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Research Article

Parameters to evaluate the immunostimulant effect of Zymosan A in head kidney leucocytes (HKL) of salmonids

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ABSTRACT. The interest in characterizing fish immune responses against pathogens or discovering new immunostimulant molecules requires the use of mass screening techniques. The classical model has been the challenge in 1000 L tanks, using a high number of fishes, resulting in high economic cost. In this work, the purpose was to minimize the costs of such tests using *in vitro* cellular models. In primary cultures of head kidney leucocytes (HKL) immune response to Zymosan A, a type of β-glucan, was evaluated. The quantification and detection of pro-inflammatory cytokines was done based on both enzymatic activity and ELISA. The activity of phagocyte oxidase and inducible nitric oxide synthase (iNOS) was measured indirectly through NBT reduction by reactive oxygen species and Griess reaction to nitric oxide, respectively. For detecting pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, monospecific antibodies generated in our laboratory were used by indirect ELISA. The immune parameters were evaluated at different concentration of Zymosan A and the evolution of the immune response over time was established. The results suggest the better time for evaluating the immunostimulant capacity of glucans and the appropriate dose of it, which is required to demonstrate the activation of macrophages (ROS and iNOS) and the production of pro-inflammatory molecules, both as indicators of good immune response. Currently, work is being conducted improving these methods, creating an ELISA sandwich and generating antibodies against total proteins of HKL for pre-absorption of induced samples. In particular, the implementation of ELISA for the characterization of cytokine secretion profiles is a biotechnological contribution in aquaculture, which has not been implemented before this work.

Keywords: head kidney leucocytes, Zymosan A, fish innate immunity. salmonids, Chile.

Parámetros para la evaluación del efecto de Zimosán A como inmunoestimulante sobre leucocitos de riñón cefálico (HKL) de salmónidos

RESUMEN. El interés en la caracterización de la respuesta inmune de peces contra patógenos o el descubrimiento de nuevas moléculas inmunoestimulantes, requiere el uso de técnicas de uso masivo y rápido. El modelo clásico ha sido el desafío en estanques de 1000 L, con un elevado número de peces, lo que tiene un alto costo económico. El objetivo de este trabajo fue reducir al mínimo los costos de dichos ensayos, utilizando modelos celulares *in vitro*. Para ello, se evaluó el efecto del inmunoestimulante de Zymosan A, un tipo de β-glucano, sobre la respuesta inmune en cultivos primarios de leucocitos de riñón cefálico (HKL), mediante la cuantificación enzimática y la detección de citoquinas pro-inflamatorias. La actividad de la fagocito oxidasa y de la óxido nítrico sintetasa inducible (iNOS), se midió indirectamente a través de la reducción de NBT, por especies reactivas de oxígeno, y la reacción de Griess para óxido nítrico, respectivamente. La detección de citoquinas pro-inflamatorias como el TNF-α, IL-6 e IL-1β, se realizó mediante ELISA indirecto, con anticuerpos monoespecíficos generados en nuestro laboratorio. Los parámetros inmunológicos fueron evaluados en diferentes concentraciones y tiempos de incubación con el Zimosán A. Los resultados sugieren las condiciones óptimas para la evaluación de glucanos con capacidad inmunoestimulante, en base a indicadores de buena respuesta inmunitaria, que considera la activación de macrófagos (ROS e iNOS) y la secreción de moléculas pro-inflamatorias. Actualmente, el trabajo está dirigido

a mejorar estos métodos, a través de la creación de un ELISA sandwich y generación de anticuerpos contra las proteínas totales de HKL para la pre-absorción de las muestras inducidas. En particular, la aplicación de ELISA para la caracterización de los perfiles de secreción de citoquinas es una contribución biotecnológica en la acuicultura, lo que no ha sido aplicado antes de este tipo de análisis.

Palabras clave: leucocitos de riñón cefálico, Zimosán A, inmunidad innata de peces, salmónidos, Chile.

