Immunomodulatory effect of cathelicidins in response to a β-glucan in intestinal epithelial cells from rainbow trout

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ABSTRACT

The aim of the present study was to characterize intestinal immune mechanisms involved in the response to β-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 β-glucan, zymosan, were assessed. The results showed that zymosan increased the production of the HDP, cathelicidin, and the cytokine, IL-1β, 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-1β in RTgutGC cells and (ii) display a synergic effect with zymosan in IL-1β upregulation. Importantly, the colocalization of both rtCATH-2 and IL-1β 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.

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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 immunomodulatory markers in response to immunostimulants. Furthermore, only a small number of studies describe their immunomodulatory properties (Brindle 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 β-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 β-glucan, zymosan, on the IECs in rainbow trout, Oncorhynchus mykiss. Significant overexpression of cathelicidins and the cytokine IL-1β was observed in the 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-1β in RTgutGC cells; and (iii) display a synergic effect with zymosan in IL-1β 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-1β 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 μg/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 x g at 20 °C for 5 min. For the purpose of gene expression analysis, 5 x 10^6 RTgutGC cells were cultured in 500 μL 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 x 10^4 RTgutGC cells were cultured in 300 μL of fully supplemented L-15 medium in an 8-well Lab-tak 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 ± 1 °C with an average of 8 ± 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 (0.5% 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 μm) 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 pellet 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 trypsin (0.05% trypsin, 0.02% EDTA in phosphate buffered saline (PBS), pH 7.4) and was added to complete intestinal tissue samples in 250 μL 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 quantitative PCR analysis, 6 x 10^4 RTgutGC cells were cultured in 300 μL of fully supplemented L-15 medium in an 8-well Lab-tak 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).

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

The 10 μl reaction consisted of 1X Brilliant II SYBR Green QPCR master mix (Stratagene), 0.5 μM of each primer and 1 μL 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 \(-2^{\Delta Ct}\) method described by Pfaffl (2001), using the measured threshold cycle (CT) values of the constitutively expressed gene EF-1α [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 differences and statistical analysis using the Kruskal–Wallis test for between samples were based on the selection criteria with a cut-off value of \( p < 0.05 \). Statistical differences between samples were based on the selection criteria with a cut-off value of \( p < 0.05 \).

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 Metα 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 amebocyte 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 (ANKEQLKSIQGQS, QKAAPNRLITTFTIQRHN and DLLNFLLESAVEEIH) from a deduced IL-1β 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 μg immunogen diluted 1:1 in PBS as a T helper cell activator, as previously described (Bethke et al., 2012) and 1:1 in Freund’s adjuvant (Thermo). The antisera 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-1β 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 chemiluminencescence with the Westar Supernova kit (Cyanagen).

2.8. Histological analysis and immunofluorescence

Fixed samples were dehydrated through an ascending ethanol series and embedded in Histoclar (Merck). Sections were cut at 6 μm 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 DMS500B 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-1β. Briefly, cells were fixed on glass slides with 4% paraformaldehyde for 10 min, 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-1β (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 × 1.25 Oil HCX PL APO CS lens (Leica Microsystems).

2.9. Antimicrobial assays

Recombinant and synthetic peptides were tested for microbiocidal activity against the Gram-negative E. coli ML35 (kindly donated by Dr. Andre Ouellette), Salmonella enterica subsp. Thypimurium (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 × 10⁶ colony forming units/mL (cfu/mL)) were exposed to different peptide concentrations in 50μL 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 antibiotic. 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-1β 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 β-defensins
β-Glucan zymosan induced the expression of trout cathelicidins and IL-1β in RTgutGC cells. Quantitative PCR analysis of the gene expression of A. Nine immune related genes in RTgutGC cells stimulated with zymosan (50 μg/mL) for 6 h. B. Cathelicidins (rtCATH-1 and rtCATH-2) and IL-1β in RTgutGC cells stimulated with 10, 25 and 50 μg/mL of zymosan. Relative expression was normalized to the gene expression of rainbow trout EF-1α and calculated using the 2−ΔΔCt method. Results are expressed as mean values ± SD. Asterisks indicate significant differences related to the control (p < 0.05).

