens which forms an important component of the innate immune system (Magnadottir 1998; Magadottir *et al.*,2010). The IgM level in serum of some fish species was reported to range between 0.25 and 23.5 mg/ml, and variation could be related to size, age, environmental condition, and disease status (Ceusta *et al*.,2004). The results in our study showed significant increase in the IgM concentration for the fish group fed the yeast supplements which is in line with previous findings where IgM level is high in fish fed probiotic diets (Allameh *et al.*,2017; Sun *et*

al.,2012) or yeast supplement diets (Huang *et al.*,2020). The improved immune response (Lysozyme and IgM) obtained with the addition of dietary yeast supplement demonstrates the improved immunity of fish in the current study, suggesting the immunomodulatory effects of the dietary yeast product.

The antioxidant defence system in fish is associated with their health status (Martínez-Álvarez *et al.*, 2005). Recent studies assessed oxidative stress by measuring their derivatives, such as the reactive oxygen metabolites (d-ROM) and BAP (Dawood *et al.*, 2016a; El Basuini *et al.*, 2016; Hossain *et al.*, 2016). Yeast dietary glucans are well known for stimulating the antioxidant enzyme activity in fish (Rodrigues *et al.*, 2020). In the present study, the combination of BAP and d-ROM values revealed that fish fed D2 (0.05%), D3 (0.1%) and D4 (0.5%) diets have improved oxidative condition compared to those receiving D5 (1%) and control group (CD1). The significantly ($p<0.05$) high BAP activity in the D2 and numerically high activity in D4 groups suggest that the spent oleaginous yeast of wheat straw fermentation is a potential feed supplement for aquatic animals. Lipid peroxidase MDA and other important antioxidant enzymes, such as SOD, CAT and GPx were analysed in this study. Lipid peroxidation of the cell membrane is measured by MDA activity (Aliahmat *et al.*, 2012). The analysis of MDA revealed a 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 defence influence that reduces the elevated level of MDA for tilapia (Xu *et al.*, 2015). The SOD activity was improved in fish group fed yeast supplemented diets especially D2 (0.05 %) compared to all test diets. A study on supplementation of yeast at 0.6% of the diet for Nile tilapia revealed 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). Conversely, catalase activity was improved in fish fed yeast supplement diets although

no significant difference was observed compared to fish fed the control diet (CD1). 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.,* 2019a; Dawood *et al.*, 2016b). The enzyme activities of CAT and GPx were higher in the liver of the fish group fed the yeast supplemented diets, suggesting an improved antioxidant defence system in which is in line with the findings of previous studies (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 assessment of tolerance against stress were conducted often through challenges tests, which demonstrate the biological and physical stress responses (Barton 2002). Amongst the stressors, low salinity is often used as an indicator of tolerance to stress for fish quality after nutritional trials (Dawood *et al*., 2017b; Dossou *et al.* 2018a; Hossain *et al*., 2016). The stress results obtained in the present study indicated that stress tolerance was significantly improved in the fish groups fed yeast supplemented diet D5 ($P<0.05$). It is appropriate to say that the presence of immune-stimulating compounds such as ß-glucan, nucleic acids and/or mannoligosaccharides in the yeast have boosted the ability of fish to survive under stressful conditions (Li and Gatlin 2006; Lokesh *et al.,* 2012).

The parameters assessed using principle component analysis (PCA) (Fig.8, table 6) revealed two clusters correlated with fish fed diet D3 and D5 suggesting that yeast supplement has boosted the growth, feed utilization and immune response of red sea bream. The fish fed D4 was in a neutral condition, however, fish fed D2 showed a strong association with antioxidant activity compared to control diet D1. This finding suggest that, yeast supplement has stimulated growth, feed utilization, antioxidant and immune of juvenile red sea bream.

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%) level dietary proportion suggest a potential probiotic for improving antioxidant activity in fish. The following experiment (II) was conducted to further investigate the optimum dietary supplementation threshold in red seabream, *P. major*.

Experiment II.: Physiological condition, digestive enzyme, blood haemato-biochemistry, antioxidant, immune and stress response of juvenile red sea bream (*Pagros major*) fed diets containing spent oleaginous yeast at ≥1% dietary proportion.

3.2.1 Abstract

A 50 days feeding trail was conducted to evaluate the efficiency of spent oleaginous yeast *Lipomyces starkeyi* by-product from wheat straw as a probiotic supplement on juvenile red sea bream (*Pagrus major*). Elements that were investigated includes physiological condition, digestive enzyme, haemato-biochemistry, antioxidant, immune and stress. Three hundred juvenile *P. major* (9.13 ± 0.05 g) were randomly distributed into 200 L polyethylene tank (20 fish per tank) in triplicate of 5 treatments; CD1 control (0 g/kg supplement), D2 (1 %, 10 g/kg supplement), D3 (1.5 %, 15 g/kg supplement), D4 (2 %, 20 g/kg supplement), D5 (2.5 %, 25 g/kg supplement). The fish were fed twice daily and sampling for weight and length were conducted at 35 and 50 days of the feeding trial. Whole body samples, digestive organs and

blood collection was done at the end of the feeding trial. The results of the combine parameters assessed by multivariate analysis revealed that fish group fed on D5 has significantly better performance followed by D4, D2 and D3 compared to the fish group fed the control diet D1 (ANOSIM, $P < 0.003$, $r \geq 0.5$). Total cholesterol (T-Cho), derivative of reactive oxygen metabolite (d-ROM), total protein (T-Pro), protease digestive enzyme (D-protease) and alanine aminotransferase (ALT) explained 77.95 % dissimilarities between fish group, suggesting the functional properties of the spent oleaginous yeast in modulating and improving these parameters. The fish fed yeast supplement diets showed considerable increase in whole body crude lipid, saturated fatty acid (SFA) and monounsaturated fatty (MUFA) acid with increasing yeast supplement while polyunsaturated (PUFA) reflected the inverse of this relationship. No significant alteration was detected on condition factor, relative condition factor and, length and weight relationship (LWR) between fish fed all test diets. Survival was not significantly different between treatments. Growth responses, digestive enzymes activity, blood health, antioxidant activity and immune response were significantly improved in fish fed yeast supplemented diets compared to fish group fed the control diet. The stress tolerance ability was significantly higher in fish group fed yeast supplement diets compared to fish group fed the control diet. This results suggest that, spent oleaginous yeast *Lipomyces starkeyi* is a potential probiotic supplement for red sea bream with an optimum supplementation at 2.5 % of dietary proportion.

Keywords:

Red sea bream (*Pagrus major*); Growth responses; Oxidative condition; Blood status;

Immune system.

3.2.2 Introduction

The Aquaculture of red sea bream has provided significant economic returns in many industrialise countries especially in eastern Asia (Kim *et al.*, 2012; Ito *et al.,* 2013). The rapid development of intensive culture practices has also led to frequent disease outbreaks resulting in considerable economic losses (Cerezuela *et al.*,2012). The conventional application of antibiotics though has boosted the growth of aquaculture industry and has resulted in increased production. On the other hand, several studies have reported on the negative impacts on the environment and the reduction in ability to resist pathogens at the species level (Lalumera *et al.*, 2004; Romero *et al.*, 2012). These findings suggest the need to investigate natural alternatives with minimal adverse impacts on the environment and animal health (Zaineldin *et al.*, 2021). The use of probiotics, prebiotics, medicinal herbs and other feed additives in fish aquaculture has been a successful approach for improving the host health condition, disease resistance and growth (Dawood *et al.*, 2016a; Van Doan *et al.*, 2020). Probiotics includes live, dead or a component of microbial cells that are supplied in the feed or via the rearing water to improve animal health and the condition of the environment (Lara-Flores, 2011). Studies have shown that the use of these natural additives have boosted organism's disease resistance, growth, feed utilization, stress tolerance and also improved the condition of the surrounding environment (Van Doan *et al.*, 2020; Merrifield *et al.,* 2010; Ringø *et al.*, 2020). Additionally, the use of additives in the modern animal feed formulation have significantly improved the utilization of non-conventional animal feed sources. There are many forms of probiotic supplement used in aquaculture, e.g. bacterial cells and yeast (Dawood *et al.*, 2019a; Gatesoupe, 1999). Yeast is often used as probiotic and prebiotic due to their unique mode of action in promoting aquatic animal health (Dawood *et al.*, 2015b; Meena *et al.*, 2013; Zhang *et al.*, 2020; Van Doan *et al.*, 2020). Yeast produces oligosaccharides, amino acids, peptides, organic acids, vitamins and other soluble molecules (Peppler, 1982; Zaineldin *et al.*, 2021). Yeast cell wall also contains polysaccharide, ß-D-glucan, chitin and mannooligosaccarides which are the

important molecules in probiotics (Bowman and Free, 2006). The supplementation of Baker's yeast cell walls in diets of Japanese seabass revealed an optimum range of 1-2 g/kg (Yu *et al.*, 2014). Whilst it's supplementation in diets tilapia (*Oreochromis niloticus*) revealed that 0.2% is the optimal ratio with beneficial effect on immune responses and the expression of related genes (Abu-Elala *et al.*, 2018). Besides, 0.4% supplementation of yeast cell wall in diets of gibel carb (*Carassius auratus gibelio*) has also improved growth and immune responses of fish (Zhang *et al.*, 2020). Recently, studies have demonstrated that diet supplementation of yeast cells could prove to be a potential protein source for fish and can be used alternatively as a fish meal replacement (Sahlmann *et al.*, 2019; Øverland and Skrede, 2017). Previous studies have suggested that oleaginous yeast derived from lignocellulose substrates were reported to be a safe ingredient for animals (Øverland and Skrede, 2017; Blomqvist *et al.*, 2018). A study of wheat straw oleaginous yeast oil effect in fish health revealed no adverse effect when replacing fish oil (Blomqvist *et al.*, 2018). The oleaginous yeast *Lipomyces* spp. is one of the main oil producing yeast in oil and brewing industry which produce >70 % of their body weight (Naganuma, 2012; Takaku *et al.*, 2020). The spent oleaginous yeast *Lipomyces starkeyi* was reported to be a useful livestock feed (Takaku *et al.*, 2020). Presently, studies on the efficacy of supplementing spent oleaginous yeast *Lipomyces starkeyi* cells derived from wheat straw by-product in diets of aquatic animals is limited. Therefore, the present study aims to evaluate the dietary efficacy of spent oleaginous yeast on the physiological condition, growth responses, feed utilization, blood haemato-biochemistry, antioxidant, immune and stress responses of juvenile red sea bream (*Pagros major*).

3.2.3 Materials and Methods

3.2.3.1 *Dietary yeast product*

The Lipomyces starkeyi spent oleaginous yeast dry cells were generously provided by Fuji Oil Holdings Inc., Japan and Toray Industries, Inc., Japan. The yeast dry cells contain 69.26 % lipid, 6.80 % protein and 3.4 % ß-glucan of the sample weight. The dry yeast cells were kept at -18 °C until use.

3.2.3.2 *Test Diet*

The formulation and chemical composition of the experimental diets is presented in Table1. The spent oleaginous yeast dry cells were supplemented at the following percentage proportion of the experimental diets: 0 % (Control diet = CD1), 1 % (D2), 1.5 % (D3), 2 % (D4), and 2.5 % (D5). The α-Cellulose powder was added to adjust the weight of the dietary proportion to 100 %. The dry yeast cells were combined with lipids ingredients such as Pollack liver oil, soybean lecithin and n-3 Highly Unsaturated Fatty Acid (HUFA) which are then added to the ingredient mixture. The combine ingredients were stirred with a spatula for a few minutes and then mix thoroughly with a food mixer for 15 minutes. Further, an amount of 30 – 40% water base on the weight of the ingredient was added and then mixed for another 15 minutes. Prior to pelleting, the pH of the diet mixture was adjusted with 4 N sodium hydroxide to neutral pH (7.2). Pelleting was conducted using a meat grinder of 1.2 mm diameter openings and then dried in a mechanical convection oven at 50 °C to less than 10 % moisture content. Choice of drying temperature was in accordance to Solomon *et al.* (2017). The dry pellets were packed in polypropylene bags and stored in a -28 °C freezer until use. The dietary proximate composition analysis presented in Table 1 showed increasing trend in crude lipid content is significantly higher ($P < 0.05$) in D5 compared to D2 and the control diet D1, and similar trend was observed for carbohydrate content. Table 2 showed the fatty acid content of the experimental diets. The Saturated fatty acid (SFA) and mono-unsaturated fatty acid (MUFA) were increased with

increasing graded levels of yeast supplement while polyunsaturated fatty acid (PUFA), n-6 PUFA and n-3 PUFA reflect an inverse relationship.

Table 3.2.1 Experimental diets ingredients and proximate composition

Ingredients	Experimental diets				
	D1	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 [4]	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	4.47	3.97	3.47	2.97
Yeast [14]	0	1	1.5	2	2.5
Total	100	100	100	100	100
Proximate composition					
Crude protein	46.85±0.76	46.41±0.98	46.95±0.27	46.52±0.66	46.16±0.89
Crude lipid	10.48±0^{b}	10.61±0.26^{ab}	11.14±0.24^{ab}	11.71±0.15^{ab}	11.94±0.38^{a}
Crude ash	13.49±0.01	13.38±0.15	13.39±0.12	13.92±0.05	13.91±0.04
Carbohydrate [15]	25.69±0.81	25.84±2.07	24.86±0.01	24.19±0.88	24.34±0.58
Gross energy (KJ/g) [16]	13.97±0.05^{b}	14.38±0.94^{ab}	14.8±0.16^{ab}	15.37±0.17^{ab}	15.59±0.34^{a}

[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 $NaHSO_3 \cdot 3H_2O$ (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 (Nicotic 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; $Na2HPO_4$,

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., Japan and 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.

Table 3.2.2 Fatty acids (mg/ 1000 mg diet) contents of experimental diets

Parameters	Experimental diets				
	D1	D2	D3	D4	D5
Fatty acids					
SFA [1]	54.9 ± 0.36^{b}	54.45 ± 0.09^{b}	53.97 ± 0.22^{b}	57.77 ± 3.74^{a}	63.63 ± 2.24^{a}
MUFA [2]	57.74 ± 0.36^{c}	58.15 ± 0.09^{cb}	58.25 ± 0.22^{b}	59.04 ± 1.74^{ab}	61.81 ± 2.24^{a}
16:2n-6+16:2n-4	1.99 ± 0.02	2.03 ± 0.01	1.97 ± 0.01	2.94 ± 0.16	2.81 ± 0.02
16:4n-3	2.03 ± 0.01	2.01 ± 0.03	1.99 ± 0.01	2.36 ± 0.07	2.34 ± 0.17
18:2n-6	24.1 ± 0.32^{a}	23.73 ± 0.16^{ab}	23.54±0.13^{ab}	24.04 ± 2.6^{ab}	21.44 ± 0.64^{b}
18:3n-6	0.8 ± 0.07	0.73 ± 0.05	0.8 ± 0.08	0.87 ± 0.11	0.81 ± 0.04
18:3A	0.24 ± 0.06	0.28 ± 0	0.27 ± 0.02	0.26 ± 0	0.26 ± 0
18:3n-3	3.11 ± 0.02	3.08 ± 0.03	3.04 ± 0.03	2.71 ± 0.7	3.19 ± 0.4
18:4n-3	3.11 ± 0.06	3.1 ± 0.03	3.07 ± 0.03	3.3 ± 0.67	2.37 ± 0.22
18:4n-1	0.31 ± 0.02	0.28 ± 0.01	0.28 ± 0.01	0.3 ± 0	0.27 ± 0
20:2n-6	0.13 ± 0.19	0.19 ± 0.12	0.14 ± 0.02	0.09 ± 0.13	0.22 ± 0.01
20:3n-6	ND	ND	0.06 ± 0.08	0.1 ± 0.15	0.15 ± 0.36
20:4n-6	1.2 ± 0	1.18 ± 0.01	1.15 ± 0.04	1.34 ± 0.04	1.23 ± 0.02
20:3n-3	0.05 ± 0.06	0.1 ± 0.01	0.11 ± 0.01	0.05 ± 0.07	0.05 ± 0.07
20:4n-3	0.98 ± 0.01	0.99 ± 0.04	0.97 ± 0	0.91 ± 0.02	0.85 ± 0.01
20:5n-3	18.52 ± 0.34	18.3 ± 0.19	18.3 ± 0.03	17.09 ± 1.12	17.29 ± 0.81
22:3n-6	0.62 ± 0.09	0.57 ± 0.02	0.62 ± 0.03	0.65 ± 0.03	0.63 ± 0.03
22:5n-3	2.19 ± 0.05	2.18 ± 0.04	2.26 ± 0.01	2.81 ± 0.15	2.7 ± 0.09
22:5n-6	0.29 ± 0.03	0.21 ± 0.01	0.2 ± 0.03	0.13 ± 0.13	0.13 ± 0.13
22:6n-3	13.93 ± 0.86	13.7 ± 0.05	14.02 ± 0.25	13.27 ± 1.82	11.01 ± 0.6

PUFA [3]	71.04 ± 1.71[a]	70.77 ± 0.22[a]	70.53 ± 0.79[a]	69.72 ± 0.39[a]	68.59 ± 1.7[b]
n-3 PUFA	41.88 ± 1.39	42.4 ± 0.11	42.04 ± 0.76	40.14 ± 2.79	37.45 ± 0.36
n-6 PUFA	27.13 ± 0.32[a]	26.61 ± 0.29[ab]	26.5 ± 0.02[ab]	27.22 ± 2.34[ab]	24.81 ± 1.17[b]
n-3/n-6 [4]	1.7 ± 0.02	1.75 ± 0.01	1.75 ± 0.03	1.67 ± 0.2	1.73 ± 0.04
22:6n-3/20:5n-3 [5]	0.75 ± 0.03	0.75 ± 0.01	0.77 ± 0.01	0.77 ± 0.06	0.6 ± 0.06

D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2.0 % yeast; D5 = the basal diet + 2.5 % yeast. Values are the means of duplicate groups represented as mean ± S.E. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of superscript letters indicates no significant difference in means between groups.[1] SFA (saturated fatty acids): 12:0; 13:0; iso-14:0; 14:0; anteiso-15:0; iso-16:0; anteiso-16:0; 15:0; 16:0; iso-17:0; anteiso-17:0; 17:0; iso-18:0; 18:0; 19:0; iso-20:0; 20:0 and 22:0.[2] MUFA (monounsaturated fatty acids): 14:1n-7; 14:1n-5; 16:1n-9; 16:1n-7; 17:1; 18:1n-11 + 18:1 n-9; 18:1n-5; 19:1; 20:1n-11; 20:1n-9; 20:1n-7; 22:1n-9 and 22:1n-7.[3] PUFA (polyunsaturated fatty acids). [4] n-3/n-6: ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids. [5] 22:6n-3/20:5n-3: ratio of docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA).

3.2.2.3 *Fish and experimental condition*

The research was conducted at the Kamoike Marine Research facility, Faculty of Fisheries, Kagoshima University, Japan. The juvenile red sea bream were purchased from commercial hatchery (Miyazaki prefecture, Japan). The juveniles were stocked in 100 L polyethylene tanks filled with 80 L of seawater in a flow-through seawater system at the rate of 1.51 L per min, supplied with continuous aeration. The juvenile fish were fed with commercial feed during a 7 days' acclimation period. The rearing water quality was maintained at the following conditions; temperature (26.1 ± 1.2 °C), pH (8.1 ± 0.5), salinity (33.1 ± 0.5 psu) and dissolve oxygen (6.1 ± 0.5 mg/L). At stocking into rearing tanks, the juvenile red sea bream (n = 300 fish) had an average initial body weights of 9.13±0.05 g. Each individual was randomly assigned into the 5 experimental groups at 20 fish per tank. A daily feeding strategy of twice per day at 8 am and

4 pm over the 50 days' trial period was adopted. Uneaten feed was siphoned after one hour of feeding and then, dried using a freeze drier.

3.2.2.4 *Sampling*

At the end of the 50-day feeding trial, all treatments were not fed 24 h prior to sample collection. The fish were anesthetized with Eugenol (4-Allylmethoxyphenol, 50 ml/L) before sampling of body weight and length. A sample of 5 fish per tank were collected and stored in a freezer at -20 °C for final whole-body analysis. Blood was extracted using heparinised (n =5) and non-heparinised (n=3) syringes. The heparinised blood was used to collect the micro-haematocrit using micro-haematocrit technique. The heparinised blood was centrifuged (3000 x g for 15 min) using a high speed refrigerated microcentrifuge and the blood plasma were obtained and kept in a freezer at -80 °C. Non-heparinised blood samples were kept at for 2 h at room temperature, and then centrifuged at 3000 x g for 15 minutes to collect serum. The viscera and liver were collected by dissecting 3 fishes from each tank and weighted to determine viscerasomatic index (VSI) and hepatosomatic indices (HSI) (El Basuini *et al.,* 2021). The liver were pooled together and stored at -80 °C for further analysis. The VSI and HSI were calculated following the equations below.

$$VSI = (\text{Viscera weight} / \text{fish body weight}) \times 100$$

$$HSI = (\text{Liver weight} / \text{fish body weight}) \times 100$$

A total of 9 fishes were collected (3 fishes from each triplicate tank) for skin mucus sample collection. Following the protocol outline by Dawood et al. (2020), the fish skin surface were washed with distilled water before a sterilized piece of cotton was rub gently on 200 mm^2 of the body surface. The collected mucus sample was immediately transferred into 1.5 mL tubes and suspended with 1 mL PBS (pH= 7.4). The samples were then centrifuged at 2,000 x g, 4 °C for 10 min. The supernatant was collected and transferred to a new 1.5 mL tubes and stored at -80 °C for analysis.

3.2.2.5 *Growth performance and feed utilization*

The growth and feed utilization indices were calculated for juvenile red sea bream following methods described by Kader et al. (2012).

Weight gain (%) = [(final weight – initial weight) /initial weight] x 100

Specific growth rate (SGR%) = [(Ln (Final weight) - Ln (initial weight))/duration (50 days)] x 100

Survival (%) = (Fish no. at 50 days/ Fish no. at the beginning of the experiment) x 100

Feed intake (FI, g / 50 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 gains) = [(final weight, g x final whole body protein content /100) – (initial weight gain, g x initial whole protein body content/100)] x 1,000 / weight gain (g)

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

The biometrics of the physiological condition of the fish was determined following the procedures described in Shadrack *et al.* (2021). Sampling for weight and length data for biometrics was conducted at 35 days and 50 days of the feeding trial. The condition factor (K) was calculated according to equation of Htun-Han (1978), and the relative condition factor (K*n*) was determined following the method of Le Cren (1951). The length and weight relationship coefficient (*b*) was determined using the equation of Froese (2006).

3.2.2.6 *Biochemical and antioxidant activity*

The analysis of proximate composition (moisture, crude protein, crude lipids) of the diets and fish whole body were done in triplicate following standard methods (Chemists, 1990). Moisture content was determined using mechanical convention oven by drying samples at 105 °C (Dk400,

Yamato Scientific CO., Tokyo, Japan) to a constant weight and loss in weight represent moisture content. Crude protein content was quantified using kjeldahl method for nitrogen level and evaluating the crude protein (Kjeltec System 1002 tecator, Sweden). Ash content was quantified by burning at 550 °C using a muffle furnace and the final weight of the product represent ash content. The crude lipids content of samples were determined by Soxhlet extraction method. Gross energy was calculated using the combustion value for carbohydrate, protein and lipid. The fatty acid of diets and fish whole body was quantified using a Gas chromatography following the procedure described by Teshima *et al*. (1986). Blood plasma chemical parameters were measured using dry chemical kit with an automated analyser (SPOTCHEM™ EZ model SP-4430, Array, Inc. Kyoto, Japan). The biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) from the blood serum were measured with an automated analyser kit (FRAS4, Diacron international s.r.l., Grosseto, Italy), following the manufacture instruction. Red blood cells (RBCs) were counted with a haemocytometer (Houston, 1990) immediately after dilution with Natt and Herrick's solution. Blood haemoglobin concentration was determined using a spectrophotometer (Multiskan Go, Themo Fisher Scientific K. K., Tokyo, Japan) following the sodium lauryl sulfate (SLS) (Wako Pure Chemical Industries Co. Japan) method by Oshiro et al. (1982) and the OD was read at 539 nm wavelength. The superoxide dismutase (SOD) activity of the serum was determined with the SOD assay Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) following the manufacturers instruction. The malondialdehyde (MDA) activity of serum was quantified using the Colorimetric TBARS microplate assay Kit (Oxford Biomedical Research, Inc., USA) following the manufactures instructions.

3.2.2.7 *Non-specific immune responses*

The lysozyme activity of serum was determined following the turbidometric assay procedure describe by Lygren *et al.* (2001). The serum and mucus total immunoglobulin (IgM) was determined following the method of Siwicki *et al.* (1994) and briefly described in Yeganeh *et al.* (2021). The oxidative radical production of neutrophils during respiratory burst was determined by the NBT assay in whole blood samples (Anderson and Siwicki, 1995). The serum protease activity was determined according to the method describe in Cordero *et al.* (2016). The total antiprotease (T-antiprotease) and α-antiprotease were determined following the methods describe in Newaj‐Fyzul *et al.* (2007). The mucus amount was quantified following the methods describe in Dawood *et al.* (2016a). The bacteria activity of serum and mucus was quantified following the method describe in El Basuini *et al.* (2020), using Escherichia coli ($1x10^8$) bacteria suspension where the OD was read at 570 nm (Multiskan Go, Themo Fisher Scientific K. K., Tokyo, Japan) and expressed as percentage inhibition of E. coli relative to the positive control. The total peroxidase activity of blood serum was measured following the method described by Salinas *et al.* (2008). Serum catalase activity was determined following previous methods (Cecchini et al., 2000, Goth, 1991).

3.2.2.8 *Digestive enzymes*

A total of 9 fish per treatment (3 fishes per tank) were sampled and dissected. Pooled intestinal samples per tank were washed with distilled water followed by rinsing in 2% sucrose solution before air dried for 15 min. To extract the crude enzyme, intestine was weighted and homogenized (1:5, w/v) in ice chilled homogenizing buffer (20 mM Tris-HCl, 1 mM EDTA,10 mM $CaCl_2$, pH 7.5) using a pellet pestle cordless motor (Sigma-Aldrich, Saint Louis city, Missouri, USA), and then centrifuged (1700 x g, at 4 °C) for 30 min. The supernatant was

collected after centrifugation and stored at -80 °C for the enzyme analysis. Protease enzyme (D-protease) activity was determined following the method of Qing and Xing (1997) with 0.5 % casein used as the substrate. The OD was measured at 680 nm using microplate reader (Multiskan Go, Themo Fisher Scientific K. K., Tokyo, Japan). Pepsin activity was determined according to the procedure of Natalia *et al.* (2004) using 2 % haemoglobin in 0.06 NH4Cl substrate. The OD was read at 280 nm using the microplate reader (Multiskan Go, Themo Fisher Scientific K. K., Tokyo, Japan). Lipase enzyme activity was determined following the procedure of Roberts (1985) where 4-ethylumbelliferyl butyrate (4 MUB) was used as substrate. The OD was taken at 450 nm emission (excitation 380 nm) with a fluorescence spectrophotometer (F-2700, Hitachi High-Tech Corp, Tokyo, Japan). Amylase activity was determined following the method of Murashita *et al.* (2018), where 1% starch solution was used as substrate. The OD was read at 540 nm using a microplate reader (Multiskan Go, Themo Fisher Scientific K. K., Tokyo, Japan).

3.2.2.9 *Low salinity stress test*

To determine stress tolerance at low salinity, 4 fish per tank (12 fishes per treatment) (modified from Dawood et al. (2020) of 5 fishes per tank) were randomly selected from the remaining population and transferred into a 20 L glass tank filled with dechlorinated low salinity water (0.2 %). The tank was then placed inside a 100 L black polyethylene tank containing water volume of a little less than 50 % of the 20 L glass tank height (Dawood *et al.*, 2017b; Dawood *et al.*, 2017a). Continuous water flow was supplied through the holding tank to keep the 20 L glass tank at ambient temperature during the test. Continuous aeration was supplied to the stress tank during the test. The number of dead fish in each tank were recorded every 10 minutes. Test was terminated when all the fish are dead. Time to 50% death was calculated according to Ren et al. (2007) and Moe *et al.* (2004) using $\log_{10}$ values in the following equation.

$$Y = aX + B$$

Where, Y= $\log_{10}$ (survival, assuming to be 100%) =2, X=time to individual death of fish (min), LT50 (X) was obtained when Y=1.7 since $\log_{10}$ (50) =1.7. Higher values indicate greater tolerance against stress.

3.2.2.10 *Statistical analysis*

The univariate statistical analysis was performed using the Paleontological Statistical Software package for Education and Data Analysis version 3.21 (Hammer *et al*. 2001). Data normality was confirmed via Kolmogorov-Smirnov test and the homogeneity of variances via Levene's test. Analysis of Variance (ANOVA) test were carried out and any significant difference were further evaluated using Tukey-Kramer range post-hoc test. The Polynomial orthogonal contrast was conducted using Microsoft excel spreadsheet to establish the relationship between increasing yeast supplement on the response variable. The Grubbs test in XLSTAT (2019) was used to remove significant outliers in fish length and weight data prior to analysis of biometrics. The linear regression function in GraphPad Prism version 8.0.1 for windows (GraphPad Software, San Deigo, California USA) was used to fit the regression model for the analysis of LWR (*b*) as describe in Shadrack *et al*. (2021) while Origin Pro 2019 default fit function was used to plot and fit the regression model for the SGR. The Principle Component Analysis (PCA) and Non-Multidimensional Scaling (NMDS) plot of important response variables were constructed using the Paleontological statistical software with the standardised data. The PCA was used to show the two dimensional distribution of each variable related to the dietary groups while the NMDS plot was used to rank the overall performance using Euclidean distance matrix. The Similarity Percentage (SIMPER) analysis was conducted using Bray Curtis dissimilarities matrix to determine the contribution (%) of each variable associated with the overall differences in dietary group. PERMANOVA (permutation - 9999) was used to determine the significance

of existing differences in the dietary groups, while ANOSIM (permutation – 9999) was used to compare the level of significance between dietary groups in a ranking order. The ANOSIM $P < 0.05$ were considered significant and the r – values > 0.5 indicate differences between group while r -values < 0.5 indicate no difference between group.

3.2.4 Results

3.2.4.1 *Growth and nutrient utilization*

The growth parameters, nutrient utilization, and survival rate presented in Table 3 showed that D5 is the optimum supplement with significant improve values for most of the response variable especially with final body weight (FBW), weight gain (WG%), specific growth rate (SGR), and feed intake (FI) ($P < 0.05$). The comparison of feed conversion ratio revealed yeast supplement group exhibit lower FCR values compared to the fish group fed on the control diet D1, although no significantly different was detected ($P > 0.05$). The survival rate was not significantly different among all fish groups although D5 had the highest survival rate ($P > 0.05$, Table 3). A significant linear relationship was observed for SGR with increasing levels of yeast supplement suggesting the optimum supplementation at 2.5% of the dietary proportion (Fig. 1).

3.2.4.2 *Whole body proximate, biometric indices and fatty acid profile*

The whole-body proximate analysis, biometric parameters and fatty acids profiles of red sea bream at the end of the feeding trial (50 days) are presented in Tables 4 and 5. No significant difference were observed in the whole body proximate values of moisture, crude ash, HSI and VSI among treatment groups ($P > 0.05$, Table 4), except for fish group fed diet D5 and D3 where whole body crude lipid and crude protein content was significantly ($P < 0.05$) different from the fish group fed on the control diet D1, respectively. The whole body fatty acid presented in Table 5 revealed a significant ($P < 0.05$) increase in SFA across supplemented group

compared to control D1. An inverse relationship with increasing yeast dietary supplement was observed for PUFAs, n-3 PUFA and n-6 PUFA. Mono-unsaturated fatty acid (MUFA) was significantly higher in fish group fed D2 and D3 compared to fish fed the control D1, D4 and D5. The biometrics of juvenile red sea bream fed the test diet for 50 days was presented in Figure 2 and 3. The logarithmic relationship between Length and weight (LWRs) of fish in each group showed a strong linear-relationship explained by the goodness of fit (R^2). The Fulton's condition factor (K) were significantly higher for fish group fed D2 compared to D4, however, no significant difference was detected among all yeast supplemented group and the control group D1 ($P < 0.05$, Fig.3, a). The relative condition factor values of fish fed the dietary group showed fish group fed D4 was significantly higher than D2, however, no significant difference was detected among all yeast supplemented group and the control group D1 ($P < 0.05$, Fig.3, b).The regression coefficient shown in Figure 2 revealed the length and weight relationship coefficient (b) where fish fed D2 showed high values compared to D4 ($P < 0.5$, Fig.3, c), however, no significant difference was found among the rest of the yeast supplemented group and the control group D1 ($P > 0.5$, Fig.3, C). The comparison of the growth coefficient of fish fed the tested diets with the standard growth coefficient (b=3) using the students t-test revealed no significant differences from the isometric growth coefficient ($P > 0.05$).

