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Impact of date palm fruits extracts and probiotic enriched diet on antioxidant status, innate immune response and immune-related gene expression of European seabass (*Dicentrarchus labrax*)

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ABSTRACT

The application of additives in the diet as plants or extracts of plants as natural and innocuous compounds has potential in aquaculture as an alternative to antibiotics and immunoprophylactics. The aim of the current study was to evaluate the potential effects of dietary supplementation of date palm fruit extracts alone or in combination with Pdp11 probiotic on serum antioxidant status, on the humoral and cellular innate immune status, as well as, on the expression levels of some immune-related genes in head-kidney and gut of European sea bass (*Dicentrarchus labrax*) after 2 and 4 weeks of administration. This study showed for the first time in European sea bass an immunostimulation in several of the parameters evaluated in fish fed with date palm fruits extracts enriched diet or fed with this substance in combination with Pdp 11 probiotic, mainly after 4 weeks of treatment. In the same way, dietary supplementation of mixture diet has positive effects on the expression levels of immune-related genes, chiefly in head-kidney of *Dicentrarchus labrax*. Therefore, the combination of both could be considered of great interest as potential additives for farmed fish.

1. Introduction

The contribution of aquaculture to world food production has increased significantly over the last few decades and this sector now supplies nearly half of the total fish and shellfish used for human consumption [1]. This industry remains as an important food producing sector in the world [2], nonetheless is challenged by several daunting issues on sustainability from biological, environmental and socio-economic points of view [3]. The downside of intensification of the farming operations have been economic losses because of the mortality, since these conditions can lead the animals to be susceptible to infections and stressors [4]. In aquaculture, one of the most promising methods of controlling diseases and stressors impact is by enhancing the defense mechanism of fish through prophylactic administration of immunostimulants [5], which are considered as a hopeful alternative to chemotherapy and vaccines [6]. All these preventive measures are aimed at enhancing the innate and/or the adaptive immune system [7], as well as, protect animals from free radicals and the effects of ROS.

The use of immunostimulants is an effective means of increasing the immuno competency and disease resistance of fish [8]. Their most proved effect is to facilitate the function of phagocytic cells and increase their bactericidal and fungicidal activities, as well as, to play an important role as natural antioxidants [see Refs. [9–12] for review]. From these reviews could be extracted than a wide variety of plant extracts have been studied as dietary additives in different fish species of interest in aquaculture, with the endeavor of fighting fish diseases, due to the fact that they have varied beneficial effects on the host, like stimulation of immunity, among others. Plant extracts have been reported as anti-stressors, growth promoters, appetite stimulators, enhancement of tonicity and immunostimulation, maturation of culture species and anti-pathogen properties in aquaculture fish due to active principles (e.g. alkaloids, terpenoids, tannins, saponins, etc.) [13,14]. Furthermore, plant extracts can be considered as an alternative to other

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substances (such as antibiotics or chemicals) used to control aquaculture diseases [15]. Moreover, these natural immunostimulants are more environmentally friendly due to the fact that they are more biodegradable than synthetic molecules and they do not produce drug resistance [16,17].

Date palm (*Phoenix dactylifera* Linn) is a valuable plant that grows in the Southwest Asia and North Africa [18]. Due to their rich beneficial health and nutritional properties, the date palm fruit has been used for many years in folk medicine in Middle East and some Asian countries for promotion of good health, treating cancer and several infectious diseases [19]. However, to the best of our knowledge only two previous studies have focused on the effects of dietary administration of palm fruits extracts in fish, revealing that date palm fruit extracts enhance the mucosal immunity and gene expression and could serve as good natural antioxidants [18,20]. In fact, there is growing interest in the search for natural antioxidants, especially those of plant origin, for replacement of synthetic antioxidants, due to their potential health benefits [21,22]. Recently, several phytochemical studies have shown that date fruits contain anthocyanins, phenolic substances, sterols, carotenoids, flavonoids, vitamins, enzymes and high amounts of carbohydrates [23,24], compounds that demonstrated its great potential as a natural immunostimulants.

To our days, it is also known that a combination of probiotics and natural immunostimulants, could prove more beneficial effects to fish than a single administration of one of them [20]. In this way, probiotics confer health benefits to the host modulating gastrointestinal microbial communities. These benefits occur at different levels, such as suppression of pathogen growth, immunological enhancement, stimulation of growth, improvement of stress tolerance, etc. [25,26]. Previous studies with *Shewanella putrefaciens* Pdp11, a bacteria isolated from the skin mucus of healthy gilthead seabream [27], have demonstrated that this probiotic improves fish gastrointestinal tract morphology and microbiota, nutrition, immune and antioxidant status, disease resistance, and also mitigates the stress response in Senegalese sole (*Solea senegalensis*) and gilthead seabream [20,28].

Taking into account all these previous considerations, the aim of this work was evaluated the effects of the dietary date palm fruit extracts administration, alone or in combination with Pdp11, on immune and antioxidant status European sea bass (*Dicentrarchus labrax*), one the major cultured fish species in the Mediterranean area. The possibility to use these natural ingredients on fish diet formulations is discussed.

2. Materials and methods

2.1. Animals

Forty-eight (40.5 ± 9.3 g weight and 16 ± 1.1 cm length) specimens of the sea water teleost European sea bass (*Dicentrarchus labrax*), obtained from the local farm (Predomar S.I., Almería, Spain), were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1} and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Preparation of microorganism

S. putrefaciens (Pdp11) was grown in tubes containing trypticase soya broth (TSB, Sigma) supplemented with 1.5% NaCl (TSBs) at 22 °C, which were continuously shook for 18 h. The absorbance was measured at 600 nm from 1 ml aliquots of bacteria cell culture every hour for 9 h, until bacteria had been growing for 24 h. Simultaneously, it was measured the number of bacterial cells present per ml of culture medium of such aliquots by plating, in order to characterise the growth curve of bacterium. Subsequently, bacterial cell cultures were centrifuged (6000 g, 15 min, 4 °C), washed them in sterile PBS (pH 7.4), counted by plating and adjusted to the required concentrations.

