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Role of Pathogen-Derived Cell Wall Carbohydrates and Prostaglandin E_2 in Immune Response and Suppression of Fish Immunity by the Oomycete *Saprolegnia parasitica*

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Saprolegnia parasitica* is a freshwater oomycete that is capable of infecting several species of fin fish. Saprolegniosis, the disease caused by this microbe, has a substantial impact on Atlantic salmon aquaculture. No sustainable treatment against saprolegniosis is available, and little is known regarding the host response. In this study, we examined the immune response of Atlantic salmon to *S. parasitica* infection and to its cell wall carbohydrates. *Saprolegnia* triggers a strong inflammatory response in its host (i.e., induction of interleukin-1β_1 [IL-1β_1], IL-6, and tumor necrosis factor alpha), while severely suppressing the expression of genes associated with adaptive immunity in fish, through downregulation of T-helper cell cytokines, antigen presentation machinery, and immunoglobulins. Oomycete cell wall carbohydrates were recognized by fish leukocytes, triggering upregulation of genes involved in the inflammatory response, similar to what is observed during infection. Our data suggest that *S. parasitica* is capable of producing prostaglanding E_2 (PGE_2) *in vitro*, a metabolite not previously shown to be produced by oomycetes, and two proteins with homology to vertebrate enzymes known to play a role in prostaglandin biosynthesis have been identified in the oomycete genome. Exogenous PGE_2 was shown to increase the inflammatory response in fish leukocytes incubated with cell wall carbohydrates while suppressing genes involved in cellular immunity (gamma interferon [IFN-γ] and the IFN-γ-inducible protein [γ-IP]). Inhibition of *S. parasitica* zoospore germination and mycelial growth by two cyclooxygenase inhibitors (aspirin and indomethacin) also suggests that prostaglandins may be involved in oomycete development.

The *Oomycota* class contains several microorganisms that have developed the ability to parasitize plants, protists, and animals (1). Typical examples are the potato pathogen *Phytophthora infestans*, which is responsible for late blight, the human pathogen *Pythium insidiosum*, and the fish parasite *Saprolegnia parasitica* (1). In order to overcome the host defenses, these organisms have evolved mechanisms that disrupt host immune pathways. These mechanisms are well studied in plant-pathogenic oomycetes, which utilize a repertoire of effector proteins to manipulate host cell processes for the pathogen's benefit (2, 3). In the group of animal-pathogenic oomycetes, little is known regarding the major molecular players involved in host-pathogen interactions.

S. parasitica is a pathogen of freshwater fish with a worldwide distribution and is responsible for major losses in aquaculture (4, 5). This parasite was efficiently controlled by the use of malachite green. However, that chemical was banned worldwide in 2002 due to its carcinogenic and toxicological effects. Currently, treatments with formalin and bronopol are the only options available (1). The infection of fish by *S. parasitica*, designated saprolegniosis, is characterized by white patches of mycelia on the skin, gills, and fins of the host. The mycelium is capable of producing and releasing motile zoospores that, after encysting, can germinate upon attachment to a new host and subsequently form new mycelial mats (4). Recently, effector proteins with the capability of self-translocating into fish host cells and proteases capable of degrading serum IgM have been described in *S. parasitica* (6, 7), giving some insight into the virulence factors employed by animal-pathogenic oomycetes.

Previous studies indicated that fish affected by saprolegniosis fail to develop an effective antibody response (8–10). A whole-fish microarray study (11) highlighted several changes of the transcriptome during the infection but without a focus on components of the immune system. A recent *in vitro* study from our group (12) demonstrated that in rainbow trout cell lines (RTG-2, RT-Gill, RTL, and RTS11) proinflammatory cytokines and chemokines (i.e., interleukin-1β_1 [IL-1β_1], IL-8, and tumor necrosis factor alpha [TNF-α]) are strongly induced upon infection, together with several components of the innate cellular response (i.e., cyclooxygenase-2 [COX-2], antimicrobial peptides, and lectins). Another *in vitro* study with the RTS-11 cell line showed similar results on proinflammatory cytokine expression and some degree of suppression of host major histocompatibility complex class II (MHC-II) molecules and associated molecules (13). Although *in vitro* systems are convenient to study host-pathogen interactions, they have several limitations, such as measuring only

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particular components of host immunological responses, with adaptive responses largely missing.

In the current study, we performed a real-time quantitative PCR (qPCR) survey of immune gene expression with a focus on immunoregulatory cytokines, antimicrobial peptides, and components of the adaptive immune response to create an immune profile of salmon experimentally infected by *S. parasitica*. Besides the expected induction of inflammatory responses, several components of adaptive immunity were found to be suppressed. This suppression was detectable even before mycelial growth in the host, indicating that *S. parasitica* possesses mechanisms to actively and remotely inhibit host defenses. *S. parasitica* was found to produce prostaglandin E_2 (PGE_2) *in vitro*, a metabolite unknown to be produced by oomycetes to date, and two enzymes involved in prostaglandin production were discovered in the oomycete genome. Prostaglandins were also found to be necessary for oomycete development and growth, as aspirin and indomethacin (COX inhibitors) prevented zoospore germination and mycelial growth. The host response to infection could be partially explained by the inflammatory response triggered by the oomycete cell wall components, and immune suppression was observed in the presence of exogenous PGE_2 *in vitro*.

MATERIALS AND METHODS

Atlantic salmon and rainbow trout. Atlantic salmon juveniles weighing approximately 25 g were kept in the aquarium facilities of the Norwegian National Veterinary Institute (NVI, Oslo, Norway). Fish were acclimatized for at least 2 weeks prior to the challenge experiments (water temperature, 12°C; 12:12 light/dark cycle).

Rainbow trout were kept in laboratory holding tanks in the freshwater aquarium facilities at the Institute of Biological and Environmental Sciences, University of Aberdeen (water temperature, 12°C; 12:12 light/dark cycle).

***Saprolegnia parasitica* and *Saprolegnia diclina*.** Stock cultures of *S. parasitica* and *S. diclina* strains were kept on potato dextrose agar (PDA; Fluka) at 18°C. To obtain zoospores and cysts, plugs of agar containing growing mycelia were inoculated either into pea broth for *in vitro* challenges (125 g $liter^{-1}$ of frozen peas that were autoclaved, filtered through cheesecloth, their volume adjusted to 1 liter, and autoclaved again) or glucose-yeast extract broth for *in vivo* challenges (GY broth; 10 g $liter^{-1}$ glucose and 2.5 g $liter^{-1}$ yeast extract). After 48 h of incubation at 18°C, the growing mycelia were washed 3 times with autoclaved tap water and then incubated with a mixture of fish tank water: tap water (autoclaved; 1:1 [vol/vol]) for a further 24 h. Zoospores and cysts were harvested by centrifugation of the liquid at 1,500 × *g* for 10 min at 4°C and counted using a hemocytometer. Their concentration was adjusted as appropriate for each of the experiments.

The strain CBS223.65 was obtained from the Centraal Bureau voor Schimmelcultures (CBS; Baarn, The Netherlands), and the strains NVI02736 and NVI05337 were obtained from the Norwegian National Veterinary Institute collection (NVI; Norway). *S. diclina* strain VS20 was obtained from the Royal Botanical Gardens collection (CSIC; Spain; a gift from J. Dieguez-Uribeondo).

***In vivo* challenge.** Atlantic salmon juveniles were used for the *in vivo* challenges. Food was withheld 2 days prior to challenge, and fish were sedated using AQUI-S (AQUI-S New Zealand, Ltd.), following the manufacturer's instructions. In groups of 5, fish were shaken in a net (amimomi technique [14]) for 1.5 min and then placed in tanks with extra aeration and without water renewal. The challenged group received an inoculum of 10^4 zoospores/cysts $liter^{-1}$. Feeding and water flow were restored 24 h postexposure. Fish status was assessed daily, and any fish found dead or moribund were removed. In fish farms, concentrations of up to 5×10^3 zoospores/cysts $liter^{-1}$ have been reported (15). The dose here used was justified by the fact that 24 h postchallenge water flow was restored and zoospores/cysts were washed out of tanks, a different picture from what happens in fish farms, where the presence of zoospores is constant. The dose employed has also been shown to cause an average of 50% mortality rates, similar to what is observed during natural outbreaks of saprolegniosis.

