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Effects of replacing fishmeal with fermented and non-fermented rapeseed meal on the growth, immune and antioxidant responses of red sea bream (*Pagrus major*)

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Abstract

The effect of rapeseed meal (RM) and *Aspergillus oryzae* fermented rapeseed meal (RM-Koji) on red sea bream (*Pagrus major*) was examined. Three groups of fish (initial weight, 4.5 ± 0.02 g) were fed a basal diet (RM0) and two test diets where half of fishmeal was replaced by RM (RM50) and RM-Koji (FRM50) for 56 days. The obtained results showed that fish fed RM0 and FRM50 exerted significantly higher growth performance, feed utilization and haemoglobin level but lower triglyceride and cholesterol than RM50 group ($p < 0.05$). Interestingly, except of antiprotease activity, all the immune parameters including lysozyme, respiratory burst (NBT) and bactericidal activities were significantly increased in fish fed RM0 and FRM50 diets compared to RM50 diet ($p < 0.05$). In addition, malondialdehyde and reactive oxygen metabolites were significantly reduced in RM0 and FRM50 groups over RM50 group ($p < 0.05$). The present results suggest that fermented RM induced better growth performance and immune responses than feeding red sea bream with non-fermented RM and both RM and RM-Koji improved the antioxidative status of fish, making RM-Koji an interesting candidate as a functional feed for aquatic animals.

KEYWORDS

antioxidant enzymes, *Aspergillus oryzae*, fermentation, innate immune responses, *Pagrus major*, rapeseed meal

1 | INTRODUCTION

Stress and disease outbreak are limiting factor in aquaculture (Eshaghzadeh, Hoseinifar, Vahabzadeh, & Ringø, 2015; Hoseinifar, Dadar, & Ringø, 2017; Hoseinifar, Eshaghzadeh, Vahabzadeh, & Peykaran Mana, 2016). In replacement to traditional disease control strategies with antibiotics and chemotherapeutics agents, new methods are being promoted to provide more eco-friendly and disease-preventive measures for a sustainable culture of fish (Dhayanithi, Ajith Kumar, Arockiaraj, Balasundaram, & Harikrishnan, 2015; Misra, Das, Mukherjee, & Pattnaik, 2006). Therefore, strengthening the defence mechanism of fish through the prophylactic administration of feed additives including pro-prebiotics (Dawood, Koshio, Abdel-Daim, & Van Doan, 2018; Hoseinifar, Ahmadi, et al., 2017; Zaineldin et al., 2018), fucoidan (Yang et al., 2014), levamisole (Kajita, Sakai, Atsuta, & Kobayashi, 1990), immunostimulants (Dawood, Koshio, & Esteban, 2018) and nucleotides (Hossain et al., 2016) is one of the most promising methods of disease control in aquaculture (Dotta et al., 2014). Nevertheless, there is a great interest in the use of natural feed additives derived from microbial (Fungi, Yeast, Bacteria)

fermentation due to the increasing demand and low cost (Couto & Sanromán, 2006; Gerzhova, Mondor, Benali, & Aider, 2015). In plant protein meals especially, microbial fermentation has been identified as an effective approach for improving biological detoxification, nutritional quality and functional properties (Jakobsen, Jensen, Erik, Knudsen, & Canibe, 2015; Shi et al., 2015; Yan et al., 2017).

In the past few years, rapeseed meal (RM) production has increased significantly to become the second most widely traded protein ingredient after soybean meal (USDA, 2017). Apart from having high biological value, rapeseed proteins were also found to possess highly interesting functional properties which impart their further utilization in the industry so as to potentially substitute animal proteins (Gerzhova et al., 2015; Ghodsvali, Khodaparast, Vosoughi, & Diosady, 2005). Recently, rapeseed protein-derived bioactive peptides were reported (Akbari & Wu, 2015) and these bioactive and functional peptides released from the primary structure of its proteins during enzymatic and microbial degradation are shown to have different biological roles including antimicrobial, antihypertensive and immunomodulatory activities (Akbari & Wu, 2015; He, Girgih, Malomo, Ju, & Aluko, 2013). In addition, certain degradation products in RM such as sinigrin and phenolic compounds have been reported to possess potent antioxidant activities associated with beneficial effects in foods (Alashi et al., 2014; Mazumder, Dwivedi, & Plessis, 2016).

