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The *in vitro* effect of probiotic *Vagococcus fluvialis* on the innate immune parameters of *Sparus aurata* and *Dicentrarchus labrax*

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ABSTRACT

In this study we evaluated the effect of the probiotic *Vagococcus fluvialis* on the cellular immune unspecific system of two different fish species of great interest in aquaculture such as gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Leucocytes from head kidney of the two fish species were extracted and concentration adjusted to 10^7 cells ml^{-1}. Phagocytic and respiratory burst activity and the peroxidase content of leucocytes were observed 30 min after incubation with the probiotic *Vagococcus fluvialis* alive or inactivated with heat shock or UV-light at different concentrations of 10^7, 10^8, 10^9 cfu ml^{-1} (final concentration 10^6, 10^7, 10^8 cfu ml^{-1}). *V. fluvialis* produced dose-dependent increments in respiratory burst in sea bream leucocytes. The respiratory burst activity of sea bream head kidney leucocytes incubated with 10^6 cfu ml^{-1} of live and inactivated bacteria was not stimulated. The highest values of peroxidase content were observed in sea bass cells with stimulation indexes higher than 1 in HK leucocytes incubated with 10^8 cfu ml^{-1} of live and inactivated bacteria. Statistical analysis revealed that differences being only significant in sea bass leucocytes where 10^8 cfu ml^{-1} bacteria denote statistically significant differences ($P < 0.05$) respect to other concentrations. Highest values of phagocytic activity were obtained in sea bass macrophages incubated with UV-light inactivated bacteria ($27.33\% \pm 1.45$), where significantly differences with sea bass HK leucocytes were detected. Our results suggest that the *in vitro* assays are a useful tool to optimize the effective dose of probiotic bacteria. Although *in vivo* studies are necessary to confirm the immunomodulatory effect of this strain.

1. Introduction

In the last few years the use of probiotics and immunostimulants has had a remarkable increase in aquaculture both due to the intensification of this area and to the general tendency of reducing the use of antibiotics [1]. Nowadays, probiotics and immunostimulants are considered as excellent candidates to control fish diseases [2–7]. There are some limitations caused by the administration of live bacteria into fish pens or cages for their potential interaction with the marine environment. The use of inactivated bacteria clearly solves such safety-related issues since they can no longer interact with other aquatic organisms. Probiotics have indeed been redefined as microbial complements, not necessarily alive, that have beneficial effects on host health [8]. Numerous microbes have been identified as probiotics for aquaculture practices, many of which differ markedly in their mode of action [5].

Among the many benefits of probiotics, modulation of the immune system could be one of the most important points to be studied. Most of the earlier studies in fish with, dealt with growth promoting and disease protective ability of probiotics [9]. However, in recent years much attention has been hitherto towards the immunomodulating effects of probiotics in aquaculture [5,6,10–14]. Clear evidence of the benefit effect of probiotics do exist; particularly on the immune system [15–19]. However, *in vitro* studies that evaluated the immune-modulator effects of bacteria on the immune cells are particularly scarce [20]. Taking into account that in most of the studies, the results obtained *in vitro* show correspondence to those obtained *in vivo* [8,13], the *in vitro* assays are being developed to reliably identify the most interesting bacterial strains [21].

The common probiotics that are used for aquaculture practices includes *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacter*, *Pseudomonas* species [15]. Many species of bacteria's are used as probiotics in aquaculture practice [22].

The present work, it is the first study including the effect of the probiotic *Vagococcus fluvialis* on the innate immune parameters of different relevant fish species for marine aquaculture such

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as: gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). The bacteria *V. fluvialis* isolated from gilthead sea bream intestine, according to the *in vitro* and *in vivo* characteristics tested in our laboratory which made them suitable as potential fish probiotic [23]. *V. fluvialis* was evaluated *in vitro* through various mechanisms of selection, such as production of antagonistic effects against pathogens [24], production of antibacterial substance [25,26], fish bile and pH resistance [25], adhesion and growth to intestinal mucus [15,27,28], and the *in vivo* study of the protective effect against *Vibrio anguillarum* European sea bass.

In the present study, the *in vitro* effects of viable or heat-killed and UV-killed bacteria were studied, such as phagocytic activity, peroxidase content and respiratory burst activities of head kidney leucocytes. Thus we do not only analyze the effect of the same strain on different fish species but we can also adjust the better concentration to feed animals in the future.

2. Materials and methods

2.1. Bacteria

The bacteria *V. fluvialis* isolated from gilthead sea bream intestine was described and identified by Sorroza et al. 2012 [23] was grown at 22 °C in tubes containing 10 ml of brain heart infusion broth (BHIB) (Pronadisa) supplemented with 1.5% NaCl, with continuous shaking for 24 h. Bacterial suspensions were washed with sterile phosphate-buffered saline (PBS) and the final concentration adjusted to 10^9 cfu ml^{-1} by spectrophotometry (Biophotometer, Eppendorf, USA). All bacterial cultures were heat-inactivated for 2 h at 60 °C and UV-light inactivated for 2.5 h and stored at −80 °C until required.

2.2. Fish

Two different species of marine fish were used: gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*) with an average body weight of 200 g. Sea bass and sea bream were obtained from a local fish farm Canexmar (Canary Island, Spain).