Fig. 1. β-Glucan zymosan induced the expression of trout cathelicidins and IL-1β in RTgutGC cells. Quantitative PCR analysis of the gene expression of A. Nine immune related genes in RTgutGC cells stimulated with zymosan (50 μg/mL) for 6 h. B. Cathelicidins (rtCATH-1 and rtCATH-2) and IL-1β in RTgutGC cells stimulated with 10, 25 and 50 μg/mL of zymosan. Relative expression was normalized to the gene expression of rainbow trout EF-1α and calculated using the 2−ΔΔCt method. Results are expressed as mean values ± SD. Asterisks indicate significant differences related to the control (p < 0.05).

The production of trout cathelicidins and IL-1β in RTgutGC cells in response to zymosan was also evaluated at the protein level using an anti-rtCATH-2 mouse antisera and anti-IL-1β rabbit antisera, respectively. The rtCATH-2 antisera 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-1β and in response to 50 μg/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-1β staining. Merged images indicate the colocalization of both rtCATH-2 and IL-1β 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-1β 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

Fig. 3. Microbicidal activity of trout cathelicidins. 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 μM). Interestingly, the rtCATH-2 variants were active against the human pathogens Salmonella typhimurium or Staphylococcus aureus, but did not display significant microbicidal activity (40 μM 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-1β was assessed by qPCR (Fig. 4A). A significant increase in the transcription of IL-1β was observed in treated cells. The relative expression of IL-1β increased up to seven-fold when cells treated with 10 μM of rtCATH-2, while no changes in the relative expression of IL-1β was observed in control cells treated with a peptide vehicle. Moreover, the upregulation of IL-1β increased in a concentration dependent manner in response to cathelicidins, ranging from 0 to 10 μM. These results showed that IL-1β 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-1β in cells treated with zymosan in the presence or absence of rtCATH-2 variants was evaluated. The induction of IL-1β by zymosan increased to a greater extent in the
cells treated in the presence of the peptides, compared to the treat-
ment with zymosan alone (p < 0.05) (Fig. 4B). This result showed
that there is a synergic IL-1β-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 synthet-
ic variant was used in subsequent assays to avoid any problems
associated with IL-1β induction by endotoxin contamination from
the recombinant peptides. Thus, RTgutGC cells were incubated with
rtCATH-2B for 6 h and the expression of IL-1β and of rtCATH-2 was
evaluated by immunofluorescence. Confocal analysis not only con-
firmed the production of IL-1β 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-1β 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-1β 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 β-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 expres-
sion of both trout cathelicidins and IL-1β was observed in vivo.
The expression of rtCATH-1, rt-CATH-2 and IL-1β was significantly in-
creased 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-1β 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-1β 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-1β 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 anal-
ysis was performed and protein expression of cathelicidins and IL-
1β 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 inter-
spersed between the epithelial cells were observed in the proximal
intestine of the treated fish (Fig. 6B), which is not evident in the distal
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-1β 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-1β. No expression of these molecules was detected in the control fish (Fig. 6E), while merged confocal images showed that rtCATH-2 and IL-1β 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-1β (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-1β 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-α (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-1β have been reported to be expressed in other rainbow trout cell lines in response to the fungus Saprolegnia parasitica (de Bruin et al., 2012) but this is the first time these molecules have been detected in the RTgutGC cell line. Thus, IL-1β 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-2α, 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 μM, whereas truncated
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-1β 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-1β 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-1β or IL-18, suggesting that salmon cathelicidins could act as a chemokine (Bridle et al., 2011). Remarkably, the lack of induction of IL-1β 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-1β in RTL-W1 trout liver cells (de Bruijn et al., 2012) and by rTNF-3α 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 B4, triggers LL-37 release from human neutrophils and in turn LL-37 promotes leukotriene B4 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...
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-1β. In addition, it reveals that IECs from rainbow trout differentially express these molecules depending on the intestinal segment analyzed, as observed for IL-1β in the intestine of Atlantic cod fed with β-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-1β 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 β-glucan in fish diets. However, assessment of the literature shows that β-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 β-glucan, the dosage and time of exposure. In this study, the significant increase in the expression of cathelicidins and IL-1β in the intestine only occurred at the last experimental timepoint 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.
5. Conclusions

The results show zymosan-induced gene and protein expression of rainbow trout cathelicidins and IL-1β in the intestinal epithelial RTgutGC cell line and in the intestinal epithelial cells from rainbow trout in response to orally administered β-glucan. Trout cathelicidins exert an immunomodulatory effect on IL-1β expression in the RTgutGC cells, and also display a synergistic 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.

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Appendix: Supplementary material

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References