Our previous studies provided the first reports on the effects of graded inclusion levels of fermented RM with *Sacharomyces cerevisiae* (Dossou, et al., 2018) and with *Aspergillus oryzae* (Dossou, et al., 2018). In both studies, up to 50% of fermented RM could replace fishmeal without compromising growth, health status, innate immune and antioxidative stress responses of red sea bream. The present study aimed to shed the light on the comparative performances of red sea bream fed with raw and fermented RM, respectively.

2 | MATERIALS AND METHODS

2.1 | Experimental diets and design

Experimental diets were composed of a basal diet (RM0), containing approximatively 495 g protein and 94 g lipid per kg diet, and two test diets where half of fishmeal was replaced by high protein RM (RM50) and fermented RM, RM-Koji (FRM50), respectively. Formulation, amino acid profile and proximate composition of the experimental diets were shown in Tables 1 and 2. Fishmeal and RM were obtained commercially while RM-Koji was prepared as previously described (Dossou, et al., 2018). Briefly, the RM was first autoclaved at 121°C for 15 min to deactivate myrosinase enzyme (Vig & Walia, 2001) and each kilogram was mixed with 1.3 L of sterile water. The soaked RM was then inoculated with the koji starter (*A. Oryzae*, Bio'c company, Uchida, Japan) at a rate of 4 g/kg RM after the mixture was incubated between 30 and 32°C for 24 hr. The mixture was agitated every 12 hr to release heat. After the apparition of white spots (sign of mycelial growth) and fruity Koji fragrance, the mixture is quickly spread on a tray, with a thickness of 2–3 cm, and covered with a warm sheet cloth to protect moisture evaporation during cultivation. From this stage, the mixture is kept for another 24 hr in a dry-air mechanical convection oven at 37°C and 95% relative humidity until the mycelia permeate the meal and hold together in a spongy white cake. After fermentation, the fermented RM (RM-Koji) was freeze-dried, ground in fine particle size and store in the freezer for further utilization.

TABLE 1 Nutritional fact of major ingredients in diets (g/kg)

	Ingredients		
Composition	**Fishmeal**	**Rapeseed meal**	**RM-Koji**
Crude protein	670	460	520
Crude lipid	82	20	14.4
Amino acid content			
Arginine	46.2	20.7	24.1
Histidine	25.8	14.6	15.9
Isoleucine	20.0	13.1	14.3
Leucine	46.8	25.5	36.1
Lysine	40.7	17.8	17.2
Methionine	17.8	6.3	9.1
Phenylalanine	31.8	21.2	15.5
Threonine	27.4	17.2	19.3
Valine	23.7	17.0	18.1

Soybean lecithin and Pollack liver oil were supplied in all diets as main lipid sources, wheat flour was used as nitrogen-free extract source, activated gluten was used as a binder to produce pellet diets, and cellulose powder was used to adjust to 100% total proportion. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 15 min; then, blended oil and water were added to form a soft dough. The dough was then pelleted (without steam injection) using a pellet mill with a (1.6–2.1 mm) diameter die (Dawood et al., 2017). Experimental feeds were finally dried at room temperature and stored in sealed plastic bags at −20°C until use.

2.2 | Fish and feeding trial

The experiment was conducted at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Red sea bream juveniles (4.5 ± 0.02 g) were obtained from a private local farm, acclimated to laboratory conditions for 1 week while fed a commercial diet (500g/kg crude protein; Higashimaru, Japan). At the beginning of the feeding trial, 25 fish per tank (three tanks per treatment) were randomly stocked into nine polycarbonate tanks with 100 L capacity (filled with 80 L of water) in a flow-through seawater system where each tank was equipped with an inlet, outlet and continuous aeration. For 56 days, fish were maintained under natural light/dark regime, fed by hand (twice daily) until apparent visual satiation level, and the main water quality parameters were as follows: temperature 25.3 ± 1.6°C, salinity 33.8 ± 0.5 g/L, dissolved oxygen

TABLE 2 Formulation and proximate composition of the experimental diets (g/kg)

