



# Immunomodulatory effect of cathelicidins in response to a $\beta$ -glucan in intestinal epithelial cells from rainbow trout

Paulina Schmitt <sup>a,\*</sup>, Jurij Wacyk <sup>b</sup>, Byron Morales-Lange <sup>a</sup>, Verónica Rojas <sup>a</sup>, Fanny Guzmán <sup>c</sup>, Brian Dixon <sup>d</sup>, Luis Mercado <sup>a,c</sup>

<sup>a</sup> Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

<sup>b</sup> Laboratorio de Biotecnología en Acuicultura (LBA), Facultad de Ciencias Agronómicas, Departamento de Producción Animal, Universidad de Chile, Santiago, Chile

<sup>c</sup> Núcleo Biotecnológico de Curauma (NBC), Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

<sup>d</sup> Department of Biology, University of Waterloo, 200 University Ave W., Waterloo, Ontario N2L 3G1, Canada

## ARTICLE INFO

### Article history:

Received 16 December 2014

Revised 16 March 2015

Accepted 17 March 2015

Available online 26 March 2015

### Keywords:

Mucosal immunity  
Host defense peptides  
Intestinal epithelial cells  
Fish IL-1 $\beta$   
Immunostimulation

## ABSTRACT

The aim of the present study was to characterize intestinal immune mechanisms involved in the response to  $\beta$ -glucans in rainbow trout. Among the immune effectors regulated in response to immunostimulants, host defense peptides (HDPs) are abundantly expressed in epithelial linings, suggesting their important role in the mucosal immune response. Therefore, the immunomodulatory properties of expressed HDPs in the epithelial intestinal cells of rainbow trout in response to the  $\beta$ -glucan, zymosan, were assessed. The results showed that zymosan increased the production of the HDP, cathelicidin, and the cytokine, IL-1 $\beta$ , in the intestinal epithelial RTgutGC cell line at the transcript and protein levels. Thus, cathelicidin-2 variants were produced and were shown to (i) induce the production of IL-1 $\beta$  in RTgutGC cells and (ii) display a synergic effect with zymosan in IL-1 $\beta$  upregulation. Importantly, the colocalization of both rtCATH-2 and IL-1 $\beta$  was detected in the intestinal epithelial cells of rainbow trout fed with a 0.3% zymosan-supplemented diet. We propose that trout cathelicidins are expressed by intestinal epithelial cells and exert immunomodulatory effects to improve the local intestinal immune response triggered by immunostimulants.

© 2015 Elsevier Ltd. All rights reserved.

## 1. Introduction

The inclusion of immunostimulants in the diet is considered one of the major strategies to increase disease resistance in fish. In this regard, polysaccharides such as  $\beta$ -glucans are one of the major microbe-associated molecular patterns (MAMPs) used in immunostimulant enriched diets to date (Bricknell and Dalmo, 2005).  $\beta$ -Glucan-supplemented diets have been shown to increase cellular and humoral immune responses in immune cells and epithelial tissues from different fish species (Chettri et al., 2012; Kühlwein et al., 2013; Lokesh et al., 2012; Skov et al., 2012). Several studies have reported enhanced resistance to a number of pathogens following  $\beta$ -glucan oral administration (Falco et al., 2012; Kiron, 2012; Palić et al., 2006). Nevertheless, very little is known about the mechanism behind these  $\beta$ -glucan effects on fish intestine.

The mucosal immune system in fish is in direct contact with the external environment and represents one of the primary barriers against pathogens. In higher vertebrates, intestinal epithelial cells (IECs) function as both physical barrier and immunocompetent cells producing a number of immune related molecules such as cytokines and host defense peptides (Gallo and Hooper, 2012). It is well established that teleost fish IECs produce a number of innate humoral defense factors (Gomez et al., 2013; Lazado and Caipang, 2014) and that they are also capable of overexpressing proinflammatory cytokines like IL-1 $\beta$  and TNF- $\alpha$  following a bacterial infection, suggesting their active contribution to the fish mucosal immune system (Komatsu et al., 2009; Mulder et al., 2007).

Among the immune-related molecules expressed by higher vertebrates in response to immunostimulants, host defense peptides (HDPs) are recognized as fundamental humoral effectors of the innate response. They are a diverse group of peptides divided into different classes based on their amino acid composition and structure. Essentially, HDPs, also known as antimicrobial peptides (AMPs), are present in virtually all organisms, where they display diverse roles in immunity (reviewed in Bulet et al., 2004). One of the most recognized roles is microbicidal activity, but there is new evidence that HDPs also display immunomodulatory functions such as chemotactic

\* Corresponding author. Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile. Tel.: +56 32 2274868; fax: +569322274837.

E-mail address: [paulina.schmitt@ucv.cl](mailto:paulina.schmitt@ucv.cl) (P. Schmitt).

activity, induction and/or inhibition of cytokine production and wound healing, among others (reviewed in [Hilchie et al., 2013](#)).

Vast evidence on the essential role of HDPs in epithelial immune responses is found in higher vertebrates ([Brogden et al., 2013](#)). In teleosts, HDPs are generally found in mucosal linings such as skin, gills and intestine, suggesting an important role in mucosal immunity ([Masso-Silva and Diamond, 2014](#); [Rakers et al., 2013](#)). However, fish HDPs have been poorly considered as potential immunological markers in response to immunostimulants. Furthermore, only a small number of studies describe their immunomodulatory properties ([Bridle et al., 2011](#); [Peter Chiou et al., 2006](#)). Several HDP families, comprising a number of variants such as cathelicidins (rtCATH-1, rtCATH-2A and rtCATHs-2B) ([Chang et al., 2006](#)) and  $\beta$ -defensins (omDB-1 to -4) ([Casadei et al., 2009](#); [Falco et al., 2008](#)), have been identified in epithelial tissues from rainbow trout. However, it is not yet clear whether these HDPs are produced by the epithelial tissues, by infiltrating phagocytes or by other cell types. Therefore, the aim of the present study was to assess the immunomodulatory properties of HDPs expressed in response to the  $\beta$ -glucan, zymosan, on the IECs in rainbow trout, *Oncorhynchus mykiss*. Significant overexpression of cathelicidins and the cytokine IL-1 $\beta$  was observed in the trout intestinal epithelial cell line RTgutGC in response to zymosan at both the transcript and protein level. Thus, rtCATH-2 peptide variants (2A and 2B) were produced and functionally characterized. The results show that both variants (i) display strong bactericidal effects against Gram-positive and Gram-negative bacteria; (ii) induce the expression of IL-1 $\beta$  in RTgutGC cells; and (iii) display a synergic effect with zymosan in IL-1 $\beta$  upregulation. The transcriptional profile observed in RTgutGC cells in response to zymosan was similar in the intestine of rainbow trout fed with a zymosan-supplemented diet. Finally, the colocalization of both rtCATH-2 and IL-1 $\beta$  in IECs from the proximal intestine supports the hypothesis that trout cathelicidins could act as immunomodulators in the local intestinal immune response triggered by immunostimulants.

## 2. Materials and methods

### 2.1. Cell culture and treatments

RTgutGC cells from rainbow trout intestinal epithelium were provided by Dr. Niels Bols (University of Waterloo, Canada) ([Kawano et al., 2011](#)). Cells were cultured and maintained in L-15 medium with glutamine (Life Technologies), supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (50  $\mu$ g/mL). Cells were harvested by trypsin treatment (0.05% trypsin, 0.02% EDTA in phosphate buffered saline (PBS), pH 7.4). Fully supplemented L-15 was then added and the cells were collected by centrifugation at  $300 \times g$  at 20 °C for 5 min. For the purpose of gene expression analysis,  $5 \times 10^5$  RTgutGC cells were cultured in 500  $\mu$ L of fully supplemented L-15 medium in 12-well culture plates at 20 °C for 16 h. After overnight plating, the cells were washed twice with PBS and induced by adding different concentrations of zymosan A (Sigma) in PBS or by adding trout cathelicidins in 0.01% acetic acid, in half supplemented L-15 medium at 20 °C for 6 h. For the immunofluorescence analysis,  $6 \times 10^4$  RTgutGC cells were cultured in 300  $\mu$ L of fully supplemented L-15 medium in an 8-well Lab-tek Chamber Slide system (Nunc). The stimulation procedure was performed as described earlier. Cell viability was evaluated using the Alamar Blue assay in accordance with the manufacturer's instructions (Life Technologies).

### 2.2. Fish, feeding trial and tissue collection

US National Research Council guidelines for the care and use of laboratory animals were strictly followed during this research ([National Research Council, 2011](#)). The protocol was approved by

the Committee on the Ethics of Animal Experiments of the *Pontificia Universidad Católica de Valparaíso* (PUCV), Chile (Fondecyt No 3130334). Pre-smolt rainbow trout (*O. mykiss*) with an average weight of 20 g were obtained from the Rio Blanco fish farm (Los Andes, Chile) and maintained in 1000 L circular flow-through tanks at  $14 \pm 1$  °C with an average of  $8 \pm 1$  mg/L of dissolved oxygen, under a 12 h/12 h light/dark cycle at the Experimental Laboratory of Aquaculture Curauma, PUCV. The fish were acclimatized for 2 weeks and fed 1% bodyweight per day during this period with the control diet before the experiment. The fish were then haphazardly distributed in 4 tanks: 2 control groups and 2 fed with a 0.3% zymosan-supplemented diet. Three fish from each tank were sampled at 1, 7, 14, 21, and 28 days and no mortality was observed either in the controls or in the treated fish. The fish were fed twice a day at a rate of 3% body weight per day during a 4-week period. The control group was fed with a diet without zymosan during the entire experimental period. After euthanizing the fish by benzocaine overdose, the complete intestine was dissected under sterile conditions. Pieces were cut from the proximal and distal regions (3 mm) and fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid) and the remaining sample was immediately conserved in liquid nitrogen. The frozen sample was then individually ground with a mortar and pestle in liquid nitrogen and the resulting frozen powder was placed in cryogenic tubes to be stored at -80 °C until RNA extraction.

