The influence of dietary β-glucans on the adaptive and innate immune responses of European sea bass (*Dicentrarchus labrax*) vaccinated against vibriosis

Alessio Bonaldo[1], Kim D. Thompson[2], Amedeo Manfrin[3], Alexandra Adams[2], Erminio Murano[4], Attilio Luigi Mordenti[1], Pier Paolo Gatta[1]

[1] Dipartimento di Morfofisiologia veterinaria e Produzioni animali. Università di Bologna, Italy
[2] Institute of Aquaculture. University of Stirling, Scotland, UK
[3] Istituto Zooprofilattico Sperimentale delle Venezie. Padova, Italy
[4] Protos Research Institute. Trieste, Italy

Corresponding author: Dr. Alessio Bonaldo. Dipartimento di Morfofisiologia Veterinaria e Produzioni Animali. Università di Bologna. Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy - Tel. +39 051 2097992 - Fax: +39 051 2097373 - Email: alessio.bonaldo@unibo.it

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ABSTRACT

The effects of feeding 1,3/1,6 β-glucans on the innate and the adaptive immune responses of European sea bass (*Dicentrarchus labrax*) was investigated. Two experiments were carried out during the study. In the first, a number of non-specific immune parameters were examined at 4, 7, 10, 14, 21 and 25 days of feeding fish with a semipurified diet containing Macrogard ©, a commercially available form of 1,3/1,6 β-glucans. The respiratory burst activity of head kidney macrophages isolated from the different groups of fish fed the immunostimulant peaked and subsequently decreased at different times during the experiment. Head kidney macrophages from fish fed 250 ppm *β*-glucans had a statistically higher level of respiratory burst activity at Day 21 of feeding compared with fish fed no immunostimulant. No statistical differences were observed in lyzozyme activity during this trial.

In the second experiment, the effect of feeding 1,3/1,6 β-glucans on the immune response of fish to an alginate-encapsulated *Vibrio* vaccine administered orally was examined. Respiratory burst of head kidney macrophages and serum lysozyme activity decreased in all fish over the course of the trial, while serum lysozyme activity was considerably lower than values obtained in the first experiment. Fish vaccinated orally had significant increases in antibody response by Week 2 post-vaccination, but *β*-glucans did not appear to affect these levels. Vaccination may have resulted in activating the immune system as a whole, thus masking any difference in immunostimulation by the β-glucans. It may be that the optimal doses and timing of *β*-glucans administration is different when the immunostimulant is administered alone or in combination with the vaccine.

In conclusion, European sea bass can be immunomodulated with oral administration of β-glucan. Optimal doses and administration times have been established when *β*-glucans are fed alone, although further studies are needed to establish ideal feeding regimes for glucans administrated in combination with vaccination.

Key words: Seabass, Glucan, Immunostimulants, Immune response, Oral vaccination.

RIASSUNTO

L'INFLUENZA DEI β-GLUCANI ORALI SUL SISTEMA IMMUNITARIO SPECIFICO E ASPECIFICO DEL BRANZINO (*DICENTRARCHUS LABRAX*) VACCINATO CONTRO LA VIBRIOSI

Questo studio è stato condotto per determinare l'effetto che i 1,3/1,6 β-glucani possono esercitare sul sistema immunitario specifico e aspecifico del branzino Dicentrarchus labrax. A questo scopo sono stati effettuati due esperimenti. Nel primo, alcuni parametri relativi al sistema immunitario aspecifico sono stati esaminati dopo 4, 7 10, 14, 21 e 25 giorni di alimentazione con diete semi-purificate contenenti il prodotto commerciale a base di β-glucani Macrogard®. L'attività relativa allo scoppio respiratorio dei macrofagi del rene craniale isolati dagli animali di tutti i gruppi sperimentali ha mostrato un picco di attività seguito da un decremento, ma con temporalità differenti a seconda del dosaggio. I macrofagi dei pesci alimentati con la dieta contenente 250 ppm di glucani hanno mostrato differenze statisticamente significative rispetto agli altri gruppi al giorno 21 di somministrazione. Nessuna differenza è stata riscontrata per quanto riguarda l'attività del lisozima. Nel secondo esperimento, gli effetti della somministrazione orale di β-glucani sono stati valutati quando somministrati insieme ad un vaccino orale contro la vibriosi. L'attività relativa allo scoppio respiratorio dei macrofagi e l'attività del lisozima sono calati lungo tutto il periodo di osservazione, con quest'ultimo valore considerevolmente più basso rispetto a quello ottenuto nel primo esperimento. I pesci vaccinati oralmente hanno mostrato un aumento della risposta anticorpale dopo 2 settimane dalla somministrazione di vaccino anche se i β-glucani non hanno mostrato effetti sul sistema immunitario specifico dei pesci. La vaccinazione, stimolando l'attività del sistema immunitario, può aver mascherato l'effetto dei β-glucani. La dose ottimale di β-glucani potrebbe essere differente quando questi vengono somministrati insieme ad un vaccino. In conclusione, il branzino sembra poter essere immunomodulato mediante la somministrazione oral dei β-glucani. È stato possibile stabilire la dose e il tempo di somministrazione ottimali quando i β-glucani vengono utilizzati singolarmente; al contrario, sono necessari ulteriori studi per stabilire la dose ottimale quando questi vengono somministrati insieme ad un vaccino.

Parole chiave: *Branzino, Glucani, Immunostimolanti, Risposta immunitaria, Vaccinazione orale.*

