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Title: Time and concentration dependency of MacroGard® induced apoptosis

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Abstract

In previous studies an effect of β-glucan on apoptosis in fish was noted and in this investigation we determine the time and concentration dependency of this effect. Primary cell cultures of pronephric carp cells were incubated for 6, 24, 48 h with various concentrations ranging from 0 – 1000 µg/ml of MacroGard® β-glucan. Apoptosis was monitored via acridine orange staining. Results indicate a clear effect of time and concentration on the induction of apoptosis *in vitro*, with only concentration ≥ 500 µg/ml causing significantly higher percentages of apoptotic cells. Apoptosis was detected after 6 h. This concentration dependent effect has to be considered when studying apoptosis in relation to immunostimulation.

Keywords

Apoptosis, beta-glucan, carp, concentration/time dependency

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Introduction

A cost effective approach to disease prevention in farming of aquatic and terrestrial animals is the administration of substances that enhance the immune system. These substances, known as immunomodulators or immunostimulants, are utilized to increase general protection against disease or to overcome the immunosuppressive effects of stress [1]. Generally substances that have immunostimulatory functions act as pathogen associated molecular patterns (PAMPs) and induce an immune response by interaction with pattern recognition receptors (PRRs). One such PAMP is β-glucan, a carbohydrate that is an essential cell wall component of fungi, bacteria, algae, oats and barley [2]. β-Glucan is an ideal target for studies on disease prevention since this substance stimulates various immune responses and enhances protection against viral and bacterial pathogens [3]. It also occurs naturally in the environment and therefore raises less concern in regards to adverse impacts on the environment and human health [4].

In humans β-glucans display various medicinal properties for example, decreases in cholesterol levels, enhancement of wound healing, and inhibition of cancer cell growth [5]. In mammals although various receptors e.g. complement receptor C3, dectin-1 and TLR1/6 have been described [6], dectin 1 is considered as major β-glucan receptor [7]. Differential responses are elicited when β-glucan binds to dectin-1 alone or together with other receptors such as TLR 2 [8]. However, dectin-1 could not be identified in fish and it has been suggested that β-glucan is detected by multiple pattern recognition receptors including toll like receptors [9]. In both mammals and fish, β-glucan recognition results in the activation of macrophages, which induces phagocytosis, leukocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species, as well as the enhancement of complement activity [9-17].

However, the immunostimulating effects of β-glucan have been shown to be dependent on dose, duration of administration, environmental temperature and the species. For example, no effect of β-glucan on stress related parameters were observed in channel catfish (*I. punctatus*) [18], whilst Jeney

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and colleagues [1] observed stress reducing effects of dietary β-glucan in rainbow trout (*O. mykiss*). This effect observed by Jeney et al. was however dose dependent and only occurred at 0.1 % β-glucan, whilst at 2 % β-glucan administered in feed led to a suppressed immune response which seemed to render the animals more susceptible to an infection. In fish, studies have shown that β-glucan dosage in the feed affects the respiratory burst activity of macrophages leading to differences in time and height of the peak respiratory burst activity [19]. These possible adverse dose effects of β-glucan have also been noted in crustaceans. For example, Hauton and Smith [20] noted that in lobster an increase in β-glucan concentration decreased the viability of granulocytes.

The possible mechanisms of the dose dependent effects of β-glucan particularly the reduction of immunostimulation at high doses have not been ascertained. However, in a recent detailed study by Kepka and coworkers [21] zymosan, a β-1,3-glucan from yeast, induced apoptosis *in vitro* and *in vivo*, which was linked to the production of ROS in carp leukocytes. Unfortunately the dose dependency of this phenomenon was not ascertained. The ability of β-glucan to induce apoptosis-related genes has previously been noted in fish by Miest et al. [22]. In fact, the β-glucan induction of apoptosis can be beneficial as β-glucan can also affect programmed cell death in human cancer cells. For example, Kim et al. [23] have shown that bacteria-derived β-glucan can induce apoptosis and that this form of cell death is involved in the tumouricidal effects of β-glucan [24, 25].

