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Intestinal morpho-physiology and innate immune status of European sea bass (*Dicentrarchus labrax*) in response to diets including a blend of two marine microalgae, *Tisochrysis lutea* and *Tetraselmis suecica*

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Abstract

The aim of this study was to investigate the effects of replacing graded levels of dietary fish meal by a blend of two marine microalgae *Tisochrysis lutea* and *Tetraselmis suecica* on intestinal morpho-physiology and innate immune response in European sea bass. Two complete diets were formulated to be iso-nitrogenous and iso-lipidic and prepared by including a blend of the two microalgae, to replace approximately 15 and 45% fish meal protein of the control diet. A fourth diet, where the microalgae mix was substituted by soybean meal, was also prepared. Each diet was offered until visual satiety over 105 days to triplicated groups of European sea bass (204 ± 12.7 g), kept in a recirculating marine water system.

The humoral and cellular innate immune parameters of E. sea bass were affected by the dietary treatment. Fish fed the microalgae-containing or the soybean rich diets, showed a significantly greater *villi* height, while the thickness of intestinal epithelium was significantly reduced in fish fed the soybean meal-rich diet. The activity of the brush border membrane enzymes, maltase, sucrase-isomaltase, γ-glutamil transferase and alkaline phosphatase was not affected by dietary treatment but changed in different intestinal tracts. The genes sucrase-isomaltase, peptide transporter 1, sodium/potassium-transporting ATPase and aminopeptidase N were overexpressed in the pyloric and proximal region of the intestine of fish fed the microalgae-including diets. In conclusion, a blend of dried marine microalgae *Tisochrysis lutea* and *Tetraselmis suecica* as alternative ingredients to dietary fish meal did not hamper gut digestive-absorptive functions of E. sea bass. Moreover, it resulted in enhanced non-specific immune response, suggesting an effective role as an immunostimulant ingredient.

Key words: serum; leukocyte; brush border; gene expression; intestinal morphology.

Introduction

In aquaculture, certain marine microalgae currently receive a growing interest as alternatives to feed ingredients such as fish oil and meal or conventional vegetable lipid and protein sources in aquafeeds for carnivorous fish species. In fact, fish oil and meal are becoming environmentally unsustainable and too expensive to be used by the aquaculture industry (Naylor et al., 2009; Tacon et al., 2011; Vargas et al., 2018). Moreover, high dietary inclusion levels of certain plant-derived ingredients have been reported to negatively affect fish growth performance, flesh quality (Mourente and Bell, 2006; Tibaldi et al., 2006; de Francesco et al., 2007; Bonaldo et al., 2008; Messina et al., 2013), stress tolerance, gut and liver integrity (Bakke-McKellep et al., 2000; Uran et al., 2008; Merrifield et al., 2009; Kokou et al., 2012), activity of the intestinal brush border membrane (BBM) enzymes (Tibaldi et al., 2006), intestinal microbioma (Bakke-McKellep et al., 2007; Merrifield et al., 2009) as well as immune response (Sitjà-Bobadilla et al., 2005; Kokou et al., 2012; Marjara et al., 2012) by physiological and molecular mechanisms which are not still fully understood.

Microalgae are considered to possess high nutritional value due to their capability to synthesize all amino acids and their high content of proteins (30 to 70 %), lipids (10 to over 20 %) in the form of triacylglycerols containing ω3 and ω6 fatty acids, such as eicosapentaenoic acid (EPA), arachidonic acid (AA) and docosahexaenoic acid (DHA), vitamins (A, B1, B2, B6, B12, C, E, biotin, folic acid and pantothenic acid), minerals (phosphorous, zinc, iron, calcium, selenium, magnesium) and antioxidant substances (Brown et al., 1997, 2002; Renaud et al., 1999; Spolaore et al., 2006; Becker, 2007; Hemaiswarya et al., 2011). In particular, marine microalgae like *Pavlova* spp. and *Tisochrysis lutea* (Prymnesiophytes) are also rich in DHA (0.2 to 11 %), *Nannochloropsis* spp. (Eustigmatophytes) and diatoms are rich in AA (up to 4%), whereas *Tetraselmis* spp. (Prasinophyte) have a significant content of EPA (Volkman et al., 1989).

Recently, the dry biomass of different marine microalgae has been shown to succesfully replace fish meal and oil in aquafeeds for European sea bass (Tulli et al., 2012; Tibaldi et al., 2015; Cardinaletti et al., 2018), gilthead sea bream (Palmegiano et al., 2009; Vizcaino et al., 2014) and Atlantic salmon (Kiron et al., 2012; Sorensen et al., 2016), while Walker and Berlinsky (2011) reported a palatability problem with negative consequencies on feed intake in Atlantic cod. Moreover, Vizcaino et al. (2018) observed, at the ultrastructural level, that dietary inclusion of the algal dried biomass of *Tisochrysis lutea* and *Nannochloropsis gaditana*, had a positive impact on the absorptive capacity of the intestinal mucosa in Senegal sole. A positive effect has been also observed by measuring the activity of certain BBM enzyme in fish fed diet including *Scenedesmus almeriensis* where an increase in leucine aminopeptidase and alcaline phosphatase activity were observed (Vizcaino et al., 2014; Vizcaino et al., 2018).

The expression of the genes involved in the intestinal nutrient uptake is affected by different factors but results are sometimes controversial depending on experimental design, diet composition, fish species and salinity (Hakim et al., 2009; Terova et al., 2009, 2013; Bucking et al., 2012; Rimoldi et al., 2015; Tang et al., 2016; Verri et al., 2017).

Besides providing nutrients, microalgae in fish diets are gaining attention for their functional properties (Spolaore et al., 2006; Yaakob et al., 2014). Their active components have already demonstrated immunostimulating activity in mammals where health promoting attributes of *Chlorella sp* or *Spirulina sp* are documented (Chou et al., 2012; Dantas et al., 1999). Amar et al. (2004) found enhanced innate immunity in rainbow trout fed a purified diet including a *Dunaniella salina* extract. Cerezuela et al. (2012) found that three orally administered microalgae (*Nannochloropsis gaditana, Tetraselmis chuii* and *Phaedactilum tricornutum*) could enhance certain immune defence mechanisms in gilthead sea bream. On the other hand, the effects of microalgae on inflammation are controversial and seem to be specie-specific. In fact, Reyes-Becceril et al. (2013), showed that dietary administration of the diatom *Navicula sp* in gilthead sea bream, induced upregulation of several genes involved both in digestion/absorption and in the inflammation response of the gut. *Chlorella vulgaris* had such an effect in Atlantic salmon (Grammes et al., 2013) while *Tetraselmis sp, Chlorella sp, Pheodactilum tricornutum* and *Nannochloropsis gaditana* in zebrafish (Bravo-Tello et al., 2017). Moreover, short or long term harmful effects on immune defences deserve particular attention as they could directly affect fish susceptibility and resistance to diseases (Metochis et al., 2016).

To date, *Tetraselmis suecica* is one of the few marine microalgae species that is currently produced for aquaculture feed due to the large amount and high quality of its intracellular protein content together with *Tisochrysis lutea* that combines medium-high level and quality of protein with high lipid and DHA contents (Brown, 2002; Tokuşoglu and Ünal, 2003).

It is not known if and to what extent a mix of the two cultured microalgae could display functional properties when included in aquafeeds.

In this context, the aim of the present study, was to evaluate the effects of graded levels of a dry mix of *Tisochrysis lutea* and *Tetraselmis suecica* in the diet on European sea bass (*D. labrax*) metabolic status, its innate immune response and intestinal morpho-physiology. The experiment, is a part of a wider study, whose results on growth performance, nutrient digestibility, muscle tissue composition and quality traits have been already published in Cardinaletti et al. (2018).

Materials and methods

Experimental diets and fish rearing conditions

Four diets were formulated to be grossly iso-nitrogenous (N, 7.5 % DM) and iso-lipidic (total lipid, 18.5 % DM). A control diet (C) was prepared in order to have a 50/50 fish to vegetable protein and fish to vegetable lipid ratios, calculated by considering the crude protein and lipid contribution of all fish-based and vegetable-based dietary ingredients. This preparation included 150 g/kg of toasted, dehulled and solvent extracted soybean meal. Two test diets, MA15 and MA45 were prepared by including a blend of *T. lutea* and *T. suecica* dried biomass in a 2:1 w:w ratio to replace 15 and 45% fish meal (FM) protein and 12 and 36% fish lipid, respectively, of the control diet. A fourth diet rich in soybean meal (SBM) was prepared with a 30:70 and 50:50 fish to vegetable protein and lipid ratios, respectively. This latter diet was obtained from the C diet through a further substitution of FM with soybean meal, so as to maintain the same fish to vegetable lipid ratio. Moreover, the SBM diet had the same fish meal content than MA45.

