

Influence of alginic acid and fucoidan on the immune responses of head kidney leukocytes in cod

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Abstract The present study investigated the immunomodulatory activities of alginic acid and fucoidan, both derived from brown seaweeds, on selected cellular immune responses and antibacterial activity of head kidney (HK) leukocytes of cod, *Gadus morhua*. Primary cultures of HK leukocytes were incubated with either 10 or 100 $\mu\text{g ml}^{-1}$ of the substances and the effects on respiratory burst, cellular proliferation, acid and alkaline phosphatase activity and cellular myeloperoxidase were measured at 3- and 24-h post-incubation. The antibacterial activity of the supernatants collected from the cell cultures incubated with 100 $\mu\text{g ml}^{-1}$ of the substances were tested against *Vibrio anguillarum* and *Aeromonas salmonicida*. Respiratory burst was significantly elevated in cells incubated with either alginic acid or fucoidan in a dose-dependent manner. Incubation with a higher dose of alginic acid and fucoidan resulted in lower cellular proliferation at 3- and 24-h, respectively. Both acid and alkaline phosphatase activities of HK leukocytes were not significantly modulated, except for a slight elevation of acid phosphatase in cells incubated with 100 $\mu\text{g ml}^{-1}$ of alginic acid for 24-h. Fucoidan, but not alginic acid significantly increased cellular

myeloperoxidase activity at a concentration of 100 $\mu\text{g ml}^{-1}$. The growth of the bacteria in both the treated and control supernatants was significantly lower than what was observed in the bacterial culture medium. However, the supernatants from the treated cells had significantly higher bacterial growth compared with supernatants of the control cells. Taken together, these results showed that at the tested concentrations, both alginic acid and fucoidan are able to differentially stimulate some cellular immune responses of cod HK leukocytes in vitro and the respiratory burst activity was significantly stimulated by these brown algal derivatives. These substances could be tested as potential immunostimulants in future in vivo studies.

Keywords Atlantic cod · Alginic acid · Fucoidan · Immune responses

Introduction

Traditional strategies to control diseases in aquaculture include the use of antibiotics and chemical disinfectants, but these are no longer recommended due to the emergence of resistant strains of the pathogens, accumulation of residues in the environment and in non-target organisms. Although the use of vaccination is an effective means of disease prevention in fish (Midtlyng et al. 1996), it could

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be expensive, time-consuming and stressful for the fish. An alternative approach is the use of immunostimulants, which is a more environment-friendly approach in disease prevention (Sakai 1999). An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the resistance of the host against diseases caused by pathogens (Bricknell and Dalmo 2005). These compounds are derived from bacteria, fungi or plants and are commonly composed of polysaccharides that activate the pattern recognition receptors of the host immune system resulting in an immune response.

Several substances such as β -glucan, chitin, algal derivatives, bacterial polysaccharides and synthetic materials like levamisole or polyinosinic:polycytidylic acid (poly I:C) have been used to stimulate immunity and disease resistance in fish (Sakai 1999; Bricknell and Dalmo 2005). Among these compounds, β -glucan has been widely used as an immunostimulant and has been reported to increase antibody production, complement activity, lysozyme level, phagocytic activity and respiratory burst in a number of fish species (Chen and Ainsworth 1992; Engstad et al. 1992; Verlhac et al. 1996; Castro et al. 1999; Bagni et al. 2005; Díaz-Rosales et al. 2007).

Algae contain several immunologically active substances that have been tested in fish (Valente et al. 2006). For example, several algal species stimulate the respiratory burst of turbot, *Psetta maxima* phagocytes in vitro (Castro et al. 2004, 2006). Some species of red, green and brown algae are able to enhance respiratory burst and chemotaxis in the phagocytes of sole, *Solea senegalensis* (Díaz-Rosales et al. 2005, 2007). Specific algal components such as carrageenan, a polysaccharide that is found in red seaweeds, induced an increase in phagocytic activity of macrophages and resistance against bacterial infections in carp, *Cyprinus carpio* during intraperitoneal injection (Fujiki et al. 1997a, b). Sodium alginate derived from brown algae enhanced non-specific immunity in carp, *C. carpio* and its resistance against *Edwardsiella tarda* (Fujiki et al. 1994; Fujiki and Yano 1997). Ergosan, an algal extract containing alginic acid, also increased non-specific defense responses of snakehead, *Channa striata* (Miles et al. 2001), rainbow trout, *Oncorhynchus mykiss* (Peddie et al. 2002), sea bass, *Dicentrarchus labrax* (Bagni et al. 2005) and beluga, *Huso huso* (Ahmadifar et al. 2009). It also increased the