3.2.4.3 *Blood status*

The blood composition of juvenile red sea bream fed the test diets for 50 days is shown in table 6. The glucose level (GLU), aspartate aminotransferase (AST) and alanine aminotransferase analysis revealed a decreasing trend with increasing yeast supplement where fish group fed on D5 had significantly low values compared to fish group fed the control diet D1 ($P < 0.05$). Total protein (T-pro) was significantly ($P < 0.05$) higher in fish group fed on D2 compared to D5, however, these values were not significantly different from the fish group fed the control diet

D1 ($P > 0.05$). The red blood cells (RBC) and mean capsular volume (MCV) were improved in fish fed yeast supplement and were significantly ($P < 0.05$) higher in fish group fed on diet D3 compared to the control group D1. No statistically variations were recorded in haematocrit (Hrt), blood urea nitrogen (BUN), haemoglobin (Hb), total glycerides (TG), total billarum (T-Bill), mean capsular haemoglobin (MCH) and mean capsular haemoglobin concentration (MCHC) of red sea bream fish fed on the experimental diets ($P > 0.05$).

3.2.4.4 *Blood and mucus immune response*

Figure 3 displays the first line of defence in the fish body such as the Immunoglobulin, lysozyme, protease, total antiprotease, α-antiprotease, NBT, bacteria activity and mucus amount between fish fed the dietary treatment. Fish yeast supplemented diets improved values of serum and mucus immunoglobulin where fish group fed D4 and D5 showed significantly high serum immunoglobulin compare to fish fed the control diet D1($P < 0.05$, Fig.4, a & b). The lysozyme activity of serum was significantly high in fish group fed yeast supplement diets ($P < 0.05$, Fig.4, c). The protease, total antiprotease and α-antiprotease activity of serum were improved in fish fed yeast supplement where fish fed D4 and D5 showed significantly higher values compared to the fish fed the control ($P < 0.05$, Fig.4, f). The NBT activity of whole blood was significantly higher in fish group fed D5 compared to the control group while no significant difference was observed between fish fed yeast supplemented group ($P < 0.05$, Fig.4, g). The mucus bacteria activity was significantly high in fish fed D3, D4 and D5 compared to the fish group fed control diet D1 ($P < 0.05$, Fig. 4, h). Serum bacteria activity was significantly high in fish fed D3 and D5 compared to the fish group fed the control diet D1 ($P < 0.05$, Fig.4, i). The mucus amount as of fish fed yeast supplement were improved in fish group fed yeast supplement where fish group fed D4 has significantly higher mucus amount compared to fish fed the control diet D1 ($P < 0.05$, Fig. 4, j). Figure 4 (k & l) shows the values of catalase and

peroxidase activities of red sea bream after 50 days of experimental period. The serum catalase activity was improved for all fish group fed the dietary supplement despite having no significa nt difference among groups ($P > 0.05$, Fig.4, k). The peroxidase activity of blood serum revealed fish fed D5 has significantly high peroxidase activity compared to fish fed D3, D2 and the control diet D1 ($P < 0.05$, Fig. 4, l).

3.2.4.5 *Antioxidant capacity*

The antioxidant activity of juvenile red sea bream fed the test diets for 50 days are presented in Table 7, and Figure 5. Fish group fed on D4 and D5 diet exhibited significantly higher values of MDA and SOD compared to control group D1 ($P < 0.05$, Table 7). Fish group fed D3 showed significantly lower d-ROM activity compared to D2, however, all fish group fed yeast supplement diet showed no significantly different value of d-ROM compared to the fish group fed the control diet D1 ($P > 0.5$, Table 7). The BAP activity significantly higher in fish group fed D3 and D4 compared to the control group ($P < 0.05$, Table 7). The combine pattern of the effect of BAP and d-ROM shown in Figure 5 reflects the balance between antioxidant and oxidative stress. Zone A reflect a good condition where D4 and D5 groups experience low oxidative stress with high tolerance ability. Zone B shows a balance between oxidative stress and tolerance ability where D2 and D3 were favoured similarly to Zone C where D1 was favoured.

3.2.4.6 *Digestive enzyme*

The digestive enzyme activity of juvenile red sea bream fed the test diets for 50 days is presented in Figure 5. The digestive protease (D-protease) enzyme activity was improved in all yeast supplemented group of which D5 was significantly higher than the control group D1 ($P < 0.05$, Fig. 6, a). The enzyme activity of lipase, amylase and pepsin were improved in fish fed

yeast supplement diets compared to the control, however, interestingly fish fed D5 showed significantly higher values compared to fish group fed the control diet D1 ($P < 0.05$, Fig. 6, b, c and d).

3.2.4.7 *Low salinity stress*

The result of the tolerance ability of juvenile red sea bream exposed to low salinity stress after 50 days of the feeding trail was presented in figure 6. The log rank 50 % mortality between all fish group fed yeast supplement (D2; D3; D4; D5) diets were significantly higher than fish fed the control diet ($P < 0.05$, Fig. 7).

3.2.4.8 *Multivariate analysis*

The principle component analysis (PCA) conducted on a set of important variables associated with the dietary groups was presented in Figure 8. The PC1 and PC2 explained 82.98 % (71.68 % and 11.4 %) of the total variation in the dataset. Most variables were distributed along the positively axis of PC1 and, positive or negative axis of PC2 which showed a strong correlation to diet D4 and D5 group. The AST, ALT, T-Chol, d-ROM and GLU which were explained by the negative axis of PC1 and, positive or negative axis of PC2 reflecting a strong association with diet D1. The diet D2 group lies along the positive access of PC2 and was strongly associated with T-Pro, BAP, T-antiprotease. The diet D3 group lies near the central axis of PC1 and PC2, showing some associated with all the variables. The closeness related to the dietary group on a ranking order was presented in Figure 9 using NMDS plot. The NMDs axis coordinate 1 and 2 explained 68.1 % of the total variation. The NMDS ranking (Euclidean distance) showed diet D5 is the optimum supplement followed by D4, D3 and D2 compared to the control which rank the least (Fig.9). The analysis of the level of differences between the dietary groups showed existing significant differences among the dietary group

(PERMANOVA, permutation = 9999, $P < 0.012$). The fish group fed on yeast supplement diet D2, D3, D4 and D5 showed significantly (ANOSIM; $P < 0.009$; r = 0.63, r = 0.52, r = 0.93, r = 1, respectively) better responses compared to fish group fed on the control diet D1 (Fig.9). No significant difference was detected between fish group fed the yeast supplement diet (ANOSIM, $P > 0.05$, r <0.5). The contribution (%) of variables explaining the differences between fish fed dietary group was presented in table 8. The variable contributed highly to the differences was T-Cho (22.0 %), d-ROM (19.1 %), T-Pro (14.17 %), D-protease (12.82 %), ALT (9.88 %), GLU (9.31 %), and FI (8.73 %). Hrt, MDA and lipase contributed the least to the differences between the dietary groups. The significant relationship assessed with polynomial contrast for the graded level of yeast supplement on several developmental parameters were presented in table 9. Interestingly, a considerable number of growth parameters (FI, WG%, SG, PG), digestive enzyme (pepsin, amylase, lipase), blood (RBC, AST, ALT), immune (Serum IgM, peroxidase, SOD) and antioxidant parameters showed significant ($P < 0.05$, $P < 0.01$) linear relationship with increasing graded level of yeast supplementation.

Table 3.2.3: Growth parameters, feed efficiency, and survival rate of red sea bream fed experimental diets for 50 days

Parameters	Experimental diets				
	D1	D2	D3	D4	D5
IBW (g/fish) [1]	9.13±0.09	9.18±0.25	9.14±0.09	9.1±0.08	9.1±0.06
FBW (g/fish) [2]	18.82 ± 0.34	19.97 ± 0.7	20.05 ± 0.93	20.08 ± 0.19	21.3 ± 0.33
WG (%) [3]	106.1 ± 3.62^{b}	117.65 ± 6.83^{ab}	119.36 ± 10.83^{ab}	120.69 ± 0.58^{ab}	134.11 ± 2.44^{a}
SGR [4]	2.41 ± 0.06^{b}	2.59 ± 0.1^{ab}	2.61 ± 0.17^{ab}	2.64 ± 0.01^{ab}	2.84 ± 0.03^{ab}
FI (g/fish/50 days) [5]	14.9±0.28^{b}	16.83±0.28^{a}	15.49±0.41^{ab}	16.35±0.71^{ab}	16.94±0.44^{a}
FCR [6]	1.54 ± 0.02	1.51 ± 0.1	1.44 ± 0.13	1.46 ± 0.05	1.39 ± 0.05
PER [7]	1.35 ± 0.02	1.36 ± 0.08	1.54 ± 0.1	1.44 ± 0.05	1.55 ± 0.06
PG [8]	229.6 ± 4.15^{b}	249.14 ± 8.77^{ab}	262.63 ± 12.24^{a}	250.31 ± 2.33^{ab}	257.74 ± 3.97^{a}
Survival rate (SR%)	91.67±13.02	95±5	91.67±1.67	96.67±3.33	96.67±1.67

D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2.0 % yeast; D5 = the basal diet + 2.5 % yeast. Values presented as means of triplicate ± S.E. Values with different superscripts indicate significant difference ($P < 0.05$). Absence of superscripts indicate no significant difference. [1] IBW (g/fish): Initial body weight; [2] FBW (g/fish/50days): Final body weight; [3] WG (%): Weight gain percentage per fish; [4] SGR(%/day): Specific growth rate; [5] FI (g/fish/50 days): Feed intake per fish per day; [6] FCR: Feed convention ratio; [7] PER: Protein efficiency ratio; 8 PG: Protein gain.

Table 3.2.4: Whole-body proximate composition and biometric indices of juvenile red sea bream fed the test diets for 50 days.

Parameter	Experimental diets				
	D1	D2	D3	D4	D5
Moisture	69.94±2.17	69.07±0.66	69.2±1.18	68.5±1.26	69.45±0.39
Crude protein	12.2±0.06^{b}	12.47±0.11^{ab}	13.1±0.52^{a}	12.47±0.07^{ab}	12.1±0.05^{b}
Crude lipid	7.73±0.5^{b}	9.34±0.1^{ab}	9.76±0.49^{ab}	9.97±0.04^{ab}	10.4±0.13^{a}
Crude ash	4±0.07	3.95±0.1	4.02±0.04	3.94±0.03	4.24±0.03
HSI [1]	1.52±0.09	1.45±0.3	1.63±0.22	1.48±0.05	1.62±0.1
VSI [2]	9.7±0.73	10±1.34	10.5±0.69	10.16±0.14	10.97±0.66

D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2.0 % yeast; D5 = the basal diet + 2.5 % yeast. Values are means of triplicate groups ± S.E. Absence of superscript letters indicates no significant difference between treatments. Crude protein, crude lipid, and ash are expressed on a wet weight basis. [1] HSI: hepatosomatic index; [2] VSI: viscerasomatic index.

Table 3.2.5: Fatty acids (mg/ 1000 mg dry weight) contents of red sea bream whole-body fed experimental diets for 50 days

Parameters	Experimental diets				
	CD1	D2	D3	D4	D5
Fatty acids					
SFA [1]	72.34±5.64[b]	81.8±2.64[ab]	76.01±5.39[ab]	89.21 ± 1.41[a]	92.92 ± 9.56[a]
MUFA [2]	65.43±1.24[ab]	66.33±6.34[ab]	72.7 ± 4.7[a]	64.18±0.93[ab]	60.84±2.61[b]
16:2n-6+16:2n-4	1.35 ± 0.02	1.01 ± 0.29	0.97 ± 0.0	0.89 ± 0.03	0.87 ± tr
16:4n-3	0.33 ± 0.02	0.3 ± 0.01	0.26 ± 0.01	0.18 ± 0.01	0.14 ± 0.03
18:2n-6	17.23 ± 2.07	15.38 ± 2.28	17.12 ± 0.78	15.33 ± 0.18	14.94 ± 0.69
18:3n-6	0.65 ± 0.05	0.27 ± 0.02	0.27 ± 0.06	1.26 ± 1.22	0.4 ± 0.17
18:3A	0.37 ± 0.24	0.65 ± 0.67	0.8 ± 0.9	0.22 ± 0.19	0.87 ± 0.57
18:3n-3	3.57 ± 0.46	3.02 ± 0.15	2.3 ± 0.07	2.08 ± 0.09	1.65 ± 0.67
18:4n-3	0.33 ± 0.29	0.72 ± 0.74	0.94 ± 0.74	0.37 ± 0.12	0.80 ± 0.66
18:4n-1	0.15 ± 1.29	0.2 ± 0.29	0.14 ± 0.2	0.83 ± 0.73	0.17 ± 0.0
20:2n-6	0.26 ± 0.37	0.27 ± 0.04	0.2 ± 0.02	0.21 ± 0.04	0.27 ± 0.14
20:3n-6	0.42 ± 0.03	0.48 ± 0.12	0.57 ± 0.08	0.47 ± 0.38	0.41 ± 0.17
20:4n-6	0.74 ± 0.32	0.55 ± 0.06	0.66 ± 0.17	0.70 ± tr	0.8 ± 0.57
20:3n-3	ND	0.32 ± 0.46	0.38 ± 0.54	0.24 ± 0.06	0.16 ± 0.12
20:4n-3	0.34 ± 0.05	0.2 ± 0.05	0.22 ± 0.06	1.14 ± 0.07	0.16 ± 0.06
20:5n-3	7.64 ± 0.08	7.82 ± 0.52	8.01 ± 1.13	7.03 ± 0.9	7.99 ± 0.18
22:3n-6	0.16 ± 0.03	0.09 ± 0.12	0.11 ± 0.02	0.18 ± 0.01	0.14 ± 0.05
22:5n-3	4.24 ± 0.17	4.46 ± 0.03	4.17 ± 0.02	5.83 ± 0.06	4.64 ± 3.05
22:5n-6	0.16 ± 0.10	0.06 ± 0.09	0.11 ± 0.16	0.14 ± 0.47	0.25 ± 0.02
22:6n-3	14.97 ± 0.54	15.14 ± 0.2	13.42 ± 0.06	12.02 ± 0.13	11.33 ± 3.77
PUFA [3]	54.59 ± 9.21	48.88 ± 1.36	47.8 ± 1.04	51.64 ± 3.49	48.6 ± 7.78
n-3 PUFA	36.1 ± 6.16	31.02 ± 0.12	29.61 ± 1.38	32.26 ± 2.56	30.81 ± 9.0
n-6 PUFA	17.96 ± .39	17.55 ± 1.25	18.93 ± 0.33	18.69 ± 0.25	17.66 ± 1.25
n-3/n-6 [4]	2.15 ± 1.68	1.93 ± 0.16	1.65 ± 0.13	1.85 ± 0.08	1.96 ± 0.66
22:6n-3/20:5n-3 [5]	1.38 ± 0.05	1.64 ± 0.1	1.69 ± 0.25	1.72 ± 0.2	1.42 ± 0.5

D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2.0 % yeast; D5 = the basal diet + 2.5 % yeast. Values are the means of duplicate groups represented as mean ± S.E. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of superscript letters indicates no significant difference in means between groups.

[1] SFA (saturated fatty acids): 12:0; 13:0; iso-14:0; 14:0; anteiso-15:0; iso-16:0; anteiso-16:0; 15:0; 16:0; iso-17:0; anteiso-17:0; 17:0; iso-18:0; 18:0; 19:0; iso-20:0; 20:0 and 22:0. [2] MUFA (monounsaturated fatty acids): 14:1n-7; 14:1n-5; 16:1n-9; 16:1n-7; 17:1; 18:1n-11 + 18:1n-9; 18:1n-5; 19:1; 20:1n-11; 20:1n-9; 20:1n-7; 22:1n-9 and 22:1n-7.

[3] PUFA (polyunsaturated fatty acids). [4] n-3/n-6: ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids. [5] 22:6n-3/20:5n-3: ratio of docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA). ND = not detected; Tr= trace (tr < 0.01).

Table 3.2.6: Blood health of red sea bream juvenile fed experimental diets for 50 days.

Parameter	Experimental diets				
	D1	D2	D3	D4	D5
Haematocrit (%)	31.33±2.96	36.67±1.86	36±0.58	35±4.16	36±1.73
Hb (mg/dl)	11.11±0.46	11.7±2.32	11.73±1.06	11.31±1.7	15.01±0.69
Glucose (mg/dl)	63.5 ± 0.5^{a}	58 ± 3.0^{ab}	55 ± 7.0^{ab}	62.5 ± 6.5^{ab}	52 ± 1.0^{b}
T-Cho (mg/dl)	162±8.0	164.0±16.0	147.0±7.0	172.5±14.5	151.0±8.0
Bun (mg/dl)	7.0±1.0	8.5±2.5	7.0±1.0	7.5±1.5	9.0±3.0
T-Bill (mg/dl)	0.7±0.3	1.1±0.7	0.45±0.05	0.6±0.3	0.45±0.05
AST (IU/L)	346.5 ± 34.5^{a}	212 ± 67^{a}	106 ± 30^{ab}	100.5 ± 41.5^{ab}	81 ± 8^{b}
ALT (IU/L)	112 ± 2.0^{a}	92.5 ± 12.5^{ab}	18.5 ± 0.5^{bc}	36.0 ± 6.0^{abc}	10.5 ± 0.5^{c}
T-Pro (g/dl)	2.75 ± 0.45^{ab}	3.4 ± 0.2^{a}	2.9 ± 0.1^{ab}	3.3 ± 0.2^{ab}	2.45 ± 0.45^{b}
TG (mg/dl)	145.5±2.5	202.5±61.5	157.5±23.5	161.5±23.5	136±5.0

RBC ($1x10^6$ mm^3)	$1.97±0.18^b$	$2.66±0.31^{ab}$	$2.83±0.24^{ab}$	$3.07±0.37^a$	$2.86±0.19^{ab}$
MCH	56.66±2.86	44.82±9.84	38.27±1.73	37.35±4.95	52.67±1.25
MCV	$173.12±33.58^b$	$141.03±14.98^{ab}$	$129.37±13.65^{ab}$	$114.71±7.75^a$	$127.35±11.67^{ab}$
MCHC	33.68±4.88	32.66±8.17	33.1±3.46	32.25±2.29	43.99±3.58

D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2.0 % yeast; D5 = the basal diet + 2.5 % yeast. Values are means of triplicates represented as means ± S.E. Different superscript letters indicate significant difference ($P < 0.05$). Absence of letters indicates no significance difference between groups. Hb: hemoglobin; T-Cho: total cholesterol; Bun: blood urea nitrogen; T-Bill: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: total glycerides; T-Pro: total protein; RBC: red blood cells; MCH: mean capsular hemoglobin; MCV: mean capsular volume; MCHC: mean capsular hemoglobin concentration.

Parameters	Experimental groupings				
	D1	D2	D3	D4	D5
MDA (nmol/ml)	124.14±0.77	125.69±1.54	123.76±3.47	128±4.63	130.32±2.32
SOD (50% inhibition)	$24.14±0.39^b$	$41.23±0.22^{ab}$	$40.72±7.88^{ab}$	$57.06±3.24^a$	$86.05±5.95^a$
d-ROMs (µMol/L)	$34.5±4.5^{ab}$	$56±4^a$	$39.5±7.5^{ab}$	$14.5±1.5^b$	$26.5±2.5^b$
BAP (U. Carr)	$88.75±11.25^c$	$284.25±16.25^{ab}$	$372.0±8.0^a$	$439.0±7.0^a$	$265.0±9.0^{ab}$

Table 3.2.7: Antioxidant capacity of red sea bream fed test diets for 50 days.

D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2.0 % yeast; D5 = the basal diet + 2.5 % yeast. The values are means of triplicate groups represented as mean ± S.E. Different superscript letters indicate significant difference ($P < 0.05$) of means. Absence of letters indicate no significance difference of means between groups.

Table 3.2.8: Contribution (%) of variable to the dissimilarity in juvenile red sea bream fed the test diet for 50 days, using Bray-Curtis dissimilarity matrix, in descending order.

Variable name	Average dissimilarity	Contribution (%)	Cumulative %
T-Cho	7.88	22.00	22.00
d-ROM	6.84	19.09	41.10
T-Pro	5.07	14.17	55.26
D-Protease	4.59	12.82	68.08
ALT	3.53	9.87	77.96
GLU	3.33	9.31	87.27
FI	3.12	8.73	96.00
α -antiprotease	2.74	7.64	103.60
FCR	2.62	7.33	111.00
Hb	2.25	6.29	117.30
Tot-protease	2.15	6.01	123.30
Peroxidase	2.04	5.71	129.00
Pepsin	1.4	3.92	132.90
SOD	1.36	3.8	136.70
Protease	0.69	1.93	138.70
BAP	0.26	0.73	139.40
PG	0.18	0.52	139.90
Amylase	0.06	0.16	140.10
Catalase	-0.24	-0.68	139.40
WG%	-0.41	-1.17	138.20
AST	-0.57	-1.61	136.60
Mucus IgM	-0.86	-2.41	134.20
SGR	-0.94	-2.62	131.60
Lysozyme	-1.27	-3.54	128.00
RBC	-1.58	-4.43	123.60
Serum IgM	-1.75	-4.88	118.70
Lipase	-2.07	-5.78	112.90
Hrt	-2.3	-6.43	106.50
MDA	-2.32	-6.49	100.00

Table 3.2.9: Polynomial orthogonal contrast showing degree of the effect of increasing yeast supplement on growth variables, blood, digestive enzyme, immune and antioxidant activity in red sea bream juvenile fed the test diet in 50 days.

Parameters	Degree of polynomial	Slope (positive +, Negative -)	Mean square (MS)	F-value	p-values	Sig. level
FI	Linear	+	3.89	6.16	0.01	** 1%
	Cubic	+	2.69	4.25	0.03	* 5%
	Quartic	+	2.69	4.24	0.03	* 5%
WG%	Linear	+	1045.35	9.50	0.002	** 1%
SGR	Linear	+	0.24	9.34	0.002	** 1%
PG	Linear	+	989.09	6.22	0.01	** 1%
Serum IgM	Linear	+	35.50	15.99	0.0002	** 1%
	Quadratic	+	17.31	7.80	0.004	** 1%
T-antiprotease	Quadratic	+	61.13	164.33	4.48E-09	** 1%
	Cubic	+	104.68	281.41	3.15E-10	** 1%
	Quartic	+	53.88	144.85	8.32E-09	** 1%
Peroxidase	Linear	+	0.01	15.36	0.000285	** 1%
SOD	Linear	+	2600.10	60.10	5.89E-07	** 1%
	Quadratic	+	157.49	3.64	0.04	* 5%
RBC	Linear	+	1.42	6.40	0.01	** 1%
ALT	Linear	-	8978.7	114.08	1.05E-07	** 1%
	Quadratic	-	601.93	7.65	0.01	** 1%
	Quartic	-	1498.67	19.04	0.0002	** 1%
AST	Linear	-	55040.83	16.45	0.0004	** 1%
BAP	Linear	+	9027.21	223.45	5.38E-09	** 1%
	Quadratic	+	570.55	14.12	0.001	** 1%
	Quartic	+	1543.89	38.22	1.18E-05	** 1%
d-ROM	Linear	-	34307.01	146.70	3.48E-08	** 1%
	Quadratic	-	54973.34	235.08	4.29E-09	** 1%
	Cubic	-	2367.41	10.12	0.002	** 1%
	Quartic	-	1798.144	7.69	0.01	** 1%
Pepsin	Linear	+	0.12	4.35	0.03	* 5%
	Cubic	+	0.13	4.73	0.02	* 5%
Amylase	Linear	+	0.81	34.27	1.87E-05	** 1%
	Cubic	+	0.12	4.96	0.02	* 5%
Lipase	Linear	+	11.52	21.97	0.0001	** 1%
	Cubic	+	2.45	4.67	0.03	* 5%

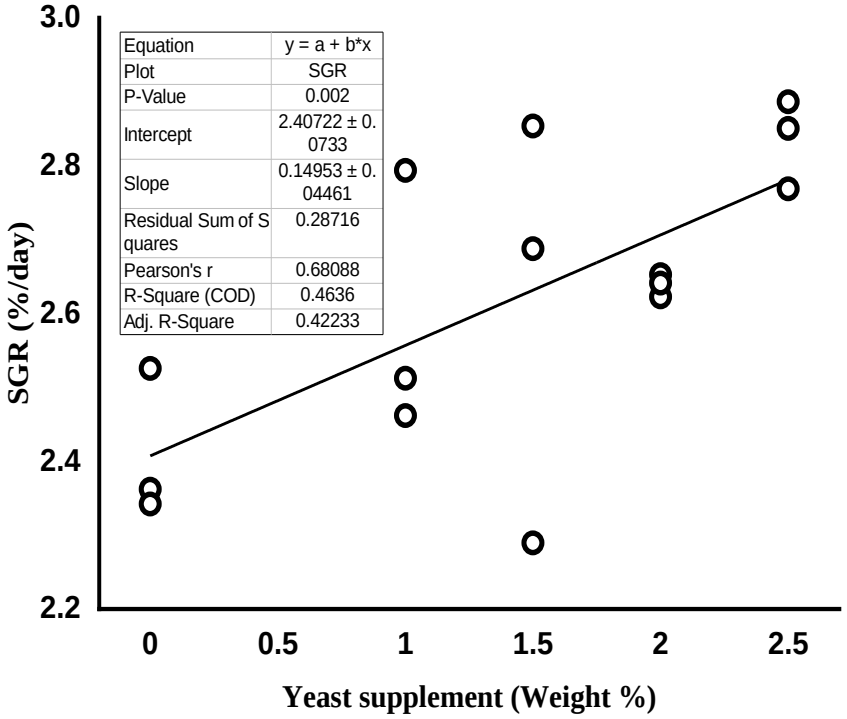


Fig. 3.2.1: The optimum yeast supplement level based on specific growth rate (SGR) for juvenile red sea bream fed the treatment diets in 50 days.

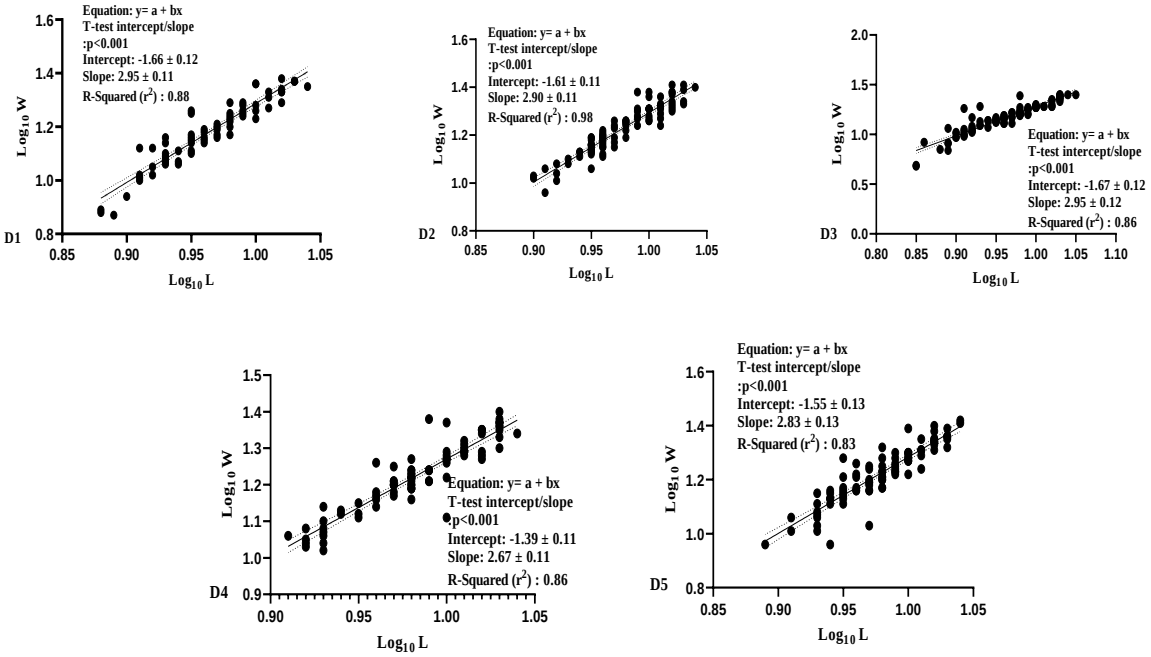


Fig. 3.2.2 Logarithm relationship between length and weight with linear regression equation for fish fed each dietary groups for 50 days. D1 (n=87), the control or basal diet with no yeast supplements; D2 (n=99), the basal diet + 1% yeast supplement; D3 (n=104), the basal diet + 1.5 % yeast supplement; D4 (n=97), the basal diet + 2.0 % yeast supplement; D5 (n=102), the basal diet + 2.5% yeast supplement.

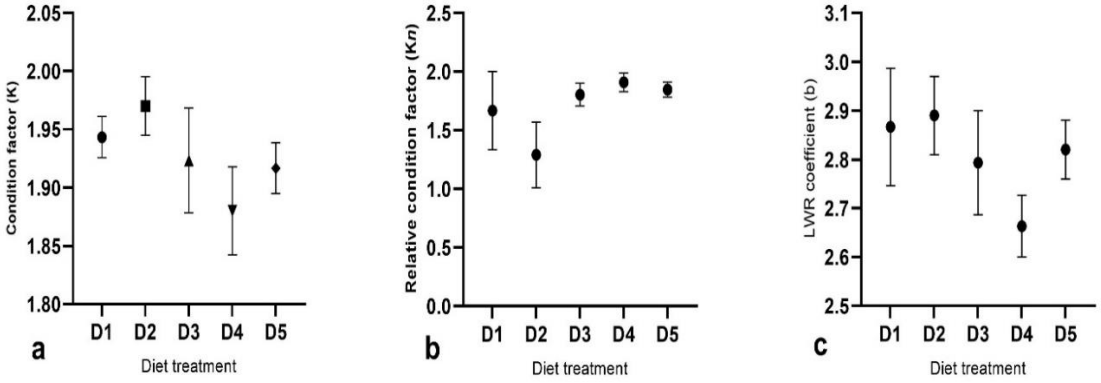


Fig. 3.2.3: A-Fulton's condition factor (K), B-Relative condition factor (K*n*) and, C-LWRs regression coefficient (*b*) for the fish fed the dietary groups for 50 days. D1 = the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast supplement; D3 = the basal diet + 1.5 % yeast supplement; D4 = the basal diet + 2.0 % yeast supplement; D5 = the basal diet + 2.5 % yeast supplement. Values presented as triplicate means ± S.E. Values with different superscripts indicate significant difference ($P < 0.05$). Absence of superscripts indicate no significant difference.

