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ORIGINAL RESEARCH

Bioconversion of fish-wastes biomass into a fish meal alternate for European seabass (*Dicentrarchus labrax*) diets

Norhan E. Saleh . Elham A. Wassef . Nagi M. El-bermawy . Khouloud M. Barakat . Heba H. Abdel-Mohsen

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Abstract The effect of replacing fish meal (FM) in juvenile European seabass (*Dicentrarchus labrax*) diets with fish wastes (FW) and fermented FW (FFW) by marine fungus *Beauveria bassiana* was investigated. Seven diets were prepared: control (CTRL) and six diets containing either FW or FFW to substitute FM at 15, 30 and 45% (FW15/FFW15, FW30/FFW30 and FW45/FFW45 each in turn). Fish (initial weight Ca. 30 g) were reared for 90 days in 21 PVC tanks (3 m^3) and fed the experimental diets to apparent visual satiation. The recorded values for growth, feed utilization efficiency, survival and amino acids composition of fish fed either FW or FFW diets, at all tested levels, were not significantly different relative to those values of control group. Carcass protein content was not influenced by diet, but lipid content of FW45 and FFW45-fed fish showed a significant elevation relative to that of other fish groups. Blood parameters indicated an improvement in general health status and liver and kidney function biomarkers of FW-fed fish up to 30% FM replacement level, relative to those of CTRL and FFW-fish groups. Fish fed the FW-diets showed higher fungal and acid fermentative bacterial counts and lower count of *Vibrio* spp. and coliform in distal intestine, suggesting better gut health as compared to FFW-fed fish. These results indicate that 30% of dietary FM can be replaced with FW meal without adverse effects on fish growth performance, feed utilization, general health status or intestinal microbiome and fermentation with *B. bassiana* has some negative influence on gut health at 45% FM substitution level.

Keywords Growth . Amino acids . Blood parameters . Fermentation . Marine fungus *Beauveria bassiana*. Intestinal microbiome

Introduction

The expansion and development of the aquaculture industry is limited by the nutritional and health factors which affect fish growth performance, quality and yield. For carnivorous fish species, aqua feeds have been largely based upon fish meal (FM) as the major protein source. The competition for FM coincided with continuous depletion of natural fisheries increase the necessity for FM-replacement by alternative sources for the sustainable growth of the industry.

Fish wastes biomass/byproducts, which are normally discarded, can be considered as a promising novel protein source in the production of aqua-feeds. Fish wastes meal is less expensive than FM, readily available, considered to be suitable and has a stable supply for replacing FM in commercial aqua-feeds industries (Baeza-Ariño et al. 2016). In general, fish waste has proved to contain amino acids, fatty acids, vitamins and minerals (Caruso 2016). Successive prior studies reported that most of fish wastes-biomass

Norhan E. Saleh (✉), Elham A. Wassef, Heba H. Abdel-Mohsen
Fish Nutrition Laboratory, Aquaculture division, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt
e-mail: nor_raafat@yahoo.com

Khouloud M. Barakat
Microbiology Laboratory, marine environment division, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt

Nagi M. El-bermawy
Fish and Animal Production Dept., Faculty of Agriculture, Saba-Basha, Alexandria University, Alexandria, Egypt

represent a rich source of biofunctional materials such as polysaccharides, polyunsaturated fatty acids (PUFA), enzymes, collagen, gelatin, and bioactive peptides (Messina et al. 2013; Pangestuti and Kim 2017). However, the biochemical composition of fish waste biomass, i.e., levels of protein, ash and lipids, differ significantly between different source-species (Le Gouic et al. 2019).

Hou et al. (2017) reported that the addition of animal byproducts, modified through chemical, enzymatic or microbial hydrolysis of proteins, prior to feeding, is an attractive mean of generating high-quality peptides that have both nutritional and physiological or regulatory functions in fish. They also added that peptides of animal-source have shown antimicrobial, antioxidant, anti-hypertensive and immune-modulatory activities beyond their nutritional value. However, the biotechnological approaches are emerging to be a better option for fish wastes utilization than conventional methods that are not ecofriendly and lead to different pollution problems (Leduc et al. 2018). A number of possibilities for bioconversion of fish- or seafood-wastes have been tried. Prior research with European seabass, have evaluated different animal protein sources as dietary FM-replacers such as; fermented prawn waste liquor for juveniles at 30% of dietary FM (Nor et al. 2011), and shrimp- and tilapia-based protein hydrolysates associated with a combination of poultry byproducts meal at 15% of FM (Robert 2014). For other marine fish species, tuna-muscles byproduct powder replaced 50% meanwhile, whole tuna byproducts replaced 30% of dietary FM without reduction in growth performance of olive flounder (*Paralichthys olivaceus*) (Uyan et al. 2006; Kim et al. 2014). Recently, Muttharasi et al. (2019) suggested that *Rastrelliger kanagurta, Sphyraena barracuda* and *Fenneropenaeus indicus* waste meals can be used as complete alternatives for FM to produce low-cost feeds for aqacultured carp, *Cyprinus carpio*.

The major goal of the present study was to evaluate the bioconversion process of fish-wastes biomass to a novel proteinous feed ingredient, by using the marine fungus *Beauveria bassiana* for the first time, to effectively retain the valuable bio-molecules of the wastes generated from fish processing. This fungus is a potent producer of chitinase (Suresh and Chandrasekaran 1998) and also considered as a source of protease and L-glutaminase enzymes (Sabu et al. 2005). The present work aimed to validate the utilization of fish wastes-biomass, in two forms (non-fermented fish waste, FW and fermented fish waste, FFW meals by marine *B. bassiana*), each as a FM-substitute at 15, 30 and 45% levels, in juvenile European seabass (*D. labrax*) diets. This research further emphasized and compared the effects of these two FM-alternates (FW and FFW) on fish performance, biochemical composition, general health status and intestinal microbiome.

