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Effect of the Administration of Date Palm Seeds (*Phoenix dactylifera* L.) in Gilthead Seabream (*Sparus aurata* L.) Diets

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Abstract

Date palms (*Phoenix dactylifera* L.) are known in traditional medicine and because of their economic importance in many countries of the North of Africa. Due its abundance in Spain we developed this study in order to analyse the possible application of date palms seeds powder in aquaculture as dietary additive for gilthead seabream (*Sparus aurata* L.). The effect of diets enriched with date palm seeds powder at 0% (control), 1.5% and 3% was studied on the growth performance, immune and antioxidant status at 15 and 30 days. No significant effects were observed on growth performance nor antioxidants liver enzymes activity or skin mucus total antioxidant activity, although fish fed with 1.5% and 3% enriched diets shows a trend to increase both of them respect control fish at 15 and 30 days. For its part, significant improvements in cellular and humoral immunity from fish fed diets containing date palm seeds were observed. Fish fed with 1.5% and especially 3% diets increased significantly the head-kidney leucocytes phagocytic ability at 15 and 30 days and trend to increase the peroxidase activity at 30 days. Finally, fish fed 1.5% diet increased significantly serum protease activity at 30 days when compared with control fish. Therefore, date palm seeds could be considered a good alternative to be included in aquaculture as additive in fish feed in order to treat or avoid fish diseases or infections by improving their immune system.

Keywords: ***Phoenix dactylifera; Immunostimulants; Antioxidants; Gilthead seabream (Sparus aurata L.); Teleosts; Aquaculture***

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1. Introduction

Medicinal plants have become in a very important part of modern aquaculture as alternative to antibiotics and chemotherapeutics in order to contributing to fish welfare and to treat or control fish diseases or infections that may take place in fish farms. The reason is that they do not have negative impacts on animal, human or environment [1-6]. The incorporation of these plants in fish feed is the most used way to administer them, which allows to treat a great number of fish at the same time and it is not stressful for them [2].

Date palm (*P. dactylifera*) is a very important plant species from an economical [7] and medicinal [8] point of view in Middle East and North Africa (MENA) countries. Furthermore, this plant presents a very high food value due to the fact that its fruit is consumed and very appreciated because of its content of vitamins, minerals, fibers, carbohydrates, proteins, fatty acids and energy [9].

Date palm seeds (DPS) which are considered a by-product or waste and they are discarded most times have been used in form of extracts in many studies in rats. Antioxidant and tissue-protective effects have been observed in all of these studies, as well as inhibition of DNA damage and antidiabetic, anti-hyperglycemic and anti-inflammatory activity after induction of oxidative stress [10-21]. More interesting is the use of DPS in livestock. DPS are habitually softened by soaking in water to feed livestock or animals (e.g. camels, sheep, goats and horses), or crushed dry and added to chicken feed, although cows, pigs, broilers, lambs and fish have been also used to study the effect of DPS as dietary additive and very positive results have been obtained [22-24].

Therefore, due to the fact that date palm is a very common plant in Spain and especially in the region of Murcia and province of Alicante, which presents the highest concentration of date palm of the European Union, its economical and medicinal properties and the common use of DPS in livestock and their positive previous results as dietary additive, we developed the present research. We used powder of DPS as dietary supplement to gilthead seabream in a try to develop a sustainable aquaculture in the MENA countries using a natural product, which is discarded after the fruit is consumed, helping also to these countries to create job, help meet global demand, and achieve their own food security aspirations [25]. The main aim of our study, which is the first study using DPS powder as dietary additive in marine fish, was to analyze the effect of dietary DPS on gilthead seabream growth performance, immune status and liver antioxidant defence. Gilthead seabream (*S. aurata* L.) was selected as a representative species of marine Mediterranean aquaculture and due its economic importance in Spain and in the region of Murcia.

2. Material and Methods

2.1 Fibre determination and chemical analysis of date palm seeds

Date palm (Deglet Nour variety) was obtained on Monastir (Tunisia) at the ripeness stage and used to dissect the seed. The DPS moisture content was measured gravimetrically in unground seeds [26]. Crude proteins were determined using a Behr analysis equipment (Behr Labor TechnikGmbh). The Kjeldahl nitrogen content [27] was converted to a protein concentration with a specific factor of 6.25. Fats were solubilized with petroleum ether using a Soxhlet apparatus [28] and total ash was determined by calcination at 550 °C [29].

Dietary fibers were estimated according to the Van Soest method [30] using a Raw Fiber Extractor 6 Channel (VELP Scientifica). The first step of the analysis involved chemical extraction with a neutral detergent solution; NDF leading to unsolvable fraction containing cellulose, hemicellulose and lignin. Then, the acid detergent fiber; ADF was estimated throw the digestion of the degradable hemicelluloses and some proteins with acid solution. The acid detergent lignin; ADL was based on solubilization of the cellulose in 72% sulfuric acid; H_2SO_4. Hemicellulose was calculated as NDF - ADF and cellulose as ADF - ADL.

2.2 Animals

Sixty specimens of gilthead seabream (*S. aurata* L.) (8.2 g ± 1.1 g) obtained from a local farm (Murcia, 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 °C ± 2 °C with a flow rate of 900 L/h and 28‰ salinity. The photoperiod was 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. Fish were allowed to acclimatise for 7 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.3 Preparation of diets and experimental design

DPS, Deglet Nour variety, at the ripeness stage were collected on Monastir (Tunisia). Seeds were isolated and grounded into powder (32 meshes). A commercial pellet diet (Skretting, Spain) was crushed and mixed with tap water before adding the correct amount of crushed DPS powder and pelleting to obtain diets supplemented with 0% (control), 1.5% and 3% DPS. All the experimental diets were allowed to dry and stored at 4 °C.

Fish were randomly assigned and divided into nine tanks (n=8 in each) thus establishing three groups and three replicates by group: control (non-supplemented diet), 1.5% DPS supplemented diet and 3% DPS supplemented diet. Animals were fed at a rate of 2% body weight/day for a month. Fifteen and thirty days after the beginning of the treatment, nine animals from each experimental group (three of each replicate tank) were sampled after sacrificing with an overdose of MS-222 (100 mg/L, Sandoz). All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.4 Sample collection

Samples of blood, skin mucus, head kidney (HK) and liver were obtained. Blood samples were collected from the caudal vein with an insulin syringe. The blood samples were left to clot at 4 °C for 4 h and later the serum was collected after centrifugation (10,000 rpm, 10 min, and 4 °C) and stored at -80 °C.

Skin mucus samples were collected using the method described by Guardiola et al. [31]. Briefly, skin mucus was collected by gentle scraping the dorso-lateral surface of specimens using a cell scraper with enough care to avoid contamination with blood and urine-genital and intestinal excretions. The skin mucus was vigorously shaken and centrifuged (400 g, 10 min, 4 ºC). The supernatant was collected and stored at -80 ºC until use.

HK leucocytes were isolated according to Esteban et al. [32]. Briefly, HK were cut into small fragments and transferred to 12 mL of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride, 2% foetal calf serum; FCS

(Gibco) 100 i.u./mL penicillin (Flow) and 100 mg/mL streptomycin (Flow)]. HK leucocytes were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 mm), washed twice (400 g, 10 min), counted in an automatic counting chamber (BioRad) and adjusted to 10^7 cells/mL in sRPMI. Cell viability was determined by the trypan blue exclusion test. Fragments of liver for antioxidant enzymes analysis were collected and stored at -80 °C.

2.5 Growth performance

The body weight of each fish was measured before the trial. Growth was monitored by obtaining the weight gain; WG%, specific growth rate; SGR% and condition factor; CF%, which were calculated for each of the treatments according to Silva-Carrillo et al. [33].

WG% = [(final weight – initial weight)/initial weight] × 100
SGR% = [(ln final weight – ln initial weight)/number days] × 100
CF% = [(weight/length^3)] × 100

2.6 Immune status

2.6.1 Total protein

Protein concentration in each sample of skin mucus and liver was determined by the dye binding method of Bradford [34], using bovine serum albumin; BSA (Sigma-Aldrich) as the standard. Briefly, 2 mg/mL solution of BSA was prepared and serial dilutions made with phosphate-buffered saline; PBS (Sigma-Aldrich) as standards. Dilutions of 5 µL of skin mucus or liver homogenates and 15 µL of PBS were prepared. Then 250 µL of Bradford reagent (Sigma-Aldrich) was added to BSA and skin mucus or liver dilutions and incubated at room temperature; RT for 10 min. The absorbance of each sample was then read at 595 nm and the results were taken and plotted onto the standard curve to obtain the total protein content of skin mucus and liver.