expression levels of cytokines such as IL-1 β , IL-8 and TNF alpha 2 in the liver of rainbow trout, *O. mykiss* (Gioacchini et al. 2008). Intraperitoneal injection of grouper, *Epinephelus coioides* with κ -carrageenan and sodium alginate resulted in the elevation of non-specific immune response and resistance against challenge with *Vibrio alginolyticus* (Cheng et al. 2007). Fucoidan, a complex sulfated polysaccharide derived from marine brown seaweeds, stimulated the expression of a phagocytosis-related gene (Deachamag et al. 2006) and enhanced resistance against infection with white spot syndrome virus (WSSV) in shrimp, *Penaeus monodon* (Chotig-eat et al. 2004).

The use of immunostimulants to enhance the defense system and increase resistance against infection with pathogens in Atlantic cod, *Gadus morhua* has been limited to their application in the larval and juvenile stages (Magnadottir et al. 2006). In another study, cod and spotted wolffish, *Anarhichas minor* fry were fed with high-M alginate diets and showed higher specific growth rates and better resistance against atypical *Aeromonas salmonicida* (Vollstad et al. 2006). However, there have been no studies on the effects of these brown seaweed-derivatives on the immune parameters of cod. The use of in vitro screening methods could elucidate the mechanisms of immune stimulation by the different test compounds, and should be followed by in vivo methods that will establish whether the benefits of immunostimulants occur in live fish (Galeotti 1998). This study followed such approach through examination of the different immune parameters in vitro using head kidney (HK) leukocytes of cod. Specifically, we evaluated some cellular immune response parameters such as respiratory burst, cellular proliferation, cellular enzymatic activity and antibacterial activity of supernatants obtained from cultures of HK leukocytes following incubation with alginic acid and fucoidan.

Materials and methods

Fish

Apparently healthy Atlantic cod with body masses ranging 150–250 g were obtained from the Marine Research Station at University of Nordland, Norway. Fish handling and experimental procedures were

approved by the National Animal Research Authority (FDU) in Norway.

Alginic acid and fucoidan

Alginic acid, consisting of mixed polymers of manuronic and guluronic acid from *Macrocystis pyrifera* and fucoidan, which was extracted from *Fucus vesiculosus* were purchased from Sigma (Steinheim, Germany). A stock solution of 10 mg ml⁻¹ was prepared for each substance using 1× PBS, aliquoted and kept at -40°C until use.

Isolation of head kidney leukocytes and incubation with brown seaweed-derived compounds

Head kidney (HK) leukocytes from Atlantic cod were prepared following Meng et al. (2003) and added with L-15 medium supplemented with 2% FBS, antibiotics (100 U ml⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin) and sodium chloride (NaCl) to a final osmolarity of 380 mOsm.

One millilitre of the cell suspension (approximately 10⁷ cells ml⁻¹) was placed in each well of the 12-well plate and incubated for 6 h at 12°C for cell attachment. The cell culture medium was aspirated to remove cell debris and unattached cells, washed with 1× PBS to remove traces of antibiotics and replaced with antibiotics-free culture medium just prior to their use. Fucoidan and alginic acid dissolved in L-15 medium were added to each well at a final concentration of either 10 or 100 µg ml⁻¹. Cells added with 1× PBS served as a control for the experiment. At 3- and 24-h post-incubation the different immune response assays were done and the cell culture medium was removed, aliquoted and kept at -80°C for the antibacterial assays.