2.3. Date palm fruit extracts

Date palm fruits were purchased freshly ripened of the *Degla* variety in a local supermarket (Murcia, Spain). To extract the water-soluble material, dried fruits (200 g) were washed using sterile distilled water, cut them into small-sized pieces, including peel and pulp, but not seeds, added 500 ml of sterilised distilled water, and incubated them for 2 h at a room temperature of 22 °C. The mixture was ground by stirring using a Moulinex machine and the supernatant was collected by centrifugation (2000 rpm, 15 min) and stored at 4 °C until use.

2.4. Experimental diets

The probiotic bacterial cells and the aqueous palm fruit extracts were added to the commercial diet according to [29]. The concentrations used in the present work are that used in our previous studies giving us better results [29,30]. Briefly, the probiotic and date palm extracts were dissolved, alone and in combination, in the least possible amount of cod oil, which was then sprayed on the pellets before feeding the animals. The non-supplemented diet (control) was sprayed only with cod oil. All the experimental diets were kept in a light protected environment and stored at 4 °C until use.

2.5. Experimental design

Fish were randomly assigned and divided into four identical tanks (n = 6) where four groups were established: 1) control, non-supplemented diet; 2) Pdp11 diet, control diet supplemented with *S. putrefaciens* Pdp11 (10^9 cfu g^{-1}); 3) date palm fruit diet, commercial diet supplemented with aqueous extracts of date palm fruits (100 g kg^{-1}); 4) mixture diet, commercial diet supplemented with Pdp11 (10^9 cfu g^{-1}) and extracts of palm fruits (100 g kg^{-1}). Six specimens were sampled from each aquarium following 2 or 4 weeks of feeding, after starving them for 24 h prior to sampling. All specimens were sacrificed by using an overdose of MS222 (Sandoz). All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.6. Sample collection

Fish were weighed and measured. Blood samples were collected from the caudal vein with an insulin syringe and the head-kidney (HK) and the gut were dissected. The blood samples were left to clot at 4 °C for 4 h and later the serum was collected after centrifugation (10,000 g, 5 min, 4 °C) and stored at −80 °C until use. Fragments of HK and gut were stored in TRIzol Reagent (Invitrogen) at −80 °C for gene expression analysis. Other HK samples were cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride, 2% foetal calf serum (FCS, Gibco), 100 i.u. ml^{-1} penicillin (Flow) and 100 μg ml^{-1} streptomycin (Flow)] [31]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh

(mesh size 100 μm), washed twice (400 g 10 min), counted (Z2 Coulter Particle Counter) and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was higher than 98%, as determined by the trypan blue exclusion test. All the cellular immune functions were performed only in viable cells.

2.7. Anti- and pro-oxidant status

Anti-oxidant and pro-oxidant status was measured spectrophotometrically (BOECO, S-22 UV/VIS) by BAP (biological antioxidant potential) and d-ROMs (reactive oxygen metabolites) tests from serum according to the procedure outlined by the manufacturer (Diacron International s.r.l., Grosseto, Italy). Briefly for BAP measurement, 50 μl of R2 reagent (ferric salt) was transferred to 1 ml of R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 μl of reagent blank, calibrator or serum sample were added to the cuvette, mixed gently and read on the previous photometer. The values were expressed as μmol antioxidant substance L^{-1} of vitamin C used as an iron-reducing agent reference. In case of d-ROMs, 5 μl of reagent blank, calibrator or serum sample serum were added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 μL R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red colour was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli units. One Carratelli unit is equal to a hydrogen peroxide concentration of 0.08% mg.

2.8. Bacteriostatic activity

Three opportunist marine pathogenic bacteria (*Vibrio harveyi*, *Vibrio anguillarum and Photobacterium damselae* subsp. *piscicida*) and one non-pathogenic bacterium for fish (*Escherichia coli*) were used to determine the bacteriostatic activity present in serum samples. Bacteria were grown in agar plates at 25 °C in the adequate media: tryptic soy (TSB, Sigma) for *V. harveyi*, *V. anguillarum* and *P. damselae*, and Luria (LB, Sigma) for *E. coli*. Then, fresh single colonies of 1–2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 16 h at 25 °C on an orbital incubator at 200–250 rpm.

The serum antimicrobial activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort [32] with some modifications. Aliquots of 100 μl of each one of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of European sea bass serum samples. The OD of the samples was measured at 620 nm at 30 min intervals during 24 h at 25 °C. Samples without bacteria were used as blanks (negative control). Samples without serum were used as positive controls (100% growth or 0% bacteriostatic activity).

2.9. Immune parameters

2.9.1. Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [33]. Briefly, aliquots of 100 μl of serum (previously diluted 1/10 in 100 mM ammonium bicarbonate buffer) was incubated with 125 μl of 100 mM ammonium bicarbonate buffer containing 2% azocasein (Sigma) for 24 h at 30 °C. The reaction was stopped by adding 10% trichloro acetic acid (TCA). The mixture was then centrifuged (6000 g, 10 min) and the supernatants were transferred to a 96-well plate in triplicate containing 100 μl $well^{-1}$ of 1 N NaOH, and the OD read at 450 nm using a plate reader. Serum was replaced by trypsin (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), or by buffer, as negative controls (0% activity).

2.9.2. Antiprotease activity

Total antiprotease activity was determined by the ability of serum to inhibit trypsin activity [34]. Briefly, 10 μl of serum samples were incubated (10 min, 22 °C) with the same volume of standard trypsin solution (5 mg ml^{-1}). After adding 100 μl of 100 mM ammonium bicarbonate buffer and 125 μl of buffer containing 2% azocasein (Sigma), samples were incubated (2 h, 30 °C) and, following the addition of 250 μl of 10% TCA, a new incubation (30 min, 30 °C) was done. The mixture was then centrifuged (10,000 rpm, 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 μl $well^{-1}$ of 1 N NaOH, and the OD read at 450 nm using a plate reader. For a positive control, buffer replaced serum and trypsin, and for a negative control, buffer replaced the serum. The antiprotease activity was expressed in terms of percentage trypsin inhibition according to the formula: % Trypsin inhibition = (Trypsin OD – Sample OD) Trypsin $OD^{-1} \times 100$.