The strain NVI02736 was used for the first challenge. Five fish were kept as controls and 10 fish were challenged. Fish were sampled when signs of saprolegniosis were detected. This was assessed through daily inspection of sedated fish under a stereomicroscope. Controls were sampled on the same day as the infected fish from the unchallenged tank. During the course of the experiment, 5 out of the 10 challenged fish developed clear signs of saprolegniosis at the 6th, 7th, 11th, 12th, and 13th day postchallenge. The experiment was terminated at 14 days postchallenge.

In the second experiment, the strain NVI05337 was used, as it showed a higher infection rate than NVI02736 in a pilot experiment (data not shown). Fifteen fish were kept as controls and 45 were challenged. Fifteen fish were sampled on the 3rd, 7th, and 12th day postexposure, with five controls sampled on each day. Unexpectedly, during the course of the experiment only 3 out of the 45 challenged fish developed clear signs of saprolegniosis. In order to allow visualization of the 3 infected fish, where the responses seen were quite different from those in uninfected fish, gene expression data are presented for each individual fish in this experiment plus averages.

Tissue harvesting and RNA extraction. Gills and head kidney (HK) were harvested from freshly killed salmon, washed thoroughly in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4; pH 7.4), and preserved in RNAlater (Sigma) following the manufacturer's guidelines. Tissues were then homogenized in TRI reagent (Sigma) using a Qiagen tissue lyser II following the manufacturer's instructions. RNA was extracted from cell cultures by adding TRI reagent directly to the wells after removing the medium. Total RNA was extracted following the manufacturer's guidelines.

***Saprolegnia parasitica* cell wall.** Cell wall components of *S. parasitica* strain CBS223.65 were prepared as described previously (16). The alkali-insoluble fraction (AIF), consisting essentially of cellulose and β-glucans with 1,3 and 1,6 linkages, was used as a representative pathogen-associated molecular pattern (PAMP) of this oomycete, as it is, overall, the major component of the *S. parasitica* cell wall (~70% of total mass) (16).

Primary head kidney leukocytes. In all experiments, primary HK leukocytes obtained from 4 rainbow trout were tested in duplicates. The HK tissue was collected aseptically from rainbow trout, and cells were pushed through a 100-μm-pore-size nylon mesh (John Stanier) with incomplete Leibovitz L-15 medium (Gibco; supplemented with penicillin and streptomycin [P/S] at 100 U/ml and 100 μg/ml, respectively, plus 1% fetal bovine serum [FBS]; Biosera) containing 10 U/ml heparin (Sigma-Aldrich). The cell suspensions were then centrifuged at 200 × *g* for 5 min, washed once with incomplete medium, and resuspended in complete medium (same as incomplete medium but with 10% FBS). Cells were counted, and 1-ml aliquots containing 2×10^6 cells were seeded into 24-well plates (Thermo Scientific) for use after 24 h of culture.

For the exposure to cell wall carbohydrates of *S. parasitica*, several concentrations of the AIF sample were used (1, 0.1, 0.01, and 0.001 mg $liter^{-1}$) to stimulate the cells for 6 h, as this was determined to be the best time point in a preliminary time course assay (data not shown). For the prostaglandin treatment, concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 μM PGE_2 (Cayman Chemical) were used, and the cells were also stimulated for 6 h. In a combinatorial test (with cell wall components and PGE_2), 0.001 mg $liter^{-1}$ of the carbohydrate preparation was used in combination with either 100 or 0.1 μM PGE_2 to stimulate the cells for 6 h. The carbohydrates were either added to the cells simultaneously with PGE_2 or after a 2-h preincubation with the prostanoid. For the challenge with *S. parasitica*, 2×10^4 cysts ml^{-1}, obtained from strains CBS223.65

and NVI02736, were used as described previously (12). RNA was harvested 24 h postinfection.

cDNA synthesis and real-time PCR. cDNA was synthesized using a Fermentas Revert Aid first-strand cDNA synthesis kit according to the manufacturer's instructions, using a minimum of 10 μg of total RNA in 45-μl reaction mixtures. Real-time PCR was performed in a LightCycler 480 (Roche) using the GoTaq qPCR master mix (Promega) in 10-μl reaction mixtures and 384-well plates, with 45 cycles (95°C for 15 s, 60°C for 60 s, and 72°C for 30 s). The primers used for each gene are reported in Table S1 of the supplemental material. The elongation factor 1α (EF-1α) gene was used as a reference gene for relative quantification of salmon and rainbow trout samples, whereas the β-tubulin chain was the reference gene used for relative quantification of *S. parasitica* samples. Crossing point (Cp) levels were determined by using the LightCycler software (Roche) with the second derivative max method. The ratios between the target and reference genes were calculated using the Pfaffl method (17) with an efficiency of 2.0 and the average Cp of the controls. Statistical analysis was performed using the REST software (18) with 2,000 iterations.

Extraction of prostaglandins from *S. parasitica* cultures. Mycelial plugs from the strains CBS223.65 and NVI02736 were inoculated into 50 ml of pea broth as previously described. As a control, sterile pea broth was used and subjected to the same procedures as the oomycete culture supernatants. After 4 days of growth at 18°C, arachidonic acid (Cayman Chemical) was added to the cultures to a final concentration of 500 μM, and 24 h later, 25 ml of the culture supernatant was collected after centrifugation at 10,000 × *g* for 10 min at 4°C. Proteins were removed from samples by addition of 1 volume (25 ml) of cold absolute ethanol followed by 2 h of incubation at −20°C and centrifugation at 10,000 × *g* for 10 min at 4°C. Protein-free supernatants were collected and acidified with 1% formic acid (>96% purity; Sigma).

Prostaglandins were extracted from samples by using a C_{18} solid-phase extraction column (DSC-18; Discovery) according to the methods described by Welsh et al. (19). Briefly, acidified samples (50 ml) were passed through activated columns that were then washed with 2 ml of 20% (vol/vol) methanol in water. Prostaglandins were eluted with 2 ml of 100% methanol, and samples were dried under an N_2 flow and stored at −80°C until analysis.

Identification of PGE_2 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Dried samples were dissolved in absolute ethanol and then applied to a Luna C_8 150- by 2-mm column (Phenomenex) maintained at 35°C and connected to a 1260 Infinity UHPLC system (Agilent). Samples (5 μl) and a standard PGE_2 control (5 μl at 100 pg/μl in absolute ethanol; Cayman Chemical) were automatically injected into the column at a flow rate of 40 μl min^{-1}. Prostaglandins were eluted in a 35-to-95% acetonitrile (0.1% acetic acid) gradient over 15 min at a flow rate of 0.5 ml min^{-1}.

An Agilent 6490 triple-quadrupole mass spectrometer with an Agilent Jet Stream electrospray ion source was used in negative ion mode. The following source parameters were used: capillary at 3,500 V; nebulizer pressure of 20 lb/in^2; gas temperature of 230°C; gas flow of 15 liters/min. For the product ion spectra of PGE_2 (parental ion at 351 *m/z*), the collision energy was 10 V.

Zoospore germination and inhibition of mycelial growth. Zoospores from strains CBS223.65, NVI02736, and VS20 were obtained as described above. Zoospores (2×10^4 ml^{-1}) were seeded into 96-well plates with pea broth or medium containing aspirin (from 7.5 mM to 3 μM; 2-fold dilutions) or indomethacin (1 mM to 0.4 μM; 2-fold dilutions) (both from Sigma), with each assay performed in triplicate. The MIC was determined and was the lowest concentration where no zoospore germination was observed after 24 h of incubation at 18°C.

For the mycelium inhibition assay, aspirin (from 7.5 mM to 0.93 mM; 2-fold dilutions) or indomethacin (1 mM to 0.06 mM; 2-fold dilutions) was added directly to the liquid agar before plate preparation. In this case, the MIC was the lowest concentration where no radial growth was observed after 24 h of incubation at 18°C.