Assays for cellular response

Respiratory burst production in cells was assayed using the nitroblue tetrazolium (NBT) (Chung and Secombes 1988). HK leukocytes (10⁷ cells ml⁻¹) in 96 well plate were added with 100 µL containing 0.4 mg ml⁻¹ NBT (Sigma) in L-15 medium. The cells were further incubated at 12°C for 1 h and the reaction was stopped by fixing the cells with 100%

methanol for 2–3 min. The cells were washed with 70% methanol, air-dried for 5 min and the reduced formazan was solubilized in 120 µl of 2 M KOH and 140 µl of DMSO. The optical density was determined at 630 nm using an automatic plate reader (Fluostar Optima, BMG Labtech GmbH, Offenburg, Germany).

Cellular proliferation was measured using the 3-(4,5 dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT) assay with some modifications (Caipang et al. 2005). HK leukocytes (approximately 10⁷ cells ml⁻¹) seeded in a 96-well plate and incubated with the different immunostimulants were added with 20 µL of fresh medium containing 0.5 mg mL⁻¹ of MTT. The plate was wrapped in aluminum foil and was further incubated for 30 min at 15°C. The crystals that formed in each well were dissolved with 125 µl of a solution containing 100 µl of dimethylsulfoxide and 25 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl [pH 10.5]) and incubated for 10 min at 15°C. Absorbance was read at 570 nm using a microplate reader (Fluostar).

Acid phosphatase (AP) activity was assayed by the method of Meng et al. (2003). HK leukocytes were lysed with 50 µl of 0.1% Triton X-100 at 4°C for 30 min, and then incubated at 37°C for 30 min to inactivate glucose-6-P. This was followed by the addition of 100 µl of 12 mM *p*-nitrophenyl phosphate in 0.2 M acetate buffer (pH 5.0). After further incubation at 15°C for 3 h, the reaction was stopped by adding 50 µl of ice-cold 0.01 M EDTA in 0.1 M NaOH and absorbance was read immediately at 410 nm using a microplate reader (Fluostar).

Alkaline phosphatase activity was determined by following the procedures described by Apines-Amar et al. (2004) with some modifications. Briefly, the cells were added with 50 µl of 4 mM *p*-nitrophenyl phosphate (Sigma) and incubated at 12°C for 30 min. This was followed by the addition of 75 µl of 3 N NaOH to terminate the reaction. Optical density was read at 405 nm using a microplate reader (Fluostar).

Cellular myeloperoxidase was determined by lysing the cells with 60 µl of 0.02% cetyltrimethylammonium bromide (CTAB, Sigma) and assayed following the procedures of Quade and Roth (1997) with some modifications. The lysed cells were added with 35 µl of fresh L-15 medium containing 20 mM 3,3',5,5'-tetramethyl benzidine hydrochloride (Sigma) and 5 mM H₂O₂. After incubation for 2 min, 35 µl of 4 M sulphuric acid was added to stop the reaction and

spectrophotometrically determined at 450 nm using a microplate reader (Fluostar).

Supernatants of the HK leukocytes incubated with $100 \mu\text{g ml}^{-1}$ of the immunostimulants were used for the antibacterial activities against *Vibrio anguillarum* strain VI-F-258-3 (O2 β serotype) and *Aeromonas salmonicida* strain NCIMB 1102. Bacterial activity was measured using the 3-(4,5 dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT; Sigma) assay following previously described procedure (Caipang et al. 2010).

All assays were done at 3- and 24-h post-incubation with the test substances and repeated twice to validate the results.

Statistical analysis

The data are represented as mean \pm SD and analyzed using one-way analysis of variance (ANOVA) (Systat ver 8). If the observed differences were significant, the Tukey's multiple comparison test was further used. Significance levels were set at $P < 0.05$.

Results

Effects on immune responses in vitro

Incubation of HK leukocytes with either 10 or $100 \mu\text{g ml}^{-1}$ of alginic acid resulted in significant increase in respiratory burst activity at 3- and 24-h post-incubation (Fig. 1a). Similar results were also observed in cells incubated with fucoidan in a dose-dependent manner (Fig. 1b).

Cellular proliferation significantly decreased when cells were incubated with fucoidan at a concentration of $100 \mu\text{g ml}^{-1}$ for 3-h (Fig. 2a). At 24-h post-incubation, no significant differences were observed in cellular proliferation of cells incubated with the two levels of concentration. There was no clear pattern in the proliferation of HK cells incubated with fucoidan for 3-h, however, at 24-h post-incubation there was a decrease in cellular proliferation at $100 \mu\text{g ml}^{-1}$ (Fig. 2b).