The ingredient composition and proximate analysis of the diets are shown in Table 1. All ingredients were ground through a 0.5 mm sieve before final mixing and dry pelleting through a 4.5 mm die in the pilot plant of the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine. The diets were stored at -20°C until used.

One hundred and forty four fish (mean body weight 204 ± 12.7 g) were randomly divided among 12 groups (12 specimen/group), kept in 300-L fiberglass tanks in a marine recirculating system ensuring optimal water condition to E. sea bass (temperature 22.8 ± 0.5°C, salinity 25.8 ± 1.3‰, dissolved oxygen 6.8 ± 0.43 mg/L, pH 8.0 ± 0.13, total ammonia nitrogen 0.04 ± 0.02 mg/L, nitrite-nitrogen 0.2 ± 0.06 mg/L).

After stocking, fish were fed diet C and adapted to the experimental conditions over 2 weeks. At the end of this period, the 12 groups were assigned to the four test diets according to a random design with triplicate groups (tanks) per treatment. Fish were hand-fed the experimental diets over 105 days, in two daily meals (9:00 am and 4:00 pm) until the first feed item was refused.

Etics

The handling procedures and sampling methods involving fish used in the trial followed the guidelines of the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Tissue sampling

At the end of the feeding trial and after 40 hours fasting period in order to avoid any effect of the meal, 4 fish per tank (12 fish per dietary treatment) were sacrificed with an overdose of the anaesthetic MS-222 (300 mg/l, tricaine methan sulphonate, Argent Laboratories, Redmont-VI, USA).

Table 1. Ingredient and proximate composition of the test diets.

	C	MA15	MA45	SBM
Ingredient Composition (g/kg)				
Chile prime fish meal	275	232	150	150
CPSP	50	50	50	50
Wheat gluten meal	200	200	200	200
Dehulled soybean meal solvent extract (48 %CP)	150	150	150	350
Gelatinized starch	130	121	90	40
Cod liver oil	56	51	45	62
Palm oil	60	57	50	62
Soy Lecithin	25	25	25	25
Freeze-dried *Tisochrysis lutea*	0	40	120	0
Freeze-dried *Tetraselmis suecica*	0	20	60	0
L-Methionine	0	0	6	7
Mineral supplement$	4	4	4	4
Vitamin supplement[#]	5	5	5	5
Na lignosulfite	30	30	30	30
Celite	15	15	15	15
Proximate composition (%)				
Dry matter	93.8	93.8	93.8	93.1
Crude protein	45.9	46.1	46.1	46.8
Total lipid	17.4	17.3	17.4	17.2
Total carbohydrate[§]	19.5	21.1	17.6	17.5
Starch	15.1	14.2	11.1	8.9
Ash	11.0	9.3	12.7	11.6

[$] *Mineral supplement composition (% mix): $CaHPO_4*2H_2O$. 78.9; MgO. 2.725 g; KCl. 0.005; NaCl. 17.65; $FeCO_3$. 0.335; $ZnSO_4*H_2O$. 0.197; $MnSO_4*H_2O$. 0.094; $CuSO_4*5H_2O$. 0.027; Na_2SeO_3. 0.067.*

[#] *Vitamin supplement composition (% mix): thiamine HCL Vit B1. 0.16; riboflavin Vit B2. 0.39; pyridoxine HCL Vit B6, 0.21; cyanocobalamine B12, 0.21; niacin Vit PP, 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05; myoinositol, 3.15; stay C Roche, 4.51; tocoferol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500UI/kg diet) 0.026; Vit D3 (2400UI/kg diet) 0.05; choline chloride, 83.99.*

[§] *Includes crude fiber and nitrogen free extract*

and 3 fish per tank were immediately subjected to blood sampling. Blood was withdrawn from the caudal vessels in heparinized and non-heparinized tubes. Plasma samples from the heparinized blood were obtained after centrifugation at 1,500 x g for 15 min at 4°C. Serum samples from non-heparinized blood were obtained after clotting for 2 hours at 4°C and centrifugation at 1,500 x g for 15 min at 4°C. Plasma and serum samples were immediately stored at -80°C for subsequent metabolic profile and analysis of immune parameters, respectively. After blood sampling, the digestive tract was removed from the open abdomen, and the head kidney (HK) was aseptically removed from 2 fish per tank (6 fish per dietary treatment) and placed in sea bass isosmolar (360 mOsm/kg) Hank's balanced salt solution (HBSS, Sigma-Aldrich, Milan, Italy) containing 0.25% heparin (Sigma-Aldrich, Milan, Italy).

The digestive tract was collected from 3 fish per tank (9 fish per dietary treatment) and divided into pyloric cecae (PC), proximal intestine (PI, section below the tract with PC until the increase in diameter indicating the start of the distal intestine) and distal intestine (DI, the terminal part of the intestine with larger diameter, untill the anus). The PI samples from 2 fish per tank (6 fish per dietary treatment) were preserved in Bouin'S solution for histological evaluation. Tissue samples from 2 fish per tank (6 fish per dietary treatment) were rinsed with iced saline, gently dried with a piece of paper, put in individual plastic tubes and stored at -20°C until the analysis of the activity of the brush border membrane (BBM) enzymes. Samples of PI, PC and DI from 2 fish per tank (6 fish per dietary treatment) were put in individual plastic tubes, frozen in liquid N and stored at -80°C for gene expression analysis.

Metabolic parameters

The plasma parameters glucose (Glu, mg dL^{-1}), cholesterol (Chol, mg dL^{-1}), triglycerides (Trig, mg dL^{-1}), total proteins (Tp, g dL^{-1}) and albumin (Alb, g dL^{-1}) were determined by an automated analyser system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy) and

commercially available kits (Biochemical Enterprise, Milan, Italy), following the manufacturer's protocols.

Humoral immune parameters

The serum lysozyme activity was determined by using the turbidimetric method according to Parry et al. (1965).

The serum antiprotease activity was measured following the method of Bowden et al. (1997) with minor modifications. Ten microliters of serum were incubated with 10 µl of 0.3% trypsin (Sigma-Aldrich, Milan, Italy) in 0.01 M Tris-HCl (Sigma-Aldrich, Milan, Italy) pH 8.2 and 500 µl of 5 mM Nα-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (BAPNA, Sigma-Aldrich, Milan, Italy), then the volume was brought up to 1 ml with 0.1 M Tris-HCl pH 8.2. Samples were incubated at 22°C for 25 min. The reaction was stopped with 150 µl of 30% acetic acid and the mixture was centrifuged at 400 x g for 5 min at 4°C. The optical density (OD) of the supernatant was read at 415 nm against a blank in a microplate reader (Sunrise, Tecan S.r.l., Milan, Italy). The inhibitory activity of antiproteases was expressed in terms of percentage of trypsin inhibition: [(OD trypsin – OD sample)/ OD trypsin] x 100 (Zuo and Woo, 1997).

Serum total myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997).

Cellular immune parameters

The HK leukocyte isolation was performed as previously described by Volpatti et al. (2014).

Histology

Tissue samples were automatically processed (TISBE tissue processor, Diapath), transversely-orientated and embedded in paraffin. One distinct paraffin block was produced for each individual sample and, from each block, a single 5 µm thickness cross-section was cut (Leica RM 2135). Sections were dewaxed and stained with Mayer's hematoxylin and eosin. The histological specimens were evaluated using an optical microscope (Leica DMRB), documented by a digital camera (Leica ICC50) and images were processed with LAS EZ software (Leica).

Slide-observation was undertaken using bright-field illumination and each slide was evaluated to highlight any degenerative and/or inflammatory phenomena. The slides were also used for quantitative image analysis to measure the villi height (40x magnification) and the epithelium thickness (100x magnification) of the intestine by mean of UTHSCA Image Tool 2.0 software (open source). All the visible *villi* of transversal sections have been measured. The epithelium height has been measured for each intact *villum* and the thickness has been measured from basal lamina to lumen profile in three different points of each *villum* (bottom, medium and top) and is equivalent to the external cellular height.

BBM enzyme activities

The stored sections of the gut were thawed and, when necessary, the remaining content was gently squeezed out. One hundred mg of gut tissue in 1 ml iced saline buffer were crushed in a tissue-lyser disruption system (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 minute. Samples were centrifuged at 13,500 x g for 10 min at 4°C and the supernatant was used to measure the BBM enzyme activities (Tibaldi et al., 2006).

The hydrolysis of maltose and sucrose, by the BBM enzyme maltase and the complex sucrase-isomaltase (SI), was determined according to Harpaz and Uni (1999).

Alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) activity was determined using commercial kits (Paramedical, Pontecagnano Faiano, Sa, Italy) as indicated by the manufacturer.

The amount of total protein in the supernatant was determined according to Bradford et al. (1976) using Bradford reagent (Sigma-Aldrich, Milan, Italy) and bovine serum albumin (Sigma-Aldrich, Milan, Italy) as a standard.

One unit (U) of enzyme activity corresponded to the amount of enzyme that transforms or hydrolyses 1 μmole of substrate ml^{-1} $minute^{-1}$. The specific enzymatic activity was calculated as U of enzyme activity per mg ml^{-1} of supernatant protein for maltase and saccarase and mU of enzyme activity per mg ml^{-1} of supernatant protein for ALP and γ-GT.

BBM enzyme gene expression

Total RNA was extracted using Aurum™ total RNA fatty and fibrous tissue kit (Bio-Rad, USA) and following the manufacturer's protocol. Tissue disruption was performed by using TissueLyser II disruption systems (Qiagen, Germany) and RNA concentration and quality was analysed by NanodropOne spectrophotometer (Thermo Scientific, USA) and by agarose gel electrophoresis. After extraction, complementary DNA (cDNA) was synthesised from 1 μg of total RNA using the iScript™cDNA Synthesis Kit (Bio-Rad, USA), diluted 1:10 in RNase-DNase free water and stored at -20 °C until quantitative real-time PCR (qPCR). An aliquot of cDNA was used to check primer pair specificity.

qPCR was used to analyse the mRNA expression of four brush border digestive enzymes: sucrase-isomaltase (SI), peptide transporter 1 (PepT-1), sodium/potassium-transporting ATPase (Na^+/K^+ATPase) and aminopeptidase N (APN). qPCR primers (Sigma-Aldrich, Germany) were designed using Beacon Designer 8.0 software (PREMIER Biosoft International, USA) based on available sequences on NCBI (Table 2). To verify correct amplification, PCR products were excised and purified using QIAquick Gel Extraction Kit (Qiagen, USA) and sequenced by Eurofins MWG Operon (Ebersberg, Germany). All sequences were confirmed by alignments and by using NCBI nucleotide BLAST software (http://blast.ncbi.nlm.nih.gov).

Six biological and two technical replicates per digestive tract and dietary treatment were analysed using CFX 96 thermal cycler (Bio-Rad, USA), including a negative control. The PCR

reaction were carried out in a final volume of 20 µL containing 6 µL of PCR-grade water, 10 µL of SsoFast

Table 2. List of primers, amplicon size and efficiency of the genes.

Gene[#]	GenBank ID	Sense/antisense (5'-3')	Amplicon size (bp)	Annealing temperatute °C
SI	AM419039	GTGACTTTCCAGCCTACT/ TAACCATAGCGACAGAGC	176	60
PepT-1	FJ237043	GGACTGGGCTGAGGAGAAA/ GGAAGAGGGATGTAGAGGAAGA	87	60
Na^+/K^+ ATPase	AM419034	CGTTACTGGAGTGGAAGA/ GTTGGCGATGATGAAGAG	118	60
APN	FJ860001	ACTGCTCTATGATGAAACCT/ AGTCATTCCACCACCTTAG	122	60
EF1A*	AJ866727	GACACAGAGACTTCATCAAG/ GTCCGTTCTTAGAGATACCA	114	60
18S*	AM419038	CGCTAGAGGTGAAATTCTTGGA/ GGAACTACGACGGTATCTGAT	125	60
ACTB*	AJ537421	TGTATGCCTCTGGTCGTA/ GTGGTGGTGAAGGAGTAG	184	60

[#] For the abbreviation of the Gene name refere to the text

* reference genes.

concentration 500 nM) of each forward and reverse primer. The qPCR program included an enzyme activation step at 95 °C (30 s) and 40 cycles each of 10 s at 95 °C and 30 s at 60 °C. The fluorescence signal was detected at the end of the 60°C reaction step. Moreover, a melt curve analysis (a heating step at 95 °C for 1 min and a final detection step from 65 to 95°C with a temperature transition rate of 0.5°C for 0.05 s) was performed to check specificity and/or absence of contaminants in the reaction.

qPCR reaction efficiency (E) for each gene assay was determined using 10-fold serial dilutions of randomly pooled cDNA. R^2 values of the standard curves were all > 0.98. Beta-actin (ACTB), elongation factor 1A (EF1A) and ribosomal protein 18S (18S) (Table 2) were evaluated as reference genes according to their stability as described previously (Pfaffl, 2004). EF1A was found to be stably expressed and was therefore used as normalisation factor. Expression of the target genes were calculated using the comparative Ct method (Schmittgen and Livak, 2008) and

expressed as fold changes of control group (values >1 indicate up-regulated genes, conversely value <1 indicate down regulated genes).

Statistical analysis

Data are presented as average value ± SD. Data were tested for normality and homogeneity of variances by using Shapiro-Wilk and Levene tests, respectively. Data of immunological and plasma metabolites parameters and of hystological measures were subjected to one-way ANOVA and Duncan's *post hoc* test (significant level of 95 %) to detect significant differences between dietary treatments. BBM enzyme activities results were analysed with two-way ANOVA test followed by Duncan's *post hoc* test (significant level of 95 %), considering the dietary treatment and intestine sections as the main factors. Gene expression results were also analysed with two-way ANOVA and differences between pairs of mean has been performed by least significant difference (LSD). All analyses were completed using the SPSS package (SPSS Inc., Chicago, IL).

Results

Fish promptly accepted all the experimental diets and during the trial no mortality was recorded. As reported earlier (Cardinaletti et al., 2018), feed intake tended to increase in response to graded levels of microalgae in the diets, resulting in the highest value in fish fed diet MA45 when compared with SBM and C diets ($P< 0.05$). Fish fed both the microalgae containing diets resulted in growth performance, FCR, and PER which were similar to those attained by fish fed the control diet ($P> 0.05$), but higher to those of the E. sea bass fed the soybean rich diet (final weight 419.1 *vs* 387.7; SGR 0.68 *vs* 0.61; FCR 1.70 *vs* 1.89; PER 1.31 *vs* 1.13; $P< 0.05$).

As shown in Table 3 dietary treatments did not affect plasma glucose, triglycerides and albumin. Relative to controls, fish fed the diets higher in microalgae (MA45) and in soybean meal (SBM) resulted in similar lower plasma cholesterol and total proteins ($P<0.05$).

Table 3. Plasma metabolic parameters measured in E. sea bass fed the test diets at the end of the 105-days feeding period.

	Dietary treatments			
Plasma parameters	**C**	**MA15**	**MA45**	**SBM**
Glu mg/dl	118.4±23.10	122.0±18.12	131.9±29.03	129.2±33.21
Chol mg/dl	347.2±61.41 ^{a}	318.8±47.24 ^{ab}	276.6±48.60 ^{bc}	263.4±44.64 ^{c}
Trig mg/dl	611.5±90.01	551.4±112.0	531.6±148.36	555.45±149.29

Tp g/dl	5.9±0.41 [a]	5.7±0.41 [ab]	5.4±0.51 [b]	5.4±0.42 [b]
Alb g/dl	2.5±0.15	2.5±0.16	2.3±0.26	2.4±0.14

Values are given as mean ± SD; (n = 3 tanks, 3 fish/tank). Different superscript lowercase letters in a row (a,b,c) indicate statistically significant differences (P<0.05) among experimental groups. Glu, glucose; Chol, cholesterol; Trig, triglicerides; Tp total protein; Alb, albumin

The humoral and cellular innate immune parameters of E. sea bass fed the experimental diets were affected by the dietary treatment. As shown in Fig 1, the serum lysozyme, antiprotease and MPO activity were significantly enhanced in fish fed the diet with the lower level of microalgae (MA15) relative to the C and the SBM diet (P<0.05). Antiprotease activity was particularly depressed in case of fish subjected to the diet high in soybean meal (P<0.05). Lastly, the RB activity of HK leukocytes stimulated with PMA was negatively affected by the lowest level of microalgae and by the SBM dietary treatments (P< 0.05) (Fig 1).

At the hystological evaluation (Fig 2) no signs of significant degenerative or inflammatory processes due to the dietary treatment were detected in the proximal intestine of E. sea bass at the end of the experiment. As shown in Table 4 and Figure 2, fish fed the microalgae-containing diets or the soybean-rich diet, showed a significantly greater *villi* height (P<0.05) compared to the control, while the thickness of intestinal epithelium was significantly reduced in fish fed the SBM diet (P<0.05) than in fish fed the control and both diets containing microalgae.