2.9.3. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [35]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and EGTA (ethylene glycol tetraacetic acid) were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22 °C, the samples were centrifuged at 400 g for 5 min at 4 °C to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (BMG Labtech). The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y $(1\text{-}Y)^{-1}$ against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental fish.

2.9.4. Serum and leukocyte peroxidase activity

The peroxidase activity in serum or leukocytes was measured according to Quade and Roth [36]. Briefly, 15 μl of serum were diluted with 135 μl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 50 μl of 20 mM 3,3′,5,5′- tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 were added. To determine the leukocyte peroxidase content, 10^6 HK leukocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (Sigma) and, after centrifugation (400 g, 10 min), 150 μl of the supernatants were transferred to a fresh 96-well plate containing 25 μl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 μl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leukocytes, respectively, were used as blanks.

2.9.5. Immunoglobulin M level

Total serum IgM levels were analysed using the enzyme-linked immunosorbent assay (ELISA) [37]. Thus, 20 μl per well of 1/100 diluted serum were placed in flat-bottomed 96-well plates in triplicate and the proteins were coated by overnight incubation at 4 °C with 200 μl of carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBT (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA, Sigma) in PBT, followed

by three rinses with PBT. The plates were then incubated for 1 h with 100 μl per well of mouse anti-seabass IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer, Sigma). After exhaustive rinsing with PBT the plates were developed using 100 μl of a 0.42 mM TMB solution, prepared daily in a 100 mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 μl of 2M H_2SO_4 and the plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody, whose OD values were subtracted for each sample value.

2.9.6. Respiratory burst activity

The respiratory burst activity of European seabass HK leukocytes was studied by a chemiluminescence method [38]. Briefly, samples of 10^6 leukocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 μl of HBSS containing 1 μg ml^{-1}phorbolmyristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added. The plate was shaken and luminescence immediately read in a plate reader (BMG labtech) for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.9.7. Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by European sea bass HK leukocytes was studied by flow cytometry [39]. Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5×10^7 cells ml^{-1} of sRPMI. Phagocytosis samples consisted of 125 μl of labelled-yeast cells and 100 μl of HK leukocytes in sRPMI (6.25 yeast cells:1 leukocyte). Samples were mixed, centrifuged (400 g, 5 min, 22 °C), resuspended and incubated at 22 °C for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 μl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 μl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leukocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) dot plots or histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the phagocytic cell population whilst the phagocytic capacity was the mean fluorescence intensity. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.10. Gene expression analysis by real-time PCR

After 2 and 4 weeks of feeding, total RNA was extracted from 0.5 g of European sea bass HK and anterior gut using TRIzol Reagent [40]. RNA was then quantified and the purity was assessed by spectrophotometry being the 260:280 ratios of 1.8–2.0. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SuperScript III reverse transcriptase (Invitrogen) with an oligo-dT_{18} primer. The expression of selected genes (Supplementary Table 1) was analysed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μl of 2 × SYBR Green supermix, 5 μl of primers (0.6 μM each) and 5 μl of cDNA template) were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample. Gene names follow the accepted nomenclature for zebrafish (http://zfin.org/).The primers used are shown in Supplementary Table 1. In all cases, each PCR was performed with triplicate samples.

2.11. Statistical analysis

All measurements were performed on three replicates. The results are expressed as mean ± standard error, SE. Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19.0 and differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Antioxidant status

Oxidative status of fish was analysed in serum of European sea bass specimens fed dietary supplementation of probiotics and palm fruits administered alone or in combination (Fig. 1). The biological antioxidant potential (BAP test) was statistically significantly increased in fish fed probiotic and palm fruit extract diets, compared with the values found in fish fed control (non-supplemented) diet and fish fed mixture diet for 2 and 4 weeks (Fig. 1A). Contrarily, the presence of reactive oxygen molecules (d-ROMs-test) not revealed any significantly variations along of the trial between the different experimental groups (Fig. 1B).

3.2. Bacteriostatic activity

Bacteriostatic activity of serum of specimens fed with the different supplement diets did not show any significant variation respect to the values found in sera from fish of the control group (non-supplement diet) or among fish from the different experimental groups (Supplementary Table 2).

3.3. Humoral immune parameters

Humoral immune parameters of European sea bass fed supplement diets were differently affected (Figs. 2 and 3). Protease and antiprotease activities were not modified by the tested diet, except for fish fed 2 weeks the mixture diet, in which this activity was statistically significantly decreased, respect to the levels of this activity recorded for the other experimental groups (Fig. 2).

The haemolytic complement activity present in the serum of specimens fed the different supplemented diets showed a statistically significant increase in fish fed for 2 weeks palm fruit extracts enriched diet, compared to the values obtained for the specimens of the other experimental groups (Fig. 3A). However, this activity was