Materials and methods

Preparation of fish-waste biomass meals

The fish-wastes biomass, used in this study, was the by-products of fresh Nile tilapia (*Oreochromus niloticus*) after processing, which included in the majority fish viscera. Such fish-waste biomass was collected fresh, as an offal and immediately transported to the laboratory (in ice box) and subjected to a steam-cooking process for 20 minutes (Maghaydah 2003) to eliminate potential pathogens and reduce excess fats. After drying in an oven at 60°C, the resulted fish wastes were homogenized and sieved to form the fish waste meal. The net weight of dried fish by-product biomass was approximately 1/5 of the fresh one.

Fungus isolation and purification

The fungus *Beauveria bassiana* was isolated from the marine sediment of Abu-Qir Bay, Alexandria, Egypt, and was chosen due to its ability to form clear lytic zones around colony on a colloidal chitin agar medium (Suresh 1996), which used for the isolation of chitinoclastic marine fungi. Chitin agar medium contained: 1g colloidal chitin, 2g agar and 100 ml of 50% aged sea water, pH 7.5. Penicillin G (0.5 mg) and streptomycin sulphate (0.5 mg) per plate were added after autoclaving. The isolate was purified repeatedly on chitin agar medium at 28°C for 7 days of growth and preserved on slants at 4°C. For inoculum preparation, 10 ml of sterile sea water was added to the 2-weeks-old hyphal agar slant. The spores were suspended by means of a sterile loop and adjusted to $1x10^6$ spores/ml suspensions to be used as the inoculums for solid state fermentation (SSF).

Solid state fermentation (SSF) culture

It was carried out using SSF medium containing 5g of solid substrate (dried fish waste meal) mixed with sea water to a solid/liquid ratio of 5:2 (w/v) and autoclaved at 121°C for 20 min (Suresh and Chandrasekaran, 1998). Solid state fermentation (SSF) medium was inoculated with 2 ml of the prepared *B. bassiana* inoculum and incubated at 27°C for 7 days at 90% relative humidity. After fermentation, the product was filtered, dried (60°C) and then re-grounded, to produce the final fermented fish wastes meal (FFW) to be tested as a FM-substitute.

Nutritional evaluation of the two fish-waste meals forms

The fish meal (FM) and the two fish-waste forms, namely non-fermented (FW) and fermented (FFW) meals were analyzed to determine their major nutrients composition and amino acids content (Table 1).

Experimental fish and facilities

One thousand two hundreds (hatchery bred) juveniles seabass with an average initial body weight of 29.0±1.0 g were transported to the Marine Fish Hatchery, El-Anfoushy (NIOF) and fed on a commercial diet (45 % CP and 17% lipids, Aller aqua, Egypt) for two weeks acclimatization period. At the beginning of

Table 1 Proximate composition (% DM) and amino acid profile (g/100 g protein) of the test ingredients

Proximate composition	Experimental ingredients		
	FM[1]	FW[2]	FFW[3]
Crude protein (CP)	65.0	40.0	41.0
Crude lipids (L)	12.0	20.5	19.0
Ash	12.0	25.0	25.2
Nitrogen Free Extract (NFE)[4]	11.0	15.0	15.6
Essential AA			
Arginine	6.73	6.55	8.34
Histidine	1.78	3.01	2.12
Isoleucine	3.42	4.90	4.02
Leucine	6.23	6.87	7.25
Lysine	5.35	7.98	6.42
Methionine	2.75	2.44	2.00
Phenylalanine	3.78	4.22	4.73
Threonine	4.67	4.14	4.11
Tryptophan	0.60	0.66	3.75
Valine	5.32	4.76	4.93
Non-essential AA			
Alanine	9.14	9.11	10.19
Aspartic acid	9.08	9.04	9.87
Cysteine	1.14	1.32	0.78
Glycine	12.26	11.75	12.02
Glutamic acid	7.54	11.19	11.26
Proline	2.10	7.50	6.99
Serine	2.89	3.36	5.00
Tyrosine	3.05	0.85	3.89

[1] Fish meal (Lab. Made); [2] Fish wastes meal; [3] Fermented fish wastes meal; [4] NFE Calculated by difference

Table 2 Composition (g/kg) and proximate analysis (%DM) of the experimental diets

Ingredient	CTRL	Experimental diets					
		FW15	FW30	FW45	FFW15	FFW30	FFW45
Fish meal (FM) [1]	550	468	385	303	468	385	303
Fish waste meal [2]	-	82	165	247	82	165	247
Soybean meal (SBM) [3]	150	150	150	150	150	150	150
Corn gluten[4]	110	140	160	200	140	160	200
Wheat flour	20	20	20	20	20	20	20
Wheat bran	50	50	50	30	50	50	30
Fish oil (FO)[5]	90	60	40	20	60	40	20
Vitamins & minerals mix[6]	30	30	30	30	30	30	30
Proximate analysis (% DM)							
Crud protein	47.83	47.86	47.80	47.74	48.11	48.00	47. 45
Lipids	16.75	16.90	17.00	17.50	16.17	17.20	16.48
Ash	14.77	14.27	13.57	13.23	13.94	13.10	14.53
Crude fiber	2.99	2.51	2.06	2. 42	2.01	2.15	2.18
Nitrogen Free Extract (NFE)[7]	16.66	19.86	18. 57	18.49	20.77	21.55	21.06
Gross Energy (MJ /Kg) [8]	20.92	20.93	21.34	21.45	21.13	21.47	22.11