In this study the dose effect of β-glucan, in the form of MacroGard®, was investigated to ascertain its relationship with apoptosis in the pronephric leucocytes of fish. The pronephros was chosen as target organ as it fulfills important immune functions such as haematopoiesis, phagocytosis and antigen processing in the fish and Verburg-van Kemenade and colleagues [30] identified lymphocytes, neutrophilic and basophilic granulocytes and macrophages in suspensions of isolated pronephric cells. This heterogenous cell suspension hence allows us to study the effect of MacroGard® on a wide variety of immune cells.

The concentrations of MacroGard® utilised corresponded to concentrations used in other investigations involving different experimental animals for example, 1 – 200 µg/ml for mouse

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macrophages [26], 100 – 800 µg/ml for porcine leucocytes [27], 1 µg/ml for macrophages of rainbow trout (*Oncorhynchus mykiss*) [28], and 0.5 – 500 µg/ml for the phagocytes of turbot (*Psetta maxima*), gilthead sea bream (*Sparus aurata*) [29] and 500 µg/ml in carp (*Cyprinus carpio*) [21].

Materials & Methods

Common carp (*Cyprinus carpio*) were obtained from Fair Fisheries, Shropshire, England and reared in black 1 m x 0.5 m tanks with 225 litres of 15 °C dechlorinated water at pH 7. The water in each tank was circulated and cleaned by a temperature regulating biological pond filter (Eheim). Fish were fed daily with commercial dry pelleted food (Tetra Pond feed) and kept on a 12 hour light/dark cycle. The head - tail length of the fish ranged from 7.9 to 18.1 cm (mean length 10.6 cm) and weight ranged from 8.2 to 89.7 g (mean weight 36.3 g). For organ sampling fish were removed from the tank by netting and sacrificed with a lethal dose (~ 0.2 %) of 2-Phenoxyethanol (Sigma Aldrich, P1126) in aquarium water.

A pronephric cell suspension was prepared using a modification of the procedure described by Verburg-van Kemenade and coworkers [30]. In brief, the organ was gently disrupted through a sterile cell strainer with 100 µm pore diameter (BD Falcon Cell strainer, Scientific Laboratory Supply, 352360) in 1 ml of modified RPMI medium. The modified cell culture medium (hereafter referred to as RPMI+) consisted of RPMI with 0.3 g/L L-glutamine (Sigma Aldrich, R7388) with 0.5 % sterile water, 0.05 % pooled carp serum, 0.05 mM β-mercaptoethanol (Sigma Aldrich, M-3148), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Sigma Aldrich, P4458). The viability of the cell population was ascertained with trypan blue staining and only cell suspensions with a viability of at least 95 % were used. The concentration of the cell suspensions was adjusted to 1 x 10^7 cells/ml with RPMI+ medium. Cells were set up in 96 well plates (Sarstedt, UK) with 100 µl per well.

MacroGard® was provided by Biorigin (Brazil), according to the certificate of analysis the MacroGard® batch (batch 250813) consisted of 67.8 % carbohydrates, 5.1 % protein (dry matter),

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14.2 % lipids, 6.5 % ash and 4.6 % moisture. The microbiological analysis was negative for *Salmonella* sp., *Escherichia coli* and Coliforms (analysed by Biotech Pharmacon).

A stock solution of MacroGard® in autoclaved deionized water (sH_2O) was sonicated (2 x 30 s at power 6, Sonics, vibra-cell). Sterility was ensured by pasteurisation of the solution in a water bath at 80 ºC for 20 min and left at room temperature to cool down. Concentrations of MacroGard® were prepared from the stock solution in sH_2O as required. Following concentrations were used: 1, 50, 100, 250, 500, and 1000 µg/ml, which correspond to 0.6, 30, 60, 150, 300 and 600 µg/ml β-glucan. In addition, three controls were set up: an untreated control (i.e. no additives), a control with H_2O as additive, and a positive UV exposed (324 J/m^2) control as described in [31]. Cells where then incubated for 6, 24 or 48 h at 20°C, and apoptosis visualized as described in [22] using acridine orange.