### 2.3. Diets

US National Research Council guidelines ([National Research Council, 2011](#)) were followed regarding trout requirements for diet formulation. All ingredients were selected to minimize alterations associated with inflammatory responses in the intestines of the fish. Both experimental diets were manufactured under the same conditions. All ingredients were ground (Waring Commercial Lab Mill) and sieved (Retsch, AS 200, <200  $\mu$ m) before mixing (VFM-10 Food Mixer). All micronutrients (vitamins and minerals) were mixed by hand *a priori* and then slowly added to the macronutrient ingredients in order to ensure a homogenous mix. For the zymosan-supplemented diet, Zymosan A from *Saccharomyces cerevisiae* (Sigma) was added at a final concentration of 0.3%. A flat matrix pelleter with a 2 mm die was used to pellet the mixture, after which the experimental diets were dried in an oven at 40 °C for 24 hours.

### 2.4. RNA extraction and reverse transcription

Total RNA was extracted from the frozen ground samples using E.Z.N.A.® Total RNA Kit I in accordance with manufacturer's instructions (Omega Biotek). Ten micrograms of the total RNA was treated with 2 units of DNase I (New England Biolabs) in DNase I reaction buffer for 10 min at room temperature and inactivated by heat for 10 min at 75 °C. The quantity and quality of the total RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis, respectively. Following heat denaturation of 1  $\mu$ g of the total RNA (65 °C for 5 min), first strand synthesis was carried out using 50 ng oligo-(dT)12–18 (Invitrogen), 1 mM dNTPs (Promega), 1 U Rnasin (Promega) and 200 U M-MLV reverse transcriptase in reverse transcriptase buffer (Promega) following the manufacturer's protocol.

### 2.5. Real-time quantitative pcr (qPCR) gene expression analysis

The 10  $\mu$ L reaction consisted of 1X Brilliant II SYBR Green QPCR master mix (Stratagene), 0.5  $\mu$ M of each primer and 1  $\mu$ L of cDNA diluted to 1/8 in sterile ultra-pure water. The primers are listed in [Table S1](#). qPCR assays were performed in triplicate in a Biorad C1000 Touch Thermocycler CFX96, and primer pair efficiencies (E) were

calculated from six serial dilutions of pooled cDNA for each primer pair. Primer pair efficiencies were calculated from the given slopes in the BioRad CFX software according to the equation:  $E = 10^{(-1/\text{slope})}$ . The assays were submitted to an initial denaturation step of 10 min at 95 °C followed by an amplification of the target cDNA (40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 5 s and extension time at 72 °C for 15 s) and fluorescence detection. After an initial 10 s denaturation step at 95 °C, a melting curve was obtained from a start temperature of 65 °C to a final temperature of 95 °C, with stepped increases of 0.06 °C/s. Relative expression was calculated using the  $-2^{\Delta\Delta Ct}$  method described by Pfaffl (2001), using the measured threshold cycle (Ct) values of the constitutively expressed gene *EF-1 $\alpha$*  [GenBank AF498320] to normalize the measured Ct values of the target genes. Calculations of means, standard deviations and statistical analysis using the Kruskal–Wallis test for expression analysis were carried out using GraphPad Prism software version 6.01 (significant value:  $p < 0.05$ ). Statistical differences between samples were based on the selection criteria with a cut-off value of >2-fold change over the reference gene ( $p < 0.05$ ).

### 2.6. Recombinant expression and chemical synthesis of rainbow trout cathelicidins

The recombinant rtCATH-2A coding peptide [GenBank AY542963] was expressed in *Escherichia coli* Rosetta (DE3) as the N-terminal His6-tagged fusion protein and produced as previously described for other host defense peptides (Figueredo et al., 2010). The primers are listed in Table S1. The replacement of Met<sub>10</sub> by Leu using site-directed mutagenesis was performed in order to avoid CNBr cleavage. The recombinant peptides were expressed at 37 °C in Luria Bertani broth by induction with 0.1 mM isopropyl- $\beta$ -D-1-thiogalactopyranoside for 6 h at 37 °C and lysed by sonication in 6 M guanidine-HCl in 100 mM Tris-Cl (pH 8.1). The His-tagged fusion peptides were purified using nickel-nitrilotriacetic acid (Ni-NTA, Qiagen) resin affinity chromatography. After cyanogen bromide cleavage, the peptides were purified by C18 reverse-phase high performance liquid chromatography (RP-HPLC). The molecular masses of purified peptides were determined using matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS). Peptide homogeneity was confirmed by acid-urea-polyacrylamide gel electrophoresis (AU-PAGE). Endotoxin presence on the purified peptides was evaluated by the Endpoint chromogenic Limulus amoebocyte lysate (LAL) Assay (Lonza). Chemical synthesis of rtCATH-2B coding peptide [GenBank AY542962] was performed by Fmoc chemistry and purchased from Proteogenix, France. The amino acid sequences of the variants are listed in Fig. 3.

### 2.7. Polyclonal antibodies and western blotting

Polyclonal antibodies were generated against a mix of 3 synthetic antigenic epitopes (ANKEQLKSISQQSD, QKAAPNRLTFTIQRHN and DLLNFLLESAAVEEHI) from a deduced IL-1 $\beta$  coding sequence [GenBank AJ223954] in New Zealand rabbits and the full-length rtCATH-2B synthetic peptide in CF-1 mice (4 weeks old). For antibody production, animals were subcutaneously injected at 1, 7 and 14 days with 150  $\mu$ g immunogen diluted 1:1 in FIS as a T helper cell activator, as previously described (Bethke et al., 2012) and 1:1 in Freund's adjuvant (Thermo). The antiserum was collected on day 18, centrifuged at 700 g for 10 min and the supernatant was stored at -20 °C. Antibody efficiency was determined by indirect ELISA and antibody specificity was determined by Western blot as described previously (Morales-Lange et al., 2014). The synthetic antigenic epitopes of IL-1 $\beta$  were conjugated to the keyhole limpet hemocyanin (KLH) carrier protein (Thermo) to evaluate the antibody specificity by Western blot, in accordance with the manufacturer's protocol. Briefly, protein samples were separated by SDS-PAGE

and transferred onto nitrocellulose membranes (BioRad). The membranes were incubated for 1.5 h with the anti-immunogen antibody (1:250 for the mouse serum and 1:1000 for the rabbit serum) and then incubated with the anti-IgG-HRP antibody 1:7000 (Thermo) for 1 h at 37 °C. Specific bands were visualized by chemiluminescence with the Westar Supernova kit (Cyanagen).

### 2.8. Histological analysis and immunofluorescence

Fixed samples were dehydrated through an ascending ethanol series and embedded in Histosec (Merck). Sections were cut at 6  $\mu$ m using a rotary microtome (Leica RM 2235), and mounted on glass slides. Paraffin sections were cleared in Neoclear (Merck) and hydrated by incubations in a descending ethanol series. The samples were stained with performic acid and Schiff reagent (PAS), a specific stain to detect polysaccharides, and counterstained with hematoxylin (Gabe, 2012). The samples were then examined under a Leica DM5000B microscope equipped with a Leica DFC450C digital camera. The examination was performed on several intestinal sections from different fish to ensure they were consistently reproducible.

Immunofluorescence analysis was carried out as described previously (Schmitt et al., 2012), using polyclonal antibodies specific for rtCATH-2 and IL-1 $\beta$ . Briefly, cells were fixed on glass slides with 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 and blocked for 2 h at room temperature with PBS/3% BSA. The slides were incubated overnight at 4 °C with both anti-rtCATH-2 (1:200) and anti-IL-1 $\beta$  (1:400) antibodies in PBS/1% BSA. A second incubation was performed for 1 h at room temperature with anti-mouse Alexa Fluor 568-conjugated (Invitrogen) and anti-rabbit Alexa Fluor 635-conjugated (Invitrogen) 1:400 in PBS/1% BSA and then incubated for 5 s with Syto 9 1:1000. The slides were then mounted in Vectashield mounting medium (Vector) and analyzed using a Leica TCS SP5 II spectral confocal microscope (Leica Microsystems). Control slides were incubated with the prebleed serum of mouse and rabbit instead the primary antibodies. The images were obtained with a Leica 40  $\times$  1.25 Oil HX PL APO CS lens (Leica Microsystems).