Introduction

β-glucans have proven to be valuable substances in the control of fish disease, and their application may be useful for controlling disease in fish culture. The immunomodulatory properties of these substances were first demonstrated in mammals, in which they were shown to induce haemopoiesis, enhance immunity and thus increase resistance to infectious pathogens (Di Luzio, 1985). Attention has since focused on the effects of these compounds on fish. Many studies have been carried out with these substances on different species such as Atlantic salmon (*Salmo salar)* (Brattgjerd *et al.*, 1994; Dalmo *et al.*, 1996; Paulsen *et al*., 2001), rainbow trout (*Oncorhynchus mykiss)* (Jorgensen *et al.*, 1993; Verlhac *et al.* 1996; Verlhac *et al.*, 1998), African catfish (*Claria gariepinus*) (Yoshida *et al.*, 1995) and snapper (*Pagrus auratus*) (Cook *et al.*, 2003). There is strong experimental evidence to suggest that β-glucans**,** administered in the diet or by intraperitoneal injection (i.p.), can modify the activity of some components of the immune system of fish (Anderson, 1996; Secombes, 1996; Galeotti, 1998; Robertsen, 1999; Sakai, 1999). Several studies have recently been reported on the effects of β-glucans in Mediterranean species, both on their innate and their adaptive immune responses. According to Castro *et al.* (1999), macrophages isolated from the head kidney of gilthead seabream (*Sparus aurata*) and incubated with high levels of b-glucans showed enhanced respiratory burst activity, followed by a period of reduced activity. Spain-Ortuño *et al.* (2002) showed that

oral administration of yeast cells (*Saccharomyces cerevisiae),* containing high levels of glucans, can increase several parameters of the innate immune response of seabream. In turbot (*Scophthalmus maximus*), serum lysozyme activity was significantly higher in fish injected with β-glucans 7, 14 and 21 days after treatment (Santarèm *et al.*, 1997). Groups of seabream fed with 1 and 10 g Kg^{-1} of glucans for short periods of time, showed a higher degree of protection against pasteurellosis than the control group (Couso *et al.*, 2003). On the other hand, no effect of β-glucans was found in turbot against *Enterococcus* sp. (Toranzo *et al.,* 1995*)*.

With regard to other Mediterranean species, few experiments are available in the literature relating to European sea bass (*Dicentrarchus labrax*) (Bagni *et al.*, 2000; 2005). The present study was therefore undertaken to investigate the effect of orally administered 1,3/1,6 β-glucan on the innate and the adaptive immune responses of sea bass. The effect of glucan treatment on the fishes' immune response to both oral and immersion vaccination against *Vibrio anguillarum* was examined.

Material and methods

Fish

European sea bass of 60.4 ± 0.5 g and 20.1 ± 0.9 g body weight were used in Experiments *I* and *II*, respectively. Animals were obtained from the Valle Cà Zuliani Srl hatchery (Pila di Porto Tolle, Rovigo, Italy) and transported to the Laboratory of Aquaculture, University of Bologna, Cesenatico (FC), Italy. Fish were acclimated to experimental conditions for a week before commencing the experiments. The experimental tanks were supplied with recirculated seawater (oxygen > 6.0 mg/l, temperature 21 ± 1 °C, salinity 30‰), with a 10/14 h L/D cycle throughout each experiment.

Table 1. Composition of semi-purified basal formula used in the Experiment I and II.

Ingredients (g 100 g^{-1} wet weight):	
Casein	45.0
Pre-gelatinised starch	20.0
Fish oil	15.0
Dextrin	10.0
Gelatine	4.5
Mineral mix	2.0
Vitamin mix	2.0
L-arginine	1.0
DL-methionine	0.5

Vaccine

Vibrio anguillarum (serotype O1, strain 43305), obtained from the American Tissue Culture Collection was grown in tryptose soya broth for 24 h. After reaching the stationery growth phase, the bacteria were inactivated with formalin, the concentration of which was 4.9 x 10^8 cells ml^{-1}. A portion of this suspension was used as an immersion vaccine, while the remainder was particulated in alginate by first freeze-drying it and then placing it in an ultra-mixer until an average particle size of less than 50 µm was obtained. Particles were dispersed in 2 L of a 3 % w/w sodium alginate solution (Protanal Pronova LF 10/60). The dispersion was then dropped into 1 M $CaCl_2$ by means of a multi-nozzle air drop generator. The resulting microspheres were recovered from the calcium bath, rinsed with distilled water and freeze dried. The dried microspheres were sieved to obtain an average particle size of up to 250 µm (Murano *et al.*, 1997).

Diets

The composition of the semipurified basal diets used in the study is given in Table 1. The formulation used was similar to that of Kaushik *et al.* (1998) which has been shown

to be nutritionally suitable for European sea bass. Vitamin-free casein, fish oil, pre-gelatinised starch and dextrin were used as dietary protein, lipid and carbohydrate sources respectively. Diets, 2.5 mm in diameter, were produced using a pelletizer machine at the Department of Veterinary Morphophysiology and Animal Production (DIMORFIPA, University of Bologna). Glucan (Macrogard© Biotec Asa, Norway) and vaccine were added to the mixture prior to pellettizing the diet at the concentrations shown below.

Experiment I set up

Sea bass, with a body weight of 60.4 ± 0.5 g, were randomly divided into four 1000 l cubic tanks with 30 fish used per tank. The tanks were part of a recirculation system consisting of a mechanical filter, a UV filter and a biological filter. Four diets containing different levels of β-glucans were administered for a 25-day period in order to evaluate their effect on the innate immune response of the sea bass. The diets used were designated as either low (L) containing β-glucan at 250 ppm; medium (M) containing β-glucan at 500 ppm; high (H) containing β-glucan at 1000 ppm or (C) the control diet containing no glucan. Animals were fed twice daily with a daily ration of 1.5 % body weight^{-1} (BW). Levels of respiratory burst activity by head kidney macrophages and serum lysozyme activity were evaluated for 5 fish per diet at Day 4, 7, 10, 14, 21 and 25 after commencing feeding.

Experiment II set up

Sea bass with a body weight of 20.1 ± 0.9 g were used in the second trial, and again 30 fish per tank were used, randomly placed in eighteen 200-l cylinder-conical tanks supplied with recirculating water. The diets used were designated as (M) containing β-glucan at 500 ppm; (H) containing β-glucan at 1000 ppm or (C) the control diet, containing no glucan. Fish were fed twice a day at 1.5 % BW throughout the experimental period.

Table 2. Antibody response in the serum of sea bass after vaccination against *Vibrio anguillarum* (*n*= 6 fish) in Experiment II.

