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Effects of a *Pseudomonas* H6 surfactant on rainbow trout and *Ichthyophthirius multifiliis: In vivo* exposure

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ABSTRACT

The *Pseudomonas* H6 lipopeptide is a surfactant which is able to eliminate various parasitic pathogens including the ciliate *Ichthyophthirius multifiliis in vitro*. This suggests an application for aquaculture purposes. However, further information on efficacy of the compound and possible immune modulation of surfactant exposed fish should be gathered before usage at farm level is considered. We performed an *in vivo* infection experiment using rainbow trout fry (mean weight 4.6 g, mean length 7.6 cm) as hosts and *I. multifiliis* theronts as the parasitic pathogen. We compared infection level, immune gene regulation and immune cell density in gills of 1) non-exposed control fish, 2) parasite exposed but untreated fish, 3) surfactant treated fish without parasite exposure, and 4) fish exposed both to parasites and surfactant. The surfactant concentration was 10 mg/L, the infection dosage 1000 theronts/fish and the exposure period 12 h. The parasite infection was recorded and samples were taken from rainbow trout gills at day 0 and 10 post-exposure. We performed an immunohistochemical investigation (detecting cells positive for MHC II, SAA, CD8, IgM, IgT and IgD) and measured the expression of genes encoding cathelidin-1, CD8, hepcidin, IFN γ, IgDs, IL-1β, IL-6 and SAA. Theront exposed fish (without surfactant treatment) became heavily infected whereas concomitant surfactant treatment (10 mg/l), along with parasite exposure, could prevent infection. A significant inflammation (upregulation of *il-1β, il6, ifn γ, cathelicidin, hepcidin*) was elicited in non-treated and parasite exposed fish but it was prevented by the surfactant treatment. When investigated 10 days after treatment no immune gene regulation was seen in fish exposed to surfactant only. The therapeutic effect may be due to a direct parasiticidal action of the surfactant, but it cannot be excluded that a modulation of the host immune reaction may influence the infection success.

1. Introduction

Parasitic infections of aquacultured fish may be controlled by application of a range of biocides and auxiliary substances (Lieke et al., 2020). Since the ban of malachite green treatments in farm settings was introduced more than 30 years ago, infections with ectoparasites such as amoebae (Dyková et al., 2010), flagellates (Chettri et al., 2014) and ciliates have accelerated the usage of formalin (Pedersen et al., 2007), hydrogen peroxide, sodium percarbonate (Buchmann et al., 2003; Heinecke and Buchmann, 2009; Jaafar et al., 2013) and peracetic acid (Meinelt et al., 2009; Straus and Meinelt, 2009; Bruzio and Buchmann, 2010; Jussila et al., 2011; Pedersen et al., 2013). However, due to the adverse reactions induced in the treated fish by these compounds a high demand for alternative and sustainable methods in disease control have been noted. Thus, formalin may disorganize the epidermis in trout skin (Buchmann et al., 2004), hydrogen peroxide elicit injuries in fish surfaces (Polinski et al., 2013; Chalmers et al., 2018; Jia et al., 2021) and peracetic acid may be lethal to the exposed fish (Straus et al., 2012; Liu et al., 2017; Soleng et al., 2019). With the discovery that a surfactant, a lipopeptide, produced by the bacterium *Pseudomonas* H6 can eliminate oomycetes such as *Saprolegnia* (Liu et al., 2015), external stages of the white spot disease agent *Ichthyophthirius multifiliis* (Al-Jubury et al., 2018) and amoebae (Jensen et al., 2020) aquaculturists have requested further information on the applicability of the compound. The studies conducted up until now have documented the antiparasitic effects by *in vitro* experiments and it is therefore relevant to investigate if the

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compound will act *in vivo* in combination with both fish and parasite. This will also indicate if the novel therapeutant has adverse effects on the fish host in an infectious environment. We have therefore performed an experimental infection of rainbow trout with theronts of the white spot disease agent *I. multifiliis* and tested if concomitant treatment with the surfactant can prevent infection. We used a surfactant dosage of 10 mg/L over 12 h, a dosage indicated as effective *in vitro* (Al-Jubury et al., 2018). By applying immunohistochemical and immune gene expression methods we have elucidated if the compound could induce regulation of immune genes and cell composition in fish gills. We examined fish before exposure and 10 d post-exposure based on the fact that the parasite does not multiplicate in the host after the theront has successfully invaded the fish skin or gill and transformed into the trophont stage. The trophonts will escape from the fish after 11 days or more (dependent on temperature) but until then the trophont number in a fish after the infection is constant (Sigh and Buchmann, 2001). Gills were used for the molecular analyses as this organ is considered more sensitive than skin (Syahputra et al., 2019; Mathiessen et al., 2021). Skin and fins were then used for microscopical analyses of parasite level.