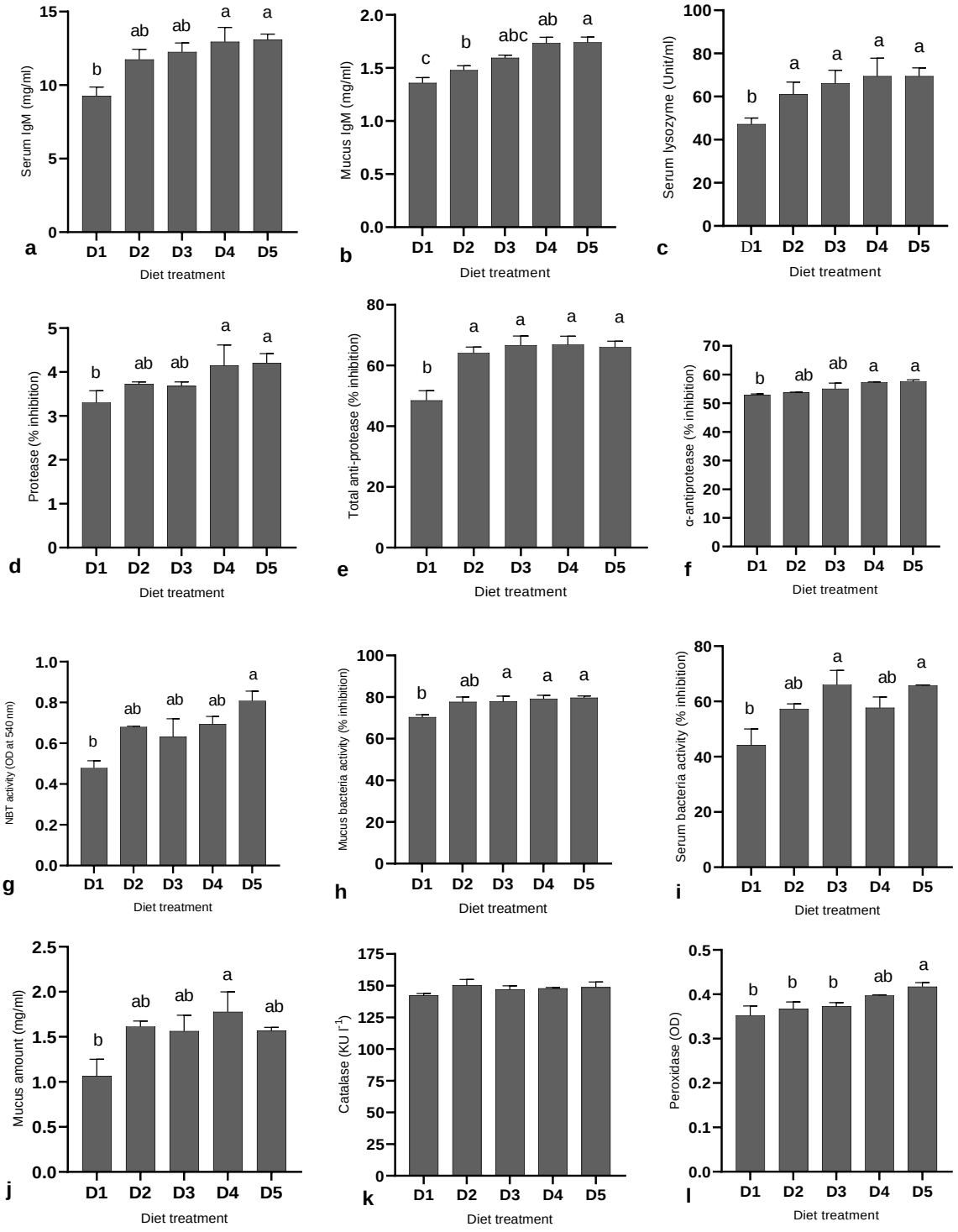


Fig. 3.2.4: Serum and mucus, immune responses of juvenile red sea bream fed test diets for 50 days. (a) Serum immunoglobulin (mg/ml); (b) Mucus immunoglobulin (mg/ml); (c) Serum lysozyme (Unit/ml); (d) Protease activity (% inhibition); (e) Total-antiprotease (% inhibition); (f) α-antiprotease (% inhibition); (g) Nitroblue tretrazolium activity in whole blood (optical density); (h) Mucus bacteria

activity (% inhibition); (i) Serum bacteria activity (% inhibition); (j) Mucus amount (mg/ml); (k) Catalase activity (kU l^{1}); (l) Peroxidase activity (optical density). Values are means (n=3) ± standard error (SE). Different superscripts indicate significant difference ($P < 0.05$) between treatment means. D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2 % yeast; D5 = the basal diet + 2.5 % yeast.

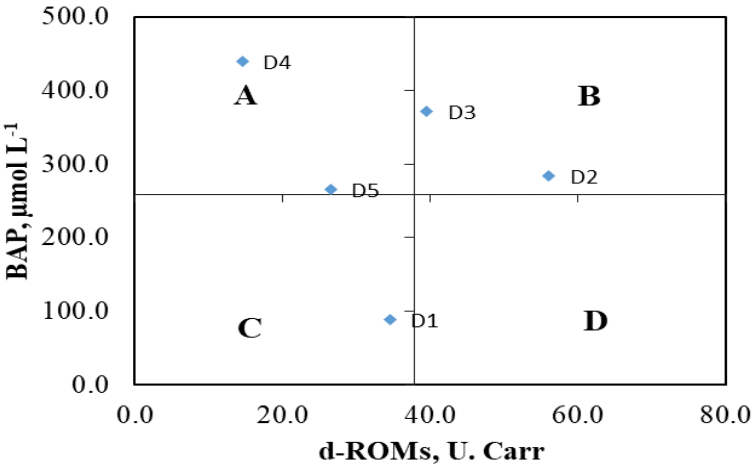


Fig. 3.2.5: Oxidative condition of red sea bream juvenile fed test diets for 50 days. Values represent the means ± SEM (n=3). The central axis is the means of both d-ROM and BAP for the dietary groups. Zone A: high antioxidant capacity and low reactive oxygen metabolites (good condition); Zone B: high antioxidant capacity and low reactive oxygen metabolite (acceptable 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). D1= the basal diet; D2= the basal diet + 1 % yeast supplement; D3= the basal diet + 1.5 % yeast supplement; D4 = the basal diet + 2 % yeast supplement; D5 = the basal diet + 2.5 % yeast supplement.

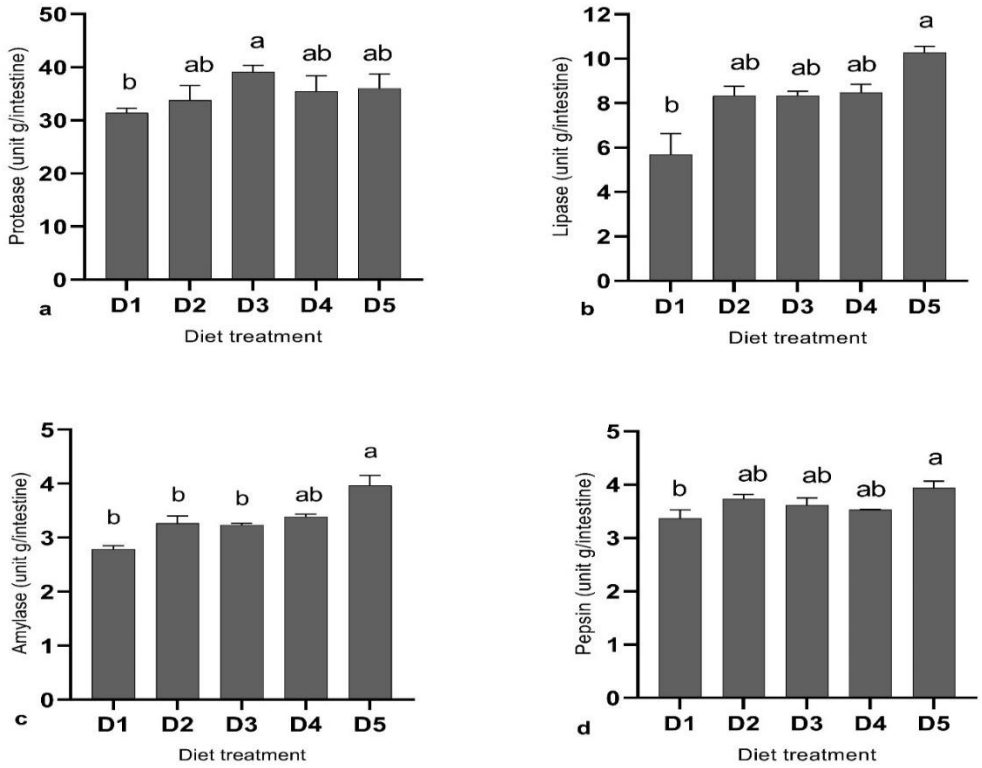


Fig. 3.2.6: Digestive enzyme activity of juvenile red sea bream fed the test diets for 50 days. (a) (Digestive) Protease activity (unit g/intestine); (b) Lipase (unit g/intestine); (c) Amylase (unit g/intestine); (d) Pepsin (unit g/intestine). Values are means ± S.E.M (n=3). D1= the basal diet; D2= the basal diet + 1 % yeast supplement; D3= the basal diet + 1.5 % yeast supplement; D4 = the basal diet + 2 % yeast supplement; D5 = the basal diet + 2.5 % yeast supplement.

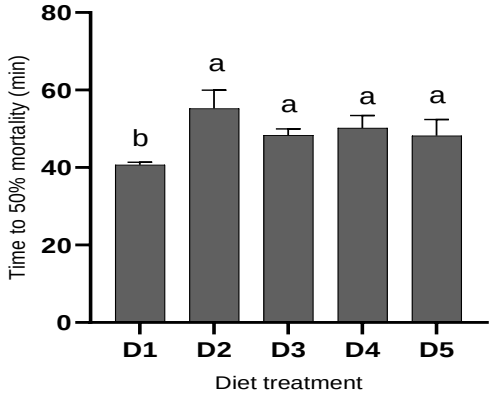


Fig. 3.2.7: Time (min) to 50% mortality of juvenile red sea bream exposed to low salinity stress after 50 days feeding test diets. The values are means ± pooled S.E. Absence of letters indicate no significant differences. D1= the control or basal diet with no yeast

supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2 % yeast; D5 = the basal diet + 2.5 % yeast.

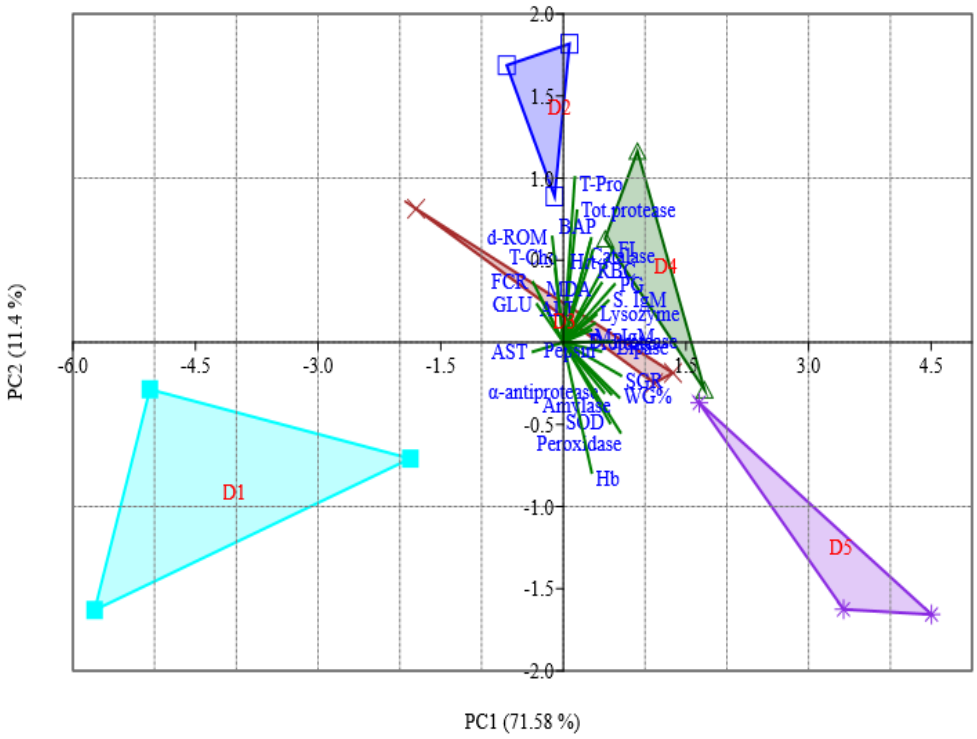


Fig. 3.2.8: The PCA plot of parameters observed in red sea bream fed the dietary groups for 50 days. The convex hull connects the region between triplicate samples per group (n=3). The PC1 and PC2 axis explained 71.58 % and 11.4 % of the total variation respectively. The direction of the green lines from the central region of the axis indicates the relationship of each variable in association with the dietary groups. D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2 % yeast; D5 = the basal diet + 2.5 % yeast.

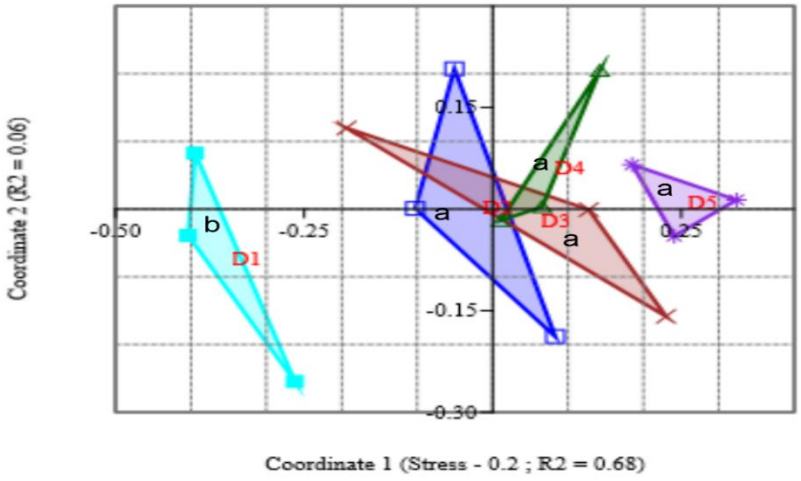


Fig. 3.2.9: The NMDS plot (stress 0.20) of rank (high to low) scores of all variables (Fig.7) in red sea bream fed the test diets for 50 days. The convex hull connects the region between triplicate samples per group (n=3). The distance (Euclidean) between the groups indicate the rank of dissimilarity related to closeness (one way ANOSIM, permutation = 9999, $P < 0.017$, global $R = 0.34$, differences with some overlap; PERMANOVA, permutation = 9999, $P < 0.02$, significant differences between dietary groups). Different letters indicate significant difference between groups. D1= the control or basal diet with no yeast supplements; D2 = the basal diet + 1 % yeast; D3 = the basal diet + 1.5 % yeast; D4 = the basal diet + 2 % yeast; D5 = the basal diet + 2.5 % yeast.

3.2.5 Discussion

Fin-fish aquaculture continues to develop innovative ways to provide alternative food production and more sustainable methods of food production to assist in the global food protein demand. However, problems with feed production and disease outbreak has often affected this industry and continued to be an ongoing problem. The use of antibiotics for disease prevention has proven a lot of success, however, it impacts on the environment and the cultured species has also been proven to be detrimental (Romero *et al.*, 2012). Thus, studies have identified the use of functional feed or additives as the better alternative method (Lara-Flores, 2011). Functional feed approaches were proved successful in the improvement of growth, nutritional

value of feed, feed utilization, support of health and enhance stress resistance, digestibility, disease resistance, intestinal health and act as an immune-stimulant agent (Encarnação, 2016). Functional feed additives include acidifiers, exogenous enzymes, probiotics, prebiotics, phytogenic, and immune –stimulants for which each is used in the diet formulation (Encarnação, 2016). Considering the use of functional feed additive in aquaculture, oleaginous yeast contains important nutritional derivatives such as lipids, glucans and mannooligosaccharides which are important for growing fish (Blomqvist *et al.*, 2018). However, little is known about the dietary effect of spent oleaginous yeast and the optimum supplementation in fish. To this end, the juveniles of red sea bream *P. major* was used in the present study to determine the efficacy of spent oleaginous yeast *Lipomyces starkeyi* on growth and wellbeing of fish.

The assessment of the *P. major* growth morphology revealed no significant alteration in length and weight relationship (b), condition factor (K) and relative condition factor (K*n*) between the test diets, alluding to the stable growth morphological condition and wellbeing of fish (Fig.2&3). The results of growth and feed utilization showed a significant increase in whole body crude lipid which reflected the similar trend observed in feed. The whole body fatty acid such as SFA, MUFA and n-6 PFA were increased with increasing yeast supplement while n-3 PUFA showed decreasing trend (Table 5). The ratio of n-3 and n-6 fatty acid in fish groups fed yeast supplemented diets decreased, which is similar to findings of Blomqvist *et al.* (2018) where yeast fed diets in sea bass decreased the n-6 fatty acid ratio in fish whole body. Having low n-3/n-6 ratio has been highlighted as important for fish as high ratio may lead to a variety of diseases (Simopoulos, 2006).The feed intake (FI), final body weight, weight gain (WG) and specific growth rate (SGR%) were significantly improved in fish group fed D5 (2.5 %), while FCR was lower in fish group fed yeast supplement diets, suggesting better growth performance and feed utilization by fish due to modulation of intestinal microflora (Rimoldi *et al.*, 2020; Navarrete and Tovar-Ramírez, 2014; Liu *et al.*, 2018) (Table 3). Further, the graded levels of

brewer yeast supplement in diet of hybrid striped bass (*Morone chrysops×M. saxatilis*) significantly improves growth (Li *et al*,2003), similar to our findings (Table 9). Also the result of significant improvement in digestive enzyme activity (amylase, lipase and pepsin) with increasing levels of dietary yeast supplements (Table 9) in our study confirms the prospective impacts of yeast supplement on growth and feed utilization (Banu *et al.*, 2020). Several studies also demonstrated the beneficial effect of yeast supplement such as improving growth and feed utilization in gibel carps (*Carassius auratus gibelio*) (Zhang *et al*., 2020) and *Lates calcarifer* (Huang *et al*., 2020). In our study, growth performance parameters (FI, WG%, SGR, PG) were improved with increasing ratio of yeast dietary supplementation (Table 9). Based on SGR, the optimum supplement in the present study is 2.5% of the dietary proportion.

Blood parameters have been highlighted as a useful indicator of fish health condition as they also improve fish ability to respond to stress conditions (El Basuini *et al*., 2016). The present study revealed low cholesterol (T-Cho) and glucose level in fish fed D3 and D5, which suggest the positive influence of yeast glucan as noted in previous studies (Robbins and Seeley, 1977; Tao et al., 2015). Fish fed yeast supplement diets showed significantly lower AST and ALT values especially in fish group fed D5 compared to control group, suggesting a better health status of fish as high values of AST and ALT is a reflection of damage in liver (Nagai *et al*., 1989; Vermeulen *et al*., 1992). Additionally, a significant inverse linear relationship was observed in AST and ALT values with increasing levels of yeast supplement (Table 9). The plasma total protein (T-Pro) values were significantly improved in fish fed yeast supplement especially for fish fed diet D2 and D4 suggesting a better health condition (Table 6). The red blood cells (RBC) were significantly increased in fish fed yeast supplement diets especially for fish fed diet D4, compared to other treatment while control diets have the lowest count (Table 6). The RBC showed a significant linear relationship with increasing level of yeast supplement (Table 9). A similar trend was also observed in tilapia fish fed diets containing baker's yeast

extract at 15 g/kg which resulted in increased red blood cell counts (Hassaan *et al*., 2018). The general trend on important blood performance indicator of health and growth such as Red blood cells (RBC), white blood cells (WBC; not recorded), haemoglobin (Hb), Haematocrit (Hrt), and total protein (T-pro) (Esmaeili, 2021) in red sea bream showed no significant health effect at all levels of yeast supplement, suggesting that it is possible to supplement the spent *L. starkeyi* up to 2.5% of the dietary proportion.

The known functional benefits of yeast in fish diets includes improving immune response by impressing on their immune cells via metabolic, neurological or endocrine pathways (Dossou *et al*., 2018). Phagocytosis activity of cells is the first line of defence mechanism in fish which is expressed in lysozyme activity and forms the non-specific immune defence mechanism of fish immune system (Cecchini *et al*., 2000; Dawood *et al*., 2016b; Dawood *et al*., 2016a). In the present study, the serum lysozyme activity of fish fed yeast supplement diets were significantly improved, suggesting the increase phagocytosis cells stimulated by dietary yeast ß-glucan from the yeast supplemented diet (Fig.3c). This finding is in accordance with the study conducted by Dawood *et al*. (2017b) where the supplementation of ß-glucan revealed an increase in lysozyme activity in *P. major*. The Immunoglobulin in blood is an adequate indicator of humoral immunity and is a significant component of blood serum (Yeganeh *et al*., 2021). The IgM in blood provides an immediate and wide range of protection against pathogens and forms an important part of the innate immune system (Magnadóttir, 1998; Magnadottir, 2010). In our study the IgM concentration was significantly enhanced in fish fed yeast supplements (Fig.4, a & b). The Serum IgM showed a significant linear relationship with increasing levels of supplement (Table 9). This finding is line with previous observation by Huang *et al*. (2020) where IgM level was enhanced in fish fed probiotic yeast supplement diets. This also suggests the immunomodulatory effects of the spent oleaginous yeast supplement in the present study.

The activity of protease and T-antiprotease reflects the capacity of the immune system to resist diseases. However, it is also important to note that the balance between protease and antiprotease is important for the proper functioning of the immune system (Cordero *et al.*, 2016). Previous studies have reported improvement in protease, T-antiprotease and α-antiprotease in fish fed probiotic supplement diets compared to fish fed the control diet (Newaj-Fyzul *et al.*, 2007). In our study, the protease, T-antiprotease and α-antiprotease activity were significantly improved in D4 and D5 yeast supplemented diets compared to the control, suggesting the functional benefits of spent yeast in enhancing the immune response of fish (Fig.4, d – f) which is consistent with the increase of anti-protease activity in Gilthead seabream (*Sparus auratus*) fed yeast ß-glucan (Reis *et al.*,2021). Additionally, T-aniprotease activity showed a significant linear relationship with increasing levels of yeast supplement (Table 9). The respiratory burst activity of blood leukocyte using Nitroblue Tetrazolium (NBT) assay is an indicator of innate immunity (Takahashi *et al.*, 2013) while mucus amount and antimicrobial serves as pivotal factor of the host to withstand pathogens (El Basuini *et al.*, 2020; Dawood *et al.*, 2017b). The supplementation of spent yeast in diets of red sea bream revealed improved anti-bacteria activity of serum and mucus, improved NBT activity and mucus amount, suggesting the beneficial effect of yeast in stimulating the red sea bream immune responses.

The health status of fish is also associated with the antioxidant status (Martínez-Álvarez *et al.*, 2005). Oxidative stress increases from the imbalance of the production and disposal of reactive oxygen species ROS (Dawood *et al.*, 2018). The superoxides dismutase (SOD), catalase (CAT), and glutathione peroxidase (PER) are vital components of the antioxidant defence which aid in removing excess ROS to sustain the cell homeostasis activity (Dawood *et al.*, 2019; Dawood *et al.*, 2016b). SOD and PER values were significantly improved and both variable showed significant linear trend with increasing level of yeast supplements (Table 7, 9). Catalase activity was numerically higher in fish fed yeast supplement diets compared to control (Table.7). This

improved antioxidant defence of CAT and PER may link to the effect of yeast glucan in stimulating the antioxidant enzyme activity in fish (Rodrigues et al., 2020). SOD, CAT and PER were also improved in Nile tilapia fish fed yeast supplement diets (Sun *et al.*, 2014; Xu *et al.*, 2015; Sohet *et al.*, 2009). The biological antioxidant (BAP) enzymes were significantly improved in all fish group fed yeast dietary supplement compared to the control (Table 7) and also showed a significant linear trend with increasing levels of yeast supplement (Table 9). The d-ROM values were significantly reducing with increasing levels of dietary yeast supplement (Table 9), suggesting the beneficial effect of yeast in lowering oxidative stress. The combination of biological antioxidant activity (BAP) and derivative of reactive oxygen metabolites (d-ROM) values revealed that fish fed D4, D3, D2 and D5 diets were in good health condition compared to those receiving control group diet (D1) (Fig.4). The high antioxidant activity for fish receiving yeast supplement suggest that the spent oleaginous yeast of wheat straw fermentation is a potential feed supplement for aquatic animals. The MDA activity is a measure of Lipid peroxidation of the cell membrane (Aliahmat *et al.*, 2012). In the present study, no significant differences were detected in MDA activity in fish fed the test diets, suggesting that fish fed the spent oleaginous maintained a balance in lipid peroxidation with improved SOD activity (Table.7).

The multivariate analysis of growth, digestive enzyme activity, antioxidant and immune responses of fish revealed a significant improvement in fish group fed the yeast supplement diet compared to fish fed the control diet (Fig. 8,9). The principle component analysis revealed that supplementation of yeast has great influence on FI, FCR, WG%, T-Pro, BAP and Hb (Fig. 8). The yeast supplemented diets were strongly associated with the cluster of variable compared to the control diets (Fig. 8) which contributed to the significant difference all response variable in fish supplement group compared to the control (Fig.9). The variables in the simper analysis model revealed that T-Cho, d-ROM, T-Pro, D-Protease and ALT were the most influential

variable associated with the variation in the fish group fed the test diets (Table 8). In this regard, yeast supplement main function in fish diets includes modulation of the intestinal microflora, thus, improve feed utilization and digestive enzyme activity (Liu *et al.*, 2018; Tovar *et al.*, 2002). Low T-Cho is associated with the effect of yeast supplements which has been similarly to reported of red yeast in lowering blood cholesterol (Cicero *et al.*, 2019). The d-ROM was significantly lower in fish group fed D4 and D5 and has significant contribution towards differences in diets (Table 8, 9). This suggest the potential influence of spent yeast in lowering the oxidative stress, indicated by lowering of the reactive oxygen metabolites (Dawood *et al.*, 2015b).

Challenge test have been popularly used in previous studies to gauge the biological and physical stress responses in fish (Wendelaar Bonga, 1997; Chadwick *et al.*, 2015). Within the different stress approaches, the low salinity indicator of tolerance to stress is a low cost procedure usually performed after nutritional trials (Hossain *et al.*, 2016). The results obtained from the low salinity stress tolerance for *P. major* revealed a significant improvement in fish group fed yeast supplement diets compared to the fish group fed the control (Fig.7). This finding indicates the influence of the immune-stimulating compounds such as ß-glucan, nucleic acids and/or mannooligosaccharides. Our results also reflect similar results in previous studies (Li and Gatlin III, 2006; Lokesh *et al.*, 2012), where yeast supplement boosted the ability of fish to survive stress conditions.

The present study highlights that dietary supplementation of spent oleaginous yeast at 1 % to 2.5 % of dietary composition significantly improves growth, well-being and antioxidant status of red seabream juvenile. This concludes that spent oleaginous yeast is a potential feed supplement for red sea bream *P. major*. The optimum supplementation level in the present study is at 2.5% of the dietary proportion therefore further study is recommended to determine the maximum supplementation threshold for this species.

Chapter IV: Effects of single or mix probiotic bacteria supplement as growth and immune stimulant for cultured fish species

Experiment I.A: Efficacy of mix probiotic bacteria strain on growth indices, physiological condition and bio-chemical composition of juvenile amberjack (*Seriola dumerili*)

Efficacy of mix probiotic bacteria strain on growth indices, physiological condition and bio-chemical composition of juvenile amberjack (*Seriola dumerili*)

4.1 Abstract

This study presents the effect of integrating mix strain (*Bacillus amyloliquefaciens* (BA)) and mix strain (*Streptococcus faecalis* (SF), *Lactobacillus plantarum* (LP), *Bacillus amyloliquefaciens* (BA) and *Bacillus mesentericus* (BM)) probiotic bacteria on growth, morphology and body composition of juvenile Amberjack (initial average body weight 5.61 ± 0.11 g). Three experimental diets were formulated, the basal diet CD1 (0% probiotic supplement), diet D2 (0.2% SF+BA supplement, $5x10^6$ cfu/g diet, $5x10^5$cfu/g diet) and diet D3 (1%, SF+LP+BM+BA supplement, $21x10^6$ cfu/g, $4x10^5$ cfu/g diet, $1x10^2$ cfu/g diet, $5x10^5$ cfu/g diet). Fifty fish were stock in each 500 L poly tank (duplicate tank per treatment) and fed the test diets for 75 days. Sampling of weights and length was conducted at 60 and 75 days, while the whole body samples were collected at the end of the feeding trial. The results showed a significantly higher condition factor (K), relatively condition factor (K*n*), and a numerically high LWRs coefficient (*b*) for fish feed SF+BA supplement compared to control and SF+LP+BM+BA group. The coefficient (*b*) values of the allometric growth trend was improved for SF+BA supplement with no significant difference from isometric growth coefficient (*b*=3). The growth parameters, feed utilization, survival (%) and whole body composition of fish fed the test diets were not significantly altered. A numerically high mono-saturated fatty acid (MUFA) and, Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) ratio was noted in fish group fed SF+BA supplement, suggesting an improve physiological condition of fish. This results demonstrated that integration of mix strain D2 at 0.2% has significantly improved physiological condition for Amberjack juvenile. Further research is recommended to determine

the dietary effects of the mix strain (SF+BA) and mix strain (SF+BM+LP+BA) on growth and health of fish.

Keywords:

Amberjack (*Seriola Dumerili*); Growth performance; condition factor; relative condition factor; Feed intake

4.2 Introduction

In recent years, aquaculture systems have expanded and appears to be one of the practical and promising tool to meet the food protein demand. Thus, increasing intensive fish farming has led to fish subjected to stress condition and weakening immune systems leading to high susceptibility to pathogens (Cerezuela *et al*,2012; Dawood *et al*,2016). Consequently, diseases leading to production losses is one of the limiting factors in intensive fish culture, hence, the use of probiotic is one of the best environmental friendly alternative approach (Dawood *et al*,2016). Probiotic bacteria's have gain wide recognition in aquaculture due to their various function such as improving nutrient absorption, growth and health status of cultured fish (Dawood *et al*. 2015a, Dawood *et al*. 2015b). Recently, some bacteria genera were discovered to be useful probiotic in aquaculture despite few contrary reports (Ringø *et al*. 2018). Probiotic as friendly additive, includes lactic acid bacteria, *Bacillus*, and *Saccharomyces*, are commonly used to improve growth performance, digestibility, immune responses, diseases resistance, gut health and well-being of culture species (Niu *et al*,2019). The dietary inclusion of BA significant improved body weight, specific growth rate, feed conversion ratio and intestinal morphology for Nile tilapia (Reda *et al*,2015), and improve disease resistance, immune response activity, and feed conversion ratio in eelfish, catla fish and catfish (Cao *et al*,2011; Ran *et al*,2012; Ridha *et al*,2012; Das *et al*,2013). Further, dietary inclusion of *Enterococcus feacalis* (Schleifer 1984) revealed protective effect against *Lactococcus garvieae*, a fish

pathogen (Baños *et al.*,2019). Similarly, addition of BM in the diet of *P.conchonius*, an ornamental fish, significantly reduce pathogenic gut inhabitants (Divya *et al.*,2012). Besides, inclusion of LP as feed additive for koi carp interestingly improve specific growth rate, weight percent, antioxidant and immune enzymes, and feed intake (Zhang *et al.*,2020). However, most study have only focus on the use of single probiotics but this can never be suitable for all host species because of the dissimilarity in physiological and physio-chemical conditions of the host or the surrounding environment (Lazado *et al.*,2015). It was suggested that combination of probiotic strain may complement or improve health benefits of an individual strain (Giri *et al.*,2014; Niu *et al.*,2019). For example, the dietary inclusion of multi-strain probiotic bacteria reduces potential adverse effects of low fishmeal diet and suggested to be a healthy immunostimulant for olive flounder (Niu *et al.*,2019). Despite the growing interest on multi-strain probiotic uses as functional feed, little information is available about the possible effect of inclusion in fish diet (Dawood *et al.*,2015; Dawood *et al.*,2018). The common methods for assess fish health condition includes morphological examination, blood and blood chemistry examination and examination of the immune function (Gatlin, 2007). The traditional method of assessing fish well-being is based on body condition (Arismendi *et al.*, 2011), length weight relationship (Le Cren, 1951), condition factor (Pennell and Barton 1996), and whole body composition (Ali *et al.*, 2005; Dempson *et al.*, 2004). Moreover, condition factor and length-weight relationship have been used previously to evaluate performance of amberjack from wild population (Kožul *et al.*, 2001; Mohamed *et al.*, 2018) and cultured environment for the effect of diet (Montero *et al.* 2004, Papadakis *et al.* 2008) and temperature regimes (Fernández-Montero *et al.*, 2018). Therefore, the main objective of this study is to evaluate the effects of single and mix strain probiotic supplement on growth responses, body condition and bio-chemical composition of juvenile amberjack.