	Week			
Treatment	0	2	4	6
C-I	0.058±0.007	0.296±0.142*	0.155±0.035*	0.178±0.066*
C-O	0.068±0.016	0.216±0.082*	0.143±0.022*	0.133±0.015*
M-Short	0.057±0.007	0.139±0.024*	0.164±0.106*	0.135±0.028*
M-Long	0.053±0.004	0.231±0.084*	0.137±0.040*	0.140±0.040*
H-Short	0.055±0.002	0.222±0.074*	0.155±0.018*	0.135±0.030*
H-Long	0.056±0.000	0.180±0.058*	0.143±0.038*	0.194±0.062*

C = control; M = 500 ppm glucans; H = 1000 ppm glucans; C-I = control vaccinated by immersion; C-O = orally vaccinated control; Short = fed immunostimulants during stage 1 and 2; Long = fed immunostimulants during stages 1, 2 and 3. Optical density of positive controls and blank was 0.696 ± 0.053 nm and 0.058.5 ± 0.009 nm, respectively. Blank has been cut off from any value shown in this table.

** denotes a significant difference from week 0 within the same treatment groups (P<0.05).*

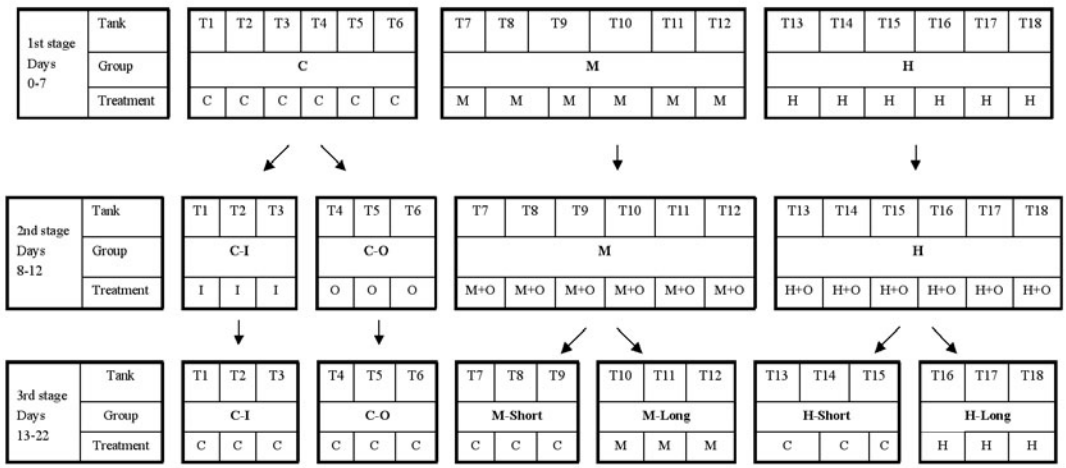
The feeding protocol over the course of the trial was divided into three different stages as shown in Figure 1. In the first stage, the diets were administered to six tanks from Day 0 until Day 7. At the end of this period, fish were sampled to determine the levels of respiratory burst by head kidney macrophages and serum lysozyme activity. During the second stage of the trial, from Day 8 until Day 12, immunostimulated groups and three of the six tanks fed the control diet were treated with oral vaccine (C-O). Pellets containing the vaccine were fed to fish in order to achieve a dose of 10^9 cells animal^{-1} over the five day feeding period. During this period glucans continued to be administered with the vaccine. At the same time, the other three tanks fed the control diet were vaccinated by immersion at Day 10 (C-I). This consisted of immersing the fish in the bacterin suspension described above (diluted 1:3 dilution) for 3 min while held in seawater. At Day 13, a second sampling was made to determine respiratory burst activity of head kidney macrophages and serum lysozyme activity. During the third stage, from Day 13 until Day 22, three tanks from each group, treated with immunostimulants, were switched to the control diet (Short) whereas the other tanks were fed with glucans for a further ten days (Long). The control fish continued to be fed diets with no immunostimulants.

The antibody response of six fish per treatment were measured at 0, 2, 4 and 6 weeks post-vaccination. From day 23 until the end of the experiment, fish were fed practical diet (Hendrix S.pA., Mozzecane, Italy) twice a day at a daily ration of 1.5 BW^{-1}.

Determination of respiratory burst activity of head kidney macrophages

Head kidney macrophage suspensions were prepared according the method of Secombes (1990), by teasing head-kidney tissue trough a 100 μm nylon mesh into Leibovitz-15 medium (L-15) (Sigma) containing 20 iu ml^{-1} of heparin. The cell suspension was then lay-

Figure 1. Feeding protocol of Experiment II.



C = control fed no immunostimulants; C-I = control vaccinated by immersion; C-O = orally vaccinated control; M = fed diets with 500 ppm glucans; H = fed diets with 1000 ppm glucans; Short = fed immunostimulants during stage 1 and 2; Long = fed immunostimulants during stages 1, 2 and 3.

ered onto a 34%/51% Percoll gradient and centrifuged at 400 x *g* for 25 min at 4°C. The macrophages at the interface of the two concentrations were collected and washed twice with L-15 medium by centrifugation at 1000 x *g* for 7 min**.** The concentration of cell suspension was adjusted to 1 x 10^7 cell ml^{-1}. Each cell suspension was placed in eight wells of a 96 well tissue culture plate (Microtest™, Bencton Dickinson Labware) at 100 µl $well^{-1}$. The cells were incubated at 23°C for 3 h before washing the plates three times with L-15 medium to remove non-adherent cells. L-15 containing 1 mg ml^{-1} Nitroblue tetrazolium (TMB) was added to three of the eight wells (100 µl $well^{-1}$). L-15 containing 1 µg ml^{-1} NBT and phorol myristate acetate (PMA) (Sigma) at 1 mg l^{-1} was added to another three of the eight wells. The plates were incubated for 60 min at 23°C before stopping the reaction with methanol. The wells were washed twice with 70% (v/v) methanol and allowed to air dry. The insoluble formazan that resulted was dissolved in 120 µl 2 M KOH and 140 µl dimethyl sulphoxide. The contents of each well were carefully mixed and air bubbles removed before reading the absorbance of the reaction at 630 nm with an ELISA reader plate reader (Labsystems Multiskan MS). The number of macrophages adhering in each well was determined from the remaining two wells by adding the lysis buffer (Secombes, 1990), and counting released nuclei on a haemocytometer. The results were then adjusted to an absorbance at 630 nm for 10^5 cells.