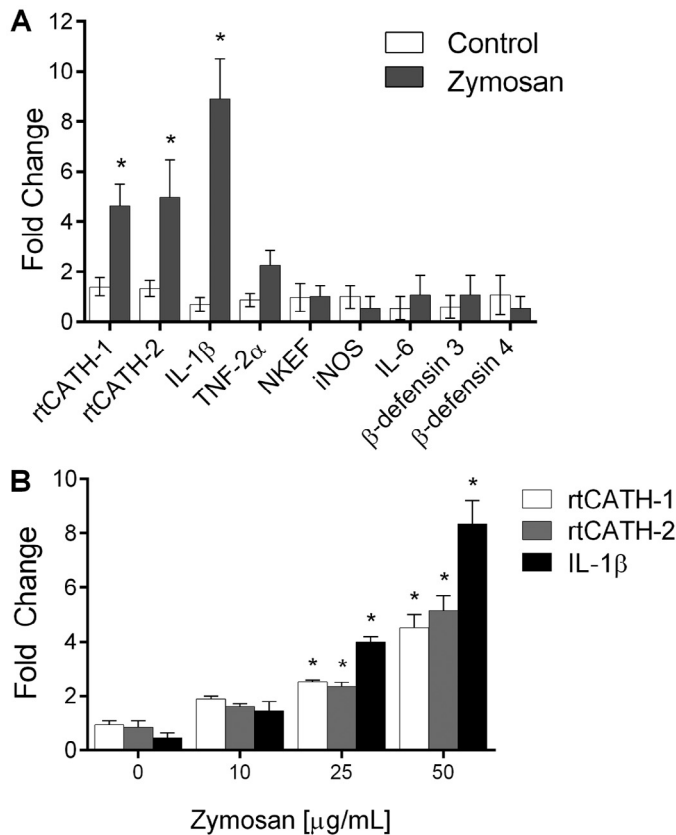
### 2.9. Antimicrobial assays

Recombinant and synthetic peptides were tested for microbicidal activity against the Gram-negative *E. coli* ML35 (kindly donated by Dr. Andre Ouellette), *Salmonella enterica* subsp. Thyphimurium (ATCC 14028), *Vibrio anguillarum* (Chilean Public Health Institute ISP 66/12), and the Gram-positive *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (Chilean Public Institute of Health ISP B7/13). Exponentially growing bacteria ( $2 \times 10^6$  colony forming units/mL (cfu/mL)) were exposed to different peptide concentrations in 50  $\mu$ L of 1% trypticase soy broth (TSB)-10 mM HEPES buffer for 1 h at 37 °C. Following exposure, the bacteria were subjected to ten-fold serial dilutions and incubated for 16 h at 37 °C in complete TSB. Surviving bacteria were quantitated as cfu/mL for each peptide concentration. The human cathelicidin LL-37 (ANASPEC) was used as a positive control.

## 3. Results

### 3.1. Zymosan-induced expression of trout cathelicidins and the cytokine IL-1 $\beta$ in RTgutGC cells

The relative expression of nine immune related genes was assessed using qPCR in the trout intestinal epithelial cell line RTgutGC in response to zymosan. The choice of these immune genes sought to represent diverse aspects of the intestinal immune response that could be detected in fish. Thus, the expression of two HDP families, cathelicidins (variants rtCATH-1 and rtCATH-2) and  $\beta$ -defensins



**Fig. 1.**  $\beta$ -Glucan zymosan induced the expression of trout cathelicidins and IL-1 $\beta$  in RTgutGC cells. Quantitative PCR analysis of the gene expression of A. Nine immune related genes in RTgutGC cells stimulated with zymosan (50  $\mu$ g/mL) for 6 h. B. Cathelicidins (rtCATH-1 and rtCATH-2) and IL-1 $\beta$  in RTgutGC cells stimulated with 10, 25 and 50  $\mu$ g/mL of zymosan. Relative expression was normalized to the gene expression of rainbow trout EF-1 $\alpha$  and calculated using the  $-2^{\Delta\Delta Ct}$  method. Results are expressed as mean values  $\pm$  SD. Asterisks indicate significant differences related to the control ( $p < 0.05$ ).

(variants 3 and 4) and the pro-inflammatory cytokines IL-1 $\beta$  and TNF-2 $\alpha$ , interleukin IL-6, Natural killer enhancing factor (NKEF) and the inducible nitric oxide synthase (iNOS) were evaluated after incubating the cells with 50  $\mu$ g/mL of zymosan for 6 h. The significant increase ( $p < 0.05$ ) in the gene expression of the HDP cathelicidins rtCATH-1 and rtCATH-2 (using universal primers amplifying both variants, rtCATH-2A and rtCATH-2B) and the IL-1 $\beta$  and was observed when compared to the control treatment (Fig. 1A). The Tumor Necrosis Factor, TNF-2 $\alpha$ , was also upregulated in response to zymosan but to a lesser extent ( $p > 0.05$ ). Other selected genes such as the HDP  $\beta$ -defensin-3 and -4, IL-6, iNOS, or NKEF did not show any significant increase in cells treated with the  $\beta$ -glucan at the time tested. Considering the multifunctional properties of vertebrate cathelicidins (Zanetti, 2004) and the key role of the proinflammatory IL-1 $\beta$  in fish (Secombes et al., 2011), the gene expression changes of these molecules in response to increased concentrations of zymosan were further monitored. The upregulation of rtCATH-1, rtCATH-2 and IL-1 $\beta$  transcription in a dose-dependent manner was detected in the RTgutGC cells, indicating that the zymosan effects are concentration dependent (Fig. 1B). The relative expression of rtCATH-1, rtCATH-2 and IL-1 $\beta$  increased approximately between five- to eight-fold when cells were treated with 50  $\mu$ g/mL of zymosan. No cell mortality was detected in treated or control cells using the Alamar Blue assay (Fig. S1).

The production of trout cathelicidins and IL-1 $\beta$  in RTgutGC cells in response to zymosan was also evaluated at the protein level using

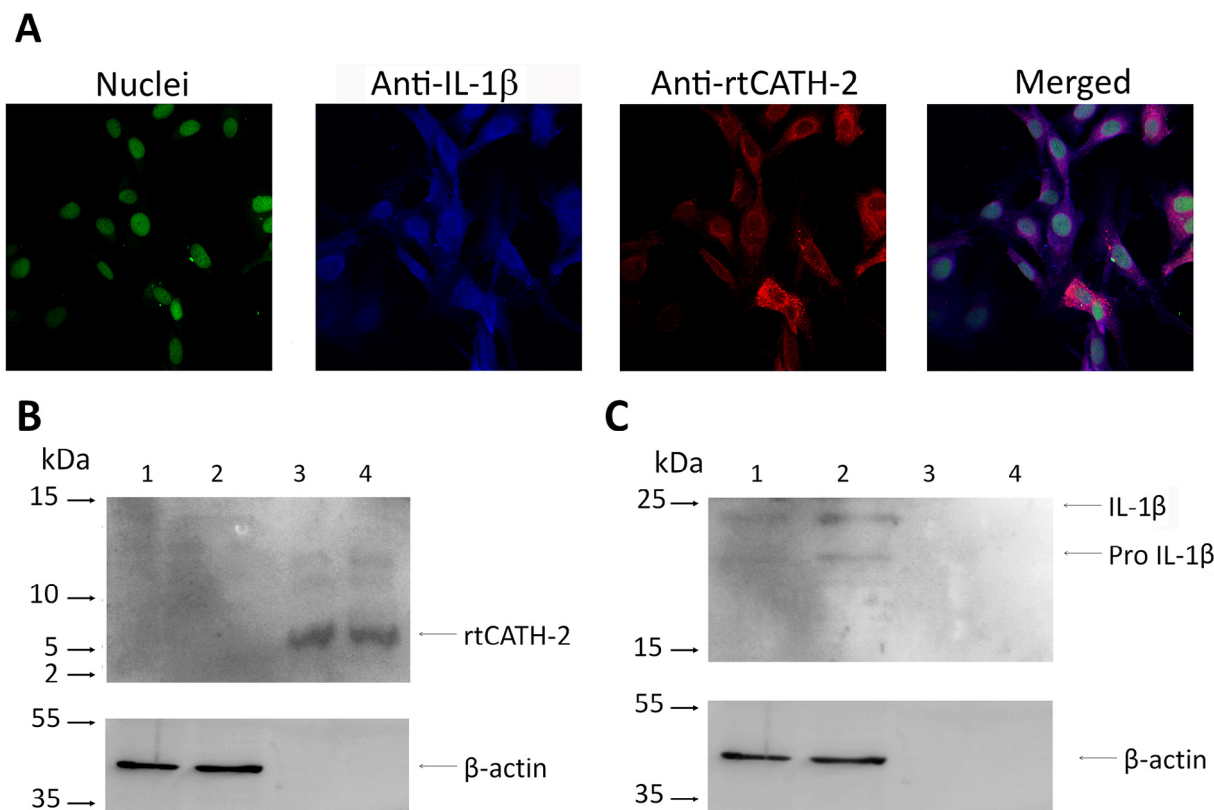
an anti-rtCATH-2 mouse antiserum and anti-IL-1 $\beta$  rabbit antiserum, respectively. The rtCATH-2 antiserum was able to recognize both rtCATH-2A and -2B variants, as determined by Western blot (Fig. S2). Immunofluorescence confirmed that RTgutGC cells produce rtCATH-2 and IL-1 $\beta$  and in response to 50  $\mu$ g/mL of zymosan (Fig. 2A). Confocal images revealed the presence of rtCATH-2 within all cells, showing a strong granular-like staining. Interestingly, rtCATH-2-positive cells were also positive for anti-IL-1 $\beta$  staining. Merged images indicate the colocalization of both rtCATH-2 and IL-1 $\beta$  inside the cells, while control cells treated with PBS buffer did not show any positive staining (data not shown). Furthermore, the precursor and the active forms of IL-1 $\beta$  were detected in cell protein extracts using Western blotting (Fig. 2B), while cathelicidin detection was stronger in the cell culture media, indicating the secretion of this HDP to the extracellular environment (Fig. 2C). The colocalization of the two molecules in RTgutGC cells and the immunomodulatory properties described for several HDPs (Hilchie et al., 2013) prompted the investigation of the multifunctional activities of the trout cathelicidins.