The consumption of amberjack is relatively high in japan due to its geographical distribution and nutritional qualities, making the species one of the important culture species which forms a famous dish in Japanese cuisine (Dawood *et al.*, 2015b). It was hypothesized that dietary inclusion mix *Streptococcus faecalis* (SF) and *Bacillis amyloliquefaciens* (BA) or a mixture of *Streptococcus feacalis* (SF), *Lactobacillus plantarum* (LP), *Bacillus amyloliquefaciens* (BA), *Bacillus mesentericus* (BM) bacteria may also have significant effect on growth indices, physiological condition and bio-chemical composition of amberjack, *Seriola dumerili*.

4. 3 Materials and Methods

4.3.1 *Probiotic bacteria*

The probiotic bacteria, *Streptococcus faecalis* T-110 and *Bacillis Amyloliquefaciens* TOA5001, and the concentration of the cells was $1x10^9$ cfu/g and $1x10^8$ cfu/g respectively, kindly provided by Toa pharmaceutical company (Japan). *Streptococcus faecalis*, *Lactobacillus plantarum*, *Bacillis Amyloliquefaciens* and *Bacillus mesentericus* was obtained from Toa pharmaceutical company (Japan), and the concentration of the dry product was $2x10^8$ cfu/g, $8x10^7$ cfu/g, $1x10^8$ cfu/g, $2x10^4$ cfu/g, respectively. These probiotic products were sealed in polypropylene bag and stored at -20 ˚C until used.

4.3.2 Experimental design and diet formulation

The dietary formulation and proximate composition of the experimental diets were shown in Table 1 and 2. All experimental ingredients were obtained commercially. The brown fish meal and soybean mean were used as main protein source, soybean lecithin as main lipid source and then 3 isonitrogenous (43.2% crude protein) and isolipidic (12.5% crude lipid) were formulated containing 0%, 0.2 % (*S. faecalis* T-110 $5x10^6$ cfu/g diet, *Bacillis amyloliquefaciens* TOA5001 $5x10^5$ cfu/g diet) and 1% (*S. faecalis* T-110 $1x10^6$ cfu/g diet, *L.plantarum* TO-A

$4x10^5$ cfu/g diet, *B. mesentericus* TO-A $1x10^2$ cfu/g diet, *B.amyloliquefaciens* $5x10^5$ cfu/g diet) in D1 (control), and D2 (mix strain) and D3 (Mix strain) supplement respectively. The choice of CFU/g diet is in accordance to Paneri *et al* (2013) who suggest that probiotic bacteria at the concentration of 10^6-10^7 CFU/ g in food is adequate while 10^9 CFU/g is optimum. The bacteria cells were quantified based on plate counting method on the diet based on the weight of cell counts per gram diet and is expressed as ratio of cell count per gram diet.

The essential amino acid (EAA) for amberjack such as methionine, lysine and taurine were supplemented to all dietary treatment at equal ratio. White flour and activated gluten were added as binding agent or source of carbohydrate at equal ratio to all diets. Then the cellulose powder was use to adjust the final product to 100% total weight proportion.

To produce pellets ingredients were mixed for 15 min using a mixing machine. Then lipid source such as Pollack liver oil and soybean lecithin were added and mixed manually with a spatula before adding to dry ingredients. The ingredients mixtures were mixed with for another 15 minutes with the mixing machine. Finally, an amount of 30% - 40% water of the ingredient weight was added and mix for 10 minutes to produce a sticky particle sufficient for production of pellets. The mixture was then passed through a grinder with diameter of 1.90 mm and the pellet obtained was dried in the oven (DK 400, Yamamoto Scientific, Tokyo, Japan) at 60 °C for 9 h to ≤ 10% moisture content. The dried pellets were stored in polyethene plastic bag at -20 °C until use.

Table 4.1 Experimental diets ingredients and proximate composition

Ingredients	Experimental diets		
	CD1	D2	D3
Brown fish meal [1]	900	900	900
Soybean meal [2]	300	300	300
Wheat flour	300	300	300
Pollack liver oil [3]	80	80	80
Soybean lecithin [4]	40	40	40
n-3 HUFA [5]	10	10	10
Methionine [6]	2.2	2.2	2.2
Lysine [7]	8	8	8
Taurine [8]	1.4	1.4	1.4
Vitamin mix [9]	80	80	80
Mineral mix [10]	80	80	80
Probio EP [11]	0	4	0
Toaraze for Aquaculture [12]	0	0	10
Igsign [13]	0	0	10
Vitamin C ester [14]	6	6	6
Activated gluten [15]	100	100	100
CMC	20	20	20
α-Cellulose [16]	72.4	68.4	52.4
Total	2000	2000	2000
Proximate composition			
Crude protein	41.7 ± 0.47	43.05 ± 0.38	44.23 ± 1.05
Crude lipid	12.79 ± 0.71	12.05 ± 0.6	12.81 ± 0.17
Crude ash	12.26 ± 0.02	12.20 ± 0.01	12.28 ± 0.01
Carbohydrate [17]	29.69 ± 1.20	29.86 ± 0.35	27.95 ± 0.86
Gross energy (KJ/g) [18]	20.0 ± 0.13	20.05 ± 0.04	20.30 ± 0.02

[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 $NaHSO_3 \cdot 3H_2O$ (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 (Nicotic 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; Na2HPO_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] Probio EP; *Streptococcus faecalis* and *Bacillis amyloliquefaciens* made by Toa Biopharma Co., Tokyo , Japan; [12,13] *Streptococcus faecalis*, *Lactobacillus plantarum*, *Bacillis amyloliquefaciens* and *Bacillus mesentericus* (Toa Biopharma Co., Tokyo , Japan);. [14]L-ascrobil-2 phosphates-Mg. [15]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name: "A-glu SS". [16]Nippon paper chemicals, Tokyo, Japan.; [17]Carbohydrate % = 100 – (crude protein % + crude lipid % + crude ash %); [18]Gross energy calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

Table 4.2 Fatty acids (FA mg/ 1000 mg diet) and total amino acid (AA g/ 100 g diet) contents of experimental diets

Parameters	Experimental diets		
	CD1	D2	D3
Fatty acids			
SFA [1]	57.08 ± 2.91	59.38 ± 1.37	58.96 ± 2.43
MUFA [2]	62.37 ± 3.35	60.1 ± 1.65	58.96 ± 1.53
16:2n-6+16:2n-4	2.04 ± 0.02	2.03 ± 0.06	2.07 ± tr
16:4n-3	2.15 ± 0.08	2.09 ± 0.05	2.25 ± 0.16
18:2n-6	25.41 ± 0.69	24.3 ± 0.71	26.55 ± 2.23
18:3n-6	0.87 ± tr	0.76 ± 0.03	0.81 ± 0.01
18:3A	0.3 ± 0.01	0.29 ± 0.01	0.3 ± tr
18:3n-3	3.24 ± 0.06	3.1 ± 0.09	3.37 ± 0.24
18:4n-3	3.23 ± 0.01	3.07 ± 0.05	3.26 ± 0.11
18:4n-1	0.32 ± 0.01	0.29 ± 0.01	0.33 ± tr
20:2n-6	0.29 ± 0.02	0.26 ± 0.04	0.05 ± 0.08
20:3n-6	ND	0.09 ± 0.13	0.15 ± 0.21
20:4n-6	1.22 ± tr	1.17 ± tr	1.26 ± 0.05
20:3n-3	ND	0.06 ± 0.08	0.05 ± 0.08
20:4n-3	1.02 ± 0.03	0.98 ± 0.01	1.03 ± 0.01
20:5n-3	16.91 ± 3.28	16.54 ± 2.98	17.49 ± 2.49
22:3n-6	ND	ND	ND
22:5n-3	2.29 ± 0.02	2.38 ± 0.03	2.42 ± 0.05
22:5n-6	0.15 ± 0.21	0.15 ± 0.21	0.11 ± 0.16
22:6n-3	14.75 ± 0.21	14.67 ± 0.31	15.37 ± 0.51
PUFA [3]	72.41 ± 2	70.23 ± 2.82	74.86 ± 0.22

n-3 PUFA	44.17 ± 3.32	43.5 ± 3.2	45.92 ± 1.6
n-6 PUFA	28.24 ± 1.32	26.73 ± 0.38	28.94 ± 1.82
n-3/n-6 [4]	1.64 ± 0.2	1.7 ± 0.15	1.66 ± 0.16
22:6n-3/20:5n-3 [5]	0.89 ± 0.16	0.9 ± 0.14	0.89 ± 0.16
Amino acids			
Arginine	4.67 ± 1.75	4.77 ± 0.03	5.16 ± 0.07
Histidine	1.71 ± 0.16	1.89 ± 0.22	2.01 ± 0.32
Isoleucine	2.03 ± 0.11	2.17 ±	2.31 ± tr
Leucine	3.78 ± 1.19	4.67 ± 0.06	4.72 ± 0.75
Lysine	4.21 ± 0.10	4.27 ± 0.23	4.47 ± 0.07
Methionine	0.45 ± 0.10	1.32 ± 0.08	1.40 ± 1.28
Phenylalanine	2.49 ± 0.03	2.48 ± 0.06	2.51 ± 0.67
Threonine	2.85 ± 0.14	2.77 ± 0.11	2.86 ± 0.58
Tryptophan	0.81 ± 0.07	0.74 ± 0.22	0.78 ± 0.06
Valine	2.28 ± 0.02	2.34 ± 0.05	2.39 ± 0.44
Taurine	0.87 ± 0.16	0.77 ± 0.03	0.81 ± tr

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values are the means of duplicate groups represented as mean ± S.D. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of superscript letters indicates no significant difference in means between groups. [1] SFA (saturated fatty acids): 12:0; 13:0; iso-14:0; 14:0; anteiso-15:0; iso-16:0; anteiso-16:0; 15:0; 16:0; iso-17:0; anteiso-17:0; 17:0; iso-18:0; 18:0; 19:0; iso-20:0; 20:0 and 22:0. [2] MUFA (monounsaturated fatty acids): 14:1n-7; 14:1n-5; 16:1n-9; 16:1n-7; 17:1; 18:1n-11 + 18:1n-9; 18:1n-5; 19:1; 20:1n-11; 20:1n-9; 20:1n-7; 22:1n-9 and 22:1n-7. [3] PUFA (polyunsaturated fatty acids). [4] n-3/n-6: ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids. [5] 22:6n-3/20:5n-3: ratio of docosahexaenoic acid to eicosapentaenoic acid (EPA).

4.3.3 *Feeding, sample collection and biochemical analysis*

Amberjack juvenile of 5.61 ± 0.11 g initial mean weight were purchase from Kagoshima prefecture and transferred to a 500L tank with a flow-through system. The fish were fed for two weeks with 50% crude protein commercial diet (Higashimaru, Japan) enabling acclimation to laboratory conditions. Prior to stocking a sum of 10 fish were pooled together and stored at -20 °C for initial whole body analysis. The fish were then stock at a density of 50 fish per 500L

in polycarbonate tank with two tanks per treatment, in a flow through system with an inlet and outlet valve each with continues aeration. The fish tanks were maintained at the natural photoperiod regime. The fish were fed to satiation twice per day at 9:00 h and 16:00 h with their respective diets for 70 days. Uneaten feed after 1 hour of feeding was siphoned into polypropylene bag, freeze dried and subtracted from the total feed intake. Regular monitoring of growth was conducted every 20 days. The siphoning of faeces was conducted once daily. The seawater supplied to the fish rearing facility was pumped from the Kagoshima bay deep sea basin, gravel filtered prior to use. The flow rate for the rearing tank was maintained at 1.5 L min-1 throughout the 75 days. The water quality parameters being monitored were as follows; water temperature 23.32 ± 0.5 °C, pH 8 ± 0.5, salinity 33.3 ± 0.5 ppt, and dissolve oxygen 6.1 ± 0.5 mg L-1. These values were considered ideal for amberjack rearing.

Sampling for weight and length was conducted twice, first at 60 (CD1, n=49,48; D2, n=48,48; D3, n=15,50) and finally at 75 days (CD1, n=30,30; D2, n=30,30; D3, n=30,30) of the feeding trial, which is in accordance to sampling strategies of Kulbicki *et al.*, (1993) who suggest that sampling more than once for a wide range of fish sizes is important for the precision in estimating a (intercept) and b (slope) of LWRs.

For the whole body composition, 3 fishes per tank were collected at 75 days of feeding in trial and stored at -20 °C for further analysis. The test diets and fish whole body were analysed for moisture, crude protein, crude lipid and crude ash in duplicate using standard methods (Chemists, 1990) describe in Kader *et al.*, (2010). The moisture content of diets was obtained by oven drying at 110 °C to constant weight while fish whole body moisture was obtained by lyophilisation to constant weight. Crude protein was obtained by the Kjeldhal method describe in Goulding *et al.* (2020), crude lipid was determined by soxhlet extraction method describe in Hewavitharana *et al.*, (2020), and crude ash by combustion in Muffle furnace at 550°C for 4 hrs as describe in Sluiter *et al.*, (2008). The amino acid profile of diets and whole body were

analysed with a high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) according to the procedures describe in Kader *et al.,* (2012). The fatty acid profile of experimental diet and fish whole body was separated and quantify using gas chromatography (GCMS) following the procedure describe by Teshima *et al.,* (1986) and Oswald *et al.,* (2019). The viability of the bacteria cells incorporated into feed was assessed by spreading onto triplicate 3M™ petrifilm aerobic count plates (Thomas Scientific, USA). Briefly, after the diet preparation was completed, 1 g of each test diet was homogenized in 10 ml PBS (0.05 M, pH 7.4). Then 1ml volume of the solution is serially diluted in 10 ml PBS to 4^{th} dilution and then, 1 ml of each dilution is spread over 3M™ petri film plates and incubated at 26 °C for 3-5 days. The bacteria colony forming unit (CFU g^{-1}) on the petri film were counted using a colony counter (ACK-3 AS ONE, Japan) as describe by Ren *et al.,* (2007).

4.3.4 *Growth, survival and feed utilization*

The following equation was used to calculate the growth response, survival and feed utilization as describe in Dawood *et al.* (2015a).

Weight gain percentage (WG, %) = (W0 – Wf) x 100/W0; specific growth rate (SGR, %/day) = {(Ln (Wf) Ln (W0))/ t (total days)} x 100; percentage survival (Sur, %)= 100 x (Nf/N0).

Where W0 and Wf represent initial and final body weight (g) respectively. The t is the number of experiment days; N0 and Nf were the initial and final fish count respectively.

Feed conversion ratio (FCR)= live weight gain (g)/ dry feed intake (g); protein efficiency ratio (PER) = live weight gain (g)/ dry protein intake (g); protein gain (PG, g/kg weight gain) = {(final weight (g) x final whole body protein content (%)/100) – (initial weight (g) x initial whole body protein content (%)/100)}/ (weight (g)) x 100; protein retention (PR, % of feed intake) = (protein gain (g.kg weight gain $^{-1}$) x 100) / protein intake (g kg weight gain $^{-1}$)

Dry feed intake (FI, g fish/ number of days) = (amount of dry feed given / dry remaining diet recovered)/ number of fish; Condition factor (CF, %) = fish weight (g)/ (length of fish)3 $(cm)^3$ x 100.

4.3.5 *Calculation of condition factor and LWR*

The length weight relationships (LWRs) in the present study was examine using the equation of Froese (2006).

$$W=aL^b$$

This equation is expressed as:

$$L_{og\ 10}\ W= a + b\ Log_{10}\ L$$

Where W, weight of fish (g), and L is the total length of fish (cm). The *a* is the intercept of the linear regression, and *b* is the exponent expressed in the LWRs. When *b* value is other than 3, weight increase is allometric (positive if $b>3$ or negative if $b<3$). If $b=3$, weight increased is isometric (Mazumder *et al.,* 2016).

The calculation for Fulton's condition factor (K) of each fish was determined as:

$$K = W/L^3 * 100.$$

The relative condition factor (*Kn*) was calculated according to (Le Cren, 1951) as.

$$Kn=W(aL^b)^{-1}$$

Where W is the weight of fish (g), L is the total length (cm), *a* is the intercept while *b* is the slope, and 100 is a factor to bring the value of *K* near unity (Mazumder *et al.,* 2016).

The exponent '*b*' value, that is equal to 3, was not used to calculate the 'K*n*' value. It was claimed that this exponent is not the real representation of the LWRs for the great majority of fish species (Shahabuddin *et al.,* 2015), therefore the '*b*' value used here was obtained from the LWRs equation (Lima-Junior *et al.,* 2002).

4.3.5 *Statistical analysis*

All growth, diet and whole body composition data were check for homogeneity of variances and normality by Levene's and Kolmogorov-smirnov test respectively. Then, the analysis of variance, ANOVA was conducted. The probability of $p< 0.05$ were considered significant and significant differences between the means were evaluated using Tukey-Kramer post hoc test. The determination of *a* and *b* was performed using linear regression model of which the fitting was carried out by linear fit function readily developed in GraphPad Prism version 8.0.1 for windows (GraphPad Software, San Deigo, California USA). The goodness of fit between L and W was evaluated using the coefficient of determination (r^2). The K, K*n* and LWRs coefficient (*b*) of log transformed data were subjected to ANOVA to determine the differences between the group means. The difference where $p<0.05$ was further evaluated using Tukey-Kramer post hoc test. For each of the diet group, the student's *t*-test (Zar, 1996) was performed on the slope of logW-logL to test whether the computed *b* values were significantly different from 3 as describe in Mazumder *et al.,* (2016), indicating the type of growth behavior.

4.4 Results

4.4.1 *Growth parameters and nutrient utilization*

Table 3. showed the growth parameters, nutrient utilization and survival rate of amberjack juvenile fed the experimental diet for 75 days. Weight gain (WG) percent and specific growth rate (SGR) were numerically higher in fish fed SF+BM+LP+BA while feed intake (FI) was higher in fish fed SF+BA supplement, compared to the fish group fed the control diet. Survival was not significantly different among dietary groups.

4.4.2 *Bio-chemical indices*

The amberjack whole-body proximate analysis, biometric parameters, fatty acids and amino acids profiles fed for 75 days are presented in Tables 4 and 5. Although no remarkable alteration

in the previous indices were observed, a high MUFA and EPA/DHA ratio was observed in fish group fed SF+BA compared to SF+LP+BM+BA and the control CD1 group. A slightly high PUFA was observed for fish group fed the control diet and SF+LP+BM+BA group.

4.4.3 *Physiological indices*

The LWRs of combine replicate data for juvenile amberjack fed the dietary groups (figure 1) was:

CD1: $W= 0.05L^{2.62}$, n = 157, $R^2 = 0.86$

D2: $W=0.05L^{2.63}$, n = 156, $R^2 = 0.76$

D3: $W=0.07L^{2.48}$, n = 125, $R^2 = 0.80$

The dietary group D2 showed better LWRs values with higher *b* compared to supplement group D3 and the control group CD1. The comparison of growth behaviour with *b*=3 (isometric growth) showed no significant difference ($p>0.05$) for CD1 (control) and D2 supplemented group indicating an isometric growth behaviour, while D3 group showed a significantly lower *b* value ($p<0.05$) suggesting a negative allometric growth behaviour.

The condition factor, relative condition factor and LWRs coefficient *b* compared between groups is shown in figure 2. The D2 group showed a significantly high ($p<0.05$) condition factor (K), relative condition factor (K*n*) and high LWRs coefficient (*b*) compared to supplemented diet group D3 and the control group CD1.

Table 4.3: Growth parameters, feed efficiency, and survival rate of amberjack fed experimental diets for 75 days

Parameters	Experimental diets		
	CD1	D2	D3
IBW (g/fish) [1]	5.60 ± 0.19	5.58 ± 0.03	5.66 ± 0.01
FBW (g/fish) [2]	55.0 ± 2.81	54.5 ± 0.26	56.0 ± 5.01
WG (%) [3]	890.74 ± 74.96	877.64 ± 8.54	891.43 ± 87.62
SGR [4]	4.3 ± 0.2	4.2 ± 0.2	4.4 ± 0.2

FI (g/fish/70 days) [5]	60.0 ± 6.66	64.0 ± 0.0	62.0 ± 0.0
FCR [6]	0.8 ± 0	0.77 ± 0	0.81 ± 0.08
PER [7]	2.24 ± 0.01	2.13 ± 0.16	2.1 ± 0.07
PG (%) [8]	1036.25 ± 13.3	980.54 ± 12.02	997.46 ± 14.03
PR (%) [9]	40.14 ± 2.8	35.59 ± 0.12	36.32 ± 4.32
Survival rate (SR%)	100 ± 0.0	98.0 ± 2.83	100 ± 0.0

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values presented as means of duplicate ± S.D. Values with different superscripts indicate significant difference ($P< 0.05$). Absence of superscripts indicate no significant difference. [1] IBW (g/fish): Initial body weight; [2] FBW (g/fish/70days): Final body weight; [3] WG (%): Weight gain percentage per fish; [4] SGR(%/day): Specific growth rate; [5] FI (g/fish/70 days): Feed intake per fish per day; [6] FCR: Feed convention ratio; [7] PER: Protein efficiency ratio; [8] PG: Protein gain; [9] PR: Protein retention.

Table 4.4: Whole-body proximate composition and biometric indices of juvenile amberjack fed the experimental diets for 75 days.

Parameter	Experimental diets		
	CD1	D2	D3
Moisture	71.59± 0.46	72.15± 0.35	71.8± 1.90
Crude protein	17.78± 0.32	17.97± 0.14	17.76± 0.95
Crude lipid	2.97± 0.3	2.82± 1.06	2.73± 0.72
Crude ash	4.41± 0.04	4.78± 0.06	4.49± 0.22
Weight and Length (60/75 days)			
Min weight (g)	36.13 – 45.36	35.88 – 43.38	36.06 – 40.65
Max weight (g)	56.64 – 67	54.15 – 62.8	56.7 – 72.09
Min length (cm)	12.3 – 13	12.8 – 13.4	12.9 – 13.0
Max length (cm)	14.8 – 15.55	14.5 – 15.1	14.9 – 16.5

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values are means of duplicate groups ± SD. Absence of superscript letters indicates no significant difference between treatments. Crude protein, crude lipid, and ash are expressed on a wet weight basis. Length and weight (Minimum and maximum) data presented for both 60 days and 75 days of feeding.

Table 4.5: Fatty acids (FA mg/ 1000 mg dry weight) and total amino acid (AA g/ 100 g-dry weight) contents of amberjack whole-body fed experimental diets for 75 days.

Parameters	Experimental diets		
	CD1	D2	D3
Fatty acids			
SFA [1]	46.82 ± 0.44	50.17 ± 4.13	46.83 ± 1.26
MUFA [2]	46.82 ± 2.93	52.57 ± 1.55	48.9 ± 1.2
16:2n-6+16:2n-4	1.01 ± 0.02	1.02 ± 0.01	0.95 ± 0.04
16:4n-3	0.4 ± 0.05	0.46 ± 0.09	0.59 ± 0.07
18:2n-6	14.11 ± 0.23	13.38 ± 1.93	14.24 ± 0.17
18:3n-6	0.42 ± 0.02	0.38 ± tr	0.44 ± 0.02
18:3A	0.14 ± 0.01	ND	0.16 ± 0.03
18:3n-3	1.18 ± 0.09	1.11 ± 0.01	1.17 ± 0.03
18:4n-3	0.39 ± 0.44	0.49 ± 0.26	0.74 ± tr
18:4n-1	ND	ND	ND
20:2n-6	0.04 ± 0.06	ND	ND
20:3n-6	0.12 ± 0.17	ND	0.1 ± 0.14
20:4n-6	0.5 ± 0.21	0.74 ± 0.04	0.74 ± 0.24
20:3n-3	0.16 ± 0.23	ND	ND
20:4n-3	ND	0.18 ± 0.25	0.23 ± 0.13
20:5n-3	5.76 ± 2.16	3.68 ± 1.37	6.57 ± 3.33
22:3n-6	ND	ND	ND
22:5n-3	1.02 ± tr	0.83 ± 0.41	0.69 ± 0.01
22:5n-6	0.06 ± 0.09	ND	ND
22:6n-3	6.55 ± 0.13	7.08 ± 1.05	7.9 ± 3.01

PUFA [3]	30.86 ± 2.11	28.33 ± 5.24	33.4 ± 6.57
n-3 PUFA	15.61 ± 2.38	13.83 ± 3.27	17.88 ± 6.32
n-6 PUFA	15.25 ± 0.27	14.5 ± 1.97	15.51 ± 0.24
n-3/n-6 [4]	1.71 ± 0.17	1.82 ± 0.1	1.91 ± 0.43
22:6n-3/20:5n-3 [5]	1.23 ± 0.48	2.01 ± 0.46	1.25 ± 0.17
Amino acids			
Arginine	5.66 ± 0.23	5.34 ± 0.59	5.31 ± 0.51
Histidine	2.12 ± 0.43	2.06 ± 0.16	2.23 ± 0.2
Isoleucine	2.57 ± 0.19	2.5 ± 0.05	2.75 ± 0.17
Leucine	5.97 ± 0.75	5.59 ± 0.13	6.24 ± 0.39
Lysine	4.72 ± 0.41	4.31 ± 0.55	4.28 ± 0.13
Methionine	1.59 ± 0.7	1.59 ± 0.2	1.47 ± 0.23
Phenylalanine	2.94 ± 0.31	3.14 ± 0.09	3.12 ± 0.15
Threonine	3.87 ± 0.07	3.71 ± 0.01	3.73 ± 0.06
Tryptophan	1.38 ± 0.1	1.47 ± 0.06	1.53 ± 0.03
Valine	2.99 ± 0.19	2.92 ± 0.12	3.22 ± 0.19
Taurine	0.97 ± 0.07	0.99 ± 0.01	1.02 ± 0.18

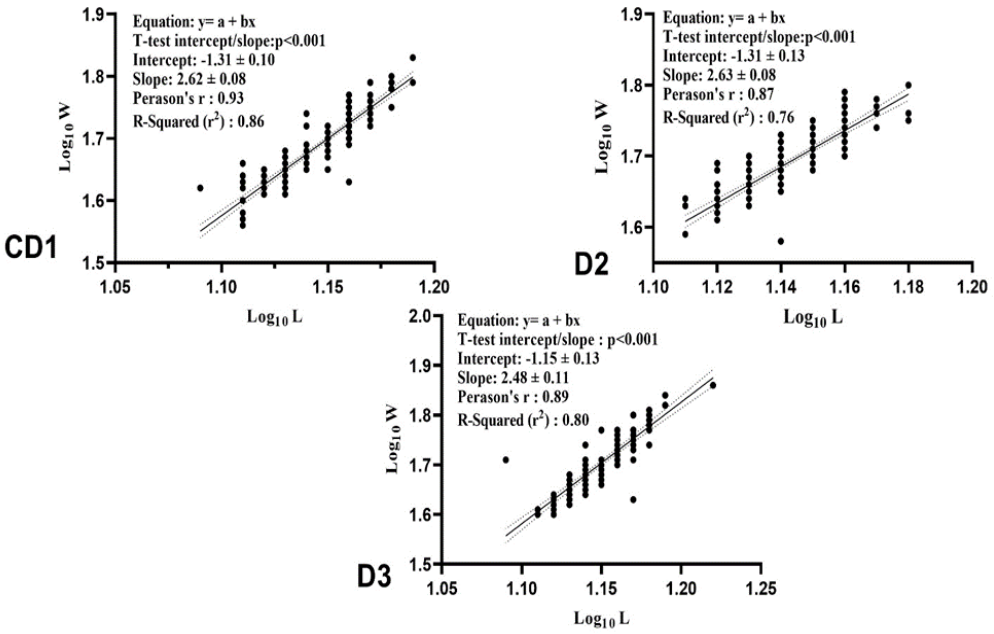


Fig.4.1: Logarithm relationship between length and weight with linear regression equation for the combine duplicate dietary groups. CD1= the basal diet (n=79,78); D2= the basal diet + a probiotic mix (SF, BA) (n=78,78); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM) (n= 45,80).

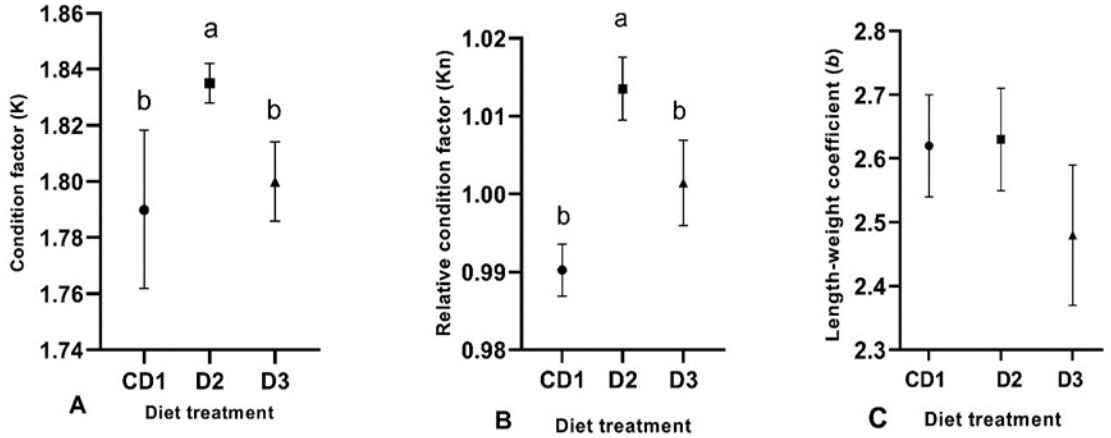


Fig.4.2: A- Fulton's condition factor (K), B-Relative condition factor (*Kn*) and, C- LWRs regression coefficient (*b*) for the fish fed the dietary groups for 75 days. CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values presented as duplicate means ± S.E. Values with different superscripts indicate significant difference ($P< 0.05$). Absence of superscripts indicate no significant difference.

4.5. Discussion

4.5.1 *Growth indices*

Probiotic bacteria main mechanism of action is to improve mucosal defences of the gastrointestinal tract including antimicrobial activity, enhancement of mucosal barrier function against ingested pathogens and immunomodulation (Paneri *et al.*,2013) which on the other hand, it contributes to improving the physical condition of fish. In this present study the survival of fish among all treatment group was 100%. Although previous studies reported improve SGR, immune response, disease resistance and health status (Cao *et al.*,2011; Diva *et al.*,2012; Ran *et al.*,2012; Ridha *et al.*,2012; Das *et al.*,2013; Reda *et al.*,2015; Baños *et al.*,2019) in fish species fed the individual strain of these probiotic bacteria (BA, BM, SF, and LP), in the present study we observed no difference in all growth indices and feed utilization among fish fed the dietary groupings. It can be acknowledging that although these probiotic bacteria strain maybe suitable for amberjack, the extent to which beneficial effect on growth indices can be detected may need further investigation. According to Lazado *et al.* (2015), no probiotic strain is suitable for all host species because of the dissimilarity in physiological and physio-chemical conditions of the host or the surrounding environment. Despite having no significant difference in growth indices among fish groups, a numerically high WG (%) and SGR was observed in fish group fed LP+BM+SF+BA, confirming the effect of probiotic bacteria such as producing growth

inhibition substances (bacteriocoins, hydrogen peroxides, diacyls etc.) (Alakomi *et al.*, 2000; Paneri *et al.*,2013; Ringo *et al.*,2018). The mix bacteria strain containing LP improves SGR confirming the findings of Zhang *et al.* (2020) where dietary inclusion of LP improves SGR for koi carp. The final body weight (FBW) and protein gain (PG) were not significantly altered among all treatment groups.