2.6.2 Immunoglobulin M level

Total serum and skin mucus immunoglobulin M; IgM levels were analysed using the enzyme-linked immunosorbent assay; ELISA [35]. For this, 100 µL per well of 1/500 diluted serum and 100/500 diluted skin mucus with 50 mM carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6) were placed in flat-bottomed 96-well plates in triplicate, and proteins were coated by overnight incubation at 4 ºC. After three rinses with 200 µL per well of PBS-Tween; PBS-T (0.1 M PBS with 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at RT with 200 µL blocking buffer (3% BSA in PBS-T), followed by three new rinses with PBS-T. The plates were then incubated for 1 h with 100 µL per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd., 1/100 in blocking buffer). Following rising, plates were incubated for 1 h with 100 µL per well of secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer, Sigma-Aldrich). After exhaustive rinsing with PBS-T the plates were developed using 100 µL per well of a 0.42 mM solution of 3,3',5,5'-tetramethylbenzidine hydrochloride; TMB (Sigma-Aldrich) prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% hydrogen peroxide. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µL 2 M H_2SO_4 and the plates were read at 450 nm in a plate reader. Serum and skin mucus samples of gilthead seabream previously tested were used as a positive control. Negative controls consisted of samples without serum and skin mucus or without primary antibody, whose optical density values were subtracted for each sample value.

2.6.3 Lysozyme activity

Serum and skin mucus lysozyme activity was measured according to the turbidimetric method described by Parry [36] with some modifications. Briefly, 20 µL of serum and skin mucus diluted 1/10 with 0.04 M NaH_2PO_4-Na_2HPO_4 buffer, pH 6.2, were placed in a flat-bottomed 96-well plate. To each well, 200 µL of freeze-dried *Micrococcus lysodeikticus* in the above buffer (0.3 mg/mL, Sigma-Aldrich) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured over 15 min at 3 min intervals at RT in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. The units of lysozyme present in serum and skin mucus were obtained from a standard curve made with hen egg white lysozyme; HEWL (Sigma-Aldrich), and the results were expressed as U/mL for the serum samples or as U/mg protein for the skin mucus samples.

2.6.4 Bactericidal activity

Two pathogenic bacteria for fish (*Vibrio harveyi* and *Vibrio anguillarum*) were used in the bactericidal assays. The two bacterial strains were grown from 1 mL of stock culture that had been previously frozen at -80 ºC. Both bacteria were cultured for 48 h at 25 ºC in Triptic Soy Agar; TSA (Difco Laboratories) and then inoculated in Triptic Soy Broth; TSB (Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v). Bacteria in TSB medium were then cultured at the same temperature, with continuous shaking (100 rpm) for 24 h. Exponentially growing bacteria were resuspended in sterile PBS and adjusted to 10^8 colony forming units/mL.

Bactericidal activity was determined following the method of Stevens et al. [37] with some modifications using the MTT assay, which is based on the reduction of the yellow soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT (Sigma-Aldrich) into a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase [38,39]. Samples of 20 µL of serum and skin mucus were added in flat-bottomed 96-well plate. PBS was added to some wells instead of the samples and served as positive control. Aliquots of 20 µL of the bacteria previously cultured were added and the plates were incubated for 5 h at 25 ºC. After that, 25 µL of MTT (1 mg/mL) were added to each well and the plates were newly incubated for 10 min at 25 ºC to allow the formation of formazan. Plates were then centrifuged (2000 g, 10 min), being the precipitates dissolved in 200 µL of dimethyl sulfoxide; DMSO and transferred to a new flat-bottom 96 well-plate. The absorbance of the dissolved formazan was measured at 570 nm in a plate reader. Bactericidal activity was expressed as percentage of no viable bacteria, calculated as the difference between absorbance of bacteria surviving compared to the absorbance of bacteria from positive controls (100%).

2.6.5 Protease activity

Protease activity of serum was quantified using the azocasein hydrolysis assay according to the method described by Ross et al. [40]. Briefly, 10 µL of serum were incubated overnight at RT and in agitation with 100 µL of ammonium bicarbonate buffer and 125 µL of 2% azocasein (Sigma-Aldrich) in sterile eppendorfs. The reaction was stopped by adding 250 µL of 10% trichloroacetic acid; TCA (Sigma-Aldrich). The mixtures were centrifuged (6000 g, 5 min), 100 µL of the supernatants transferred to a flat-bottomed 96-well plate, and 100 µL of 1 N sodium hydroxide (NaOH) added. Optical density was read at 450 nm using a plate reader. Serum samples were replaced by trypsin (5 mg/mL, Sigma-Aldrich) for the positive controls (100% of protease activity) or by ammonium bicarbonate buffer for the negative controls (0% of protease activity). Activity for each sample was expressed as % protease activity in relation to the controls.

2.6.6 Antiprotease activity

Antiprotease activity of serum was determined by the ability of serum to inhibit trypsin activity [41]. Briefly, 10 µL of serum samples were incubated (10 min, 22 ºC) with the same volume of standard trypsin solution (5 mg/mL). After adding 100 µL of 100 mM ammonium bicarbonate buffer and 125 µL of buffer containing 2% azocasein (Sigma-Aldrich), samples were incubated (2 h, 30 ºC) and, following the addition of 250 µL of 10% TCA, a new incubation (30 min, 30 ºC) 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 of 1 N sodium hydroxide (NaOH), and the optical density 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 ¼ (Trypsin OD – Sample OD)/Trypsin OD × 100

2.6.7 Head-kidney leucocyte phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by HK leucocytes was studied by flow cytometry according to Esteban et al. [42]. Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate; FITC (Sigma-Aldrich), washed and adjusted to 10^8 yeast cells/mL of sRPMI. Phagocytosis samples consisted of 60 µL of labelled-yeast cells and 100 µL of HK leucocytes in sRPMI. Samples were mixed, centrifuged (400 g, 5 min and 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 50 µL ice-cold trypan blue (0.5% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes 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 5,000 cells, which were acquired at a rate of 300 cells/s. Data were collected in the form of two-parameter side scatter; SSC (granularity) and forward scatter; FSC (size), 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. A quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.6.8 Head-kidney leucocyte respiratory burst activity

The respiratory burst activity of HK leucocytes was studied by a chemiluminescence method [43]. Briefly, 100 µL of HK leucocytes suspension were placed in triplicate in the wells of a 96-well flat-bottomed plate. Then, 100 µL of Hank's balanced salt solution; HBSS (Gibco) containing 1 mg/mL phorbolmyristate acetate; PMA (Sigma-Aldrich) and 10^{-4} M luminol (Sigma-Aldrich) were added to each well. The plate was shaken and immediately read in a plate reader for 1 h at 2 min intervals. The kinetic of the reactions was analysed and the maximum slope of each curve calculated. Backgrounds of luminescence were calculated using reactant solutions containing luminol but not PMA.

2.6.9 Head-kidney leucocyte peroxidase activity

The peroxidase activity in serum, skin mucus and HK leucocytes was measured according to Quade and Roth [44]. 10^7 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide; CTAB (Sigma-Aldrich) 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 addition, 5 µL of serum and 10 µL of skin mucus were diluted with 45 µL and 40 µL, respectively, of HBSS (Gibco) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. As substrates, 100 µL of 20 mM TMB and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µL of 2 M H_2SO_4 and the optical density was read at 450 nm in a plate reader. Standard samples without HK leucocytes, serum or skin mucus were used as blanks.

2.7 Antioxidant status

2.7.1 Liver antioxidant activities

Samples of liver were used for determining the activities of glutathione reductase; GR, superoxide dismutase; SOD and catalase; CAT. GR was measured by the method modified by Carlberg and Mannervik [45]. The reaction was initiated by adding 0.1 mM nicotinamide adenine dinucleotide phosphate; NADPH to the mixture of enzyme in 50 mM potassium phosphate buffer pH 7.0 containing 2 mM ethylenediaminetetraacetic acid; EDTA and 0.5 mM glutathione disulphide or oxidized; GSSG. The change in absorbance was monitored at 340 nm for 3 min by a UV–Vis Thermo Scientific model Evolution 300 dual beam spectrophotometer. One unit of GR activity is defined as the amount of enzyme that catalyzes the reduction of 1 µmol of NADPH per minute (ε340 nm for NADPH/6.22 mM cm).

SOD activity was determined by the method of McCord and Fridovich [46] based on the inhibition of the reduction of cytochrome C in the presence of SOD at 550 nm. The SOD-like activity of the complexes was studied at 25 ºC by an indirect method using cytochrome C as superoxide oxidant (indicator). The superoxide radical anion was generated *in situ* by the xanthine oxidase reaction and detected spectrophotometrically by monitoring the formation of the reduced form of cytochrome c at 550 nm in a Thermo Scientific Evolution 300 dual beam spectrophotometer. CAT activity was measured by the method of Aebi [47] monitoring the consumption of H_2O_2 at 240 nm. This method is based on the principle that the absorbance will decrease due to the decomposition of H_2O_2 by catalase at 240 nm. H_2O_2 solution (10 mM), liver extract and 50 mM phosphate buffer (pH=7) were pipetted into a cuvette. The reduction of H_2O_2 was followed at a wavelength of 240 nm for 4 min against a blank containing 50 mM phosphate buffer. All the enzyme activities were expressed in units of enzyme/mg protein.