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INTRODUCTION

Immunostimulatory capacity of β-glucan has been demonstrated in fish, which is a powerful tool applied to its farming, as it strengthens the immunity, resistance to diseases and stress (Bagni *et al.*, 2000). Innate immunity experiences the greatest reinforcement mediated by this type of pathogen-associated molecular patterns (PAMPs), therefore its presence can increase the number and activity of macrophages, phagocytosis, respiratory burst, cytotoxicity and activity of lysozyme, which has been evaluated in different fish species such as, *Pagrus auratus*, *Sparus aurata* and *Dicentrarchus labrax* (Cook *et al*., 2003; Rodríguez *et al*., 2003; Vazzana *et al*., 2003; Kudrenko *et al*., 2009). Different routes have been used for administration of β-glucan to fish, been the most common intraperitoneal injection (ip). *Danio rerio* has demonstrated a remarkable decrease in mortality and increased survival when they have been treated via ip and then challenged with *Aeromonas hydrophila* (Rodríguez *et al*., 2003). Furthermore, the combined use of PAMPs such as β-glucan/LPS has also shown improvement in resistance to bacterial pathogens in *Cyprinus carpio*, and also it has been shown that ip and oral applications are more effective than applications by bathing (Selvaraj *et al*., 2006).

Although the effect of glucans on immunity in fish is known, both cellular and molecular pathways involved have not been characterized. In general terms, β-glucans are part of a heterogeneous group of glucose polymers, major constituents of the walls of fungi and bacteria, and hence derives its ability to activate the immune system of both invertebrates and vertebrates (Kataoka *et al*., 2002). The molecular mechanisms that are activated by this type of glucans, vary from the complexity of the organism and the structural characteristics of the β-glucans (Tsoni & Brown, 2008). For example, only β-glucans that have ramifications with β-1→3 and β-1→6 bonds in its structure trigger the immune system. In addition, the extension of the structure of these molecules influences the immune response; in this way, large particles (such as Curdlan and Zymosan) are able to directly activate leukocytes (Brown & Gordon, 2005). Intermediate particles as phosphoglucans, have immunostimulatory activity *in vivo*, but not *in vitro*. On the other hand, small particles or with low molar mass, as laminarin, are biologically inactive both *in vitro* and *in vivo* (Adams *et al*., 2008; Tsoni & Brown, 2008). The effect of glucans on immunity has been best established in higher vertebrates, for example Curdlan, a linear β-glucan has shown the ability to activate macrophages in rats by stimulating the nuclear transcription factor kB, leading to an overexpression of iNOS , TNF-α and MIP-2 (Kataoka *et al*., 2002; Ferwerda *et al*., 2008). For its part, Zymosan A, a β-glucan constituent of the wall of yeasts induced a number of cytokines and membrane markers comparable to lipopolysaccharide (Dowling *et al*., 2008).

In fish farming, β-glucan has been used as an immunostimulant, however, it is required to improve the quality of these applications, which involves looking for glucans with high immunostimulatory capacity. This can be evaluated in tanks with challenged fish, leading to high costs, impeding efficient molecule screening processes. This work presents a proposal for evaluating *in vitro* glucan molecules with immunostimulatory activity. The model presents two advantages; first, the lower cost of implementation, because of the use of primary cultures of macrophages from head kidney of trout and second, as a new proposal to assess the immunostimulatory capacity only at phenotypic level by conventional enzymatic quantifications and through the detection of cytokines secreted into the culture medium. The analysis of the results suggests inducer concentrations and incubation times for the evaluation *in vitro* of β-glucan on immunity of salmonids, with reference to standard Zymozan A.

MATERIAL AND METHODS

Fish

Rainbow trout (*O. mykiss*) from Rio Blanco fish farm were stabilized in the Laboratorio Experimental de Acuicultura (LEDA) in 1000 L tanks with filtered fresh water recirculation at a temperature between 17 and 20°C with constant aeration and commercial diet once daily until they reached the weight of 130 g.

Isolation of leucocytes from head kidney

The method described by Braun-Nesje *et al*. (1981) was used. Briefly, head kidney was removed aseptically and macerated through a 100 µm nylon mesh, using L-15 medium. The resulting suspension was placed on a discontinuous Percoll density gradient of 34/51%. The gradient was centrifuged at 800 g for 40 min. The interface cells were recovered and washed twice with 1X Hank's Balanced Solution for 5 min at 400 g. The concentration and cell viability was estimated with trypan blue. The cells were adjusted to 5 x 10^6 cells mL^{-1} with L-15 medium supplemented with 2% FBS and antibiotics (100 U mL^{-1} of penicillin/100 mg mL^{-1} streptomycin). 100 µL of cell suspension was seeded to a 96-well plate. The cells were stabilized at 17°C overnight (ON) prior induction.

