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Differential expression of immune-related biomarkers in primary cultures from Atlantic salmon (*Salmo salar*) exposed to processed *Paecilomyces variotii* with or without inactivated *Moritella viscosa*

Dominic Duncan Mensah [a,*], Byron Morales-Lange [a], Margareth Øverland [a], Kartik Baruah [b], Liv Torunn Mydland [a,**]

[a] Department of Animal and Aquaculture Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Oluf Thesens Vei 6, 1433, Ås, Norway
[b] Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Ulls Väg 26, 75007, Uppsala, Sweden

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ABSTRACT

Paecilomyces variotii (a filamentous fungus), is a promising novel protein source in fish feeds due to its high nutritional value. Also, *P. variotii* has Microbial-Associated Molecular Patterns (MAMPs) such as glucans and nucleic acids that could modulate the host's immune response. To understand the potential bioactive properties of this fungus in Atlantic salmon (*Salmo salar*), our study was conducted to evaluate the gene expression of immune-related biomarkers (e.g., cytokines, effector molecules and receptors) on primary cultures from salmon head kidney (HKLs) and spleen leukocytes (SLs) exposed to either UV inactivated or fractions from *P. variotii* with or without inactivated *Moritella viscosa* (a skin pathogen in salmonids). Moreover, the effect of the fermentation conditions and down-stream processing on the physical ultrastructure and cell wall glucan content of *P. variotii* was characterized. The results showed that drying had a significant effect on the cell wall ultrastructure of the fungi and the choice of fermentation has a significant effect on the quantity of β-glucans in *P. variotii*. Furthermore, stimulating Atlantic salmon HKLs and SLs with *P. variotii* and its fractions induced gene expression related to pro-inflammatory (*tnfα*, *il1β*) and antimicrobial response (*cath2*) in HKLs, while response in SLs was related to both pro-inflammatory and regulatory response (*tnfα, il6 and il10*). Similarly, the stimulation with inactivated *M. viscosa* alone led to an up-regulation of genes related to pro-inflammatory (*tnfα*, *il1β*, *il6*) antimicrobial response (*cath2*), intra-cellular signalling and recognition of *M. viscosa* (*sclra*, *sclrb*) and a suppression of regulatory response (*il10*) in both HKLs and SLs. Interestingly, the co-stimulation of cells with *P. variotii* and *M. viscosa* induced immune homeostasis (*il6, tgfβ*) and antimicrobial response (*cath2*) in SLs at 48h. Thus, *P. variotii* induces immune activation and cellular communication in Atlantic salmon HKLs and SLs and modulates *M. viscosa* induced pro-inflammatory responses in SLs. Taken together, the results from physical and chemical characterization of the fungi, along with the differential gene expression of key immune biomarkers, provides a theoretical basis for designing feeding trials and optimize diets with *P. variotii* as a functional novel feed ingredient for Atlantic salmon.

1. Introduction

Salmonid aquaculture industry faces several challenges, including infectious diseases and stressful environmental conditions, which cause high mortalities and economic losses [1] as well as challenges related to sustainability of feed ingredients [2]. These factors are a driving force in the pursuit for alternative feed ingredients.

Microbial ingredients (MI), such as fungi, have been identified as promising alternative feed ingredients that could address both challenges [3] since in addition to their high nutritional value, MI possess Microbial-Associated Molecular Patterns (MAMPs) like β-glucans, mannans oligosaccharides (MOS), chitin and nucleic acids, which are documented immunostimulants and health promoters in aquaculture species [4–6]. The recognition of these MAMPs by host pattern recognition receptors (PRRs) on fish cells can trigger the immune response, including respiratory burst activity [7] and the up-regulation of

* Corresponding author.
** Corresponding author.
E-mail addresses: dominic.duncan.mensah@nmbu.no (D.D. Mensah), liv.mydland@nmbu.no (L.T. Mydland).