[1] 999 LT Denmark (70% CP)
[2] Processed fish wastes meals: (non-fermented, FW) or fermented (FFW)
[3]local product, Alexandria Company
[4] 65% protein
[5]FO, Iceland SR, produced from fresh capelin (*Mallotusvillosus*), herring (*Clupeaharengus*) + and/or blue whiting (*Micromesistius poutassou*)
[6] local vitamin & mineral premix; (AGRE–VET, Co,) Each 1 kg contains: Vit A (12,000000 IU.), Vit D (2500000 IU), Vit E (10000 mg) Vit K3 (500 mg) Vit B1 (1000 mg), Vit B2 (5000 mg), Vit B6 (1500 mg), Vit B12 (50 mg), Biotin (150), Folic acid (1000 mg), Pantothenic acid (10000 mg), nicotinic acid (30000 mg). Magnesium (60000 mg), Copper (4000 mg), Iron (30000 mg), Zinc (4000 mg), Cobalt (200 mg), Iodine (300 mg).
[7] NFE: calculated by difference: 100- (crude protein + lipids+ crude fiber + ash).
[8] GE, calculated on the basis of 23.6, 39.4 and 17.2 kJ /g for protein, lipids and carbohydrate respectively (NRC, 2011).

the experiment, 5 fish were sacrificed for initial biochemical analysis. Fish were distributed (50 fish /tank) into 21 PVC cylindrical tanks (3 m^3 each), replicated three times and fed the pelleted experimental diets 3 times a day (9.00 -13.00-16.00) to apparent visual satiety, 7 days a week for 90 days. Accumulated solids at tank-bottom were siphoned out daily in the morning before first feeding. Tanks were supplied with a continuous flow of filtered and UV-treated fresh seawater and the total volume of water was renewed daily. Salinity was 38‰ (Portable Refract Meter, model GG-201/211), temperature maintained at 24.0± 1.3°C, pH was 7.28±0.28, dissolved oxygen was 7.4±0.85 mg/l and ambient light regime was 13h light: 11h dark.

Diet preparation

Diet ingredients were all purchased from the local market except for fish waste meal. All experimental diets are produced in Fish Nutrition Laboratory, NIOF, Alexandria as previously described by Abdel-Mohsen et al. (2018). Seven diets were prepared: namely control diet (CTRL) and six diets containing either non-fermented fish-wastes meal (FW) or fermented fish-wastes meal (FFW) each as a partial substitute of FM at 3 levels: 15, 30 and 45%, designated as FW15/FFW15, FW30/FFW30 and FW45/FFW45 each in turn. The diets were iso-nitrogenous (~ 48% CP), iso-lipidic (~17% L) and iso-energetic (~21 MJ/kg) and were formulated to fulfill the nutritional requirements of the species (NRC 2011). The biochemical and amino acids composition of the experimental diets are illustrated in Tables 2 and 3 respectively.

Data collection

At end of the feeding trial, 24h after last meal, fish of each tank were bulk–weighed and counted to monitor growth and survival rate. Five fish from each tank were randomly sampled, pooled for final whole body composition analysis. Ten fish were taken from each tank at random and slightly anesthetized with clove oil

Table 3 Amino acids composition (g/100 g protein) of experimental diets

Amino Acids (AA)	Experimental diets						
	CTRL	FW15	FW30	FW45	FFW15	FFW30	FFW45
Essential AA							
Arginine	6.12	6.21	6.26	6.13	6.23	6.60	6.01
Lysine	8.62	8.36	8.12	8.30	8.60	8.30	7.40
Histidine	2.63	2.96	2.28	2.20	2.78	2.82	2.84
Isoleucine	4.62	4.50	4.41	4.35	4.46	4.50	4.90
Leucine	7.12	6.12	7.31	7.01	7.40	7.10	7.32
Methionine	2.55	2.70	2.84	2.88	2.66	2.74	2.90
Phenylalanine	4.12	4.33	4.40	3.92	4.32	3.25	3.56
Threonine	4.22	4.52	4.36	4.60	4.50	4.46	4.42
Tryptophan	0.55	0.74	0.69	0.68	0.73	0.72	0.79
Valine	4.75	5.10	5.01	4.83	4.72	4.73	4.40
Non-Essential AA							
Alanine	6.32	6.10	6.63	5.86	5.40	5.42	5.06
Aspartic acid	9.30	9.30	10.30	9.70	7.16	8.26	8.21
Cysteine	0.80	0.70	0.82	0.93	8.70	9.10	8.88
Glycine	7.01	7.10	7.40	7.41	6.04	6.56	7.01
Glutamic acid	15.04	14.50	15.01	15.30	14.01	13.10	13.01
Proline	4.31	4.36	4.50	4.46	4.16	4.12	4.02
Serine	4.90	4.60	4.58	4.50	4.40	4.01	4.16
Tyrosine	3.35	3.32	3.01	3.36	3.01	3.14	3.02

(20 mg/L) (Mylonas et al. 2005) for 3 min and blood samples were immediately obtained, from caudal vein, for haematological analyses. After blood sampling, fish were individually weighted and total length (cm) was measured, then killed by sudden icing and dissected to remove liver and viscera which was weighed separately for fish biometry records. All applicable institutional guidelines for the care and use of animals were followed by the authors.