### 3.2. The rtCATH-2 variants display microbicidal activity against gram-positive and gram-negative bacteria

In contrast to rtCATH-1, rtCATH-2 was previously shown to be constitutively expressed in healthy trout and specifically induced in the intestine during bacterial infection (Chang et al., 2006). Thus, the full-length variants of the two predicted isoforms of rtCATH-2 were synthesized or expressed as recombinant peptides in *E. coli* to investigate their functional bioactivity. Circular dichroism analysis reveals a  $\beta$ -turn structure for the rtCATH-2A variant, while rtCATH-2B appeared to display a non-structured random coil (Fig. S3). The antimicrobial properties of these peptides were evaluated by the liquid growth bactericidal assay. Minimal bactericidal concentration (MBC) results show that both cathelicidins display a similar spectrum of activity, with activity seen against both Gram-positive and Gram-negative bacteria (Fig. 3). The rtCATH-2B variant showed slightly better activity against Gram-negative bacteria, approximately two-fold compared to rtCATH-2A and closer to the MBC values observed for the human cathelicidin LL-37 (10–20  $\mu$ M). Interestingly, the rtCATH-2 variants were active against the human pathogens *Salmonella typhimurium* or *Staphylococcus aureus*, but did not display significant microbicidal activity (40  $\mu$ M and above) against the salmonid pathogen *Vibrio anguillarum* (Fig. S4). The availability of biologically active cathelicidin variants prompted an evaluation of whether rtCATH-2 could also display an immunomodulatory effect.

### 3.3. Rainbow trout cathelicidins exerted an immunomodulatory effect on RTgutGC cells

In order to identify any immunomodulatory activity of rtCATH-2, RTgutGC cells were treated with the full-length variants of trout cathelicidins separately for 6 h, and the expression of IL-1 $\beta$  was assessed by qPCR (Fig. 4A). A significant increase in the transcription of IL-1 $\beta$  was observed in treated cells. The relative expression of IL-1 $\beta$  increased up to seven-fold when cells were treated with 10  $\mu$ M of rtCATH-2, while no changes in the relative expression of IL-1 $\beta$  was observed in control cells treated with a peptide vehicle. Moreover, the upregulation of IL-1 $\beta$  increased in a concentration dependent manner in response to cathelicidins, ranging from 0 to 10  $\mu$ M. These results showed that IL-1 $\beta$  transcription is upregulated by both rtCATH-2 variants in RTgutGC cells. In order to analyze the effect of rainbow trout cathelicidins on zymosan-induced gene expression, the levels of IL-1 $\beta$  in cells treated with zymosan in the presence or absence of rtCATH-2 variants was evaluated. The induction of IL-1 $\beta$  by zymosan increased to a greater extent in the



**Fig. 2.** Zymosan induced the production of rtCATH-2 and IL-1 $\beta$  in RTgutGC cells. A. Immunofluorescence analysis on cells stimulated with zymosan (50  $\mu$ g/mL) for 6 h. Syto 9 was used as the nucleic acid stain, Alexa 568 goat anti-mouse for rtCATH-2 detection and Alexa 635 goat anti-rabbit for IL-1 $\beta$  detection. Immunostained cells were photographed under confocal microscopy. Scale bar, 25  $\mu$ m. B. Western blot analysis for rtCATH-2 (B) and IL-1 $\beta$  (C) detection in protein extracts from control cells (1), zymosan-treated cells (2); and conditioned media from control cells (3) and zymosan-treated cells (4).

cells treated in the presence of the peptides, compared to the treatment with zymosan alone ( $p < 0.05$ ) (Fig. 4B). This result showed that there is a synergic IL-1 $\beta$ -inducing effect from zymosan and rtCATH-2 variants in RTgutGC cells.

The immunomodulatory effect of rainbow trout cathelicidins at the protein level was then examined. Only the rtCATH-2B synthetic variant was used in subsequent assays to avoid any problems associated with IL-1 $\beta$  induction by endotoxin contamination from the recombinant peptides. Thus, RTgutGC cells were incubated with rtCATH-2B for 6 h and the expression of IL-1 $\beta$  and of rtCATH-2 was evaluated by immunofluorescence. Confocal analysis not only confirmed the production of IL-1 $\beta$  in RTgutGC cells in response to rtCATH-2B but also revealed self-induction (Fig. 4C). Confocal images showed the presence of rtCATH-2 and IL-1 $\beta$  within the cells and merged images showed the colocalization of both molecules. Strong fluorescent immunolabeling for both molecules was observed in the cells; it was also found to be uniformly distributed throughout the cytoplasm. When the cells were treated with PBS or the peptide vehicle, no expression of rtCATH-2 or IL-1 $\beta$  was detected.

#### 3.4. Differential expression of immune related genes observed in the intestine of rainbow trout fed with 0.3% zymosan-supplemented diet

Results from the *in vitro* analysis led us to investigate the effects of zymosan on the mucosal immune response of the rainbow trout through the oral administration of this  $\beta$ -glucan. The expression of the chosen immune related genes was evaluated for the whole fish intestine during a 4-week feeding trial with a 0.3% zymosan-supplemented diet (Fig. 5). The results from the two biological replicates from treated fish, as well as from the control fish, did not

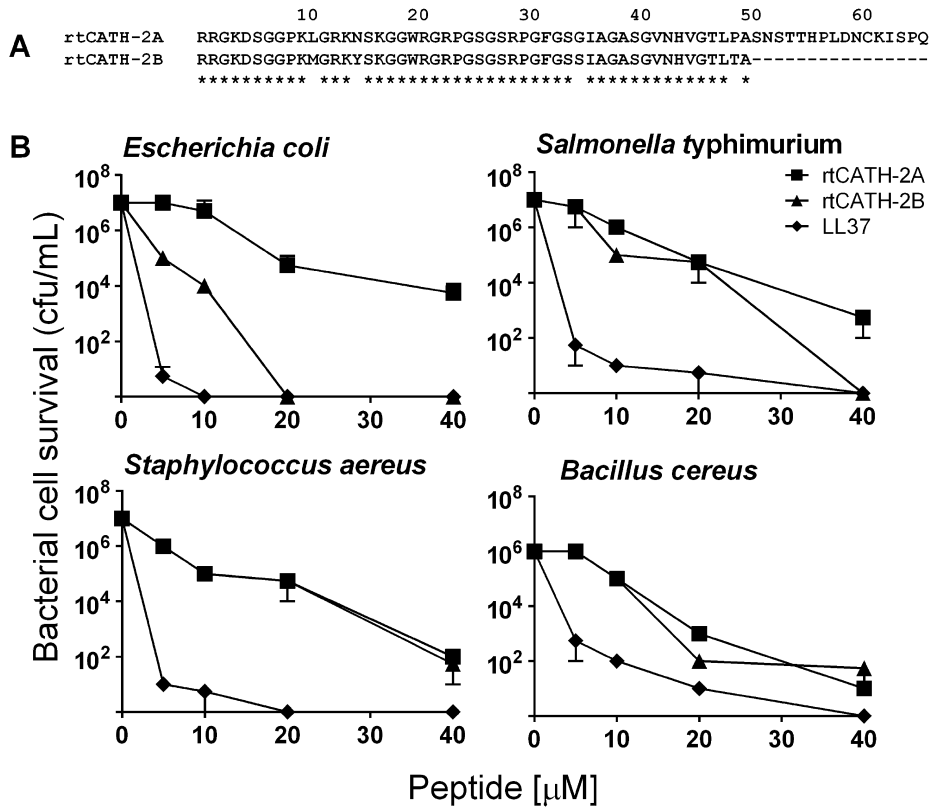
vary significantly and no differences in the growth performance were observed during the trial between treated and control fish (Fig. S5).

The results show that in response to the zymosan the expression of both trout cathelicidins and IL-1 $\beta$  was observed *in vivo*. The expression of rtCATH-1, rt-CATH-2 and IL-1 $\beta$  was significantly increased in the whole fish intestine after 3 weeks of feeding with the zymosan-supplemented diet. At day 28, the expression of rtCATH-1, rtCATH-2 and IL-1 $\beta$  increased between 4- and 6-fold in the intestine of the trout fed with the zymosan-supplemented diet, while no effect was observed in the intestine of fish fed with the control diet (Fig. 5). No significant changes were observed in the other analyzed genes, remaining at a low basal level during the 4-week trial.