RESULTS

Immune response to saprolegniosis. In order to investigate the host immune response to infection, in the first experiment fish with obvious signs of saprolegniosis were sampled within 24 h of detection, although this occurred at various times. Thirty-nine out of the 53 (73%) genes analyzed had significant expression changes in the gills, while 41 (77%) were differentially expressed in the HK of infected fish (Fig. 1; see also Table S2 in the supplemental material). The expression profiles were very similar between these two tissues, with only 12 genes showing tissue-specific behavior. Saprolegniosis induced a strong proinflammatory response in the gills and HK of the host, and this was characterized by a strong induction of IL-$1\beta_1$, IL-6, IL-8, IL-22, TNF-α_2, and COX-2 (Fig. 1A and C). This was associated with a strong activation of innate immunity, with the antimicrobial peptides β-defensin 3, cathelicidin-2, and hepcidin highly induced by the infection. Hepcidin was the most strongly induced gene in the gills, with an average 1,134-fold change in comparison to controls. Upregulation of serum amyloid A and haptoglobin was also observed. These proteins have been previously associated with acute-phase responses during infection (20). The anti-inflammatory cytokines IL-10_a and TGF-β were only induced in the head kidney cells (Fig. 1C), as were the two paralogues of IL-17C. Lastly, the cell surface receptors CD80 and CD209 also exhibited higher expression caused by the infection in both tissues, with upregulation associated with activation of T cells by antigen-presenting cells in mammals.

A remarkable number of genes (28, i.e., 52%) had lower expression levels in infected fish in at least one of the two tissues studied (Fig. 1B and D). Most of the suppressed genes were cytokines associated with T-helper cell-type responses, such as IL-4/13A, IL-17A/F_2, IL-21, and the two paralogues IFN-$\gamma_{1/2}$. IFN-γ_1 was the most suppressed gene in both tissues (95% and 92% suppression in the gills and HK, respectively). All the components of the antigen presentation machinery that were analyzed exhibited some degree of suppression (i.e., MHC-I and -II, β2-microglobulin, TAPBP, PA28β, and Iclp), as well as T- and B-cell antigen receptors and coreceptors (i.e., CD-$8_{\alpha/\beta}$, T-cell receptor α/β [$TCR_{\alpha/\beta}$], membrane IgM [mIgM], membrane and soluble IgD [m/sIgD], and m/sIgT). Regarding transcription factors associated with adaptive immunity, GATA3 and T-bet expression levels were also lower in infected fish, which further suggests a compromised Th1 and Th2 response in the *S. parasitica*-infected fish.

Immune response to exposure to *S. parasitica*. Aiming to investigate the immune changes hosts undergo after exposure to *S. parasitica*, in a second experiment we sampled fish at defined time points postexposure, including fish with and without symptoms of saprolegniosis. A smaller selection of immune-related genes (27 genes) was chosen, based on the results obtained in the previous experiment. After 3 days of exposure, several genes were differentially expressed in the gills and head kidney of the exposed fish, although a greater number changed in the mucosal tissue (18 genes) than in the HK (8 genes), probably due to the fact that the gills are in direct contact with the oomycete and the external medium. The antimicrobial peptides cathelicidin-1 and β-defensins 1 and 2 exhibited higher expression in the gills of the challenged fish, whereas only β-defensin 1 was significantly upregulated in

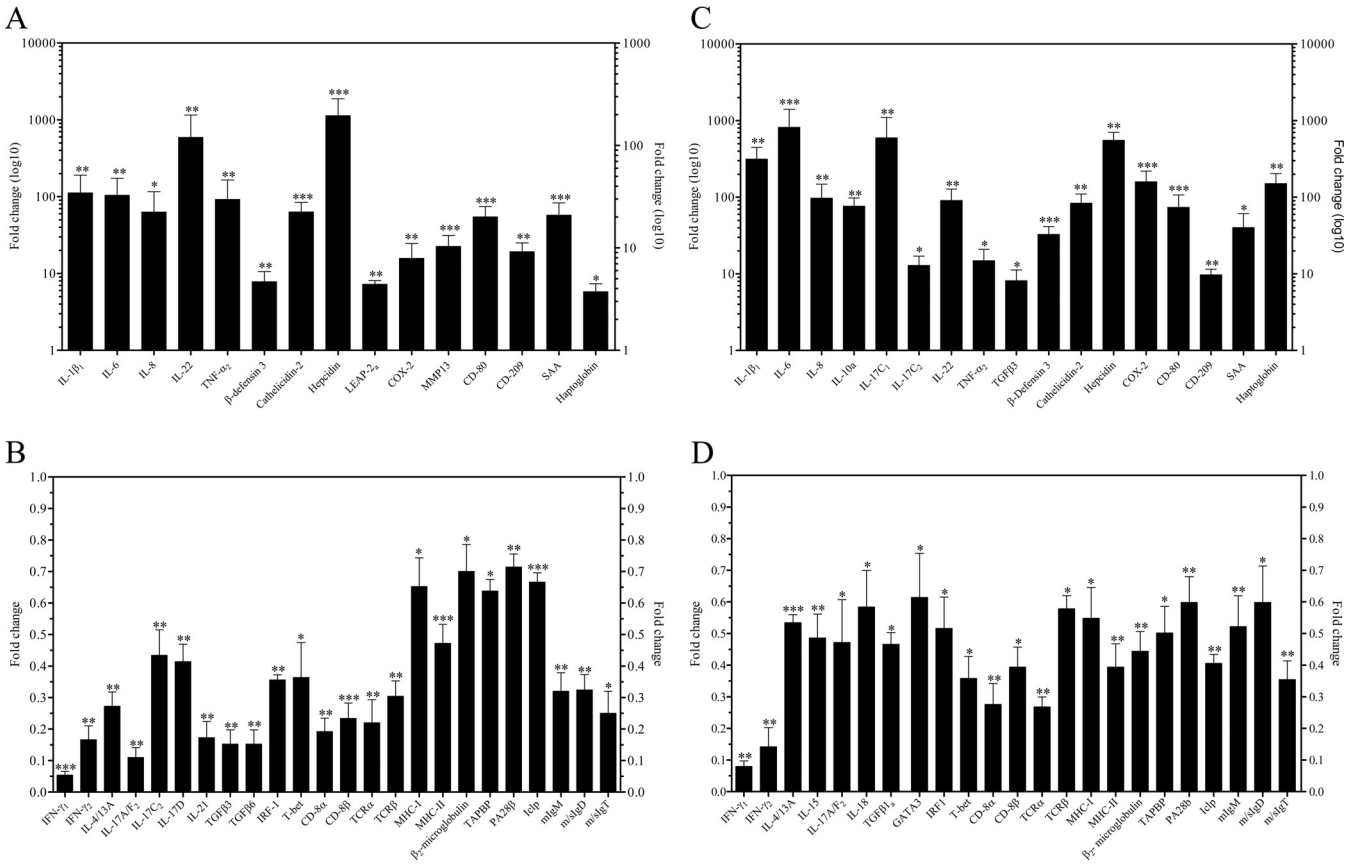

FIG 1 Immune response of Atlantic salmon presmolts to *S. parasitica* infection. Real-time PCR was used for the relative quantification of the expression of several immune genes in the gills (A and B) and head kidney (C and D) of *S. parasitica*-infected salmon. (A and C) The fold change (average + standard error of the mean [SEM]) of genes significantly upregulated in gill and head kidney samples, respectively. (B and D) The fold change (average + SEM) of genes significantly downregulated in gill and head kidney samples, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the HK (Fig. 2A and B). In contrast, the expression levels of several proinflammatory markers were lower than in the controls, with a significant downregulation of IL-6 and TNF-α_2 in the gills and of COX-2 in the HK. Although no challenged fish had yet developed any signs of infection, many genes associated with the adaptive immune response, including IL-4/13A, IL-21, IL-22, IFN-$\gamma_{1/2}$, T-bet, RORγ, mIgM, m/sIgD, and m/sIgT showed significantly lower expression in one of the two tissues analyzed, in a similar fashion as in infected fish in the previous experiment.