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cytokines such as *tnfα*, *il1β*, *il8*, *il10* [8]. This in turn, can increase the resistance of fish against pathogens [9,10]. For instance, Caspian trout (*Salmo trutta*) fed diets containing β-glucan and MOS showed increased expression of cytokines (*tnfα*, *il1β*, *il8*) and effector molecules (increased activity of lysozymes, immunoglobulin M and alternative complement) [11]. Moreover, at the *in vitro* level, salmon head kidney leukocytes (HKLs) from primary cultures induced with hydrolyzed yeast products from *Debaromyces hansenii* showed higher protein levels of TNFα, IL10 after 6 and 24 h post induction, (respectively) [12].β-glucans have also demonstrated their ability to induce resistance and increase survival of salmonids against several pathogens [13,14]. Therefore, the use of immunostimulants presents an interesting alternative to address fish mortalities caused by multi-stressor conditions, such as pathogens and environmental stress during the prodction cycle.

Like other fungal species, filamentous fungi *P. variotii* is a high protein MI with potential health benefits for salmonids owing to the MAMPs it contains. *P. variotii* has been used in the past as protein source for pigs [15] and chicken [16] but not fish. With renewed interests to test the suitability of *P. variotii* in diets for salmonids, it is imperative to also understand its potential immunostimulatory or cytotoxic effects on fish. *In vitro* mammalian models have shown that exopolysaccharides of *Paecilomyces* spp induce immunological responses in cells. Moreover, Osaku et al. [17] showed that linear β-(1,6)-linked-D-glucan was the predominant exopolysaccharide in the cell wall of *P. variotii*. Their study further demonstrated that refined β-glucans caused an immunostimulatory activity on murine peritoneal macrophages by increasing *tnfα* and *il6* transcripts at 6 and 48 h. Similarly, He et al. [18] observed that exposing macrophages from RAW 264.7 cell line to exopolysaccharides of *Paecilomyces lilacinus* induced an inflammatory response, characterized by the up-regulation of *il1β*, *tnfα*, and increased nitric oxide production and phagocytic capacity of the stimulated cells compared to non-stimulated cells. Thus, this implies that the β-glucans from *P. variotii* could possess immunostimulatory properties, which can stimulate the immune system of salmonids and provide protection against salmonid pathogens, thereby increase their robustness and resilience. However, data related to the immune-modulatory effects of *P. variotii* in Atlantic salmon is non-existent, necessitating this study.

A prominent strategy to test the effectiveness of an immunostimulant is in the presence of a pathogen such as *Moritella viscosa,* a gram-negative bacterium and the aetiological agent of winter ulcers disease in fish during low temperature seasons in regions such as Norway [19,20]. This disease causes large skin ulcerations leading to significant mortalities and economic losses in Atlantic salmon, rainbow trout and infections in other species such as Atlantic cod [21]. It has been reported that the extracellular products (ECP) of *M. viscosa* causes mortalities in Atlantic salmon similar to infected fish, as well as cytotoxic effects [21]. The ECP including esterase, metalloproteins and siderophores, which sequester iron from their host contribute to their pathogenicity and cytotoxicity [22]. Cell culture of Atlantic salmon keratocytes have shown that these cells are able to internalize bacteria such as *Aeromonas salmonicida*, *Carnobacterium piscicola* and *Pseudomonas fluorescens* [23] but not *M. viscosa* [19]. This may imply that *M. viscosa* uses different chemotactic, tropism and interaction with their host in their virulence. In Atlantic salmon macrophage-like cell line (SHK-1), heat-killed *M. viscosa* induces the expression of pro-inflammatory cytokines interleukin 8 (*il8*) [24]. Again, stimulating rainbow trout intestinal cell line (RTgutGC) and macrophage-like cell lines (RTS11) with β-glucans and *A. salmonicida* increased the response to the inactivated bacteria in both cell lines and strong bactericidal effects in rainbow trout HKLs [25]. Despite these positive results of co-stimulation bactericidal effects in salmonids, few studies have documented these effects on salmon cells stimulated with immunostimulants and *M. viscosa*. In the study of de O. Roberti Filho et al. [26], β-1,3/1,6-glucan (MacroGard®) supplemented diets decreased mortality of Atlantic salmon post-smolts challenged with *M. viscosa* by 55.4% compared with non-supplemented control dietary group. Hence, understanding the how *P. variotii* modulates *M. viscosa* induced stress on salmonids is a step towards designing control measures for the bacteria.