	Test diets		
Ingredients (g/kg)	**RM0**	**RM50**	**FRM50**
Fishmeal[a]	570	285	285
Rapeseed meal[b]	0	285	0
RM-Koji[c]	0	140	285
Wheat flour	140	140	140
Soybean lecithin[d]	20	30	30
Polack liver oil[e]	20	30	30
Vitamin mix[f]	30	30	30
Mineral mix[g]	30	30	30
Amino mix[h]	3	8.6	9.3
Stay-C[i]	0.8	0.8	0.8
Activated gluten[j]	50	50	50
α-cellulose	136.2	110.6	109.9
Proximate composition			
Crude protein	494.6	497.7	492.9
Crude lipid	93.8	96.9	93.7
Gross energy (kJ/g)[k]	190.2	191.3	193.4

[a]Nippon Suisan Co. Ltd., Tokyo, Japan. [b]Rapeseed meal obtained from J-Oil Mills Inc., Japan. [c]Fermented rapeseed meal with Tane Koji (*Aspergillus Oryzae*, Bio'c company, Uchida, Japan). [d]Kanto Chemical Co., Inc. Tokyo, Japan. [e]Riken Vitamin, Tokyo, Japan. [f]Vitamin mixture (mg/kg diet): ß-carotene (0.10), vitamin D_3 (0.01), menadione $NaHSO_3{\cdot}3H_2O$ (K_3) (0.05), DL-α-tocopherol acetate (E) (0.38), thiamine-nitrate (B_1) (0.06), riboflavin (B_2) (0.19), pyridoxine-HCl (B_6) (0.05), cyanocobalamin (B_{12}) (0.0001), biotin (0.01), inositol (3.85), niacin (nicotinic acid) (0.77), Ca Pantothenate (0.27), folic acid (0.01), choline chloride (7.87), ρ-aminobenzoic acid (0.38) and cellulose (1.92). [g]Minerals' mixture (mg/kg diet): $MgSO_4$ (5.07), Na_2HPO_4 (3.23), K_2HPO_4 (8.87), Fe Citrate (1.1), Ca Lactate (12.09), Al $(OH)_3$ (0.01), $ZnSO_4$ (0.13), $MnSO_4$ (0.03), Ca $(IO_3)_2$ (0.01) and $CoSO_4$ (0.04). [h]Amino-mix: RM0 (L-lysine 0, Methionine_0, Betaine_0.3), RM50 (L-lysine 0.34, Methionine_0.22, Betaine_0.3), FRM50 (L-lysine 0.49, Methionine_0.14, Betaine_0.3). [i]Stay-C 35: L-Ascrobyl-2-phosphate-Mg. [j]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS". [k]Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

6.23 ± 0.3 mg/L and pH 7.5. These ranges were considered within optimal values for juvenile red sea bream (El Basuini, et al., 2016).

2.3 | Sample collection, blood and biochemical analysis

At the end of the feeding trial, all fish were fasted for 24 hr. The total number and individual body weight of fish from each tank were measured to calculate the survival, growth performance and feed utilization of fish fed the test diets according to the following formulae:

$$\text{Survival (\%)} = 100 \times (\text{final no. of fish}/\text{initial no. of fish})$$

$$\text{Weight gain (\%)} = (\text{final weight} - \text{initialweight}) \times 100/\text{initial weight}$$

$$\text{Specific growth rate (\%/day)} = \frac{\text{Ln(final weight)} - \text{Ln(initial weight)}}{\text{duration(56 days)}} \times 100$$

$$\text{Feed intake (g fish/56days)} = \frac{\text{dry diet given} - \text{dry remaining diet recovered}}{\text{no. of fish}}$$

$$\text{Feed efficiency ratio(FER)} = \frac{\text{live weight gain(g)}}{\text{dry feed intake(g)}}$$

$$\text{Protein efficiency ratio(PER)} = \frac{\text{live weight gain(g)}}{\text{dry protein intake(}g\text{)}}$$

Five fish per tank were randomly selected, and their blood was collected by puncture of the caudal vein using heparinized (1,600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes with a 26-gauge needle and pooled. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyse the haematocrit, haemoglobin and respiratory burst (NBT) levels while plasma and serum were obtained by centrifugation at 3,000 *g* for 15 min under 4°C and then stored at −80°C until the analysis.

Haematocrit was determined using the microhaematocrit technique, and haemoglobin concentration was measured using cyanmet-haemoglobin method (Haemoglobin B blood test kit, Wako Pure Chemical Industries, Japan) following the manufacturer's instructions. Total serum protein and plasma chemical parameters were measured spectrophotometrically with an automated analyser (SPOTCHEM TM EZ model SP-4430, Arkray, Inc. Kyoto, Japan).