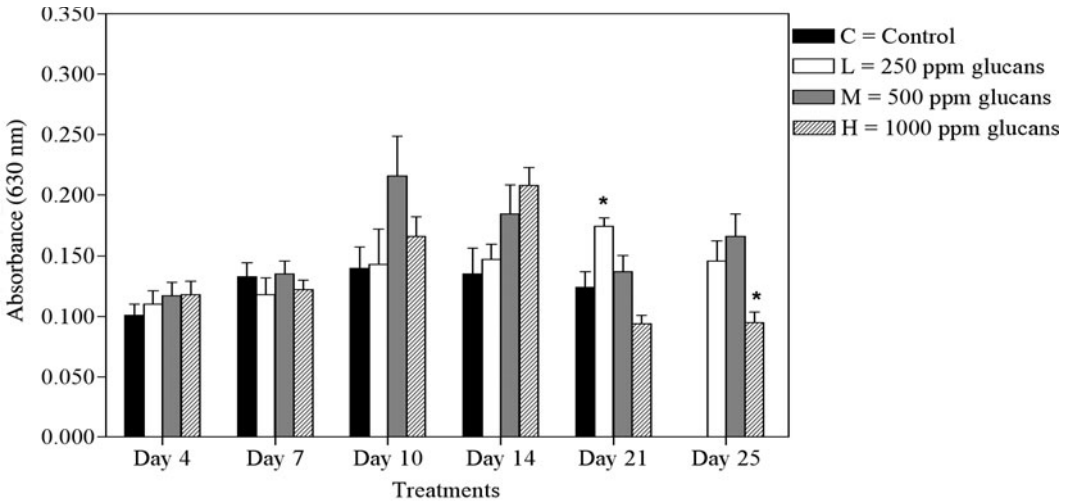
Determination of serum lysozyme activity

Serum lysozyme activity was measured using a turbidity assay in which 0.2 $mgml^{-1}$ lyophilised *Micrococcus lysodekticus* in 0.04 M sodium phosphate buffer (pH 6.1) was used as a substrate (Parry *et al*., 1965). One hundred µl of serum was added to 2 ml of the bacterial suspension and the reduction in absorbance at 540 nm determined after incubating between 0.5 and 6.5 min at 22°C.

Determination of anti-Vibrio anguillarum antibodies by ELISA

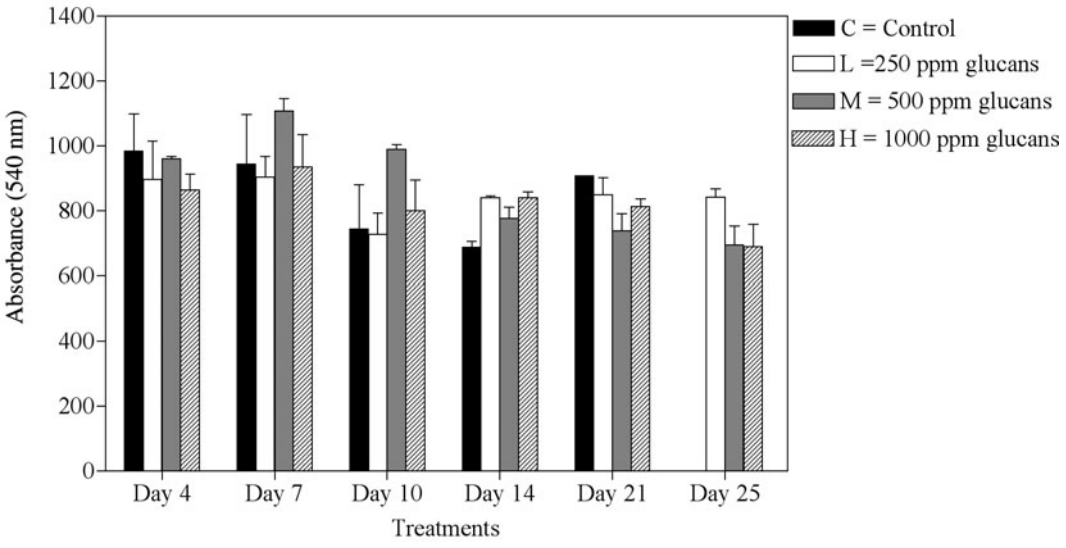
The antibody levels against *V. anguillarum* in sera from experimental fish were determined by an enzyme-linked immunosorbent assay (ELISA). *V. anguillarum,* prepared as described above, was diluted to 20 µg ml^{-1} with PBS, and added to microplates (Polysorp, Nunc) at 50 µl $well^{-1}$. The plates were incubated overnight at 22° ± 2. The following day, the plates were washed four times with phosphate buffer saline [PBS-T: 0.01 M phosphate containing 0.05 % Tween-20, (Sigma)], and non-specific binding sites on the plates were blocked with 3 % bovine serum albumin (BSA)(Sigma) in PBS-T for 30 min at 22°C. After washing the plate four times, sea bass sera diluted 1:100 in BSA PBS-T, were added to the plate at 50 µl $well^{-1}$ and incubated for 2 h at 25°C. Positive controls were included in the assay by adding 100 µl $well^{-1}$ sea bass serum, previously shown to be positive in the ELISA when diluted 1:100 in BSA PBS-T, to duplicate wells, while blank value was determined using duplicate wells of 100 µl $well^{-1}$ BSA PBS-T. After another four washes with PBS-T, rabbit anti-sea bass Ig (obtained from rabbits injected with sea bass Ig and prepared by the Istituto Zooprofilattico delle Venezie, Italy) diluted 1:500 in BSA PBS-T, was added to the plate at 50 µl $well^{-1}$. After incubating for 90 min at 25 °C, the plate was again washed and 50 µl $well^{-1}$ peroxidase-conjugated goat anti-rabbit antiserum (1:1000 dilution in PBS) (Sigma) was added. The plates were then incubated for a further 90 min at 25 °C and washed again as above. Next, 100 µl $well^{-1}$ of substrate (0.4 mg/ml *o*-phenylenedi-

Figure 2. Nitroblue tetrazolium reduction by head-kidney macrophages after stimulation with PMA in Experiment I. Results are expressed as the mean absorbance of 10^5 cells at 630 nm for triplicate wells ± SD for five fish.