2. Methods

2.1. Parasites

Ichthyophthirius multifiliis were obtained from infected rainbow trout reared in a Danish commercial trout farm, Jutland, Denmark. For production of infective theronts, infected rainbow trout were euthanized by immersion into 300 mg/L MS222 (Sigma-Aldrich, Denmark), transferred to a plastic tray with tank water where trophonts over 4 h were allowed to escape the fish surface as tomonts. They were collected by pipette and incubated in a plastic tray containing 0.2-μm filtered tank water (22 °C), allowing their transformation into tomocysts which released theronts after 24–30 h. Theronts were collected and a suspension of theronts established. Theront density was recorded in subsamples by enumerating theronts in five droplets (20 μL) of the suspension on a glass plate (thickness 6 mm) with concave wells (diameter 25 mm, depth 3 mm, maximum water capacity 2000 μL) under a dissection microscope (40× magnification). A final concentration of 1740 theronts/mL was used for exposure of fish to an infection of 1000 theronts per fish (Sigh and Buchmann, 2001).

2.2. Pseudomonas H6 surfactant (PS)

An extract of the *Pseudomonas* H6 lipopeptide surfactant (in the following abbreviated SPH6) was prepared and lyophilized (Liu et al., 2015) and stored at −20 °C until initiation of experiments. In brief, bacteria grown on King's Medium B Base (KMB) agar plates were flooded by sterile demineralized water. The culture supernatant was sterile filtered and acidified (pH 2, HCl) whereafter the precipitate was dissolved in sterile distilled water. The pH was adjusted to pH 8 by use of NaOH. Following lyophilization and storage at −20 °C a stock solution of 2 mg/mL was prepared by dissolving 80 mg of the lyophilized surfactant in 40 mL sterile distilled water followed by 15 min stirring. Thereafter 7.5 mL stock solution was added into each fish tank containing 1.5 L municipal water to reach 10 mg/L, the therapeutic concentration (Al-Jubury et al., 2018),which was used for exposure of five fish.

2.3. Fish

A total of 40 rainbow trout (mean size: body weight 4.6 g, body length 7.6 cm) hatched from disinfected eyed eggs originating from trout farm Hallesø (Jutland) and reared under disease free conditions at the Bornholm salmon hatchery, Nexø Denmark, were acclimatized for 14 d at the University of Copenhagen facility and used for the experiment. The fish were randomly allocated into 8 tanks containing 5 L municipal tap water at pH 7.6 and temperature 15 °C in a temperature controlled room. Fish were kept at a 12 h light/12 h dark cycle and fed commercial pelleted feed (INICIO 917, BioMar A/S) at a feeding rate of 1% of their body mass once daily. Water was continuously aerated and recycled by internal biofilters (AS2012, EHEIM, Germany) with a 50% water exchange daily to avoid ammonia accumulation.

2.4. Experimental design

Fish were randomly divided into four groups (each with two replicates of five fish): 1) non-treated negative control fish (No SPH6, No Infection), 2) exposed to both parasites and surfactant (Plus SPH6, Plus Infection), 3) exposed to surfactant only (Plus SPH6, No Infection), 4) exposed to parasites only (No SPH6, Plus Infection). During the exposure the water volume was reduced to 1.5 L. Duplicate groups were applied in order to confirm absence of tank effects and the number of fish (2 × 5) was needed to secure statistical sound analyses. The surfactant was used at a final concentration of 10 mg/L, and each fish was exposed to theronts (Sigh and Buchmann, 2001). The exposure period was 12 h whereafter the fish were relocated to similar sized fish tanks with a water volume of 5 L. Ten days post infection/exposure, when small trophonts (white spots) were visible in the surface of infected fish, fish were euthanized by 300 mg/L Ms-222 (tricaine methane sulphonate, Sigma-Aldrich, Denmark) and samples were taken.

2.5. Parasite infection

Infection success was evaluated by microscopy and molecular tools. The number of white spots (trophonts) in the fish skin epidermis and the fins was counted on each fish at 10 days post-challenge (dpc) by use of a dissection microscope (magnification 40 x, Leica, Germany). The infection recorded visually was further confirmed by measuring transcripts of the gene encoding the parasite i-antigen IAG52A in gill samples (see section 2.8 on gene expression analysis). The number of parasites in the gills were not enumerated by microscopy because this procedure (manipulation) could induce regulation of one or more genes. The microscopical parasite enumeration of skin and fins were thereby supplemented the molecular parasite estimation in the gills.

2.6. Sampling organs

The gills on the left side of the fish were fixed for immunohistochemistry (IHC) and gills on the right side were preserved for quantitative realtime PCR assays (qPCR). The gill samples to be processed for IHC were fixed in 4% neutral formaldehyde (24 h at 4 °C) and then transferred to 70% EtOH until processing. Gill samples for qPCR were immediately after excision preserved in RNAlater (R0901, Sigma-Aldrich, Denmark) and incubated at 4 °C overnight and then stored at −20 °C until further use.

Samples of skin and fins were only taken for microscopy and not taken for molecular analysis because the counting procedure under the microscope could bias the qPCR. Gills were used for gene expression analyses due to their higher sensitivity to stimulation (Syahputra et al., 2019).