At 24-h post-incubation, the level of acid phosphatase activity was significantly enhanced in cells incubated with $100 \mu\text{g ml}^{-1}$ of alginic acid (Fig. 3a). On the other hand, there were no significant differences in the acid phosphatase activity in cells

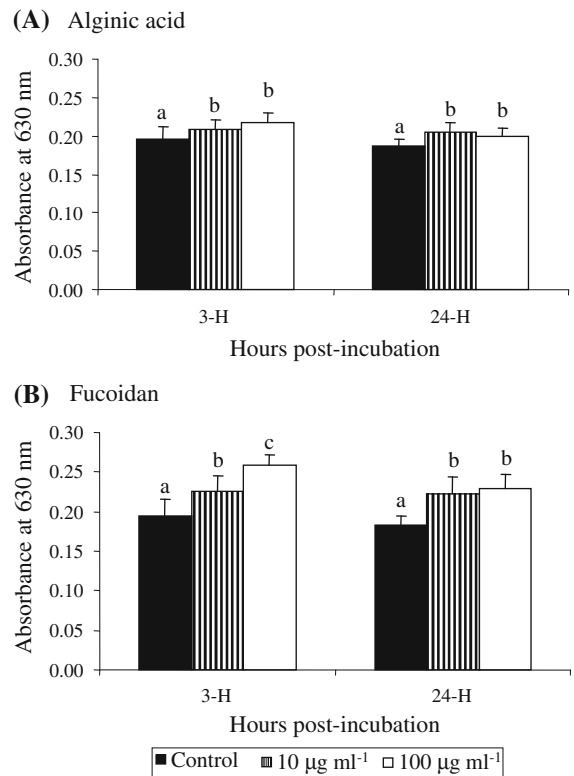


Fig. 1 Respiratory burst activity in head kidney leukocytes incubated with either 10 or $100 \mu\text{g ml}^{-1}$ of **a** alginic acid and **b** fucoidan for 3- and 24-h. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean \pm SD, N = 8

incubated with different concentrations of fucoidan at different incubation times (Fig. 3b). The levels of alkaline phosphatase in HK cells were not affected by the concentrations of either alginic acid (Fig. 4a) or fucoidan (Fig. 4b) as well as the length of the incubation period.

When HK cells were incubated with alginic acid, no significant differences in the activity of cellular myeloperoxidase were observed regardless of the concentration used and the duration of incubation (Fig. 5a). On the other hand, HK cells incubated with fucoidan at a concentration of $100 \mu\text{g ml}^{-1}$ for 3- or 24-h resulted in significant elevation of myeloperoxidase activity (Fig. 5b).

Bacterial proliferation cell culture supernatants

The growth of two bacterial pathogens, *V. anguillarum* and *A. salmonicida* in supernatants obtained