Table 4. Height of villi and thickness of intestinal epithelium in the proximal intestine of E. sea bass fed the test diets at the end of the 105 days feeding period.

	Dietary treatments							
Histological parameters	**n**	**C**	**n**	**MA15**	**n**	**MA45**	**n**	**SBM**
Villi height (µm)	201	523±216 [c]	189	698±250 [a]	124	660±176 [a]	254	608±235 [b]
Thickness (µm)	213	41±13 [a]	89	44±10 [a]	112	43±10 [a]	231	36±11 [b]

Data are presented as means ± SD. Row means with different superscript lowercase letters indicate significant differences among dietary treatments (a, b, c, P < 0.05). n= total number of readings coming from 6 transversal sections.

As shown in Figure 3, the activity of the BBM enzymes maltase, SI and γ-GT varied according to the different intestinal tracts (P<0.05) but were unaffected by dietary treatments and no significant interaction between dietary treatment and intestinal tract was found.

The PC was the major site of disaccharases activity, while the highest activity of γ-GT was found in the distal intestine, thus confirming the functional differences of the tracts. The activity of the alkaline phosphatase was little affected by both dietary treatments and intestinal tract (P>0.05).

The results of the qPCR indicated that the expression of all the genes was differently affected by dietary treatments according to the intestinal sections.

As shown in Table 5, all genes were significantly upregulated in the pyloric and proximal intestinal regions, in fish given the microalgae-containing diets (P<0.05) with a nearly positive dose dipendent trend as the microalgae level was increased in the diet. This allowed a significant difference to be attained for diet MA45 over diet MA15 for gene expression of SI, APN and Na^+/K^+-ATPase in the proximal intestine.

On the opposite, in the distal portion there was a down regulation of all genes in fish fed diets MA15, MA45 and SBM compared to the control. In case of Na^+/K^+-ATPase the difference was significant only with the diet MA45.

Table 5. Gene expression values of brush border membrane enzymes in the intestinal tracts of E. sea bass fed the test diets at the end of the 105-days feeding period.

	Dietary treatments			
Enzymes and digestive tracts	C	MA15	MA45	SBM
SI				
pyloric caeca	1.00±0.09 ^{d}	1.21±0.08 ^{cd}	1.35±0.09 ^{bc}	0.86±0.22 ^{e}
proximal intestine	1.00±0.05 ^{d}	1.50±0.12 ^{b}	1.92±0.14 ^{a}	1.12±0.12 ^{d}
distal intestine	1.02±0.21 ^{d}	0.59±0.19 ^{f}	0.52±0.12 ^{f}	0.69±0.23 ^{ef}
PepT-1				
pyloric caeca	1.00±0.10 ^{c}	1.31±0.07 ^{ab}	1.30±0.08 ^{b}	1.02±0.10 ^{c}
proximal intestine	1.00±0.08 ^{c}	1.39±0.13 ^{ab}	1.47±0.11 ^{a}	1.06±0.02 ^{c}
distal intestine	1.03±0.26 ^{c}	0.73±0.11 ^{d}	0.60±0.19 ^{d}	0.49±0.08 ^{e}
APN				
pyloric caeca	1.01±0.04 ^{d}	1.24±0.04 ^{c}	1.26±0.05 ^{c}	0.91±0.12 ^{d}
proximal intestine	1.02±0.09 ^{d}	1.87±0.02 ^{b}	1.99±0.24 ^{a}	1.09±0.12 ^{d}
distal intestine	1.00±0.08 ^{d}	0.75±0.09 ^{e}	0.58±0.06 ^{f}	0.64±0.15 ^{ef}
Na^+/K^+ATPase				
pyloric caeca	1.00±0.03 ^{d}	1.46±0.05 ^{bc}	1.53±0.14 ^{b}	0.90±0.15 ^{d}
proximal intestine	1.03±0.28 ^{d}	1.67±0.13 ^{b}	2.05±0.07 ^{a}	1.40±0.19 ^{c}
distal intestine	1.02±0.24 ^{d}	0.87±0.06 ^{d}	0.62±0.13 ^{e}	0.76±0.17 ^{de}

SI, sucrase-isomaltase; PepT-1, peptide transporter 1; APN, aminopeptidase N; Na^+/K^+ATPase. Values are given as mean ±SD; (n = 3 tanks, 2 fish/tank) and represent fold changes relative to the control group and whithin each gene. Means values sharing different superscript letters are significant different (a, b, c, d, e, f; $P<0.05$). Interaction between main factors diet *vs* intestinal tract is for $P<0.05$.

Discussion

The inclusion of a blend of *Tisochrysis lutea* and *Tetraselmis suecica* in the diet of E. sea bass (*D. labrax*) did not adversely affect growth performance and feed conversion efficiency, while the diet high in soybean resulted in significantly depressed growth, feed and protein conversion efficiency when compared to C diet and both microalgae-containing diets (Cardinaletti et al., 2018).

The results obtained in the present study demonstrated that the blend of microalgae included in the diet positively enhanced the innate immune response and did not adversely affect intestine morphology and functionality.

At the end of the feeding trial, the physiological status of fish, as assessed through the analysis of certain blood biochemical parameters (Coz-Rakovac et al., 2005), have shown that E. sea bass fed the diet containing the highest level of microalgae or high in soybean meal had significantly lower levels of plasma total proteins compared to control fish. The present findings could reflect lower crude protein apparent digestibility of the same diet, as reported by Cardinaletti et al. (2018) or an impaired liver protein synthesis, as total plasma protein concentration is closely related to the rate of protein synthesis in the liver. Since the level of circulating albumin was similar in all fish groups, we can also suppose that the synthesis of hepatic globulins may have been affected by dietary treatments. To what extent this would possibly involve immunoglobulins or other globulins remains to be addressed.

While plasma levels of circulating glucose and triacylglycerol were not affected by dietary treatments, the diets MA45 and SBM resulted in a significant hypocholesterolemic effect. This is only partially a consequence of the lack of cholesterol in the vegetable ingredients used to replace dietary fish meal. In case of soybean meal, it is well known that its hypocholesterolemic effect goes beyond this and claims for the action of specific mechanisms reducing blood and tissue cholesterol levels in different animal models including E. sea bass (Messina et al., 2013). The hypocholesterolemic effect of MA45 in the present work confirm what has been previously reported in Japanese flounder fed graded levels of *Chlorella ellipsoidea* (Kim et al., 2002). In addition, lower blood triglycerides and cholesterol have been observed in yellow croacker (*P. crocea*) fed *Haematococcus pluvialis* enriched diets (Li et al., 2014) and in olive flounder (*P. olivaceus*) fed *Eucheuma denticulatum* supplemented diets (Ragaza et al., 2015). This wide

evidence on the hypocholesterolemic effects should be attributable to specific substances in the microalgae such as fucoxanthin, contained in *Tisochrysis*, that may act as mediator in lipid metabolism (Maeda, 2015) and/or to unknown mechanisms that still need to be investigated. In this study, dietary treatments led to changes in certain innate immune parameters of E. sea bass. We observed an increase in serum lysozyme, antiprotease and myeloperoxidase activities in fish fed the diet including the lower level of microalgae mixture compared to the other groups. Our findings are in agreement with previous studies, showing an increased serum lysozyme, MPO activities and antiprotease in Pacific red snapper fed a *Navicula* sp. supplemented diet (Reyes-Becerril et al., 2014) and a significant decrease of lysozyme and complement activity in fish fed diets containing high levels of soybean meal (Geay et al., 2011; Peng et al., 2013). An enhancement of serum lysozyme and MPO activity has also been reported in olive flounder (*P. olivaceus*) fed diets containing the marine brown alga *Ecklonia cava* (Kim et al., 2008). Similar results in serum lysozyme has also been observed by Amar et al. (2004) in rainbow trout fed diet added with *Dunaniella salina*. On the contrary, Reyes-Becerril et al. (2013) failed to find any modulation of MPO activity in gilthead sea bream fed *Navicula* sp. supplemented diet. Taking into consideration other innate immune parameters such as respiratory burst, Cerezuela et al. (2012) reported an increase of the latter in gilthead seabream fed over two weeks a diet supplemented with 50-100g/kg of *Pheodactylum tricornutum*. In the present study, the respiratory burst was impaired only in fish fed the lowest level of microalgae and the soybean meal containing diet. These evidences suggest that changes in certain innate immune parameters in fish fed diets including microalgae are specie-specific depending on the microalgae species and on their level of inclusion in the diet.

Interestingly, we observed that the serum antiprotease activity and the respiratory burst were significantly reduced in fish fed SBM diet. These immune parameters have been poorly investigated in fish in response to diet rich in soybean meal therefore further investigation is needed to clarify the possible mechanisms behind these results.