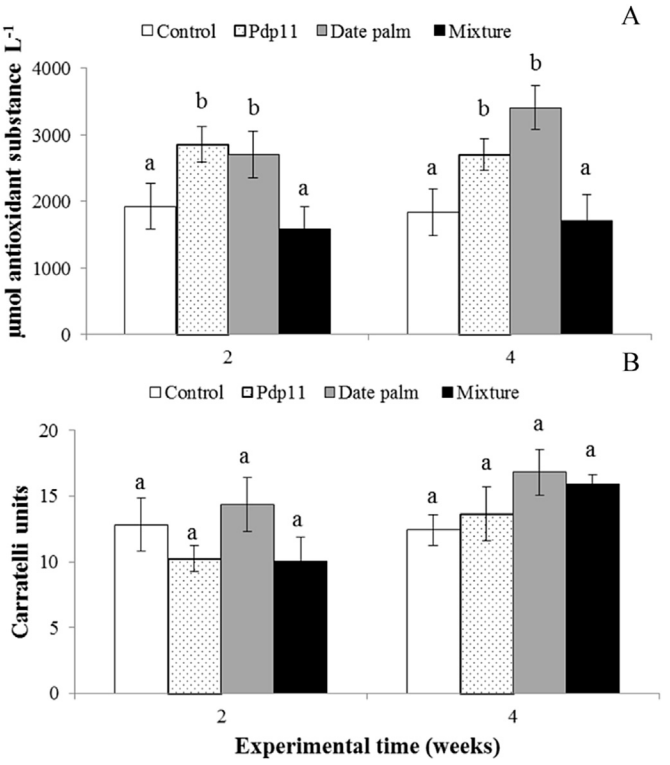

Fig. 1. Biological antioxidant potential (expressed as µmol antioxidant substance L^{-1}) (**A**) and reactive oxygen metabolites (expressed as Carratelli units) (**B**) in serum of European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 and 4 weeks of experiment. Bars represent the mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P \leq 0.05$).

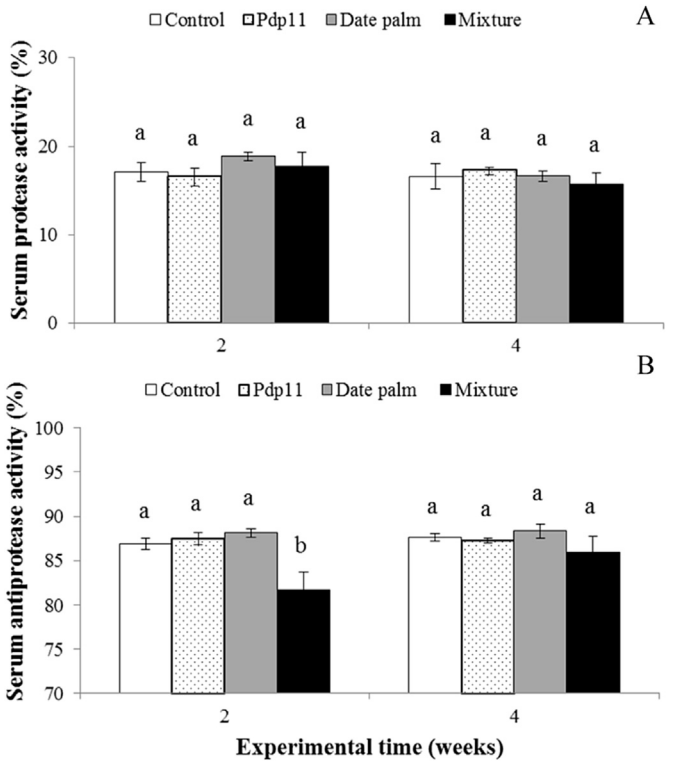

Fig. 2. Serum protease (**A**) and antiprotease (**B**) activities, expressed as %, of European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 and 4 weeks of experiment. Bars represent the mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P \leq 0.05$).

statistically significant decreased in fish fed the mixture diet for 4 weeks, but respect to the specimens fed date palm fruit extract diet (Fig. 3A). Finally, no significant variations were found on the seric peroxidase activity and total IgM level of European sea bass specimens after being fed with the tested experimental diets for 2 or 4 weeks (Fig. 3B and C).

3.4. Cellular immune parameters

Cellular immune parameters were modulated by the experimental diets. Respiratory burst activity of HK leucocytes was not significantly affected by any diet after 2 weeks, while a statistically significant decrease of this activity in fish fed with probiotic supplement diet (both alone or in combination with date palm fruits) was observed after 4 weeks of diet administration (Fig. 4A).

Peroxidase activity of European sea bass HK leukocytes did not show any significant variations throughout the experiment in any experimental group, respect to the values found in leucocytes from fish of the control group (Fig. 4B). On the contrary, phagocytic activity of HK leucocytes of European sea bass was increased in both terms, percentage of phagocytic cells (phagocytic ability) and number of particles ingested per phagocyte (phagocytic capacity). Thereby, phagocytic ability of HK leucocytes from fish fed mixture diet was increased after 4 weeks of diet administration, respect to the values obtained from leucocytes from specimens of control and Pdp11 groups (Fig. 4C). Similarly, phagocytic capacity was statistically significant enhanced in HK leucocytes from European sea bass fed mixture diet, respect to values of control group (non-supplement diet) and compared to all experimental groups after 2 and 4 weeks, respectively (Fig. 4D).

3.5. Gene expression

The expression of genes in the HK of European sea bass was differently affected by the different assayed diets (Fig. 5). A statistically significant up-regulation of *rbl* and *sod* genes was observed in fish fed probiotic supplement diet, as well as, in the expression of *il-1β* and *hep* genes in fish fed probiotic supplement diets (both alone or in combination with date palm fruit extracts) after 2 weeks (Fig. 5A). However, when fish were fed for 4 weeks, the *fbl* and *hep*genes, *rbl*, *il-1β* and *hep* genes, and *sod* and *lyz* genes expression was up-regulated in HK of fish fed probiotic diet, mixture diet and date palm fruit diet, respectively (Fig. 5B).

In the case of gut, a statistically significant down-regulation of *rbl* gene expression was observed in specimens fed with all supplement diets for 2 weeks, while a statistically significant up-regulation of *hep* and *lyz* genes was observed in fish fed probiotic diet, respect to the values found for HK leucocytes from control fish (Fig. 6A). However, after 4 weeks of trial, the expression of *fbl* and *sod* genes was statistically significant up-regulated in fish feddate palm fruit diet and probiotic diets, respectively. Contrarily, *hep* gene expression was significantly decreased in all experimental groups, respect to the values recorded for gut from control fish (Fig. 6B).