Despite the fact that the same challenge methodology as in the first experiment was used and a strain of higher virulence was employed, infection rates at 7 days postchallenge were lower than expected (3 out of 15 fish infected, in comparison to 5 out of 10 in the first experiment). Nevertheless, a clear change in the expression profile could be observed in the group as a whole. Noticeably, IL-4/13A and IFN-$\gamma_{1/2}$ expression levels were no longer inhibited and IL-22 expression was now significantly elevated at 7 days postchallenge in gill samples (Fig. 2C and D). The expression level of antimicrobial peptides was again higher in the challenged group (cathelicidin 1, hepcidin, and β-defensin 3 in the gills and β-defensin 4 in the HK) with the exception of β-defensin 1, which exhibited a suppressive state. At this sampling point, only three fish had detectable skin lesions where *S. parasitica* mycelia could be observed. Interestingly, these infected fish showed higher expression of proinflammatory cytokines (e.g., IL-1β_1, IL-6, and TNF-α_2) and reduced levels of genes associated with the adaptive immune response (e.g., IL-4/13A, IFN-γ_1, T-bet, MHC-I/II, and m/sIgM/T) relative to other fish in the same group (Fig. 2C and D, triangles versus circles).

Twelve days after exposure to zoospores of *S. parasitica*, several of the adaptive immune response genes, which had significantly lower levels in the previous samples, were still suppressed (MHC-II, m/sIgT, RORγ, and T-bet). However, expression of a few key genes was now significantly increased (IL-4/13A and IL-21) (Fig. 2E and F). Interestingly, none of the sampled fish had any signs of disease, although *S. parasitica* mycelia were observed colonizing the surface of the tank and feed remains. A suppression of the inflammatory response was also observed in the gill samples, as IL-1β_1, IL-6, and TNF-α_2 expression was significantly lower. In addition, some of the antimicrobial genes (i.e., hepcidin in gills and β-defensin 4 in HK) had decreased expression relative to control levels. These data suggest that these fish were resistant to the disease, as they did not exhibit any sign of saprolegniosis and the immune profile was substantially different from that of the infected fish.

***In vitro* production of PGE_2 by *S. parasitica*.** Although previous evidence suggested the existence of eicosanoid metabolism in oomycetes (21, 22), the production of such molecules by these organisms has not been reported before. We investigated if *S. parasitica* produces PGE_2 *in vitro*. As previous literature indicated

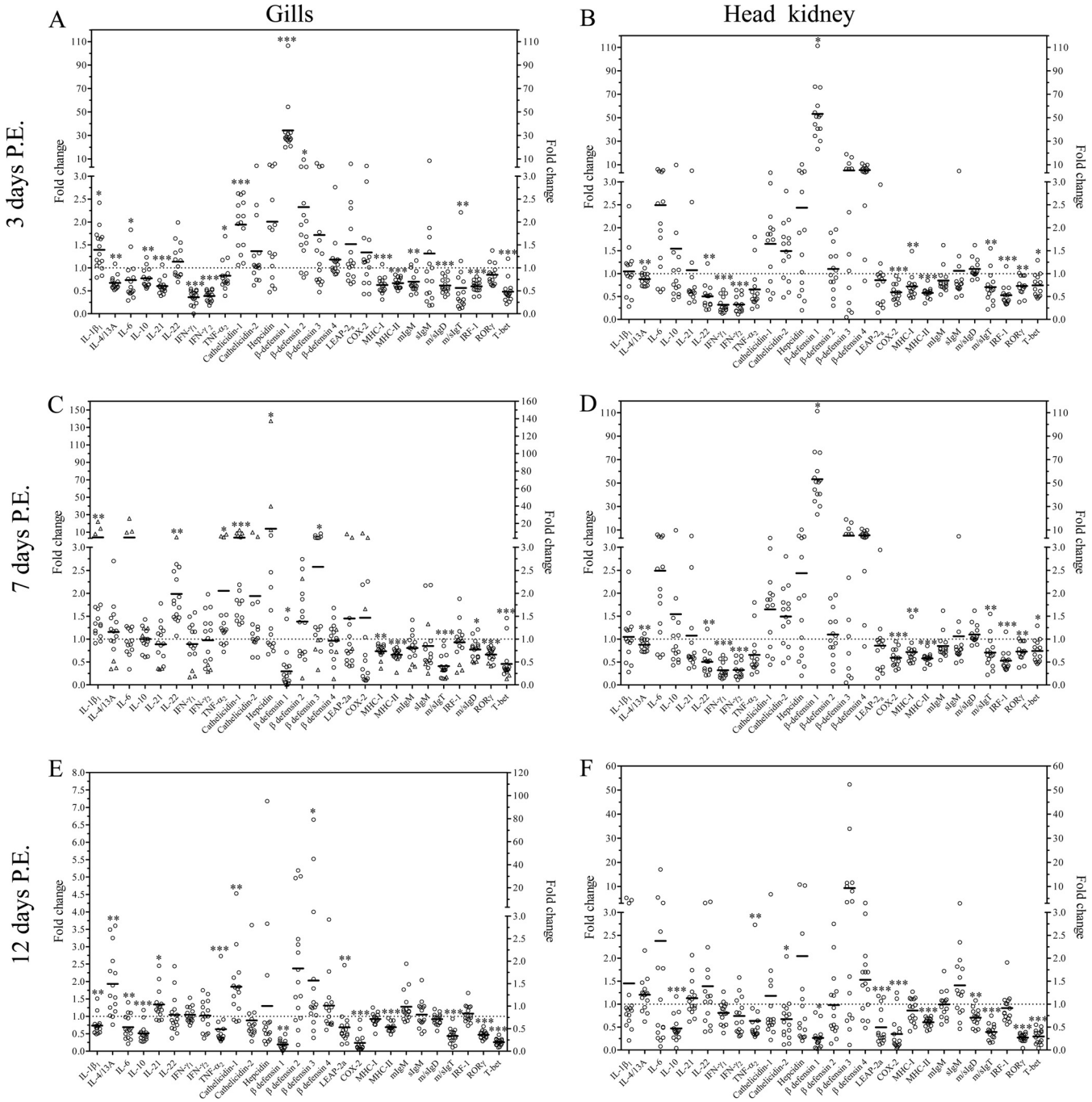

FIG 2 Immune response of Atlantic salmon presmolts to *S. parasitica* exposure. Real-time PCR was used for the relative quantification of the expression of several immune genes in the gills and head kidney of salmon exposed to *S. parasitica* for 3 (A and B), 7 (C and D), or 12 (E and F) days. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P.E., postexposure. The data are presented as the fold change for individual fish, with bars showing the averages. ○, challenged fish with no signs of lesions; △, challenged fish with skin lesions.

that microbial PGE_2 production is dependent on the presence of arachidonic acid (23), we analyzed culture supernatants of the strains CBS223.65 and NVI02736 for the presence of PGE_2 by using LC-MS/MS after 24 h of incubation with 500 μM arachidonic acid. Both cultures presented a detectable peak that eluted at 3.10 min, a similar retention time as that observed for the PGE_2 standard (Fig. 3A and B). Fragmentation of the PGE_2 parent ion at *m/z* 351 from the standard sample generated ions at *m/z* 333, 315, 271, 233, and 193 (Fig. 3C), and this spectrum was identical to the ones obtained from CBS223.65 and NVI02736 supernatants (Fig. 3D and E), confirming the presence of PGE_2 in the media of the oomycete cultures. No PGE_2 was detected when sterile pea broth was analyzed (data not shown).