#### 3.5. Zymosan induced the expression of rtCATH-2 and IL-1 $\beta$ in epithelial cells and increased the number of mucus-secreting cells from the rainbow trout intestine

Since significantly increased gene expression of cathelicidins and IL-1 $\beta$  was observed in the whole intestine from the treated fish, further investigation was initiated to determine whether this was restricted to a specific portion of the organ. Thus, histological analysis was performed and protein expression of cathelicidins and IL-1 $\beta$  was determined by immunofluorescence on the proximal and distal intestine separately at day 28 of the feeding trial.

Histological analysis using PAS staining showed differences in the intestine between the control and treated fish (Fig. 6, upper panels). An increased number of mucus-secreting cells interspersed between the epithelial cells were observed in the proximal intestine of the treated fish (Fig. 6B), which is not evident in the distal



**Fig. 3.** Trout cathelicidins display strong microbicidal activity against Gram-positive and Gram-negative bacteria. **A.** Amino acid alignment of rtCATH-2 trout cathelicidin variants. **B.** Antimicrobial activity of trout cathelicidins against Gram-positive and Gram-negative bacteria. The bactericidal effect is reported as a reduction in bacterial cell survival as a function of peptide concentration. Surviving bacteria are expressed as cfu/mL for each peptide concentration. Human cathelicidin LL37 was used as a positive control.

portion of the intestine (Fig. 6D). Positive reactions with PAS revealed the presence of mucine, glycogen, and/or neutral mucopolysaccharides.

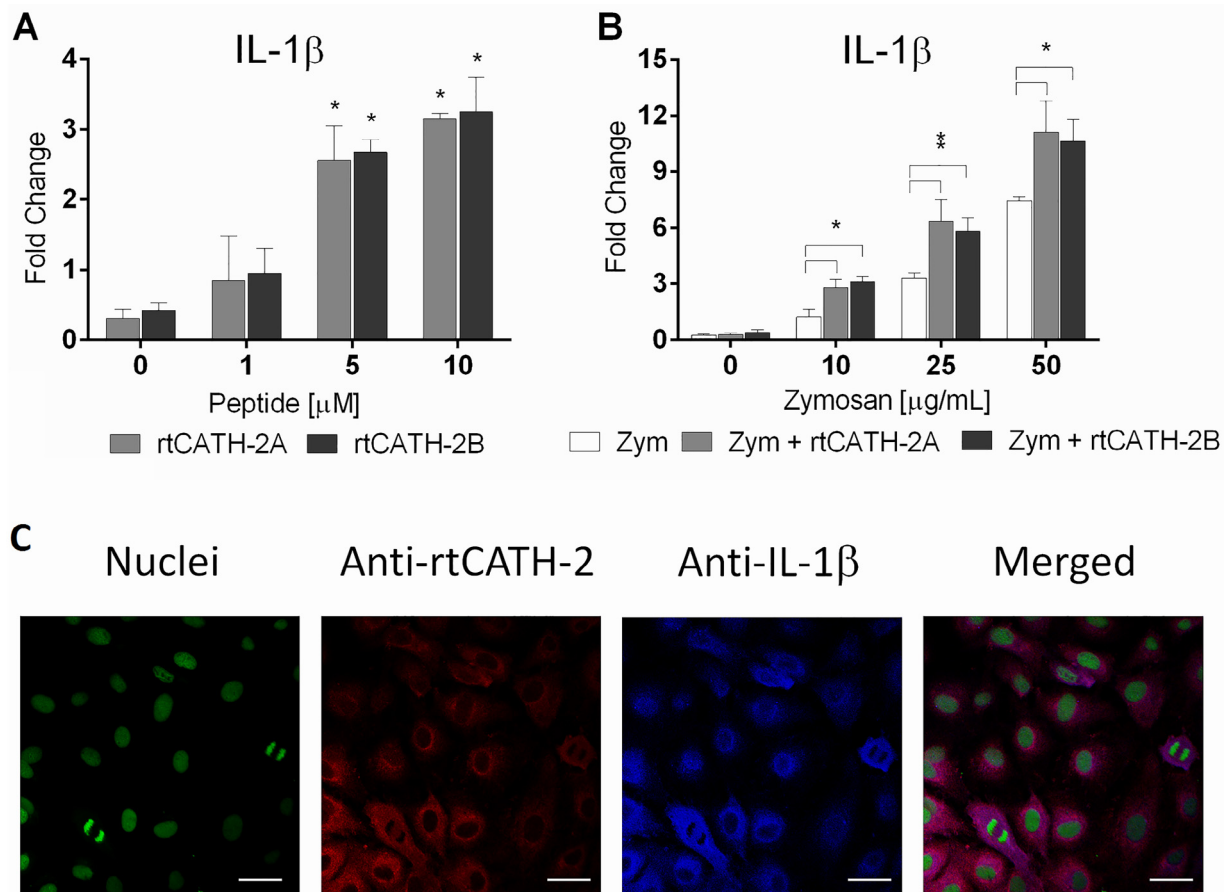
The immunodetection of rt-CATH-2 and IL-1 $\beta$  also revealed differences between intestinal portions (Fig. 6, lower panels). Immunofluorescence analysis showed a high number of cathelicidin-expressing IECs only in the proximal intestine from the treated fish. The immune reactivity of positive IECs expressing rtCATH-2 was abundant, distributed around the perinuclear region of the cells with strong granular-like staining. Furthermore, some IECs were expressing cathelicidins together with IL-1 $\beta$ . No expression of these molecules was detected in the control fish (Fig. 6E), while merged confocal images showed that rtCATH-2 and IL-1 $\beta$  fluorescence signals overlapped at times, demonstrating cellular colocalization (Fig. 6F). Positive rtCATH-2 staining was also observed in cells infiltrating the lamina propria as well as the epithelium in the proximal intestine of the treated fish (Fig. 6F). In contrast, IECs from the distal intestine showed constitutive expression of cathelicidins in both treated and control fish, with no detection of IL-1 $\beta$  (Fig. 6G, H). Interestingly, all samples from the distal intestine of the treated fish display cytoplasmic granule-like staining, which is yet to be clarified. Taken together, results from the *in vivo* approach showed increased colocalized production of rtCATH-2 and IL-1 $\beta$  and an increase in the number of mucus-secreting cells in the proximal IECs of the rainbow trout in response to a zymosan-supplemented diet.

**4. Discussion**

An essential tool for gaining new insight into the mucosal immune response in fish is the availability of well characterized *in*

*vitro* system models. Currently, several immortalized cells derived from various epithelial lining tissues are available for studying immune gene expression in trout (Bols et al., 1994; Kawano et al., 2011). Recently, the RTgutGC cell line from the intestinal epithelia of *Oncorhynchus mykiss* was shown to express several immune related molecules, such as TNF- $\alpha$  (Kawano et al., 2011), tapasin (Sever et al., 2014), MHC class I alpha chain and beta-2-microglobulin (Kawano et al., 2010), suggesting behavior comparable to IECs. Therefore, RTgutGC cells were considered a representative model that may be used to address the *in vitro* effect of zymosan in the intestinal immune response of the rainbow trout. Previously, trout cathelicidins and IL-1 $\beta$  and have been reported to be expressed in other rainbow trout cell lines in response to the fungus *Saprolegnia parasitica* (de Bruijn et al., 2012) but this is the first time these molecules have been detected in the RTgutGC cell line. Thus, IL-1 $\beta$  was considered for further study due to its earlier expression and central role in mediating proinflammatory responses (Secombes et al., 2011). In the present study, other analyzed genes were not modulated by zymosan, but the possibility of their modulation at different time points or higher zymosan concentrations could not be ruled out. There was in fact a slight increase in the expression of TNF-2 $\alpha$ , which was shown to be expressed in RTgutGC cells in response to LPS after 24 h (Kawano et al., 2011).

The *in vitro* microbicidal properties of full-length rtCATH-2 variants were characterized in order to obtain evidence of their previously known biological functionality and then, as potential immunomodulators. Results from the antimicrobial tests were consistent with previous data on fish cathelicidins (Bridle et al., 2011; Chang et al., 2006). Surprisingly, full-length cathelicidins showed no activity against *Vibrio anguillarum* up to 40  $\mu$ M, whereas truncated



**Fig. 4.** Trout cathelicidins induced the expression of IL-1 $\beta$  in RTgutGC cells and display synergy with zymosan. Quantitative PCR analysis of the gene expression of IL-1 $\beta$  in A. RTgutGC cells treated with 1, 5 and 10  $\mu$ M of rtCATH-2A (light gray bars) and rtCATH-2B (dark gray bars) for 6 h. B. RTgutGC cells treated with 10, 25 and 50  $\mu$ g/mL of zymosan only (white bars) or in the presence of 5  $\mu$ M of rtCATH-2A (light gray bars) or rtCATH-2B (dark gray bars) for 6 h. Relative expression was normalized to the gene expression of rainbow trout EF-1 $\alpha$  and calculated using the  $-2^{\Delta\Delta C_t}$  method. Results are expressed as mean values  $\pm$  SD. Asterisks indicate significant differences ( $p < 0.05$ ) in comparison to the control (A) or to the zymosan treatment alone. C. Immunofluorescence analysis on cells stimulated with 5  $\mu$ M of rtCATH-2B for 6 h. Syto 9 was used as the nucleic acid stain, Alexa 568 goat anti-mouse for rtCATH-2 detection and Alexa 635 goat anti-rabbit for IL-1 $\beta$  detection. Immunostained cells were photographed under confocal microscopy. Scale bar, 25  $\mu$ m.