Inductions

After overnight (ON) period, unattached cells were washed with L-15 medium. The attached cells were stimulated with commercial Zymosan A (SIGMA) at 0.1/0.5/1.0/2.0 mg mL^{-1} To quantify nitric oxide, cells were incubated from 2 to 96 h. For respiratory burst, they were incubated for 1 h. Meanwhile, to assess cytokine induction, incubation was performed between 2 and 10 h.

Quantification of nitric oxide

We used the method of Greiss reaction (Neumann *et al*., 1995), which quantifies the nitrite content in cell supernatants, since NO is unstable and is oxidized to nitrite and nitrate. After incubation at 17°C, 100 µL of each well supernatant were taken and left in a separate 96-well plate; then 100 µL of Greiss reagent (sulfaniamide 1% and 0.1% ethylenediamine naftil acid 2.5% phosphoric acid) was added. Absorbance was measured at 540 nm in a Versamax plate reader. Nitrite concentration in the sample was calculated from a calibration curve of sodium nitrite at known concentrations.

Respiratory burst

The methodology used was reduction of NBT to formazan (Selvaraj *et al*., 2005), which forms a blue precipitate inside the cells. Induced monolayer was washed 3 times with L-15 medium. 100 µL of NBT (1 mg mL^{-1}) was added and incubated for 1 h. Then, washed 3 times with Hank's 1X and cells were fixed with methanol 100% for 3 min. The cells were washed twice with 70% methanol and allowed to air dry. The formazan produced was solubilized by adding 120 µL of 2M KOH and 140 µL 100% DMSO. Absorbance was read at 630 nm using KOH/DMSO as blank.

Indirect ELISA

The cytokines IL-1β, IL-6 and TNF-α were tested with antibodies produced and obtained in our laboratory. Polyclonal antibodies developed in mouse were obtained against synthetic epitope sequences of the respective cytokines tested. For the indirect ELISA assay, 100 µL of cell culture medium (50 ng μL^{-1} in 0.06 M carbonate buffer) were loaded and incubated at 4°C ON in 96 well ELISA plates. Then, washed three times with PBST 0.05% and blocked with BSA (1%) for 2.5 h at 37°C. Afterwards, washed and incubated with first antibody (100 µL, 1.5 mg mL^{-1}) for 2 h at 37°C. The second antibody anti-mouse IgG HRP conjugate (100 µL, 1:7000) was incubated for 1.5 h at 37°C. The plate was revealed incubating with TMB for 30 min at room temperature and reaction was stopped with 50 µL of sulfuric acid. Finally, absorbance was read at 450 nm.

For statistical analysis, calculations of means, standard deviations and analysis of variance (ANOVA) of all the assays was carried out using Microsoft Excel 2007. Differences were considered significant if the *P*-value was < 0.05.

RESULTS

HKL centrifugation on gradient yielded an interface, between 34% and 51% of Percoll, enriched in viable macrophages which were stabilized in culture. After 24 h of culture, HKL showed typical spherical cell morphology in the early hours, and then the majority of the cells, due to its characteristics of macrophages began to adhere to the bottom of the well. On day 5 the cells were largely adhered and stabilized (Fig. 1).

HKL cells stabilized in culture for 5 days, hereinafter macrophages, were induced to generate iNOS in response to 1 mg mL^{-1} of Zymosan A. Initially the production of NO tends to increase slightly at 4 h of induction; however the peak was reached after 72 h of incubation, beginning a descent at 96 h (Fig. 2a). Evaluating the dose-response relationship in the activation of glucan-dependent iNOS, it was found that this is only significant at 72 h using 1 mg mL^{-1} of inducer (Fig. 2b).