Prostaglandin metabolism in *S. parasitica*. The role of prostanoids in oomycete metabolism is unknown, but previous studies have suggested that prostaglandin-like substances are required for

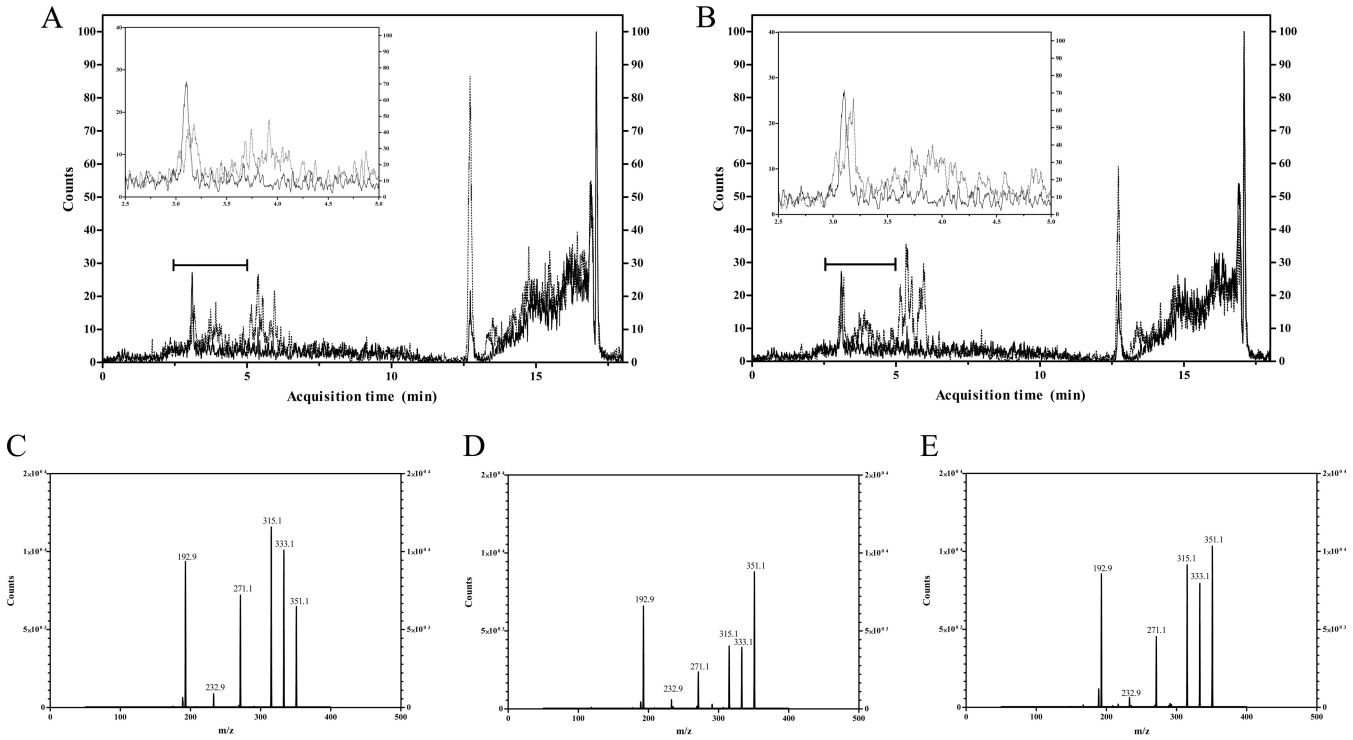

FIG 3 PGE_2 production by *S. parasitica*. (A and B) Chromatogram for 500 pg of standard PGE_2 (full lines) and extracted eicosanoids from CBS223.65 (A) and NVI02736 (B) culture supernatants (dashed lines) after overnight incubation with 500 μM arachidonic acid. Inserts depict in further detail the elution time of PGE_2 at approximately 3 min. (C, D, and E) MS/MS results for PGE_2 parental ion (*m/z* 351.1) from standard PGE_2, CBS223.65, and NVI02736 culture supernatants, respectively.

mycelial growth of *Achlya* and *Saprolegnia* species (21). A survey of the *S. parasitica* genome database (Broad Institute) revealed enzymes that could be involved in the production of prostanoids: a putative phospholipase (SPRG_10331) and a putative prostaglandin E_2 synthase (SPRG_13907).

Although SPRG_10331 shows low identity (approximately 30%) with the *Salmo salar*-secreted phospholipase A_2 (PLA_2; GI 209737974), it presents several characteristics of a secreted phospholipase, including a signal peptide, 14 cysteine residues, and the active site canonical sequence C-C-{P}-X-H-{LGY}-X-C-Y (24) (Fig. 4A). The avirulent strain CBS223.65 and the highly pathogenic strain NVI02736 express this phospholipase during normal growth (data not shown) and during infection of rainbow trout head kidney leukocytes (Fig. 4B).

No enzyme homologous to cyclooxygenases could be found in the *S. parasitica* genome (data not shown), but by interrogating the genome with the zebrafish prostaglandin E synthase (GI 41053638), a single gene was identified: SPRG_13907. Although this gene presents an overall low identity with the teleost PGES (approximately 30%), it harbors the conserved active site C-X-X-C (25), and its C-terminal region shows high identity to the teleost enzyme (Fig. 5A). This putative PGES is also expressed by the strains CBS223.65 and NVI 02736 during normal growth (data not shown) and during infection of rainbow trout head kidney leukocytes (Fig. 5B).

Aspirin and indomethacin, two well-known COX inhibitors, were tested for their capacities to inhibit zoospore germination and mycelial growth. Two strains of *S. parasitica* (CBS223.65 and NVI02736) and a strain of *Saprolegnia diclina* (VS20), a salmonid egg parasite, were studied. All three strains were susceptible to both inhibitors, with similar sensitivities (Table 1). Aspirin inhibited germination of the three isolates at a minimal concentration of 3.75 mM; indomethacin was effective at 0.125 mM against CBS223.65 and NVI20736, while 0.250 mM was required for inhibition of the germination of strain VS20. Mycelial growth was inhibited by aspirin at a minimal concentration of 1.875 mM for the *S. parasitica* species, while 3.75 mM was required for inhibition of *S. diclina*. Indomethacin was inhibitory for mycelial growth at a minimal concentration of 0.125 mM for the three strains tested.

Immune response to cell wall carbohydrate components of *S. parasitica* and exogenous PGE_2. The pathogen cell wall is typically one of the first points of contact with the host immune system. To assess the role of oomycete cell wall carbohydrates on the activation of the fish immune system, we utilized the AIF from the *S. parasitica* cell wall, which consists essentially of cellulose and β-glucans with 1,3 and 1,6 linkages (16), to stimulate rainbow trout HK leukocytes *in vitro* for 6 h and then measured the expression of selected immune genes. The 27 genes previously analyzed were assessed also in this experiment, together with MX-2 and γIP, two genes that are modulated by IFN-γ, and IL-17A/F_1 and IL-17A/F_3, recently identified paralogues of salmonid IL-17A/F (T. Wang et al., unpublished data). Where a response was observed, there was typically a dose effect, with the lowest concentration used (0.001 mg ml^{-1}) having no effect or a smaller effect than the other (higher) doses (Fig. 6A). Incubation with the cell wall carbohydrates caused a strong induction of inflammatory markers (IL-1β_1, IL-6, IL-8, TNF-α_2, and COX-2) that was similar

