Fish & Shellfish Immunology (2008) **24**, 663–668

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SHORT COMMUNICATION

Effects of inulin on gilthead seabream (*Sparus aurata* L.) innate immune parameters

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Received 10 July 2007; revised 6 September 2007; accepted 5 October 2007
Available online 25 October 2007

KEYWORDS
Inulin;
Leucocytes;
Innate immune system;
Gilthead seabream;
Fish;
Teleost

Abstract Inulin, a fructooligossacharide, is a prebiotic that plays an important role in the immune function in mammals, but it has never been assayed in other vertebrate groups. Thus, we have studied the inulin effects on the gilthead seabream (*Sparus aurata* L.) innate immune response both *in vitro* and *in vivo*. For the *in vitro* study, head-kidney leucocytes were incubated with inulin (ranging from 0 to 1000 $\mu g\ ml^{-1}$) for 30, 90, 180 and 300 min and 24 h and any effect was observed on leucocyte viability or the main innate cellular immune responses (leucocyte peroxidase, phagocytic, respiratory burst and natural cytotoxic activities). For the *in vivo* study, seabream specimens were fed for 1 or 2 weeks with a commercial diet supplemented with inulin: 0 (control), 5 or 10 g inulin kg^{-1} diet (0.5 and 1%, respectively). Inulin produced a significant inhibition in phagocytosis and respiratory burst in leucocytes from specimens fed diets containing 0.5% or 1% of inulin for 1 week.
Based on the present results, inulin does not seem to be a good immunostimulant for seabream, though its effects in other species and combined with other immunostimulans (i.e. probiotics) might be of great interest.

Prebiotics are non-digestible food ingredients that beneficially affect the host. Among their effects, they selectively stimulate the growth and/or activity of bacteria in the colon [1,2], while among potential prebiotics, the effects of fructooligossacharides (FOS) have been widely investigated in humans and other higher vertebrates. Inulins are naturally occurring FOS. They are produced by many types of plants and belong to a class of carbohydrates known as fructans, so that they are mainly comprised of fructose units and, typically, have a terminal glucose [3]. Fructans are non-digestible oligossacharides which are fermented by the gut microflora, particulary, bifidobacteria and lactobacilli [4]. Several *in vitro* and *in vivo* studies have demonstrated that inulin or FOS provide an effective means of promoting

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1050-4648/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2007.10.002

bifidobacterium and lactobacillus growth, while selectively arresting the growth of pathogenic microorganisms [5—12].

It is know that inulin, particularly long chain molecules thereof, stimulates the human immune system by binding to specific lectin-like receptors on leucocytes and increasing macrophage proliferation [13]. Moreover, mice fed inulin show an increased percentage of NK cells and/or macrophage response kinetic [14]. It has also been demonstrated that the insoluble gamma form of inulin is able to activate the alternative complement pathway by triggering presence of complement C3 receptors on the surface of macrophages [15]. These results underline the effect of inulin on the mammalian immune system, although, in fact, very little work has been carried out to understand such immunomodulatory actions.

To date, numerous studies have demonstrated the benefits of immunostimulants on the fish immune system. However, there is very little information regarding the effect of prebiotics in fish [16], although they have been successfully used for more than ten years in other cultivated species [17—21]. Thus, the present study aims to determine the effects of inulin on the innate immune system of gilthead seabream (*Sparus aurata*, L.), a very important fish species in the Mediterranean aquaculture, both *in vitro* and *in vivo*, while the potential use of the inulin as fish immunostimulant will be discussed.

Forty-five specimens (175 g mean bw) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from CULMAREX S.A. (Murcia, Spain), were kept in 4 running seawater aquaria (450—500 l, flow rate 1500 l h^{-1}) at 28‰ salinity, 20 °C and a 12 h light: 12 h dark photoperiod. The animals were fed with a commercial pellet diet (Skretting) at a rate of 1% bw day^{-1}. Animals were acclimated for 15 days prior to the experiments. The Bioethical Committee of the University of Murcia approved the studies described. For sampling, specimens were anaesthetised with 100 mg l^{-1} MS222 (Sandoz), bleed and head-kidney (HK) leucocytes isolated from each fish under sterile conditions [22]. Briefly, blood was allowed to clot at 4 °C and the serum removed and stored at 80 °C. The head-kidney was excised, cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 2% foetal calf serum (FCS, Gibco), 100 i.u. ml^{-1} penicillin (Flow) and 100 μg ml^{-1} streptomycin (Flow)] [22]. 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 and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was determined by the trypan blue exclusion test.

For *in vitro* study, HK leucocytes were dispensed into wells of flat-bottomed 96-well microtitre plates (Nunc) and incubated with inulin (0-control-, 7.81, 15.625, 31.25, 62.5, 125, 250, 500 or 1000 μg ml^{-1}) for 30, 90, 180 or 300 min or 24 h at 22 °C, with 85% relative humidity and 5% CO_2 atmosphere. Leucocyte viability and cellular immune activities were determined. For the *in vivo* study, thirty fish were placed into 3 aquaria and each group received a diet consisting of a non-supplemented commercial diet (Skretting) (control group) or the same diet supplemented with 0.5% or 1% of inulin. To prepare the diets, a commercial pellet diet was crushed, mixed with the appropriate inulin concentration and water, and made again into pellets, which were allowed to dry and stored at 4 °C until use. Control diets were prepared adding only water. Fish were fed at a rate of 10 g dry diet kg^{-1} biomass, for 1 or 2 weeks. Five specimens from each group were sampled to measure the humoral and cellular immune activities. No mortality was observed during the experiment.

Firstly, to determine whether inulin affects HK leucocyte viability, they were incubated with inulin and 30 μl of propidium iodide (PI) (400 μg ml^{-1}, Sigma) were added to each sample. The samples were then analysed in a FACScan (Becton Dickinson) flow cytometer with an argon-ion laser adjusted to 488 nm. Analyses were performed on 5000 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 red fluorescence (FL2) dot plots or histograms were made on a computerised system. Dead cells were estimated as the percentage of cells with propidium iodide (red-PI fluorescent cells, PI^+).

Among humoral immune activities, the natural haemolytic complement and peroxidase activities were assayed receiving the diet. Briefly, 15 μl of serum were diluted with 135 μl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates [23]. Then, 50 μl of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB) (Sigma) and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 μl of 2 M sulfuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum were used as blanks. Finally, the activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [24]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and EGTA 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. 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 $Y/(1-Y)$ 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 group.

Among the cellular immune activities, the phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry [25]. 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 leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 × *g*, 5 min, 22 °C), resuspended and incubated at 22 °C for 30 min. At the end of