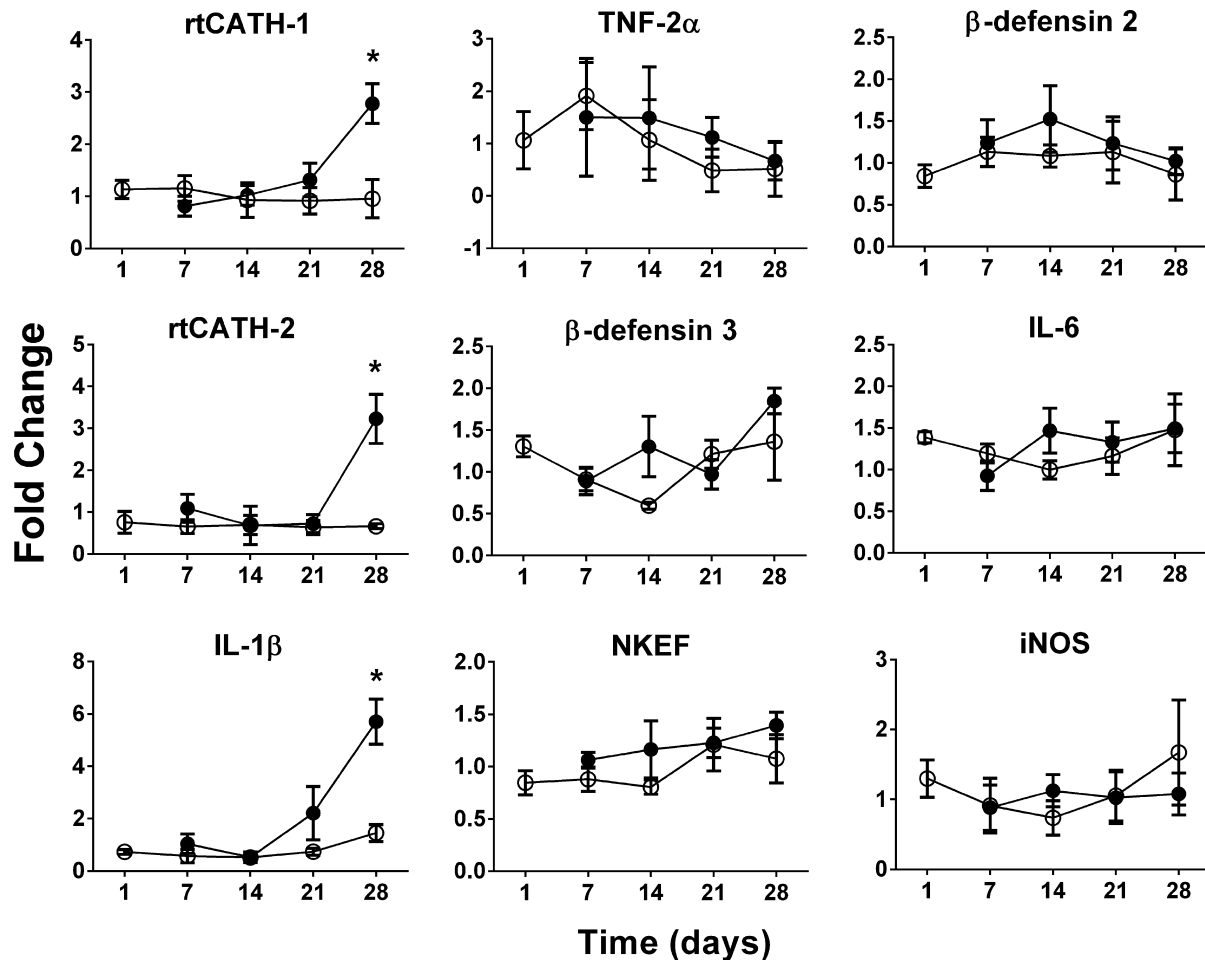
variants of salmon and trout cathelicidins showed bactericidal activity against this fish pathogen (Bridle et al., 2011; Chang et al., 2006). This discrepancy with the previously reported activity could be due to the different lengths of the variants as well as the specific strain of the tested pathogen. Indeed, cathelicidins from other fish species and even between variants from the same species showed significant variability regarding *in vitro* antibacterial activity, probably due to the high sequence diversity within this group of effector molecules (Scocchi et al., 2009).

In vertebrates, cathelicidins are among the HDPs that have been described as immunomodulators, acting in both innate and adaptive immune responses. Their roles include both proinflammatory and anti-inflammatory effects depending on the agonist (Hilchie et al., 2013). In this study rtCATH-2 variants display upregulation of IL-1 $\beta$  in trout RTgutGC cells and a synergic effect when tested together with zymosan. Proinflammatory effects of cathelicidins have been described previously, such as chicken fowlicidin-1, which induces IL-1 $\beta$  in RAW-264.7 cells (Bommineni et al., 2014). Additive effects between proinflammatory mediators have also been reported previously for cathelicidins (Zheng et al., 2007). Although most of the known immunomodulatory activity of HDPs has been characterized in mammals, there is some evidence from fish pointing in a similar direction. Atlantic salmon cathelicidins (asCATHs) upregulate the expression of IL-8 in peripheral blood leukocytes, but not the expression of IL-1 $\beta$  or IL-18, suggesting that

salmon cathelicidins could act as a chemokine (Bridle et al., 2011). Remarkably, the lack of induction of IL-1 $\beta$  by asCATHs in salmon leukocytes suggests the existence of different mechanisms involved in the immunomodulatory processes for distinct cell populations, such as between macrophages and enterocytes or even between the same cell types at different locations. Indeed, differences in the phagocytic activity and natural cytotoxic activity have been described between intestinal leukocytes and head kidney or peripheral blood leukocytes from rainbow trout (Martin et al., 2012).

Several cytokines are involved in the induction of trout cathelicidins. Gene expression of trout cathelicidins has been observed after induction with recombinant rIL-6 in the macrophage-like RTS-11 cell line (Costa et al., 2011), by rIL-1B in RTL-W1 trout liver cells (de Bruijn et al., 2012) and by rTNF-3 $\alpha$  in both RTS-11 and RTL-W1 cells (Hong et al., 2013). Altogether, the results presented here suggest some feed forward mechanisms occurring between fish cytokines and HDPs, as observed in higher vertebrates (Zheng et al., 2007). For instance, the inflammatory mediator produced by leukocytes, leukotriene B<sub>4</sub>, triggers LL-37 release from human neutrophils and in turn LL-37 promotes leukotriene B<sub>4</sub> production in these cells (Wan et al., 2011). Future research must be oriented toward elucidating the mechanisms underlying these regulation processes in fish.

HDPs produced by epithelial cells are stored in cytoplasmic granules and released into the extracellular environment (Gallo and



**Fig. 5.** Induced expression of trout cathelicidins and IL-1 $\beta$  in the intestine of rainbow trout fed with 0.3% zymosan-supplemented diet. Quantitative PCR analysis of the gene expression of several immune related genes in the intestine of the rainbow trout during an oral immunostimulation with 0.3% zymosan-supplemented diet for 28 days. Relative expression was normalized to the gene expression of rainbow trout EF-1 $\alpha$  and calculated using the  $-2^{\Delta\Delta Ct}$  method. Results are expressed as mean values  $\pm$  SD. Open circles, fish fed with control diet. Black circles, fish fed with zymosan-supplemented diet. Asterisks indicate significant differences related to the control ( $p < 0.05$ ).

Hooper, 2012). Hence, the immunolabeling for rtCATHs in cytoplasmic granules of RTgutGC cells and IECs, together with the *in vitro* release of the peptides to the extracellular media observed by Western blot, strongly evidence rainbow trout IECs as HDP producers.