** marks the group which differs from the others within day (P<0.05).*

Figure 3. Lysozyme serum activity of sea bass in Experiment I. Results are expressed as the mean units min^{-1} ± SD for five fish.



amine in 0.05 M citrate phosphate pH 5 to which was added 0.3 μl ml^{-1} of 30 % H_2O_2) was added and left to react in the dark for 30 min at 22°C. The enzyme reaction was stopped by adding 50 μl $well^{-1}$ of 2 M H_2SO_4 and measured at an absorbance of 492 nm with a microplate reader (Multiskan MS, Labsystem). All stages of the assay were carried out in a humidified atmosphere. The antibody response is expressed as the mean absorbance at 492 nm for a 1/100 dilution of serum value, minus the blank value from six fish.

Statistical analysis

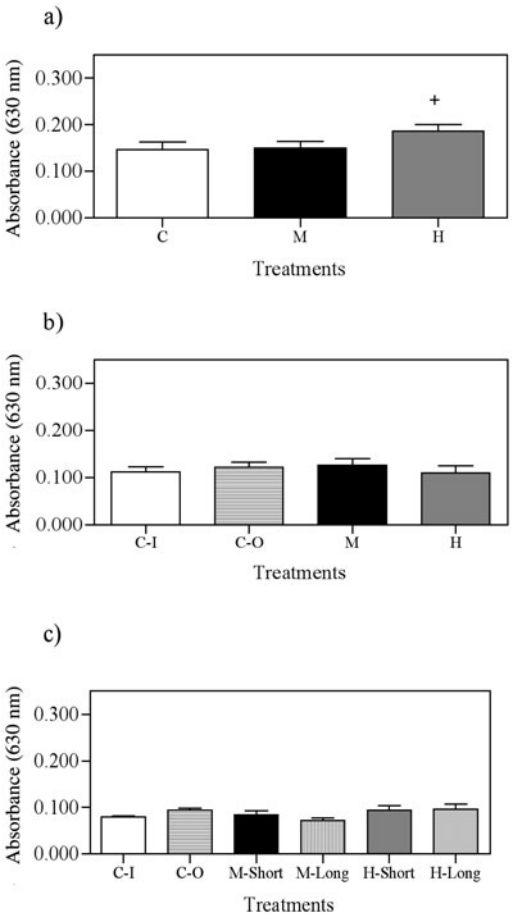
Data at any one sampling time were analysed using Grubb's test in order to reject outliers and were then subjected to a one-way ANOVA. Differences between means were compared using the Newmann-Keuls multiple comparison test (GraphPad Prism 4.00; Graph Pad Software, San Diego, California, USA). In all statistical testing, differences at $P<0.05$ were considered as significant.

Results and discussion

Experiment I

Fish accepted the semipurified diets and consumed the pellets fed to them in both experiments. In Experiment *I*, sampling of fish took place on Days 4, 7, 10, 14, 21 and 25 to evaluate their innate immune response after feeding the *β*-glucans. No analysis of fish from Group C was made at Day 25 due to technical problems. Group L showed a statistically higher level in respiratory burst activity of head kidney macrophages at Day 21 compared with the other three groups. On the other hand, at the end of the first experiment (at Day 25), the respiratory burst activity of macrophages isolated from the group of fishes fed with the highest level of glucans was signif-

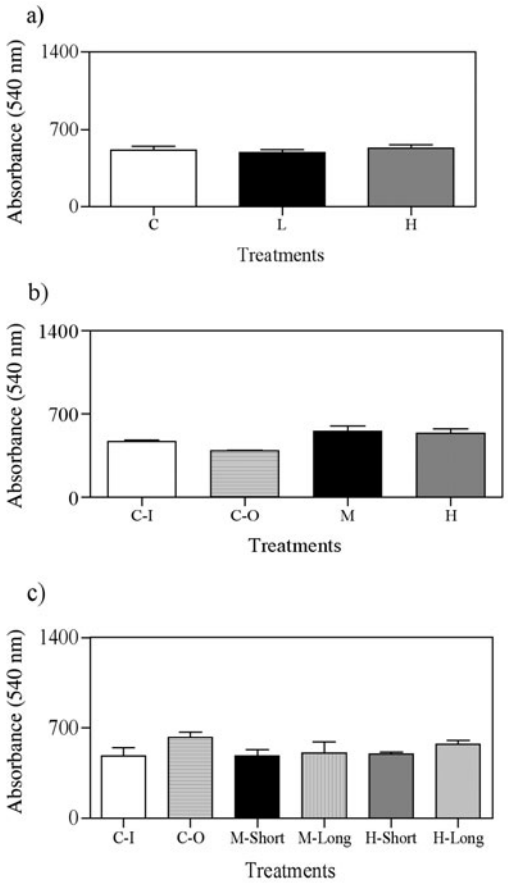
Figure 4. Nitroblue tetrazolium reduction by head-kidney macrophages after stimulation with PMA in Experiment II.



Results are expressed as the mean absorbance of 10^5 cells at 630 nm for triplicate wells ± SD: a) first stage; b) second stage; c) third stage. C = control; M = 500 ppm glucans; H = 1000 ppm glucans C-I = control vaccinated by immersion; C-O = orally vaccinated control; Short = fed immunostimulants during stage 1 and 2; Long = fed immunostimulants during stages 1, 2 and 3.

+ marks the group which marginally differs from the others (P<0.10).

Figure 5. Lysozyme serum activity of sea bass in Experiment II.



Results are expressed as the mean units min^{-1} ± SD: a) first stage; b) second stage; c) third stage. C = control; M = 500 ppm glucans; H = 1000 ppm glucans C-I = control vaccinated by immersion; C-O = orally vaccinated control; Short = fed immunostimulants during stage 1 and 2; Long = fed immunostimulants during stages 1, 2 and 3.

icantly lower than the other two glucan-fed groups (Figure 2). Each group showed a peak in macrophage respiratory burst activity which subsequently decreased, but at different times during the experiment. The highest peak in activity was seen with macrophages isolated from the group fed Diet M at Day 10, even though this value was not statistically different to that of the control group. No statistical differences were observed in lyzozyme activity during this trial (Figure 3).

Experiment II

In this trial, three consecutive feeding periods were carried out. During the first stage of the experiment, experimental diets containing the immunostimulants were administered to the fish; during the second stage, fish were vaccinated either by immersion or orally through their diet, some of which were still fed the immunostimulants; and in the third stage fish in some of the tanks were switched to the control diet while the others continued to be fed on the immunostimulants diet for a further ten days.