Macrophages in culture responded to stimulation of Zymosan A via increasing destructive activity by phagocyte oxidase, which was evidenced indirectly through quantification of reactive oxygen species. There was no dose-response to glucan, using 0.5 mg

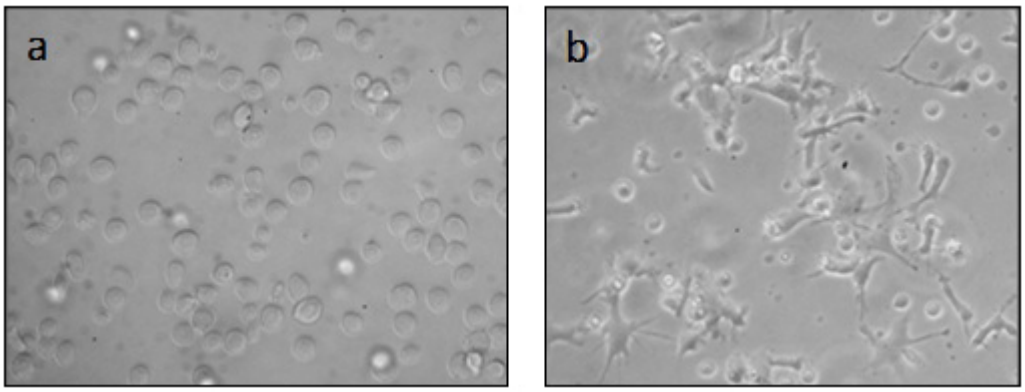

Figure 1. Contrast phase microphotography of cultured HKL, (a) cells 24 h post stabilization are observed spherical and refractile, (b) after five days of stabilization in culture, they were observed adhering to the plate, with notable extensions (400x).

Figura 1. Microfotografía en contraste de fase de HKL en cultivo, (a) células post 24 h de estabilización se observan esféricas y refringentes, (b) luego de cinco días de estabilización en cultivo se observan adheridas a la placa, con notorias prolongaciones (400x).

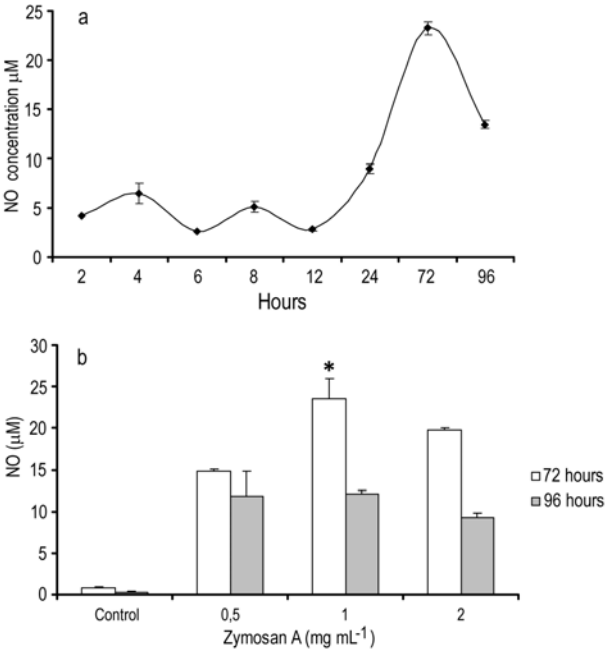

Figure 2. Quantification of nitric oxide produced by HKL in culture, (a) induced with 1 mg mL^{-1} of Zymosan A, production peaks at 72 h. (b) Dose-effect response quantified in the two periods of increased detection of NO produced by cultured cells. Values correspond to mean ± SD (* $P < 0.05$).

Figura 2. Cuantificación de oxido nítrico producido por HKL en cultivo (a) inducidos con 1 mg mL^{-1} de Zymosan A, alcanza el máximo de producción a las 72 h. (b) Efecto dosis respuesta cuantificado en los dos tiempos de mayor detección de NO producido por las células en cultivo. Los valores graficados corresponden al promedio ± SD (* $P < 0,05$).

mL^{-1} of inducer and four times this concentration, a significantly ROS level was quantified compared to control, but showing no significance between them (Fig. 3).