2.7.2 Total antioxidant activity

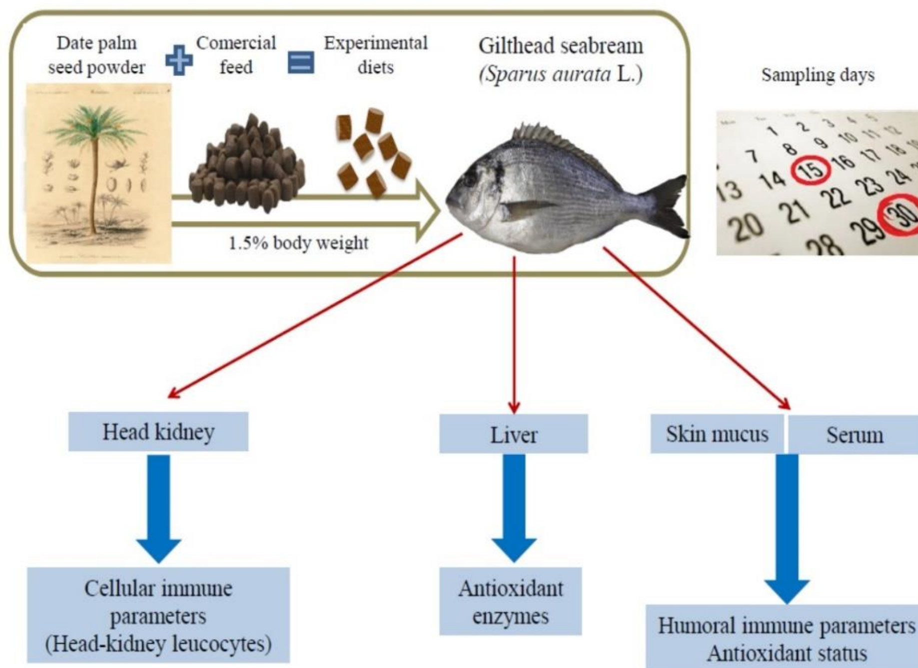
The total antioxidant activity; TAA of skin mucus was analysed by the 2,2'-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid); ABTS method described by Arnao et al. [48], which is based on the ability of the antioxidants in the sample to reduce the radical cation of ABTS, as determined by the decolouration of ABTS·+, and measuring the quenching of the absorbance at 730 nm. This activity is calculated by comparing the values of the sample with a standard curve of ascorbic acid and expressed as ascorbic acid equivalents (mmol)/mg protein.

Samples of 50 µL of skin mucus were added to 950 µL of cation ABTS+ and the decrease of absorbance was measured in a spectrophotometer (BOECO S-22 UV/Vis, Germany), using as blank of reaction PBS. The samples were analyzed in triplicate.

A standard curve was done with ascorbic acid (Sigma-Aldrich) and the antioxidant capacity of serum and skin mucus interpolated from the adjusted curve.

2.8 Statistical analyses

The results are expressed as means ± SEM. The normality of the variables was confirmed by the Shapiro-Wilk test and homogeneity of variance by the Levene test. Statistical differences among the four groups of treatments were assessed by one-way ANOVA analyses, followed by the Tukey or Games Howell test, depending on the homogeneity of the variables. The significance level was 95% in all cases ($P<0.05$). All the data were analysed by the computer application SPSS for Windows® (version 15.0, SPSS Inc., Chicago, USA).



Graphical Abstract.

3. Results

3.1 Fibre determination and chemical analysis of DPS

Fibre and chemical composition of selected DPS was determined and the results indicated that hemicelluloses were the most abundant fibres (26.5 ± 0.001% dry-matter basis; db) followed by celluloses (24.1 ± 0.1% db) and lignin (21.2 ± 0.001% db). Results from the chemical analysis revealed that fat was more abundant (11.2 ± 0.1% db) than proteins (6.2 ± 0.01% db) while ash represents the 1.5 ± 0.1% db.

3.2 Growth performance

No significant differences were obtained for any parameter at any assayed time in fish fed 1.5% or 3% DPS compared with control fish, although a decrease in WG and SGR of fish fed 3% DPS diet was observed (FIG. 1).

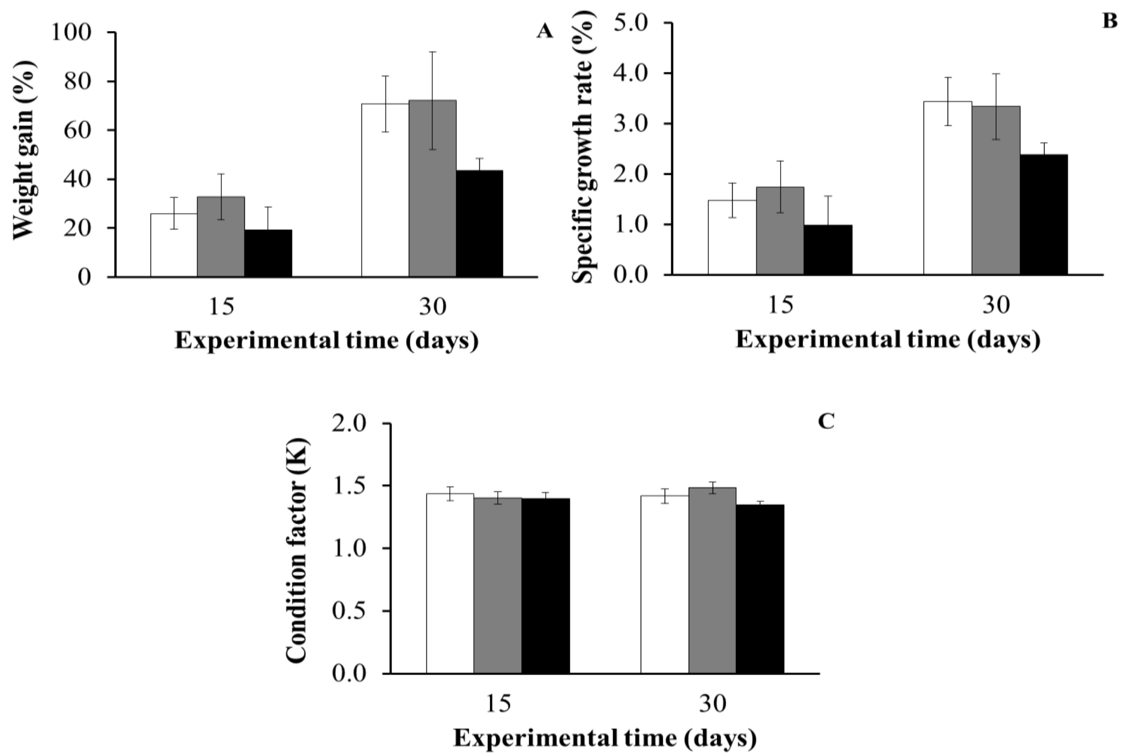


FIG. 1. **Weight gain (A), specific growth rate (B) and condition factor (C) of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

3.3 Humoral immune parameters

3.3.1 IgM level

No significant variations compared with the control group were observed in serum or skin mucus IgM levels at 15 or 30 days of feeding DPS diets, respect to control group (FIG. 2).

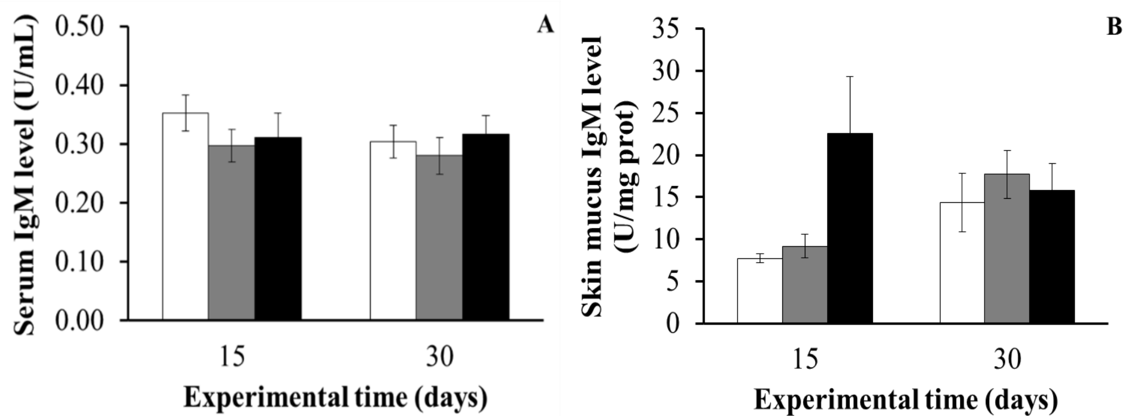


FIG. 2. **IgM levels in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

3.3.2 Lysozyme activity

Similarly, no significant differences were observed in serum or skin mucus lysozyme activity at 15 or 30 days in fish fed DPS diet compared with control group, although fish fed 3% DPS diet showed an increased serum lysozyme activity at 15 days while fish fed with 1.5% and 3% DPS diets showed a dose-dependent decreases in skin mucus lysozyme activity (FIG. 3).