To understand the immunological response induced by *P. variotii* when included in the diet of salmon, the suitability of primary culture from the head kidney leukocytes (HKLs) and spleen leukocytes (SLs) may provide valuable insights in future research. Therefore, the main objective of this study was to evaluate the potential differential expression of immune-related biomarkers in HKLs and SLs of Atlantic salmon when stimulated with *P. variotii* (either UV inactivated or cellular fractions) and *M. viscosa*. Results obtained from this trial could become relevant in the event of an outbreak of the bacterium during future salmon feeding trials with novel functional feeds.

Furthermore, it has been reported that several factors (e.g., carbon source, pH, temperature and duration), affect the proportion of fungal cell wall polysaccharides, including β-glucans [27]. Specifically, Osaku et al. [17] reported that pH of 11.8 (with ammonium nitrate concentration of 0.26% and glucose at 0.96%) was the optimal condition to increase production of β-glucans in *P. variotii*. Also, an earlier study Cheng et al. [28] demonstrated that using 24.5 °C, an initial pH of 7.46 and a fermentation duration of 73.9 h was an optimal condition to achieve a high yield of intracellular polysaccharides in *Paecilomyces cicadae*. Again, a major setback for inclusion of fungi in fish feed is their recalcitrant cell walls and the lack of appropriate enzymes to digest these cell walls by fish. Notwithstanding, earlier studies have disclosed that the choice of down-stream processing has a significant effect on physical properties and nutrient digestibility [29,30]. Hence, a secondary objective was to determine the effect of the fermentation and downstream processing on the physical ultrastructure and glucan and protein composition of *P. variotii* produced on different substrates.

2. Materials and methods

2.1. Analysis of fungal cell wall glucans and protein content

To analyse fungal cell wall glucans, *P. variotii* (provided by Enifer, Espoo, Finland) was produced on one of three substrates: sulphite stillage (SS), glucose (Glc) or glycerol (Gly). In addition, *P. variotii* from SS was further prepared by mechanical lysis using a tissue lyser (Qiagen, Retsch GmbH & co, Germany) for 15 cycles (30 sec^{-1}) per 1 min each. Then, the samples were centrifuged at 15,000×*g* for 10 min at 4 °C. The resulting pellet and the supernatant, referred to as insoluble fraction (IF) and soluble fraction (SF), respectively, were freeze dried (Alpha 1–4 LSC basic, Christ, Germany) for 24 h. After, 100 mg of the intact *P. variotii* was evenly spread on a weighing boat and deactivated with ultraviolet irradiation in an MSC-Advantage Class II Biological safety cabinet (ThermoFisher) for 2 h. Both the UV inactivated and the lyophilized samples were stored at 4 °C until use.

The total glucans (α-glucan and β-glucan) contents of *P. variotii* grown on different substrates were quantified using the β-glucan Assay Kit (Megazyme, K-YBGL) following the manufacturer's recommendations. Briefly, 9 mg and 10 mg of each dried and pulverized samples were used to determine the total and α-glucan, respectively (in duplicate). Thereafter, the optical density of each sample was determined at 510 nm using a VersaMax plate reader (Molecular Devices). The β-glucan content was calculated as the difference between the total and α-glucan measurements.

The nitrogen (N), carbon (C), and sulphur (S) content of *P. variotii* products were analysed by CHNS Elemental Analyzer (Vario El Cube Elemental Analyzer system GmbH, Hanau, Germany). Crude protein was calculated as N × 6.25.