Analytical procedures

Blood and serum assays

Blood was sampled with heparinized syringes, collected into micro-tubes for each tank and the haematological profiles of fish were measured immediately. Major blood constituents of each blood sample were determined (by a Fully Automatic Blood Cell Counter, model PCE-210 N, Erma, Inc. India). Red blood cells count (RBC, 10^6/µl), haemoglobin concentration (Hb, g/dl), haematocrit value (PCV, %), mean corpuscular volume (MCV, fl), mean corpuscular haemoglobin (MCH, pg), MCH concentration (MCHC, g/dl) and white blood cells count (WBC, 10^3/µl) were measured. The rest of blood specimens, which were sampled with non-heparinized syringes, are left to clot at 4°C and the coagulated blood samples are centrifuged at 4000 g for 10 min and then the obtainable sera are stored at–80°C for serum biomarkers assay. The following biomarkers were determined; aspartate amino transferase (AST) and alanine amino transferase (ALT) according to Reitman and Frankel (1957), lipid profile: cholesterol (Atamanalp et al. 2003), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) by using kits (BioMed-Cholesterol, Chem. for Lab Technology, Cairo, Egypt) and serum triglyceride (mg/dL) by the colorimetric method (Chawla 2003). Serum creatinine was measured according to the method of Henry (1974) by using

kits (Pasteur Lab, France). Total protease activity was assayed according to the procedure of Anson (1938), using casein (Sigma-Aldrich, Shanghai, China) as a substrate which reacts with Folin reagent. Lipase activity was determined adopting the method of Mckellar and Cholette (1986) as modified by Versaw et al. (1989) and application of β-naphthyl caprylate (Sigma-Aldrich, Shanghai, China) as a substrate.

Growth performance, feed utilization and fish biometry

At end of the feeding trial, growth and feed utilization indices were calculated using the following indicators: weight gain (WG, g / fish) = final body weight (FBW, g) – initial body weight (IBW, g); average daily gain (ADG, g / fish / d) = WG / t; specific growth rate (SGR, % /d) = 100 (ln FBW – ln IBW) / t where "t" is the number of feeding trial days. Feeding rate (FR, %BW / d) = 100 x (feed intake / (IBW + FBW) / 2 / t); feed conversion ratio (FCR) = dry feed consumed (g) / WG; protein efficiency ratio (PER) = (WG) / (protein intake, g). Protein productive value (PPV) = 100 (protein gain, g) / protein fed (g). The biometric indices were calculated as follows: hepatosomatic index (HSI, %) = 100 x liver weight (g) /body weight (g); viscerosomatic index (VSI, %) = 100 x viscera weight (g) /body weight (g) and Fulton condition factor (K) = 100 (body weight (g) /total length^3 (cm)).

Proximate composition

Proximate composition of feed ingredients, diets and fish were all determined according to the standard methodology of AOAC (2005).

Determination of amino acids (AA)

Fish meal, fish-waste meals in the two forms (FW and FFW), experimental diets and fish samples from each treatment were hydrolyzed using the methane disulfonic acid method (Simpson et al.1976) and the high-performance liquid chromatography (HPLC, Shimadzu Model LC-10AT, Japan) analysis was carried out to determine quantitative individual amino acids.

Statistical analysis

Data are presented as means ± SE, standard error (n=3 per treatment). Data were subjected to the Shapiro–Wilk test and univariate procedure, and the results indicated that the data were distributed normally (Shapiro–Wilk test, WC 0.90). Differences between dietary groups for any given parameter are analyzed by two-ways analysis of variance (ANOVA) followed by Duncan's multiple range tests to separate the means. Statistical significance between means was tested at the 0.05 probability level. All statistical tests were performed using the Standard Version of SAS (2004) software package for Windows.

Results

At end of the experiment, replacement of FM by either FW or FFW, at all replacement levels, did not significantly (P>0.05) affect growth or feed utilization criteria (Table 4). Weight gain (WG), average daily gain (ADG) and specific growth rate (SGR) were all comparable to those of CTRL fish. However on absolute terms, the highest WG and SGR values were recorded in fish fed FW30 diet, meanwhile the lowest among all were those for FFW45-fed fish. No significant variations (P>0.05) in feed intake (FI) among dietary groups indicating that fish almost equally consumed the tested diets. Similarly, diet did not significantly influence neither protein efficiency ratio (PER) nor protein productive value (PPV) of fish. Survival ranged between 98 and 100% with no pronounced variations among treatments. Condition factor (K), hepatosomatic index (HSI) and viscerosomatic index (VSI) values are given in Table (4) and results revealed that the values of both HSI and VSI tend to increase numerically with the increase of FM-replacement level in both fish groups.

Table 4 Growth, feed utilization and biometric indices (mean± SE) of European sea bass (*D. labrax*) fed the experimental diets

Performance indices	Experimental fish groups						
	CTRL	FW15	FW30	FW45	FFW15	FFW30	FFW45
IBW (g)	29.83±0.81	29.27±0.29	30.40±0.01	29.78±1.41	29.34±0.09	27.87±0.52	28.61±0.46
WG (g)	22.58±3.18	23.08±2.94	24.95±0.34	23.11±0.28	24.20±0.52	23.88±4.16	20.52±0.93
ADG (g/fish/d)	0.25±0.53	0.26±0.34	0.28±0.10	0.26±0.09	0.27±0.05	0.27±0.54	0.23±0.15
SGR (% /d)	3.45±0.16	3.48±0.14	3.67±0.15	3.49±0.01	3.54±0.00	3.51±0.20	3.36±0.05
FR (%BW / d)	34.89±0.00	33.33±1.7	33.40±2.44	36.12±1.34	35.44±2.53	34.84±278	35.04±2.55
FCR	1.95±0.59	1.69±0.15	1.36±0.01	1.75±0.06	1.64±0.22	1.63±0.05	1.75±0.05
PER	1.31±0.22	1.49±0.21	1.69±0.07	1.28±0.03	1.38±0.11	1.38±0.09	1.19±0.03
PPV (%)	25.25±1.59	23.14±1.40	25.73±2.17	24.89±0.32	23.38±1.33	23.60±1.5	22.70±1.0
S (%)	100.0 ±0.21	99.02±0.14	99.02±0.14	99.02±0.14	98.12±0.47	100.0 ±0.21	99.02±0.14
K	1.22±0.18	1.23±0.11	1.17±0.09	1.17±0.11	1.28±0.10	1.42±0.16	1.39±0.18
HSI	1.88±0.14^{b}	1.91±0.17^{b}	2.31±0.13^{ab}	2.68±0.35^{a}	2.11±0.41^{ab}	2.22±0.10^{ab}	2.89±0.51^{a}
VSI	8.49±0.28^{c}	8.56±0.34^{c}	10.11±0.57^{ab}	10.66±0.14^{ab}	10.10±1.07^{ab}	9.77±0.46^{b}	11.73±1.13^{a}

Means in the same row with different superscripts are significantly different (P <0 .05).