Prior to statistical analysis the data were arcsin transformed to meet the assumption of normal distribution. The data were analysed for the influence of exposure time (i.e. 6, 24, and 48 h) and the various treatments (i.e. controls and MacroGard® concentrations) with 2-way repeated ANOVA. For the analysis of the distinct effects the treatments had on apoptosis a 1-way ANOVA was performed for the individual time points with a subsequent Turkey's post-hoc test. Analyses were performed using Minitab Release 14 and GraphPad Prism 4.

Results

The concentration of MacroGard® ($F = 12.39$, $p < 0.0001$) and the exposure time ($F = 20.73$, $p < 0.0001$) had a significant effect on apoptosis levels. Additionally an interaction was found between the main effects ($F = 1.98$, $p < 0.05$). As this global test indicated that the effect of dose varied with time, a series of one-way ANOVAS and post-hoc tests was conducted to examine where these differences lie (Figure 1).

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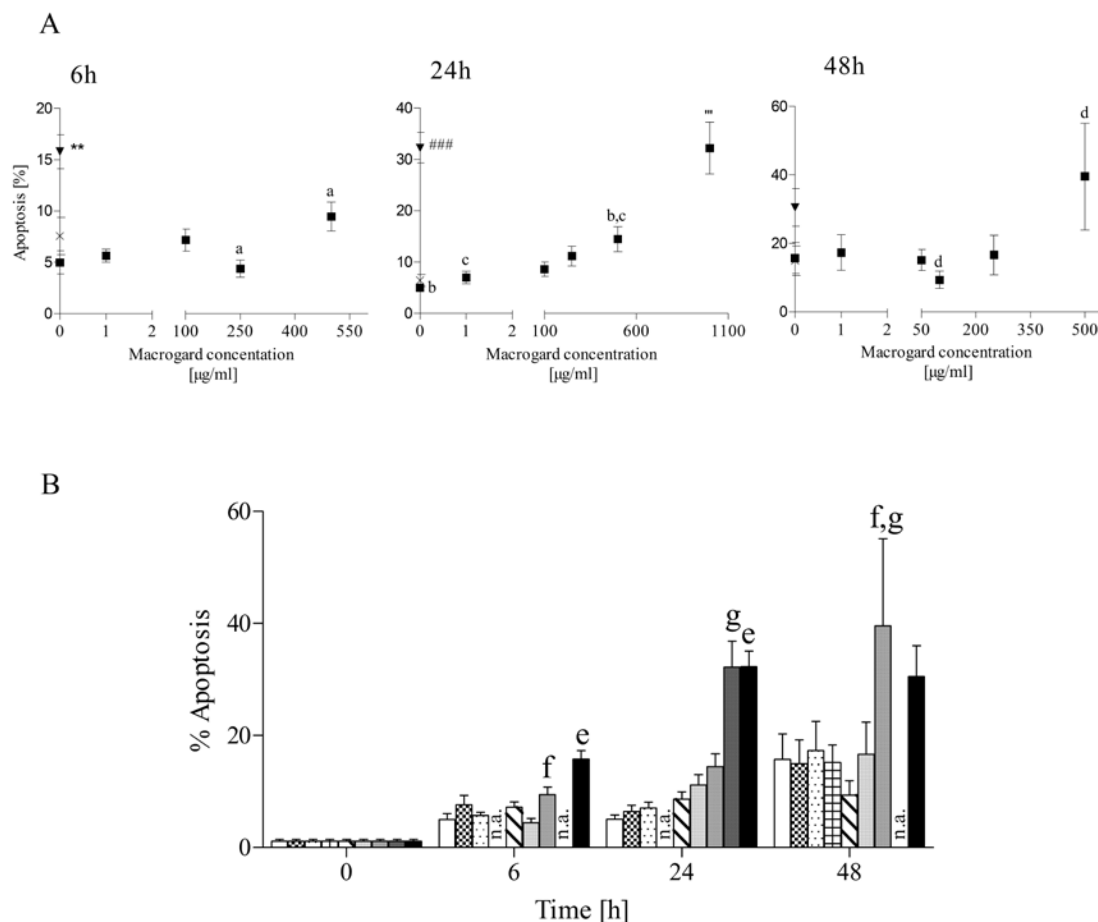