2.7. Immunohistochemistry (IHC)

The gill samples were dehydrated through a series of increasing ethanol (70%, 85%, 96%, and 99.9%), cleared in two changes of xylene substitute and embedded in paraffin. Gills were then cut into 4 μm sections on a microtome (Leica RM2135, Leica Microsystems, Germany) and collected on adhesive microscope slides which subsequently were dried for 24 h at 30 °C. After deparaffinizing in xylene substitute and rehydration in decreasing grades of ethanol, slides were incubated in Tris Buffered Saline (TBS) and then transferred to 1.5% H_2O_2 in TBS for 10 min (to quench endogenous peroxidase activity) followed by 5-min

washing with tap water. To retrieve antigens, the slides were boiled in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0) for 5 × 3 min in a microwave oven, cooled at room temperature for 1 h and then transferred to TBS at room temperature. Slides were blocked by 2% BSA in TBS, and then incubated at 4 °C overnight with primary antibody MHC II 1:2000, SAA 1:5, CD8 1:200, IgM 1:400, IgT 1:300 and IgD 1:10000 (Table 1) as described by Chettri et al. (2014). For negative controls, incubation with 1% BSA in TBS with no primary antibody was used. Isotype controls were used for validation (Olsen et al., 2011; Chettri et al., 2014). Following incubation, unbound primary antibody was rinsed off by 5 min incubation in TBS at room temperature, and the tissue was covered for 30 min with anti-mouse EnVision System HRP (K4001, Dako, Denmark) as the secondary antibody. Then the sections were rinsed by 2 min in TBS and 2 min in tap water, whereafter the positive cells were visualized by incubating slides in AEC substrate (Sigma-Aldrich, USA) for 30 min followed by tap water washing for 5 min. All slides were counterstained with Mayer's hematoxylin (Dako, Denmark) for 3 min and subsequently mounted in water soluble mounting medium Aquamount (Merck, UK). The slides were examined using a Leica DMLB microscope (Leica Microsystems, Germany) and photomicrographs taken with Leica CD 300 (Leica Microsystems, Germany). The density of positive cells was counted based on the photomicrographs. For each fish the number of positive cells, located in the epithelial lining of gill lamellae, was counted from 30 lamellae (five pairs of lamellae from three gill filaments).

2.8. Gene expression analysis

Gills were homogenized by incubating in homogenization buffer with 2-mercaptoethanol (Sigma-Aldrich) on a TissueLyser II (Qiagen, USA) for 6 min, after which total RNA was extracted using the GenEluteTM mammalian RNA kit (RTN350-KT, Sigma-Aldrich) according to the manufacturer's instruction and subsequently treated with DNase I (Cat. No. EN0521, Thermo Scientific, USA). Total RNA concentration and purity were determined applying a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and the quality was assessed using 2% agarose gel electrophoresis. The extracted RNA was kept at −80 °C until cDNA synthesis in the T100™ Thermal Cycler (BioRad, USA) using 20 μl reaction volume with 1000 ng of RNA, oligo d(T)16 primer and TaqMan® reverse transcription reagents (N8080234, Thermo Fischer Scientific, Denmark) (25 °C for 10 min, 37 °C for 60 min, 94 °C for 10 min). The synthesized cDNA was diluted ten times using RNase free water (10,977,049, Thermo Fischer Scientific, Denmark) and subsequently stored at −20 °C until Quantitative PCR assays which were performed in an AriaMx Real-Time PCR system (Agilent Technologies, USA) in a 12.5 μL total reaction volume contained 2.75 μL DNase/RNase free water, 6.25 μl of Brilliant® II QPCR master mix (Agilent Technologies, USA), 1.0 μL primer mixture (forward and reverse primer 10 μM each, TaqMan® probe 5 μM) and 2.5 μl cDNA template (5 ng/μL). Primers and corresponding TaqMan probes (synthesized at TAG Copenhagen AS, Denmark) for a total of 26 immune-related genes of rainbow trout are shown in Supplementary material Fig S1. Besides, the gene encoding the *Ichthyophthirius multifiliis* IAG52A I-antigen was also quantitatively analyzed to estimate the parasite burden according to Jaafar et al. (2020). Negative controls without template and reverse transcriptase minus were set for every plate to detect contamination during assays. The qPCR reactions were carried out as the following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 5 s; 60 °C for 15 s. Genes encoding elongation factor (ELF) 1-α, β-actin and ARP were used as endogenous control (reference genes).

Table 1
Monoclonal antibodies used for immunohistochemical analysis. Reactivity, dilution and origin.

Antibodies	Against	Dilution	Reference
SaSa MHC II beta (F1–6)	Beta 2 domain of MHC II beta chain in Atlantic salmon	1:2000	Hetland et al. (2010)
Onmy SAA (F1–4)	of SAA in rainbow trout	1:5	Chettri et al. (2014)
SaSa CD8 (F1–29)	The membrane distal Ig-like domain of CD8 alpha chain in Atlantic salmon	1:200	Hetland et al. (2010)
IgM (F1–18)	Anti-trout IgM	1:400	Jørgensen et al. (2011)
Onmy IgT (F1–8)	The second constant domain of IgT heavy chain in rainbow trout	1:300	Olsen et al. (2011)
IgD	Anti-trout IgD	1:10000	Ramirez-Gomez et al. (2012)

2.9. Data analysis

2.10. Ethics

The experiment was performed and approved by the Experimental Animal Inspectorate, Committee for Experimental Animals, Ministry of Environment and Food, Denmark under license no. 2019-15-0201-00388 and followed the ethical guidelines of the University of Copenhagen.