4. Discussion

Many studies have indicated that supplement feeding with probiotics and natural immuno stimulants, such as plant extracts, are beneficial to aquaculture of many different species of vertebrates and invertebrates [12,41,42]. In fact, an increasing interest in the use of plant extracts as fish immuno stimulants has emerged in the last decade [43,44,18]. Previous works developed by our team reported different effects of date palm fruit extracts [18,20,45], as

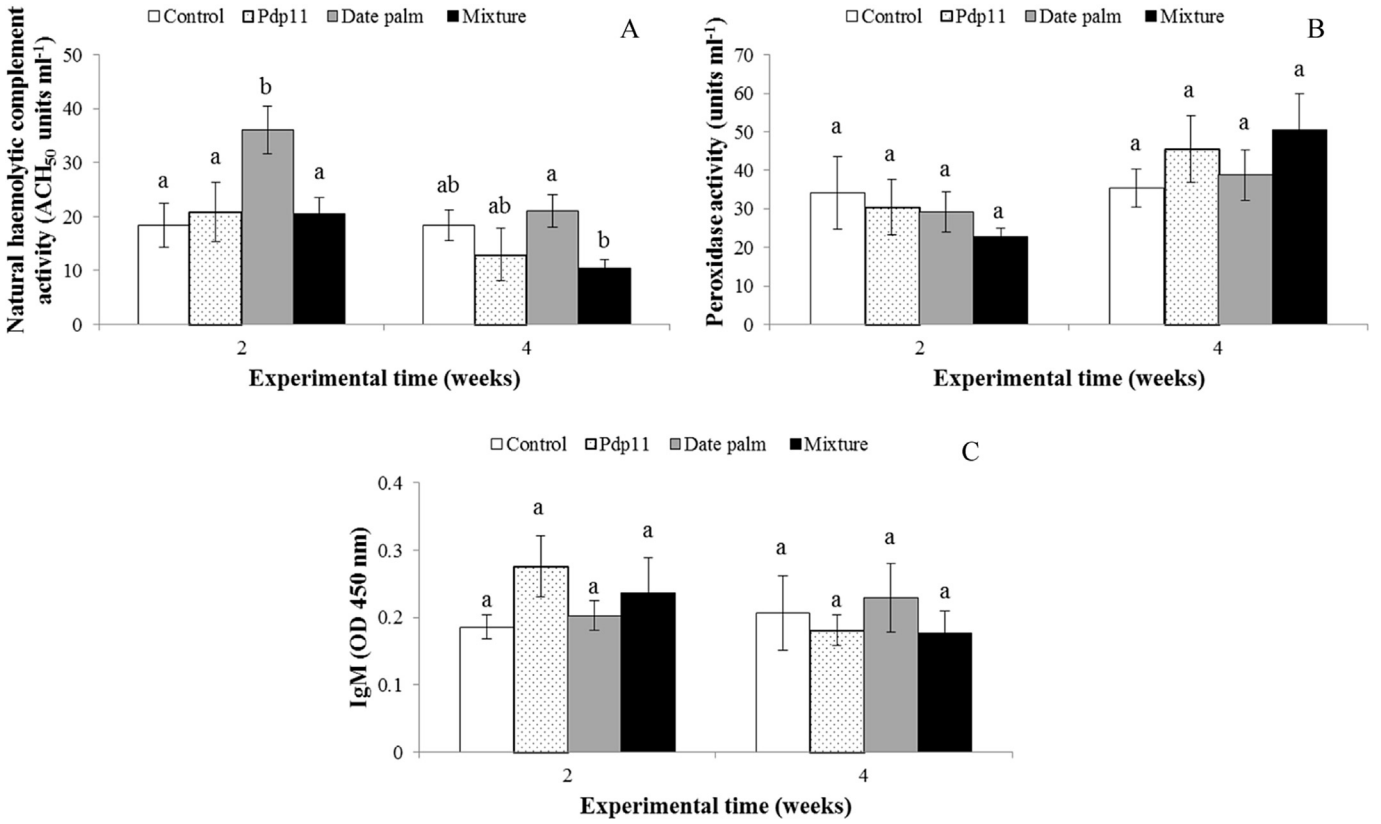

Fig. 3. Humoral immune parameters in the serum of European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 and 4 weeks of experiment. **A**. Natural haemolytic complement activity. **B**. Peroxidase activity. **C**. Total immunoglobulin M. Bars represent the mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P \leq 0.05$).

well as Ppd11 [28,46–48] on fish.

To achieve the reduction of oxidative stress increase the levels of antioxidants could be a solution [49]. Antioxidants are essential nutrients necessary for optimal growth, development, and reproduction in animals [50,51]. In fact, several synthetic antioxidants are authorized for use as feed additives in the European Union [52] However, there is growing interest in the replacement them by natural antioxidants, as a strategy to prevent oxidative damage in various health disorders, in which oxidative stress is known to play a part. In fish, works focus on immuno stimulants (reviewed by Ref. [53]) are more numerous than those focus on antioxidant properties of natural products. Present results corroborate previous data obtained in gilthead sea bream [20], evidencing the antioxidant properties of both date palm fruit extracts and the probiotic Pdp11, but when administering both in an individual way. However, when a mix diet with two probiotics (Pdp11 and *Bacillus*) and date palm fruit extracts was administrated to fish, it was observed an important increase in the antioxidant protein (*nkefa*) expression in skin after 2 weeks of feeding [18]. The reason of this fact deserves further investigations to validate its antioxidant effects and fully appreciate its potential as candidate additive for fish diet.

Regarding humoral innate immune system, various peptides such as antiprotease, protease, lysozymes, antibodies, complement factors, bactericidal components and other lytic factors are present in serum, where they prevent adherence and colonization of microorganisms [54] leading to prevention of infection and disease [55]. In the present study, we found no significant variations in the seric total IgM levels, protease and peroxidase activities, whilst the antiprotease and the haemolytic complement activity in the serum were altered. Antiprotease activity in serum inhibits the activity of the proteases used by certain bacteria to invade the host [42]. Besides, it has been reported that the hydrolysis of protein *in vivo* is regulated as a result of trypsin inhibition activity which further stimulates the defense against pathogens [56,57]. Thereby, antiproteasecontributes to the innate immunity of animals by its bactericidal and anti-inflammatory properties [55]. In our study, this activity decreased in fish fed mixture diet for 2 weeks, while no variations were observed for any other experimental groups and sample times. Contrarily with our results, several studies have demonstrated the enhancements of the antiprotease activity following feeding fish with diet containing garlic, ginger, neem leaf or *Mentha piperita* [58–63]in several fish species. In the same way, certain probiotics [64] or diet enriched with plants extracts and/or probiotics [65,66] can successfully elevate this activity in fish. These studies demonstrated clearly that high antiprotease activity protect fish from infection and disease which increase their survival.