Besides the effects on innate immunity, it is known that vegetable ingredients and certain marine microalgae such as *Phaeodactylum tricornutum* and *Tetraselmis chuii*, may adversely affect the integrity of the digestive system (Atalah et al., 2007; Escaffre et al., 2007; Cerezuela et al., 2012). In the present study no negative effects were noted in the proximate intestine of E. sea bass fed microalgae-containing diets as previously observed in sea bream fed *S. almeriensis*, *T. suecica* and *T. lutea*, by Vizcaino et al. (2014, 2016) and in senegalese sole fed *T. lutea* and *N. gaditana* (Vizcaino et al., 2018). In particular, fish fed the MA15 and MA45 diets showed greater *villi* height compared to fish fed the control diet and this result could be considered a sign of an increased absorption ability. On the contrary, the soybean meal-rich diet reduced the *villi*

thickness as it has been observed in salmonids and other fish species (Van den Ingh et al., 1991; Boonyaratpalin et al., 1998).

With the aim at evaluating possible diet-induced changes in digestive-absorptive processes, we have also evaluated in different intestinal tracts, the activity/gene expression of several digestive enzymes located in the BBM of enterocytes, responsible for the final stages of nutrient digestion and assimilation. The present study, has investigated the effect of dietary microalgae on the activity of a panel of BBM enzymes which resulted not affected by dietary treatments. On the contrary, several studies have reported that changes in diet nutrient or ingredient composition can significantly modulate the intestinal BBM enzymes activity in fish (Krogdahl et al., 1999, 2003; Tibaldi et al., 2006). To explain the lack of effects due to the diet on BBM enzyme activity, it should be noted that in this study, according to Adamidou et al. (2009), tissue sampling time was set at 36-40 hours after the last meal to ensure a nearly complete emptying of intestine. This could have contributed to minimize possible changes in BBM enzyme activity due to the diet. For the same reason no apparent correlation was found between enzyme activities and the apparent nutrient or energy digestibility values reported for the same diets by Cardinaletti et al. (2018).

Significant differences in maltase, SI, γ-GT and ALP activity were detected along the gut, mirroring the functional differences of the intestinal tract. The activity of both the disaccharidases in the final degradation of carbohydrates to glucose, was higher in the pyloric caeca and significantly decreased in the remaining intestinal tracts, in agreement with previous studies (Harpaz et al., 2005; Krogdahl et al., 1999; Tibaldi et al., 2006).

γ-GT is one of the major enzymes in the intestinal microvilli and plays an essential role in the final digestion and absorption of dietary proteins. The microalgae-containing diets did not affect its activity in agreement with protein digestibility values and efficiency ratio as already reported in Cardinaletti et al. (2018). The enzyme showed its highest level of activity in the distal intestine as already reported by Tibaldi et al. (2006) in E. sea bass and by Harpaz and Uni (1999) in various fish species thus confirming its physiological role.

Lastly, the alkaline phosphatase of the intestinal brush border is often used as a marker of intestinal integrity as it is expressed in mature enterocytes. In the present study its activity was not affected by the diet, indicating that the dietary substitution of fish meal with microalgae did not cause major functional changes in the integrity of the gut in E. sea bass, as also confirmed by histology.

Regarding the results of gene expression, we found that the mRNA levels of the selected BBM enzymes SI, PepT-1, APN and Na^+/K^+ATPase varied significantly in relation to the dietary treatment in different intestinal tracts, as previously demonstrated in zebrafish, eel, seabass,

seabream, cod, waterloach and rainbow trout (Verri et al., 2003, 2008; Rønnestad et al., 2007; Terova et al., 2009, 2013; Ostaszewska et al., 2010; Rimoldi et al., 2015; Tang et al., 2016).

The mechanisms that underlies changes in gene expression in response to changes in dietary composition are not fully elucidated (Hooton et al., 2015). SI is known to be involved in the final digestion and absorption of starch hydrolysate and sucrose (Goda, 2000) and in the present study, its mRNA level was significantly up-regulated in fish fed diets including the microalgae mixture in the PC section and proximal intestine.

Templeton et al. (2012) and Laurens et al. (2012) reported that structural carbohydrates represent a large fraction of the algal biomass ranging from 6% to 18% depending on algal species considered. Based on total carbohydrate and starch contents of the diets, we can speculate that SI gene expression could be associated to the type of carbohydrates supplied by microalgae which have not been analyzed in this study and which will be worth studying in future research. Furthermore, microalgae provide intermediate metabolites in carnivorous fish (Hemre et al., 2002; Krogdahl et al., 2005) and in mammals (Hooton et al., 2015) and these together with other products derived from monosaccharides metabolism might likely have induced such an up-regulation of SI mRNA as previously observed in mammals (Yasutake et al., 1995). Moreover, due to a lack of correlation between mRNA gene expression and enzyme activity of sucrase in our study, the existence of post-transcriptional mechanisms cannot be ruled out and need further investigation.

Several methods have been used to determine protein digestibility in fish species but only a few studies try to correlate protein absorption with the expression levels of intestinal amino acid and oligopeptide transporters (Liu et al., 2014; Ostaszewska et al., 2010; Terova et al., 2013; Rimoldi et al., 2015). It is known that PepT-1 is involved in di- and tri- peptide transport in enterocites and its expression level and function are very responsive to dietary treatments (Gilbert et al., 2008; Verri et al., 2017). In fish, this was shown to be affected by different plant protein sources in the diet. In particular, in seabream fed diets including lupin and chick pea, Terova et al. (2013) observed an upregulation of the PepT-1 gene, positively related with fish growth and FCR. In the present study, we obtained similar results by including microalgae in the diet. In fact, the upregulation of PepT-1 mRNA observed here in the pyloric and proximal intestinal sections was positively related with growth and PER values (see Cardinaletti et al., 2018). In fish fed the microalgae-including diets, the modulation of PepT-1 gene expression could be ascribed to the observed increase of feed consumption due to a compensatory response to attain a same digestible protein intake as a consequence of a parallel slight decrease in crude protein apparent digestibility (Cardinaletti et al., 2018).

More interesting might be the hypothesis of an upregulation of PepT-1 due to the salt concentration of microalgae diets (the Na^+ content of microalgae is 1.5%, while in fish meal is 0.4-1.1 % and in soybean meal is 0.02%), that could increase the expression of this oligotransporter, as recently observed by Rimoldi et al. (2015). In the present study, the expression of Na^+/K^+ATPase was also investigated in order to see the potential effect of dietary manipulation on a gene that codify for an enzyme which is crucial in maintaining the intracellular homeostasis. Na^+/K^+ATPase also provides the driving force to support several Na^+-dependent transport processes. In particular, the mRNA expression level of Na^+/K^+ATPase show similar pattern of those of SI, PepT-1 and APN as also observed by Hakim et al. (2009) in fasted/refeed seabass. The results obtained in the present study, have demonstrated the high expression of the Na^+/K^+ATPase gene in the proximal portion of intestine that is the major site of nutrient absorption (Almansa et al., 2001).

Finally, we have also evaluated the crucial role of APN mRNA expression, a BBM enzyme that catalyzes the cleavage of amino acids from the protein terminus (Taylor, 1993; Gonzales and Robert-Baudouy, 1996). In this work, the APN mRNA expression level was affected by different dietary treatments. In fact, the APN mRNA was higher in fish fed both the microalgae-containing diets compared to the other groups. These results are consistent with previous data from carp (Hakim et al., 2009) and chicken (Gilbert et al., 2010) fed different dietary protein sources, so it is possible to assume a role of the protein source on the expression of APN in fish.

Conclusions.

The results of this study show that a blend of dried marine microalgae *Tisochrysis lutea* and *Tetraselmis suecica* biomass might be used as alternative ingredients to fishmeal in the diets for E. sea bass without hampering plasma homeostasis of certain metabolites and gut digestive-absorptive functions which were improved relative to those of fish given a soybean meal-rich diet. Moreover, dietary microalgae inclusion resulted in an enhanced non-specific immune response, suggesting that microalgae can be effectively used in aquaculture as immunostimulant ingredients.

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References

Adamidou, S., Nengas I., Alexis, M., et al. 2009. Apparent nutrient digestibility and gastrointestinal evacuation time in European seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes. Aquaculture. 289 (1-2), 106-112. http://doi.org/106112.10.1016/j.aquaculture.2009.01.015

Almansa, E., Sanchez, J.J., Cozzi, S., et al. 2001. Segmental heterogeneity in the biochemical properties of the Na+-K+-ATPase along the intestine of the gilthead seabream (*Sparus aurata* L.) J. Comp. Physiol. B-Bioch. Systemic and Environ. Physiol. 171, 557-567. PMID: 11686614.

