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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