Detection of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in the culture medium showed a peak of secretion from macrophages after 4 h of induction with Zymosan A, for all cytokines (Fig. 4). Six hours after induction, secretion of IL-1β and TNF-α tended to stabilize, except when the dose of inducer was

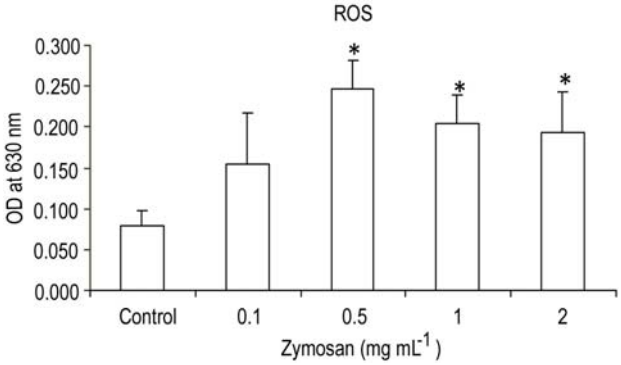

Figure 3. Quantification of reactive oxygen species produced by cultured HKL induced with different concentrations of Zymosan A, over 0.5 mg mL^{-1} of inductor the production of ROS is significant compared to control. Values correspond to mean ± SD (*$P < 0.05$).

Figura 3. Cuantificación de especies reactivas de oxígeno producidas por HKL en cultivo inducidos con diferentes concentraciones de Zymosan A, sobre 0,5 mg mL^{-1} de inductor la producción de ROS es significativa con respecto a control. Los valores graficados corresponden al promedio ± SD (* $P < 0,05$).