Diets' composition was analysed in triplicate according to standard AOAC methods (AOAC, 1990). Moisture was analysed by oven-drying at 110°C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550°C for 4 hr.

2.4 | Immunological assays

A turbidimetric assay using lyophilized cells of *Micrococcus luteus* (Sigma, USA) was used to determine serum lysozyme activity (Lygren, Sveier, Hjeltnes, & Waagbø, 1999), serum bactericidal activity (BAT) was performed according to Yamamoto and Iida (1995), and the serum antitrypsin activity was measured following Ellis (1987). Oxidative radical production by neutrophils during respiratory burst was measured through the nitro blue tetrazolium assay as described by Kumari and Sahoo (2005). The total peroxidase activity in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum was diluted with 35 µl of Hank's buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3,30,5,50-tetramethylbenzidine

hydrochloride TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 2 min. The colour developing reaction in serum samples was stopped by adding 50 µl of 2 M sulphuric acid, and the OD (450 nm) was measured in a plate reader. HBSS was used as a blank instead of serum.

2.5 | Antioxidant potential

The malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation in fish serum and measured using Colorimetric TBARS Microplate Assay Kit (Oxford Biomedical Research, Inc., USA) according to the manufacturer's instructions. The absorbance was read at 532 nm, and the MDA level was expressed as nmol per ml serum. Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were measured spectrophotometrically at 505 nm from blood plasma with a dedicated analyser FRAS4, Diacron International s.r.l., Grosseto, Italy, by following the method described previously (Morganti et al., 2002). In the BAP test, the plasma samples were mixed with a coloured solution obtained by mixing a ferric chloride solution with a thiocyanate derivate solution that causes a discoloration, the intensity of which is measured photometrically and is proportional to the ability of the plasma to reduce ferric ion. For d-ROMs test, reactive oxygen metabolites (primarily hydroperoxides) of the sample generated alkoxyl and peroxyl radicals, in the presence of iron released from plasma proteins by an acidic buffer, following the Fenton reaction. Such radicals then oxidized an alkyl-substituted aromatic amine, thus producing a pink-coloured derivative, which is photometrically quantified.

2.6 | Statistical analysis

The normality of data and homogeneity of variances were confirmed by Kolmogorov–Smirnov test and Levene's test, respectively, before ANOVA analysis. All data were presented as means values ± standard error of mean (*SEM*, $n = 3$). Data were subjected to statistical verification using Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $p < 0.05$ were considered significant, and significant differences between means evaluated using Duncan's multiple range test.

3 | RESULTS

3.1 | Growth, feed utilization and survival

Growth, feed utilization and survival of red sea bream fed experimental diets are given in Table 3. After 56 days of feeding trial, groups fed with RM50 diet showed significantly reduced ($p < 0.05$) weight gain and specific growth rate when compared to those fed RM0 and FRM50 diets. Feed conversion efficiency and protein efficiency ratio followed the same trend, while on the contrary, a significant change was not detected for the same parameters in case of fish fed FRM50 diet, when compared to control. Feed intake and survival of test fish over the feeding period were unaffected by dietary treatments.

3.2 | Haematocrit, haemoglobin and blood chemical parameters

The haematocrit, haemoglobin and blood chemical parameters in red sea bream are presented in Table 4. When compared to control, no significant effects of the test diets were found on haematocrit, glucose, serum protein, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT) levels of test fish ($p > 0.05$). Replacing fishmeal by RM (RM50) significantly decreased ($p < 0.05$) haemoglobin and negatively affected ($p < 0.05$) triglyceride in test fish, whereas cholesterol was gradually improved with the inclusion of RM and RM-Koji, respectively, in fish diets.

3.3 | Humoral immune responses

At the end of the feeding trial, except of antiprotease activity, which did not change among treatments ($p > 0.05$; Table 5), all the immune parameters were significantly reduced in fish fed RM50 diet comparing to those fed FRM50 and control diets ($p < 0.05$). Oxidative radical production by neutrophils (NBT, Figure 1) and serum BAT

TABLE 3 Growth performance, feed utilization and survival in red sea bream fed test diets for 56 days