3. Results

3.1. Infection parameters

The number of white spots (trophonts), in the fish skin and fins at 10 dpc, was confirmed visually under the dissection microscope. A mean of 40 trophonts per fish (range 10–140) were counted by microscopy in the surface of skin and fins of un-treated fish exposed to parasites. This infection was significantly higher than in fish exposed to theronts and surfactant treated at the same time (Student's *t*-test $P = 0.0098 < 0.05$) (Fig. 1A). The presence of parasites was further confirmed by measuring transcripts of the gene encoding the parasite i-antigen IAG52A in gill samples. The expression of the IAG52A gene in the gills was clearly detected in the exposed but non-treated fish (Fig. 1B). No expression of the IAG52A gene was detected in parasite exposed but surfactant treated fish as well as in the non-exposed control fish.Figs. 1 and 2

3.2. Immunohistochemistry (IHC)

3.2.1. Detection of MHC II+ cells

A significantly lower immune-reactivity was observed in the uninfected and surfactant treated fish compared to infected, non-surfactant treated fish (Fig. 2). In all samples, MHC II positive cells were localized along the epithelium of gill lamellae and in the inter-branchial septum between the filaments (the intraepithelial lymphoid tissue) (Fig. 3A-C). In addition, MHC II positive cells were concentrated at the sites where trophonts had penetrated (Fig. 3D) and elicited hyperplasia, lamellar fusion and clubbing of filaments. Thus, the infected but untreated fish (Fig. 2) displayed numerous MHC II immune-reactive cells encircling the *I. multifiliis* trophonts. In addition, reactivity was also detected within the parasite's food vacuoles (Fig. 3D).

3.2.2. Detection of SAA+ cells

SAA positive cells were rarely observed in the gills of control fish but a positive staining was scattered throughout the filaments and lamellae of parasite exposed but surfactant treated trout gills (Fig. 3G). In parasitized, non-surfactant treated trout gills, SAA staining was noted surrounding the trophonts (Fig. 3H). Significant differences were found between groups but the densities were low in the epithelial lining. However, when compared to un-infected groups, a significant difference in the number of SAA positive cells was seen both in infected, non-surfactant treated and infected, surfactant treated trout gills,

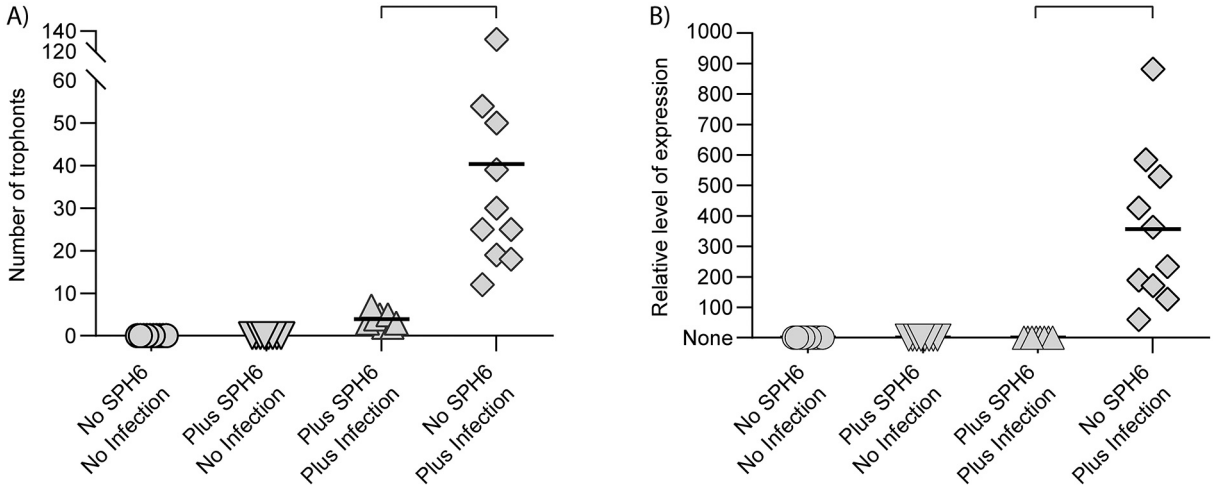

Fig. 1. *Ichthyophthirius multifiliis* burden in skin and fins of rainbow trout 10 days post infection. A: the total number of trophonts in the fish skin and fins (visual enumeration by microscopy). Bracket indicate significant difference between groups (Student's *t*-test, $p < 0.05$). B: Molecular estimation of the infection level of *I. multifiliis* in gills as represented by the i-antigen IAG52A gene transcript level relative to the reference gene expression (EF1α, Arp and β-actin).