Figure 1: Time and concentration dependency of MacroGard® influence on apoptosis

Percentage of apoptotic cells in the cell suspension was analysed with acridine orange. Cell suspensions were treated with different concentrations of MacroGard® and incubated over 3 different time periods. Significance was defined as $p \leq 0.05$. Symbols for significant differences: * = significantly different to all other samples with ** = $p \leq 0.01$; ### = significantly different to all other samples except 1000 µg/ml with $p \leq 0.001$; ''' = significantly different to all other samples except UV with $p \leq 0.001$, same letters indicate differences between samples (with a, c, d, f = $p \leq 0.05$, b = $p \leq 0.01$, f, g = $p \leq 0.001$). n.a. = not analysed. Data are shown as mean ± SEM with n = 8. A) Concentration dependency of MacroGard® effects at different time points. X = water control, ▼ = UV control, n = 6 – 8. B). Time dependency of various MacroGard® concentrations. ▬ = UV, ▬ = 1000 µg/ml, ▬ = 500 µg/ml, ▬ = 250 µg/ml, ▬ = 100 µg/ml, ▬ = 50 µg/ml, ▭ = 1 µg/ml, ▩ = water, ▭ = 0 µg/ml.

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At all the time points there was no significant difference detected in apoptosis levels of the two negative controls (i.e. untreated and with added water). By running a positive control (i.e. UV exposure) alongside the samples it was established that apoptosis could be induced in the tested cell population. This UV exposure caused higher percentages of apoptosis positive cells after 6 hours (15.8 ± 1.54 %) and 24 hours (32.3 ± 2.8 %) when compared to the non-treated control (both $p \leq 0.001$). When cells were exposed to MacroGard® for 6 hours none of the tested concentrations (1 – 500 µg/ml) induced apoptosis in the pronephric carp leucocytes. Most effects were observed after 24 h of MacroGard® exposure. At this time point 500 µg/ml induced significantly higher apoptosis in the cell culture (i.e. 14.5 ± 2.4 %) compared to the non-treated control (5.1 ± 0.7 % apoptosis) ($p \leq 0.01$) and the 1 µg/ml MacroGard® concentration (7.0 ± 1.1 % apoptosis) ($p \leq 0.05$ and 0.001 respectively).

At 24 h, a concentration of 1000 µg/ml of MacroGard®, included in the experimental design to test if concentrations > 500 µg/ml induced significantly higher apoptosis (32.2 ± 5.0 %, $p \leq 0.001$), induced similar to apoptosis levels as the positive UV control. Because of the relatively high levels of apoptosis therefore this concentration was not tested at the other time points utilised. We therefore included an additional concentration of 50 µg/ml at the 48 h time point. As at 24 h 500 µg/ml MacroGard® induced significantly higher apoptosis levels (39.56 ± 15.57 %, $p \leq 0.05$ compared to 100 µg/ml). Additionally, there was a significant difference between the apoptosis levels caused by 500 µg/ml at 48 h and 24 h and 6 h (both $p \leq 0.001$). None of the other tested concentrations were significantly different.

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Discussion

The 2010 report by the Food and Agriculture Organisation of the United Nations on fisheries and aquaculture [32] stated that aquaculture will overtake capture fisheries as the primary source of food fish in the future. However the growth of this food-producing sector is impaired by disease outbreaks, which both reduce productivity in fish farms and pose a problem for biosecurity. Immunostimulation is an important tool in aquaculture to increase resistance to pathogens [3] especially since substances, such as β-glucan, occur naturally in the environment and are thus less likely to raise concerns about residues in food fish and the environment [4]. Previous studies on β-glucan have highlighted a time and dose dependency of the effects [1, 33-39].