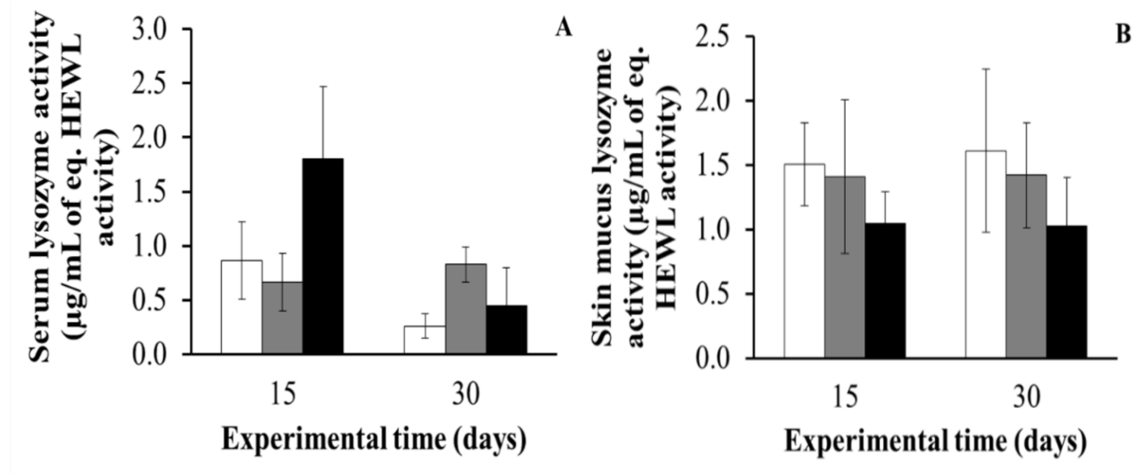


FIG. 3. **Lysozyme activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

3.3.3 Skin mucus bactericidal activity

No significant differences were detected against *V. harveyi* or *V. anguillarum* at any time comparing the results with control group (FIG. 4), although a trend to increase in the bactericidal activity against *V. harveyi* with the increase of DPS in diets was observed at 15 days.

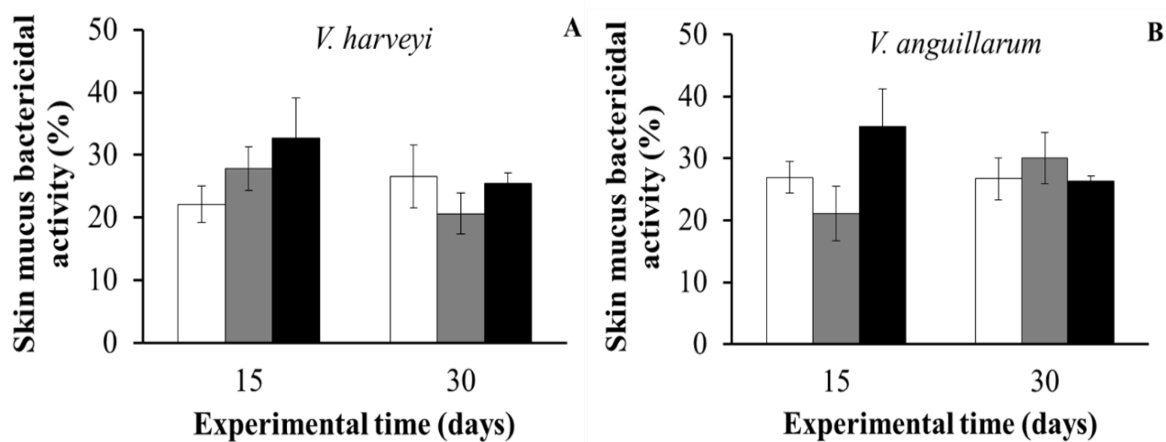


FIG. 4. **Bactericidal activity against *Vibrio harveyi* (A) or *Vibrio anguillarum* (B) in the skin mucus of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

3.3.4 Serum protease and antiprotease activities

Regarding serum protease activity, fish fed 1.5% DPS diet showed significant increases in comparison with control fish at 30 days (FIG. 5A), while no statistically significant differences were observed in serum antiprotease activity between any different treatments at any assayed time (FIG. 5B).

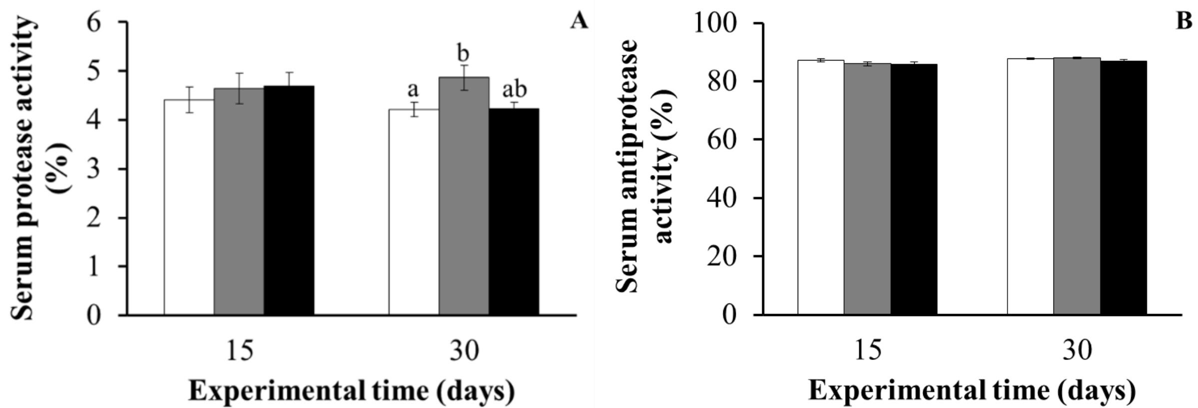


FIG. 5. **Protease activity (A) and antiprotease activity (B) in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups (P < 0.05).**

3.3.5 Peroxidase activity

No significant variations were observed in serum or skin mucus peroxidase activity at 15 or 30 days of feeding DPS in comparison with control group. However, fish fed 1.5% DPS diet showed higher serum peroxidase activity at 15 and 30 days compared with control group, while fish fed 3% DPS diet showed higher activity at 30 days. For its part, fish fed 3% DPS diet for 15 days also showed higher skin mucus peroxidase activity than fish fed control diet (FIG. 6).

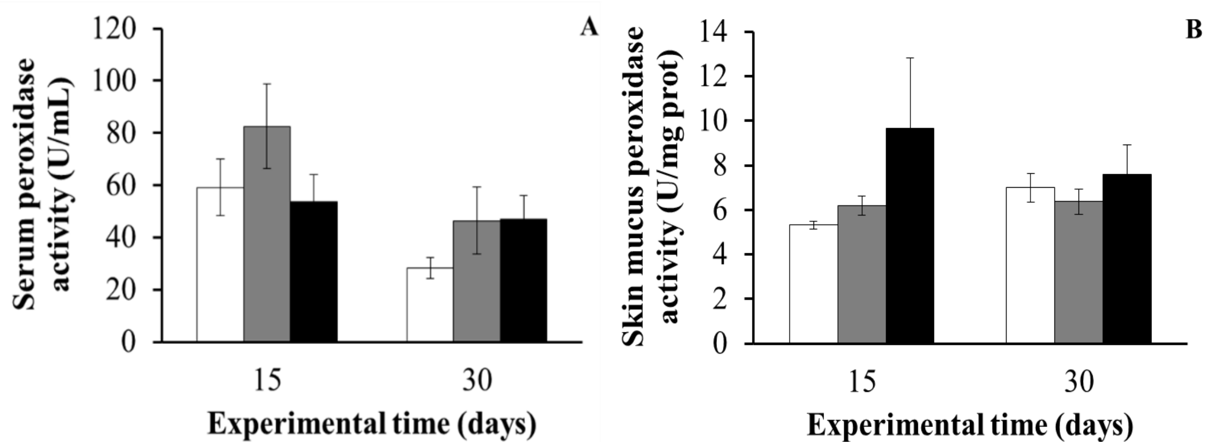


FIG. 6. **Peroxidase activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

3.4 Cellular immune parameters

Regarding HK leucocyte phagocytic activity, fish fed 1.5% DPS diet showed a significant increase of HK leucocyte phagocytic ability when compared with control group at 15 days, while fish fed 3% DPS diet showed a significant increase of phagocytic ability at 15 and 30 days in comparison with control fish (FIG. 7A). On the other hand, no differences in HK leucocyte phagocytic capacity, respiratory burst activity and peroxidase activity were observed between any assayed treatments at any assayed time (FIG. 7B, 7C, 7D), although fish fed 1.5% and 3% DPS diets showed higher peroxidase activity at 30 days compared with control fish.