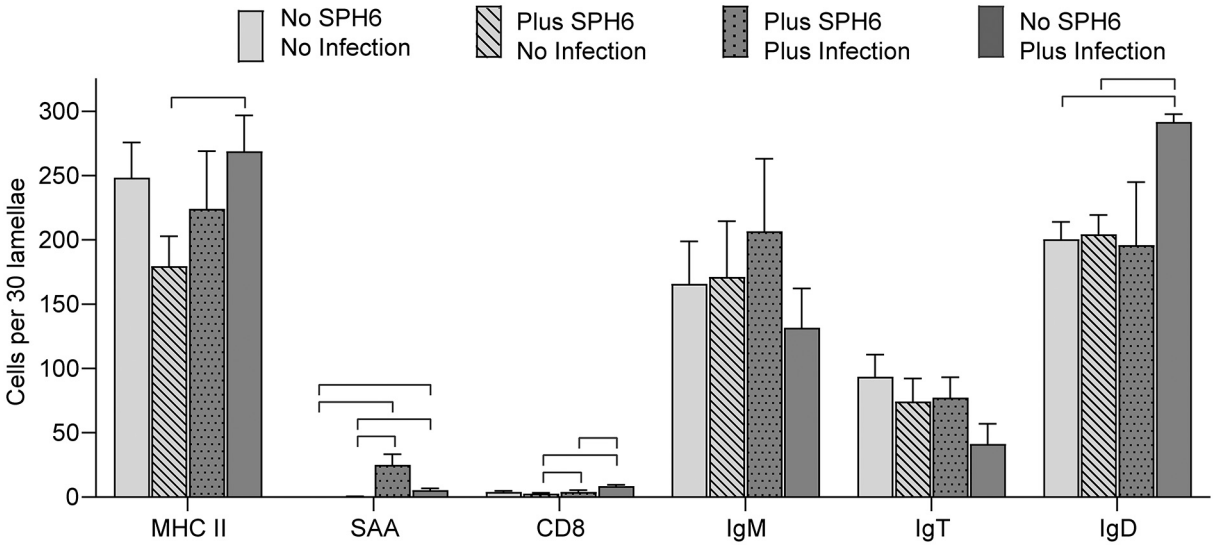

Fig. 2. IHC positive cells in gill tissue of rainbow trout presented as the total number of immuno-stained cells in 30 lamellae. Mean ± SD (6 replicates). Brackets above indicate significant differences between indicated groups (for MHC II, IgM, IgT, IgD, SAA, CD8). Brackets above columns indicate significant ($p < 0.05$) differences between groups.

respectively (Fig. 2).

3.2.3. Detection of CD8+ cells

CD8 positive cells were observed in low numbers in the filaments, lamellar epithelium and the intraepithelial lymphoid tissue of all fish but were frequent in the tissues surrounding the trophonts (Fig. 3I-L) and occasionally staining was discernable within some trophonts (Fig. 3L). Significant differences were found between groups (Fig. 2).

3.2.4. Detection of IgM

The IgM localization in sections was primarily confined to lymphocytes in the lamellar capillaries, especially at the distal portion of the lamellae (Fig. 3M-O). In a few cases epithelial staining was also detected in the epithelial lining (Fig. 3P) and in the intraepithelial lymphoid tissue (Fig. 3N). There were no significant difference in the distribution and abundance of IgM positive cells between treated groups (Fig. 2). However, when *I. multifiliis* trophonts were present in the gills of infected, non-surfactant treated trout a marked accumulation of IgM was found associated with the trophonts and noted in food vacuoles in the parasite (Fig. 3P).

3.2.5. Detection of IgT

IgT immunoreactivity was only located in the epithelioid cells covering the lamellae, filaments and interbranchial septum (Fig. 3Q-S). Numerous squamous pavement cells and rounded cells stained IgT positive (Fig. 3Q-S). No accumulation of IgT positive cells was seen around the parasites and inside the parasitic food vacuoles (Fig. 3T). The *I. multifiliis* infected but untreated trout gills tended to carry fewer IgT cells compared to the control fish although the difference was not significant (Fig. 2).

3.2.6. Detection of IgD

IgD positive cells were detected throughout the lamellae, filaments and intraepithelial lymphoid tissue (Fig. 3U-W). Besides, numerous IgD positive cells were surrounding the trophonts and within food vacuoles in the parasites in the infected, non-surfactant treated fish gills (Fig. 3X)