However for many substances used as immunostimulants, including LPS and β-glucan, effectiveness is dose dependent, such that high concentrations often lead to adverse effects, including immunosuppression [19, 38, 40, 41]. As a result the resistance of the animals to pathogens is not enhanced or is even decreased at high concentrations, and hence it is important for both feed manufacturers and fish farmers that the dietary dose of the immunomodulating substance utilized does not induce such negative effects. In the present study, as well as in several other studies, it has been demonstrated that the effects of β-glucan are dose dependent [20, 29, 35]. For instance the cell viability in lobster granulocytes decreased with an increase from 50 to 250 µg/ml in MacroGard® concentration [42]. The respiratory burst activity in response to MacroGard® exposure is also dependent on the applied concentration as demonstrated by Castro and coworkers [29]. In their report concentrations ranging from 0.5 to 5 µg/ml had no effect on the production of ROS while concentrations of 7.5 to 500 µg/ml significantly induced the respiratory burst. In addition, the authors investigated the stimulatory effects of MacroGard® and found that low concentrations (i.e. 1 – 2.5 µg/ml) led to higher ROS production after secondary stimulation with another ROS inducing agent compared to cell cultures that were not pre-treated with MacroGard®. In the same study higher concentrations of MacroGard® (i.e. 10 – 500 µg/ml) inhibited the ROS response to the secondary stimulation, which was interpreted by the authors as a sign of exhaustion of the cells. However in regard to the findings described in this manuscript it is possible that the lower ROS production could

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be due to the onset of apoptosis and thus lower cell numbers in the culture. Reactive oxygen species are known for their cytotoxicity in fish [43, 44] and hence it is possible that the higher β-glucan concentrations induced ROS, causing apoptosis which then results in lower respiratory burst activity due to the reduced cell numbers.

Nonetheless, even though reports have shown that β-glucan has dose-dependent immunostimulating and immunosuppressing effects, up to now the mechanisms behind these differential effects have not been elucidated. It is interesting however that Kepka et al. [21] showed that *in vitro* 500 µg/ml zymosan and *in vivo* 40 µg zymosan/g body weight can induce apoptosis in pronephric granulocytes and macrophages (*in vitro*), as well as peritoneal leukocytes (*in vivo*) in carp.

The findings of our study and the work conducted by Kepka et al. [21] suggest that some of the immunosuppression noted may be associated with the induction of apoptosis in immune cells, which are exposed to particular high doses of β-glucan. In our studies the pro-apoptotic effect was noted to be time and dose dependent with only concentrations of ≥ 500 µg/ml causing apoptosis in pronephric leucocytes *in vitro.* This is perhaps not surprising as most substances are toxic above a certain threshold [45]. This concentration dependency is therefore an important factor to be considered during dietary administration of an immunostimulant. In aquaculture situations it is therefore important to administer β-glucan at concentrations which are high enough to stimulate the immune response, but also low enough to avoid any possible adverse effects due to high doses. This supports our previous observations [10, 11, 13, 16, 22] in which we showed that MacroGard® administered at the producer recommended (10 - 15 mg/kg bodyweight) dose does not induce apoptosis in pronephric cells but stimulates the immune response.

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References

1. Jeney G, Galeotti M, Volpatti D, Jeney Z, Anderson DP. Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. Aquaculture. 1997 154:1-15.

2. Volman JJ, Ramakers JD, Plat J. Dietary modulation of immune function by β-glucans. Physiology & Behavior. 2008 94:276-84.

3. Sakai M. Current research status of fish immunostimulants. Aquaculture. 1999 172:63-92.

4. Gannam AL, Schrock RM. Immunostimulants in fish diets. In: Lim C, Webster CD, editors. Nutrition and Fish Health: Food Products Press; 2001, p. 235-66.

5. Petravic-Tominac V, Zechner-Krpan V, Grba S, Srecec S, Panjkota-Krbavcic I, Vidovic L. Biological effects of yeast β-glucans. Agriculturae Conspectus Scientificus. 2010 75:149-58.

6. Dalmo Ra, Bøgwald J. β-Glucans as conductors of immune symphonies. Fish & Shellfish Immunology. 2008 25:384-96.

7. Brown GD, Gordon S. Fungal [beta]-Glucans and Mammalian Immunity. Immunity. 2003 19:311-5.

8. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2. J Exp Med. 2003 197:1107-17.

9. Pietretti D, Vera-Jimenez NI, Hoole D, Wiegertjes GF. Oxidative burst and nitric oxide responses in carp macrophages induced by zymosan, MacroGard® and selective dectin-1 agonists suggest recognition by multiple pattern recognition receptors. Fish & Shellfish Immunology. 2013 35:847-57.