Parameters	Test diets		
	RM0	RM50	FRM50
Initial body weight (g)	4.50 ± 0.00	4.50 ± 0.00	4.51 ± 0.00
Final body weight (g)	19.51 ± 0.29^{b}	17.02 ± 0.41^{a}	18.94 ± 0.16^{b}
Weight gain (%)	333.66 ± 6.39^{b}	278.38 ± 9.36^{a}	320.05 ± 4.39^{b}
Specific growth rate (%/day)	2.62 ± 0.02^{b}	2.37 ± 0.04^{a}	2.56 ± 0.01^{b}
Feed intake (g fish/56 day)	13.22 ± 0.17	13.77 ± 0.26	13.29 ± 0.10
Feed efficiency ratio	1.13 ± 0.007^{b}	0.91 ± 0.01^{a}	1.08 ± 0.01^{b}
Protein efficiency ratio	2.45 ± 0.01^{b}	1.99 ± 0.10^{a}	2.38 ± 0.02^{b}
Survival (%)	81.33 ± 4.80	80 ± 2.3	84 ± 0.00

Notes. Data represent means ± *SEM* ($n = 3$). Values with different letters are significantly different ($p < 0.05$). Absence of letters indicates no significant difference between treatments.

TABLE 4 Haematocrit, haemoglobin and blood chemistry in red sea bream fed test diets for 56 days

	Test diets		
Parameters	**RM0**	**RM50**	**FRM50**
Haematocrit (%)	38.66 ± 1.20	36 ± 1.00	37.33 ± 1.20
Haemoglobin (mg/dl)	3.58 ± 0.11^{b}	2.46 ± 0.01^{a}	3.19 ± 0.13^{b}
Glucose (mg/dl)	49.5 ± 4.50	46.33 ± 4.09	46.33 ± 8.41
Serum protein (g/dl)	2.66 ± 0.24	2.56 ± 0.08	2.96 ± 0.32
Cholesterol (mg/dl)	276.33 ± 1.22^{c}	260.33 ± 3.42^{b}	239.50 ± 1.34^{a}
Triglyceride (mg/dl)	156 ± 3.00^{a}	244 ± 15.00^{b}	190 ± 3.00^{a}
GOT (UI/l)	150.66 ± 13.86	131.66 ± 16.22	98 ± 14.74
GPT (UI/l)	48.67 ± 5.23	28.50 ± 5.50	28.50 ± 4.50

Notes. Data represent means ± *SEM* (n = 3). Values with different letters are significantly different ($p < 0.05$). Absence of letters indicates no significant difference between treatments.
GOT: glutamic oxaloacetic transaminase; GPT: glutamic pyruvate transaminase.

TABLE 5 Antiprotease activity and antioxidant potential of red sea bream fed test diets for 56 days

	Test diets		
Parameters	**RM0**	**RM50**	**FRM50**
Antiprotease activity (%)	74.5 ± 0.15	73.66 ± 0.63	74 ± 0.25
MDA (nmol/ml)	1.52 ± 0.17^{b}	1.09 ± 0.14^{a}	1.06 ± 0.15^{a}
d-ROMs (μMol/L)	71 ± 1.00^{b}	50 ± 5.00^{a}	60 ± 3.00^{ab}
BAP (U.Carr)	3900.66 ± 297.32	3653.50 ± 156.5	3,815 ± 222

Notes. Data represent means ± *SEM* (n = 3). Values with different letters are significantly different ($p < 0.05$). Absence of letters indicates no significant difference between treatments.
BAP: biological antioxidant potential; d-ROMs: reactive oxygen metabolites; MDA: malondialdehyde concentration.

(Figure 2) were significantly higher ($p < 0.05$) in fish fed control diet comparing to those fed FRM50 diet. However, lysozyme (Figure 3) and peroxidase enzyme (Figure 4) levels in test fish were not significantly affected ($p > 0.05$) within these two groups.

3.4 | Antioxidant potential

Inclusion of RM and RM-Koji in diets induced significant changes in antioxidative status of red sea bream (Table 5). Indeed, MDA concentration and d-ROMs were significantly reduced in fish fed RM50 and FRM50 diets ($p < 0.05$) when compared to control. However, the overall BAP of test fish was not affected by any dietary treatment. Figure 5 shows the pattern of combined effects of d-ROMs and BAP. FRM50 group was in zone A reflexing moderate intensity of oxidative stress and higher tolerance ability against oxidative stress. Further, RM0 groups located in zone B reflexing higher d-ROMs values and higher BAP values. However, RM50 groups located in zone C reflexing lower d-ROMs values and lower BAP values.