Table 5 Fish biochemical composition (% wet weight, mean± SE) of European sea bass (*D. labrax*) fed the experimental diets

Parameter (%)	Dietary fish groups						
	CTRL	FW15	FW30	FW45	FFW15	FFW30	FFW45
Protein	17.27±0.16	17.83±0.05	18.14±1.04	18.46±0.30	19.06±0.19	17.37±0.39	17.44±0.31
Lipid	6.09±0.47^{bc}	5.83±0.95^{c}	6.40±0.12^{b}	7.09±1.08^{a}	6.57±0.11^{b}	6.85±0.75^{ab}	7.37±0.58^{a}
Ash	4.46±0.09	4.15±0.45	4.32±0.15	4.32±0.35	4.48±0.23	4.48±0.11	4.03±0.07
Moisture	71.04±0.19	71.86±0.35	70.53±0.2	69.79±0.17	70.23±0.2	71.49±0.08	70.95±0.13

Means in the same row with different superscripts are significantly different (P <0 .05).

Fish whole body proximate analysis, at the end of the feeding trail, is given in Table (5), and showed no significant effect of FM replacement on protein content among dietary groups. However, fish fed FW45 or FFW45 have significantly higher (P<0.05) lipid content than those fish fed the other diets.

The amino acids (AA) composition of seabass fed the provided diets at the end of the feeding trial showed slight significant alterations (P<0.05) among dietary treatments, only among the essential amino acid (EAA) tryptophan increased significantly in FW-fed fish relative to that of other diets-fed fish (Table 6).

The major blood constituents of seabass provided the experimental diets are illustrated in Table (7). Hemoglobin (Hb), haematocrit (PCV), red blood cells (RBC) and white blood cells (WBC) count in fish fed the FW-containing diets, at all inclusion levels, are significantly higher than the corresponding for fish fed either CTRL or FFW diets. As for white blood cells differentiation, fish fed the FW diets have higher lymphocytes and monocytes count relative to fish fed the FFW diets at all inclusion levels. Oppositely, basophils, eosinophils and neutrophils count is relatively higher in fish fed the FFW diets than those fed the FW diets.

Serum biomarkers analysis (Table 8) showed that aspartate amino transferase (AST) and alanine amino transferase (ALT) enzymes concentration in FW-fed fish were lower, at all inclusion levels (P<0.05), than in fish fed the other diets. The present results also illustrate that total cholesterol, low-density lipoproteins (LDL) and triglycerides levels in fish fed the FW15 diet are the lowest among all dietary groups (P<0.05). Serum creatinine, as an indicator for kidney function, showed significant variations among all dietary treatments. The highest creatinine level was recorded in fish fed the FFW15 diet and the least value was that of FW15-fed fish. Additionally, analyses of fish serum illustrate the priority of FW15 fish in lipase and protease activities comparing with fish of the other dietary groups and these activities were reduced to reach the lowest values in fish fed FFW15 diet.

The total count of the distal intestine microbial load, *Vibrio* spp., fecal coliform, total fungal and acid fermentative bacterial count of fish fed the experimental diets, are all demonstrated in (Table 9). Results indicated significant diet-induced changes in seabass microbiome composition. FW-fed fish, at all substitution levels, showed higher fungal and acid fermentative bacteria count and lower count of

Table 6 Fish amino acids composition (g/100 g protein) of European sea bass (*D. labrax*) fed the experimental diets

Amino Acids (AA)	Dietary groups						
	CTRL	FW15	FW30	FW45	FFW15	FFW30	FFW45
Essential AA							
Arginine	7.47±0.81	6.21±1.03	6.36±1.22	7.25±2.33	7.33±0.08	7.60±1.20	7.58±1.31
Lysine	9.31±2.09	8.36±2.25	8.12±1.77	8.87±2.21	8.60±3.01	8.30±1.97	9.05±2.12
Histidine	2.15±0.14	2.86±0.41	2.38±0.11	2.16±0.33	2.78±0.10	2.82±0.17	2.54±0.12
Isoleucine	4.80±1.01	4.50±1.44	4.41±0.37	5.03±2.79	4.46±1.22	4.50±1.24	4.79±2.11
Leucine	7.99±1.01	7.12±1.14	7.31±2.17	8.03±3.19	7.40±1.22	7.1±2.36	8.16±2.63
Methionine	2.73±0.57	2.50±0.71	2.84±0.13	2.88±0.21	2.66±0.36	2.74±0.17	2.90±0.71
Phenylalanine	4.27±0.11	4.13±0.91	4.40±0.72	4.46±0.36	4.32±0.11	4.25±0.14	5.14±0.91
Threonine	5.20±1.41	4.32±1.17	4.36±1.23	5.39±0.97	4.50±1.30	4.46±2.37	5.22±1.21
Tryptophan	3.84±1.01^{ab}	3.54±0.22^{b}	4.69±0.13^{a}	4.73±0.33^{a}	3.83±0.51^{ab}	3.72±0.14^{ab}	4.16±0.33^{a}
Valine	5.79±2.04	4.90±1.11	5.04±1.15	4.89±2.24	4.72±1.11	4.73±1.19	5.71±1.14
Non-Essential AA							
Alanine	8.85±0.01	8.10±0.32	8.63±0.01	8.74±0.12	8.61±0.25	8.32±0.14	8.59±0.32
Aspartic acid	10.79±2.25	9.40±1.11	10.30±3.07	10.86±2.21	9.56±2.41	10.96±2.31	10.27±3.14
Glutamic acid	14.83±3.11^{ab}	13.50±4.25^{b}	14.0±2.32^{ab}	15.29±4.36^{a}	14.40±2.32^{ab}	13.80±3.14^{b}	15.18±4.21^{a}
Tyrosine	3.15±0.33	3.32±0.32	3.16±0.14	3.36±0.21	3.41±0.21	3.34±0.36	3.32±0.32
Glycine	9.09±2.37^{a}	7.10±1.14^{b}	7.40±1.32^{b}	8.46±1.24^{ab}	7.14±0.87^{b}	7.06±2.18^{b}	7.12±1.71^{b}
Proline	4.11±1.14	4.36±0.94	4.50±1.33	4.46±0.94	4.66±1.25	4.52±1.14	4.60±1.36
Serine	4.63±1.33	4.40±0.32	4.48±1.12	4.84±1.04	4.80±0.92	4.70±1.17	4.79±0.21
Cysteine	0.60±0.17	0.7±0.31	0.82±0.12	0.83±0.18	0.76±0.35	0.80.36	0.97±0.18