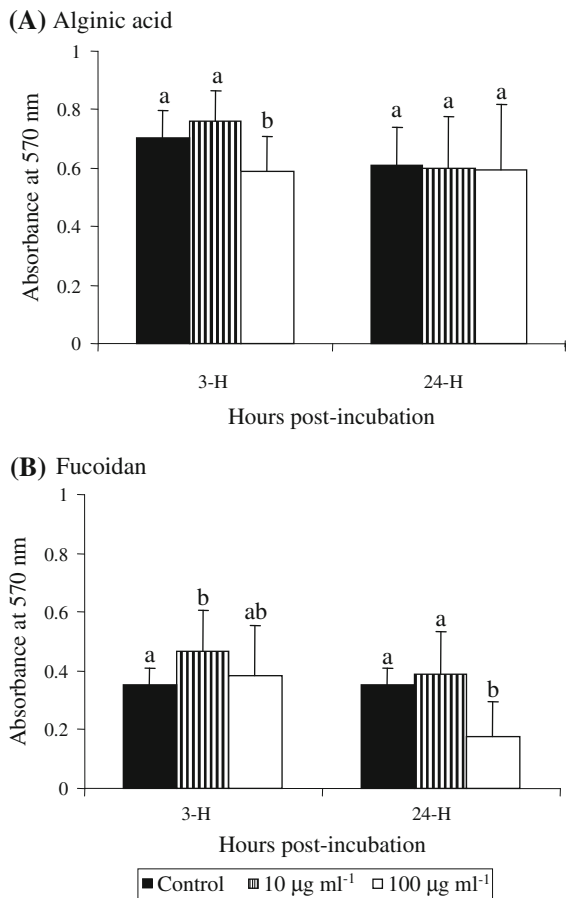


Fig. 2 Proliferation of head kidney leukocytes (as measured by the MTT assay) incubated with either 10 or 100 $\mu\text{g ml}^{-1}$ of **a** alginic acid and **b** fucoidan for 3- and 24-h. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean \pm SD, $N = 8$

from HK leukocytes incubated with 100 $\mu\text{g ml}^{-1}$ of either alginic acid or fucoidan is shown in Fig. 6. When compared with the bacterial culture medium, there was significantly lower proliferation of *V. anguillarum* in supernatants obtained from cells incubated with either alginic acid (Fig. 6a) or fucoidan (Fig. 6b) as well in the control (added with $1 \times$ PBS). However, the bacterial growth in supernatants obtained from cells treated with the test substances was significantly higher than in the supernatant of the control cells. Similar observations were also obtained for the growth of *A. salmonicida* in supernatants of the treated cells (Fig. 6c, d).

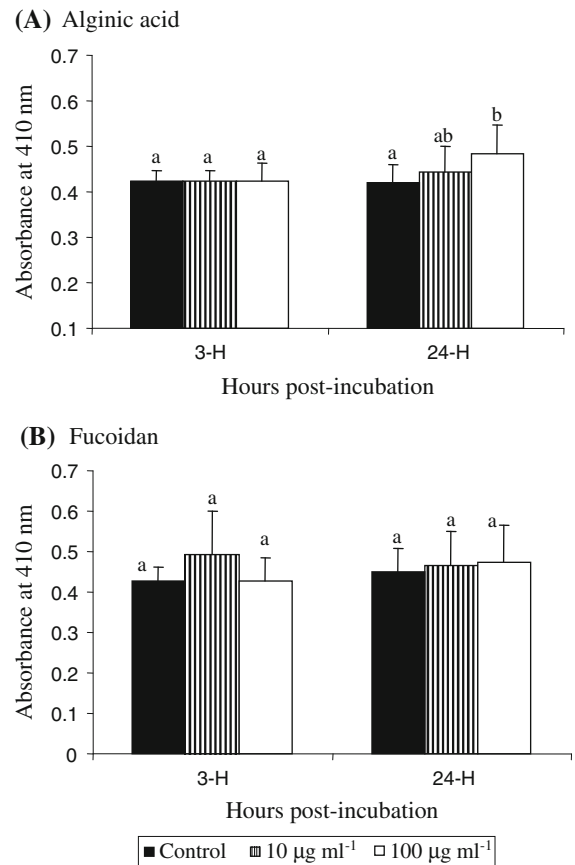


Fig. 3 Acid phosphatase activity of head kidney leukocytes incubated with either 10 or 100 $\mu\text{g ml}^{-1}$ of **a** alginic acid and **b** fucoidan for 3- and 24-h. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean \pm SD, $N = 8$

Discussion

Two substances, alginic acid and fucoidan, which are derived from brown seaweeds were evaluated for their ability to stimulate the cellular immune responses of cod HK leukocytes. Both substances were able to stimulate cellular respiratory burst in a dose-dependent manner, with a clearer pattern observed in cells incubated with fucoidan. No clear effect on cellular proliferation was observed, hence, it is likely that the concentrations of the substances used in the present study were not cytotoxic to the HK leukocytes. The cellular enzymes, acid and alkaline phosphatase were not apparently modulated by the test substances. However, cellular