2.2. Fungal cell ultrastructure, morphology and mannan-specific concanavalin a (ConA) in cell wall of P. variotii

The ultrastructure and morphology of *P. variotii* subjected to downstream processing were analysed using scanning electron microscopy

(SEM) and transmission electron microscopy (TEM) according to Straume et al. [31] and Agboola et al. [29]. Moreover, the mannan composition of the cell wall was determined using confocal microscopy.

Samples processed for SEM were coated with Pt-p1 to a thickness of 25 nm using a Leica EM ACE200 sputtering machine (Leica biosystems, USA) and observed with a Zeiss EVO50 EP scanning electron microscopy (Zeiss international, Germany) with an accelerating voltage of 10 kV in the secondary emission mode. In addition, samples for TEM were sectioned at 50 nm with a Leica EM UC6 (Leica biosystems, USA) and examined using a JEM-2100 Plus (JEOL, Japan) transmission electron microscope (equipped with TVIPS camera and JEM-2100 optic system to take photographs). All electron microscopy was conducted at the Imaging centre of the Faculty of Biosciences, Norwegian University of Life Sciences (NMBU, Campus Ås). The ratio of the cell wall thickness to cell area was determined on five randomly selected, evenly sectioned TEM images using imageJ v1.52a. For each image, the area (μm^2) and five random measurements of the cell wall thickness (μm) were taken and recorded.

For confocal microscopy, 2 mg of differently processed *P. variotii* was resuspended in PBS (1x) for 5 min at room temperature (RT) and centrifuged at 1,000×*g* for 5 min at 4 °C. For fluorescence detection of mannan conA lectin in the cell wall and nuclei, each sample was incubated in the dark for 40 min with conA-conjugated Alexa Fluor™ 594 (Invitrogen, C11253) diluted 1:50 (in 0.1 M $NaHCO_3$) and Sytox™ green nucleic acid stain (1:2500 in DMSO. Invitrogen, S7020) at RT. Following this, the samples were washed three times with PBS-T (PBS with 0.05% Tween20) and then gently layered on glass slide and allowed to dry before mounting with 80% glycerol. Fluorescence was detected at 590/617 nm using a Zeiss LSM800 confocal microscope (Zeiss international, Germany).

2.3. Primary cell cultures

2.3.1. Fish

Six Atlantic salmon post-smolts (*ca.* 2 kg) were obtained from the Norwegian Institute of Water Research (NIVA, Solbergstrand, Norway). In the research facility, the fish were fed commercial diets in flow through concrete tanks according to approved EU and Norwegian protocols and methods by animal welfare and research ethics bodies. The fish were euthanized by a sharp blow to the head and immediately transported on ice to a lab at the Norwegian University of Life Sciences.

2.3.2. Head kidney and spleen primary cultures

The head kidney and spleen of each fish was aseptically harvested and collected in L15 culture media (Gibco) supplemented with 1% Penicillin-streptomycin (pen-strep). Thereafter, each organ was homogenized through a 70 μm^2 nylon mesh and washed into a falcon tube using L15 medium. Mononuclear cells from both organs were obtained using the methods described by Morales-Lange et al. [32]. Briefly, 1 mL of cell solution was carefully layered on a 34/51% Percoll (GE Healthcare, Sweden) gradient prepared in L-15 medium. All the tubes were centrifuged (without brake) at 800×*g* for 40 min at 15 °C. The interface layer between the two gradients was carefully collected and washed with L15 medium (13 mL). The residual Percoll was removed by centrifugation at 200×*g* for 7 min at 15 °C, and the cell pellets were finally resuspended in L15 medium containing 10% foetal bovine serum (FBS) and 1% pen-strep (ultimate medium).