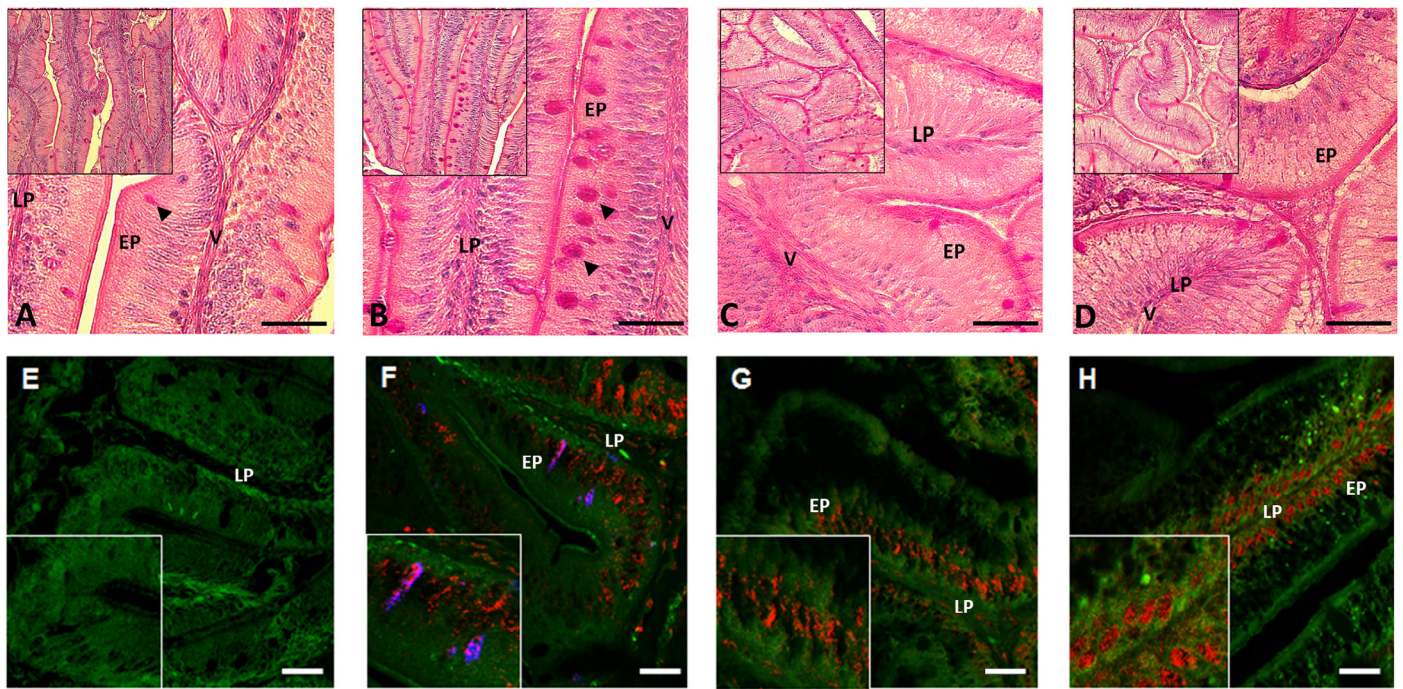
This study also showed that feeding a 0.3% zymosan-supplement diet to rainbow trout for 4 weeks increased the number of mucus-secreting cells and induced the expression of immune genes, notably, trout cathelicidins and IL-1 $\beta$ . In addition, it reveals that IECs from rainbow trout differentially express these molecules depending on the intestinal segment analyzed, as observed for IL-1 $\beta$  in the intestine of Atlantic cod fed with  $\beta$ -glucan (Lokesh et al., 2012). The overexpression of certain immune genes and the increased number of mucus-secreting cells only in the proximal intestine could be related to a higher absorption capacity of this portion of the intestine (Halver and Hardy, 2002). It could also be associated to regional specialization within the intestinal immune system as described in higher vertebrates (Mowat and Agace, 2014).

The colocalization of cathelicidins and IL-1 $\beta$  in the IECs from the treated fish supports the hypothesis of the potential immunomodulatory properties of these HDPs in the intestine of the rainbow trout. Furthermore, cathelicidins concentration could be increased in the fish intestine due to rtCATH-expressing immune cells, as suggested by the positive staining observed in cells infiltrating the lamina propria and intestine epithelium of the treated

fish. These cells could be related to eosinophilic granular cells (EGCs), which are found in epithelial linings and are known to express HDPs in teleosts (reviewed in Esteban, 2012). It may also be noted that previous studies have analyzed the expression of these genes by qPCR using samples from the complete organ, though failing to identify the cell type involved in the expression (Casadei et al., 2013). Thus, the production of HDPs and cytokines by rainbow trout IECs contributes to current knowledge on intestinal immune response in salmonids.

Numerous studies have expanded our understanding of the effect of  $\beta$ -glucan in fish diets. However, assessment of the literature shows that  $\beta$ -glucans may mediate both proinflammatory (Lokesh et al., 2012) or anti-inflammatory (Skov et al., 2012) effects after a bacterial challenge in a pattern that is not consistent or predictive, possibly depending on the composition of the  $\beta$ -glucan, the dosage and time of exposure. In this study, the significant increase in the expression of cathelicidins and IL-1 $\beta$  in the intestine only occurred at the last experimental time point and the induction of other genes at later times cannot be ruled out. Nevertheless, one of the purposes of an immunostimulant diet is to circumvent a general and sustained induction of the immune response, which may be detrimental to the organism in the long term. Thus, care must be taken when evaluating these kinds of functional diets and overdosing must be avoided.





**Fig. 6.** Zymosan-supplemented diet induced the expression of rtCATH-2 and IL-1 $\beta$  and increases the number of mucus-secreting cells. Upper panels, histological analysis of proximal (A, B) and distal intestine (C, D) of the rainbow trout fed with the control diet (A, C) and the zymosan-supplemented diet (B, D) for 28 days, stained with PAS-hematoxylin. Arrow shows Goblet cells (magnification 400 $\times$ , insert 200 $\times$ ). Lower panels, merged confocal images for detection of rtCATH-2 and IL-1 $\beta$  in proximal (E, F) and distal intestine (G, H) of the rainbow trout fed with the control diet (E, G) and the zymosan-supplemented diet (F, H) for 28 days by immunofluorescence (magnification 400 $\times$ , insert 800 $\times$ ). In red, rtCATH-2 detection. In blue, IL-1 $\beta$  detection. In pink, colocalization of rtCATH-2 and IL-1 $\beta$ . EP: Intestinal epithelium, V: blood vessel. LP: lamina propria. Scale bar, 25  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 5. Conclusions

The results show zymosan-induced gene and protein expression of rainbow trout cathelicidins and IL-1 $\beta$  in the intestinal epithelial RTgutGC cell line and in the intestinal epithelial cells from rainbow trout in response to orally administered  $\beta$ -glucan. Trout cathelicidins exert an immunomodulatory effect on IL-1 $\beta$  expression in the RTgutGC cells, and also display a synergic effect with the zymosan. Rainbow trout cathelicidins, as a multifunctional HDP and an early response expression effector, may be a useful immunological marker to assess intestinal health status.

## Acknowledgments

We gratefully acknowledge Mr. James Conaghan for his English review assistance, Mr. Patricio Cataldo from the Experimental Laboratory of Aquaculture Curauma/PUCV and Mr. Ramon Ramirez from Fraunhofer Chile for technical assistance. Recombinant peptides were produced at André Ouellette laboratory at the University of Southern California, US. P.S. was supported by FONDECYT Postdoctoral Fellowship No 3130334.

## Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2015.03.007.

## References

- Ángeles Esteban, M., 2012. An Overview of the Immunological Defenses in Fish Skin. *ISRN Immunol.* 2012 1–29. doi:10.5402/2012/853470.
- Bethke, J., Rojas, V., Berendsen, J., Cárdenas, C., Guzmán, F., Gallardo, J.A., et al., 2012. Development of a new antibody for detecting natural killer enhancing factor (NKEF)-like protein in infected salmonids. *J. Fish Dis.* 35, 379–388. doi:10.1111/j.1365-2761.2012.01354.x.
- Bols, N., Barlian, A., Chirino-Trejo, M., Cadwell, S., Goegan, P., Lee, L., 1994. Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *J. Fish Dis.* 17, 601–611. doi:10.1111/j.1365-2761.1994.tb00258.x.
- Bommineni, Y.R., Pham, G.H., Sunkara, L.T., Achanta, M., Zhang, G., 2014. Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide. *Mol. Immunol.* 59, 55–63. doi:10.1016/j.molimm.2014.01.004.
- Bricknell, I., Dalmo, R., 2005. The use of immunostimulants in fish larval aquaculture. *Fish Shellfish Immunol.* 19, 457–472. doi:10.1016/j.fsi.2005.03.008.
- Bridle, A., Nosworthy, E., Polinski, M., Nowak, B., 2011. Evidence of an antimicrobial-immunomodulatory role of Atlantic salmon cathelicidins during infection with *Yersinia ruckeri*. *PLoS ONE* 6, e23417. doi:10.1371/journal.pone.0023417.
- Brogden, K.A., Johnson, G.K., Vincent, S.D., Abbasi, T., Vali, S., 2013. Oral inflammation, a role for antimicrobial peptide modulation of cytokine and chemokine responses. *Expert Rev. Anti Infect. Ther.* 11, 1097–1113. doi:10.1586/14787210.2013.836059.
- Bulet, P., Stöcklin, R., Menin, L., 2004. Anti-microbial peptides: from invertebrates to vertebrates. *Immunol. Rev.* 198, 169–184.
- Casadei, E., Wang, T., Zou, J., González Vecino, J.L., Wadsworth, S., Secombes, C.J., 2009. Characterization of three novel beta-defensin antimicrobial peptides in rainbow trout (*Oncorhynchus mykiss*). *Mol. Immunol.* 46, 3358–3366. doi:10.1016/j.molimm.2009.07.018.
- Casadei, E., Bird, S., Vecino, J.L.G., Wadsworth, S., Secombes, C.J., 2013. The effect of peptidoglycan enriched diets on antimicrobial peptide gene expression in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 34, 529–537. doi:10.1016/j.fsi.2012.11.027.
- Chang, C., Zhang, Y., Zou, J., Nie, P., Secombes, C.J., 2006. Two Cathelicidin genes are present in both rainbow trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*). *Antimicrob. Agents Chemother.* 50, 185–195. doi:10.1128/AAC.50.1.185.
- Chettri, J.K., Raida, M.K., Kania, P.W., Buchmann, K., 2012. Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*. *Dev. Comp. Immunol.* 36, 463–474. doi:10.1016/j.dci.2011.08.014.
- Costa, M.M., Maehr, T., Diaz-Rosales, P., Secombes, C.J., Wang, T., 2011. Bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: effects on macrophage growth and antimicrobial peptide gene expression. *Mol. Immunol.* 48, 1903–1916. doi:10.1016/j.molimm.2011.05.027.
- de Bruijn, I., Belmonte, R., Anderson, V.L., Saraiva, M., Wang, T., van West, P., et al., 2012. Immune gene expression in trout cell lines infected with the fish pathogenic oomycete *Saprolegnia parasitica*. *Dev. Comp. Immunol.* 38, 44–54. doi:10.1016/j.dci.2012.03.018.