In the case of complement, which is one of the most important serum factors in innate immunity [67],an increase of this activity was observed in fish feddate palm fruits extracts for 2 weeks. Similar results have been obtained with glycyrrhizin in yellow tail [68] and with *Viscum album coloratum* and *Nyctanthes arbortristis* in Nile tilapia [69,70]. Furthermore, this effect was also observed in Mozambique tilapia when water soluble fraction of *Tinosporacordifolia* was intraperitoneal injected to fish [71]. Regarding probiotics, different works reported their ability to increase complement activity when administer dietary alone or in combination with plant extracts [65,72]. New studies should be performed to deepen in the knowledge of the effects of these substances on humoral immune components as well as their precise role against pathogen bacteria.

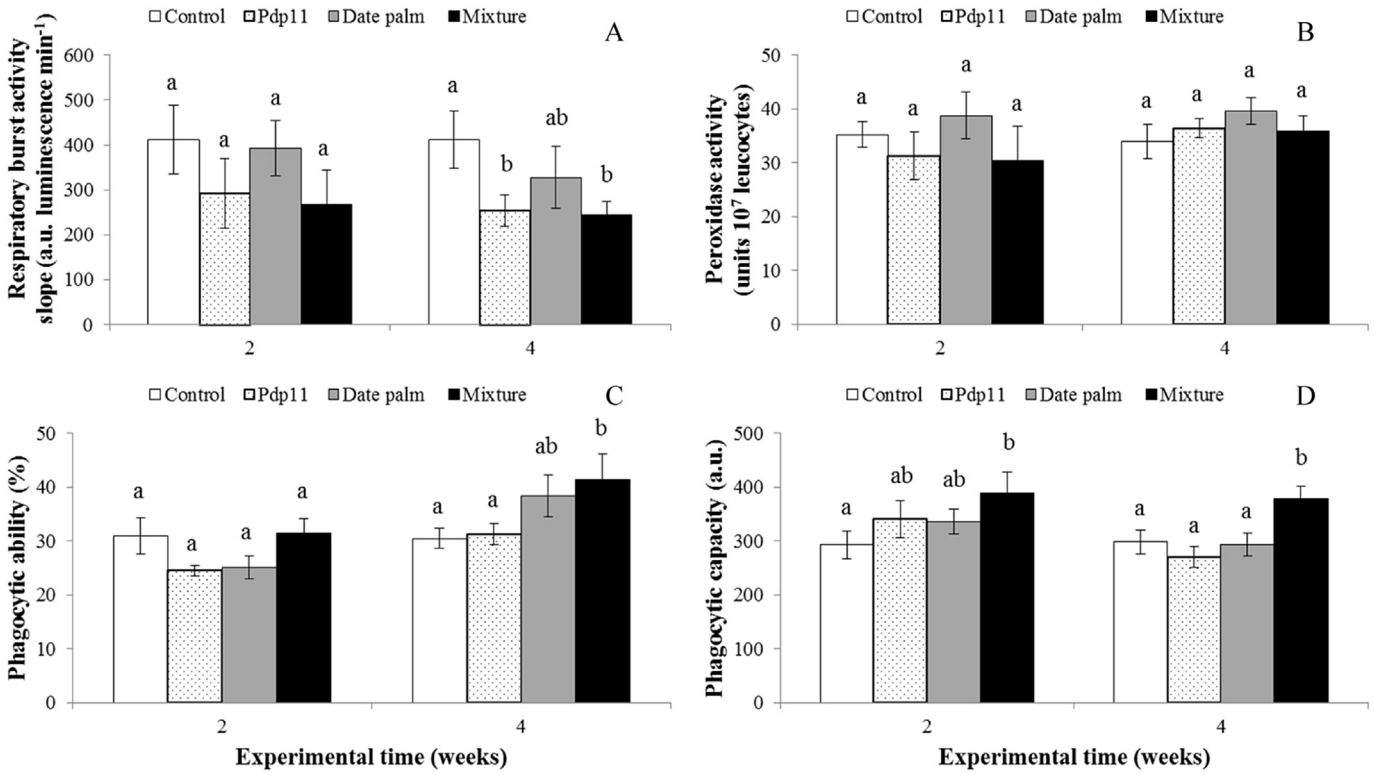

Fig. 4. Cellular innate immune parameters of head-kidney leucocytes from European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 and 4 weeks of experiment. **A**. Respiratory burst. **B**. Peroxidase activity. **C**. Phagocytic ability. **D**. Phagocytic capacity. Bars represent the mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P \leq 0.05$).

Regarding cellular immunity, respiratory burst activity significantly decreased in fish fed for 4 weeks probiotic supplement diet (alone or in combination with date palm fruits extracts). Respiratory burst is a crucial effect or mechanism for limiting the growth of fish pathogens [73]. Contrarily to present results, Pdp11 stimulated the leukocyte respiratory burst and peroxidase activities of gilthead seabream and Senegalese sole [30,74,75]. These activities were no modified in fish fed date palm fruit extracts diet. In this regards, the available results related to the effects of immunostimulants and/or probiotic on respiratory burst activity are contradictory because sometimes this activity was decreased [76]while in other occasions increased [16,77–85]. The detected differences could be attributed to different methodologies applied as well as to different plant species and different nature of the extracts used in the assays.