10. Pionnier N, Falco A, Miest J, Frost P, Irnazarow I, Shrive A, et al. Dietary β-glucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection. Fish & Shellfish Immunology. 2013 34:819-31.

11. Pionnier N, Falco A, Miest JJ, Shrive AK, Hoole D. Feeding common carp *Cyprinus carpio* with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection. Fish & Shellfish Immunology. 2013 39:285-95.

12. Vera-Jimenez NI, Nielsen ME. Carp head kidney leukocytes display different patterns of oxygen radical production after stimulation with PAMPs and DAMPs. Molecular Immunology. 2013 55:231-6.

13. Falco A, Miest JJ, Pionnier N, Pietretti D, Forlenza M, Wiegertjes GF, et al. β-Glucan-supplemented diets increase poly(I:C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (*Cyprinus carpio*). Fish & Shellfish Immunology. 2014 36:494-502.

14. Hashimoto T, Ohno N, Adachi Y, Yadomae T. Enhanced production of inducible nitric oxide synthase by β-glucans in mice. FEMS Immunology and Medical Microbiology. 1997 19:131-5.

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15. Vetvicka V, Vashishta A, Saraswat-Ohri S, Vetvickova J. Immunological effects of yeast- and mushroom-derived β-glucans. Journal of Medicinal Food. 2008 11:615-22.

16. Falco A, Frost P, Miest J, Pionnier N, Irnazarow I, Hoole D. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements. Fish & Shellfish Immunology. 2012 32:1051-7.

17. Vera-Jimenez NI, Pietretti D, Wiegertjes GF, Nielsen ME. Comparative study of β-glucan induced respiratory burst measured by nitroblue tetrazolium assay and real-time luminol-enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.). Fish & Shellfish Immunology. 2013 34:1216-22.

18. Welker TL, Lim C, Yildirim-Aksoy M, Shelby R, Klesius PH. Immune response and resistance to stress and *Edwardsiella ictaluri* challenge in channel catfish, *Ictalurus punctatus*, fed diets containing commercial whole-cell yeast or yeast subcomponents. Journal of the World Aquaculture Society. 2007 38:24-35.

19. Bonaldo A, Thompson KD, Manfrin A, Adams A, Murano E, Mordenti AL, et al. The influence of dietary β-glucans on the adaptive and innate immune responses of European sea bass (*Dicentrarchus labrax*) vaccinated against vibriosis. Italian Journal of Animal Sciences. 2007 6:151-64.

20. Hauton C, Smith VJ. *In vitro* cytotoxicity of crustacean immunostimulants for lobster (*Homarus gammarus*) granulocytes demonstrated using the neutral red uptake assay. Fish & Shellfish Immunology. 2004 17:65-73.

21. Kepka M, Verburg-van Kemenade BM, Homa J, Chadzinska M. Mechanisms involved in apoptosis of carp leukocytes upon *in vitro* an *in vivo* immunostimulation. Fish & Shellfish Immunology. 2014 39:386-95.

22. Miest JJ, Falco A, Pionnier NPM, Frost P, Irnazarow I, Williams GT, et al. The influence of dietary β-glucan, PAMP exposure and *Aeromonas salmonicida* on apoptosis modulation in common carp (*Cyprinus carpio*). Fish & Shellfish Immunology. 2012 33:846-56.

23. Kim MJ, Hong SY, Kim SK, Cheong C, Park HJ, Chun HK, et al. β-Glucan enhanced apoptosis in human colon cancer cells SNU-C4. Nutrition Research and Practice. 2009 3:180-4.

24. Kobayashi H, Yoshida R, Kanada Y, Fukuda Y, Yagyu T, Inagaki K, et al. Suppressing effects of daily oral supplementation of beta-glucan extracted from *Agaricus blazei* Murill on spontaneous and peritoneal disseminated metastasis in mouse model. Journal of Cancer Research and Clinical Oncology. 2005 131:527-38.

25. Zhang M, Chiu LCM, Cheung PCK, Ooi VEC. Growth-inhibitory effects of a β-glucan from the mycelium of *Poria cocos* on human breast carcinoma MCF-7 cells: Cell-cycle arrest and apoptosis induction. Oncol Rep. 2006 15:637-43.