4 | DISCUSSION

The nutritional value of rapeseed protein can be improved by microbial fermentation, thus providing an alternative ingredient for fishmeal substitution in aquafeed. Microbial fermented meals have already been reported to modulate the innate immune system, the antioxidative health status and general performance of fish (Ashida & Okimasu, 2005; Kim et al., 2009; Kim, Pham, Kim, Son, & Lee, 2010; Lee, Mohammadi, & Hoon, 2016). Our previous reports focused on graded replacement of fermented RMs in diets for red sea bream (Dossou, et al., 2018; Dossou, et al., 2018). Therefore, a comparative immunomodulatory effect of simple RM and RM-Koji along with their respective influence on growth, blood parameters and antioxidant enzyme activities was investigated in the present study.

The results of this trial showed that RM-Koji successfully replaced 50% fishmeal without compromising body weight gain and specific growth rate of juvenile red sea bream. Meanwhile, fish fed with RM50 recorded significantly lower body weight gain and specific growth rate when compared to RM0 and FRM50 fed groups ($p < 0.05$). Thus, confirming our previous report on the inclusion level of RM-Koji in diet for red sea bream (Dossou, et al., 2018) and supporting the assertion that the fermentation process might have improved the digestibility and nutritional efficiency of the RM. Indeed, increasing nutrient digestibility in various fermented meal has already been reported previously (Bartkiene, Krungleviciute, Juodeikiene, Vidmantiene, & Maknickiene, 2015; Koo, Kim, & Nyachoti, 2018; Lim et al., 2010). In addition, Kim et al. (2013) reported that *A. oryzae*-mediated fermentation improves

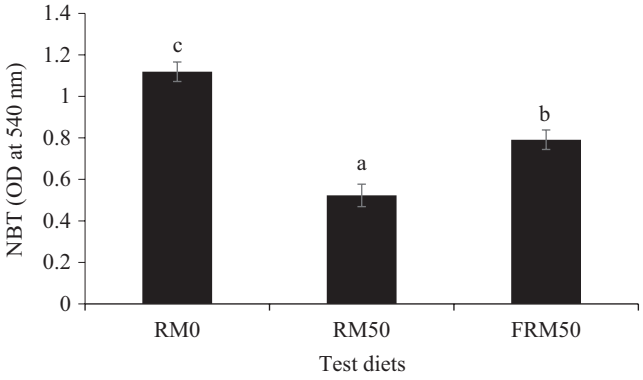

FIGURE 1 Respiratory burst activity in red sea bream fed test diets for 56 days. Data represent means ± *SEM* (n = 3). Values with different letters are significantly different (p < 0.05)

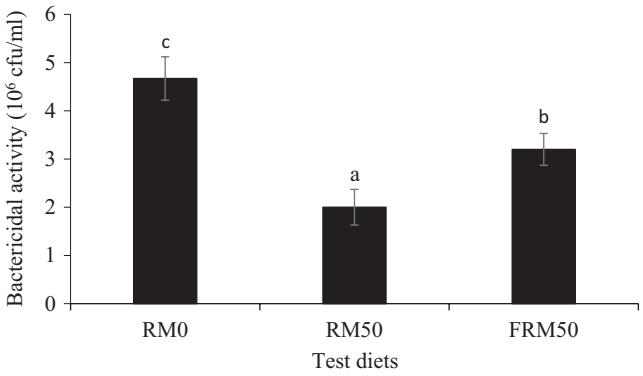

FIGURE 2 Bactericidal activity of red sea bream fed test diets for 56 days. Data represent means ± *SEM* (n = 3). Values with different letters are significantly different (p < 0.05)

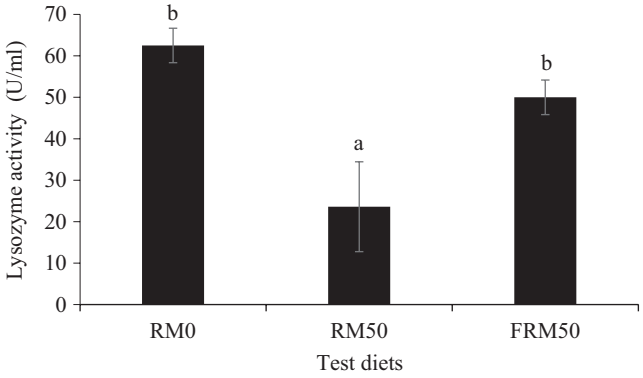

FIGURE 3 Lysozyme activity of red sea bream fed test diets for 56 days. Data represent means ± *SEM* (n = 3). Values with different letters are significantly different (p < 0.05)