Means in the same row with different superscripts are significantly different (P <0 .05).

Table 7 Fish complete blood count analysis of European sea bass (*D. labrax*) fed the experimental diets

Parameter	Dietary fish groups						
	CTRL	FW 15	FW 30	FW45	FFW 15	FFW 30	FFW 45
RBCs ($\times10^6$/L)	2.87±0.04^{bc}	3.90±0.03^{a}	3.60±0.01^{a}	3.32±0.05^{a}	2.68±0.05^{b}	3.15±0.03^{a}	2.32±0.03^{c}
Hb (g/dL)	9.25±0.02^{b}	13.94± 0.06^{a}	13.67±0.05^{a}	11.81±0.08^{b}	8.43±0.04^{c}	10.36±0.03^{b}	8.86±0.04^{c}
PCV (%)	22.40±0.03^{c}	30.18±2.04^{a}	28.41±3.04^{a}	26.29±1.09^{b}	20.26±2.03^{c}	24.48±1.04^{b}	19.05±1.17^{d}
WBCs ($\times10^3$/L)	1.63±0.04^{c}	3.20±0.01^{a}	2.76±0.47^{b}	2.24±0.36^{b}	1.58 ±0.04^{c}	1.79±0.06^{c}	1.36±0.22^{c}
Lymphocytes (%)	53.50±0.50^{c}	68.00±1.01^{a}	65.00 ±1.0^{a}	60.50±0.50^{b}	53.00±1.0^{c}	56.50±0.5^{b}	54.50±0.5^{bc}
Monocytes (%)	2.00±0.00^{c}	4.00±0.00^{a}	4.00±0.00^{a}	3.00±0.0^{b}	1.50±0.50^{d}	2.50±0.50^{bc}	2.00±0.0^{c}
Basophils (%)	12.00±0.0^{b}	9.50±0.50^{c}	10.00±0.0^{c}	13.50±0.50^{b}	16.50±0.00^{a}	14.0±0.0^{b}	17.00±00^{a}
Eosinophils (%)	12.50±0.50^{b}	9.50±0.50^{c}	11.50±0.50^{b}	13.50±0.50^{ab}	15.50±0.50^{a}	12.50±0.5^{b}	15.00± 1.0^{a}
Neutrophils (%)	20.00±1.00^{a}	9.00±1.00^{c}	9.50±0.50^{c}	10.50±1.50^{bc}	13.50±3.50^{bc}	14.50±0.50^{ab}	11.50±1.50^{bc}

Means in the same row with different superscripts are significantly different (P <0 .05).

Table 8 Serum biomarkers analysis (Mean ± SE) in European sea bass *(*D*. labrax*) fed the experimental diets

parameter	Dietary fish groups						
	CTRL	FW15	FW30	FW45	FFW15	FFW 30	FFW45
AST (U/l)	32.75±0.52^{ab}	20.94±0.4^{d}	23.36±0.87^{d}	29.04±0.43^{c}	35.29±1.10^{a}	30.9±0.63^{bc}	34.29±1.02^{a}
ALT (U/l)	25.29±0.84^{a}	15.77±0.51^{c}	19.7±0.43^{bc}	22.3±1.12^{b}	25.86±0.57^{a}	24.22± 1.10^{a}	26.61±0.33^{a}
Creatinine (mg/dl)	0.49± 0.02^{c}	0.31±0.0^{d}	0.36±0.01^{d}	0.43±0.00^{c}	0.70±0.01^{a}	0.44±0.01^{c}	0.59±0.03^{b}
Total cholesterol (mg/dl)	254.81±5.34^{b}	213.32±6.05^{d}	223.94±1.2^{c}	228.49±5.77^{c}	278.91±3.73^{a}	239.76±2.17^{c}	265.30±1.66^{a}
HDL (mg/dl)	45.08±0.85^{b}	60.68±0.94^{a}	55.34±0.43^{a}	48.13±0.44^{b}	27.95±0.69^{d}	36.19±0.28^{c}	32.56±1.28^{cd}
LDL (mg/dl)	21.55±1.28^{b}	14.84±0.55^{d}	17.04±0.45^{c}	18.94±0.67^{c}	30.53±1.04^{a}	20.05±0.91^{b}	24.94±0.33^{b}
Triglycerides (mg/dl)	132.20±0.99^{b}	91.23±2.06^{d}	103.02±0.46^{d}	118.51±0.73^{cd}	153.45±4.18^{a}	125.99±3.48^{c}	138.67±2.56^{b}
Lipase (U/mg)	2.35±0.08^{c}	2.79±0.01^{a}	2.53±0.04^{b}	2.29±0.02^{c}	1.51±0.02^{e}	2.56±0.05^{b}	1.89±0.03^{d}
Protease (U/mg)	4.40±0.11^{c}	6.81±0.12^{a}	5.35±0.01^{b}	4.79±0.02^{c}	2.72±0.03^{d}	4.58±0.03^{c}	3.19±0.03^{cd}

Means in the same row with different superscripts are significantly different (P <0 .05).