The cell count and viability for each sample was assessed using the trypan blue exclusion test in a TC20 Automated Cell Counter (Bio-Rad, Norway). The observed cell viability was above 95% for all samples. Then, the cells were readjusted to 10^6 cells mL^{-1} (in ultimate medium) and seeded into 24-well plates. The cells were stabilized for 2 h at 20 °C and then non-adherent cells and media were removed. The adherent mononuclear cells were reconstituted with 1 mL of ultimate media and cultured overnight at 20 °C. The following day, the cells were exposed to 100 μg mL^{-1} of either the insoluble fraction (IF), soluble fraction (SF) or UV inactivated *P. variotii* (PEK) resuspended in L15-medium. In addition, cells were also incubated with total proteins from inactivated *M. viscosa* (at 100 μg mL^{-1}), as well as a combination of *M. viscosa* and one of the cell fractions or UV inactivated *P. variotii* (100 μg mL^{-1} of each) in L15 medium.

M. viscosa was heated at 85 °C for 10 min, vortexed and sonicated, followed by centrifugation and protein quantification using Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Negative control for each fish individual was maintained with no addition of any of the stimulants above (only L15 medium). The exposure duration was set at 6, 24 or 48 h. The choice of induction concentration (100 μg mL^{-1}) was based on a previous in-house study (data not presented) and the work of He et al. [18]. At the end of each induction period, the medium was removed, and the cells were lysed with 500 μL of Qiazol lysis reagent (QIAGEN, 79306, Germany) and immediately frozen at −80 °C until RNA extraction. Inductions were conducted in duplicates.

2.4. RNA extraction and reverse transcription

Total RNA was extracted using RNeasy® plus mini kit 250 (QIAGEN, 74136, Germany) following the manufacturer's instruction. Genomic DNA was eliminated using gDNA eliminator to avoid genomic contamination. Subsequently, the RNA concentration and purity were measured using a Nanodrop spectrophotometer (ThermoFisher, Nanodrop 8000). After quantification, the RNA was stored at −80 °C.

Reverse transcription was done using Superscript™ IV Reverse Transcriptase (Invitrogen, 18090200). The reactions were performed in a thermocycler (AB Applied Biosystems, GeneAmp PCR system 9700) with a 20 μL reaction volume. Preheating was done at 65 °C for 5 min. Afterward, the samples were kept on ice for at least 1 min and then the reverse transcriptase was added. The reaction then proceeded in the following order: incubation at 55 °C for 10 min and subsequent inactivation at 80 °C for 10 min. Finally, the cDNA was diluted three times by adding RNase-free water and then stored at −20 °C until further use.

2.5. Quantitative real time polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using CFX Opus 384 real-time PCR instrument (BioRad, Norway). The list of primers used is shown in Table 1. For each reaction, a total volume of 10 μL: 5 μL of SYBR green, 0.375 μL forward primer (10 μM), 0.375 μL reverse primer (10 μM), 1.25 μL RNase free water and 3 μL template cDNA was used. Each biological sample was run in triplicates per target gene. The thermocycler protocol was 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Afterward, a melting curve was obtained. To determine the changes in relative gene expression, Elongation factor 1-alpha (*ef1a*) was used as housekeeping gene to normalize the expression of the target genes related to pro-inflammatory response (*tnfα*, *il1β*, *il6*), anti-inflammatory response (*il10*, *tgfβ)*, effector molecules (*inos*, arg*1*, *cath2*), and receptors (*sclra*, *sclrb*) by the $2^{-\Delta\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* from the Ct value of the target gene described by Livak and Schmittgen [33]. Negative controls with no cDNA template were also included.

2.6. Data analysis

GraphPad Prism v8.0.2 was used for graphical representation (bar plots) and statistical analysis of gene expression data and glucan content of the fungus (means, SEM, Shapiro-Wilk test for normality, natural log transformation, *t*-test). Differences were considered significant when *p*-value was <0.05. Moreover, a Principal component analysis (PCA) was performed using the RT-qPCR data per experimental condition from HKLs by FactoExtra package in Rstudio v2022.12.0.

Table 1
List of primers for RT-qPCR. Accession no: National Center for Biotechnology Information.