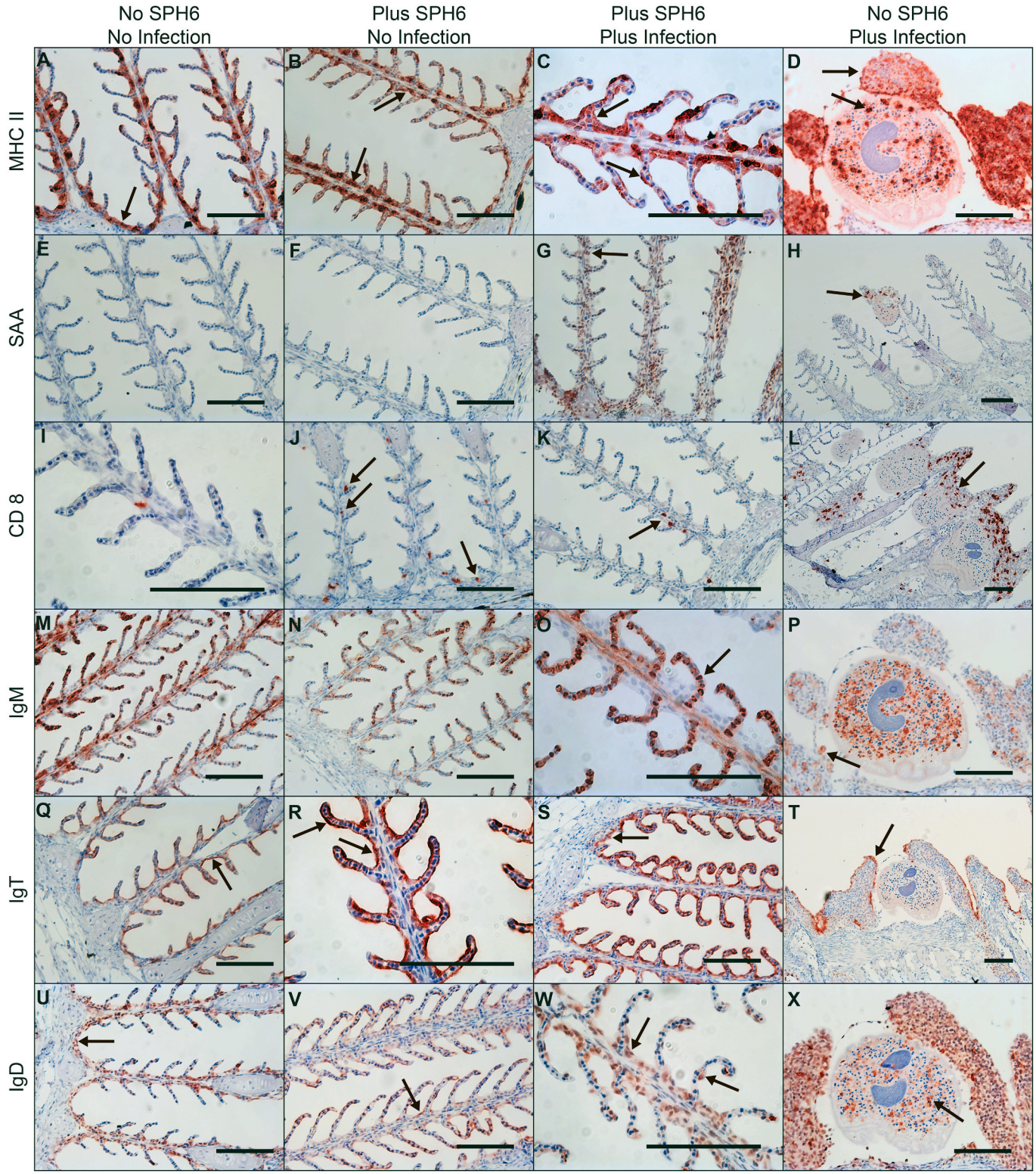

Fig. 3. Immunohistochemical localization of cells (stain reddish-brown) in the gills of rainbow trout 10 days post *I. multifiliis* infection and/or SPH6 treatment or non-exposed negative control fish. The four experimental fish groups are corresponding to the four columns. Each row represents the immune molecule detected by IHC. Arrows indicate representative examples of positive staining using the specific antibody. Scale bar: 100 μm in all micrographs. Magnifications: 100×: G, H, L, T; 200×: A, B, C, D, E, F, J, K, M, N, P, Q, S, U, V, X; 400×: C, I, O, R, W. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