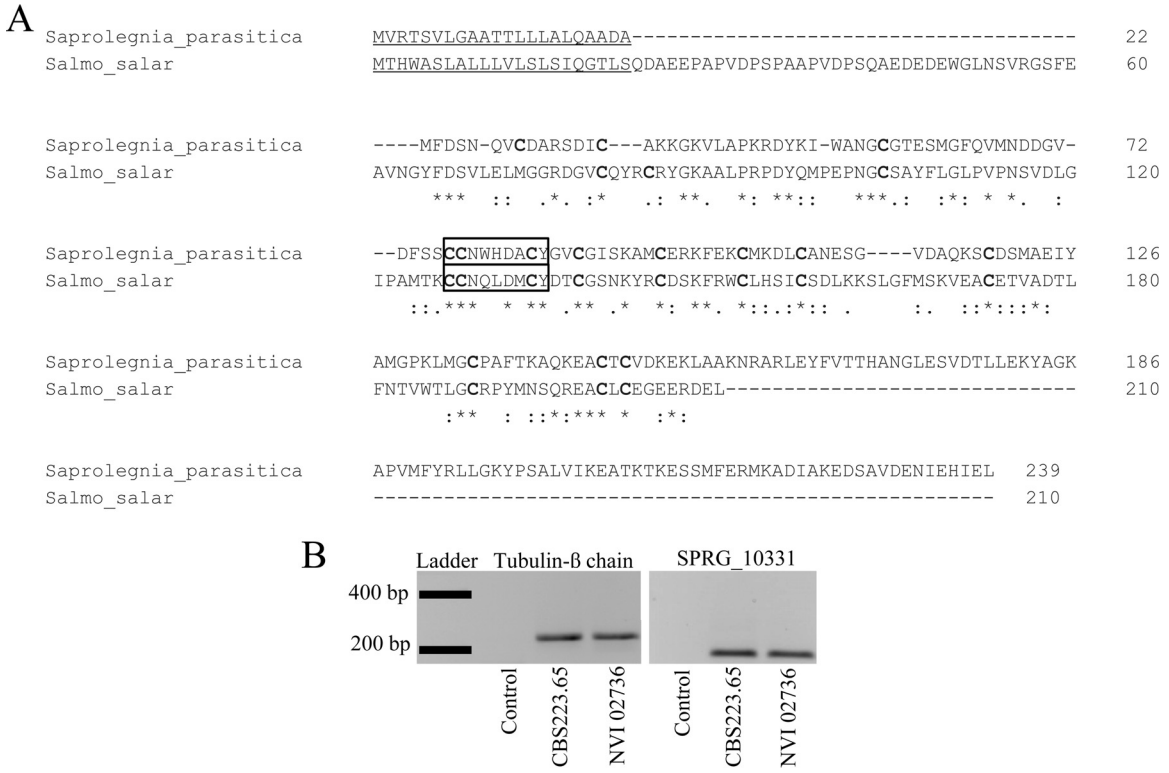

FIG 4 Putative phospholipase from *S. parasitica*. (A) Alignment of the putative oomycete phospholipase (SPRG_10331) with the sequence for *S. salar* PLA_2 (GI 209737974). Underlined, signal peptide; boxed, active site; boldface, cysteine residues. (B) Reverse transcription-PCR (RT-PCR) results for rainbow trout head kidney leukocytes after 24 h of *in vitro* infection with strain CBS223.65 or NVI02736. Uninfected cells were used as controls for the RT-PCR. The mRNA for SPRG_10331 was detected in both strains.

to that observed during the *in vivo* challenges. IL-10 family members (IL-10_a and IL-22) were also induced, as were IL-17A/F_1 and IL-$17C_1$ and, to a lesser extent, RORγ and cathelicidin 1. No changes in the expression of IL-4/13A, IFN-γ_1, MHC-I and –II, m/sIgM, and m/sIgT were detected, in contrast to the suppression that was observed during the infection process. However, γIP was significantly downregulated at all carbohydrate concentrations tested.

PGE_2 was used to study the effect of exogenous prostaglandin on the immune response of rainbow trout HK leukocytes. After a short exposure time (6 h), all the concentrations used in the experiment were capable of causing significant changes for at least one of the genes analyzed. Overall, the incubation of HK leukocytes with PGE_2 induced the expression of IL-6, IL-10_a, and COX-2 while suppressing the expression of IFN-γ_1, TNF-α_2, mIgM, m/sIgT, and γIP (Fig. 6B), somewhat similar to the effect caused by *S. parasitica* infection *in vivo*. No changes were detected for most of the other genes analyzed (IL-$1\beta_1$, IL-4/13A, IL-8, IL-17A/$F_{1,2,3}$, IL-$17C_1$, IL-21, IL-22, T-bet, GATA3, RORγ, MHC-I and -II, sIgM, m/sIgD, cathelicidin 1 and 2, β-defensin 3, hepcidin, IRF-1, and MX-2).

A coincubation assay was designed to test whether the variations in expression observed during oomycete infection could be mimicked. For this purpose, a low dose of cell wall carbohydrates (0.001 mg ml^{-1}) was added to the cells either simultaneously with 100 μM or 0.1 μM PGE_2 or after a 2-h preincubation with the prostanoid. The combination of glucan with PGE_2 triggered a stronger induction of genes associated with the inflammatory response than that seen with these compounds individually (Fig. 7). A significant difference in gene expression levels of IL-6, IL-8, IL-17A/F_1, and COX-2 was detected in samples that received both components. The coincubation also suppressed the expression of IFN-γ_1 and γIP. Preincubation with the prostaglandin did not cause any significant additional changes compared to the same dose simultaneously applied with the cell wall carbohydrates.

DISCUSSION

The suppression of the immune response of vertebrate hosts during oomycete infection, although suggested in the literature (8, 13, 26, 27), has never been clearly demonstrated *in vivo*. Our results reveal the immune response of salmonids to oomycete infection after exposure to *S. parasitica*. In agreement with *in vitro* data (12, 13), fish that are infected with *S. parasitica* show a very strong induction of proinflammatory cytokines and antimicrobial peptides (AMPs), both locally, as observed in gill samples, and systemically, as this induction is also detected in HK samples. The AMP cathelicidin 2 has been shown previously to exert an inhibitory activity against *S. parasitica* maturation, and the induction of such molecules implies a role of AMPs in the first line of defense against oomycetes (12). Although strong, this combinatorial response is not sufficient to clear the infection, as affected fish succumb to the pathogen if no treatment is provided. The failure of the immune system to respond adequately can be explained by the severe suppression of several components of adaptive immunity seen in this study. Besides the suppression of cytokines associated with T-helper cell responses, many genes associated with antigen

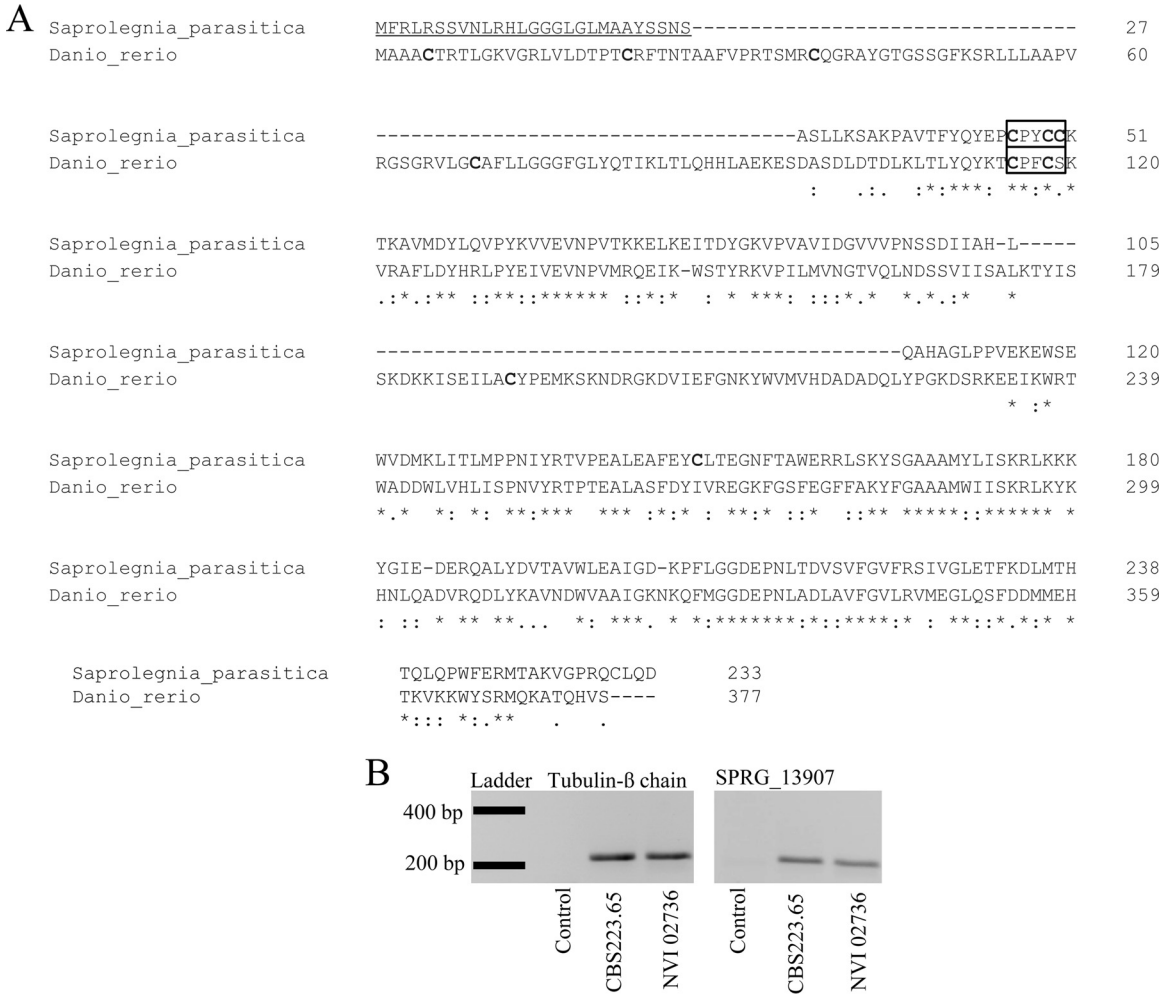

FIG 5 Putative PGE_2 synthase from *S. parasitica*. (A) Alignment of the putative oomycete PGE_2 synthase (SPRG_13907) sequence with the sequence for *Danio rerio* PGE synthase (GI 41053638). Underlined, signal peptide; boxed, active site; boldface, cysteine residues. (B) Reverse transcription-PCR (RT-PCR) results for rainbow trout head kidney leukocytes after 24 h of *in vitro* infection with strain CBS223.65 or NVI02736. Uninfected cells were used as controls for the RT-PCR. The mRNA for SPRG_13907 was detected in both strains.