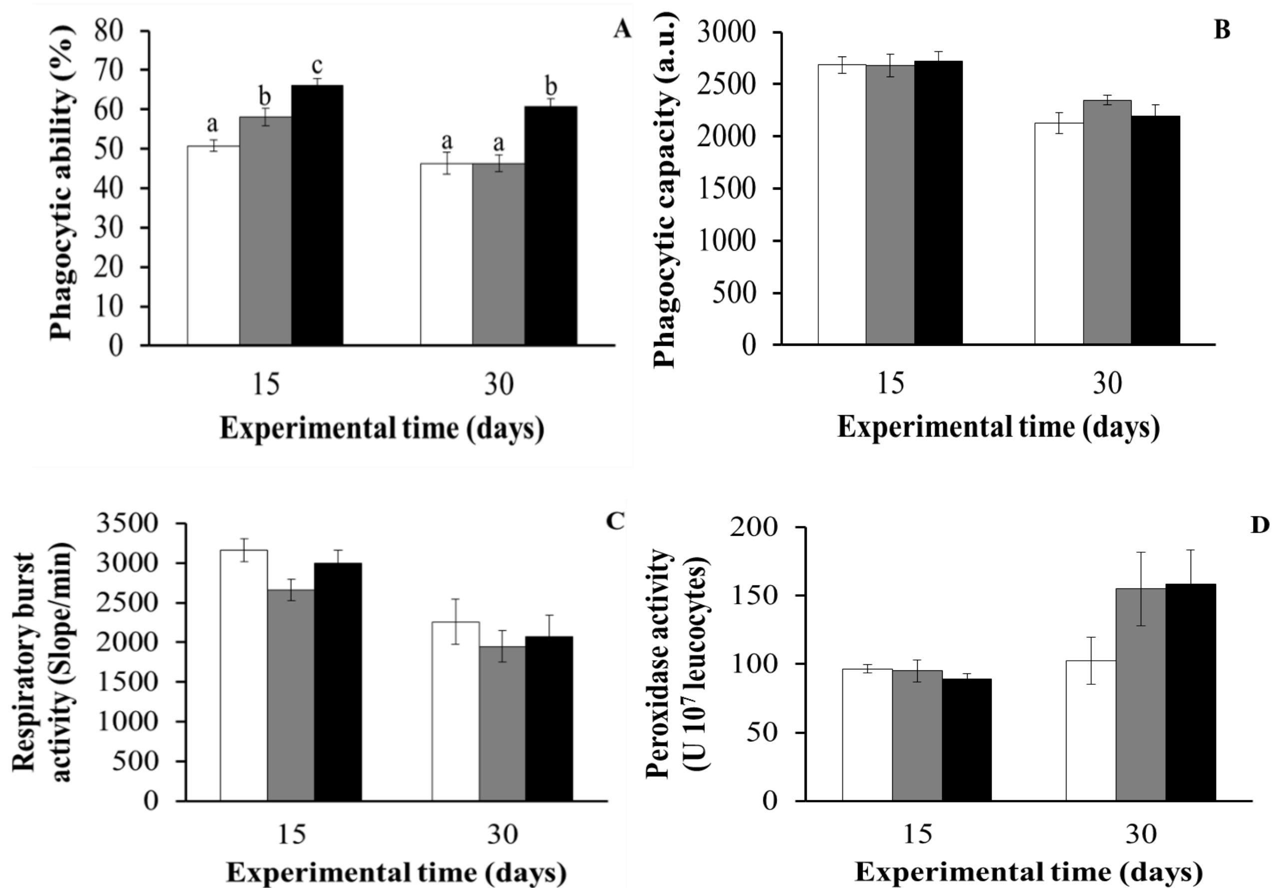


FIG. 7. **Phagocytic ability (A) and capacity (B), respiratory burst (C) and peroxidase (D) activities in the head-kidney leucocytes of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups (P < 0.05).**

3.5 Liver antioxidant enzyme activities

No significant differences in any enzyme activity were detected between treatments at any assayed time (FIG. 8), although a DPS dose-dependent increase of GR activity is observed at 15 days in comparison with control group, while 1.5% DPS diet showed strong but not significant increase at 30 days. Curiously, a DPS dose-dependent increase of CAT activity is also observed at 15 and 30 days in comparison with control fish.

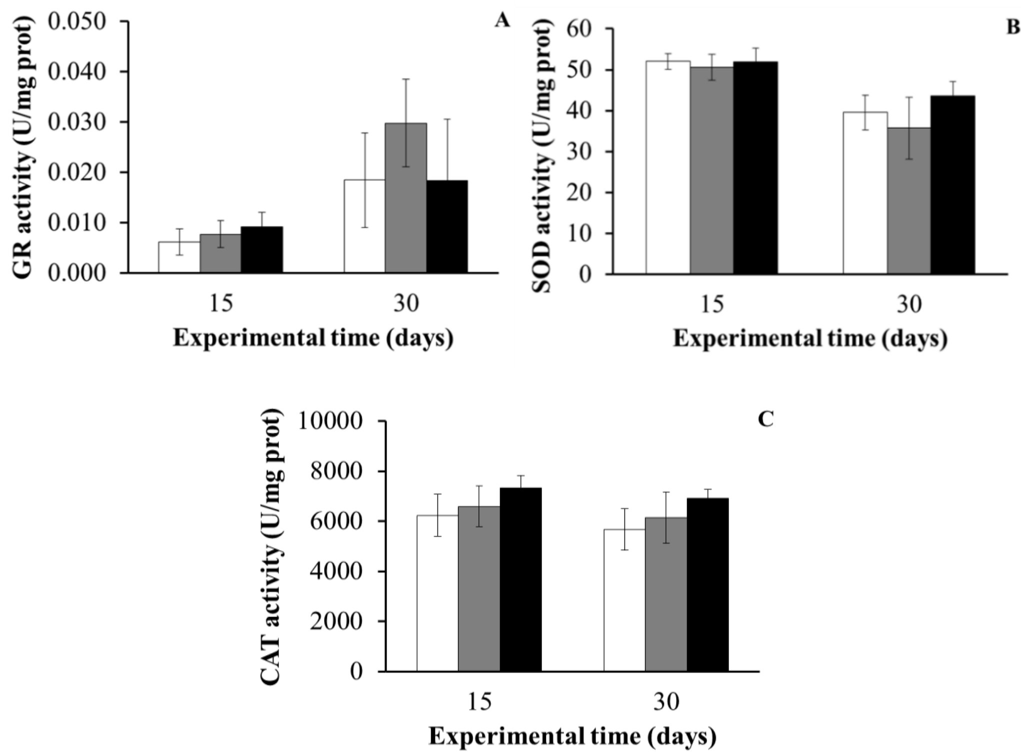


FIG. 8. **Glutathione reductase (A), superoxide dismutase (B) and catalase (C) activities in the liver of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

3.6 Skin mucus total antioxidant activity

No significant variations were observed in the skin mucus TAA, although fish fed 3% DPS diet for 30 days showed an increased activity respect to control fish (FIG. 9).