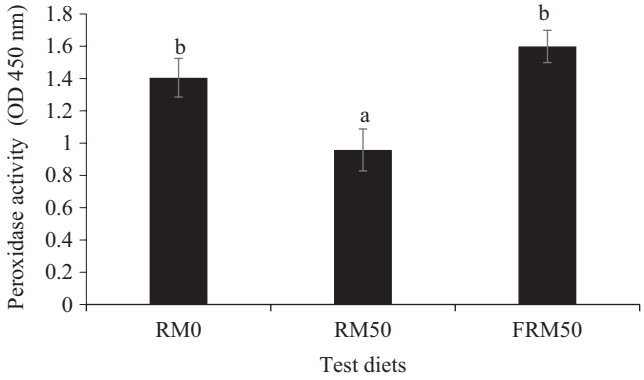

FIGURE 4 Peroxidase activity of red sea bream fed test diets for 56 days. Data represent means ± *SEM* (n = 3). Values with different letters are significantly different (p < 0.05)

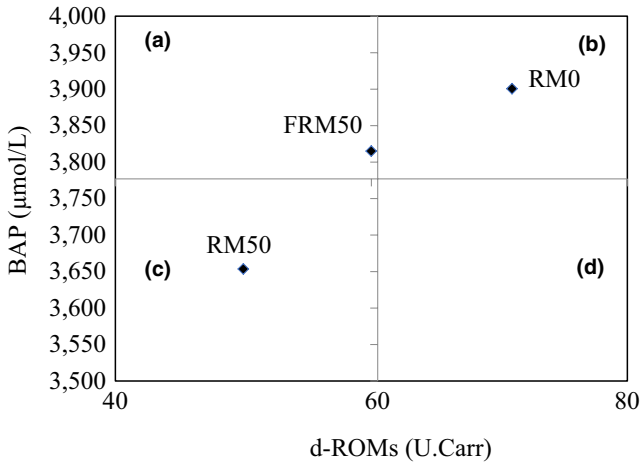

FIGURE 5 Oxidative status in red sea bream fed test diets for 56 days. Values are expressed as mean ± *SEM*. Central axis based on mean values of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) from each treatment. Zone (a) High BAP and low d-ROMs (good condition); Zone (b) High BAP and high d-ROMs (acceptable condition); Zone (c) Low BAP and low d-ROMs (acceptable condition); and Zone (d) Low BAP and high d-ROMs (stressed condition)

metabolites content by modifying their availability. Improvement of growth performance in broiler chicks fed with fermented RM-based diet was reported (Ashayerizadeh, Dastar, & Shams, 2017). However, changes in growth parameters in the present study could not be attributed to any improved palatability induced by the RM-Koji since feed intake was not affected among all treatments. This agrees with the findings of previous reports suggesting that replacing fishmeal by fermented cottonseed meal (Sun et al., 2015) or soybean meal (Zhou et al., 2011) may not affect the palatability and acceptability of the diets. Our previous report also showed similar trends (Dossou, et al., 2018) in opposition with cases reported by Kim et al. (2009) and Lee et al. (2016) where fermentation of soybean meal significantly improved diet palatability for juvenile parrot fish (*Oplegnathus fasciatus*) and rockfish (*Sebastes schlegeli*), respectively. In the present study, fish survival was not affected by the dietary treatment. However, almost 20% mortality occurred in control and test groups. This could be imputable to the stress due to the change in feed and experimental condition at the beginning of the trial since most of the mortality occurred during the first 2 week of the feeding trial.