4.5.2 Bio-chemical composition

It was well documented that body composition is also a good indicator of the physiological condition of fish although relatively time consuming to measure (Ali *et al.*, 2005). The percentage of water from proximate analysis is a good indicator of the relative contents of energy, proteins and lipids (Ali *et al.*, 2005). The lower the percentage of water, the greater the lipid and protein content, reflecting a high energy density of the fish (Dempson *et al.*, 2004). Fatty acids and amino acids are macromolecule known for regulating the health condition and nutritional quality of fish (Sarma *et al.*,2013). The omega-6 polysaturated fatty acid (PUFAs) and omega-3 (PUFAs) are the important lipid mediator in signalling molecule for regulating inflammation in fish (Patterson *et al.*, 2012), and have potential effect on diseases prevention in human (Jobling and Leknes 2010). However, the values vary within and between species dues to size, sexual condition, feeding and physical activity (Ali *et al.*, 2005). In the present study fish whole body proximate composition was not significantly different among all treatment groups, indicating a stable energy profile of fish (Dempson *et al.*, 2004). The whole body fatty acid and amino acid profile among groups showed no significant alteration. Nonetheless, a numerically high MUFA, and DHA/EPA ratio was observed in fish fed the mix strain bacteria (SF+BA) compared to control and mix strain group (SF+LP+BM+BA), suggest

an improved essentail fat for growth (Emery *et al.*, 2016), and physiological condition of fish (AbuMweis *et al.*, 2018,).

4.5.3 *Physiological condition*

The condition factor (K*n*) is a good indicator of the body condition, growth rate and, length and age structures of the fish, which also serves as firsthand information on the general wellbeing of fish (Dutta 1994; Kohler *et al.* 1995). Datta *et al.* (2013) emphasis that condition factor is a good indicator of the robustness or wellbeing of experimented fish, after observing the growth of spotted snakehead *Channa punctate* fed under different feeding regime. Mazzola *et al.*, (2000) compared the performance of amberjack fed two test diet using body condition indices whereby of these two diets tested one was reported to have allometric growth trend similar with that of the wild population. Similarly, Sotoyama *et al.*, (2018), evaluated the growth responses, condition factor and body composition of Yellowtail (*Seriola quinqueradiata*), a species of jackfish, fed under two water temperature regime (25°C and 30°C) of which results showed favourable growth trend for fish reared at 25 °C. In the present study, the fish group fed SF+BA bacteria supplement showed significantly high K and K*n* values compared to fish group fed the control diet and SF+LP+BM+BA bacteria supplement, suggesting that condition factor is also useful in assessing the beneficial effect of probiotic supplement on amberjack body condition. The values of K and K*n* reported herein are within the range reported for wild caught amberjack of similar size range (Mohamed *et al.*, 2018).

The coefficient (*b*) values of the allometric relationship computed between the dietary group showed numerically high value for fish group fed SF+BA supplement with no significant difference from isometric *b* value (*b*=3), suggesting that D2 group exhibit isometric growth behaviour while D3 group is of a negative allometric growth behaviour. This allometric trend for D2 group is similar to typical wild population growth trend reported in Mazzola *et al.*,2000.

Thus, these findings demonstrate that SF+BA supplement is a potential candidate for improving growth behaviour and physiological condition of culture species, especially for amberjack.

In conclusion, the integration of mix strain SF+BA and mix strain SF+LP+BM+BA probiotic bacteria has improved condition factor (K), relative condition factor (K*n*), growth behaviour (*b*) and body composition for amberjack with no negative alteration. We recommend further research on the efficacy of these live probiotic bacteria strain with the focus on growth responses, blood chemistry, immune responses and stress responses to fully determine their mode of action in aquatic species.

Experiment I.B: Growth performance, blood chemistry, oxidative status, immune response, and intestinal morphology of juvenile amberjack, *Seriola dumerili* fed mix strain probiotic bacteria.

4.6.1 Abstract

The probiotic supplement has an important role in animal welfare especially in addressing production, disease, health responses and environmental conditions. This paper presents the result of a 60-day feeding trial on the dietary effect of single or mix bacteria strain on blood health, immune response, oxidative status and intestinal condition in amberjack juvenile (5.6 ± 0.1 g; Number = 300). Three experimental diets (≃42.9% crude protein and 12.6% crude lipid) were formulated with no additives in the control group (CD1) or *Streptococcus faecalis* and *Bacillus amyloliquefaciens* (SF+BA; 2g/kg; $5x10^6$ cfu/g diet, $5x10^5$ cfu/g diet) at 0.5% inclusion in the second group (D2), or a mixture of *Streptococcus faecalis* T110 (SF*)*, *Lactobacillus plantarum* TOA (LP), *Bacillus amyloliquefaciens* TOA5001 (BA) and *Bacillus*

mesentericus (BM) TOA (SF,$1x10^6$ cfu/g diet; LP, $4x10^5$ cfu/g diet; BM,$1x10^2$ cfu/g diet; BM $5x10^5$ cfu/g diet) at 1% inclusion in the third group (D3). Results showed no significant differences in growth indices, survival rate, whole-body composition and blood health among fish fed the dietary groups ($p>0.05$). The liver lysozyme activity, superoxide dismutase (SOD), peroxidase activity and biological antioxidant (BAP) were significantly higher ($p<0.05$) in fish group fed supplemented diet D2 compared to fish fed the control diet (CD1). The fish group fed supplemented diet D3 showed numerically high ($p>0.05$) SOD and BAP activity compared to fish groups fed the control diets. The higher ($p<0.05$) content of intestinal lactic acid bacteria was observed in fish group fed supplemented diet D2, with improve intestinal histological structure such as increase villi length, cryptal depth, and the number of goblet cells compared to fish fed the control diet (CD1) and D3 group. In conclusion, these results suggest that incorporation of mix strain probiotic bacteria SF+BA at 2g/kg (0.5%), has improved the blood health, immune response and oxidative status of amberjack fish.

Keywords:

Amberjack (*Seriola dumerili*); Growth performance; Oxidative status; Blood health; Immune response; Intestine histology

4.6.2 Introduction

Aquaculture is one of the prominent sectors in food production and has expanded influentially from small scale extensive farming to large scale intensive farming system and progressively spreading across the globe. It provides an alternative avenue to address food security and meet protein demand. The rapid growth of intensive aquaculture has brought a lot of challenges especially diseases outbreak and stressors (El Basuini et al., 2021). Chemotherapy including antibiotics have been used for decades as growth and health promoters, but with rising anxiety

about the consequences of their implementation (e.g., development of resistant pathogen strains, suppression of the immune system, accumulation of toxic residues, and environmental hazards), it has been disallowed in many countries (El Basuini et al., 2020).

Functional feed additives in terms of probiotics, prebiotics, synbiotics, medicinal herbs, and their extracts represent alternatives promising natural strategies to antibiotics (Dawood et al., 2019b, Dawood et al., 2018, Mohammadi et al., 2021, Paray et al., 2021). The effectiveness of probiotics in aquaculture sustainability is well documented (Dawood et al., 2019a). The incorporation of either lives or killed probiotics forms in aqua feeds increases the success of aquatic organisms' production (Díaz-Rosales et al., 2006, Salinas et al., 2008, Rodriguez-Estrada et al., 2013). Probiotic bacteria are an important additive in aquaculture due to their various functional benefits including promote growth, modulate digestive enzyme activities, improve nutrient absorption, modification of gut microbial community, immunostimulant, and improve tolerance to stress and unfavourable environmental conditions (Hirose et al., 2006, Hirose et al., 2009, Thanh Tung et al., 2010, Dawood et al., 2015a, Ringø et al., 2018). The combination of probiotic bacteria strains may complement and improve health of individual strains which may have beneficial effect on the host organism or the surrounding environment (Niu et al., 2019). According to these observation, it was hypothesis that mix strained probiotic bacteria supplement would effectively benefit the blood health, immune responses, antioxidant and intestinal bacteria content in amberjack, *Seriola dumerili*. The information on blood health, oxidative status, immune function and intestinal condition in fish is still is limited. Therefore, it is necessary to established whether or not the same response is observed on other health related parameters which will contribute to the complete understanding of the efficacy of single and mix probiotic bacteria strain for this species. Further, the findings of this study will also contribute to alternative probiotic supplement for amberjack to improve production due to its delicacy and comparative high market value in Japan (Dawood et al., 2015).

In light of this, the current paper presents the affirmative impacts of mix strain *Streptococcus faecalis* T-110 and *Bacillis Amyloliquefaciens* (SF, BA) and mix strain *Streptococcus faecalis* T-110, *Lactobacillus plantarum* TO-A, Bacillus *mesentericus* TO-A, Bacillus *amyloliquefaciens* (SF, LP, BM, BA) probiotic bacteria on growth, blood health, immune response, oxidative status and intestinal condition of the juvenile amber jack.

4.6.3 Materials and methods

4.6.3.1*: Ethical approval*

Rules of Animal Experiment in Kagoshima University does not apply to a fish. However, the protocol of this study was due consideration for the animal care (number of fish, fish handling etc.).

4.6.3.2: *Test diets*

Tables 1 show the dietary formulation and the proximate composition of the experimental diets. Three experimental diets (≃42.9% crude protein and 12.55% crude lipid) were formulated with no additives in the control group (CD1) or mix SF (*Streptococcus faecalis* T-110 $5x10^6$ cfu/g diet) and BA (*Bacillis amyloliquefaciens* TOA5001 $5x10^5$ cfu/g diet) ® Toa Biopharma Co., Ltd., Tokyo, Japan in the second group (D2) at 0.5% proportion, or a mixture of SF (*S. faecalis* T-110 $1x10^6$ cfu/g diet), LP (*Lactobacillus plantarum* TO-A $4x10^5$ cfu/g diet), BA (*Bacillus mesentericus* TO-A $1x10^2$ cfu/g diet), BA (*B.amyloliquefaciens* $5x10^5$ cfu/g diet) ® Isign Toa Biopharma Co., Ltd., Tokyo, Japan in the third group (D3) at 1% proportion. The experimental diets were made by thoroughly mixing the dried ingredients using a food mixing machine for 15 minutes. Lipid sources (Pollack liver oil and soybean lecithin) were added to the dry ingredients. About 35% water was added to the mixture and blended for 10 minutes to produce a sticky particle suitable for the production of pellets. The final pellets were obtained with a

grinder (1.90 mm) and then dried in the oven (DK 400, Yamamoto Scientific, Tokyo, Japan) at 40 °C for about 9 hours. The dried pellets were stored in a polyethylene plastic bag at -20 °C until use.

Table 4.6.1: Experimental diets ingredients and proximate composition.

Ingredients, g	Experimental diets		
	CD1	D2	D3
Brown Fish meal [1]	450	450	450
Soybean meal [2]	150	150	150
Wheat flour	150	150	150
Pollack liver oil [3]	40	40	40
Soybean lecithin [4]	20	20	20
n-3 HUFA [5]	5	5	5
Methionine [6]	1.1	1.1	1.1
Lysine [7]	4	4	4
Taurine [8]	0.7	0.7	0.7
Vitamin mix [9]	40	40	40
Mineral mix [10]	40	40	40
Activated gluten [11]	50	50	50
CMC	10	10	10
Vitamin C stay [12]	3	3	3
Probio EP [13]	0	2	0
Toaraze for Aquaculture [14]	0	0	5
Igsign [15]	0	0	5
α – cellulose [16]	36.2	34.2	26.2
Total (g)	1000	1000	1000
Proximate composition [17]			
Crude protein %	41.7 ± 0.47	43.05 ± 0.38	44.23 ± 1.05
Crude lipid %	12.79 ± 0.71	12.05 ± 0.6	12.81 ± 0.17
Crude ash %	12.26 ± 0.02	12.20 ± 0.01	12.28 ± 0.01
Carbohydrate [18]	29.69 ± 1.20	29.86 ± 0.35	27.95 ± 0.86
Gross energy (KJ/g) [19]	20.0 ± 0.13	20.05 ± 0.04	20.30 ± 0.02

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). [1]Nihon Suisan Co. Ltd (Tokyo, Japan); [2]J. Oil Mills, Japan; [3,4,] Riken Vitamines, 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^{-1} diet): β-carotene, 0.10; Vitamin D3, 0.01; Menadione $NaHSO_3 \cdot 3H_2O$ (K_3), 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 (Nicotic 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−1 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] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name: "A-glu SS"; [12] L-ascrobil-2 phosphates-Mg; [13] Probio EP; *Streptococcus faecalis* and *Bacillis amyloliquefaciens* made by Toa Biopharma Co., Tokyo , Japan; [14,15] *Streptococcus faecalis*, *Lactobacillus plantarum*, *Bacillis amyloliquefaciens* and *Bacillus mesentericus* (Toa Biopharma Co., Tokyo , Japan); [16] Nippon paper chemicals, Tokyo, Japan; [17] Values are means of duplicate groups ± SEM of the mean; [18] Carbohydrate (%): 100 – (crude protein + crude lipid + crude ash); [19] Gross energy: Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

4.6.3.4: *Fish experiment and feeding*

Amberjack juvenile (*Seriola dumerili*) was purchase from Kagoshima Creation of Abundance Sea Association (Kagoshima, Japan) and transferred to the aquatic laboratory of Kamoike Marine Production units, Faculty of Fisheries, Kagoshima University, Japan. Juveniles were

acclimated to the experimental conditions at a 500L tank with a flow-through system (1.5 L/ min) with continuous aeration under natural photoperiod regime. A total of 300 amberjack juveniles with an average initial body weight of 5.6 ± 0.1 g were randomly allocated into 6 tanks (500 L) at a density of 50 fish per tank. All groups were fed to satiation 2 times (9:00 h and 16:00 h) per day with their respective diets for 60 days. The uneaten feed was siphoned from tanks, freeze-dried, and subtracted from the total feed intake. The water quality parameters being monitored were as follows: temperature=23.32 ± 0.5 °C; pH=8 ± 0.5; salinity= 33.3 ± 0.5 psu; dissolve oxygen= 6.1 ± 0.5 mg/ L. These water parameter values were within the ideal condition for amberjack juveniles.

4.6.3.5 *Performance variables*

The growth performance parameters, feed utilization, and survival parameters of amberjack fed the test diets were determined according to the formulas describe in Dawood et al. (2015a).

Weight gain percentage (WG, %) = $(W_f - W_0) \times 100/W_0$

Specific growth rate (SGR, %/day) = $[\text{Ln } W_f - \text{Ln } W_0/ t] \times 100$

Survival rate % (SR %)= $100 \times (N_f/N_0)$.

Where W_0 and W_f represent initial and final body weight (g), respectively; t is the number of experiment days; N_f and N_0 were the initial and final fish count, respectively.

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

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

Protein gain (PG, g/kg weight gain) = {(final weight (g) x final whole body protein content (%)/100) – (initial weight (g) x initial whole body protein content (%)/100)}/ (weight (g)) x 100

Protein retention (PR, % of feed intake) = (protein gain (g.kg weight gain $^{-1}$) x 100) / protein intake (g/kg weight gain)

Dry feed intake (FI, g fish/ number of days) = (amount of dry feed given / dry remaining diet recovered)/ number of fish

4.6.3.6: *Sample collection and chemical analysis*

Before the final sampling, fish were fasted for 24 h to slow the metabolic process related to stress. During sampling, all fish have anesthetized with 50 mg/L eugenol (4-allylmethoxylphenol, Wako Pure Chemical Ind., Japan), then body weight and length were measured according to tank numbers. Three fish from each tank were collected and stored at -20 °C for whole-body analysis. The blood from the caudal fin of 5 fishes per tank was collected using a 2 ml heparinised syringe. A fraction of the blood was analysed for haematocrit using the haematocrit technique. The sample heparinized blood was centrifuged at 3000 x g for 15 min at 4 °C using a high-speed microcentrifuge (MX-160, Tomy Tech USA., Tokyo, Japan) to obtain plasma (stored at -80 °C until analysis). The plasma chemical parameters were measured spectrophotometrically with dry chemistry analyser (SPOTCHEM EZ model SP-4430, Arkray, Inc., Kyoto, Japan) (Tatsumi *et al.*, 2000). Reactive oxygen metabolites (d-ROM) and Biological antioxidant potential (BAP) were measured spectrophotometrically from blood plasma using an automated analyser (FRAS4, Diacron International s.r.l., Grosseto, Italy) following the method of Morganti et al. (2002) and Kader *et al.* (2012).

Three fish from each duplicate tanks were dissected for liver and viscera. The liver and viscera were removed and weigh for viscerasomatic index (VSI) and hepatosomatic index (HSI). The VSI and HSI index were determined according to the following equations:

Viscerasomatic index (VSI, %) = Viscera weight (g) /body weight (g) × 100

Hepatosomatic index (HSI, %) = Weight of liver /weight of fish×100.

The digestive tract from three dissected fish per tank was cut into small pieces, wash with pure water, polled together, and stored at -80°C. The liver from three dissected fish per tank was

pooled together and stored at -80°C. The dietary ingredients, test diets, and fish whole body were analysed for moisture, crude protein, crude lipid, and crude ash in duplicate using standard methods (Chemists, 1990) describe in Kader *et al.* (2010). The viability of bacteria cells in the test diet and intestinal content was determined using total bacteria and lactic acid bacteria kit (3M foot safety, USA). Briefly, 1 g of test diet was homogenized in 10 ml PBS (0.05 M, pH 7.4). One ml volume at each dilution was transferred onto the 3DM Petri film (anaerobic and aerobic for total and lactic acid bacteria respectively) surface, spread evenly, and incubate at 26 °C for 3-5 days (Nikoskelainen et al., 2003). The bacteria colony form on the Petri film was counted using colony counter (ACK-3 AS ONE, Japan) according to Ren *et al.* (2007). In similar manner, the intestinal bacteria count was determined.

4.6.3.7: *Analysis of non-specific immune responses*

The lysozyme activity of plasma and liver were determined as described by Dawood et al. (2015a). The oxidative radical production of neutrophils during respiratory burst was determined from whole blood using the nitro blue tetrazolium (NBT) method of Anderson and Siwicki (1995), and Kumari and Sahoo (2005). The total peroxidase in plasma was measured according to the method of Salinas *et al.* (2008).

4.6.3.8: *Analysis of antioxidant activity and lipid peroxidation*

The catalase enzyme activity of plasma was determined by measuring the optical density of the reaction mixture of hydrogen peroxide (H_2O_2) and ammonium molybdate forming a stable complex (Goth, 1991). The yellow complex colour formation of ammonium molybdate and hydrogen peroxide was measured at 405 nm with a spectrophotometer (Spectronic 200, Thermo Fisher Scientific K. K., Tokyo, Japan). In the case of tissue, 25 mg of sample was homogenized

in 250 µl RIPA buffer (containing protease inhibitor 1l per ml). The content was centrifuged at 3000 x g in 10 minutes and 50 µl of the supernatant was used for analysis.

The superoxide dismutase (SOD) activity of plasma was measured as percentage reaction inhibition rate of the enzyme with WST (Water Soluble Tetrazolium dye) substrate and xanthine oxide, as described by Dawood *et al.* (2016). The analysis follows the instruction from the SOD kit-WST (Dojindo, Lab., Kumamoto Japan). For tissue sample, 25 mg of tissue was homogenized in RIPA buffer (containing 1 µl/ml protease inhibitor) and centrifuge at 3000 x g in 10 mins at 4°C. The supernatant was used for the analysis.

The lipid peroxidation of plasma was determined by measuring the malondialdehyde (MDA) as a biomarker. The MDA was measured calorimetrically with TBARs microplate assay Kit (Oxford Biochemical Research, Inc., USA) following the manufacturer's instruction. The absorbance of the reaction mixture was measured at 532 nm. For tissue samples, 25 mg of sample was homogenized in 250 µl of RIPA buffer (containing protease inhibiter at 1µl/ml) and centrifuge at 3,000 x g in 10 min at 4 °C. The supernatant was used in the analysis.

4.6.3.9: *Intestine histological assessment*

The intestine was cut and immersed in Bousin solution for 12 h. Fixing was rapidly done by rinsing in alcohol every 24 h until clear. The tissue was embedded in paraffin blocks, section, deparaffinised and rehydrated. A rotary microtome (RM 2135, Leica, Nussloch, Germany), was used to obtained sagittal sections (5 µm). The section was placed on glass slide, rehydrated, and stained with hematoxylin and eosin. The slides were then mounted (Entellan, EMD Millipore, Billerica, MA, USA) permanently and examine under light microscope (BX41, Olympus, Tokyo, Japan).

4.6.3.10: *Statistical analysis*

The statistical analysis was performed using the Paleontological statistical software package for education and data analysis version 3.21 (Hammer *et al.,* 2001). The normality of the data was confirmed by the Kolmogorov-Smirnov test and homogeneity of variances by Levene's test before ANOVA analysis. Probabilities of $p < 0.05$ were considered significant, and significant differences between means were evaluated using the Tukey-Kramer post hoc test.

4.6.4 Results

4.6.4.1: *Performance variables and whole-body proximate analysis*

The effect of probiotic bacteria dietary groups on growth, feed utilization, survival rate, biometric indices, and final whole-body proximate analysis of amberjack juvenile after 60 days feeding period are shown in Table 2. Results showed no significant ($p>0.05$) difference in all means of growth parameters, feed utilization, survival rate, biometric indices, and whole-body composition between control and probiotic supplemented diet groups. The total bacteria count and lactic acid bacteria count in feed shown in Figure 4.b, revealed significantly high ($p<0.05$) lactic acid bacteria count for D2 probiotic supplement compared to control and D3 probiotic supplement.

4.6.4.2: *Blood chemical parameters and hematocrit*

The blood status of amberjack juvenile fed experimental diets for 60 days is shown in Table 4. No significant ($p>0.05$) changes in blood profile was observed in all the fish groups fed the tested. Total protein (TP) and Haematocrit are numerically higher in diet group D2 and D3 compared to the control diet although there was no significant difference among means ($p>0.05$).

4.6.4.3: *Immune response*

Figure 1 displays amberjack immunological responses after 60 days of the experimental period. Liver lysozyme activity (U/mg) was significantly higher ($p<0.05$) in the D2 group followed by the D3 group and the lowest value was in the control group. Plasma lysozyme activity and NBT activity showed no significant alteration among all fish group fed the supplement diets (D2, D3) and the control diet (CD1).

4.6.4.4: *Antioxidant potential*

At the end of the 60-days feeding period, the antioxidant potentials of amberjack juveniles are presented in Table 5 and Figs. 2 and 3. Fish in the D2 group showed a higher SOD value ($p<0.05$) and the lowest value was in the control group (Table 5). Biological antioxidant potential (BAP) activity was significantly higher ($p<0.05$) in the D2 group compared to D3 and control group (Table 5). No significant ($p>0.05$) modulations were noticed among experimental groups for MDA, d-ROM (Table 5), while peroxidase activity was significantly higher in fish fed D2 compared to the control CD1 ($p<0.05$: Fig. 2, a & b). Catalase activities were numerically higher in fish fed probiotic supplement compared to fish fed the control diet ($p>0.05$; Fig. 2, c). Figure 3 shows the trend of the combined effect of d-ROM and BAP. D2 group was in Zone A (good condition) indicating higher tolerance ability to oxidative stress, diet CD1 (control diet) is Zone C (acceptable condition) indicating lower d-ROM and lower BAP values, while diet D3 is in Zone D (poor condition) reflexing higher tolerance of d-ROM and low BAP values.

4.6.4.5 *Intestinal microflora and histology*

The bacterial count in the intestine of amberjack juvenile fed test diets for 60 days is shown in Fig. 4. Lactic acid bacterial count in diet was significantly higher ($p<0.05$) in diet D2 supplement compared to diets D3 group and the control diet (CD1). Lactic acid bacteria count

in fish intestine was significantly higher ($p<0.05$) in fish group fed the D2 supplement compared to fish fed D3 and control diet (CD1). Figure 5 presents the cross-section in the small intestine of amberjack juvenile fed on the experimental diets for 60 days. The histological structure of the Amberjack juvenile's intestine in all experimental groups displayed a normal and intact structure in terms of serosa, tunica muscularis, lamina propria, and simple columnar epithelium villi lining around the connective tissue core. The cross-section in the small intestine of fish groups fed on D2 and D3 diets showing an increase in villi length, cryptal depth, and the number of goblet cells compared to the control group, although no quantitative analysis was performed.

Table 4.6.2: Amberjack juvenile performance variables (growth performance, feed utilization, survival rate, and biometric indices) and whole-body proximate analysis after 60 days feeding period.

Items	Experimental diets		
	CD1	D2	D3
IBW, g/fish [1]	5.60 ± 0.19	5.58 ± 0.03	5.66 ± 0.01
FBW, g/fish [2]	46.03 ± 0.82	47.23 ± 0.14	47.08 ± 2.38
WG, % [3]	725.17 ± 8.95	737.95 ± 9.83	732.86 ± 15.48
SGR [4]	3.52± 0.02	3.54 ± 0.4	3.53 ± 0.10
FI, g/fish /60 days [5]	43.40 ± 0.71	45.0 ± 2.83	44.5 ± 0.71
FCR [6]	0.82 ± 0.03	0.76 ± 0.00	0.81 ± 0.09
PER [7]	1.98 ± 0.06	1.77 ± 0.10	1.83 ± 0.15
PG [8]	791 ± 66.59	779.78 ± 38.02	802.99 ± 7.70
PR [9]	31.65 ± 0.03	28.30 ± 1.13	29.29 ± 0.41
SR, % [10]	100 ± 0.00	98 ± 2.83	100 ± 0.00
HSI [11]	0.70 ± 0.40	0.60 ± 0.10	0.60 ± 0.20
VSI [12]	4.80 ± 0.80	3.90 ± 0.00	4.10 ± 0.20
Whole-body proximate analysis [13]			
Moisture	74.36 ± 0.60	76.61±1.57	76.62±0.74
Crude protein	17.55 ± 0.08	16.50± 1.0	16.31 ± 0.66

Crude lipid	2.00 ± 0.01	1.94± 0.07	1.89 ± 0.11
Crude ash	3.82 ± 0.25	3.83± 0.37	4.13 ± 0.20

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values are the means of duplicate groups ± SEM of the mean. The absence of superscript letters indicates no significant difference in means between groups.

[1] IBW (g/fish): Initial body weight; [2] FBW (g/fish/60days): Final body weight; [3] WG (%): Weight gain percentage per fish; [4] SGR(%/day): Specific growth rate; [5] FI (g/fish/60 days): Feed intake per fish per 60 days; [6] FCR: Feed convention ratio; [7] PER: Protein efficiency ratio; [8] PG: Protein gain; [9] PR: Protein retention; SR: [10] Survival rates %; [11] HSI: hepatosomatic index (%); [12] VSI: viscerasomatic index; [13] Whole-body proximate analyses are expressed on a wet weight basis.

Table 4.6.3: Blood status of amberjack juvenile fed experimental diets for 60 days.

Items	Experimental diets		
	CD1	D2	D3
Haematocrit (%)	40.2 ± 0.85	44.0 ± 1.13	43.2 ± 1.13
Glucose (mg/dl)	128.5 ± 9.19	134.5 ± 24.75	137.0 ± 24.04
T-Cho (mg/dl)	186.5 ± 20.51	201 ± 9.90	222.50 ± 38.89
Bun (mg/dl)	13.5 ± 2.12	13.0 ± 1.41	14.5 ± 3.54
T-Bill (mg/dl)	1.10 ± 0.28	1.65 ± 0.35	1.40 ± 0.71
AST (IU/L)	69.00 ± 28.28	164.50 ± 36.06	162.50 ± 31.82
ALT (IU/L)	36.0 ± 5.66	55.5 ± 21.92	70.5 ± 20.51
TG (mg/dl)	68.00 ± 4.24	84.50 ± 36.06	54.0 ± 7.07
TP (g/dl)	3.30 ± 0.28	4.20± 0.85	3.95 ± 0.78

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values are means of triplicates represented as means ± S.E.M. Absence of letters indicates no significant difference between groups. T-Cho: total

cholesterol; Bun: blood urea nitrogen; T-Bill: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: total glycerides; TP: total protein.

Table 4.6.4: Antioxidant potential of amberjack juvenile fed test diets for 60 days.

Items	Experimental diets		
	CD1	D2	D3
MDA (nmol/ml)	1.78±1.36	3.01±1.62	1.83±0.61
SOD (50% inhibition)	31.72±12.83^{b}	82.41±17.47 ^{a}	59.22±18.20 ^{ab}
d-ROMs (µMol/L)	176.50±13.2^{b}	200.0±15.9^{ab}	285.5±20.5^{a}
BAP (U. Carr)	376.50±25.0 ^{c}	1578.0±14.10 ^{a}	587.5±42.50 ^{b}

CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM). Values are means of triplicates represented as means ± S.E.M. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of letters indicates no significant difference between groups.

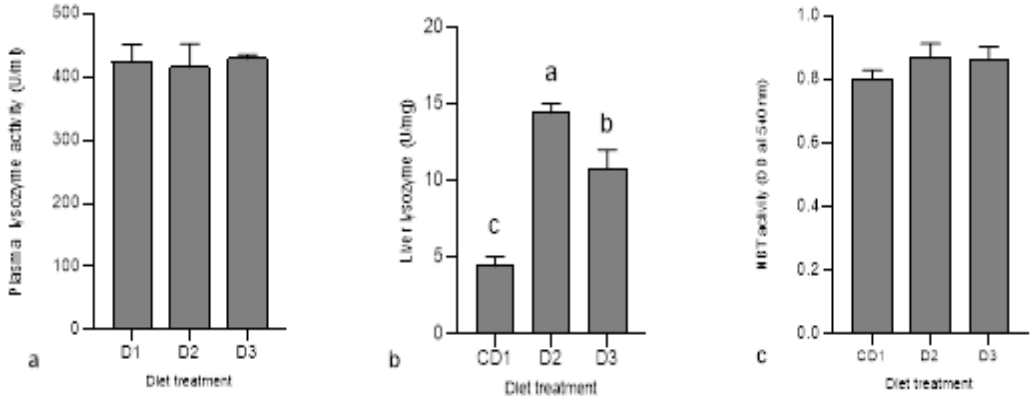


Fig.4.6.1: Amberjack immunological responses after 60 days of the experimental period. (a) Plasma lysozyme activity (unit/ml); (b) Liver lysozyme activity (U/mg); (c) NBT activity (540 nm optical density). Values are means ± SEM (n=3). Different superscripts indicate a significant difference ($p<0.05$) between treatment means. The absence of letters indicates no

significant differences. CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM).

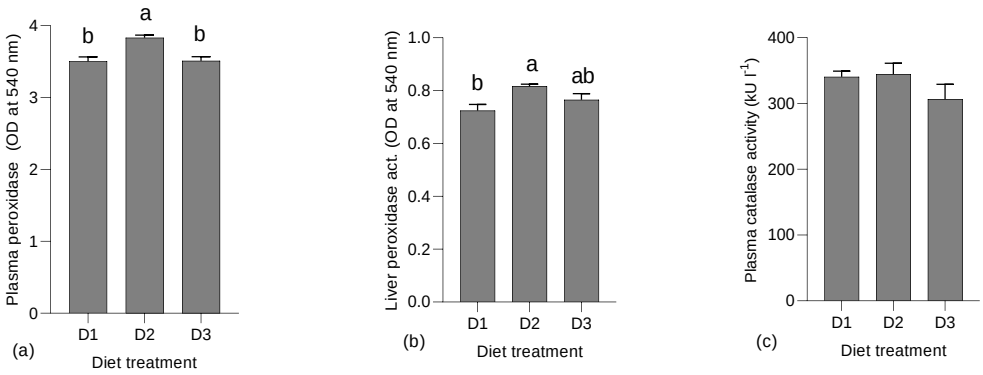


Fig.4.6.2: Peroxidase (a&b) and catalase (c) activities of amberjack juvenile fed test diets for 60 days. Values are means ± SEM (n=3). The absence of letters indicates no significant differences. CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM).

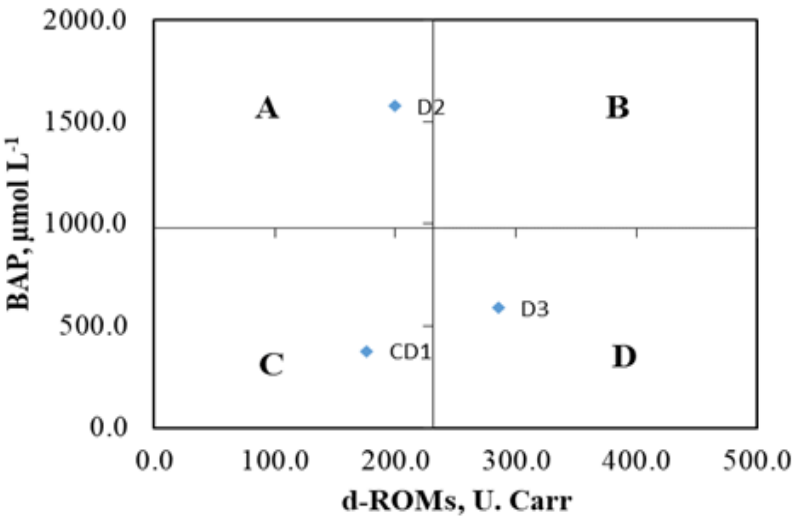


Fig.4.6.3: Oxidative condition of amberjack juvenile fed test diets for 60 days. Values represent the means ± SEM (n=3). The central axis is the means of both d-ROM and BAP for the dietary groups. Zone A: high antioxidant capacity and low reactive oxygen metabolites (good condition); Zone B: high antioxidant capacity and low reactive oxygen metabolite (acceptable

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 basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM).