presentation were found to be expressed at lower levels during infection. A similar effect on the host has been observed in the case of the amoebic parasite *Neoparamoeba perurans*, the agent of amoebic gill disease, where the same components of the antigen presentation machinery are suppressed in infected tissues (MHC-I and -II, β2-microglobulin, TAPBP, and PA28β) (28). Another pathogen with the capacity to suppress the fish immune system is the salmon louse *Lepeophtheirus salmonis* (29, 30). In this case, the parasite saliva contains biologically active molecules, such as PGE_2, that suppress the host immune response (31). Immunoglobulin production is also impaired in infected fish, including the mucosa-specific IgT, which plays an important role in defense against pathogens that use this route to establish an infection (32).

TABLE 1 MICs of aspirin and indomethacin for *Saprolegnia* germination and growth

Life stage and strain	MIC (mM)	
	Aspirin	Indomethacin
Zoospores		
S. parasitica CBS223.65	3.750	0.125
S. parasitica NVI 02736	3.750	0.125
S. diclina VS20	3.750	0.250
Mycelia		
S. parasitica CBS223.65	1.875	0.125
S. parasitica NVI 02736	1.875	0.125
S. diclina VS20	3.750	0.125

Infected fish have a similar expression profile as those exposed to the oomycete for 3 days when no mycelial growth is visible. This indicates that the immune suppression occurs either before the establishment of the pathogen or at very early stages of this interaction and persists during the course of the disease. Another interesting finding was obtained from fish that were exposed to *S. parasitica* for 12 days and did not show any signs of infection, where induction of cytokines of adaptive immunity was observed and no suppression of the antigen presentation machinery was detected. These fish also showed no induction of proinflammatory genes. Some of these latter genes were actually found to be

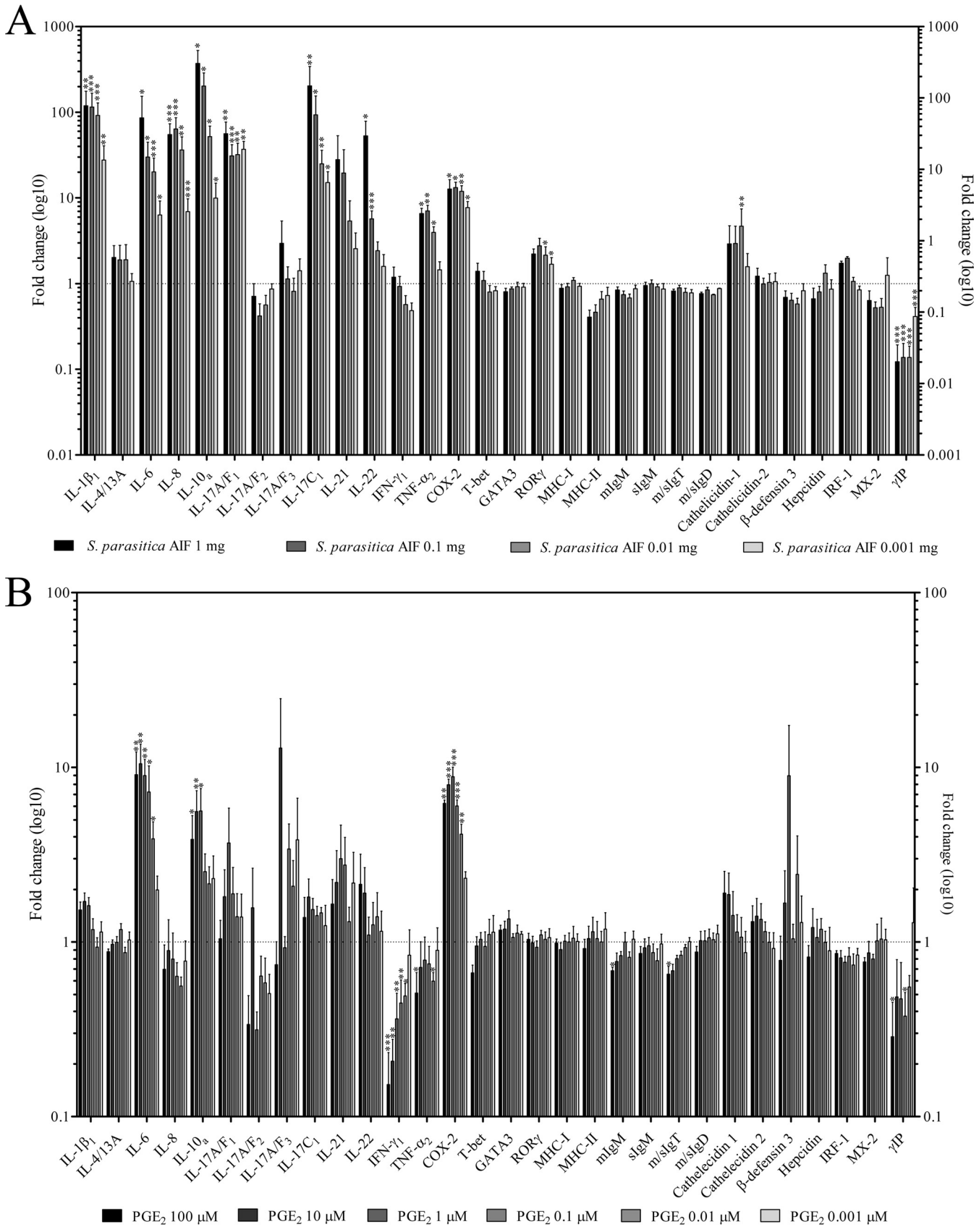

FIG 6 Immune response of rainbow trout head kidney leukocytes to *S. parasitica* cell wall carbohydrates or PGE_2. (A) Fold change (average + standard error of the mean [SEM]) of immune gene expression levels, quantified by real-time PCR after 6 h of incubation with 1, 0.1, 0.01, and 0.001 mg ml^{-1} of *S. parasitica* cell wall carbohydrates. (B) The fold change (average + SEM) of immune gene expression levels, quantified by real-time PCR after 6 h of incubation with 100, 10, 1, 0.1, 0.01, and 0.001 μM PGE_2. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