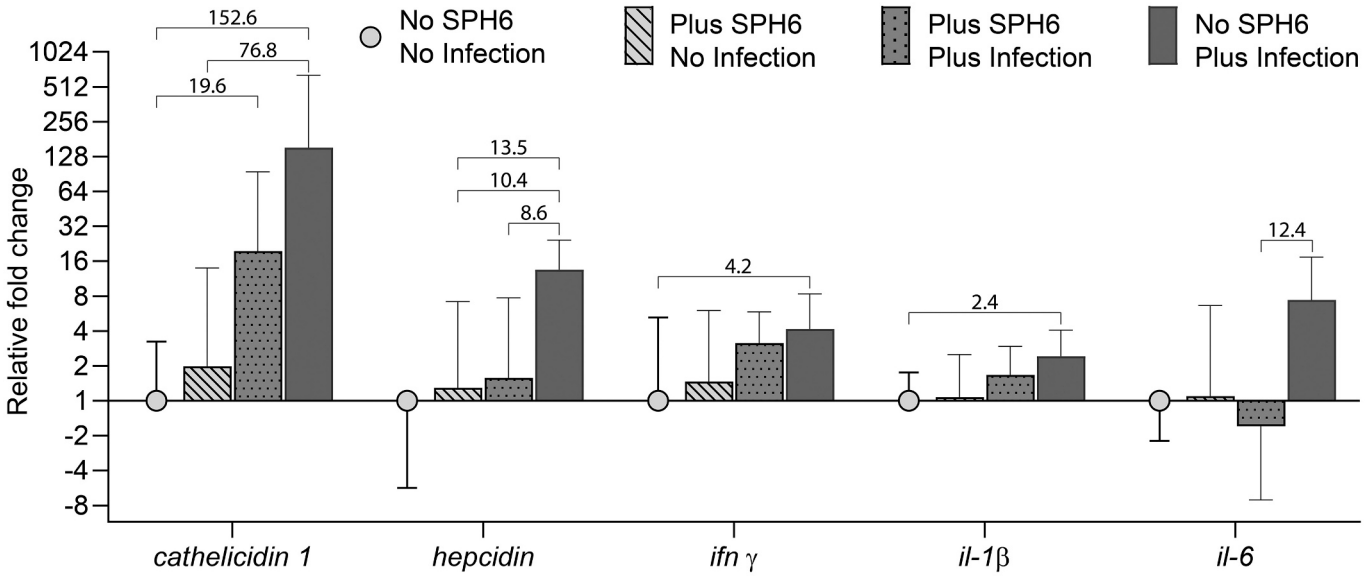

Fig. 4. Expression of immune-relevant genes in rainbow trout gills 10 days after exposure. Effects of SPH6 exposure alone and in combination treatment with *I. multifiliis* exposure. Brackets and the numbers above indicates significant and corresponding fold changes, respectively.

(Fig. 2).

3.3. Rainbow trout immune gene expression

The cathelicidin-1, hepcidin, IFN γ, IL-1β, IL-6 and SAA genes were only significantly upregulated in the infected but un-treated gills (Fig. 4). Expression of genes encoding the pro-inflammatory cytokine IL-1β and the effector molecule SAA was significantly higher in infected but untreated compared to infected and surfactant-treated fish. The expression of the inflammatory cytokine IL-6 gene was significantly lower in non-infected but treated gills compared to infected and non-surfactant treated gills. No significant regulation was detected for CD8 and IgDs expression with regard to treatments compared to the control. Downregulation of CD8 and IgDs genes were observed in the infected compared to the non-infected fish. Details of the qPCR investigations are found in Supplementary files S1 and S2.

4. Discussion

The surfactant produced by the bacterium *Pseudomonas* H6 has the ability to kill various pathogens *in vitro* including oomycetes, amoebae and ciliates (Liu et al., 2015; Al-Jubury et al., 2018; Jensen et al., 2020). The membrane of the target cell seems to be injured by the bacterial lipopeptide whereby the cytoplasmic constituents are released from the cell (Al-Jubury et al., 2018). This antiparasitic effect suggested that the product could find application for control of parasitic diseases in aquaculture. However, any future use depends on its efficacy, also under *in vivo* conditions, and that the effect on the fish host is negligible when compared to pathologies associated with the infection. We conducted a controlled study on infection with *I. multifiliis* theronts and treatment with the surfactant. It was found that a treatment with the surfactant in the concentration (10 mg/L) (based on *in vitro* studies suggested to be the therapeutic dosage (Al-Jubury et al., 2018), prevented infection of the fish concomitantly exposed to infective theronts. Only few trophonts established in the surfactant treated fish whereas non-treated fish, exposed to the same infection procedure, obtained a high infection (measured both by molecular and microscopical techniques). This suggests that the lipopeptide can kill the theront before it enters the surface (gills, fins, skin) of the fish, corroborating results from *in vitro* testing (Al-Jubury et al., 2018). The free-living infective theront, released from the tomocyst, is the infective stage in the *I. multifiliis* life cycle, and we here show that SPH6 treatment can at least partly control the infection. The direct influence of the surfactant on the rainbow trout, 10 days after exposure, was demonstrated to be absent or weak, as judged from our immune gene expression analyses and immunohistochemical studies. However, it cannot be excluded that the surfactant could modulate expression of other immune genes in skin or gills and thereby add to prevention of infection. It was previously demonstrated that the SPH6 surfactant did not induce regulation of selected immune genes at early time points (<24 h) post-treatment but the release of mucus from mucous cells in the fins was clearly induced by the compound (Mathiessen et al., 2021). We recorded that the *I. multifiliis* infection, in non-treated fish, induced a strong up-regulation of inflammation associated genes in the host gills, corroborating previous studies (Syahputra et al., 2019). In contrast, the fish treated at the same time as they were subjected to infection, merely obtained a low infection. The inflammation induced in the gills of these fish, as judged from expression of *il1β, il-6, ifnγ, cathelicidin and hepcidin,* was low to negligible.

