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Dietary administration effects of fenugreek seeds on skin mucosal antioxidant and immunity status of gilthead seabream (*Sparus aurata* L.)

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ABSTRACT

Improving fish defense through the preventive administration of immunostimulants has an important role in controlling the outbreak of the disease in aquaculture. As a continuity of our previous studies, this paper describes the effects of dietary fenugreek (*Trigonella foenum graecum*) seeds on the skin mucosal antioxidant status and immune response of gilthead seabream (*Sparus aurata* L.). Fish were fed with four experimental diets: one a basal diet (control) and three diets with powdered fenugreek seeds incorporated in the fish feed at 1%, 5%, and 10%. After eight weeks of feeding, free radicals scavenging and antioxidant assays were assessed in skin mucus by measuring the peroxidation of phospholipid liposomes, hydroxyl radical and hydrogen peroxide scavenging, measurement of total antioxidant activity and the determination of antioxidant activity in a linoleic acid system. The skin mucosal immune response was evaluated by measuring the IgM levels and some enzymatic activities (peroxidase, antiprotease, protease, esterase, and ceruloplasmin). Our results demonstrated that fenugreek inclusion improved the hydroxyl radical scavenging capacity and conferred very high antioxidant activity. Besides, only the highest supplementation level (10%) was able to augment the peroxidase and protease activities confronted by a general decrement in the antiprotease activity in the experimental fed groups with 1% and 10%. These results suggest that the dietary administration of fenugreek at the higher inclusion dose enhances the skin mucosal immunity response and the antioxidant status of gilthead seabream a species with one of the highest rates of production in marine aquaculture.

1. Introduction

To date, very few studies have studied the effects of immunostimulants plant administration on the fish skin mucosal immunity [1–12]. These studies have been reported that administration of several herbal dietary supplements improved the skin mucosal immune responses of important species related to aquaculture such as common carp, rainbow trout, and gilthead seabream, among others. In fish as in other vertebrates, the skin is the outermost organ separating the individual from its environment and forming the first line of defence against biological, chemical, or physical hazards [13]. The fish skin is covered by a mucus layer involved in several important physiological functions such as immune protection against pathogenic invasion [14]. This activity is managed by an array of immune-related substances present within the mucus layer such as numerous antimicrobial peptides, different enzymes (e.g., peroxidase, protease, antiproteases, and lysozyme), agglutinins, immunoglobulins and C-reactive proteins [15]. In fact, during an infection in teleost fish, the mucosal barriers perform inherent, innate immune responses which are known to be the first, quickest and strongest mechanisms of defence [16].

The immune response is a key feature on the fish health, numerous additives are being included in fish feeds as immune-modulating

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ingredients which can boost the immune system of farmed animals. In recent years, the preventive use of herbal immunostimulants in aquaculture is very often and can overcome many of the problems associated with fish diseases [17]. In addition to their important activity against a broad spectrum of pathogens, herbal immunostimulants or their extracts are often locally available, inexpensive, easily biodegradable, and hence environment-friendly [18]. Also, their high contents of several active compounds such as alkaloids, terpenoids, tannins and saponins, among others, play a crucial role in increasing both innate and adaptive immune response and antioxidant defence see the reviews [19,20]. Concerning to antioxidant defence, the cellular antioxidant action is strengthened by the presence of dietary antioxidants [21,22] which have a protective role from oxidative damage during oxidative stress. The efficacy of many natural antioxidants, especially those of plant origin, have already been demonstrated recently in several fish species [4,7,23–30] due to their potential health benefits.

Among medicinal plants, seeds of fenugreek (*Trigonella foenum-graecum* L.) have a wide range of properties such as hypoglycemic, hypocholesterolemic, lactation aid, antibacterial, antifungal, anticancer and particularly antioxidant and immunological [31]. On our previous works, the antioxidant and immunological properties of fenugreek were tested by the inclusion on the fish diets and have been demonstrated the protective effect on lipid peroxidation and enzymatic antioxidant in muscle or liver [29]. The best results enhancing of the mucosal skin immunity in gilthead seabream were obtained on a three weeks trial when it was added in combination with one probiotic strain (*Lactobacillus plantarum*) [5]. However, the promising effects reported on the fish when the fenugreek was supplied encouraged further research endeavours to explore the mucosal immune-modulatory functions and the antioxidant capacity of skin mucus after a longer period of administration. To the best of our knowledge, there are no previous studies available focused on the skin mucus antioxidant capacity after dietary administration of herbal immunostimulants.