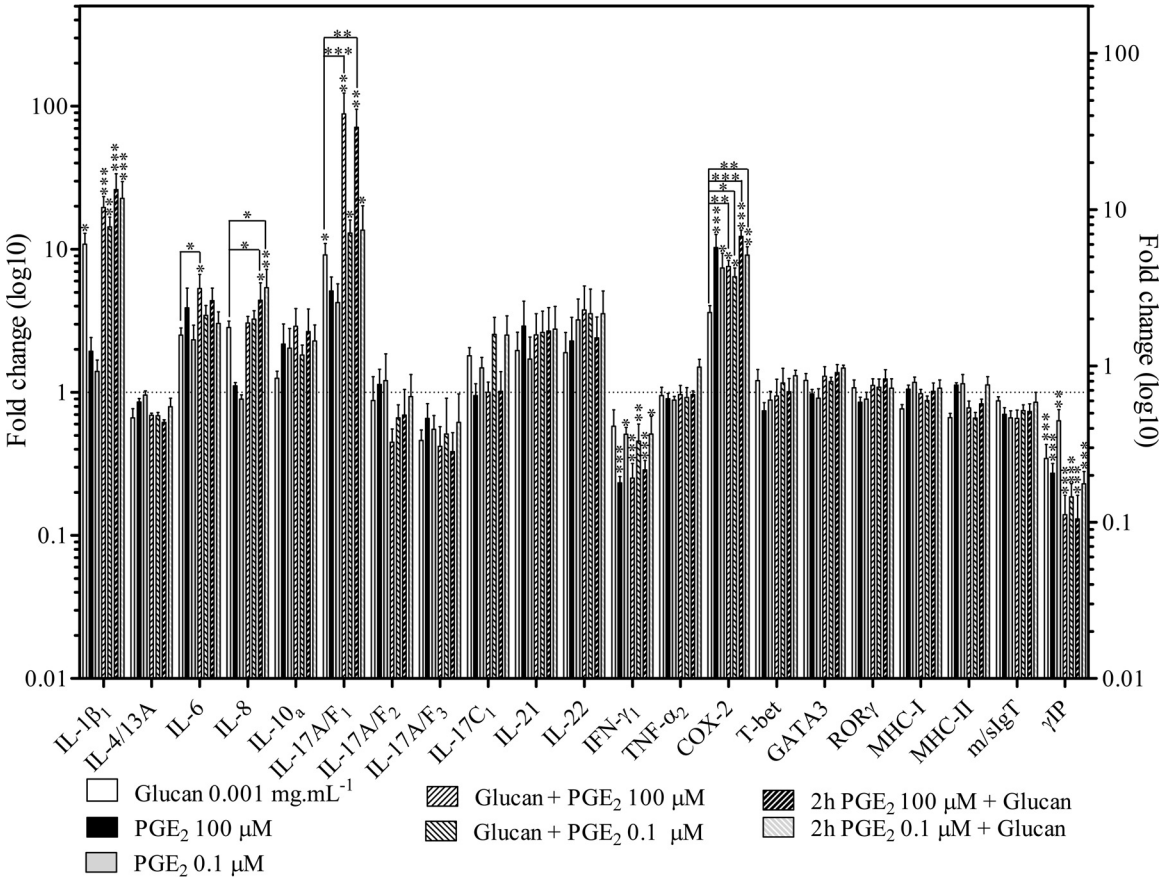

FIG 7 Immune response of rainbow trout head kidney leukocytes to *S. parasitica* cell wall carbohydrates and PGE_2. Head kidney leukocytes were incubated with 0.001 mg ml^{-1} of the cell wall AIF sample and either 100 μM or 0.1 μM PGE_2 simultaneously (white hashed bars) or after 2 h of preincubation with the prostaglandin (black and gray hashed bars). The data are presented as the fold change (average + standard error of the mean) of immune gene expression levels quantified by real-time PCR after 6 h of incubation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

suppressed (IL-1β_1, IL-6, and TNF-α), perhaps indicating the beginning of a Th2 response. It is not possible to determine if these fish were capable of mounting an efficient immune response or if the pathogen failed to infect the host due to some underlying cause. Nevertheless, these fish presented an immune profile completely different from that seen in infected fish and the findings suggested that IL-4/13A and IFN-$\gamma_{1/2}$ could be associated with resistance to saprolegniosis. IL-4/13A is highly expressed in the thymus, skin, and gills of salmon, and this has been associated with a role in early development of T cells, similar to that observed in mice, where IL-4 is a major Th2 response cytokine (33). Fish IFN-γ has several functional properties in common with mammalian IFN-γ, including the ability to enhance respiratory burst activity, nitric oxide production, and phagocytosis of bacteria by macrophages (34–36). The role of these two cytokines during infection by *S. parasitica* needs to be further studied, but our data suggest they could be interesting markers for saprolegniosis resistance.

In vertebrates, prostanoids, including prostaglandins and thromboxanes, are synthesized from arachidonic acid by the action of COX (37). The synthesis of prostaglandin-like molecules by microbes has been widely reported (38), and although previous publications suggested the presence of eicosanoid metabolism in oomycetes (21, 22), to our knowledge no clear evidence for the production of prostaglandins by these organisms has been presented. In this study, we were able to detect PGE_2 being produced *in vitro* by two strains of *S. parasitica*. A phospholipase-like enzyme (SPRG_10331) and a prostaglandin-like synthase (SPRG_13907), homologous to vertebrate proteins involved in the prostaglandin biosynthesis pathway, were also discovered in the oomycete genome and found to be expressed during normal growth and infection *in vitro*. Homologues of these two proteins are also found across many oomycete species, indicating the presence of eicosanoid metabolism across the oomycetes. While in higher vertebrates prostanoids play a vital role in mediating inflammation and many cell-cell signaling events (37), there is no available information on the function of these molecules in oomycetes. However, they appear to be important metabolites, since we found that cyclooxygenase inhibitors prevented hyphal growth and zoospore germination of *S. parasitica*, even in the absence of a clear COX homologue. This apparent paradox has also been reported in true fungi, such as *Candida* spp., where although no COX homologue is known, COX inhibitors prevent PGE_2 production, the yeast-hypha transition, yeast and hyphae growth, and biofilm formation (23, 39–42). The exact role of these lipids in oomycete biology needs to be further addressed, as off-target effects of aspirin and indomethacin cannot be ruled out at this point.

Oomycetes and fungi have biochemically distinct cell walls. Different from the chitin-based scaffold that is found in true fungi (43), oomycetes contain very small amounts of this carbohydrate (<5% for *S. parasitica* [16]) but have a high proportion of cellulose and other glucans (16, 44). Derivatives of fungal cell walls are regularly used as immune stimulants in aquaculture and can trigger the activation of the immune system and increase resistance to infections (45), but no information is available regarding the immunogenicity of glucans originating from oomycetes. The interaction between the host and the oomycete is likely to start with the recognition of the *Saprolegnia* cell wall, as it is this component of the pathogen that is in direct contact with fish tissues. Hence, we assessed the response *in vitro* of rainbow trout HK leukocytes to various concentrations of cell wall-derived glucans from *S. parasitica*. The oomycete cell wall glucans were recognized by the leukocytes, and this triggered a strong inflammatory response similar to those described for zymosan and β-glucan from fungal sources (46). Antimicrobial peptide expression was insensitive to the stimulus, which contrasts with the strong induction that was observed *in vivo* during infection.

In salmonids, PGE_2 is reported to exert an immunosuppressive action (47–49), as confirmed by our *in vitro* assay, where a short exposure to PGE_2 caused a moderate inflammatory type of response, with suppression of trout IFN-γ_1, m/sIgT, mIgM, and γIP transcription, possibly indicating an impairment of adaptive responses. When cells were exposed to PGE_2 and *S. parasitica* cell wall-derived glucans, the suppression of IFN-γ_1 and γIP was maintained and a stronger induction of proinflammatory genes occurred, as seen with IL-6, IL-8, IL-17A/F_1, and COX-2. This profile is more similar to the one observed during *in vivo* infection relative to the responses triggered by the glucans or prostanoid individually.

In conclusion, our investigations provide compelling evidence for active suppression of fish adaptive immunity during infection by *S. parasitica*. This suppression occurs before host colonization and persists during the course of infection. Fish that did not show disease symptoms 12 days postchallenge appeared to initiate a Th-2 like response, and this might be protective against the oomycete. We have provided sound evidence that *S. parasitica* can produce and secrete PGE_2, which has not been reported before for any oomycete. Putative enzymes involved in prostaglandin biosynthesis were identified in the *S. parasitica* genome by homology searches. With homologues found across several oomycete species, and expression seen during *in vitro* growth and infection, eicosanoid metabolism appears to be necessary for oomycete growth, as inhibitors of this pathway (aspirin and indomethacin) prevent zoospore germination and mycelial growth. Cell wall carbohydrates of *S. parasitica* are quickly recognized by fish leukocytes, triggering the induction of proinflammatory genes. When combined with *S. parasitica* cell wall carbohydrates, the addition of exogenous PGE_2 to rainbow trout leukocytes increases the proinflammatory response while suppressing IFN-γ_1, m/sIgT, and mIgM, partially mimicking the immune profile seen during infection.

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