Table 9 Distal intestinal microbial count (mean ±SE) of European sea bass *(D. labrax*) fed fish experimental diets

Microbial count (CFU/ml)	Dietary groups						
	CTRL	FW15	FW30	FW45	FFW15	FFW30	FFW45
Total count	25.00±2.65^{bc}	16.67±3.711^{c}	20.33±0.88^{c}	41.00±3.78^{a}	32.00±3.51^{ab}	35.00±2.89^{ab}	38.67±2.33^{ab}
Vibrio spp.	17.00±3.79	16.00±3.06	20.00±3.61	22.33±6.36	25.33±3.84	44.67±5.17	46.00±4.93
Fecal coliform	8.00±0.58	6.00±1.53	6.33±1.20	18.33±2.03	8.33±0.33	10.00±0.57	19.67±2.91
Fungal count	32.33±5.36^{b}	27.67±3.53^{b}	100.00±0.58^{a}	16.00±3.21^{c}	8.00±1.15^{cd}	16.67±2.84^{c}	1.33±0.89^{d}
A. F. bacteria	11.33±2.60^{c}	138.67±8.10^{a}	116.00±1.52^{b}	14.33±3.84^{c}	11.00±2.08^{c}	12.67±2.60^{c}	8.00±1.73^{c}

Means in the same row with different superscripts are significantly different (P <0 .05).

Vibrio spp. and coliform as compared to the corresponding substitution levels in FFW-fed fish. The highest significant count of both yeast and acid fermentative bacterial count are recorded in in FW30-fed fish.

Discussion

The present results showed that neither the form of FM-replacer (non-fermented or fermented) nor the level (%) of FM-substitution revealed any pronounced effect on the overall growth or feed utilization criteria relative to CTRL fish. This indicated nutritionally-balanced diets fulfilling the essential amino acid requirements for juvenile's European seabass (FAO 2018) with no negative effect on feed intake or fish performance. To the authors' knowledge, no reports on the effects of using fish–waste meals on European seabass performance are available in the literature for comparison purpose. According to Sotolu (2009), dietary fish waste meal at 15% inclusion level is better than fish meal for African catfish (*Clarias gariepinus*). Similarly, good results are obtained with FM replacement by 75% processed meal from knife fish (*Chita laornata*) in juvenile Nile tilapia, *O. niloticus* diets (Abarra et al. 2017). Fish-offal meal was also evaluated in diets for Indian major carp (*Labeo rohita*) and catfish (*Heteropneustes fossilis*) and both fish grew better on diets replacing up to 50% of FM, possibly through a protein-sparing effect related to the higher lipid content of the fish-offal meal (Satpathy et al. 2003; Mondal et al. 2008). In addition, fermented blend of fish offal and slaughter house blood within Indian major carp feed, replaced up to 75% of FM with no negative impact on growth (Samaddar et al. 2015). In contrast, fermented and non-fermented tuna hydrolysate-containing diets at both 50 and 75% FM-replacement levels significantly reduced growth of juvenile barramundi (*Lates calcarifer*) as compared to the control fish (Siddik et al. 2018).

However, fish growth rate should not be the single criterion to evaluate the performance of a novel dietary protein source. It is also necessary to study the response of fish metabolism to ensure that modifications in the diet will not induce significant metabolic disturbances that could affect fish health status or resistance to their environment.

In general, our results showed no statistically different changes in the whole fish amino acid composition at termination of the trial, only the EAA tryptophan increased in FW-fed fish, at all substitution levels, relative to those of fish fed the CTRL diet. The same observation was recorded by Alam et al. (2002) for olive flounder (*Paralichthys olivaceus*). Moreover, similar findings are more recently reported by Kim et al. (2014) when tuna byproduct meal inclusion level had increased in the diets of juvenile olive flounder. This similarity in sea bass amino acid profile among our dietary treatments was also reported by Yamamoto et al. (2000) for rainbow trout (*Oncorhynchus mykiss*) and they explained the similarity by that body proteins are synthesized based on the genetic information from DNA, so that amino acid composition of specific body proteins is the same irrespective of the diet.

Generally, proximate composition of seabass was mostly affected by the various alternative animal and/or plant protein sources for dietary FM (Kaushik et al. 2004; Rimoldi et al. 2016). In general, the major nutrients and individual amino acid composition of seabass juvenile, in the present results, are relatively similar to the values earlier reported by Roncarati et al. (2006) and Baki et al. (2015). The results of the present study showed that body protein and ash contents are not significantly different among treatments

indicating that juvenile's seabass were not negatively affected by any of the novel FW/FFW dietary ingredients and efficient protein utilization for all experimental diets. However, fish lipid content was significantly modified and using FW as a FM replacer has led to an increase in fish lipid content. A direct correlation between the fish lipid content and level of dietary FW was observed. The relatively higher carcass lipid content, as well as VSI in FW45 and FFW45-fed fish as compared to the other fish groups may indicate massive accumulation of lipids in viscera. The increase in seabass lipid content can be explained by the higher lipids in fish offal and differences in fatty acids profile relative to those in FM used. The overall data suggest that FW in the two forms affect fat deposition and the lipogenic potential in European seabass, and this result is in agreement with those of Dias et al. (2005) for the same species. Similarly, juvenile olive flounder (*Paralichthys olivaceus*) fed tuna byproduct meal as a FM substitute showed variations in the whole body proximate composition (Kim et al. 2014). Also, Lu and Ku (2013) reported that juvenile cobia (*Rachycentron canadum*) fed shrimp waste meal (SWM) up to 25% FM replacement level, recorded no significant differences in the protein and ash concentration of their muscle, but contradict with that lipid content was low when fish fed diets with high SWM level.