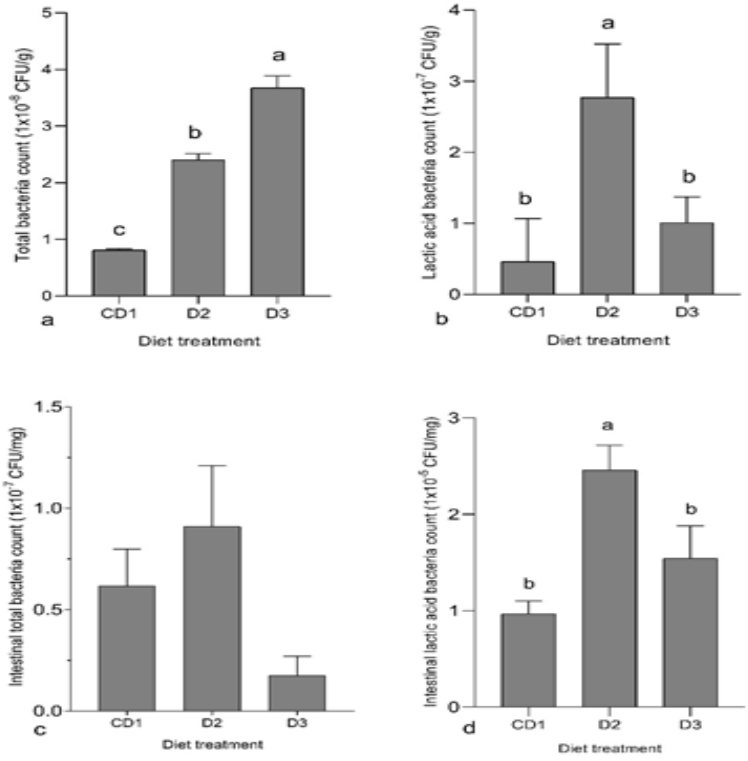
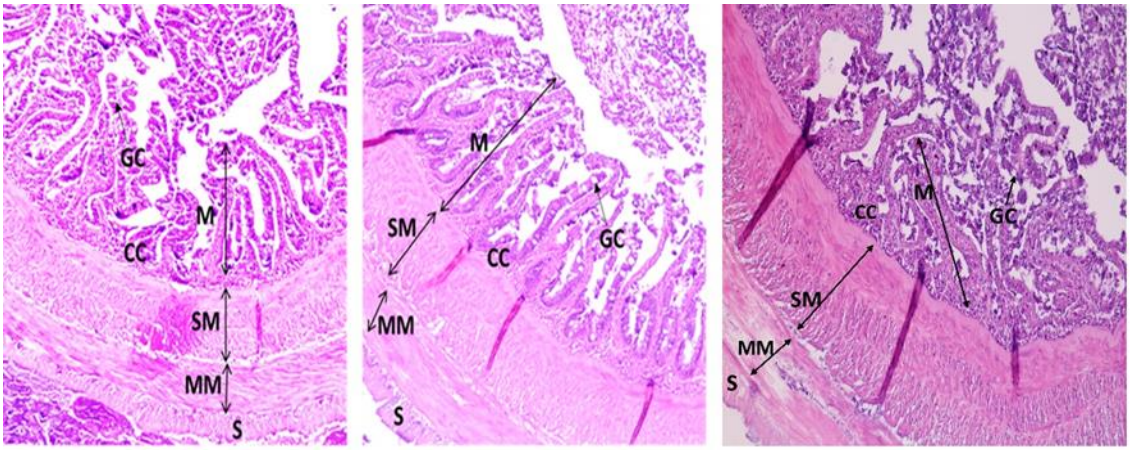


Fig.4.6.4: The bacterial count in experimental diets and intestine of amberjack juvenile fed test diets for 60 days. (a) Total bacteria count in experimental diets. (b) Lactic acid bacteria in experimental diets. (C) Intestinal total bacteria count. (d) Intestinal lactic acid bacteria count. Values are means ± SEM (n=3). CD1= the basal diet; D2= the basal diet + a probiotic mix (SF, BA); D3= the basal diet + a probiotic mixture (SF, LP, BA and BM).



A B C

Fig.4.6.5: The cross-section (60x magnification) in the small intestine of amberjack juvenile fed on the experimental diets for 60 days (H & E stain, x100). Where, M= Mucosa; SM= Submucosa; MM= Mascularis mucosa; S= Serosa; GC= Goblet cell; CC= Crypts cell. A = CD1 (the basal diet); B = D2 (the basal diet + a probiotic mix (SF, BA)); C = D3 (the basal diet + a probiotic mixture (SF, LP, BA and BM)).

4.6.5 Discussion

Aquaculture is currently growing consistently to address food protein demand in the phase of rapid population growth and environmental crises. Intensive farming systems to boost production has been the current focus, however, challenges with intensive systems require better strategies to mitigate the effects on production and quality. Several approaches were investigated including the use of functional feed such as nucleotide supplementation (Hossain *et al.*, 2021), fermented feed (Dossou *et al.*, 2018), and inclusion of bacteria as probiotics in the feed (Dawood *et al.*, 2015b). From this perspective, feed additive and immunostimulant are regarded as a useful method to enhance growth and improve the disease resistance of cultured species (Oliva-Teles, 2012). Probiotic bacteria are known to be promising feed additive in aquaculture regardless of limited contradictory reports (Ringø *et al.*, 2018). It was well documented that probiotic bacteria vary in their immunological effects depending on species

and host (Ringø *et al.*, 2018). Hence, in our study amberjack juvenile was used to test for the effects of probiotic bacteria in modifying the gut microflora and influencing growth and health status of fish. The species SF, BA, LP and BM was supplemented following the manufacturing requirement at 0.5 g/ 100g diet.

The result of growth and feed utilization showed an improved SGR and FI for the D2 group with slightly low FCR suggest a better nutrient uptake and metabolism though no significant difference was observed among treatment. This result demonstrates enhanced feed intake stimulated by D2 (SF, BA) supplement causing the modulation of intestinal microflora as similar observation reported in Ringø *et al.* (2003). Similarly, BA was reported to increase SGR in Nile tilapia (Reda and Selim, 2015), improve disease resistance, immune responses and FCR for in eel, catla and catfish (Cao *et al.*, 2011, Ran *et al.*, 2012, Ridha and Azad, 2012, Das *et al.*, 2013). The final body weight (FBW) and protein gain (PG) were not significantly different among dietary supplemented groups (D2, D3). The growth performance and feed utilization for fish fed the supplemented diets (D2-D3) were not significantly different from the control group, however, previous studies reported that probiotic bacteria are a useful growth promoter due to its functions in the gastrointestinal tract of fish such as producing growth inhibition substances (bacteriocins, hydrogen peroxides, diacyl, etc.) (Alakomi *et al.*, 2000). Thus, it is acknowledging that the dietary supplementation ratio at which beneficial effect on growth can be detected may need further investigation. Survival was found to be not significantly different among treatment groups over the 60 days of a rearing period. The growth and survival reported herein were in agreement with the previous findings of Shadrack *et al.* (2021).

Blood conditions are good indicators of the welfare and health status of fish, hence are direct reflectors of stressors and external stimuli (El Basuini *et al.*, 2021). Following previous research (Dawood *et al.*, 2015b, Kader *et al.*, 2013), blood parameters obtained in the present study were considered to be within the normal range for juvenile amberjack. The results showed high GOT

and T-Cho for D2 and D3 supplemented group though no significant difference was established among groups (<0.05), implying the alteration that needed further attention or investigation. A numerically high Haematocrit and TP for D2 and D3 dietary supplement group, suggest the improved health status. Similar findings were reported for amberjack and rainbow trout where high hematocrit was observed when fish were fed with heat kill-lactobacillus (Dawood *et al.*, 2015a) and *Enterococcus feacalis* supplement diets (Rodriguez-Estrada *et al.*, 2013), respectively. It was reported that high hematocrit indicates evenly distributed ions without any reduction in the synthesis of haemoglobin (Dawood *et al.*, 2015c). A low level of TG was observed for diet group D3 which shows the effect of mix probiotic bacteria strain in producing essential enzymes responsible for regulating the blood health and maintains a low level of plasma triglycerides. The measure of oxidative stress was normally determined through assessment of free radicals using analytical systems using derivatives such as reactive oxygen metabolites, d-ROM test, and antioxidant potential (BAP) in plasma (Dawood *et al.*, 2015a). It is a comparative measurement where the imbalance between oxidants and antioxidants is revealed when the oxidant activity exceeding the neutralizing capacity of antioxidants (Dawood *et al.*, 2015a, Celi *et al.*, 2010). Several studies reported that d-ROM and BAP are reliable parameters for determining oxidative stress conditions in fish (Gao *et al.*, 2012). From the results presented here, it was concluded that the D2 group was in less oxidative stress condition compared to the control and D3 group. Lipid peroxidase MDA and other important antioxidant enzymes such as SOD were also analysed in this study. Results showed a significantly high SOD and high MDA activity for D2 compared to CD1 (control) and D3 group, where the high activity of these two parameters indicate improved antioxidant status of fish (Xu *et al.*, 2015). The first line of defence mechanism for fish is phagocytosis expressed in lysozyme activity and respiratory burst activity (Nitroblue Tetrazolium, NBT) (Dawood *et al.*, 2016, Cecchini *et al.*, 2000). Lysozyme activity is measured as a non-specific immune defence in fish while nitro

blue tetrazolium (NBT) is monitored as an indicator of innate immunity (Cecchini *et al*., 2000, Miyazaki, 1998). Lysozyme is an important molecule in the innate defence system in fish expressed in body fluid (mucus, blood) and tissues (liver, kidney etc.) (Saurabh and Sahoo, 2008). The result of the present study showed a significantly high activity of liver lysozyme in fish fed D2 and D3 compared to control, which demonstrates the enhancement of the phagocytosis cells stimulated by LAB supplementation (Saurabh and Sahoo, 2008). The NBT activity was numerically high in fish fed probiotic supplement D2 and D3 compared to control ($p>0.5$), suggest immune stimulating effect of bacteria supplement in the diet of fish which is similar to findings of Abmughaid *et al*. (2020) where supplementation of probiotic bacteria in diets of tilapia (*Oreochromis niloticus*) significantly improves NBT activity. The significantly high level of peroxidase (GPx) and improved catalase (CAT) activity observed in fish group fed D2 and D3 dietary supplement is in line with the findings of Salinas *et al*. (2008) and Dawood *et al*. (2015a) where supplementation of probiotic heat kill bacteria in diets of red sea bream and amberjack causes a high level of these parameters. From these results, it can be concluded that the non-specific immune response for amberjack was enhanced by probiotic bacteria supplementation where the D2 group outperformed the control (CD1) and D3. Similarly, Irianto and Austin (2003) and Dawood *et al*. (2015b) elucidate the supplementation of inactivated bacteria and heat-killed Lactobacillus in stimulating the innate immune parameters of rainbow trout (*Oncorhynchus mykiss*) and amberjack (*Seriola dumerili*) respectively.

Probiotic bacteria are favourable microorganisms due to their ability to stimulate host gastrointestinal (GI) development, digestive function, mucosal tolerance, stimulating immune responses and improved disease resistance (Ringø *et al*., 2018, Dawood *et al*., 2015c). The analysis of lactic acid bacteria in feed and intestinal content of fish revealed less LAB in control and D3 group compared to significantly high LAB count for D2 group, hence the improve

immune and antioxidant activity. This result suggests that inclusion of D2 probiotic strain has regulate the microflora in favour of the beneficial LAB strain which is in accordance with the report of Kuebutornye *et al*. (2020). The histological features are useful indicators of inflammatory and pre-inflammatory alteration induce by biotic and abiotic factors in aquatic environment (Velmurugan *et al*., 2020). The supplementation of probiotic bacteria improves the intestinal features such as increase villi length, goblet cells and crypt depth which is in line with previous findings reported in Ringø *et al*. (2018). Similarly, the descriptive observation (60x magnification) of the micrograph of fish intestine in this study revealed an increase in villi length, cryptal depth, and the number of goblet cells for supplemented group (D2-D3) compared to the control group (CD1). This demonstrate the benefits of probiotic bacteria supplement which is useful for stimulating GI in fish, thus, improving fish health status.

In conclusion, the present study shows that mix probiotic bacteria SF, BA (D2) supplemented to the diet of amberjack has improved blood health, immune responses, oxidative status and intestinal condition of fish. Fish group fed on D2 should significantly higher liver SOD, lysozyme activity and BAP activity compared to fish fed the control diet. Lactic acid bacteria in the diet and intestinal content were significantly higher in fish fed D2 supplemented diet compared to D3 supplement diet and control diet (CD1). This results suggest that D2 bacteria supplement is a potential probiotic supplement for improving immune and antioxidant activity in amberjack fish.

Experiment II: Effects of single and mix probiotic bacteria supplements on growth, digestive activity, blood haemato-biochemistry, immune and growth-related gene, antioxidant activity and stress tolerance of juvenile red sea bream, *Pagrus major*

4.7.1 Abstract

A 50-day feeding trial was conducted to evaluate the efficiency of single and mix strains of probiotic bacteria supplements on juvenile red sea bream (*Pagrus major*). The elements investigated include growth, digestibility, haemato-biochemistry, antioxidant, immune, immune, and growth gene expression and stress responses. 300 juvenile *P. major* (21.56 g± SE) were randomly distributed into fifteen 200 L polyethylene tanks (20 fishes per tank) in triplicate of 5 treatments; D1= the basal diet; *Streptococcus faecalis* T-110 $5x10^6$ cfu/g diet and *Bacillis amyloliquefaciens* TOA5001 $5x10^5$ cfu/g diet in the second group (D2); mix *Streptococcus faecalis* T-110 $1x10^6$ cfu/g diet, Lactobacillus plantarum TO-A $4x10^5$ cfu/g diet, Bacillus mesentericus TO-A $1x10^2$ cfu/g diet, Bacillus amyloliquefaciens $5x10^5$ cfu/g diet in the third group (D3); mix *Streptococcus faecalis* T-110 $1x10^6$ cfu/g diet, Lactobacillus plantarum TO-A $4x10^5$ cfu/g diet, Bacillus mesentericus TO-A $1x10^2$ cfu/g diet in fourth group (D4), single strain *Bacillis amyloliquefaciens* TOA5001 $5x10^5$ cfu/g diet in the fifth group (D5). The results of the combined parameters revealed that D2 and D3 groups exhibit better performance followed by D4, D5 and control group D1. This finding demonstrated that the use of mix strain probiotic bacteria (D3) has improved immune response, antioxidant enzymes, immune and growth-related gene expression of juvenile red sea bream compared to the mix strain D5, single strain D4, and the control group D1. The single strain BA also exhibits better overall performance and can be used alone in diets of juvenile red sea bream. Therefore, we recommend the use of single strain (D2) and mix strained (D3) group as potential probiotic bacteria supplement for red sea bream *Pagrus major*, and may be useful also for other aquatic species.

Further studies may consider a comparable CFU/g diet to determine the efficacy of these bacteria strains in aquafeed in a more comprehensive manner.

Keywords:

Red seabream (*Pagrus major*); Growth performance; Oxidative status; Blood health; Immune response

4.7.2 Introduction

Aquaculture of marine species has been a long tradition in the Southeast Asia region and has contributed significantly to the production of animal protein in human diets (Naylor *et al.*, 2021). Due to increased demand for finfish in domestic and global markets, aquaculture systems were intensified to meet the supply-demand. The increased intensive aquaculture systems encounter ongoing problems with fish subjected to stress conditions resulting in weakening of the immune system and high susceptibility to pathogens and is still an ongoing problem (Shadrack *et al.*, 2021a, Naylor *et al.*, 2021). Past investigations recognized that probiotics were the best approach to improve species condition, tolerance ability to environmental stressors, and ability to resist pathogens (Dawood *et al.*, 2015c, Dawood *et al.*, 2015a). Probiotic bacteria were recently proven to be useful feed supplements for boosting growth, immune function, and resistance to pathogens (Ringø *et al.*, 2018). Probiotics as friendly additives include lactic acid bacteria, *Bacillus*, and *Saccharomyces* (Niu *et al.*, 2019). The dietary inclusion of *Bacillus amyloliquefaciens* (BA) significantly improves condition factor (K) and relative condition factor in red sea bream (Shadrack *et al.*, 2021a), improve specific growth rate (SGR) and feed conversion ratio (FCR) in Nile Tilapia (Reda and Selim, 2015), improves disease resistance, immune response in eel fish, catla fish and catfish (Cao *et al.*, 2011, Ran *et al.*, 2012, Ridha and Azad, 2012, Das *et al.*, 2013) and improve survival of white-leg shrimps (*Litopenaeus vannamei*) challenged with *Vibrio parahaemolyticus* (Imaizumi *et al.*, 2021). The individual

strain of probiotic bacteria such as *Lactobacillus plantarum* (LP) was reported to improved feed intake (FI), SGR, weight percent, antioxidants, and immune enzyme in koi carp (Zhang *et al.*, 2020). It was reported that the combination of probiotic bacteria strains may complement or improve the health of an individual strain (Niu *et al.*, 2019, Giri *et al.*, 2014) because the use of a single probiotic can never be suitable for all host species due to dissimilarity in the physiological and physio-chemical status of a host of the surrounding environment (Lazado *et al.*, 2015). The inclusion of multi-strain probiotic bacteria in diets of olive flounder reduces the potential adverse effects of low fishmeal diets (Niu *et al.*, 2019). Although some studies have already investigated the use of multi-strain probiotics in aquaculture, there is still limited information on its effect across many species (Dawood *et al.*, 2018b). Thus, in the present study, we investigate the effect of single mix of strain *Streptococcus faecalis* and *Bacillis amyloliquefaciens* TOA5001 (SF+BA) or mix strain *Streptococcus faecalis* T110, *Lactobacillus plantarum* TOA, *Bacillus mesentericus* TOA, *Bacillis amyloliquefaciens* TOA5001 (SF+LP+BM+BA), mix strain *Streptococcus faecalis* T110, *Lactobacillus plantarum* TOA, *Bacillus mesentericus* TOA (SF+LP+BM) and single strain *Bacillus amyloliquefaciens* TOA5001 (BA) and on growth, digestive activity, blood haemato-biochemistry, immune and growth related gene, antioxidant and stress response of juvenile red sea bream, *Pagrus major*.

4.7.3 Material and Methods

4.7.3.1: *Bacteria strain and Ethics*

The bacteria strains (BA, SF, LP, and BM) were kindly provided by Toa pharmaceutical company (Japan) and the concentration of the dry products were *Streptococcus faecalis* T-110 $2x10^8$ cfu/g, *Lactobacillus plantarum* TO-A $8x10^7$ cfu/g, *Bacillus mesentericus* TO-A $2x10^4$ cfu/g, *Bacillus amyloliquefaciens* $1x10^8$ cfu/g. These bacteria strains and the cfu/g were decided

by the manufacturer. These probiotic products were sealed in polypropylene bags and stored at -20 ˚C for further use.

Rules of Animal Experiment in Kagoshima University do not apply to a fish. However, the protocol of this study was due to consideration for animal care (number of fish, fish handling, etc.).

4.7.3.2: *Experimental design and diet formulation*

The experiment was conducted based on 5 treatment groups. The formulation and proximate composition of the experimental diets were shown in Tables 1 and 2. The bacteria supplement was supplemented following the percentage proportion of the experimental diets: 0% (control diet = D1), 0.2 % (D2 = the basal diet + SF $5x10^6$ cfu/g diet, BA $5x10^5$ cfu/g diet), 1% (D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet), 0.5 % (D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet), and 0.5 % (D5 = the basal diet + BA $5x10^5$ cfu/g diet). The weight of the dietary proportion was adjusted to 100% by adding cellular powder. The dry bacteria cells were combined with lipid sources such as Pollack liver oil or soybean lecithin and stir gently prior to adding to the ingredient mixture. The ingredients were stirred with a spatula for 5 min and then with a food mixture for 15 min. Then, based on the weight of the ingredient, 30-40 percent water was added and stirred for the next 10 min. Before pelleting, the pH of the ingredients was adjusted to neutral (pH 7.2) using 4N sodium hydroxide. Pelleting was made using a meat grinder with a 1.2 mm diameter opening and then dried in a convection oven at 45 degrees to less than 10% of the moisture content. Finally, the dry pellets were packed in polypropylene bags and stored in a freezer at -28 ℃ until use.

The proximate composition of the diets presented in Table 1 showed no significant difference in crude lipid, crude protein, crude ash, moisture, carbohydrate, and energy content between all test groups ($P > 0.05$).

Table 4.7.1: Experimental diets ingredients and proximate composition.

Ingredients, g	Experimental diets				
	CD1	D2	D3	D4	D5
Brown Fish meal [1]	900	900	900	900	900
Soybean meal [2]	300	300	300	300	300
Wheat flour	300	300	300	300	300
Pollack liver oil [3]	80	80	80	80	80
Soybean lecithin [4]	40	40	40	40	40
n-3 HUFA [5]	10	10	10	10	10
Methionine [6]	2.2	2.2	2.2	2.2	2.2
Lysine [7]	8	8	8	8	8
Taurine [8]	1.4	1.4	1.4	1.4	1.4
Vitamin mix [9]	80	80	80	80	80
Mineral mix [10]	80	80	80	80	80
Probio EP [11]	0	4	0	0	0
Toaraze for Aquaculture [12]	0	0	10	10	0
Igsign [13]	0	0	10	0	10
Vitamin C [14]	6	6	6	6	6
Activated gluten [15]	100	100	100	100	100
CMC [16]	20	20	20	20	20
α – cellulose [17]	72.4	68.4	52.4	62.4	62.4
Total	2000	2000	2000	2000	2000
Proximate composition [17]					
Crude protein %	40.68±0.16	42.74±4.85	40.3±0.62	40.84±0.28	40.17±0.11
Crude lipid %	12.71±0.1	14.16±2.01	12.99±0.11	13.13±0.16	13.07±0.34
Crude ash %	11.8±0.03	11.66±0.11	11.92±0.1	11.84±0.07	11.78±0.1
Carbohydrate [18]	29.39±0.18	26.16±2.26	30.3±0.45	29.41±0.04	29.79±0.29
Gross energy (KJ/g) [19]	19.08±0.1	19.52±0.1	19.2±0.07	19.27±0.02	19.15±0.09

D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet. [1]Nihon Suisan Co. Ltd (Tokyo, Japan); [2]J. Oil Mills, Japan; [3,4,] Riken Vitamines, 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–[1] diet): β-carotene, 0.10; Vitamin D3, 0.01; Menadione $NaHSO_3 \cdot 3H_2O$ (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 (Nicotic 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–1 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] Probio EP; *Streptococcus faecalis* and *Bacillis amyloliquefaciens* made by Toa Biopharma Co., Tokyo , Japan; [12,13] *Streptococcus faecalis, Lactobacillus plantarum, Bacillis amyloliquefaciens* and *Bacillus mesentericus* (Toa Biopharma Co., Tokyo , Japan); [14] L-ascrobil-2 phosphates-Mg; [15] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name: "A-glu SS"; [16] Nippon paper chemicals, Tokyo, Japan; [17] Values are means of triplicate groups ± SEM of the mean; [18] Carbohydrate (%): 100 – (crude protein + crude lipid + crude ash); [19] Gross energy: Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

The viability of the bacteria cells incorporated in feed were assessed by spreading onto 3M™ petrifilm aerobic count plates (Thomas Scientific, USA) in triplicate per treatment. The lactic acid bacteria content in feed were determine by spreading onto 3M™ petrifilm anaerobic count plate (Thomas Scientific, USA) in triplicate per treatment. Briefly, after the diets were prepared, 1 g of the test diet was homogenized in 10 ml PBS (0.05m, pH 7.4). then 1 ml of the solution was serially diluted in 10 ml PBS buffer to 4th dilution. Finally, 1 ml of each dilution was spread over the 3M™ petrifilm plate and incubated at 26 °C for 3-5 days. The bacteria colony forming unit (CFU g^{-1}) were counted using a colony counter (ACK-3 AS ONE, Japan) as describe in

Ren et al. (2007). In similar manner, the total bacteria count and lactic acid bacteria count were determining from fish intestinal content.

4.7.3.3: *Feeding and experimental conditions.*

The experiment was conducted at the Kamoike Marine Research facility, Faculty of Fisheries, Kagoshima University, Japan. The juvenile red sea bream were purchased from a commercial hatchery (Miyazaki prefecture, Japan). Juvenile fish were stocked in 100 L polyethylene tanks filled with 80 L of seawater in a flow-through seawater system at 1.51 L per minute, with continuous aeration. The juvenile fish were fed with commercial feed for 7 days' acclimation period. The rearing water condition was maintained as follows; (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).

After the acclimation period, juvenile fish (n=300) of 2.56 g average initial body weight were stocked into the rearing tank. Each fish was assigned randomly into 5 experimental groups at 20 fish per tank (triplicate tank per treatment). Feeding was conducted twice daily at 8 am and 4 pm over the 50 days' trial period. Uneaten feed was siphoned after one hour of feeding, then dried using a freeze drier. The weight was used later for the calculation of feed intake (FI).

4.7.3.4: Sampling

At the end of the 50 day of the feeding trial, fish were starved for 24 hours prior to sample collection. During sampling, Eugenol (4- Allylomethoxyphenol, 50 ml/Ml) was used to anesthetize the fish for weight and length data collection. Five juvenile fish were collected per tank and stored at -20 °C for the final whole body analysis. Blood was collected using heparinized (n=5) and non-zheparinized (n=3) syringes. A small fraction of the heparinized blood was used to determining haematocrit by following the micro-hematocrit technique. The heparinized blood was centrifuged at 3000 x g for 15 minutes using a high-speed refrigerated

microcentrifuge and the plasma obtained was kept in a freezer at -80 °C until used. The non-heparinized blood was kept at room temperature for 2 hours and then centrifuged at 3000 x g for 15 minutes to collect serum which was then stored at -80 °C until used. A total of 3 fish per tank were dissected and, liver and viscera weight were taken for the calculation of viscerasomatic index (VSI) and hepatosomatic indices (HSI). All livers per tank were pooled together and stored at -80 °C for further analysis. VSI and HSI were calculated according to the following equation:

VSI = (Viscera weight / fish body weight) × 100

HSI = (Liver weight / fish body weight) × 100

Nine fish (3 fish per tank) were collected and the skin was washed with PBS and distilled water, followed by gentle rubbing with a sterilized piece of cotton over 200 mm^2 of the body surface according to the protocol described in (Dawood et al., 2020b). The cotton containing the mucus was transferred into a 1.5 mL tube and suspend in 1ml PBS (pH=7.4). Then the samples were centrifuged at 2000 x g, 4 °C for 10 min. The supernatant was collected and transferred into new 1.5 ml tubes and stored at -80 °C.

4.7.3.5: *Performance and feed utilization*

The growth performance and feed utilization indices were calculated following the equations describe in Kader et al. (2012).

Weight gain (%) = ((final weight – initial weight)/ initial weight) x 100

Specific growth rate (SGR%) = ((Ln (Final weight) – Ln (initial weight))/ duration of feeding (days) x 100

Survival (%) = (final no. of fish at 50 days/ total no. of fish at stocking) x 100

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

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

4.7.3.6: *Biochemical and antioxidant activity*

The diet and whole-body proximate composition were determined following standard procedures outline in Chemists (1990). A mechanical convection oven (Dk400, Yamato Scientific CO., Tokyo, Japan) was used to dry the samples at 105 ℃ to constant and the weight loss represents moisture content. Crude protein was quantified following the Kjeldahl nitrogen method (Kjeltec System 1002 tecator, Sweden) and evaluating the protein content. Ash content was quantified by burning at 550 ℃ in a muffle furnace for 4 hours and the final product represents ash content. Crude lipid content was determined following the Soxhlet extraction method. Gross energy was calculated using combustion values for protein, lipid, and carbohydrate.

Blood plasma chemical parameters were measured using dry chemical kits with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Array, Inc. Kyoto, Japan). The biological antioxidant potential (BAP) and derivative of reactive oxygen metabolites (d-ROM) from blood serum were determined with an automated analyzer using a chemical kit (FRAS4, Diacron international s.r.l., Grosseto, Italy), according to the manufactures instruction. The red blood cells (RBCs) were counted with a haemocytometer (Houston, 1990), immediately after dilution with Natt and Herrick's solution. The blood haemoglobin concentration was determined using a dry chemical kit with an automated analyzer (Lygren *et al.*, 2001).

The superoxide dismutase (SOD) activity of blood serum or liver was measured using the SOD assay kit ((Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instruction. The malondialdehyde (MDA) in blood serum or liver were

measured using the Colorimetric TBARS microplate assay Kit (Oxford Biomedical Research, Inc., USA) according to the manufactures instruction.

4.7.3.7: *Non-specific immune responses*

The serum lysozyme activity was measured following the turbidometric assay technique describe in Lygren *et al.* (2001). Serum and mucus Immunoglobulin (IgM) activity were measured according to the procedure of Siwicki *et al.* (1994). The oxidative radical production of neutrophils during respiratory burst was measured according to the NBT assay in whole blood (Anderson and Siwicki, 1995). The protease activity of serum was measured following the procedure described in Cordero *et al.* (2016). The total antiprotease (T-antiprotease) and α-antiprotease of serum was measured following the methods described in Newaj-Fyzul *et al.* (2007). The amount of mucus as an indicator of immune response was quantified following the method describe in Dawood *et al.* (2016). The serum and mucus bacteria activity was quantified following the method of El Basuini *et al.* (2020), using Escherichia coli ($1x10^8$) bacteria suspension and the OD was read at 570 nm (Multiskan Go, Themo Fisher Scientific K. K., Tokyo, Japan) and was expressed as percentage inhibition of E. coli relative to the positive control. The serum peroxidase (GPx) activity was quantified according to the method described in (Salinas *et al.*, 2008). Catalase (CAT) activity of serum was quantified following previous methods (Cecchini *et al.*, 2000, Goth, 1991).

4.7.3.8: *Real-time PCR analysis*

Livers were obtained from dissecting the fish, weighed, and placed in fivefold of RNAlater (Invitrogen; Thermo Fisher Scientific K. K., Tokyo, Japan) solution and stored at -80 °C until analysis. The RNA from liver samples was extracted using the RNeasy Mini Kit 50 (Qiagen; Hilden, Ger- 321 many). Briefly, 30 mg liver was placed in a sterilized tube (1.5 ml),

homogenized, and centrifuged at 12,000 rpm for 15 s. The supernatant was collected and mixed with 70% ethanol. After completing the RNA extraction, the Prime 324 ScriptTM RT Master Mix Kit (Takara Bio Inc. Shiga, Japan) was used to obtain the cDNA following the manufacturer protocol. Finally, the PCR analysis was performed using the SYBR Master Mix kit (Thermo Fisher Scientific K. K., Tokyo, Japan) using the primers presented in Table 2. The elongation factor (β-Actin) was tested for stability and used as a housed-keeping gene (Hossain *et al*., 2016). Amplification of genes was made with CFD-3120 Mini Opticon Real-Time PCR System (BIO-RAD, Singapore) according to the following steps: 2 min denaturation at 95 °C, 40 cycles at 95 °C for 15 s, and 65 °C for 30 s. Each assay was performed in triplicate 0 °C for 30 s.

4.7.3.9: *Low salinity stress assessment*

Four juvenile fish per tank were randomly selected and were placed in 20 L transparent glass aquaria containing 18 L of dechlorinated water. The test was conducted in triplicate for each experimental treatment. Time taken to reach 50% death was expressed as tolerance limit and was calculated according to the following equation from Moe *et al*. (2004).