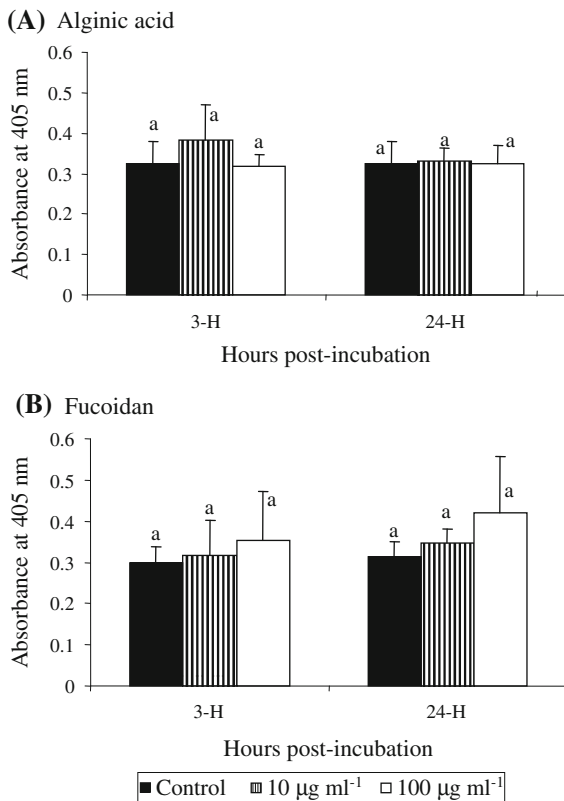


Fig. 4 Alkaline phosphatase activity of head kidney leukocytes incubated with either 10 or 100 $\mu\text{g ml}^{-1}$ of **a** alginic acid and **b** fucoidan for 3- and 24-h. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean \pm SD, $N = 8$

myeloperoxidase was enhanced by fucoidan at a higher dose. Although the supernatants obtained from cells incubated with the test substances reduced bacterial growth in comparison with the bacterial culture medium, the bacterial levels were still higher when compared with supernatants obtained from the control cells. The aforementioned results clearly indicate that using HK leukocytes as a model in an in vitro study, the effects of these test substances on selected cellular immune responses could be observed. Hence, these data could provide baseline information in subsequent in vivo studies to evaluate the effects of these substances as immunostimulants.

The increased respiratory burst in immune-related cells of different fish species as a result of administration of alginate or alginic acid has been well-documented. For example, intraperitoneal injection of Ergosan containing 1% of alginic acid extract from the brown alga *Laminaria digitata* resulted in

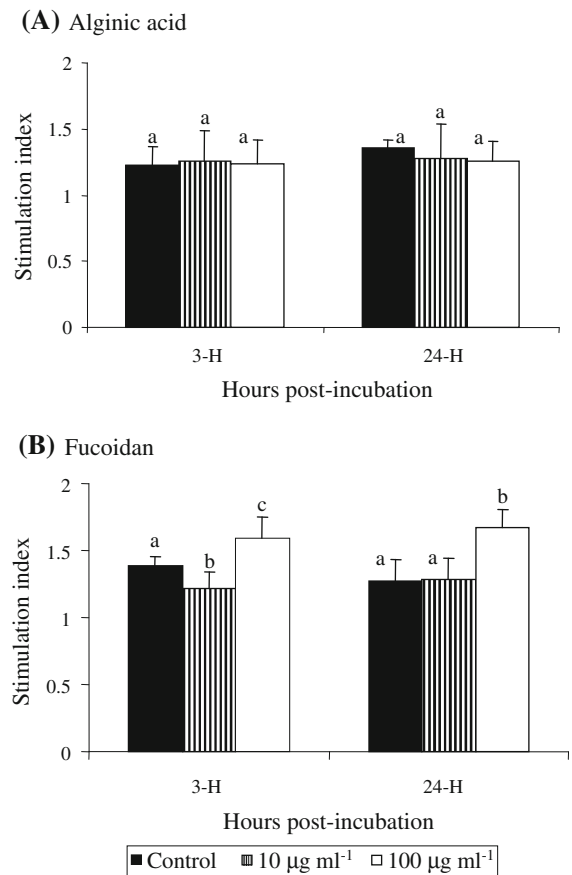


Fig. 5 Stimulation index of myeloperoxidase in head kidney leukocytes incubated with either 10 or 100 $\mu\text{g ml}^{-1}$ of **a** alginic acid and **b** fucoidan for 3- and 24-h. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean \pm SD, $N = 8$