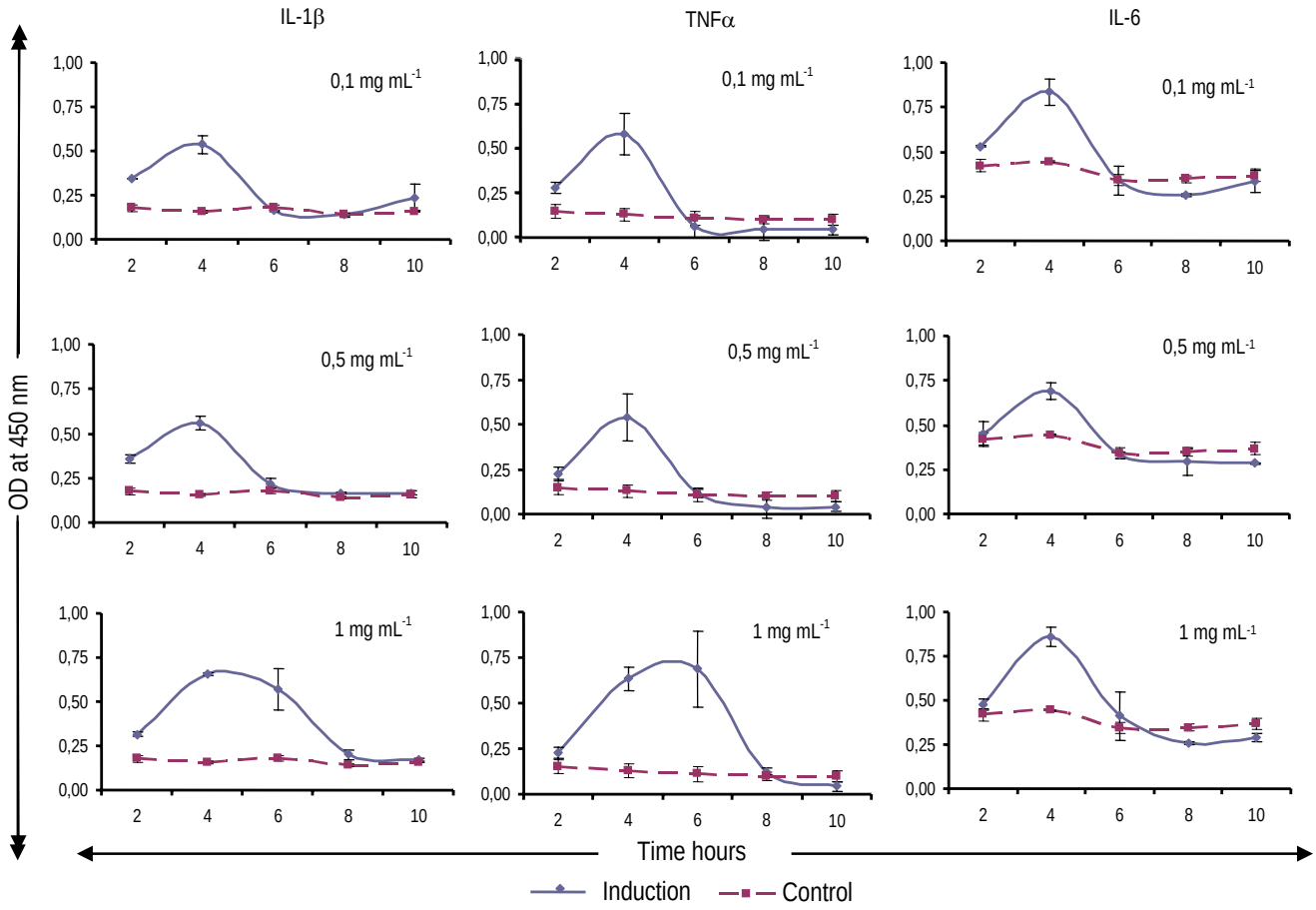

Figure 4. Indirect ELISA detection curves of pro-inflammatory cytokines produced by HKL over time in response to different concentrations of Zymosan A. Values correspond to mean ± SD (*$P < 0.05$).

Figura 4. Curvas de detección por ELISA indirecto de citoquinas pro-inflamatorias producidas por HKL a través del tiempo, en respuesta al inductor Zymosan A aplicado en diferentes concentraciones. Los valores graficados corresponden al promedio ± SD (*$P < 0,05$).

higher (1 mg mL^{-1}), secretion levels maintained as detected at 4 h. For IL-6 in all inducer concentrations tested, the stabilization arises from hour 6, where the levels detected are similar to the control situation. All assays showed no dose-response relationship, since the peak of secretion detected at 4 h, showed no significant differences for the same cytokine induced with different concentrations of Zymosan A (Fig. 4).

DISCUSSION

The study of fish immune responses requires optimal molecular markers to characterize the involvement of cellular and humoral components of both innate and adaptive responses (Randelli *et al*., 2008; Narváez *et al*., 2010). Moreover, the generation of *in vitro* models has allowed the identification of key pro-inflammatory cytokines expressed in response to challenges with PAMPs and pathogens. Therefore, it has been necessary to establish cell lines (Rojas *et al*., 2010) or the implementation of laboratory primary cell cultures, in both cases the central idea is to generate *in vitro* models for the evaluation of immune responses, with the aim of projecting it to the fish and its productive management in aquaculture. In the case of this work, we have tested basic conditions for implementing an *in vitro* system to assess glucan type immunostimulants, known as potent inducers of the immune response that protects the fish against pathogens (Brown & Gordon, 2005).

From head kidney was obtained a phase of leukocytes (HKL) enriched in macrophages, which were grown successfully. This phase corresponds to a Percoll interface between 34% and 51%, which has been previously characterized in fish and particularly in rainbow trout (Chettri *et al*., 2010, 2011). This

phase is used for *in vitro* induction, both at 24 h of stabilization, as well as days later, where the population of cells adhering to the plate is in the 80% of the total cells grown at the beginning. HKL culture cells are capable of responding to the challenge using different PAMPs such as LPS, flagellin and Poly I:C, in the case of Zymosan A there has been an up regulation of pro-inflammatory genes like TNF-α, IL-1β, IL-6 and the key enzyme in the pathway of prostaglandins, COX-2 (Chettri *et al*., 2011).

Our results confirm the ability of HKL to respond to the challenge with Zymosan A, in all the tested assays, both at enzyme level as in ELISAs. NO has potent toxic effects and, as such, it is an important component of the available arsenal to animal hosts, their production depending on the activation of iNOS, an enzyme that is normally under the regulation of pro-inflammatory cytokines (Rieger & Barreda, 2011). A classical approach to establish the level of immune response, associated with this path, is the quantification of NO production, which has been previously established in fish macrophages (Tafalla & Novoa, 2000; Jurecka *et al*., 2009). There are no bibliographic records that indicate the level of NO in response to challenge with Zymosan in HKL of rainbow trout, our results show that at 72 h of incubation, the highest level of quantification is reached, and doses of 1 mg mL^{-1} are the ones which generate significantly higher NO production, showing the highest level of response to Zymosan A. Currently, in our laboratory, we are implementing an ELISA to quantify iNOS directly, test that can be performed in shorter induction time.