Amar, E.C., Kiron, V., Satoh, S., et al. 2004. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish Shellfish Immunol. 16, 527-537. http://doi.org/10.1016/j.fsi.2003.09.004.

Atalah, E. Cruz, C.M.H., Izquierdo, M.S., et al. 2007. Two microalgae *Crypthecodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for seabream (*Sparus aurata*). Aquaculture 270 (1-4), 178-185. http://doi.org/10.1016/j.aquaculture.2007.04.009

Bakke-McKellep, A.M., Press, C.M., Baeverfjord, G., et al. 2000. Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. J Fish Dis. 23, 115-127. http://doi.org/10.1046/j.1365-2761.2000.00218.x.

Bakke-McKellep, A.M., Penn, M.H., Salas, P.M., et al. 2007. Effects of dietary soybean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). Br J Nutr. 97, 699-713. http://doi.org/10.1017/S0007114507381397. PMID: 17349083.

Becker, E.W. 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207-210. http://doi.org/10.1016/j.biotechadv.2006.11.002. PMID: 17196357.

Bonaldo, A., Roem, A.J., Fagioli, P., et al. 2008. Influence of dietary levels of soybean meal on the performance and gut histology of gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) Aquac Res. 39, 970-978. http://doi.org/10.1111/j.1365-2109.2008.01958.x.

Boonyaratpalin, M., Suraneiranat, P., Tunpiball, T. 1998. Replaicement of fish meal with types of soybean products in diets for the Asia sea bass (*Lates calcarifer*). Aquaculture. 161, 67-78.

Bowden, T.J., Butler, R., Bricknell, I.R., et al. 1997. Serum trypsin-inhibitory activity in five species of farmed fish Fish. Shellfish Immunol. 7, 377-385. http://doi.org/10.1006/fsim.1997.0092.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bravo-Tello, K., Ehrenfeld, N., Solís, C.J., et al. 2017. Effect of microalgae on intestinal inflammation triggered by soybean meal and bacterial infection in zebrafish. PLoS ONE 12(11): e0187696. http://doi.org/10.1371/journal.pone.0187696 .

Brown, M.R., Jeffrey, S.W., Volkman, J.K., et al. 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151, 315-331. http://doi.org/10.1016/S0044-8486(96)01501-3.

Brown, M.R. 2002. Nutritional Value and Use of Microalgae in Aquaculture. In: Cruz-Suárez L.E., Ricque-Marie D., Tapia-Salazar M., Gaxiola-Cortés M.G., Simoes N. (ed). Avances en Nutrición Acuicola VI. Memorias del VI Simposium Internacionale de Nutrición Acuicola. 3-6-septiembre 2002. Cancun, Quintana Roo, Mexico. pp 281-292.

Bucking, C. and Schulte, P.M., 2012. Environmental and nutritional regulation of expression and fuction of two peptide tranporter (PepT1) isoforms in a euryhaline teleost. Comp. Biochem. Physiol. Part A. 161, 379-387.

Cardinaletti, G., Messina, M., Bruno, M., et al. 2018. Effects of graded levels of a blend of *Tisochrysis lutea* and *Tetraselmis suecica* dried biomass on growth and muscle tissue composition of European sea bass (*D. labrax*) fed diets low in fish meal and oil. Aquaculture. 485, 173-182. http://doi.org/10.1016/j.aquaculture.2017.11.049.

Cerezuela, R., Guardiola, F.A., Meseguer, J., et al. 2012. Enrichment of gilthead seabream (*Sparus aurata* L.) diet with microalgae: effects on the immune system. Fish Physiol. Biochem. 38,1729–1739. http://doi.org/10.1007/s10695-012-9670-9. PMID: 22678706.

Chou, N., Cheng, C., Wu, H., et al. 2012. Chlorella sorokiniana-Induced Activation and Maturation of Human Monocyte-Derived Dendritic Cells through NF-kappa B and PI3K/MAPK Pathways. Evid-based Compl Alt Article Number: 735396. http://doi.org/10.1155/2012/735396.

Coz-Rakovac, R., Strunjak-Perovic, I., Hacmanjek, M., et al. 2005. Blood chemistry and histological properties of wild and cultured sea bass (*Dicentrarchus labrax*) in north Adriatic sea. Vet Res Comm. 29, 677-687. http://doi.org/10.1007/s11259-005-3684-z. PMID: 16369882.

Dantas, D.C.M., Kaneno, R., Queiroz, M.L.S., 1999. The effects of *Chlorella vulgaris* in the protection of mice infected with *Listeria monocytogenes*. Role of natural killer cells. Immunopharm Immunot. 21, 609-619. http://doi.org/10.3109/08923979909007129.

de Francesco, M., Parisi, G., Pérez-Sanchez, J., et al. 2007. Effects of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. Aquac Nutr. 13, 361-372. http://doi.org/10.1111/j.1365-2095.2007.00485.x.

Directive 2010/63/EU. Official Journal of the European Union. Directive of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes.

Escaffre, A., Kaushik, S., Mambrini, M. 2007. Morphometric evaluation of changes in the digestive tract of rainbow trout (*Oncorhynchus mykiss*) due to fish meal replacement with soy protein concentrate. Aquaculture. 27, 3127–138. http://doi.org/10.1016/j.aquaculture.2007.09.028.

Geay, F., Ferraresso, S., Zambonino-Infante, J.L., et al. 2011. Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. BMC Genomics. 12, 522. http://doi.org/10.1186/1471-2164-12-522. PMID: 22017880.

Gilbert ER, Wong EA, Webb KE, Jr. 2008. Board-invited review: Peptide absorption and utilization: Implications for animal nutrition and health. J. Anim. Sci. 86, 2135-2155. http://doi.org/10.2527/jas.2007-0826. PMID: 18441086.

Gilbert, E.R., Li, H., Emmerson, D.A., et al. 2010. Dietary protein composition influences abundance of peptide and amino acid transporter messenger ribonucleic acid in the small intestine of 2 lines of broiler chicks. Poultry Sci. 89, 1663-1676. http://doi.org/10.3382/ps.2010-00801 .

Goda,T. 2000. Regulation of expression of carbohydrate digestion/absorption-related genes. Bri J Nutr. 84, Suppl.2: S245-S248. http://doi.org/10.1079/096582197388626. PMID: 11242478.

Gonzales, T. and Robert-Baudouy, J. 1996. Bacterial aminopeptidases: properties and functions. FEMS Microbiol. Rev. 18, 319–344. http://doi.org/10.1016/0168-6445(96)00020-4 . PMID: 8703509.

Grammes, F., Reveco, F.E., Romarheim, O.H., et al. 2013. *Candida utilis* and *Chlorella vulgaris* Counteract Intestinal Inflammation in Atlantic Salmon (*Salmo salar* L.) PLOS ONE. 8 (12), e83213. http://doi.org/10.1371/journal.pone.0083213. PMID: 24386162.

Hakim, Y., Harpaz, S., Uni, Z. 2009. Expression of brush border enzymes and transporters in the intestine of European sea bass (*Dicentrarchus labrax*) following food deprivation. Aquaculture. 290, 110-115. http://doi.org/10.1016/j.aquaculture.2009.02.008.

Harpaz, S., Hakim, Y., Slosman, T., et al. 2005. Effects of adding salt to the diet of Asian sea bass *Lates calcarifer* reared in fresh or salt water recirculating tanks, on growth and brush border enzyme activity. Aquaculture. 248, 315-324. http://doi.org/10.1016/j.aquaculture.2005.03.007.

Harpaz, S. and Uni, Z. 1999. Activity of intestinal mucosal brush border membrane enzymes in relation to the feeding habits of three aquaculture fish species. Comp Biochem Physiol Part a Mol Integr Physiol. 124, 155-160. http://doi.org/10.1016/S1095-6433(99)00106-3.

Hemaiswarya, S., Raya, R., Ravi, K.R, et al. 2011. Microalgae: a sustainable feed source for aquaculture. World J Microbiol Biotechnol. 27, 1737-1746. http://doi.org/10.1007/s11274-010-0632-z.

Hemre, G.I., Mommsen, T.P., Krogdahl A. 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquaculture Nutrition. 8, 175-194. http://doi.org/10.1046/j.1365-2095.2002.00200.x.

Hooton, D., Lentle, R., Monro, J., et al. 2015. The secretion and action of brush border enzymes in the mammalian small intestine. Rev Physiol, Biochem, Pharmacol. 168, 59-118. http://doi.org/10.1007/112_2015_24. PMID: 26345415.