As suggested by Faggio Fedele Arfuso Panzera and Fazio (2014), Fazio et al. (2012) and Fazio et al. (2013), blood parameters can be used to estimate the health status of fish. In addition, liver enzyme level can also help understanding liver function (Johnston, 1999). In the current study, no significant effects of the test diets were found on haematocrit, glucose, serum protein, GOT and GPT levels of test fish. However, replacing fishmeal by RM negatively reduced blood haemoglobin and significantly decreased serum triglyceride in test fish. Meanwhile, values recorded for fish fed the RM-Koji-based diet did not change significantly with those fed control diet. In rainbow trout fed rapeseed protein concentrate, Hermann, Reusch, and Hanel (2016) explained the decreasing blood haematology to iron deficiency. According to the authors, with the increasing level of antinutrients factors (ANFs) in diet and due to the iron-chelating effects of phytate, the reduced transcription rates may have induced alteration of haematocrit in fish. In the present study, ANFs in diets were not calculated but it is well known that the fermentation process can lower ANFs, especially phytate in ingredients (Gatlin et al., 2007; Greiner & Konietzny, 2006). Cholesterol was gradually improved with the inclusion of RM and RM-Koji, respectively, in fish diets. Hypocholesterolaemic effect of plant proteins is well documented in fish (Shafaeipour, Yavari, Falahatkar, Maremmazi, & Gorjipour, 2008; Sitjà-Bobadilla et al., 2005), and these observations are consistent with those of our previous studies (Dossou, et al., 2018; Dossou, et al., 2018).

Immunity in fish like that in all vertebrates plays a major role in protection against pathogens (Burgos-Aceves, Cohen, Smith, & Faggio, 2016; Kiron, 2012; Swain et al., 2007). For this purpose, fish rely on specific but also on non-specific mechanisms, which increase in stressed fish is important for resistance against many diseases. Several immunostimulants are being used as a promising alternative to antibiotics and have been reported to have a role in the control of diseases in aquaculture (Park & Jeong, 1996; Yan, Guo, Dawood, & Gao, 2017) but could not be used efficiently because of some factors such as high cost (Dawood, Koshio, Ishikawa, & Yokoyama, 2015; Dügenci, Arda, & Candan, 2003). The present study confirmed once more the efficacy of RM-Koji to maintained/enhance innate immune responses in fish. At the end of the feeding trial, except of antiprotease activity, which did not change among treatments, oxidative radical production by neutrophils (NBT), serum BAT, lysozyme and peroxidase enzyme activities were significantly reduced in fish fed the unfermented RM diet comparing to those fed control and RM-Koji diets. These findings joined our previously reported data (Dossou, et al., 2018) and are consistent with the study of Ashayerizadeh et al. (2017) where fermented RM has been effectively used to control salmonella contamination in broiler chicks. Several other reports have also suggested that fish immune responses could be elevated using microbial fermented soybean meal as fishmeal replacement (Kim et al., 2009, 2010; Kokou, Rigos, Henry, Kentouri, & Alexis, 2012; Lee et al., 2016). The antioxidant defence system is also associated with health and immune system in fish (Faggio, Pagano, Alampi, Vazzana, & Felice, 2016; Hoseinifar, Zoheiri, & Caipang, 2016; Martínez-Álvarez, Morales, & Sanz, 2005). Indeed, in juvenile crucian carp (*Carassius auratus gibelio*♀ × *Cyprinus carpio*♂) fed RM diets, Cai et al. (2013) have linked the weakening of fish immunity to the decrease in antioxidant potentials in fish. Surprisingly, we noticed an improvement of the oxidative status in fish fed both unfermented and fermented RM when compared to control. MDA concentration and d-ROMs in those groups were significantly reduced compared to control. This might explain partially why the antiprotease activity in test fish was not significantly affected. In addition, fungal fermentation of numerous food materials has been recently reported to improve their antioxidant benefits and enhancing the availability of potential bioactive peptides presents in the untreated meal (He et al., 2013; Kim et al., 2013; Lee et al., 2016). Accepting with Ding, Zhang, Ye, Du, and Kong (2015) that antioxidant enzymes are important indicators of animals' physical health and reaction in response to external stimuli, these observations confirm once again the functional role of rapeseed and fermented derivate in aquaculture and even human nutrition.

5 | CONCLUSION

The ability of RM-Koji, over simple RM, to replace fishmeal in practical fish diet formulations for red sea bream without compromising growth performance, health and innate immune status is supported by the findings of this study. Fish NBT, BAT, lysozyme and peroxidase activities were significantly increased in group fed RM-Koji diet in comparison with RM fed group. In addition, combining BAP and d-ROMS (Figure 5), fish fed RM-Koji showed better antioxidative status than those fed with control and RM. Considering the lower price of rapeseed products compared to fishmeal, together with the high value addition aspects of RM-Koji over RM, the obtained results revealed the biological and economic benefits of *A. oryzae*-mediated RM in red sea bream.

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