Taking into account all these considerations, the main objective of the present study was to evaluate the *in vivo* potential effects of dietary supplementation with fenugreek on skin mucosal antioxidant status and immune response in gilthead seabream after two months of feeding. Innate immunity is known to be the first, quickest and strongest mechanisms of defense [16]. The results are discussed on if the fenugreek dietary had any negative influence or if, on the contrary, enhances the fish defence mechanisms when is administered for a period longer than the one previously studied.

2. Material and methods

2.1. Animals

Eighty (45.47 ± 9.5 g weight and 13.96 ± 0.9 cm length) specimens of the seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from the 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 ± 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 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. Experimental diets

Fenugreek (*T. foenum-graecum*) seeds, obtained from a local market (Cairo, Egypt) were crushed. In the laboratory, the seeds were added to a commercial pellet diet [D-2 Optibream AE 1P: crude protein (48%), fat (18%), ash (6.3%) and cellulose (3.6%)] (Skretting, Spain) at 1, 5, and 10 g 100^{-1} g of fenugreek seed powder. Briefly, the commercial pellet diet was crushed and mixed with distilled water containing the adequate amount of fenugreek seed powder or not supplemented (control diet). The final products were introduced into a meat grinder at room temperature and re-pelleted. The resulting pellets were dried in a forced-air oven at 37 °C for 24 h before packaging in polypropylene bags and storing at 4 °C until use.

2.3. Experimental design

Fish were randomly distributed into four identical tanks (20 fish per group) where the following groups were established: 1) control, non-supplemented diet (0%); 2) 1%, diet supplemented with 1 g 100^{-1} g of fenugreek seed; 3) 5%, diet supplemented with 5 g 100^{-1} g of fenugreek seed; and 4) 10%, diet supplemented with 10 g 100^{-1} g of fenugreek seed. The fish were fed at a rate of 2% body weight day^{-1} during eight weeks. At the end of the trial, all specimens were starved for 24 h before sampling and then they were sacrificed by using an overdose of MS-222 (Sandoz, 100 mg L^{-1} water) and sampled.

2.4. Sample collection

Twenty specimens of each group were sampled at the end of the trial. Skin mucus samples were collected from naïve specimens using the method described by Ref. [32]. Briefly, skin mucus was collected by gentle scraping the dorsolateral surface of seabream specimens using a cell scraper with sufficient care to avoid contamination with blood and genitourinary and intestinal excretions. Collected mucus samples were vigorously shaken, centrifuged (3000 × g, 10 min, 4 °C) and frozen at −20 °C until use.

2.5. Free radicals scavenging and antioxidant assays

All the chemicals we used were of chromatographic grade and were purchased from Sigma Chemical Co. (Poole, Dorset UK). The food additive compiled in Alimentarius Codex [33] propyl gallate (PG, E−310) was used as antioxidant standard (at the permitted concentration of 100 mg g^{-1}).

2.5.1. Peroxidation of phospholipid liposomes

The ability of skin mucus samples to inhibit lipid peroxidation at pH 7.4 was tested by using ox brain phospholipid liposomes, as described by Ref. [34]. The experiments were conducted with a phosphate buffer solution (PBS) (3.4 mM Na_2H-PO_4-NaH_2-PO_4, 0.15 M NaCl), pH 7.4. The assay mixtures were made up to a final volume of 1 ml, with PBS, 0.5 mg ml^{-1} phospholipid liposomes, 100 μM $FeCl_3$, 100 μl samples or 100 μl of common food additive dissolved in water, and 100 μM ascorbate (added to start the reaction). After incubation (37 °C, 60 min), 1 ml each of 1% (w/v) thiobarbituric acid (TBA) and 2.8% (w/v) trichloroacetic acid were added to each mixture. The solutions were heated in a water bath (80 °C, 20 min) to develop the malondialdehyde thiobarbituric adduct ($(TBA)_2$-MDA). The $(TBA)_2$-MDA chromogen was extracted into 2 ml of butan-1-ol and the extent of peroxidation was measured in the organic layer as absorbance at 532 nm.

2.5.2. Hydroxyl radical scavenging

The deoxyribose assay was used to detect possible scavengers of hydroxyl radicals. In a final volume of 1.2 ml, the reaction mixtures contained the following reagents: 10 mM KH_2PO_4-KOH buffer (pH 7.4), 2.8 mM H_2O_2, 2.8 mM deoxyribose, 50 μM $FeCl_3$ premixed with 100 μM EDTA before addition to the reaction mixture, and 100 μl of tested samples, or 100 μl of common food additive dissolved in water. Ascorbate (100 μM) was added to start the reaction. The tubes were incubated at 37 °C for 1 h. The products of the hydroxyl radical (OH) attack on deoxyribose were measured with thiobarbituric acid [35].