No statistically significant difference was observed in respiratory burst activity of head kidney macrophages between treatments during the three feeding periods (Figure 4). However, at the end of first period, macrophages from Group H fish, fed the diet containing 1000 ppm glucans, had a higher respiratory burst activity compared with control fish (P = 0.0567). Respiratory burst activity was observed to decrease over the course of the trial in all groups, from the first feeding phase (OD_{630} ranging from 0.147 to 0.186) to the third feeding phase, during which respiratory burst values were all under an OD_{630} of 0.100.

No significant differences were seen in the lysozyme activity during the course of Experiment *II* (Figure 5), however values were considerably lower compared with those obtained in Experiment *I*.

The antibody response of the fish immediately before vaccination was low (Table 2). Each group showed a significant increase their antibody response by Week 2 post-vaccination compared with initial levels; how-

ever no statistical difference was noted between treatments. The highest antibody values were obtained in animals vaccinated by immersion at Week 2 post-vaccination (OD_{492} = 0.296), while within the orally vaccinated groups, treatment M-Long showed the highest value at Week 2 (OD_{492} = 0.231), even though differences among groups were not statistically different.

Recent findings relating to the effects of β-glucans, administered orally, on the immune response of certain species of Mediterranean fish, have lead to the question of their effect on European sea bass. There are only two reports available in the literature relating to β-glucans fed to sea bass. In the first report Bagni *et al.* (2000) fed glucans together with increased doses of vitamins long-term. They were able to demonstrate increased activation of the alternative complement pathway and increased serum lysozyme activity in these fish, although they did not see any difference in their serum protein content, including albumin and globulin. Unfortunately, it was not possible to establish the effect of the β-glucans on the immune response of the fish in their study since the two immunostimulants were fed simultaneously. In their second study, Bagni *et al.,* (2005) compared the effects of Macroguard with Ergosan, a commercial algal extract containing alginic acid. Long term feeding of these substances did not appear to affect the innate or adaptive immune responses measured. The immunoenhancing properties of β-glucans on respiratory burst activity of head kidney macrophages have been reported elsewhere in a number of fish species, however. For instance, Macrogard© delivered orally has been shown to enhance macrophage activity in turbot (de Baulny *et al*., 1996). Snapper fed on a diet supplemented with β-glucan-based Ecoactiva© and maintained at a winter temperature, had significantly higher macrophage activity compared to control fish and these levels remained elevated for 56 days (Cook *et al.*, 2003). Rainbow trout (Jeney and Anderson, 1993) and Atlantic salmon (Jorgensen and Robertsen, 1995) are two other species shown to have enhanced macrophage activity following incorporation of β-glucans into their diets.

The respiratory burst activity of the macrophages appeared to be influenced both by the duration of feeding and the level of β-glucans fed. In fact each group of fish in the first trial showed an increase in respiratory burst activity, which subsequently decreased over the course of the trial. Macrophages from Groups M and H fish, appeared to have the highest respiratory burst activity at Days 10 and 14 respectively, whereas macrophages from Group L fish, fed with the lowest dose of immunostimulants (i.e. 250 ppm) peaked later at Day 21. Furthermore, at the end of the first experiment (at Day 25), the respiratory burst activity of macrophages isolated from the group fed with the highest level of glucans was significantly lower than the other two glucan-fed groups. We can thus assume from Experiment *I* that β-glucans administered orally to fish enhanced the respiratory burst activity of the macrophages, but this enhancement was strictly time and dose dependent since after a specific period of administration at a particular dose i.e. 1000 ppm for 25 days, a decrease in activity occurred.

Prolonged application of another β-glucans based product, Vitastim©, resulted in decreased macrophage activity in fish fed with the product compared to fish fed with a control diet containing no glucans (Efthimiou, 1996). In another trial, this time using African catfish fed Macrogard©, macrophage activity initially peaked, but then decreased back to initial levels by Day

45 of the study (Yoshida *et al.*, 1995). Although the reason for the decrease in immune response in fish fed immunostimulants long-term is still unclear, negative feedback systems may by functioning to lower the stimulated immune response to pre-stimulation levels (Sakai, 1999). Other points to consider is that Munoz *et al*. (1998) added NaCl to their culture medium to make it isohosmotic (350 mOsm kg"') for sea bass which was not done in the present study. No significant differences were observed in serum lysozyme activity between the dietary groups in either of the two experiments carried out here. Several authors have failed to obtain a positive effect on serum lysozyme activity when glucans are administered orally (De Baulny *et al.* 1996; Verlhac *et al.* 1996; Jeney *et al.* 1997). On the other hand, Engstad *et al.* (1992) and Paulsen *et al.* (2003) observed an increase in plasma lysozyme in Atlantic salmon injected with glucan. The variation in lysozyme activity obtained in the different studies suggests that Macrogard has a greater effect on lysozyme activity after injection rather than by oral administration. The difference between the two routes of administration may be due to the inflammatory reaction induced by i.p injection, which may, in turn, enhance the fish's immune response (de Baulny *et al.*, 1996). The degradation and absorption of the glucan in the digestive tract of the fish after oral administration must also be taken into account, as this may modify the effect of the molecule on the immune system. Further work is needed to establish the time-dose response β-glucans uptake in the gut of sea bass.

In Experiment *II,* β-glucans were used simultaneously with an oral vaccine against Vibriosis in order to evaluate the effects of the glucans on the innate and adaptive immune responses of the sea bass. There are no reports concerning oral immunisation against Vibriosis in sea bass. The vaccine used was protected in alginate before inclusion into the feed, and particles had an average diameter of around 20 μm. The antibody response of fish treated with the oral vaccine increased by Week 2 in all groups compared with values at Day 0 in pre-vaccinated fish. The results of our study suggest that oral administration of the vaccine was able to induce a systemic antibody immune response in the fish. However, the aim of this study was not to evaluate the vaccine but to examine the effects of the glucans on vaccination, and a non-vaccinated control group of fish was not included in the experimental layout. Therefore it was not possible to evaluate the protection elicited by the vaccine, and further studies are necessary to evaluate this route of vaccine administration in sea bass by testing the efficacy of the vaccination with experimental challenges.

Respiratory burst activity in Group H at the end of the first period showed a higher value in comparison with the control (P = 0.0567), partially reflecting the trend that occurred in Experiment *I*. However, after vaccination, i.e. at the end of second and third periods, respiratory burst activity appeared substantially decreased in all groups. Also lysozyme activity values were low if compared with those obtained in Experiment *I*.