Kim, K.W., Bai, S.C., Koo, J.W., et al. 2002. Effects of dietary *Chlorella ellipsoidea* supplementation on growth, blood characteristics, and whole-body composition in juvenile Japanese flounder *Paralichthys olivaceus*. Journal of the World Aquaculture Society, 33, 425-431. http://doi.org/10.1111/j.1749-7345.2002.tb00021.x.

Kim, S.S. and Lee, K.J. 2008. Effects of dietary kelp (*Ecklonia cava*) on growth and innate immunity in juvenile olive flounder *Paralichthys olivaceus* (Temminck et Schlegel). Aquac Res. 39, 1687-1690. http://doi.org/10.1111/j.1365-2109.2008.02046.x.

Kiron, V., Phromkunthong, W., Huntley, M., et al. 2012. Marine microalgae from biorefinery as a potential feed protein source for Atlantic salmon, common carp and whiteleg shrimp. Aquac. Nutr. 18, 521-531. http://doi.org/10.1111/j.1365-2095.2011.00923.x.

Kokou, F., Rigos, G., Henry, M., et al. 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture. 364, 74-81. http://doi.org/10.1016/j.aquaculture.2012.08.009.

Krogdahl, Å., Nordrum, S., Sorensen, M., et al. 1999. Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L. Aquac Nutr. 5, 121-133.

Krogdahl, Å., Bakke-McKellep, A.M., Baeverfjord, G. 2003. Effects of graded levels of standard soybean meal on intestinal structure mucosal enzyme activities and pancreatic response in Atlantic salmon (*Salmo salar* L.). Aquacult. Nutr. 9, 361-371. http://doi.org/10.1046/j.1365-2095.2003.00264.x.

Krogdahl, A., Hemre, G.I., Mommsen, T.P. 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquac Nutr. 11, 103-122. http://doi.org/10.1111/j.1365-2095.2004.00327.x .

Laurens, L.M.L., Dempster, T.A., Jones, H.D.T., et al. 2012. Algal Biomass Constituent Analysis: Method Uncertainties and Investigation of the Underlying Measuring Chemistries. Anal. Chem. 84, 1879–1887. http://doi.org/10.1021/ac202668c. PMID: 22242663.

Li, M., Wu, W., Zhou, P., et al. 2014. Comparison effect of dietary astaxanthin and *Haematococcus pluvialis* on growth performance, antioxidant status and immune response of large yellow croaker *Pseudosciaena crocea*. Aquaculture. 434, 227-232. http://doi.org/10.1016/j.aquaculture.2014.08.022.

Liu, Z., Zhou, Y., Liu, S., et al. 2014. Characterization and dietary regulation of oligopeptide transporter (PepT1) in different ploidy fishes. Peptides, 52, 149-156. http://doi.org/10.1016/j.peptides.2013.12.017. PMID: 24406900.

Maeda, H., 2015. Nutraceutical effects of fucoxanthin for obesity and diabetes therapy: a review. J. Oleo. Sci. 64 (2), 125-132.

Marjara, I.S., Chikwati, E.M., Valen, E.C., et al. 2012. Transcriptional regulation of IL-17A and other inflammatory markers during the development of soybean meal-induced enteropathy in the distal intestine of Atlantic salmon (*Salmo salar* L.). Cytokine. 60, 186-196. http://doi.org/10.1016/j.cyto.2012.05.027. PMID: 22795954.

Merrifield, D.L., Dimitroglou, A., Bradley, G., et al. 2009. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, *Oncorhynchus mykiss* (Walbaum) J Fish Disease. 32, 755-766. http://doi.org/10.1111/j.1365-2761.2009.01052.x. PMID: 19490393.

Messina, M., Piccolo, G., Tulli, F., et al. 2013. Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax* L.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. Aquaculture. 376, 6-14. http://doi.org/10.1016/j.aquaculture.2012.11.005.

Metochis, C.P., Spanos, I., Auchinachie, N., et al. 2016. The effects of increasing dietary levels of soy protein concentrate (SPC) on the immune responses and disease resistance (furunculosis) of vaccinated and non-vaccinated Atlantic salmon (*Salmo salar* L.) parr. Fish & Shellfish Immunology. 59, 83-94. http://org.doi/10.1016/j.fsi.2016.10.016. PM ID: 27742588.

Mourente, G. and Bell, J.G. 2006. Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass (*Dicentrarchus labrax* L.): effects on flesh fatty acid composition, plasma prostaglandins E-2 and F-2 alpha, immune function and effectiveness of a fish oil finishing diet. Aquac Nutr. 11, 25-40. http://doi.org/10.1016/j.cbpb.2006.08.012. PMID: 17055762.

Naylor, R.L., Hardy, R.W., Bureau D.P., et al. 2009. Feeding aquaculture in an era of finite resources Proceedings of the National Academy of Sciences of the United States of America. 106 (36), 15103-15110.

Ostaszewska, T., Kamaszewski, M., Grochowski, P., et al. 2010. The effect of peptide absorption on PepT1 gene expression and digestive system hormones in rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol A: Mol Integr Physiol. 155, 107–114. http://org.doi/10.1016/j.cbpa.2010.03.013.

Palmegiano, G.B., Gai, F., Gasco, L., et al. 2009. Partial replacement of fish meal by *T-Iso* in gilthead sea bream (*Sparus aurata*) juveniles diets. Ital. J. Anim. Sci. 8 (Suppl.2), 869-871. http://org.doi10.4081/ijas.2009.s2.869.

Parry RM, Chandan RC, Shahani RM. 1965. A rapid sensitive assay of muramidase. Proceedings of the Society for Experimental Biology and Medicine. 119, 384–386.

Pfaffl, M.W., Tichopad, A., Prgomet, C., et al. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations Biotecn Letters. 26, 509-515. http://doi.org/10.1023/B:BILE.0000019559.84305.47 . PMID: 15127793.

Peng, M., Xu, W., Ai, Q., et al. 2013. Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scophthalmus maximus* L.) Aquaculture. 392-395, 51-58. http://doi.org/10.1016/j.aquaculture.2013.02.002 .

Quade, M.J., Roth, J.A. 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet Immunol Immunopathol. 58 (3-4), 239-248. http://doi.org/S0165-2427(97)00048-2 .

Ragaza, J.A., Koshio, S., Mamauag, R.E., et al. 2015. Dietary supplemental effects of red seaweed *Eucheuma denticulatum* on growth performance, carcass composition and blood chemistry of juvenile Japanese flounder, *Paralichthys olivaceus*. Aquaculture Research. 46, 647-657. http://doi.org/10.1111/are.12211

Renaud, S.M., Thinh, L.V., Parry, D.L. 1999. The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. Aquaculture. 170, 147-159. http://org.doi/10.1016/S0044-8486(98)00399-8.

Reyes-Becerril, M., Guardiola, F., Rojas, M., et al. 2013. Dietary administration of microalgae Navicula sp. affects immune status and gene expression of gilthead seabream (*Sparus aurata*). Fish & Shellfish Immunol 35, 883-889. http://org.doi/10.1016/j.fsi.2013.06.026.

Reyes-Becerril, M., Angulo, C., Estrada, N., et al. 2014. Dietary administration of microalgae alone or supplemented with Lactobacillus sakei affects immune response and intestinal morphology of Pacific red snapper (*Lutjanus peru*). Fish & Shellfish Immunol. 40, 208-216. http://doi.org/10.1016/j.fsi.2014.06.032 . PMID: 25014313.

Rimoldi, S., Bossi, E., Harpaz, S., et al. 2015. Intestinal B0AT1 (SLC6A19) and PEPT1 (SLC15A1) mRNA levels in European sea bass (*Dicentrarchus labrax*) reared in fresh water and fed fish and plant protein sources. J Nutr Sci. 4, e21. http://doi.org/10.1017/jns.2015.9 .

Rønnestad, I., Gavaia, P.J., Viegas, C.S., et al. 2007. Oligopeptide transporter PepT1 in Atlantic cod (*Gadus morhua* L.): cloning, tissue expression and comparative aspects. J Exp Biol. 210, 3883–3896. http://doi.org/10.1242/jeb.007898 .

Sitja-Bobadilla, A., Pena-Llopis, S., Gomez-Requeni, P., et al. 2000. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture. 249, 387-400. http://doi.org/10.1016/j.aquaculture.2005.03.031 .