Respiratory burst has also been a classical evaluation to establish the level of activation of macrophages in response to a stimulus (Cook *et al*., 2003). This parameter has been previously established and the test conditions of rainbow trout HKL, determined (Chettri *et al*., 2010). Our results, in this regard, are a positive control of the Zymosan A induction established *in vitro* over HKL. The reference concentration of Zymosan A to achieve generation of ROS, in a clearly detectable way, is 1.6 mg $mL^{-1.}$ Our assays proved that over 0.5 mg mL^{-1}, HKL are able to increase the production of reactive oxygen species significantly with respect to control, as well as up to 2 mg mL^{-1} tested.

The main objective of this work focused on establishing an analytical model of HKL response to Zymosan A, as a reference to test immunostimulants in salmonids, considering a high-capacity screening system. For this, cytokines secreted into the culture medium were detected in an *in vitro* assay that has not previously been proposed or established for salmonid HKLs. Normally, fish cytokine assessments are conducted at a transcriptional level, and very few papers consider the use of antibodies as indicators of cytokine production. In trout, there is only one reference of the detection of TNF-α, where it has been shown that LPS stimuli can induce cytokine secretion, without increasing the transcriptional activity of the gene that encodes for it (Roher *et al*., 2011).

Our results confirm that increased transcriptional activity, previously demonstrated, that occurs in trout HKL in response to Zymosan A (Chettri *et al*., 2011), also causes increased secretion of pro-inflammatory cytokines TNF-α, IL-1β and IL-6. The result that best represents what has been described at the transcriptional level, is the secretion of IL-6, which at four hours of induction, achieved the highest detection in the culture medium, coinciding exactly with the previously demonstrated at transcriptional level, with the highest level of expression at the same time.

In the case of IL-1β and TNF-α, transcriptional activity described by Chettri *et al*. (2011), increases to 12 h of incubation which, at protein level, does not correlate with our results, since at 10 h, basal levels of the cytokine in the culture medium are already detected. As to the concentration of inductor, it was proved that low doses of Zymosan A (0.1 mg mL^{-1}) generate secretion of three cytokines, as well as it was shown previously occurring at transcriptional level. It is interesting to note that the samples evaluated by indirect ELISA, considered 50 ng uL^{-1} of total proteins in the culture medium, which is a highly sensitive, replicable and massified method.

In summary we propose a model for evaluating the glucan immunostimulant ability in salmonids, using HKL primary cultures, and evaluating the secretion of pro-inflammatory cytokines in the culture medium. The test dose of inducer can be 0.1 mg mL^{-1} and the incubation time 4 h. As a positive control of induction, quantification of reactive oxygen species is proposed, through NBT reduction in cells treated with 0.5 mg mL^{-1} of inducer. Finally, as evidence of the destructive capacity of macrophages, we propose the use 1 mg mL^{-1} of inducer for 72 h for the quantification of NO.

The widespread of such screening techniques of potentially useful molecules as immunostimulants, have the potential to increase in a short-term period the supply of aquaculture products with a high capacity to improve the fish farm production.

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REFERENCES

Adams, E.L., P.J. Rice, B. Graves, H.E. Ensley, H. Yu, G.D. Brown, S. Gordon, M.A. Monteiro, E. Papp-Szabo, D.W. Lowman, T.D. Power, M.F. Wempe & D.L. Williams. 2008. Differential high affinity interaction of Dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side chain branching. J. Pharmacol. Exp. Ther., 325(1): 115-123.

Bagni, B, L. Archetti, M. Amadori & G. Marino. 2000. Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*). J. Veter. Med., 47: 745-751.

Braun-Nesje, R., K. Bertheussen, G. Kaplan & R. Seljelid. 1981. Salmonid macrophages: separation, *in vitro* culture and characterization. J. Fish Dis., 4: 141-151.

Brown, G.D. & S. Gordon. 2005. Immune recognition of fungal β-glucans. Cell. Microbiol., 7(4): 471-479.

Chettri, J.K., L. Holten-Andersen & K. Buchmann. 2010. Factors influencing *in vitro* respiratory burst assays with head kidney leucocytes from rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis., 33(7): 593-602.