Surfactants isolated from other bacteria within the genus *Bacillus* possess an ability to regulate inflammation-associated genes (Byeon et al., 2008; Giri et al., 2016; Devi et al., 2019), which suggests that bacterial surfactants may have an immunomodulatory effect (Zhang et al., 2015). However, the SPH6 applied in this study showed merely a weak and non-significant effect on rainbow trout when examined 10 days after treatment, as judged by qPCR and IHC. A previous study demonstrated an early effect (less than 24 h after exposure) of SPH6 treatment on mucous cell function in trout fins (Mathiessen et al., 2021). Further, it is noteworthy that we in this work found that the density of MHCII cells in gills were lower in surfactant treated fish compared to infected fish. Thus, it cannot be excluded that the surfactant activated antiparasitic immune mechanisms in the fish during the early exposure to surfactant and parasites. This could have contributed to the prevention of infection as we saw it both by microscopical and molecular investigations. Inflammatory reactions were associated with the parasite infection. This was previously described as regulation of genes encoding inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and interferon γ (INF γ) (Sigh et al., 2004; Titus et al., 1991; Syahputra et al., 2019; Paul et al., 2021) followed by acute-phase responses (Jenab et al., 2020). IL-1β is mainly produced by activated macrophages and may act as an initiator and driver of the inflammatory responses in fish (Gonzalez et al., 2007; Heinecke and Buchmann, 2013; Herath et al., 2016) and the gene *il1β* is upregulated in fish skin or gills when exposed

to pathogens (Heinecke and Buchmann, 2013; Lindenstrøm et al., 2003; Sigh et al., 2004; Neary et al., 2012; Dash et al., 2017; Moreira et al., 2017; Paul et al., 2021). The cytokine gene *il-6* is up-regulated in fish when exposed to various pathogens and the cytokine IL-6 itself initiates proliferation of macrophage in rainbow trout (Costa et al., 2011; Syahputra et al., 2019; Jaafar et al., 2020), catfish (Moreira et al., 2017) and rohu (Paul et al., 2021). Likewise, IFNγ induces production of macrophage-derived antimicrobials, nitric oxide and elicits respiratory burst (Zou et al., 2005; Arts et al., 2010; Grayfer et al., 2010; Peng et al., 2018). These cytokines are also associated with the induction of acute phase reactant release including Serum amyloid A protein (SAA) and hepcidin (Jørgensen et al., 2000; Costa et al., 2011; Cuesta et al., 2008). Extra-hepatic SAA production is involved in the local host defense against *I. multifiliis* (Gonzalez et al., 2007; Olsen et al., 2011; Chettri et al., 2014; Kovacevic et al., 2015). In this study the changes induced were not significant but we found the gene encoding hepcidin upregulated in infected fish. The molecule play a role in fish innate immunity by exhibiting antimicrobial activity (Hsieh et al., 2010) and by regulating iron homeostasis in the host during infection (Ganz, 2003; Hilton and Lambert, 2008; Xie et al., 2019). Cathelicidins are members of the AMP family, and are usually found in neutrophils and on mucosal surfaces where they display a broad spectrum of antimicrobial activities affecting bacteria, enveloped viruses and fungi (Zanetti et al., 2002; Chang et al., 2006). The gene *cathelicidin1* was the most significantly upregulated gene in response to *I. multifiliis* infection (152.6 and 19.6 folds increase in two infected group compared to the control) in our study. This supports data of Jaafar et al. (2020), who indicated this AMP as part of the first line defense in trout against *I. multifiliis*. The expression of IgDs and CD8 α + genes in infected (non-surfactant treated) trout was not regulated.

The immunomodulatory role of microbial surfactants on the cellular adaptive immune mechanisms is still controversial as both inhibition (Park and Kim, 2009; Gao et al., 2014) and enhancement (Gao et al., 2012; Xu et al., 2016) of adaptive immune molecules, such as CD8, CD4 and MHC II, have been presented. In addition, the VS16 biosurfactant, isolated from *Bacillus licheniformis,* enhanced immunoglobulin levels (Giri et al., 2017) but in the present study we did not find evidence that SPH6 induced any regulation of Ig genes. However, it should be framed that we examined fish at day 10 after treatment and it cannot be excluded that the SPH6 could have induced regulation shortly after exposure. We did not show any parasite-related regulation of IgM and IgT transcripts. However, these immunoglobulins classes were previously shown involved in the host reaction towards the parasite (Olsen et al., 2011; Heinecke and Buchmann, 2013; Syahputra et al., 2019; Jaafar et al., 2020), and we demonstrated (by IHC) that IgM and IgT molecules clearly were clustered around the developing trophont, as previously noted by Olsen et al. (2011) and Jørgensen et al. (2011).

The results presented here demonstrated that administration of the surfactant SPH6, by bath treatment, prevented infection of rainbow trout even when exposed to a high *I. multifiliis* infection pressure. This was reflected by the significant reduction of parasite load in biosurfactant treated fish. It may be explained by a direct parasiticidal effect of the compound as it can be speculated that the theronts were killed before they could penetrate the fish surface. The biosurfactant treatment, administered alone, did not trigger modulations of any of the genes investigated in uninfected trout when examined after 10 d. However, it cannot be excluded that early induction of innate immune genes after treatment could have contributed to the antiparasitic effect. In addition, other immune genes, which were not included in the present study, could be stimulated by the surfactant. Further studies should therefore include additional immune genes and elucidate a possible combined effect of SPH6 on immune modulation and direct killing of parasites by SPH6.

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Declaration of Competing Interest

Boqian Wu is employed by the company Sundew producing the surfactant tested in the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737479.

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