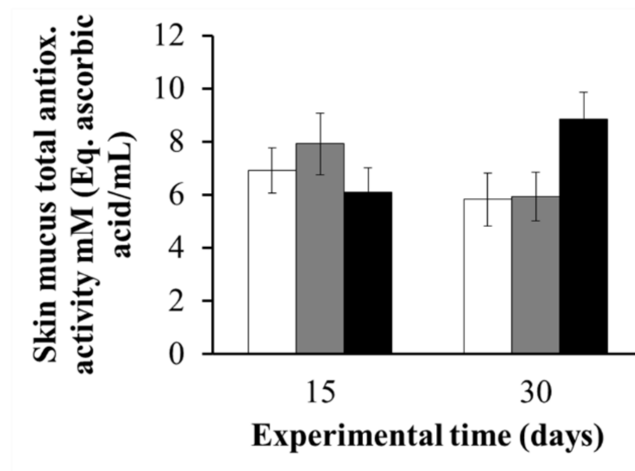


FIG. 9. **Total antioxidant activity in the skin mucus of gilthead seabream specimens fed diets supplemented with 0 (control, white), 0.5% (grey) or 1% (black) date palm seeds for 15 and 30 days. Data are presented as means ± SEM (n = 10).**

4. Discussion

Due the very high intensity that take place in fish farms, its usual the apparition of some problems such as environmental pollution, stress and increase of opportunistic pathogens which can compromise the immune status of fish and makes them susceptible to infections and diseases and even die [3,49]. For this reason and according to Food and Agriculture Organization of the United Nations (FAO), who established as one of their fundamental pillars the development of modern aquaculture by using natural products and immunostimulants through prophylactic administration in order to strength and stimulate fish immune system [50]. In the last years phytotherapy has gained importance as eco-friendly immunostimulant alternative to antibiotics and chemotherapeutics to treat or control fish diseases or infections due they are capable to stimulate both, innate and adaptive immune system including humoral and cellular responses [1-6]. In addition, these plants can promote the maturation of the cultivated species [2,3,51]. Therefore, we developed this study in order to ascertain whether DPS could potentially be considered as a functional food ingredient for farmed fish due this plant is very common in the south of Spain, its known economical and medicinal properties and its common use in livestock and positive previous results as dietary additive, in an attempt also to revalorize this product which is usually discarded after the fruit is consumed.

According to the percentages used in our study, both of them are low because, in general, low concentrations are more practical than high concentrations, because the former could lead to immunosuppression [52,53]. Furthermore, three studies carried out in fish used concentrations of DPS between 0.5% and 5% [54-56]. Besides this, due that date palms are rich in vitamins, minerals, carbohydrates, proteins, fatty acids and energy [9], and medicinal plants are able to stimulate the appetite and the growth of fish when they are administered in the food [1,51], firstly we studied the effect of incorporation of DPS in fish feed on growth performance. No significant differences were obtained in fish fed DPS diets and fish fed control fed. Previous studies in which DPS has been incorporated in animal diets provided variable results as regards growth performance [22-24]. In studies using sheep as animal model, the incorporation of DPS and date fruits increased growth performance by improving feed utilization [57-59] while its use in cows did not present adverse effects [60,61]. In addition, some studies in broilers found a negative effect of DPS on growth performance and feed utilization [62-64], while other studies showed no effect [65] or positive results [66]. Respect to this, it was established that the way DPS are added and the age of broilers are crucial for obtaining positive or negative results [67]. Similarly, as regards the effect of DPS on fish growth performance, the concentration used seems to be important. In this sense, Yousif et al. [68] demonstrated that the incorporation of dates and DPS at 15% in tilapia (*Oreochromis aureus*) decreased WG, SGR and feed utilization compared with fish fed a control diet containing no date pulp or DPS. Similar results were obtained in Nile tilapia, where low concentrations of DPS increased WG and high concentrations (15% - 30% or more) decreased it [22,54,69,70,71]. Low concentrations (0.5% - 2%) of DPS also increased WG, SGR and feed utilization in African catfish (*Clarias gariepinus*) [55]. However, the results for common carp (*Cyprinus carpio*) are contradictory since 0.25% DPS diet decreased WG, SGR and feed utilization, while a 0.5% DPS diet increased them [56].

Furthermore, DPS are rich in dietary fibres. The significance of dietary fibres in animal feeding is their influence on the speed of passage, mucosal functionality and the crucial role they play as substrate for gut microbiota, which is related to performance and digestive health [72]. Taking into account that, at present, most marine farmed fish are carnivores [73] and that indigenous microbiota play a critical role in the lives of their vertebrate hosts [74], it would be very interesting to carry out *in vivo* studies to determine the possible effects of dietary fibre supplementation on fish gut microbiota composition and also to support the

hypothesis that fish gut microbiota could make the fermentation of these fibres found in DPS extracts with potential benefits to the host.

Continuing with our study, the next objective was to analyze the effect of DPS diets on fish immune status, which regarding literature, are very scarce. For this reason, in recent years, our research team has evaluated many and varied effects caused by the dietary administration of date palm fruit extracts (alone or in combination with probiotic bacteria) on growth performance, mucosal and systemic immunity and antioxidant status in several fish species, finding marked immunostimulant effects on common carp (*Cyprinus carpio*) [75], European sea bass (*Dicentrarchus labrax*) [76] and gilthead seabream [77,78]. In the studies carried out in gilthead seabream, a commercial diet used as control diet was enriched with probiotic *Shewanella putrefaciens* (Pdp11), probiotic *Bacillus sp*., aqueous date palm fruit extracts (4%) or a combination of Pdp11 + *Bacillus sp* + extracts and given to fish for 4 weeks. In the study carried out in European sea bass, the diet with probiotic *Bacillus sp.* was not used, while in the study carried out in common carp only date fruit aqueous extract at 200 mL/kg was tested for 8 weeks. However, the effect of DPS powder alone on immune and antioxidant status of fish has not been previously studied.

As to our results of DPS diets on humoral immune status, no differences were observed in serum or skin mucus IgM level, although skin mucus IgM in fish fed the 3% diet showed a non-significant increase at 15 days, which is in line with previous results pointing to non-significant variations in serum IgM level in European sea bass [76] or a significant increase in skin mucus IgM of gilthead seabream [78] after 2 or 4 weeks of treatment with date fruit aqueous extracts alone or with probiotic, as well as in skin mucus total Ig of common carp [75] after 8 weeks of treatment with date fruit extract. Furthermore, skin of gilthead seabream specimens fed with date extract alone or with probiotic significantly increased the expression of *ighm* and *ight* genes after 2 and 4 weeks, respectively [78]. Regarding lysozyme activity, although results showed an increase of serum lysozyme activity in fish fed DPS diets at 15 and 30 days, neither serum nor skin mucus showed significant increases with respect to the control group at any assayed time, while a marked increase in skin mucus lysozyme activity was observed after 8 weeks of treatment with date extract in common carp [75], as well as lysozyme gene expression in HK of European sea bass fed with date extract for 4 weeks [76]. As in the case of IgM and lysozyme activity, no differences in bactericidal activity of skin mucus samples against *V. harveyi* or *V. anguillarum* were obtained after feeding fish with DPS powder, although a non-significant dose-dependent increase was observed against *V. harveyi* at 15 days, as in the study of Guardiola et al. [76], where European sea bass fed with date extract alone or with probiotic did not show any difference at any assayed time in bactericidal activity against *V. harveyi*, *V. anguillarum*, *P. damselae* and *E. coli* with respect to the control group.

For its part, fish fed 1.5% DPS diet showed a significantly increase in serum protease activity at 30 days, which is totally in line with previous results, where skin mucus protease activity was significantly higher in gilthead seabream fed a diet enriched with date extract alone or with probiotic at 2 and 4 weeks [78], as well as in common carp at 8 weeks [75]. However, serum antiprotease activity did not vary between the different treatments and control group at any assayed time, which is contrary to the results obtained in previous studies, where skin mucus of gilthead seabream specimens fed with date extract showed decreased antiprotease activity at 2 weeks and increased levels at 4 weeks compared with the control group [78]. Similar results were obtained in European sea bass specimens fed with date extract with probiotic showed a decrease in serum antiprotease activity at 2 weeks [76]. As to peroxidase activity, although non-significant differences were obtained, fish fed with DPS diets showed higher activity, contrary to previous results, in which diets enriched with date extract alone or with probiotic decreased

peroxidase activity in skin mucus of gilthead seabream specimens at 2 and 4 weeks [78] or had no effect in serum of European sea bass at any assayed time [76].

In addition to humoral immune status, cellular immune status was also analyzed and positive results were obtained. Starting with phagocytic activity of HK leucocytes, specimens fed with DPS enriched diets showed a significant increase in phagocytic ability at 15 and 30 days, especially with the 3% diet, while phagocytic capacity was not affected. Our results agreed with those obtained in European sea bass fed date extract with probiotic had higher phagocytic ability and capacity at 2 and 4 weeks. However, in the case of respiratory burst, no differences were obtained between different treatments and control group at any assayed time, while in the study of sea bass specimens fed date extract with probiotic showed a decrease in this activity at 4 weeks [76]. Finally, fish fed the DPS diets showed a non-significant dose-dependent increase in HK leucocyte peroxidase activity at 30 days, in agreement with the data obtained in the sea bass trial [76].