Chettri, J., M. Raida, L. Holten-Andersen, P. Kania & K. Buchmann. 2011. PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol., 35: 476-482.

Cook, M., P. Hayball, W. Hutchinson, B. Nowak & J. Hayball. 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 Immunol., 14: 333-345.

Dowling, D., C.M. Hamilton & S. O'Neill. 2008. Comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with LPS compared with a panel of TLR ligands. Cytokine, 41: 254-262.

Ferwerda, G., F. Meyer-Wentrup, B.J. Kullberg, M.G. Netea & G.J. Adema. 2008. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cell. Microbiol., 10: 2058-2066.

Jurecka, P., I. Irnazarow, J. Stafford, A. Ruszczyk, N. Taverne, M. Belosevic, H. Savelkoul & G. Wiegertjes. 2009. The induction of nitric oxide response of carp macrophages by transferrin is influenced by the allelic diversity of the molecule. Fish Shellfish Immunol., 26(4): 632-638.

Kataoka, K., T. Muta, S. Yamazaki & K. Takeshige. 2002. Activation of macrophages by linear (1-3)-β-D-Glucans. Implications for the recognition of fungi by innate immunity. J. Biol. Chem., 277(39): 36825-36831.

Kudrenko, B., N. Snape & A. Barnes. 2009. Linear and branched ß (1–3) D-glucans activate but do not prime teleost macrophages in vitro and are inactivated by dilute acid: Implications for dietary immunostimulation. Fish Shellfish. Immunol., 26(3): 443-450.

Narváez, E., J. Berendsen, F. Guzmán, J.A. Gallardo & L. Mercado. 2010. An immunological method for quantifying antibacterial activity in *Salmo salar* (Linnaeus, 1758) skin mucus. Fish Shellfish Immunol., 28: 235-239.

Neumann, F., D. Fagan & M. Belosevic. 1995. Macrophage activating factors secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. Dev. Comp. Immunol., 19(6): 473-482.

Randelli, E., F. Buonocore & G. Scapigliati. 2008. Cell markers and determinants in fish immunology. Fish Shellfish Immunol., 25: 326-340.

Rieger, A. & D. Barreda. 2011. Antimicrobial mechanisms of fish leukocytes. Dev. Comp. Immunol., 35(12): 1238-1245.

Rodríguez, A., A. Cuesta, J. Ortuño, M.A. Esteban & J. Meseguer. 2003. Immunostimulant properties of a cell wall-modified whole *Saccharomyces cerevisiae* strain administered by diet to seabream (*Sparus aurata* L.). Vet. Immunol. Immunopathol., 96: 183-192.

Roher, N., A. Callol, J.V. Planas, F.W. Goetz & S.A. Mackenzie. 2011. Endotoxin recognition in fish results in inflammatory cytokine secretion not gene expression. Innate Immunity, 17: 16-28.

Rojas, V., N. Galanti, N.C. Bols, V. Jiménez, R. Paredes & S.H. Marshall. 2010. *Piscirickettsia salmonis* induces apoptosis in macrophages and monocyte-like cells from rainbow trout. J. Cell Biochem., 110: 468-476.

Selvaraj, V., K. Sampath & V. Sekar. 2005. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. Fish Shellfish Immunol., 19: 293-306.

Selvaraj, V., K. Sampath & V. Sekar. 2006. Adjuvant and immunostimulatory effects of β-glucan administration in combination with lipopoly-saccharide enhances survival and some immune parameters in carp challenged with *Aeromonas hydrophila*. Vet. Immunol. Immuno-pathol., 114: 15-24.

Tafalla, C. & B. Novoa. 2000. Requirements for nitric oxide production by turbot (*Scophthalmus maximus*) head kidney macrophages. Develop. Comp. Immun., 24: 623-631.

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Tsoni, V. & G. Brown. 2008. β-Glucans and Dectin-1. Ann. New York Acad. Sci., 1143: 45-60.

Vazzana, M., D. Parrinello & M. Cammarata. 2003. Chemiluminescence response of glucan stimulated leukocytes isolated from different tissues and peritoneal cavity of *Dicentrarchus labrax*. Fish Shellfish Immunol., 14: 423-434.