$Y = aX + b$ (8)

where Y = log10 (survival), X = time to individual death of fish (min). LT50 (X)

obtained when Y = 1.7 as log10 (50) = 1.7.

4.7.3.10: *Digestibility assessment*

The digestibility of crude protein, crude lipid, and dry matter was measured indirectly using chromium oxide as an inert marker. Fish were fed with a diet containing chromium oxide for 5 days to be accustomed to the feed. Thereafter, feeding to satiation was conducted twice per day and feces were collected 3 hours after each feeding session using a siphon and a fine-mesh

nylon net. Feces were freeze-dried and milled to powder form. The quantification of chromium oxide in diet and feces were made following the method of Furukawa (1966). The following formula was used to determine the apparent digestibility coefficients.

ADC nutrient (%) = 100 - (% Cr_2O_3 diet/ %Cr_2O_3 faeces ×% nutrient faeces /% nutrient diet

ADC dry matter (%) = 100 – (100 - (% Cr_2O_3 diet/ % Cr_2O_3 faeces)

4.7.3.11: *Statistical analysis*

The Kolmogorov-Smirnov test and Shapiro-Wilk test were performed to verify the normality of the data and homogeneity of variance was confirmed using Levene test. One-way analysis of variance ANOVA was performed using Palaeontology Statistical software version 3.21 (Hammer et al., 2001). The significant differences were further evaluated using Tukey- Kramer post hoc test. The principle component analysis (PCA and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical algorithm were employed to show variables correlated with the dietary group and the ranking. The single linkage clustering using Bray-Curtis dissimilarity matrix was conducted to identify fish group with similar overall performance. All data were standardized prior to PCA and UPGMA analysis using the aforementioned statistical software.

4.7.4 Results

4.7.4.1: *Growth and feed utilization*

The growth parameters, nutrient utilization, and survival rate presented in Table 3 revealed no existing differences ($P > 0.05$) between all dietary groups although final body weight (FBW), weight gain (WG%), specific growth rate (SGR) were numerically improved in D2 and D3 supplemented diets compared to D4, D5, and the control group. The comparison of feed conversion ratio revealed bacteria supplement diet D2 exhibit lower FCR values compared to

the fish group fed other supplemented diets (D2, D4, D5) and the control diet D1, although no significant difference was detected ($P > 0.05$). The survival rate was numerically improved in fish-fed bacteria supplemented diets compared to the control ($P > 0.05$). No significant difference was established between the whole-body proximate composition amongst the fish group fed the test diets ($P > 0.05$).

4.7.4.2: *Blood evaluation*

The blood composition of juvenile red sea bream fed the test diets for 50 days is shown in Table 5. Haematocrit (Hrt) and Red blood cells (RBC) count was numerically higher in the fish group fed the bacteria supplemented diets compared to the control group. The Fish group fed on supplemented diet D2 showed significantly lower values of total cholesterol (T-Cho), glucose (GLU), aspartate aminotransferase (AST) compared to the fish group fed the control diet ($P < 0.05$). The total glycerides (TG) and ALT values showed the lowest peak in the fish group fed D2 compared to the fish group fed other mixes (D3, D5) and single (D4) probiotic supplement diets and the control diet D1 ($P > 0.05$). No significant differences were observed in other blood parameters such as total protein (T-Pro), hemoglobin (Hb), total bilirubin (T-Bill), blood urea nitrogen (Bun), mean capsular haemoglobin (MCH), mean capsular volume (MCV), and mean capsular hemoglobin concentration (MCHC) ($P > 0.05$).

4.7.4.3: *Immune response assessment*

Figure 2 displays the first line of defense in the fish body such as the Immunoglobulin, lysozyme, protease, total antiprotease, α-antiprotease, NBT, bacteria activity, mucus amount, catalase, and peroxidase in fish fed the test diets. Fish-fed bacteria supplement D2 group exhibit high values of serum and mucus immunoglobulin (IgM), serum lysozyme, total-antiprotease (T-antiprotease), NBT activity, mucus and serum bacteria activity, and mucus amount

compared to fish fed the control diets ($P < 0.05$). High values of protease were observed in groups fed D4 and D5, α-antiprotease in D5, and high catalase (CAT) and mucus bacteria activity in all fish group fed supplemented diet (D2-D5) compared to the control D1 ($P < 0.05$). Peroxidase (GPx) activity was higher in all fish fed probiotic bacteria supplemented diets accept for D4 which is lower and significantly different from D5 ($P < 0.05$).

4.7.4.4: *Antioxidant activity*

The antioxidant activity of juvenile red sea bream fed the test diets for 50 days is presented in Table 6, and Figures 3. Fishes fed on D2 and D3 diets exhibited significantly lower values of MDA compared to other bacteria supplement groups (D4, D5) and the control group D1 ($P < 0.05$, table 6). SOD values were not significantly different amongst fish fed all test diets ($P < 0.05$). The BAP activity was significantly higher ($P < 0.05$) in the D3 group followed by D2 compared to the control group and other bacteria supplement groups (Table 6). The d-ROM activity was significantly ($P < 0.05$) lower in the control group compared to supplemented group (D2, D3). The combined pattern of the effect of BAP and d-ROM showed in figure 3 reflects the balance between antioxidant and oxidative stress. Zone A reflects a good condition with low oxidative stress and high tolerance ability. Zone B shows a balance between oxidative stress and tolerance ability where D2 and D3 were favoured similarly to Zone C where D1 and D4 were favoured. Zone D represent high oxidative stress condition and low tolerance ability where D5 was favoured.

4.7.4.5: *Relative growth and immune gene expression*

A significantly high hepatic expression of *IGF-1* and *IGF-2* mRNA was found in fish fed D2 and D3 compared to fish fed D4, D5, and the control diet D1 ($P < 0.05$, Fig.5). A significantly high hepatic expression of *TNF-* α and *IL-1* β was found in the fish group fed probiotic

supplemented diets compared to fish fed the control diets ($P < 0.05$, Fig.5). Fish group fed D3 showed the highest expression of *IGF-1, IGF-2* and *TNF-*α compared to all fish groups fed the supplement diets and control diet diets.

4.7.4.6: *Digestibility*

The digestibility of nutrients presented in Table 5 showed a significantly high crude protein and crude lipid digestibility coefficient for the D2 group compared to fish fed the control group D1 ($P < 0.05$). A high digestibility coefficient of dry matter was observed in fish groups fed D4 followed by D2 and D5 while D3 and D4 being the least. The crude protein digestibility coefficient was generally higher for fish groups fed all probiotic supplemented diets (D2-D5) compared to the control diet D1.

4.7.4.7: *Salinity stress*

The tolerance ability of juvenile red sea bream exposed to low salinity stress after 50 days of the feeding trial was presented in Figure 6. The log-rank 50 % mortality (LT_{50}) between fish group revealed a significantly higher tolerance ability in fish fed D5 supplement compared to the control group D1 ($P <0.05$). In general, fish-fed bacteria supplement (D2-D4) showed better tolerance ability compared to the fish group fed the control diet D1 ($P > 0.05$).

4.7.4.8: *PCA and UPGMA analysis*

The PCA and UPGMA analysis results of several important growths, blood, immune, antioxidant and relative gene expression of juvenile red sea bream fed the test diets in 50 days is shown in Figures 5 & 6. The PC1 and PC2 explained 71.5 % (42.46 % and 29.04 %) of the existing correlation between variables and the test diets. Immune (*TNF-*α) and growth (*IGF-1, IGF-2*) relative gene expression, and RBC were strongly related to the fish group fed

supplemented diet D3. Immune enzymes like IgM, Lysozyme, Protease, T-antiprotease, CAT, and antioxidant enzyme (BAP) were strongly correlated to the fish group fed diet D2. The blood parameters such as AST, TG, and ALT were strongly correlated to fish-fed diet D4. The fish group fed diet D5 showed a strong correlation with FCR, T-Cho, T-Pro, and GLU while fish fed the control diet D1 showed a strong correlation with SOD and MDA. The UPGMA cluster analysis revealed 3 clusters such as D1 and D5 (Cluster 1) were closely related, D4 (Cluster 2) was closer to cluster 1 but far from D3 in cluster 2. The fish group fed D2 forms a single but higher cluster (Cluster 3) which is more different to cluster 1 (D5, D1) and cluster 2 (D3, D4). The variables related to the cluster revealed, D2 and D3 groups showed a strong positive association to most variables related to better performance in the cluster analysis (Fig. 6). The assessment of similarity index using Bray-Curtis dissimilarity index in Single linkage clustering algorithm revealed fish group fed D2 (BA) and D3 (SF, LP, BM) were relatively similar compared to D4, D5 and the control group D1 (Fig. 7).

Table 4.7.2: Forward (F) and Reverse (R) primers used for growth and immune mRNA quantification with quantitative real-time PCR.

Primer name	Primer Sequence (5'-3')	Accession number
ß-actin-F	TCTGTCTGGATCGGAGGTC	JN226150.1
ß-actin-R	AAGCATTTGCGGTGGACG	
TNF-α-F	CCAAACAGAAGCACTAACCAAGA	AY314010.1
TNF-α-R	CTAAATGGATGGCTGCCTTG	
IL-1ß-F	CGAGTACCAAACAGCATGGA	AY257219.1
IL-1α-R	GTGTAGGGGGCAGGTAGGTC	
IGF-1-F	TAAACCCACACCGAGTGACA	AB050670.1
IGF-1-R	GCGATGSSGAAAAGCTACGG	

IGF-2-F	CGGCAAACTAGTGATGAGCA	AB360966.1
IGF-2-R	CAGTGTCAAGGGGGAAGTGT	

Where: *ß-actin* – housekeeping gene; *TNF-α* – Tumor Necrosis Factor; *IL-1β* – Interleukim-1b; *IGF-1* – Insulin-Like Growth Factor 1; *IGF-2* – Insulin-Like Growth Factor 2.

Table 4.7.3: Red sea bream juvenile performance variables (growth performance, feed utilization, survival rate, and biometric indices) and whole-body proximate analysis after 50 days feeding period.

Items	Experimental diets				
	CD1	D2	D3	D4	D5
IBW, g/fish [1]	21.6±0.69	21.38±0.47	21.45±0.46	21.66±0.03	21.72±0.07
FBW, g/fish [2]	45.48±0.23	46.39±1.44	45.84±2.05	45.01±3.12	44.5±3.16
WG, % [3]	110.47±13.73	116.97±3.98	113.65±6.42	107.78±14.68	104.84±13.96
SGR [4]	2.48±0.22	2.58±0.06	2.53±0.1	2.53±0.1	2.38±0.23
FI, g/fish /60 days [5]	31.51±0.51	28.8±4.43	32.83±2.48	28.94±3.89	33.09±4.05
FCR [6]	1.34±0.22	1.16±0.23	1.36±0.19	1.25±0.2	1.46±0.07
PER [7]	1.87±0.28	2.07±0.37	1.86±0.27	1.99±0.3	1.71±0.08
PG [8]	656.3±53.2	642.43±19.92	597.32±26.71	604.5±41.95	587±41.7
PR [9]	51.25±4.88	53.1±8.79	45.41±5.42	51.7±6.97	44.38±2.75
SR, % [10]	76.92±15.38	79.49±8.88	84.62±13.32	79.49±4.44	82.05±16.01
HSI [12]	0.95±0.23	1.2±0.43	1.12±0.27	0.99±0.21	0.87±0.15
VSI [13]	7.35±1.04	7.33±1.03	6.54±1.13	6.12±0.35	6.51±0.56
Whole-body proximate analysis [14]					
Moisture	68.74±1.23	69.2±1.63	69.19±1.42	69.82±0.5	69.62±0.62
Crude protein	14.43±0.73	13.85±0.21	13.08±0.16	13.43±0.3	13.19±0.33
Crude lipid	7.98±1.17	7.99±0.39	7.95±0.28	8.37±0.06	8.15±0.12
Crude ash	5.63±0.13	5.08±0.02	4.66±0.13	4.73±0.02	5.23±0.05

D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet. Values are the means of triplicate groups ± SEM of the mean. The absence of superscript letters indicates no significant difference in means between groups. [1] IBW (g/fish): Initial body weight; [2] FBW (g/fish/50days): Final body weight; [3] WG (%): Weight gain percentage per fish; [4] SGR(%/day): Specific growth rate; [5] FI (g/fish/50 days): Feed intake per fish per 50 day; [6] FCR: Feed convention ratio; [7] PER: Protein efficiency ratio; [8] PG: Protein gain; [9] PR: Protein retention; SR: [10] Survival rates %; [11] CF: condition factor, [12] HSI: hepatosomatic index (%); [13] VSI: viscerasomatic index; [14] Whole-body proximate analysis are expressed on a wet weight basis.

Table 4.7.4: Apparent digestibility coefficient (%) of nutrients in juvenile red sea bream fed the test diets in 50 days.

Parameters	D1	D2	D3	D4	D5
Dry matter	74.07 ± 5.52^{b}	80.48 ± 2.31^{ab}	73.37 ± 0.01^{b}	84.64 ± 0.19^{a}	79.77 ± 0.28^{ab}
Total lipid	87.22 ± 0.35^{c}	94.94 ± 0.02^{a}	88.93 ± 1.04^{b}	94.59 ± 0.5^{a}	91.78 ± 0.56^{ab}
Crude protein	88.54 ± 1.09^{b}	93.53 ± 0.82^{a}	90.3 ± 1.56^{ab}	92.6 ± 0.35^{ab}	92.74 ± 0.88^{ab}

D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet. Values are the means of triplicate groups ± SEM of the mean. The absence of superscript letters indicates no significant difference in means between groups.

Table 4.7.5. Blood status of juvenile red sea bream fed the experimental diets for 50 days.

Parameter	Experimental diets				
	D1	D2	D3	D4	D5
Haematocrit (%)	41.33±8.14	46.0±0.1	43.67±7.51	46.33±0.58	44.0±7.0
Hb (mg/dl)	6.6±1.77	6.4±1.57	7.1±1.73	6.23±1.15	5.6±2.07
Glucose (mg/dl)	123.5 ± 6.36^{b}	54.2 ± 0.01^{a}	88.5 ± 7.78^{b}	72 ± 4.24^{ab}	71 ± 2.83^{ab}
T-Cho (mg/dl)	239.5 ± 10.61^{b}	167.5 ± 12.02^{a}	227 ± 14.14^{ab}	218.5 ± 14.85^{ab}	240.5 ± 4.95^{b}
Bun (mg/dl)	21±2.83	16.5±0.71	22±7.07	18±1.41	18±2.83
T-Bill (mg/dl)	0.4±0.14	0.35±0.07	0.4±0.14	0.35±0.07	0.55±0.07
AST (IU/L)	35 ± 1.41^{c}	12.5 ± 0.71^{a}	60 ± 7.07^{bc}	56 ± 1.41^{bc}	55 ± 8.49^{c}
ALT (IU/L)	13 ± 4.24^{a}	12 ± 2.83^{a}	30 ± 5.66^{ab}	53.5 ± 10.61^{b}	21 ± 1.41^{a}
TP (g/dl)	4.9±1.41	3.25±0.07	5.15±1.63	4.15±0.64	4.95±0.64
TG (mg/dl)	126 ± 11.31^{ab}	105 ± 8.49^{b}	138 ± 7.07^{a}	133.5 ± 9.19^{ab}	137.5 ± 6.36^{ab}
RBC(1x106 mm^3)	6.53±1.05	7.13±2.93	8.17±3.1	7.3±0.22	8.72±3.13
MCH	10.58±4.5	9.41±1.65	6.43±0.52	8.57±1.76	6.52±1.04
MCV	65.58±22.19	72.23±29.08	57.05±14.15	63.55±2.74	53.78±13.92
MCHC	15.82±1.44	13.91±3.42	13.23±3.39	13.44±2.42	12.49±3.01

D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet. Values are means of triplicates represented as means ± S.E. Different superscript letters indicate significant difference ($P < 0.05$). Absence of letters indicates no significance difference between groups. Hb: hemoglobin; T-Cho: total cholesterol; Bun: blood urea nitrogen; T-Bill: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: total glycerides; TP: total protein; RBC: red blood cells; MCH: mean capsular hemoglobin; MCV: mean capsular volume; MCHC: mean capsular hemoglobin concentration.

Table 4.7.6: Antioxidant potential of red sea bream juvenile fed test diets for 50 days.

Parameters	Experimental groupings				
	D1	D2	D3	D4	D5
Serum MDA (nmol/ml)	48.51±4.21^{bc}	46.43±2.28^{bc}	37.32±3.98^{ab}	21.95±0.19^{a}	60.98±3.94^{c}
Liver MDA (nmol/mg)	36.59±2.89^{c}	7.76±0.31^{a}	29.93±0.24^{bc}	12.65±1.66^{a}	28±0.01^{b}
Serum SOD (50% inhibition)	48.57±0.23	47.87±1.13	45.3±0.35	41.85±0.81	44.56±1
Liver SOD (50 % inhibition)	23.89±0.43	20.87±1.68	26.82±4.01	24.38±5.28	25.09±2.94
d-ROMs (µMol/L)	4.5±0.71^{a}	12.5±7.78^{b}	11.5±6.36^{b}	7±5.66^{ab}	9.5±4.95^{ab}
BAP (U. Carr)	1145±21.21^{b}	1814±8.49^{ab}	1850±15.56^{a}	986.5±6.36^{c}	1003±16.97^{c}

D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet. Values are means of triplicates represented as means ± S.E.M. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of letters indicates no significant difference between groups.

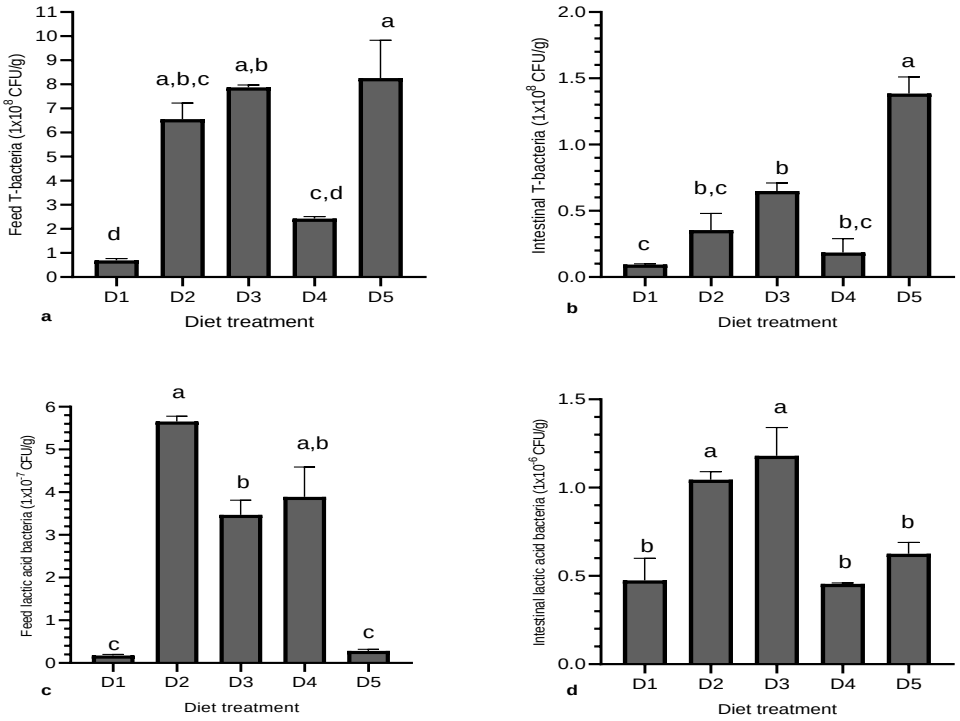


Figure 4.7.1: Total and Lactic acid bacteria count in test diets and intestine of juvenile red sea bream fed for 50 days. a – total bacteria in feed; b- total bacteria in the intestine; c – lactic acid bacteria in feed; d – lactic acid bacteria in the intestine. D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

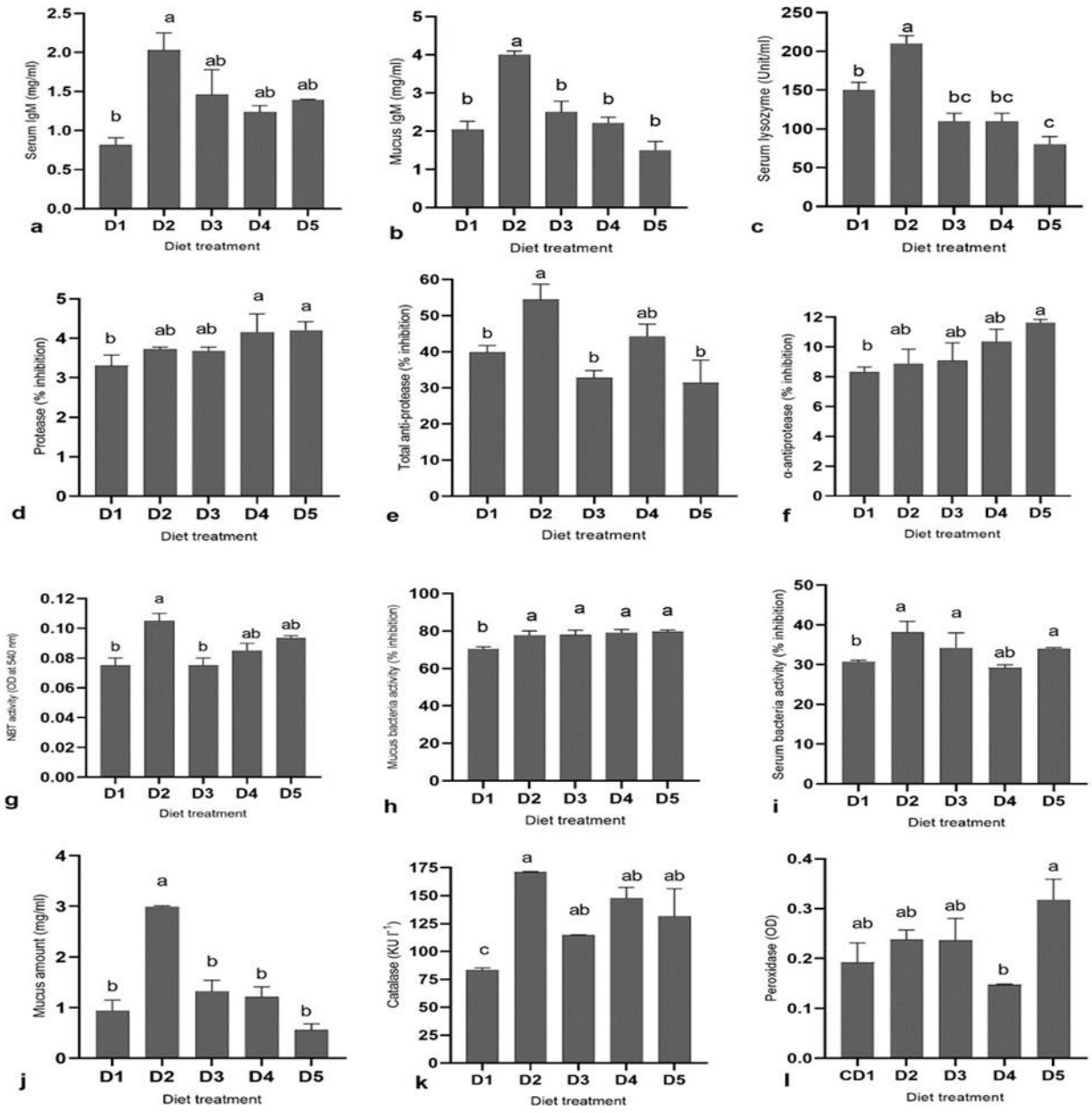


Fig.4.7.2: Serum and mucus, immune responses of juvenile red sea bream fed test diets for 50 days. (a) Serum immunoglobin (mg/ml); (b) Mucus immunoglobin (mg/ml); (c) Serum lysozyme (Unit/ml); (d) Protease activity (% inhibition); (e) Total-antiprotease (% inhibition); (f) α-antiprotease (% inhinbition); (g) Nitroblue tretazolium activity in whole blood (optical density); (h) Mucus bacteria activity (% inhibition); (i) Serum bacteria activity % inhibition; (j) Mucus amount (mg/ml); (k) Catalase activity (kU l^{-1}); (l) Peroxidase activity (optical densitiy).

Values are means (n=3) ± standard error (SE). Different superscripts indicate significant difference ($p<0.05$) between treatment means. D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

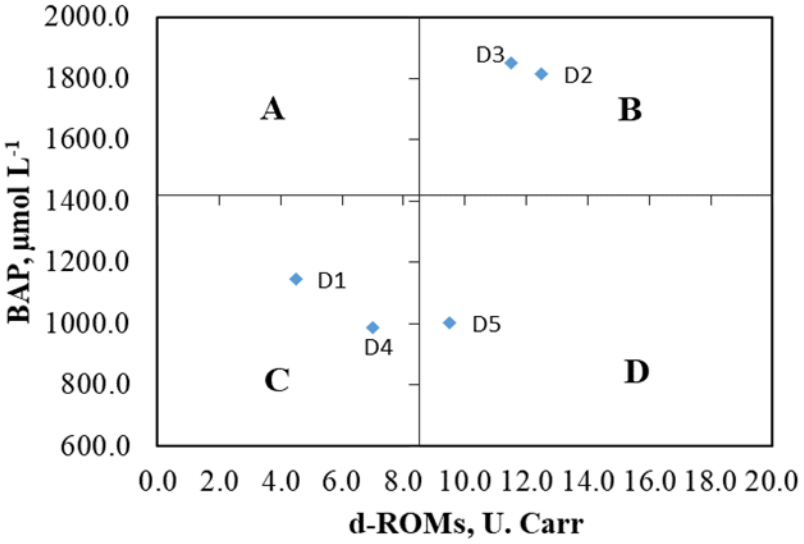


Fig.4.7.3: Oxidative condition of red sea bream juvenile fed test diets for 50 days. Values represent the means ± SEM (n=3). The central axis is the means of both d-ROM and BAP for the dietary groups. Zone A: high antioxidant capacity and low reactive oxygen metabolites (good condition); Zone B: high antioxidant capacity and low reactive oxygen metabolite (acceptable 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). D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

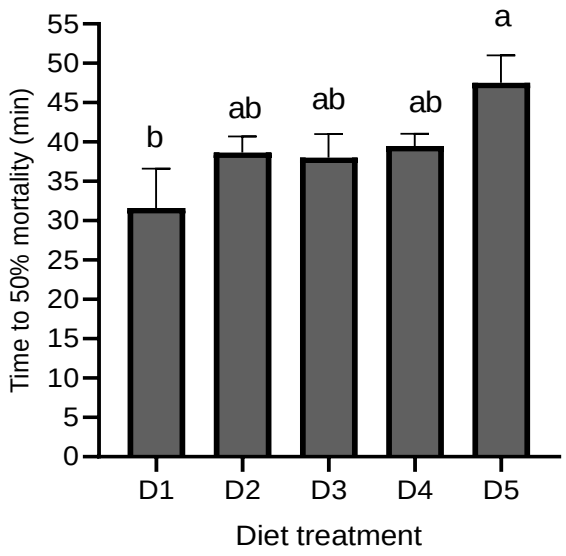


Fig.4.7.4: Time (min) to 50% mortality (LT_{50}) of juvenile red sea bream exposed to low salinity stress after 50 days feeding test diets. The values are means ± pooled S.E. Absence of letters indicate no significant differences. D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

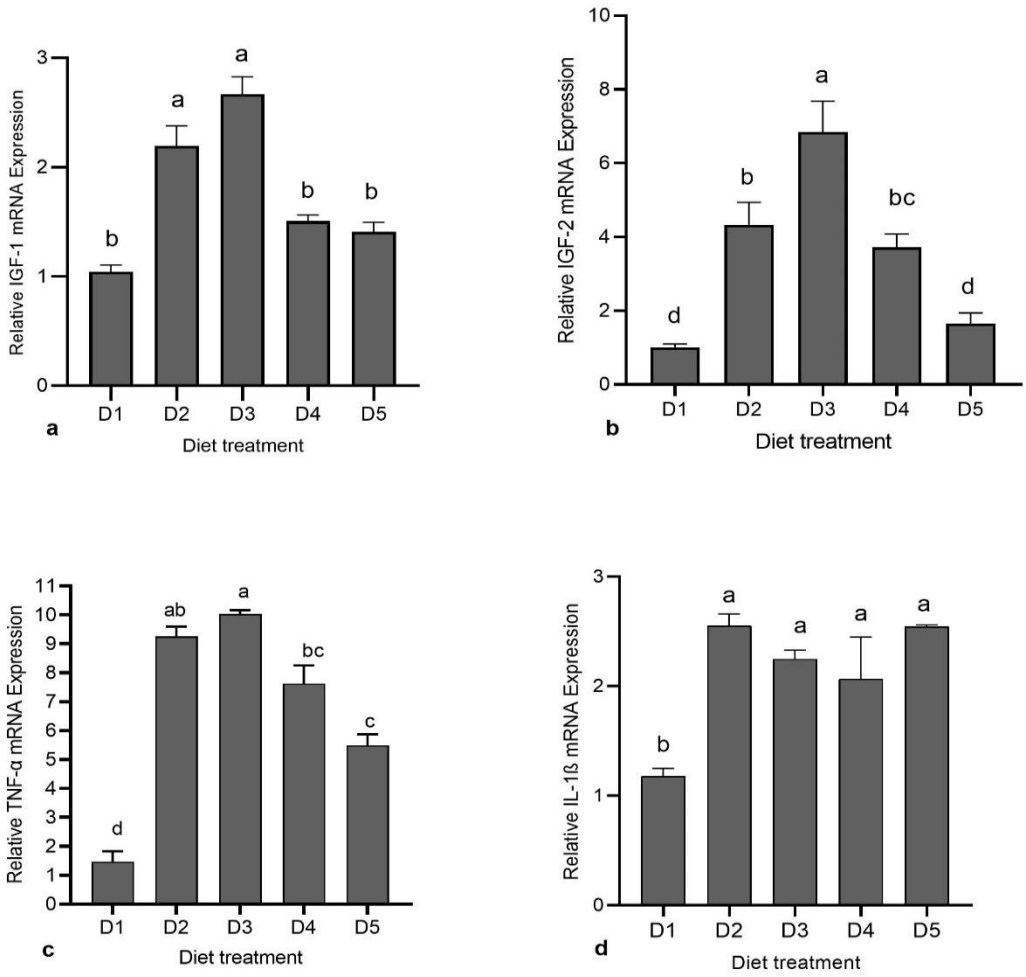


Fig.4.7.5: Relative mRNA expression of growth (*IGF-1*, *IGF-2*) and immune (*TNF-α*, *IL-1ß*) gene for juvenile red sea bream fed the test diets in 50 days. The values are means ± pooled S.E. Absence of letters indicate no significant differences. D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

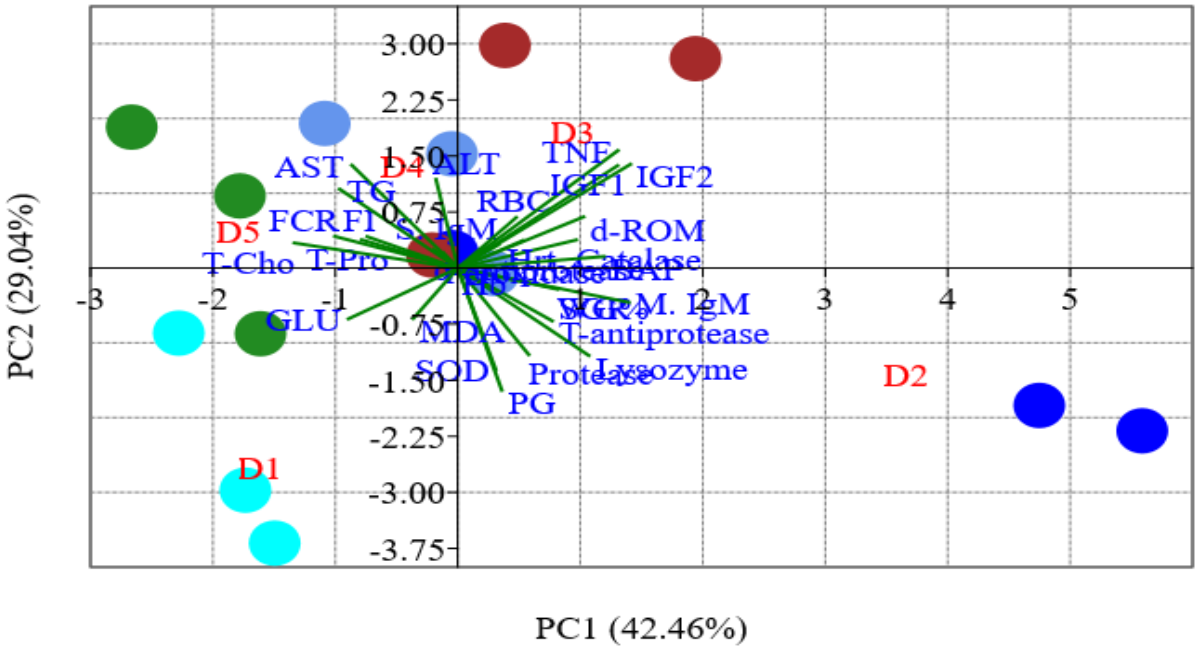


Fig.4.7.6: The PCA plot of parameters observed in red sea bream fed the dietary groups for 50 days. The convex hull connects the region between triplicate samples per group (n=3). The PC1 and PC2 axis explained 42.46 % and 29.04 % of the total variation respectively. The direction of the green lines from the central region of the axis indicates the relationship of each variable in association with the dietary groups. The filled color circles represent replicate per treatment group. D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