Finally, the effect of DPS on the antioxidant status of fish was also studied. In the case of liver antioxidant enzymes, although none of the enzymes showed significant differences between DPS diets and the control group, an increase in GR activity in fish fed the 1.5% diet at 30 days and a dose-dependent increase in CAT activity at 15 and 30 days was observed. In previous studies in fish, the expression of antioxidant enzymes GR, CAT and SOD was studied in gut, skin and gills [77], where no differences for any enzyme were obtained in gut samples of fish fed date extract with respect to the control fish. However, GR and CAT gene expression increased in skin of fish fed date extract alone while fish fed date extract with probiotic had increased GR but decreased SOD expression levels at 4 weeks. In the case of gill samples, fish fed date extract alone or with probiotic showed a significant increase in GR and CAT expression at 4 weeks. SOD expression increased significantly in fish fed date extract alone or with probiotic at 2 weeks, while this increase was not significant at 4 weeks [77], as in the study of Guardiola et al. [76], where a significant up-regulation of SOD expression was recorded in HK from European sea bass specimens fed date extract. As to TAA, 3% DPS diet increased TAA in skin mucus, although not in a statistically significant way, which is in line with results obtained also in sea bass [76]. Therefore, all these results suggest date palm seeds could be considered a good alternative to be included in aquaculture as additive in fish feed by enhancing humoral and cellular immune activity, which could suggest a preventive use in order to avoid fish diseases and/or infections.

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6. Abbreviations

ADF: Acid detergent fiber; ADL: Acid detergent lignin; BSA: Bovine serum albumin; CAT: Catalase; CTAB: Cetyltrimethylammonium bromide; CF: Condition factor; DPS: Date palm seeds; DMSO: Dimethyl sulfoxide; db: Dry-matter basis; ELISA: Enzyme-linked immunosorbent assay; EDTA: Ethylenediaminetetraacetic acid; FAO: Food and Agriculture Organization of the United Nations; FCS: Foetal calf serum; FITC: Fluorescein isothiocyanate; FSC: Forward scatter; GSSG: Glutathione disulphide or oxidized; GR: Glutathione reductase; HBSS: Hank's balanced salt solution; HK: Head kidney; HEWL: Hen egg white lysozyme; H_2O_2: Hydrogen peroxide; IgM: Immunoglobulin M; MENA: Middle East and North Africa;

NADPH: Nicotinamide adenine dinucleotide phosphate; NDF: Neutral detergent solution; PMA: Phorbolmyristate acetate; PBS: Phosphate-buffered saline; PBS-T: PBS-Tween; RT: Room temperature; SGR: Specific growth rate; H_2SO_4: Sulfuric acid; TCA: Trichloroacetic acid; TSA: Triptic Soy Agar; TSB: Triptic Soy Broth; SSC: Side scatter; SOD: Superoxide dismutase; WG: Weight gain; ABTS: 2,2'-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid); MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TAA: Total antioxidant activity; TMB: 3,3',5,5'-tetramethylbenzidine hydrochloride.

7. Conflict of Interest

The authors declare no conflict of interest.

REFERENCES

1. Citarasu T. Herbal biomedicines: a new opportunity for aquaculture industry. Aqua Int. 2010;18(3):403-14.
2. Harikrishnan R, Balasundaram C, Heo M. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. Aquaculture. 2011;317(1-4):1-15.
3. Bulfon C, Volpatti D, Galeotti M. Current research on the use of plant-derived products in farmed fish. Aqua Res. 2015;46(3):513-51.
4. Reverter M, Bontemps N, Lecchini D, et al. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. Aquaculture. 2014;433:50-61.
5. Van-Hai N. The use of medicinal plants as immunostimulants in aquaculture: a review. Aquaculture. 2015;446:88-96.
6. Awad E, Awaad A. Role of medicinal plants on growth performance and immune status in fish. Fish Shellfish Immunol. 2017;67:40-54.
7. Jain SM, Al-Khayri JM, Johnson DV. Date palm biotechnology. Dordrecht: Springer, Netherlands; 2011.
8. El-Hadrami A, Al-Khayri JM. Socioeconomic and traditional importance of date palm. Emir J Sci Food Agri. 2012;24(5):371-85.
9. Zaid A, de-Wet PF. Date palm cultivation. In Chapter II: Origin, geographical distribution and nutritional values of date palm. Rome United Nations FAO Plant Production and Protection. 1999;156.
10. Al-Qarawi A, Mousa H, Ali BH, et al. Protective effect of extracts from dates (Phoenix dactylifera L.) on carbon tetrachloride–induced hepatotoxicity in rats. Int J Appl Res Vet Med. 2004;2:176-80.
11. Al-Qarawi AA, Abdel-Rahman H, Ali BH, et al. The ameliorative effect of dates (Phoenix dactylifera L.) on ethanol-induced gastric ulcer in rats. J Ethnopharmacol. 2005;98(3):313-7.
12. Habib HM, Ibrahim WH. Effect of date seeds on oxidative damage and antioxidant status in vivo. J Sci Food Agric. 2011;91(9):1674-9.
13. Abdelaziz DHA, Ali SA. The protective effect of Phoenix dactylifera L. seeds against CCl4-induced hepatotoxicity in rats. J Ethnopharmacol. 2014;155(1):736-43.
14. Ahmed AF, Al-Qahtani JH, Al-Yousef HM, et al. Proanthocyanidin-rich date seed extract protects against chemically induced hepatorenal toxicity. J Med Food. 2015;18(3):280-9.
15. Waly MI, Al-Ghafri BR, Guizani N, et al. Phytonutrient effects of date pit extract against azoxymethane induced oxidative stress in the rat colon. Asian Pac J Cancer Prev. 2015;16(8):3473-7.

16. Attia H, Al-Rasheed N, Mohamad R, et al. The antifibrotic and fibrolytic properties of date fruit extract via modulation of genotoxicity, tissue-inhibitor of metalloproteinases and nuclear factor- kappa B pathway in a rat model of hepatotoxicity. BMC Complement Altetrn Med. 2016;16(1):414.
17. Hasan M, Mohieldein A. In vivo evaluation of antidiabetic, hypolipidemic, antioxidative activities of Saudi date seed extract on streptozotocin induced diabetic rats. J Clin Diagn Res. 2016;10(3):FF06-12.
18. Khan F, Khan TJ, Kalamegam G, et al. Anti-cancer effects of Ajwa dates (Phoenix dactylifera L.) in diethylnitrosamine induced hepatocellular carcinoma in Wistar rats. BMC Complement Altern Med. 2017;17:418.
19. Abdel-Magied N, Gharib AA, Zid NA. Possible ameliorative effect of aqueous extract of date (Phoenix dactylifera) pits in rats exposed to gamma radiation. Int J Radiat Biol. 2018;94(9):815-24.
20. Abdel-Salam AM, Al-Hemaid WA, Afifi AA, et al. Consolidating probiotic with dandelion, coriander and date palm seeds extracts against mercury neurotoxicity and for maintaining normal testosterone levels in male rats. Toxicol Rep. 2018;5:1069-77.
21. Khan TJ, Kuerban A, Razvi SS, et al. In vivo evaluation of hypolipidemic and antioxidative effect of ‘Ajwa’ (Phoenix dactylifera L.) date seed extract in high-fat diet-induced hyperlipidemic rat model. Biomed Pharmacother. 2018;107:675-80.
22. Belal IEH. Evaluating fungi-degraded date pits as a feed ingredient for Nile tilapia (Oreochromis niloticus L). Aqua Nutr. 2008;14(5):445-52.
23. El-Hadrami A, Daayf F, El-Hadrami I. Secondary metabolites of date palm. In: Jain SM, Al-Khayri JM, Johnson DV, editors. Date palm biotechnology. Dordrecht: Springer, Netherlands; 2011. 653-74 p.
24. Hossain MZ, Waly MI, Singh V, et al. Chemical composition of date–pits and its potential for developing value-added product - a review. Pol J Food Nutr Sci. 2014;64(4):215-26.
25. Aquaculture market in the MENA Region in 2016, Middle East's largest and leading trade exhibition dedicate to the aquaculture industry. Ventures Middle East. Dubai. 2017.
26. AOAC 945.15. Moisture in cereals, moisture in corn, moisture of grain and seeds. Official Methods of Analysis of AOAC International. 17th ed. Gaithersburg, Maryland, USA. 2010.
27. AOAC 2001.11. Protein (crude) in animal feed, forage (plant tissue). Official Methods of Analysis of AOAC International. 18th ed. Gaithersburg, Maryland, USA. 2005a.
28. AOAC 920.39. Fat (crude) or ether extract in animal feed. Official methods of AOAC International. 16th ed. Gaithersburg, Maryland, USA. 1997.
29. AOAC 942.05. Determination of ash in animal feed. Official Methods of Analysis of AOAC International. 18th ed. Gaithersburg, Maryland, USA. 2005b.
30. Nabili A, Fattoum A, Passas R, et al. Extraction and characterization of cellulose from date seeds (Phoenix dactylifera L.). Cell Chem Tech. 2016;50(9-10):1015-23.
31. Guardiola FA, Cuesta A, Arizcun M, et al. Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (Sparus aurata). Fish Shellfish Immunol. 2014;36(2):545-51.
32. Esteban MA, Mulero V, Muñoz J, et al. Methodological aspects of assessing phagocytosis of Vibrio anguillarum by leucocytes of gilthead seabream (Sparus aurata L.) by flow cytometry and electron microscopy. Cell Tissue Res. 1998;293(1):133-41.