enhanced respiratory burst activity in rainbow trout, *O. mykiss* (Peddie et al. 2002). Grouper, *E. coioides* exhibited significant increase in respiratory burst at HK phagocytes during intraperitoneal injection (Cheng et al. 2007) or oral administration of sodium alginate (Yeh et al. 2008). The results of our in vitro studies could be tested using in vivo studies in cod to confirm whether a similar response could be elicited at the organism level. On the other hand, fucoidan also stimulated respiratory burst in cod HK leukocytes. There are limited studies on the use of fucoidan as immunostimulant in fish, but in mammalian cells, fucoidan was found to enhance hydrogen peroxide and nitrite production in macrophages (Choi et al. 2005) as well as nitric oxide (Nakamura et al. 2006). The production of these substances contributes to

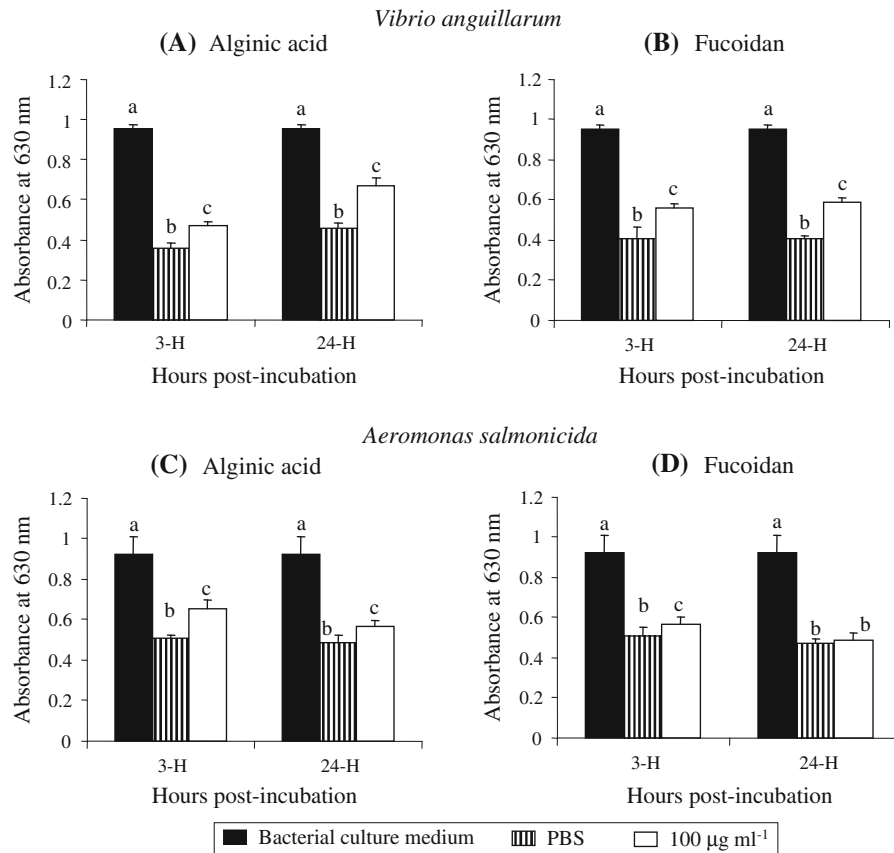


Fig. 6 Proliferation of *Vibrio anguillarum* and *Aeromonas salmonicida* in supernatants obtained from head kidney leukocytes incubated with 100 µg ml⁻¹ of either alginic acid

or fucoidan for 3- and 24-h. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean \pm SD, $N = 8$

enhanced respiratory burst in mammalian macrophages. A similar mechanism could take place in fish as demonstrated by our results, and this needs further studies.

There was no clear negative effect on proliferation of cod HK leukocytes during incubation with the test substances. This suggests that the doses of the substances used in the present study were not cytotoxic. This has an implication when these test substances are used in in vivo studies, i.e., if administered in therapeutic dose there is a minimal risk of toxic effects on the host organism.