Immuno stimulant administration mainly facilitates the function of fish phagocytic cells [9,83,86–90]. However, phagocytic activity of macrophages was not enhanced in yellowtail fed with glycyrrhizin [68]. Our results showed increases in the phagocytic ability and capacity of HK leucocytes of fish fed the mixture diet. Thus, the combination of date palm fruits extracts and Pdp 11 probiotic provided a synergistic effect on European sea bass phagocytic cells.

One of the most practical immune functions is the direct ability to kill bacteria and this is also important in the serum which contains bactericidal components and other lytic factors. In our study, bactericidal activity of serum of European sea bass was no significantly modified due to the supplemented diets administration. However, several studies displayed potential serum anti-bacterial properties in fish fed with enriched plant diets. For example, *Curcuma longa*, *Allium sativum*, *Achyranthes aspera, M. piperita*, garlic and ginger enriched diet increased the serum bactericidal activity [60,63,86,91]. In the case of probiotic diet supplementation, several studies have also reported enhancements of serum bactericidal activity in fish [92,93]. The fact that ours results not revealed significant differences between the experimental groups, seem to be correlated to those obtained in the innate humoral parameters evaluated, which are directly associated to bactericidal activity, where only occasional increases were observed depending on the experimental groups and time.

To evaluate whether the dietary supplementation influenced the expression of immune-related genes, the expression of carbohydrate-binding proteins (*fbl* and *rbl*), antimicrobial peptides (*hep* and *lys*), proinflammatory cytokine (*il-1β*) and protein related with oxidative metabolism (*sod*) genes were evaluated in HK and gut of experimental fish. In the case of HK, an up-regulation of most of the studied genes was observed in fish fed mixture diet while only *sod* and *lyz* gene expression was up-regulated in fish fed date palm fruits extracts for 4 weeks. Among them, RBLs recognize various kinds of pathogens in inflammatory site and enhance their phagocytosis by binding on the leukocyte surface [94]; interleukin 1β plays a central role in the regulation of immune and inflammatory responses to infections [95], while hepcidin is a small cysteine-rich protein with antimicrobial activity that plays an important role in iron homeostasis and in defense against infections [96]. Likewise, lysozymes are important proteins for the defense against bacterial infection and superoxide dismutase can prevent oxidative stress though catalyzing the dismutation reaction of ROS into O_2 and H_2O_2 in living organisms [97]. Ours results are contrary to those found by Hoseinifar et al. [45]in common carp where demonstrated that the expression of *il-1β*in HK was considerably decreased in fish fed with date palm fruit extracts diet, as well as, the expression of *lys* gene remained similar compared to common carp fed with control diet.

Respect to the expression of these same genes in the gut, we

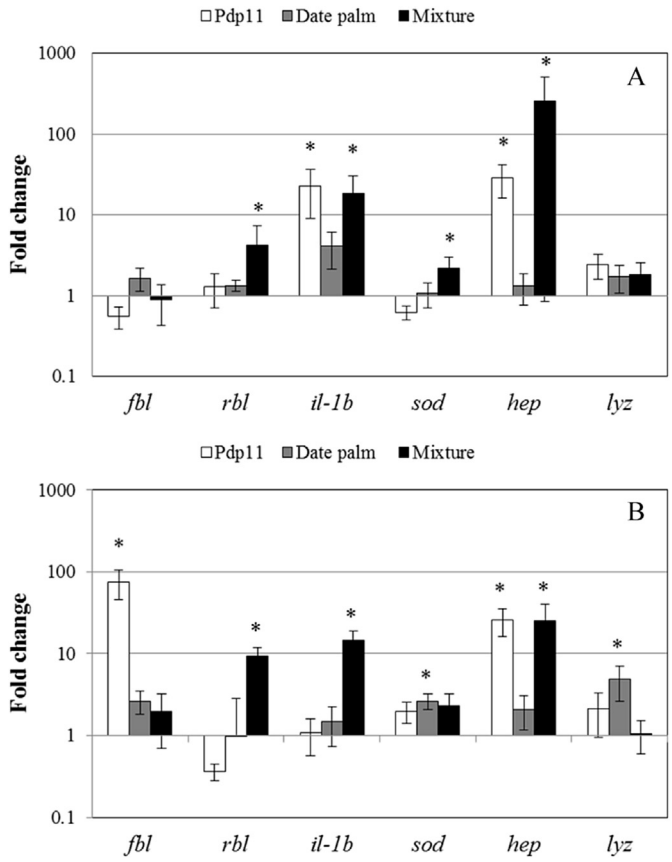

Fig. 5. Gene expression, determined by real-time PCR, in head-kidney of European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 (**A**) and 4 (**B**) weeks of experiment. Bars represent the mean ± SEM (n = 6) fold change relative to control. Asterisks denote significant differences between treatment groups ($P \leq 0.05$).