33. Silva-Carrillo Y, Hernandez C, Hardy RW, et al. The effect of substituting fish meal with soybean meal on growth, feed efficiency, body composition and blood chemistry in juveniles potted rose snapper Lutjanus guttatus (Steindachner, 1869). Aquaculture. 2012;364-365:180-5.
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
35. Cuesta A, Meseguer J, Esteban MA. Total serum immunoglobulin M levels are affected by immunomodulators in seabream (Sparus aurata L.). Vet Immunol Immunopathol. 2004;101(3-4):203-10.
36. Parry R, Chandan RC, Shahani KM. A rapid and sensitive assay of muramidase. Exp Biol Med. 1965;119(2):384-6.
37. Stevens MG, Kehrli ME, Canning PC. A colorimetric assay for quantitating bovine neutrophil bactericidal activity. Vet Immunol Immunopathol. 1991;28(1):45-56.
38. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophy. 1993;303(2):474-82.
39. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;89(2):271-7.
40. Ross NW, Firth KJ, Wang A, et al. Changes in hydrolytic enzyme activities of naïve Atlantic salmon Salmo salar skin mucus due to infection with the salmon louse Lepeophtheirus salmonis and cortisol implantation. Dis Aquat Organ. 2000;41(1):43-51.
41. Hanif A, Bakopoulos V, Dimitriadis GJ. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (Sparus aurata) larvae. Fish Shellfish Immunol. 2004;17(5):411-35.
42. Rodríguez A, Esteban MA, Meseguer J. Phagocytosis and peroxidase release by seabream (Sparus aurata L.) leucocytes in response to yeast cells. Anat Rec A Discov Mol Cell Evol Biol. 2003;272(1):415-23.
43. Bayne CJ, Levy S. Modulation of the oxidative burst in trout myeloid cells by adrenocorticotropic hormone and catecholamines: mechanisms of action. J Leukoc Biol. 1991;50(6):554-60.
44. Quade MJ, Roth JA. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet Immunol Immunopathol. 1997;58(3-4):239-48.
45. Carlberg I, Mannervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem. 1975;250(14):5475-80.
46. Mccord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem. 1969;244(22):6049-55.
47. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121-6.
48. Arnao MB, Cano A, Acosta M. Methods to measure the antioxidant activity in plant material. Free Radic Res. 1999;31(Suppl):S89-96.
49. Holmes AH, Moore LS, Sundsfjord A, et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387(10014):176-87.
50. Magnadottir B. Immunological control of fish diseases. Mar Biotechnol (NY). 2010;12(4):361-79.
51. Chakraborty SB, Hancz C. Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. Rev Aquaculture. 2011;3(3):103-19.

52. Jian J, Wu Z. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, Pseudosciaena crocea (Richardson). Aquaculture. 2003;218(1-4):1-9.
53. Jian J, Wu Z. Influences of traditional Chinese medicine on non-specific immunity of Jian Carp (Cyprinus carpio var. Jian). Fish Shellfish Immunol. 2004;16(2):185-91.
54. Assem H, Khalifa A, Salhia MEL. Physiological and microbiological indices as indicators of evaluating dietary fungi degraded date pits as a probiotic for cultured Nile tilapia Oreochromis niloticus fingerling and its effect on fish welfare. Egypt J Aquatic Res. 2014;40(4):435-41.
55. Sotolu AO, Kigbu AA, Oshinowo AJ. Supplementation of date palm (Phoenix dactylifera) seed as feed additive in the diets of juvenile african catfish (Burchell, 1822). J Fish Aqua Sci. 2014;9(5):359-65.
56. Ahmed VM, Abdulrahman NM, Ameen SAH, et al. Impacts of date palm seeds (Phoenix dactyliferous L.) on growth indices and nutrient utilization of common carp Cyprinus carpio L. J Agric Sci Technol B. 2017;7(4):280-4.
57. Al-Kinani LMZ, Alwash AH. Study of different proportions of date stones in the ration for fattening. Iraq J Agric Sci. 1975;10:53-62.
58. Elgasim EA, Al-Yousef YA, Humeida AM. Possible hormonal activity of date pits and flesh fed to meat animals. Food Chem. 1995;52(2):149-52.
59. Al-Owaimer AN, El-Waziry AM, Koohmaraie M, et al. The use of ground date pits and Atriplex halimus as alternative feeds for sheep. Aus J Bas Appl Sci. 2011;5:1154-61.
60. Alwash AH, de Peters EJ. The use of date stones for feeding and fattening ruminant animals. World Rev Anim Prod. 1982;18:29-32.
61. Rezaeenia A, Naserian AA, Valizadeh R, et al. Effect of dietary inclusion of date seed (Phoenix dactylifera L.) on intake, digestibility, milk production, and milk fatty acid profile of Holstein dairy cows. Trop Anim Health Prod. 2018;50(7):1427-33.
62. Sharifi M, Bashtani M, Naserian AA, et al. The effect of increasing levels of date palm (Phoenix dactylifera L.) seed on the performance, ruminal fermentation, antioxidant status and milk fatty acid profile of Saanen dairy goats. J Anim Physiol Anim Nutr (Berl). 2017;101(5):e332-41.
63. Najib H, Al-Yousef YM, Hmeidan M. Partial replacement of corn with dates in layer diets. J Appl Anim Res. 1994;6:91-6.
64. Al-Bowait M, Al-Sultan S. The effect of partial replacement of maize by alkali-treated date pits on broiler growth, survival rate and economic returns. Livest Res Rural Dev. 2007;19(9):1-12.
65. Vandepopuliere JM, Al-Yousef Y, Lyons J. Dates and date pits as ingredients in broiler starting and Coturnix quail breeder diets. Poult Sci. 1995;74(7):1134-42.
66. El-Far AH, Ahmed HA, Shaheen HM. Dietary supplementation of Phoenix dactylifera seeds enhances performance, immune response, and antioxidant status in broilers. Oxid Med Cell Longev. 2016;2016:5454963.
67. Hussein AS, Alhadrami GA, Khalil YH. The use of dates and date pits in broiler starter and finisher diets. Bioresour Technol. 1998;66(3):219-23.
68. Yousif OM, Osman MF, Alhadrami GA. Evaluation of dates and date pits as dietary ingredients in tilapia (Oreochromis aureus) diets differing in protein sources. Bioresour Technol. 1996;57(1):81-5.
69. Belal IEH, Al-Owaifer MA. Incorporating date pits Phoenix dactylifera and their sprouts in semi-purified diets for Nile tilapia Oreochromis niloticus (L.). J World Aquaculture Soc. 2004;35(4):452-9.

70. Gaber MM, Labib EH, Omar EA, et al. Effect of partially replacing corn meal by date stone on growth performance in Nile tilapia (Oreochromis niloticus) fingerlings, diets supplemented with digestarom. Sci Rep. 2012;1(10):1-5.
71. Gaber MM, Labib EH, Omar EA, et al. Effect of partially replacing corn meal by date stone on growth performance in Nile tilapia fingerlings, diets supplemented with marjoram: effects of date stone on growth Nile tilapia. Green J Agric Sci. 2014;4(1):001-008.
72. Gidenne T. Dietary fibres in the nutrition of the growing rabbit and recommendations to preserve digestive health: a review. Animal. 2015;9(2):227-42.
73. FAO. The state of world fisheries and aquaculture. 2016.
74. Llewellyn MS, Boutin S, Hoseinifar SH, et al. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front Microbiol. 2014;5:207.
75. Hoseinifar SH, Khalili M, Rufchaei R, et al. Effects of date palm fruit extracts on skin mucosal immunity, immune related genes expression and growth performance of common carp (Cyprinus carpio) fry. Fish Shellfish Immunol. 2015;47(2):706-11.
76. Guardiola FA, Porcino C, Cerezuela R, et al. 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). Fish Shellfish Immunol. 2016;52:298-308.
77. Esteban MA, Cordero H, Martínez-Tomé M, et al. Effect of dietary supplementation of probiotics and palm fruits extracts on the antioxidant enzyme gene expression in the mucosae of gilthead seabream (Sparus aurata L.). Fish Shellfish Immunol. 2014;39(2):532-40.
78. Cerezuela R, Guardiola FA, Cuesta A, et al. Enrichment of gilthead seabream (Sparus aurata L.) diet with palm fruit extracts and probiotics: effects on skin mucosal immunity. Fish Shellfish Immunol. 2016;49:100-9.