The activity of cellular enzymes, acid and alkaline phosphatases seemed to be not affected by the test substances used. Acid phosphatase is an important enzyme that is involved in the degradation of phagocytized pathogens, thereby preventing their growth and multiplication in the host (Attwood et al. 1996). Previous studies in fish have shown that

alginic acid and/or alginate enhanced phagocytic ability (Peddie et al. 2002; Cheng et al. 2007; Yeh et al. 2008), and would likely trigger an increase in the production of acid phosphatase. In our present study, acid phosphatase in HK leukocytes was not apparently affected by the test substances and this could be due to the concentration used. Additional studies are needed to determine whether increasing the dose of these test substances would trigger an enhanced activity of acid phosphatase in HK leukocytes.

Alkaline phosphatase is another lysosomal enzyme that has a protective role in fish during the initial stages of wound healing (Iger and Abraham 1994). This cellular enzyme was not modulated by the test substances used, and this is not surprising because the different doses of the test substances were not cytotoxic to HK leukocytes; hence, there is no immediate need for cellular repair. Whether or not

increasing the dose of the test substances would result in enhanced alkaline phosphatase activity as a response to higher cytotoxicity is an interesting topic in future studies.

Cellular myeloperoxidase increased in HK leukocytes incubated at a higher dose ($100 \mu\text{g ml}^{-1}$) of fucoidan. In fish, myeloperoxidase activity is a measure of neutrophil antimicrobial activity (Swain et al. 2007). Together with halide ions and H_2O_2 , myeloperoxidase kills bacteria by halogenation of the bacterial cell walls and by the production of bactericidal hypohalite ions (Klebanoff and Clark 1978). Crude fucoidan was also found to inhibit the growth of *Vibrio harveyi*, *Staphylococcus aureus* and *Escherichia coli* (Chotigeat et al. 2004). This suggests that fucoidan could be involved in the antibacterial response in HK leukocytes when administered at therapeutic doses. In the case of alginic acid, higher doses could be tested in future studies to ascertain whether the levels of myeloperoxidase could be enhanced.

Supernatants obtained from HK cells incubated with the test substances were able to inhibit bacterial growth. However, the levels of bacterial growth in the supernatants from treated cells were significantly higher than growth observed in supernatants obtained from untreated cells (incubated with PBS). A similar phenomenon was also observed in supernatants from HK cells treated with crude bacterial phytase (Lazado et al. 2010). The possibility of the presence of contaminants that could act as immunostimulant, e.g., lipopolysaccharide (LPS) in PBS, which was used to incubate the control cells, is ruled out because we have used PBS in aliquot volumes and discarded the unused amount during the experiment. Several immune factors including antibacterial substances could have been secreted by HK leukocytes in the course of incubation with PBS, and this is not surprising because cod produces a variety of these compounds (Magnadóttir et al. 1999a, b). However, the higher proliferation the bacterial pathogens in the supernatants of the treated cells could be attributed to the presence of residual substances, which are mainly polysaccharides, and could have provided a good enrichment medium for the bacteria to grow. Even if the presence of polysaccharides in the cell culture medium favored bacterial growth, the high respiratory burst activities in the treated cells may have produced significant amounts of oxygen radicals that

could have kept the bacterial population in the supernatants at a minimum level.

In summary, we have shown that both alginic acid and fucoidan at concentrations of 10 and $100 \mu\text{g ml}^{-1}$ are able to stimulate selected cellular immune responses of cod HK leukocytes. Even if the observed differences in the immune parameters studied between the treated and control cells were slight, statistical tests have shown that there was an increased respiratory burst activity and no apparent cytotoxic effects using the different concentrations in the present study. Fucoidan resulted in enhanced cellular myeloperoxidase activity, which could be important in the antibacterial response. Supernatants obtained from cells incubated with the test substances decreased bacterial growth but were significantly higher than in supernatants from control cells. Although not all immune parameters were significantly upregulated by the test substances, nevertheless, we have provided evidence from our in vitro studies that both alginic acid and fucoidan have potential use as immunostimulants in cod as a health management strategy. Future in vivo studies are necessary to evaluate and compare the different immune response mechanisms in cod as a result of their exposure to these algal derivatives with other established immunostimulants such as β -glucans and lipopolysaccharides. Proper therapeutic doses of these algal derivatives should also be determined in order to prevent possible side-effects on the host.

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