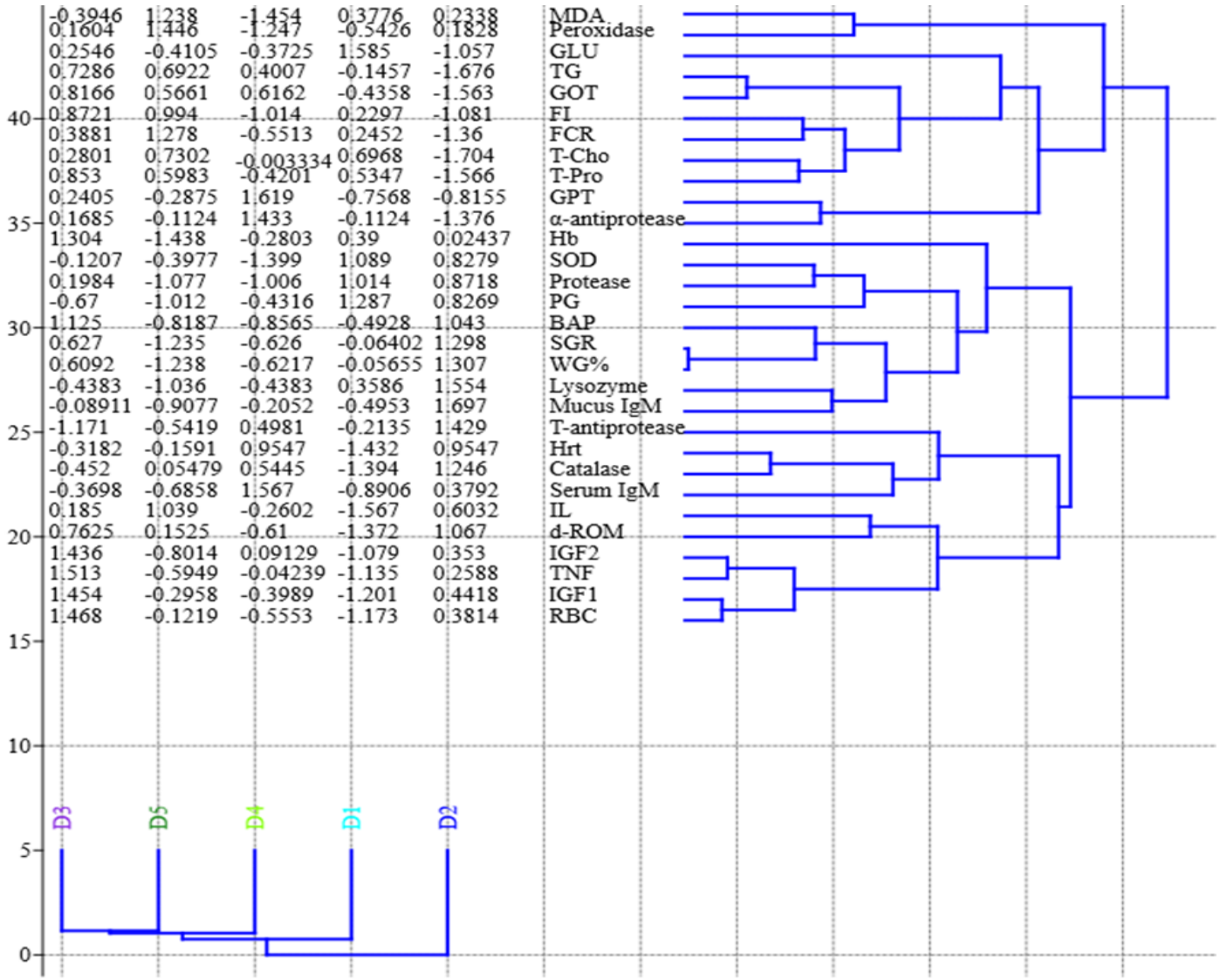


Fig.4.7.7: The UPGMA clustering dendrogram of growth performance variable, blood, immune, antioxidant enzyme, and gene-related expression of juvenile red fed the test diets for 50 days. The numbers on the bottom left corner of the dendrogram represent dietary cluster groups 1-5 (D1-D5). D1 = the basal diet; D2 = the basal diet + SF $5x10^6$ cfu/g diet + BA $5x10^5$ cfu/g diet); D3 = the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet + BA $5x10^5$ cfu/g diet; D4= the basal diet + SF $1x10^6$ cfu/g diet + LP $4x10^5$ cfu/g diet + BM $1x10^2$ cfu/g diet; D5 = the basal diet + BA $5x10^5$ cfu/g diet.

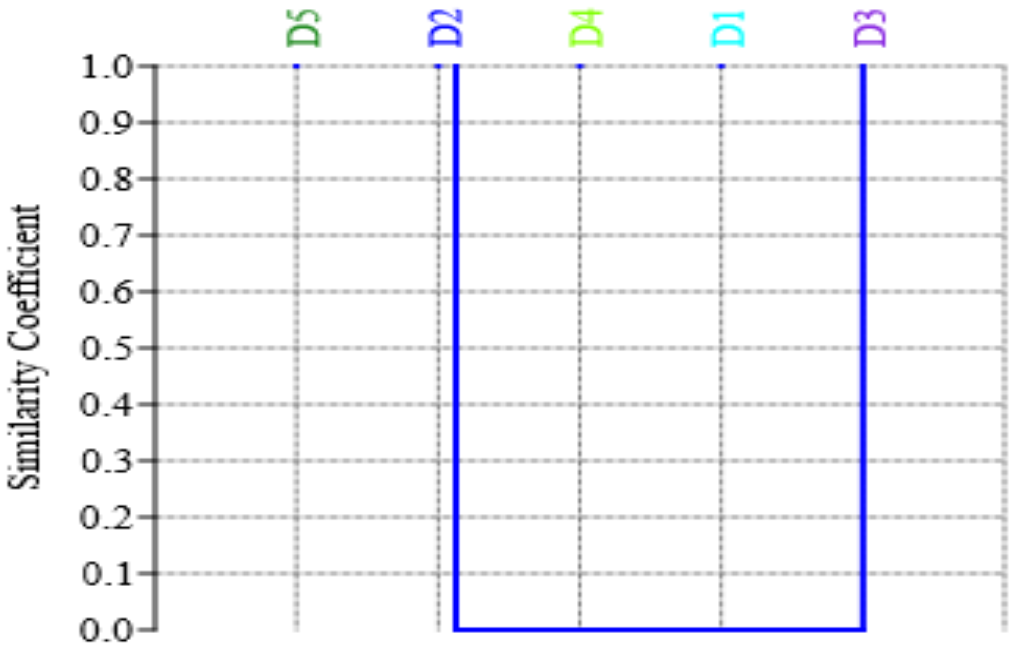


Fig.4.7.8. Single linkage Cluster of growth performance parameter's, blood health, immune and antioxidant activity (Fig. 6) using Bray-Curtis similarity matrix for fish group fed the test diets in 50 days. Dissimilarity coefficient score of zero (0) indicated similarities between groups while score of one (1) indicated differences between groups.

4.7.5 Discussion

Aquaculture has been contributed significantly to meet animal protein demand in the phase of rapid population growth and environmental crises (Maulu *et al.*, 2021). Although many approaches are being investigated individually to solve production problems, challenges with culture systems require better strategies to mitigate the effects on production and investigate the mechanism involved in improving culture and production chain (Maulu *et al.*, 2021). At the stage of animal welfare, the use of functional feed was used to boost production by promoting the growth and health status of the animal (Leal and Calado, 2019). Several of the namely functional feed includes nucleotide supplementation (Hossain *et al.*, 2021), fermented feed (Dossou *et al.*, 2018), and probiotic bacteria supplement (Dawood *et al.*, 2015b). Functional feeds types have specific responses in the animal such as stimulating immune and growth response, and also as enhances disease resistance in cultured species (Oliva‐Teles, 2012).

Probiotic bacteria are known to be promising feed additive and the use of each strain vary in their immunological effects depending on species and host (Ringø *et al.*, 2018). The use of multi-strain probiotic bacteria supplementation was proven to be useful in some species compared to a single strain as multi-strain may promote a better environment or improve the health of individual strain benefiting the host (Niu *et al.*, 2019). Hence, in our study red sea bream juvenile was used to test for the effects of single and mix probiotic bacteria in modifying the gut microflora and influencing growth and health status of fish. The bacteria were supplemented following the manufacturing requirement of 0.2-0.5% per kilogram diet (SF, LP, BA and BM).

The common beneficial effect of probiotic bacteria supplement in feed is the stimulation of animal growth by modulation of intestinal microflora for better feed utilization (Ringø *et al.*, 2003). Single strain probiotic supplement BA was reported to increase SGR in Nile tilapia (Reda and Selim, 2015) improve disease resistance, immune responses, and FCR for in eel,

Catla, and catfish (Cao *et al.*, 2011, Ran *et al.*, 2012, Ridha and Azad, 2012, Das *et al.*, 2013). In the present study, final body weight (FBW), WG%, and SGR were higher in fish-fed yeast probiotic supplemented diet D2 and D3 compared to the control group D1 and mix strain (D5) or mix strain (D4) ($P < 0.05$, Table 3). The relative expression of growth gene IGF-1 and IGF-1 were significantly higher in fish groups fed D2 and D3 compared to other supplemented groups (D4, D5) and the control group D1 ($P < 0.05$, Fig.5). Probiotic bacteria are a useful growth promoter due to their functions in the gastrointestinal tract of fish such as producing growth inhibition substances (bacteriocins, hydrogen peroxides, diacyl, etc.) (Alakomi *et al.*, 2000). Thus, it is acknowledging that supplementation of single strain BA or Mix strain SF+BA or SF+ LP+BA+BM or SF+LP+BM can boost growth response while mix strain (SF+LP+BM) or single strain (BA) alone cannot stimulate better growth response unless otherwise combine for better effect on the host which is in line with previous reports on the use of mix strain probiotic bacteria in aquaculture (Niu *et al.*, 2019). The findings of growth response confirm with our previous findings where mix strain D2 (SF+BA) and mix strain D3 (SF+LP+BA+BM) numerically improves growth performance parameters in juvenile amberjack *Seriola dumerili* (Shadrack *et al.*, 2021a). A significant growth response may be achieved if the period of trial is extended as the gene expression showed favourable growth responses.

Blood conditions are useful indicators of stressors and external stimuli in fish health (El Basuini *et al.*, 2021). The assessment of blood parameters revealed significantly lower values of Glucose, T-Cho, AST, ALT, and TG in the fish group fed D2 supplement compared to the control D1 ($P < 0.05$, Table 5), implying the positive alteration in the health status of fish. A numerically high Hrt in the fish group fed the probiotic supplement diets, suggests the improved health status as high haematocrit indicates evenly distributed ions without any reduction in the synthesis of haemoglobin (Dawood *et al.*, 2015c). Haematocrit was reported to be improved in

amberjack and rainbow trout fed heat kill-lactobacillus (Dawood *et al.*, 2015c) and Enterococcus feacalis supplement diets (Rodriguez-Estrada *et al.*, 2013), respectively.

Oxidative stress was usually measured through assessment of free radicals by analytical systems using derivatives such as reactive oxygen metabolites, d-ROM test, and antioxidant potential (BAP) in plasma (Dawood *et al.*, 2015a). The measurement was used to revealed the imbalance between oxidants and antioxidants, and if the neutralizing capacity of antioxidants is exceeded (Celi *et al.*, 2010, Dawood *et al.*, 2015c). Further, d-ROM and BAP are known to be reliable parameters for determining oxidative stress conditions in fish (Gao *et al.*, 2012). The result of the current study concluded that D2 and D3 were in less oxidative stress condition compared to the control D1, D4, and D5 groups. The Lipid peroxidase MDA and other antioxidant enzymes such as SOD were also important reflectors of antioxidant defence mechanisms in fish (Xu *et al.*, 2015). Results of the present study showed significantly lower SOD and high MDA activity for D2 compared to D1 (control) or D3, D4, and D5 group, suggesting that fish fed D2 group showed better response to antioxidant activity and may be a useful feed supplement for fish.

The phagocytosis defence mechanism in fish expressed in lysozyme activity and respiratory burst activity (NBT) was used as the first line of defence in fish (Saurabh and Sahoo, 2008, Dawood *et al.*, 2016). The result of the present study showed a significantly high activity of lysozyme and NBT in the fish group fed D2 compared to fishes fed all other probiotic supplement diets (D3-5) and the control diet D1. The increase in NBT activity demonstrates the immune-stimulating effect of bacteria supplement bacteria supplement BA in diet D2 as probiotic bacteria has been reported to increase NBT in tilapia (*Oreochromis niloticus*) (Abomughaid, 2020). Significantly high CAT activity was observed in fish groups fed all probiotic supplemented diets (D2-D5) compared to the control diet D1 while peroxidase (GPx)

activity was numerically higher in fish groups fed supplemented diets compared to control diets, except for the D4 group which is significantly lower than fish fed D5 ($P < 0.05$, Fig. 2). These findings on CAT and GPx were in line with the findings of Salinas *et al*. (2008) and Dawood *et al*. (2015a) on the beneficial effect of probiotic heat kill bacteria in diets of red sea bream and amberjack.

The Protease and T-antiprotease were reflectors of the capacity of fish to resist diseases, however, the balance between these two immune enzymes is important for the proper functioning of the immune system (Cordero *et al*., 2016). The use of probiotic supplements in fish diets was reported to improve protease, T-antiprotease, and α-antiprotease in fish (Newaj-Fyzul *et al*., 2007). In our study, the protease, α-antiprotease activities were higher in groups fed probiotic supplement diets (D2-D5) compared to the group fed control diet D1 (Fig.2, d-f). Fish group fed diet D2 (SF+BA) supplement showed generally better activity of protease, T-antiprotease, and α-antiprotease enzyme compared to fish fed other mix probiotic supplement or single strain (BA) and the control group. Skin mucus is the first line of defence against microorganism fish and a component of innate immunity that protects fish from infection (Guardiola *et al*., 2014). In the present study, supplementation of probiotic bacteria significantly improved skin mucus amount in the fish group fed D2 diet compared to other probiotic supplement diets (D3-D5) and the control diet D1. Bacteria activity in skin mucus and serum is related to the ability of these body fluids to stimulate production of special molecules in the innate immune system that affect the antimicrobial responses and kill pathogenic bacteria (Guardiola *et al*., 2014). The evaluation of mucus bacteria activity against *Enrichia coli* (E. coli) in the present study revealed significantly better response in fish group fed probiotic supplements diets (D2-D5) compared to the control diet D1 (Fig. 2, h). The serum bacteria activity was significantly improved in fish groups fed probiotic bacteria supplement diets (D2,

D3, D5) except for D4 which was not significantly different from the control group ($P < 0.05$, Fig.2, i).

Probiotic bacteria supplements can stimulate host GI development, digestive function, mucosal tolerance, stimulating immune responses, and improved disease resistance (Ringø *et al.*, 2018, Dawood *et al.*, 2015c). The total bacteria count in diet and intestinal content of fish were generally higher in bacteria supplemented groups (D2-D5) compared to the control D1, however, significant differences were also observed between supplemented groups ($P < 0.05$, Fig.1 a & b). A higher quantity of lactic acid bacteria was observed in the feed and intestine content of fish fed D2 and D3 groups compared to control D ($P < 0.05$, Fig. 1 c & d). Less amount of lactic acid bacteria was present on the intestinal content of fish fed D4, D5, and the control diet D1. This result suggests that the inclusion of a single strain D2 and D3 mix probiotic strain has regulated the microflora in favouring the condition that stimulates or improves the growth of LAB, however, such findings have already been reported (Kuebutornye *et al.*, 2020). The digestibility of nutrients revealed that the fish group fed D2 supplement exhibit better digestibility of crude protein and crude lipid compared to the fish group fed the control diet D1 ($P < 0.05$, Table 4). Additionally, fish fed D3, D4 and D5 showed better digestibility of crude protein and crude lipid compared to the control D1. An investigation of the retention of probiotic bacteria BA in the digestive tract of penaeid shrimp revealed their presence in all sections of the digestive tract (Imaizumi *et al.*, 2021), suggest better digestive activity when combine with other strain (D2) as reported in the present study.

Challenge tests have been often used to gauge the biological and physical stress responses in fish (Wendelaar Bonga, 1997, Dawood *et al.*, 2020a). The challenge test includes salinity as a physiological indicator of fish tolerance to stress and was performed usually after nutritional trials (Hossain et al., 2016). In the current study, stress tolerance ability (LT_{50}) for juvenile red

sea bream was higher in all fish groups fed the bacteria supplemented diets (D2-D5). Similarly, red sea bream feed immunostimulant showed greater tolerance to salinity (Dawood *et al.*, 2015c) which could be related to improved feed utilization due to improved microvilli alignment, thus, energy and other nutrients will be available to synthesis adrenal steroids to respond to physical stressors (Soleimani *et al.*, 2012).

The multivariate analysis helps to explain the correlation of variables with the dietary groups in a two-dimensional for a better understanding of the effect of single and mix probiotic supplements. The PCA analysis revealed most variables were correlated to fish groups fed D2 and D3 compared to D5, D4 and the control group D1 (Fig.5). Several variables such as AST, ALT, T-Cho, MDA, and TG were correlated D4, D5, and control group D1, suggesting a lower performance compared to D2 and D3 groups. The clustering using UPGMA revealed 3 clusters where D3 and D5 were closer to cluster 2 (D4 and D1) compared to D2. Cluster 3 (D2) is highly different from the two sets of clusters (1&2), suggesting that D2 has better performance followed by D3, D5, D4, and D1 (Fig. 6). The effect of single strain D5 or mix strain D4 does not show better fish performance compared to combine effect of D3 which complement the findings of Niu *et al.* (2019) where mix strains probiotic bacteria can improve health of individual strains for better response in fish. In the present study, the single linkage cluster also revealed that D2 and D3 were relatively similar based on the overall performance (Fig. 7).

The supplementation of probiotic strain D2, D3, D4 and D5 showed improved immune responses and antioxidant defence mechanisms in juvenile red sea bream. The use of mix strain D4 and single strain D5 does not show better performance compared to when combining all four strains in D3 or two strains in D2. The fish group fed D2 and D3 showed better responses which complement the use of these strains in diets of amberjack as reported previously, suggesting that mixed bacteria strains are useful probiotic supplements in diets of aquatic species.

Chapter V: General Discussion

5. General Discussion

The two important cultured species such as red sea bream, *Pagros major* and amberjack, *Seriola dumerili* had been contributed significantly to animal protein in human diet. Although there are many variety of species being successfully cultured in developed countries, aquaculture of marine species in developing countries could only relied heavily on larvae or juveniles of wild brood stock for which milkfish (*Chanos chanos*) is one of the potential species (Shadrack *et al.*,2021b).

In recent years red sea bream and amberjack aquaculture has been skyrocket due to the demand in fish protein, particularly in the east Asia region (Dawood *et al.*,2015a). Challenges in intensive aquaculture production is usually related to stress which negatively affect growth and immune responses (Wang *et al.*, 2008a, b). Although chemotherapeutics and antibiotics have been popularly used to address diseases in intensive farming, the emerging disease resistance to pathogens, negative alteration of immune system, environmental hazards and food safety problems are major concerns (Bachère *et al.*,2003). The works on finding alternative means of solving intensive aquaculture problems have become the main research target (Miranda *et al.*,2002). Recently, studies have demonstrated the positive impacts of functional feed additives on growth performance, feed utilization, digestibility activity, disease resistance and immune enhancement of aquatic animals (Encarnação 2016; Hoseinifer *et al.*,2017; Bharathi *et al.*,2019). The use of probiotic in aquaculture was recognized as a sustainable way of improving gut microbiota, performance and health condition of fish. Probiotics include live, dead or a component of microbial cells that are supplied to feed or via the rearing water to improve animal health and the condition of the environment. Some contrary reports on the use of oral administration of probiotic in fish aquaculture were due to introduction of live bacteria into the aquatic environment, as a result, the recent probiotic studies mainly aimed at exploring the beneficial impact of inactivated bacteria as they do not interact with the aquatic environment

(Salinas *et al.*, 2008). The dietary supplementation of probiotic bacteria showed considerable improvement on growth, immune response and health status of fish (Dawood *et al.*,2019). The use of mix bacteria strains as probiotic have been proven to be an alternative approach to single strain as a mixture of strain may help to improve health condition of an individual strain (Niu *et al.*, 2019). Previous research efforts have also proved yeast as potential probiotic in aquaculture (Gatesoupe 1999; Zaineldine *et al.*, 2021). The use of yeast as probiotic was related to their unique mode of action such as promoting aquatic animal health (Meena *et al*, 2013; Zhang *et al.*, 2020; Van Doan *et al.*, 2020).

Functional feed additives are beneficial to fish as they improved growth performances, immune responses and tolerance ability to stressful conditions (Irianto and Austin 2003; Wang *et al.*,2008; Encarnação *et al.*,2016). Yeast on the other hand is a well-known probiotic supplement for aquatic animal (Dawood *et al.*,2019; Gatesoupe, 1999). Yeast cell is an important probiotic supplement for aquatic animals as it contained molecules that promotes better feed utilization and growth responses in aquatic animals (Peppler, 1982; Zaineldin *et al.*, 2021; Bowman and Free, 2006). In the present study, supplementation of spent oleaginous yeast has significantly improved growth responses parameters and feed utilization in red sea bream compared to fish fed the control diet. Previous study also reported improved growth and immune responses of aquatic species fed yeast supplemented diets such as in Japanese sea base (Yu et al., 2014), tilapia (*Oreochromis niloticus*) (Abu-Elala *et al.*, 2018) and gibel carb (*Carassius auratus gibelio*) (Zhang *et al.*, 2020). Although various yeast strains have been reported on their beneficial effect in aquatic animal, little is known about the effect of oleaginous yeast (Overland and Skrede, 2017; Blomqvist *et al.*, 2018). In the present study growth performance and feed utilization were higher in fish fed supplemented diets compared to the control group which reflect an improved intestinal microflora (Rimoldi *et al.*, 2020; Navarrete and Tovar-Ramírez, 2014; Liu *et al.*, 2018). Lower ratio of n-3 and n-6 in fish group

fed supplemented diet suggested improve health condition similar to sea bass fed yeast supplemented diets (Blomqvist *et al.*, 2018). Blood condition were improved such as lowering of ALT and AST in supplemented group suggesting less prone to liver disease (Nagai *et al.*, 1989; Vermeulen *et al.*, 1992). Serum immune responses parameters were improved which indicate the functional properties of yeast in stimulating the immune responses (Dawood *et al.*,2017a; Magnadottir 1998; Magnadottir 2010). The current findings indicate an optimum level of 2.5 % of in diets of red sea bream with achievable benefits.

Probiotic bacteria are one of the best option for addressing animal welfare and improve health status. In the present study, growth performance, growth related gene expression and nutrient utilization of protein were higher in fish group feed probiotic diets. These results were similar to those of previous studies where addition of bacteria cells improves growth performance of red sea bream *Pagros major* (Dawood *et al.*, 2015a, b), amberjack *Seriola dumerili* (Dawood *et al.*,2015), Rainbow trout *Oncorhynchus mykiss* (Bagheri *et al.*, 2008; Rodriguez-Estrada *et al.*, 2013), Cobia *Rachycentron canadum* (Xing *et al.*, 2013), Tilapia *Oreochromis niloticus* (Standen *et al.*, 2013) and grouper *Epinephelus coioides* (Huang et al., 2014). Mix strain probiotic bacteria in diets of fish have been proved in several studies to improved performance of fish compared to a single strain as multiple strains have the capacity to improve health of an individual thus, benefiting the host (Dawood *et al.*, 2018; Giri *et al.*, 2014; Niu *et al.*,2019). In the present study, the fish group fed single strain bacteria do not show better growth, immune response and feed utilization compared to mix strain. However, some probiotic bacteria as single strain does not require mixing to show beneficial effect on the host as observed in the present study. Our study descriptively shows that probiotic bacteria helps improved fish intestinal condition such as increased villi length, mucosa, submucosa, mascularis mucosa, sarosa and number of goblet cells and crypts cells, which suggest an improved microflora benefiting the host similar to findings reported in Ringo *et al.* (2018). Although no significant

difference in growth performance was observed on fish group fed probiotic supplement diets, an extensive feeding period would benefit the host as growth relative mRNA expression was significantly high compared to the fish group fed the control diet. The blood condition of fish fed (Single or mix) probiotic supplement were improve similarly to improved blood condition of amberjack and rainbow trout fed heat kill-lactobacillus (Dawood *et al.*, 2015a) and *Enterococcus feacalis* supplement diets (Rodriquez-Estrada *et al.*, 2013), respectively. The immune response parameters such as lysozyme, serum and mucus bacteria activity, peroxidase activity, serum and mucus immunoglobulin were improved in fish fed probiotic supplement diets. Previous studies proved that beneficial bacteria cells interact with intestinal epithelia cells in the host gut and regulate the physical and immunological properties of intestine such as tightening of epithelial junction, production of antimicrobial peptides, production of mucosal immunoglobulin, and modulating inflammatory reaction which triggers cascades of reaction, thus, stimulating the immune response (Thomas and Versalovic, 2010; Wells, 2011; Liu, 2013; Shida and Nanno, 2008).

With the current interest in *P. major* and *S. dumerili* in Asia region, in the western pacific, milkfish *Chanos chanos* is one of the alternative option for low tech extensive aquaculture systems because fry or juveniles can be obtained from the wild as hatcheries are expensive to maintained. The establishment of fry seasonality in the pacific island is important for the development of aquaculture industry to support local economy and food security due to arising need for food fish (Bell *et al.*, 2015). Many island countries have enchanted in milkfish aquaculture based on wild fry (Sulu *et al.*, 2016; Pickering *et al.*, 2013; FAO 2011; SPC 2010). Vanuatu is an archipelago of islands in the tropical south pacific island with no quantitative record of milkfish fry occurrence. There is a need to explore the possibility of cultivating this species to add to the alternative sources of fish food. In the present study (third paper), milkfish fry seasonality was from November to April with two fry occurrence peak, one in November

and the other in April. The long seasonality is advantages as fry maybe available at an extended period for collection to supply local aquaculture farmers. This seasonality period is similar to Fiji (9 month of fry occurrence) with two seasonality peaks, aiding the establishment of milkfish aquaculture (Napulan *et al*., 2012). The abundance of fry in the present study was low compared to findings in Fiji and Solomon Islands. Therefore, the present study concluded that further research is needed to identify abundant fry collection site and establish a local feed production industry to support the aquaculture of milkfish in Vanuatu. The use of functionally feed additives could also improve performance of *C. chanos* and boost their tolerance to environmental stress due to effects of climate change in the tropical pacific island countries. Further study may trial these functional feed additives on performance, immune responses, blood health and stress tolerance ability in milkfish.

The present study links with previous research on the use of functional materials such as probiotic bacteria cells and yeast cells supplementation in diets of aquatic animal. Further studies are recommended to understand the relation and mechanism of the bacteria and yeast examined in the current study for their effect on performance and immune response in aquatic animals. The knowledge on these feed additives and experimental conditions can be utilized further to improve growth performance and feed utilization in fish. The seasonality of milkfish *C. chanos* presents the bassline data for the development of milkfish aquaculture in Vanuatu. The knowledge gained from using functional feed additives in *P. major* and *S. dumerili* can be utilized in milkfish aquaculture to improve performance and response to stress condition in the current phase of increasing food protein demand and climate change.

The present work illustrated a significant interaction on enhancing growth, blood chemistry, antioxidant parameters and immunological parameters of amberjack, *S. dumerili* and red sea bream, *P. major*. These findings are useful to those engage in aquaculture research and the fish farming industry. This study recommends the utilization of spent *L. starkeyi* and the probiotic

bacteria, SF+BA or SF+BA+LP+BM as potential probiotic supplement in aqua feed development.

Summary and conclusion

7. Summary and conclusion

The is an increased knowledge on the beneficial effect of natural functional feed additives regarding its influence on balancing gut microbiota in aquatic animals and thus, improving the health status.

The present study has obtained the following concluding remarks.

1. Oleaginous yeast, *Lipomyces starkeyi* significantly improved growth performance and feed utilization of red sea bream.
2. The antioxidant and immune enzyme in *P. major* were improved, thus providing them with improved stress tolerance ability.
3. The optimum supplementation of spent oleaginous yeast for *P. major* is 2.5% of the dietary proportion.
4. The oleaginous yeast significantly improved digestive enzyme activity in *P. major*.
5. Blood chemistry of *P. major* were generally improved with oleaginous yeast supplementation in the diet.
6. whole body crude lipid in *P. major* increase with increasing oleaginous yeast dietary supplement.
7. The supplementation of *Bacillus amyloliquefaciens* (BA) significantly improved physiological condition and intestinal lactic acid bacteria count in amberjack *Seriola dumerili*.
8. Mix strain *Streptococcus faecalis* (SF), *Lactobacillus plantarum* (LP) and *Bacillus mesentericus* (BM) supplementation improved health status of *S. dumerili* reflected on improved whole body fatty acid profile.
9. Improved intestinal cells were found in *S. dumerili* feed probiotic single and mix bacteria strains.

10. Antioxidant and immune response of *S. dumerili* were improved with probiotic single and mix strain supplement.
11. Blood condition of *S. dumerili* were improved with BA supplementation.
12. The probiotic bacteria supplement significantly improved growth and immune related gene expression of *P. major*.
13. Digestibility of protein, lipid and dry matter were improved in *P. major* fed single and mix strain probiotic bacteria
14. Digestibility of protein were significantly improved in red sea bream fed BA supplement.
15. Mix strain (LP, BM) probiotic supplement significantly improved stress tolerance ability for *P. major*.
16. Mix strain (LP, SF, BM) significantly improved growth and immune related gene expression of *P. major* compared to single strain (SF) or mix of strain (LP, BM) supplement group.
17. Single strain BA supplement showed best overall performance followed by mix of three strains (LP, SF, BM) in *P. major*
18. A potential marine species for aquaculture in developing nation of Vanuatu is milkfish, *Chano chanos*.
19. The *C. chonos* fry seasonality was from October to May with two seasonal peaks.
20. Two seasonality peaks of *C. chanos*, one in November and the other in April.
21. The milkfish SST threshold for fry occurrence along the coast of Pacific island nations was 26 °C.
22. Establishment of fry collection site considering the abundance is recommended for trailing *C. chanos* aquaculture in Vanuatu.

23. The use of these functional feed additives in the present study can be used to boost *C. chanos* aquaculture in Vanuatu in the near future.
24. The use of spent oleaginous yeast and single or mix strain probiotic bacteria both improved fish performance, immune response and health status. Thus, there mechanisms responsible for this effects will be an interesting study.
25. The use of spent *Lipomyces starkeyi* yeast and mix probiotic bacteria (SF+BA or SF+BA+LP+BM) were recommended as potential probiotic supplement to be utilize in aqua feed development, in particular for red sea bream and amberjack aquaculture industry.

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