Schmittgen, T.D., and Livak, K.J. 2008. Analyzing real-time PCR data by the comparative C-T method. Nature protocols. 3(6),1101-1108. http://doi.org/10.1038/nprot.2008.73

Sorensen, M., Berge, G.M., Reitan, K.I., et al. 2016. Microalga *Phaeodactylum tricornutum* in feed for Atlantic salmon (*Salmo salar*) - Effect on nutrient digestibility, growth and utilization of feed Aquaculture. 460, 116-123. http://doi.org/10.1016/j.aquaculture.2016.04.010.

Spolaore, P., Joannis-Cassan, C., Duran E., et al. 2006. Commercial applications of microalgae. J. Biosc. Bioeng. 101. 87-96. http://doi.org/10.1263/jbb.101.87 . PMID: 16569602.

Tacon, A.G.J., Hasan, M.R., Metian, M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects.. FAO Fisheries and Aquaculture Technical Paper No. 564: 87.

Tang, J., Qu, F., Tang, X., et al. 2016. Molecular characterization and dietary regulation of aminopeptidase N (APN) in the grass carp (*Ctenopharyngodon idella*). Gene. 582, 77–84. http://doi.org/10.1016/j.gene.2016.01.046. PMID: 26828613.

Taylor A. 1993. Aminopeptidases: towards a mechanism of action. Trends Biochemistry in Science. 18, 167–171.

Templeton, D.W., Quinn, M., Van Wychen, S., et al. 2012. Separation and quantification of microalgal carbohydrates. Journal of Chromatography A. 1270, 225– 34. http://doi.org/10.1016/j.chroma.2012.10.034 . PMID: 23177152.

Terova, G., Corà, S., Verri, T., et al. 2009. Impact of feed availability on PepT1 mRNA expression levels in sea bass (*Dicentrarchus labrax*). Aquaculture. 294, 288–299. http://org.doi/10.1016/j.aquaculture.2009.06.014 .

Terova, G., Robaina, L., Izquierdo, M., et al. 2013. PepT1 mRNA expression levels in sea bream (*Sparus aurata*) fed different plant protein sources. SpringerPlus 2, 17. http://doi.org/10.1186/2193-1801-2-17 .

Tibaldi, E., Hakim, Y., Uni, Z., et al. 2006. Effects of the partial substitution of dietary fishmeal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). Aquaculture. 261, 182–193. http://doi.org/10.1016/j.aquaculture.2006.06.026 .

Tibaldi, E., Chini Zittelli, G., Parisi, G., et al. 2015. Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried *Isochrysis sp.*(T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. Aquaculture. 440, 60-68. http://doi.org/10.1016/j.aquaculture.2015.02.002 .

Tokuşoglu, Ö. and Ünal, M.K. 2003. Biomass Nutrient Profiles of Three Microalgae: *Spirulina platensis, Chlorella vulgaris*, and *Isochrisis galbana*. J Food Sci. 68, 1144-1148. http://doi.org/10.1111/j.1365-2621.2003.tb09615.x .

Tulli, F., Chini Zittelli, G., Giorgi, G., et al. 2012. Effect of the Inclusion of Dried *Tetraselmis suecica* on Growth, Feed Utilization, and Fillet Composition of European Sea Bass Juveniles Fed Organic Diets. J. Aquat. Food Prod T. 21, 1-11. http://doi.org/10.1080/10498850.2012.664803 .

Uran, P.A., Goncalves, A.A., Taverne-Thiele, J.J., et al. 2008 Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio L.*). Fish & Shellfish Immunology. 25 (6): 751-760. http://doi.org/10.1016/j.fsi.2008.02.013.

Van den Ingh, T.S., Krogdhal, A., Olly, J.J., et al. 1991. Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. Aquaculture. 94(4), 297-305.

Vargas, A., Randazzo, B., Riolo, P., et al. 2018. Rearing Zebrafish on Black Soldier Fly (*Hermetia illucens*): Biometric, Histological, Spectroscopic, Biochemical, and Molecular Implications. Zebrafish. 15 (4). http://doi.org/10.1089/zeb.2017.1559

Verri, T., Kottra, G., Romano, A., et al. 2003. Molecular and functional characterisation of the zebrafish (*Danio rerio*) PEPT1- type peptide transporter. FEBS Lett. 115–122. http://doi:10.1016/S0014-5793(03)00759-2.

Verri, T., Danieli, A., Bakke, S., et al. 2008. A rapid and inexpensive method to assay transport of short chain peptides across intestinal brush-border membrane vesicles from the European eel

(*Anguilla anguilla*). Aquaculture Nutrition. 14, 341-349. http://doi.org/10.1111/j.1365-2095.2007.00538.x

Verri, T., Barca, A., Pisani, P., et al. 2017. Di- and tripeptide transport in vertebrates: the contribution of teleost fish models. Comparative Physiology, B, 187, 395–462. http://doi.org/10.1007/s00360-016-1044-7 . PMID: 27803975.

Vizcaíno, A.J., López, G., Sáez, M.I. et al. 2014. Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream. *Sparus aurata* juveniles. Aquaculture. 431, 34-43. http://doi.org/10.1016/j.aquaculture.2014.05.010 .

Vizcaino, A.J., Saez, M.I., Lopez, G., et al. 2016. *Tetraselmis suecica* and *Tisochrysis lutea* meal as dietary ingredients for gilthead sea bream (*Sparus aurata* L.) fry. J. App. Phycol. 28 (5), 2843-2855. http://doi.org/10.1007/s10811-016-0845-0

Vizcaíno, A.J., Rodiles, A., López, G., et al. 2018. Growth performance, body composition, and digestive functionality of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles fed diets including microalgae freeze-dried biomass. Fish Physiol Biochem. https://doi.org/10.1007/s10695-018-0462-8 .

Volkman, J.K., Jeffrey, S.W., Nichols, P.D., et al. 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. J Exp Mar Biol Ecol. 128, 219-240. http://doi.org/10.1016/0022-0981(89)90029-4 .

Volpatti, D., Bulfon, C., Tulli, F., et al. 2013. Growth parameters, innate immune response and resistance to *Listonella* (*Vibrio*) *anguillarum* of *Dicentrarchus labrax* fed carvacrol supplemented diets. Aquac Res. 45, 31–44. http://doi.org/10.1111/j.1365-2109.2012.03202.x .

Walker, A.B. and Berlinsky, D.L. 2011. Effect of Partial Replacement of fish meal protein by Microalgae on Growth, Feed Intake, and Body Composition of Atlantic Cod. N Am J Aquac. 73, 76-83. http://doi.org/10.1080/15222055.2010.549030 .

Yaakob, Z., Ali, E., Zainal, A., et al. 2014. An overview: biomolecules from microalgae from animal an aquaculture. J Biol Res. 21, 6. http://doi.org/10.1186/2241-5793-21-6 .

Yasutake, H., Goda, T., Takase, E., 1995. Dietary regulation of sucrose-isomaltase gene expression in rat jejunum. Biochimica et Biophysica Acta. 676, 108-112. http://doi.org/10.1016/0304-4165(94)00143-L . PMID: 7873573.

Zuo, X., Woo, P.T.K. 1997. In vivo neutralization of Cryptobia salmositica metallo-protease by a 2-macroglobulin in the blood of rainbow trout *Oncorhynchus mykiss* and in brook charr *Salvelinus fontinalis*. Dis. Aquat. Org. 29, 67-72. http://doi.org/10.3354/dao029067 .

Fig 1. Serum lysozyme, antiprotease and myeloperoxidase activity (n = 3 tanks, 4 fish/tank), and respiratory burst activity (n = 3 tanks, 2 fish/tank) in E. sea bass at the end of the 105-days feeding period. Data are presented as means ± SD. Different letters indicate significant differences among dietary treatments (P<0.05).

Fig 2. Morphological appearance of the caudal portion of the proximal intestine of E. sea bass fed control (A), SBM (B), MA15 (C) and MA45 (D) diets at the end of the 105-days feeding period. H-E staining.

Fig. 3. Enzymatic activity of maltase, sucrase, ALP and γ-GT in PC, PI and DI of E. sea bass at the end of the 105-days feeding period. Data are presented as means ± SD (n= 3 tanks, 2 fish/tank). Different letters indicate significant differences among intestinal tracts of the same diet (P<0.05).

1) Graded levels of a mixture of *Tisochysis lutea* and *Tetraselmis suecica* dried biomass were tested in European sea bass diets low in fish meal and oil and compared with a soybean meal –rich diet.
2) The proximal intestine of fish fed diets including microalgae showed a greater villi height and thickness compared to fish fed the soybean meal–rich one.
3) Including the dried microalgae mixture in the diet, increased the gene expression of certain brush border membrane enzymes without affecting the activity of those involved in the final stages of the digestive-absorptive processes.
4) Certain innate immune response parameters improved in fish fed diets with moderate levels of dietary microalgae mixture.