26. Hetland G, Sandven P. β-1,3-Glucan reduces growth of *Mycobacterium tuberculosis* in macrophage cultures. FEMS Immunology and Medical Microbiology. 2002 33:41-5.

27. Sonck E, Stuyven E, Goddeeris B, Cox E. The effect of β-glucans on porcine leukocytes. Veterinary Immunology and Immunopathology. 2010 135:199-207.

28. Novoa B, Figueras A, Ashton I, Secombes CJ. *In vitro* studies on the regulation of rainbow trout (*Oncorhynchus mykiss*) macrophage respiratory burst activity. Developmental & Comparative Immunology. 1996 20:207-16.

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29. Castro R, Couso N, Obach A, Lamas JÚS. Effect of different β-glucans on the respiratory burst of turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) phagocytes. Fish & Shellfish Immunology. 1999 9:529-41.

30. Verburg-van Kemenade B, Groeneveld A, van Rens B, Rombout J. Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). Journal of Experimental Biology. 1994 187:143-58.

31. Vidal MC, Williams G, Hoole D. Characterisation of a carp cell line for analysis of apoptosis. Developmental & Comparative Immunology. 2009 33:801-5.

32. FAO. The State of World Fisheries and Aquaculture. Rome; 2010, p. 197.

33. Jørgensen JB, Sharp GJE, Secombes CJ, Robertsen B. Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. Fish & Shellfish Immunology. 1993 3:267-77.

34. Volpatti D, D'Angelo L, Jeney G, Jeney Z, Anderson DP, Galeotti M. Nonspecific immune response in fish fed glucan diets prior to induced transportation stress. Journal of Applied Ichthyology. 1998 14:201-6.

35. Ai Q, Mai K, Zhang L, Tan B, Zhang W, Xu W, et al. Effects of dietary β-1, 3 glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*. Fish & Shellfish Immunology. 2007 22:394-402.

36. Gopalakannan A, Arul V. Enhancement of the innate immune system and disease-resistant activity in *Cyprinus carpio* by oral administration of β-glucan and whole cell yeast. Aquaculture Research. 2010 41:884-92.

37. Lin S, Pan Y, Luo L. Effects of dietary β-1, 3-glucan, chitosan or raffinose on the growth, innate immunity and resistance of koi (*Cyprinus carpio koi*). Fish & Shellfish Immunology. 2011.

38. Misra C, Das B, Mukherjee S, Pattnaik P. Effect of long term administration of dietary β-glucan on immunity, growth and survival of *Labeo rohita* fingerlings. Aquaculture. 2006 255:82-94.

39. Brattgjerd S, Evensen O, Lauve A. 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. 1994 83:288.

40. Misra CK, Das BK, Mukherjee SC, Meher PK. The immunomodulatory effects of tuftsin on the non-specific immune system of Indian Major carp, *Labeo rohita*. Fish & Shellfish Immunology. 2006 20:728-38.

41. Nayak SK, Swain P, Nanda PK, Dash S, Shukla S, Meher PK, et al. Effect of endotoxin on the immunity of Indian major carp, *Labeo rohita*. Fish & Shellfish Immunology. 2008 24:394-9.

42. Hauton C, Smith VJ. Changes in immune gene expression and resistance to bacterial infection in lobster (*Homarus gammarus*) post-larval stage VI following acute or chronic exposure to immune stimulating compounds. Molecular Immunology. 2007 44:443-50.

43. Risso-de Faverney C, Devaux A, Lafaurie M, Girard JP, Bailly B, Rahmani R. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species. Aquatic Toxicology. 2001 53:65-76.

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44. Xiang L, Shao J. Role of intracellular Ca 2+, reactive oxygen species, mitochondria transmembrane potential, and antioxidant enzymes in heavy metal-induced apoptosis in fish cells. Bulletin of Environmental Contamination and Toxicology. 2003 71:114-22.

45. Turner SM. Chemical risk: a primer. ACS information pamphlet. Washington: American Chemical Society, Department of Government Relations and Science Policy; 1996.