Anderson *et al*. (1989) reported increased innate and adaptive responses in rainbow trout when the immunostimulant levamisole was administered during or after immunisation with the *Yersinia ruckeri* O-antigen, but no increase was observed in the innate response when levamisole was given prior to immunisation.

The apparent inability of β-glucans to improve the innate immune response of sea bass after vaccination could partially be explained by the concomitant vaccination,

which may have played a role in exhausting macrophages of their respiratory burst activity giving a different result to those obtained in Experiment *I* for the same dose and time of administration. For this reason, it might be that the optimal dose and timing of β-glucans administration is different if the immunostimulant is administered alone or together with a vaccine.

As with the study of Bagni *et al.,* (2005), β-glucans did not appear to exert any substantial effect on the adaptive immune response of fish in our trial. According to Verlhac *et al.* (1996), the binding of glucan particles to specific membrane receptors on the macrophage can modify the structure of the membrane in such a way that the macrophages becomes more efficient at processing and presenting antigen. However, the effect of β-glucans on the adaptive immune response of fish appears somewhat contradictory in the literature. Verlhac *et al.* (1998) found that trout vaccinated against Enteric Red Mouth (ERM) disease by i.p. injection and fed glucans developed a higher antibody response than fish not fed with the immunostimulant. Several other studies have demonstrated that glucans have adjuvant properties when injected simultaneously with a vaccine resulting in increased antibody responses (Rørstad *et al.*, 1993; Aarke *et al.*, 1994). Raa *et al.* (1992) reported that oral administration of yeast glucan to Atlantic salmon increased protection against *V. anguillarum* and *V. salmonicida*. On the other hand, de Baulny *et al.* (1996) did not observe any reduction in mortality in fish fed β-glucans for a prolonged period after challenging the fish with a virulent strain of *V. anguillarum*. In channel catfish fed β-glucan, an enhanced innate immune response was measured, but no increased resistance to *Edwardsiella ictaluri* was observed (Duncan and Klesius, 1996). Thompson *et al.* (1995) also reported that rainbow trout injected with yeast glucan did not show any enhanced protection against *V. anguillarum* infection.

In conclusion, it is possible to include European sea bass amongst the fish species, which can be immunomodulated with oral administration of β-glucan. Innate immune responses appeared particularly influenced by both the duration and the dosage of the treatment, although further studies are needed to establish ideal feeding regimes for the administration of glucans in combination with vaccination for sea bass. The antibody response of fish did not appear to be enhanced through this method of dietary immunostimulation. Vaccination possibly resulted in activating the immune system as a whole, thus masking any difference in respiratory burst activity between the different groups of fish as a result of immunostimulation by the glucans.

Conclusions

The results of this study suggest that dietary β-glucan may be beneficial in European sea bass when administered at a certain dose, still to be verified, and fed for no more than three weeks. Further investigation of the use of β-glucans on the enhancement of the immune response of sea bass is necessary, including a challenge with *V. anguillarum* to determine the potential of β-glucans in resistance to Vibriosis.

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REFERENCES

AAKRE, R., WERGELAND, H.I., AASJORD, P.M., ENDRESEN, C., 1994. Enhanced antibody responses in Atlantic salmon (*Salmo salar* L.) to *Aeromonas salmonicida* cell wall antigens using a bacterin containing beta-1,3-M-glucan as adjuvant. Fish Shellfish Immun. 4:47-61.

ANDERSON, D.P., 1996. Environmental factors in fish health: immunological aspects. In: G. Iwawa and T. Nakanishi (eds.) The Fish Immune System: Organism, Pathogen and Environment. Academic Press, San Diego, CA, USA, pp 289-310.

ANDERSON, D.P., SIWICKI, A.K., DIXON, O.W., LIZZIO, E.F., 1989. Immunostimulation by levamisole in rainbow trout (*Salmo gairdneri*) *in vivo*. In: W.Ahne and E.Kurstak (eds.) Viruses of lower vertebrates. Springer-Verlag, Berlin, Germany, pp 469-478.

BAGNI, M., ARCHETTI, L., AMADORI, M., MARINO, G., 2000. Effect of long-term administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*). J. Vet. Med B. 47:745-751.

BAGNI, M., ROMANO, N., FINOIA, M.G., ABELLI, L., SCAPIGLIATI, G., TISCAR, P.G., SARTI, M., MARINO, G, 2005. Short- and long-term effects of a dietary yeast beta-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). Fish Shellfish Immun. 18:311-325.

BRATTGJERD, S., EVENSEN, O., LAUVE, A., 1994. Effect of injected yeast glucan on the activity of macrophages in Atlantic salmon, *Salmo salar* L:, as evaluated by *in vitro* hydrogen peroxide production and phagocytic capacity. Immunology 83:288-294.

CASTRO, R., COUSO, N., OBACH, A., LAMAS, J., 1999. Effect of different β-glucans on the respiratory burst of turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) phagocytes. Fish Shellfish Immun. 9:529-541.

COOK, M.T., HAYBALL, P.J., HUTCHINSON, W., NOVAK, F., HAYBALL, J.D., 2003. Administration of a commercial immunostimulant preparation, EcoActiva© as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider) in winter. Fish Shellfish Immun. 14:333-345.

COUSO, N., CASTRO, R., MAGARIÑOS, B., OBACH, A., LAMAS, J., 2003. Effect of oral administration of glucans on the resistance of gilthead seabream to Pasteurellosis. Aquaculture 219:99-109.

DALMO, R.A., BØGWALD, J., INGEBRIGTSEN, K., SELJELD, R., 1996. The immuno-modulatory effect of laminaran [β (1-3)-D-glucan] on Atlantic salmon, *Salmo salar* L., anterior kidney leucocytes after intraperitoneal, peroral and peranal administration. J. Fish Dis. 19:449-457.

DE BAULNY, M., QUENTEL, C., FOURNIER, V., LAMOUR, F., LEGOUVELLO, R., 1996. Effect of long term oral administration of Beta-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot *Scophtalmus maximus*. Dis. Aquat. Organ. 2:139-147.

DI LUZIO, N., 1985. Update on the immunomodulating activities of glucans. Springer Semin. Immun. 8:387-400.