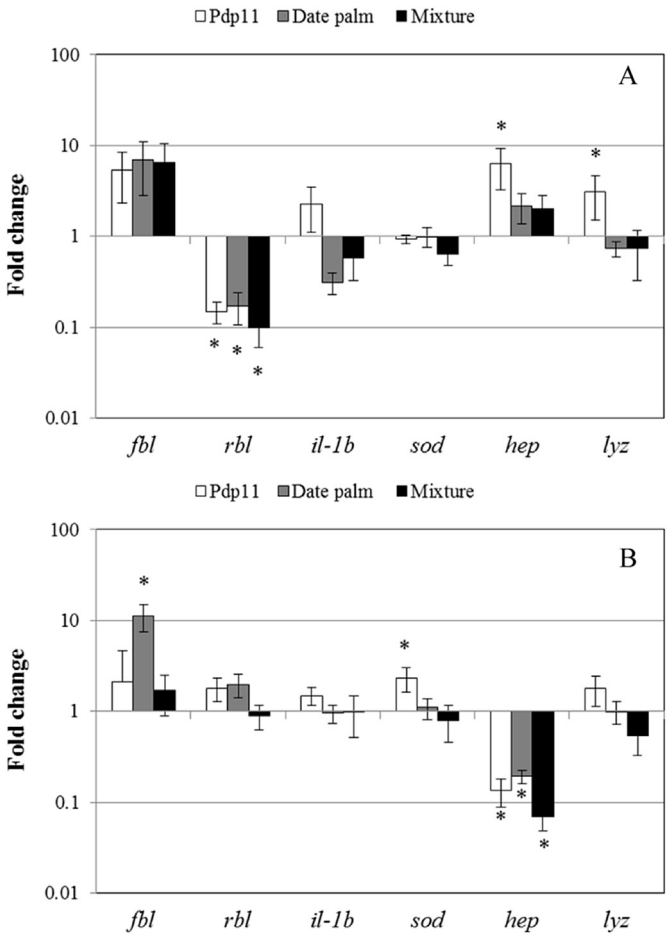

Fig. 6. Gene expression, determined by real-time PCR, in gut of European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 (**A**) and 4 (**B**) weeks of experiment. Bars represent the mean ± SEM (n = 6) fold change relative to control. Asterisks denote significant differences between treatment groups ($P \leq 0.05$).

could highlight a down-regulation of *rbl* and *hep* expression genes in specimens fed all supplement diets for 2 and 4 weeks, respectively. Contrarily, the expression of *hep* and *lyz* genes in fish fed for 2 weeks with probiotic enriched diet were up-regulated. Likewise, the expression of *fbl* and *sod* genes was also up-regulated in fish fed for 4 weeks with date palm fruits extracts and probiotic diets, respectively. Thus, the increased of this lectin could be involved in the protection against pathogens since is known that FBK is also able to opsonize bacteria [98]. In relation to *sod* gene expression, the results obtained are opposite to those observed in the gut of gilthead seabream fed with the same probiotic but they are in agreement with those obtained for the expression of this gene in fish feddate palm fruits diets, where no significant variations were observed [20]. These results also could be correlated to those obtained in freshwater shrimp fed with anthraquinone extracted from *Rheum officinale*, one of the herbs commonly used in traditional Chinese medicine formulae against cancer, where have reported that diet increased the gene expression of stress-related gene [99]. Comparing our results of *sod* gene expression in gut with other mucosal surfaces (gills and skin), few similarities were observed. Only results related to 4 weeks are available. Furthermore, any variations in *sod* gene expression in gut and gills of fish fed with date palm fruits and mix diets were detected [20]. A deeper characterization of the general response in fish fed with these substances is needed by analysing the expression of the genes directly or indirectly involved in the physiological activities, in order to understand the effects related to these plant extracts and/or probiotics.

In conclusion, the present results demonstrate that addition of palm fruits extracts alone enhances several of the evaluated activities while only enhances the expression of immune-related genes when is administrated in combination with Pdp 11 probiotic. In our previous studies it was confirmed that these additives seems to be good natural antioxidants and improved the mucosal immunity [18,20]. Present results throw more light on beneficial effects of date palm fruit extracts and probiotic supplementation on antioxidant status and innate immune parameters of European sea bass. Therefore the mixture diet could potentially be considered of great interest as immunostimulant diet to use as food additive for farmed fish. Future research is needed to obtain enough information about synergistic effect of plant extracts and probiotics in farmed fish and the possible protective effects after *in vivo* challenges to pathogen bacteria.

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Suplementary Table 1. Primers used for real-time PCR.

Gene name	Gene abbreviation	GenBank number	Primer sequences (5′→3′)
Elongation factor 1α	*ef1α*	AF184170	CTGTCAAGGAAATCCGTCGTTGACCTGAGCGTTGAAGTTG
Fucose-binding lectin	*fbl*	EU877448	TGCCTACAGCGCTATTGATGCTCCAGCAGGTCAACTCTCC
Rhamnose-binding lectin	*rbl*	FL486165	CTGACTGTGCACATCCAACCCACAGGGGTCTCCAAACACT
Superoxide dismutase	*sod*	FJ860004	TGTTGGAGACCTGGGAGATGATTGGGCCTGTGAGAGTGAG
Hepcidine	*hep*	DQ131605	CCAGTCACTGAGGTGCAAGAACAGACACAAGCGTCACAGC
lysozyme	*lys*	FN667957	ATTTCCTGGCTGGAACACAGTGATGTTGTTGCCAGAGCTC
Interleukin-1β	*il-1β*	AJ269472	CAGGACTCCGGTTTGAACATTTGTCCCCTTTTGAATGGAC

SuplementaryTable 2. Bacterial growth in the serum of European sea bass specimens fed with different diets (control, Pdp11, date palm or mixture) after 2 and 4 weeks of experiment. Data represent the mean ± SEM (n = 6). Different letters denote significant differences between treatment groups (P ≤ 0.05).

Experimental time (weeks)	Experimental groups	Bacteria			
		Vibrio harveyi	*Vibrio anguillarum*	*Photobacteriumdamselae*	*Escherichia coli*
2	Control	50.98 ± 2.46a	71.76 ± 1.47a	93.33 ± 0.88a	50.41 ± 2.43a
	Pdp11	54.42 ± 4.38a	69.66 ± 2.44a	90.18 ± 3.64a	54.46 ± 2.35a
	Date palm	58.51 ± 1.91a	71.83 ± 0.46a	93.45 ± 1.39a	44.46 ± 1.33a
	Mixture	57.25 ± 1.24a	68.68 ± 1.59a	89.83 ± 2.09a	49.52 ± 6.09a
4	Control	61.34 ± 2.28a	75.51 ± 1.19a	94.72 ± 1.01a	45.94 ± 4.24a
	Pdp11	56.38 ± 2.25a	77.31 ± 0.73a	96.16 ± 1.73a	47.38 ± 2.76a
	Date palm	59.69 ± 0.65a	68.97 ± 0.74a	91.12 ± 1.31a	45.43 ± 4.07a
	Mixture	59.33 ± 1.77a	74.24 ± 1.17a	93.44 ± 2.28a	47.79 ± 7.34a

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