2.3. Isolation of head kidney leucocytes and tested with the bacteria

Head–kidney leucocytes (HK leucocytes) were isolated from each fish analyzed ($n = 30$) under sterile conditions following the technique described by Secombes [29] with some modifications. Briefly, head kidney was excised, cut into small fragments and transferred to 8 ml of supplemented cell culture medium (Leibovitz L-15) (Gibco, Gaithersburg, MD, U.S.A). Cell suspensions were obtained by forcing fragments of the organ through a 100 μm nylon mesh. After centrifugation (400×*g* for 10 min), cells suspension was layered on a Ficoll gradient (Limphoprep) suspension and centrifuged at 1100×*g* for 30 min at 4 °C. The interface layer was harvested and diluted in 1 ml of supplemented L-15 medium and again centrifuged at 450×*g* for 10 min at 4 °C to remove residual Ficoll and finally resuspended in L-15 medium supplemented with P/S/G. Viable cells were stained with trypan blue and counted in a Neubawer's camera. For the study to the respiratory burst and peroxidase content, aliquots of 100 μl containing 1×10^6 cells ml^{-1} in L-15 medium supplemented with P/S/G were added to 96-well microtitre plates (Nunc, Roskilde, Denmark) and aliquots of 500 μl containing 1×10^7 cells ml^{-1} in L-15 medium supplemented with P/S/G were seed onto 20 mm diameter glass coverslips in 6-well plates (Nunc, Roskilde, Denmark). After 3 h incubation at 22 °C, non-adherent cells were removed and medium was substituted by L-15 and P/S/G supplemented with 2% FCS. Monolayers were incubated overnight at 22 °C.

Live and inactivated bacterial were adjusted in PBS to 10^7, 10^8, 10^9 cfu ml^{-1} and 100 μl of each suspension was added to samples of 100 μl of HK Leucocytes after 30 min of incubation. The peroxidase content and respiratory burst activities were measured. To study phagocytic activity a MOI 1:1 technique was realized after 1 h incubation with live and inactivated bacteria.

2.4. Peroxidase content

The total peroxidase content present inside leucocytes was measured according to Quade and Roth [30]. To estimate the leucocyte peroxidase content, 10^6 HK leucocytes in L-15 medium per well were dispensed and fixed into round-bottomed 96-well plates. Live and inactivated bacteria, adjusted in PBS to 10^7, 10^8, 10^9 cfu ml^{-1} and 100 μl of each suspension was added to samples of 100 μl of HK leucocytes and incubated for 30 min. After incubation, leucocytes were lysed with 75 μl of 0.02% cetyl trimethyl ammonium bromide (CTAB, Sigma–Aldrich, Germany). Afterwards, 50 μl of 10 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, Sigma–Aldrich, Germany) and 25 μl of 5 mM H_2O_2 were added producing a colour-change reaction. This reaction was stopped after 2 min by adding 50 μl of 2 M sulphuric acid (H_2SO_4) and the optical density was read at 450 nm in a multiscan spectrophotometer (MultiskanFc, Thermo, Chicago) reader. Controls consisted of leucocytes incubated with 100 μl of PBS without bacterial cells.

2.5. Respiratory burst activity

The generation of intracellular superoxide radicals by phagocytes was determined by the reduction of nitro-blue tetrazolium (NBT) according to the technique described by Secombes [29] and Boesen et al. [31]. 100 μl NBT, dissolved at 1 mg ml^{-1} in HBSS, were added to the wells and the phagocytes incubated at 22 °C for 30 min. Wells containing phagocytes were incubated with live and inactivated probiotic bacteria at different concentrations (10^6, 10^7, 10^8 cfu ml^{-1}) and used to determine the response of phagocytes to the probiotic bacteria. As a positive control, we used phorbol myristate acetate (PMA, Sigma) (1 μg ml^{-1}). After incubation, cells were fixed in 70% methanol and reduced formazan within phagocytes was solubilised by adding 120 μl 2 M KOH and 140 μl dimethylsulfoxide (DMSO) (Sigma). Finally, absorbance was read at 620 nm in a multiscan spectrophotometer (MultiskanFc, Thermo, Chicago). All experiments were carried out by triplicate. Respiratory burst activity of isolated phagocytes from assayed was expressed as stimulation index, which was calculated as the ratio between the absorbance obtained with phagocytes from fish incubated with bacterial cells and the absorbance of the controls phagocytes incubated with PMA.

2.6. Phagocytic activity

The phagocytic activity was measured as described by Puangkaew et al. [32] Phagocyte monolayer was incubated with 10 μl of 10^9 cfu ml^{-1} (MOI 1:1; bacteria/macrophage cell ratio) of live and inactivated probiotic for 1 h at 22 °C. After washing with PBS, the cells were stained with Diff Quick solution (Panreac, Spain). One hundred macrophages per slide were counted and the phagocytic capacity was determined as the percentage of macrophages with phagocytic ability.

2.7. Statistical analysis

The results are expressed as the stimulation index (mean ± SE). Thus, values higher than 1 were mean activation, while lower values reflecting inhibition. All statistical analyses were carried out