In our study, HSI values are within the range reported earlier for the species (Wassef et al. 2016, 2017). Lu and Ku (2013) recorded significant increase in HSI values when 20-25% of FM was replaced by SWM in juvenile cobia diet. Condition factor (K) values greater than 1 imply that fish are in good physiological state of well-being. The K values reported in our trial, independently from the treatments, are greater than one, and this coincided with the recorded fish growth parameters. These findings are in agreement with those K values previously obtained for the species (Kousoulaki et al. 2015).

Haematological and serum biochemical parameters in cultured fish species are important tools in the evaluation of a novel dietary ingredient and values of haemoglobin and hematocrit are indicators of general health status of fish where their values may change in response to deficiencies in essential nutrients (Fazio et al. 2018). The recorded major blood constituents of the current research are within the normal range previously reported by Fazio et al. (2018) for the species. In the present work, an increase in the total number of circulating RBC count coincided with an increase in both Hb and PCV values was noticed in sea bass fed the FW-containing diets relative to those of fish from the other dietary treatments. The elevation of WBC count in FW-fed fish as compared with those of fish fed the other diets may be related to the activity-enhancing defense mechanism induced by utilization of FW meal but still the values of WBCs are within the recorded average normal reference range for cultured seabass (*D. labrax*) (Filiciotto et al. 2012). In general, haematological analysis indicates no pathological signs in fish fed the provided diets indicating good general health status.

Serum enzyme activity may also provide important information on the functional state of different organs (Peres et al. 2014). Blood enzymes' values (AST and ALT) and the levels of energetic metabolites (cholesterol and triglycerides) of fish are considered important diagnostic characters as well. Their values are used in estimating the health and condition of fish and in identifying and assessing the impact stressors in nature (Coz-Rakovac et al. 2005). The elevated activities of serum AST and ALT are generally related to liver damage, as a mark of liver necrosis (Wang et al. 2014). In the present study, a reduction in ALT and AST enzymes activities was recorded in fish fed the FW containing diets, at all inclusion levels, relative to fish from the other dietary groups indicating that FM-replacement by FW meal had certain positive effect on the liver function. Besides, the lowest total cholesterol, LDL and triglycerides levels, in the present study, in FW-fed fish in comparison with the other two dietary groups give a positive sign of fish health. Similarly, total cholesterol was influenced by dietary substitution of FM with seafood byproduct and soybean protein for red seabream (Kader and Koshio 2012). Furthermore, the present results showed an elevation in lipid function indicators (cholesterol and triglycerides) and a reduction in lipase in fish fed FFW diets, relative to the corresponding's of FW-fed fish groups. This may be related to that fish metabolism was becoming relatively slower. Also, the increase in serum LDL and triglycerides and the decrease of HDL in fish fed the FFW diets relative to the other dietary fish groups may indicate an increase in lipids accumulation in fish blood vessels (Paruruckumani et al. 2015). However, all basal serum triglycerides and cholesterol levels, reported in the present study, are in agreement with previously obtained for the species (Peres et al. 2014). In conclusion, the present results further indicate that dietary protein sources and treatments affect the regulation of lipid metabolism in juvenile seabass in fish. The lowest value of creatinine noticed in fish fed the FW diets, in the present study, indicating an enhancement in kidney function in this fish group relative

to the other dietary groups. Unfortunately, no reference for creatinine level in seabass blood was available for the comparison purpose.

Data of the intestinal microbiome count demonstrated that the FW30 diet had a remarkable reduction impact on the pathogenic bacteria as well as an elevation in the beneficial microbes within seabass distal intestine, which indicate improving gut-health functionality and fish immune response thereafter. Generally, a beneficial effect on intestinal microbiome was detected in FW-fed fish.

In the current study, it was hypothesized that the fermentation of FW biomass via fungal treatment, would improve its nutritive value and promote growth of seabass in comparison to CTRL fish, because the marine fungus *B.bassiana* secretes chitinase, protease and L-glutaminase extracellular enzymes, but it did not. In contrast, application of *B. bassiana* has negatively affected the intestine beneficial microbiome, where it reduced the beneficial fungal count and acid fermentation bacteria whilst elevated the harmful *Vibrio* spp. and fecal coliform relative to those in fish of the other dietary treatments. This negative influence may be attributed to the presence of *B. bassiana* metabolites in FFW meal. To the authors knowledge there are no published reports on the effects of marine *B. bassiana* metabolites on fish or even other aquatic organisms. The present results suggest that the interaction between *B. bassiana* and fish is therefore complex, contradictory and context dependent. Additional studies are therefore required on marine *B. bassiana* metabolites and their effects on fish performance, particularly gut health.

Conclusion

Up to 30% of fish meal in the diet of juvenile European seabass can be replaced with fish waste meal without adverse effects on growth performance, feed utilization or general health status. Fermentation of FW meal with *B. bassiana* has some negative impacts on fish health and intestinal integrity at 45% FM substitution level.

Conflict of interest None of the authors have any conflict of interest regarding the research described in this article

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