DUNCAN, P.L., KLESIUS, P.H., 1996. Dietary immunostimulants enhance non specific immune responses in channel catfish but not resistance to *Edwarsiella ictaluri*. J. Aquat. Anim. Health. 8:241-248.

EFTHIMIOU, S., 1996. Dietary intake of Beta-1,3/1,6 glucans in juvenile Dentex (*Dentex dentex*), Sparidae - effects on growth performance, mortalities and non-specific defence mechanism. J. Appl. Ichthyol. 12:1-7.

ENGSTAD, R.E., ROBERTSEN, B., FRIVOLD, E., 1992. Yeast glucan induces increase in lysozyme activity and complement-mediated haemolytic activity in Atlantic salmon blood. Fish Shellfish Immun. 2:287-290.

GALEOTTI, M., 1998. Some aspects of the application of immunostimulants and a critical review of methods for their evaluation. J. Appl. Ichthyol. 14:189-199.

KAUSHIK, S.J., GOUILLOU-COUSTANS, M.F., CHO, C.Y., 1998. Application of the recommendations on vitamin requirements of finfish by NRC (1993) to salmonids and sea bass using practical and purified diets. Aquaculture 161:463-474.

JENEY, G., ANDERSON, D.P., 1993. Glucan injection or bath exposure given alone or in combination with a bacterin enhance the non-specific defence mechanism in rainbow trout (*Onchorhyncus mykiss*). Aquaculture 116:315-329.

JENEY, G., GALEOTTI, M., VOLPATTI, D., JENEY, Z., ANDERSON , D.P., 1997. Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucans. Aquaculture 154:1-15.

JØRGENSEN, J.B., ROBERTSEN, B., 1995. Yeast β-glucan stimulates respiratory burst activity of

Atlantic salmon (*Salmo salar* L.) macrophages. Dev. Comp. Immunol. 19:43-57.

JØRGENSEN, J.B., LUNDE, H., ROBERTSEN, B., 1993. Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. Fish Shellfish Immun.3:267-277.

MURANO, E., GATTA, P.P., MAZZOLINI, E., GIORGETTI, G., BAUCE, G, PERBELLINI, A., 1997. Oral immunisation of rainbow trout *Oncorhynchus mykiss* against vibriosis with microincapsulated vaccin. Proc. 6th Int. Workshop on Bioencapsulation "from fundamental to industrial applications", Barcelona, Spain, (abstr.).

MUÑOZ, P., CALDUCH-GINER, J.A., SITJÀ-BOBADILLA, A., ALVAREZ-PELLITERO, P., PÉREZ-SÁNCHEZ, J. 1998. Modulation of the respiratory burst activity of Mediterranean sea bass (*Dicentrarchus labrax* L.) phagocytes by growth hormone and parasitic status. Fish Shellfish Immun. 8:25-36.

PARRY, R.M., CHANDAN, R.C., SHANANI, K.M., 1965. A rapid and sensitive assay of muramidase. P. Soc. Exp. Biol. Med. 119:383-386.

PAULSEN, S.M., ENGSTAD, R.E., ROBERTSEN, B., 2001. Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast β-glucan and bacerial lipopolysaccharide. Fish Shellfish Immun.11:23-37.

PAULSEN, S.M., LUNDE, H., ENGSTAD, R.E., ROBERTSEN, B., 2003. *In vivo* effects of β-glucans and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immun. 14:39-54.

RAA, R., RØRSTAD, G., ENGSTAD, R., ROBERTSEN, B., 1992. The use of immunostimulants to increase resistance of aquatic organism to microbial infections. In: M. Shariff, R.P Subasighe and J.R Arthur (eds.) Diseases in Asian Aquaculture. Fish Health Section, Manila, Philippines, pp 39-50.

ROBERTSEN, B., 1999. Modulation of the non-specific defence of fish by structurally conserved microbial polymers. Fish Shellfish Immun.9:269-290.

RØRSTAD, G., AASJORD, P.M., ROBERTSEN, B., 1993. Adjuvant effect of a yeast glucan in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immun. 3:179-190.

SAKAI, M., 1999. Current research status of fish immunostimulants. Aquaculture 172:63-92.

SANTARÈM, M., NOVOA, B., FIGUERAS, A., 1997. Effects of β-glucans on the non specific immune responses of turbot (*Scophthalmus maximus*). Fish Shellfish Immun.7:429-437.

SECOMBES, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberston and W.B. van Muiswinkel (eds.) Techniques in Fish Immunology. Fair Haven, SOS Publications, NJ, USA, pp 137-154.

SECOMBES, C.J., 1996. The non-specific immune system: cellular defences. In: G. Iwawa and T. Nakanishi (eds.) The Fish Immune System: Organism, Pathogen and Environment. Academic Press, San Diego, CA, USA, pp.63-103.

SPAIN.ORTUÑO, J., CUESTA, A., RODRÍGUEZ, A., ESTEBAN, M.A., MESEGUER, J., 2002. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). Vet. Immunol. Immunop. 85:41-50.

THOMPSON, K.D., CACHOS, A., INGLIS, V., 1995. Immunomodulating effects of glucans and oxytetracycline in rainbow trout, *Oncorhynchus mykiss*, on serum lysozyme and protection. In: M. Shariff, R.P. Subasighe and J.R. Arthur (eds.) Diseases in Asian Aquaculture Vol. 11 Fish Health Section. Asian Fisheries Society, Manila, Philippines, pp 433-439.

TORANZO, A.E., DEVESA, S., LAMAS, J., RIAZA, A., LEIRO, J., BARJA, J.L., 1995. Efficacy of intraperitoneal and immersion vaccination against *Enterococcus* sp. in turbot. Aquaculture 134:17-27.

VERLHAC, V., GABAUDAN, J., OBACH, A., SCHÜEP, W., HOLE, R., 1996. Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 143:123-133.

VERLHAC, V., OBACH, A., GABAUDAN, J., SCHÜEP, W., HOLE, R., 1998. Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immun. 8:409-424.

YOSHIDA, T., KRUGER, R., INGLIS, V., 1995. Augmentation of non-specific protection in African catfish, *Clarias gariepinus* (Burchell), by the long term oral administration of immunostimulants. J. Fish Dis. 18:195-198.