- Falco, A., Chico, V., Marroquí, L., Perez, L., Coll, J.M., Estepa, A., 2008. Expression and antiviral activity of a beta-defensin-like peptide identified in the rainbow trout (*Oncorhynchus mykiss*) EST sequences. *Mol. Immunol.* 45, 757–765. doi:10.1016/j.molimm.2007.06.358.
- Falco, A., Frost, P., Miest, J., Pionnier, N., Irnazarow, I., Hoole, D., 2012. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with  $\beta$ -glucan supplements. *Fish Shellfish Immunol.* 32, 1051–1057. doi:10.1016/j.fsi.2012.02.028.
- Figueredo, S., Mastroianni, J.R., Tai, K.P., Ouellette, A.J., 2010. Expression and purification of recombinant  $\alpha$ -defensins and  $\alpha$ -defensin precursors in *Escherichia coli*. In: Giuliani, A., Rinaldi, A.C. (Eds.), *Antimicrobial Peptides. Methods and Protocols*. Humana Press, pp. 47–60. doi:10.1007/978-1-60761-594-1\_4.
- Gabe, M., 2012. *Histological Techniques*, first ed. Springer, NY.
- Gallo, R.L., Hooper, L.V., 2012. Epithelial antimicrobial defence of the skin and intestine. *Nat. Rev. Immunol.* 12, 503–516. doi:10.1038/nri3228.
- Gomez, D., Sunyer, J.O., Salinas, I., 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol.* 35, 1729–1739. doi:10.1016/j.fsi.2013.09.032.
- Halver, J.E., Hardy, R.W., 2002. *Fish Nutrition*, third ed. Academic Press.
- Hilchie, A.L., Wuertth, K., Hancock, R.E.W., 2013. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* 9, 761–768. doi:10.1038/nchembio.1393.
- Hong, S., Li, R., Xu, Q., Secombes, C.J., Wang, T., 2013. Two types of TNF- $\alpha$  exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF- $\alpha$ 3 in rainbow trout *Oncorhynchus mykiss*. *J. Immunol.* 191, 5959–5972. doi:10.4049/jimmunol.1301584.
- Kawano, A., Kales, S.C., Fujiki, K., DeWitte-Orr, S.J., Dixon, B., Lee, L.E.J., et al., 2010. A comparison of rainbow trout cell lines for their expression of the major histocompatibility complex genes and the induction of beta-2-microglobulin by dsRNA. *Fish Shellfish Immunol.* 29, 312–318. doi:10.1016/j.fsi.2010.04.007.
- Kawano, A., Haiduk, C., Schirmer, K., Hanner, R., Lee, L.E.J., Dixon, B., et al., 2011. Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide. *Aquac. Nutr.* 17, e241–e252. doi:10.1111/j.1365-2095.2010.00757.x.
- Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. *Anim. Feed Sci. Technol.* 173, 111–133. doi:10.1016/j.anifeedsci.2011.12.015.
- Komatsu, K., Tsutsui, S., Hino, K., Araki, K., Yoshiura, Y., Yamamoto, A., et al., 2009. Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. *Dev. Comp. Immunol.* 33, 499–506. doi:10.1016/j.dci.2008.09.012.
- Kühlwein, H., Merrifield, D.L., Rawling, M.D., Foe, A.D., Davies, S.J., 2013. Effects of dietary  $\beta$ -(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.). *J. Anim. Physiol. Anim. Nutr. (Berl)* 1–11. doi:10.1111/jpn.12078.
- Lazado, C.C., Caipang, C.M., 2014. Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol.* 39, 78–89. doi:10.1016/j.fsi.2014.04.015.
- Lokesh, J., Fernandes, J.M.O., Korsnes, K., Bergh, O., Brinchmann, M.F., Kiron, V., 2012. Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or  $\beta$ -glucan and challenged with *Vibrio anguillarum*. *Fish Shellfish Immunol.* 33, 626–631. doi:10.1016/j.fsi.2012.06.017.
- Martin, E., Verlhac Trichet, V., Legrand-Frossi, C., Fripiat, J.-P., 2012. Comparison between intestinal and non-mucosal immune functions of rainbow trout, *Oncorhynchus mykiss*. *Fish Shellfish Immunol.* 33, 1258–1268. doi:10.1016/j.fsi.2012.09.019.
- Masso-Silva, J.A., Diamond, G., 2014. Antimicrobial peptides from fish. *Pharmaceuticals (Basel)* 7, 265–310. doi:10.3390/ph7030265.
- Morales-Lange, B., Bethke, J., Schmitt, P., Mercado, L., 2014. Phenotypical parameters as a tool to evaluate the immunostimulatory effects of laminarin in *Oncorhynchus mykiss*. *Aquac. Res.* doi:10.1111/are.12426.
- Mowat, A.M., Agace, W.W., 2014. Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* 14, 667–685. doi:10.1038/nri3738.
- Mulder, I.E., Wadsworth, S., Secombes, C.J., 2007. Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*. *Fish Shellfish Immunol.* 23, 747–759. doi:10.1016/j.fsi.2007.02.002.
- National Research Council, 2011. *Guide for the Care and Use of Laboratory Animals*, eighth ed. The National Academies Press, Washington, D.C.
- Palić, D., Andreasen, C.B., Herolt, D.M., Menzel, B.W., Roth, J.A., 2006. Immunomodulatory effects of beta-glucan on neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820). *Dev. Comp. Immunol.* 30, 817–830. doi:10.1016/j.dci.2005.11.004.
- Peter Chiou, P., Khoo, J., Bols, N.C., Douglas, S., Chen, T.T., 2006. Effects of linear cationic alpha-helical antimicrobial peptides on immune-relevant genes in trout macrophages. *Dev. Comp. Immunol.* 30, 797–806. doi:10.1016/j.dci.2005.10.011.
- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45.
- Rakers, S., Niklasson, L., Steinhagen, D., Kruse, C., Schaubert, J., Sundell, K., et al., 2013. Antimicrobial peptides (AMPs) from fish epidermis: perspectives for investigative dermatology. *J. Invest. Dermatol.* 133, 1140–1149. doi:10.1038/jid.2012.503.
- Schmitt, P., de Lorgeril, J., Gueguen, Y., Destoumieux-Garzon, D., Bachère, E., 2012. Expression, tissue localization and synergy of antimicrobial peptides and proteins in the immune response of the oyster *Crassostrea gigas*. *Dev. Comp. Immunol.* 37, 363–370. doi:10.1016/j.dci.2012.01.004.
- Scocchi, M., Pallavicini, A., Salgaro, R., Bociek, K., Gennaro, R., 2009. The salmonid cathelicidins: a gene family with highly varied C-terminal antimicrobial domains. *Comp. Biochem. Physiol. B Biochem Mol. Biol.* 152, 376–381. doi:10.1016/j.cbpb.2009.01.003.
- Secombes, C.J., Wang, T., Bird, S., 2011. The interleukins of fish. *Dev. Comp. Immunol.* 35, 1336–1345. doi:10.1016/j.dci.2011.05.001.
- Sever, L., Vo, N.T.K., Bols, N.C., Dixon, B., 2014. Expression of tapasin in rainbow trout tissues and cell lines and up regulation in a monocyte/macrophage cell line (RTS11) by a viral mimic and viral infection. *Dev. Comp. Immunol.* 44, 86–93. doi:10.1016/j.dci.2013.11.019.
- Skov, J., Kania, P.W., Holtén-Andersen, L., Fouz, B., Buchmann, K., 2012. Immunomodulatory effects of dietary  $\beta$ -1,3-glucan from *Euglena gracilis* in rainbow trout (*Oncorhynchus mykiss*) immersion vaccinated against *Yersinia ruckeri*. *Fish Shellfish Immunol.* 33, 111–120. doi:10.1016/j.fsi.2012.04.009.
- Wan, M., Godson, C., Guiry, P.J., Agerberth, B., Haeggström, J.Z., 2011. Leukotriene B4/antimicrobial peptide LL-37 proinflammatory circuits are mediated by BLT1 and FPR2/ALX and are counterregulated by lipoxin A4 and resolvin E1. *FASEB J.* 25, 1697–1705. doi:10.1096/fj.10-175687.
- Zanetti, M., 2004. Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukoc. Biol.* 75, 39–48. doi:10.1189/jlb.0403147.
- Zheng, Y., Niyonsaba, F., Ushio, H., Nagaoka, I., Ikeda, S., Okumura, K., et al., 2007. Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human alpha-defensins from neutrophils. *Br. J. Dermatol.* 157, 1124–1131.