GENE	FUNCTION	FORWARD	REVERSE	SIZE	TM	ACCESS. NO
ef1α	House keeping	GCAGTGGCAGTGTGATTTCG	GTAGATCAGATGGCCGGTGG	131	62	NM_001123629.1
tnfα	Pro-inflammatory	GCAGCCATCCATTTAGAGGGTGAA	CTAAACGAAGCCTGGCTGTAAACG	170	64	NM_001123589.1
il1β	Pro-inflammatory	AGGACAAGGACCTGCTCAACT	CCGACTCCAACTCCAACACTA	72	61	NM_001123582.1
il6	Pro-inflammatory	AGACCAGATGAAGGCTGCTG	TTGGTGTCAACCAAGGAGGTT	153	64	XM_014143031.2
il10	Anti-inflammatory	ACAACAGAACGCAGAACAACC	GCATAGGACGATCTCTTTCTTCAG	89	63	XM_045705802.1
tgfβ	Anti-inflammatory	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	191	63	XM_014129261.2
arg1	Effector molecule	TGCGTATCAGCCAAAGACATAG	CCACCTCAGTCATGGAGTAAAC	109	64	NM_001141316.2
inos	Effector molecule	AGGTGCTGAATGTGTTGCAC	GTATTCTCCTGCCTGGGTGA	140	64	XM_014214976.2
cath2	Effector molecule	TCGGACAAGAAGAGGCAAGC	CTTCCGCTAGCTCCAGCAAT	141	63	XM_014140493.2
sclra	Receptor	GACAACACAACACTGACAAACAAG	GTGATCCTCCTGACTGATGATT	76	64	NM_001123579.1
sclrb	Receptor	TGGACAACACAACGCTCACA	AGATGCGGCGGTAGGTAAAG	159	63	NM_001123580.1

3. Results

3.1. Fungal cell wall glucans and protein content

A significant increase in total glucan levels were observed in *P. variotii* produced using batch fermentation in comparison to the continuous fermentation method (Table 2). The highest composition of both total glucans and β-glucans were recorded in *P. variotii* fermented on Gly (21.19 %) and Glc (16.74 %) as substrates.

Notably, when Gly was used as substrate, the levels of total glucans and β-glucans were twice as high as those in *P. variotii* grown on SS (10.52 %). The opposite trend was observed for nitrogen and protein content with higher values recorded for *P. variotii* grown on SS (57.16 %). Regarding the alpha glucan content, this was highest in *P. variotii* from Glc (2.12 %). Interestingly, a higher level of β-glucans (but not α-glucans) was recorded in IF (16.82 %) than in SF (1.58 %).

3.2. Microscopy

The electron and confocal micrographs shows filamentous fungi *P. variotii* before (Fig. 1A) and after drying (Fig. 1B) with fluidized bed. Moreover, the scanning electron micrographs of the wet sample (before drying) displayed a micro fungus with a smooth surface (Fig. 1a), while after drying (Fig. 1b), the sample exhibited surfaces that had shrunk and developed pockets on the cell surface. The observations made on the SEM images transcends onto the TEM images shaping the thickness of the cell wall.

The TEM images (Fig. 1c and d) displayed a cross section of *P. variotii* before and after drying. The images showed a clearly distinct cell wall and a cytoplasm with organelles. The cell wall of *P. variotii* was thicker before drying (Fig. 1c) but comparatively thinner after drying (Fig. 1d). Moreover, the confocal images also illustrated that the drying process may compromise the integrity of the fungal cell wall showing a clear outline with less visible mannan stain (in red) but visible nuclei acid stains in green (Fig. 1f). The wet sample (before drying) on the other hand had a well-defined cell wall with cytoplasm and nucleic acid stains in green. Confocal imaging of IF (not presented) showed that the choice of processing had negligible effect on the ingredient, which exhibited an intact cell wall and cytoplasmic content in the images observed. The ratio between the cell wall thickness and cell area (Fig. 2) of wet *P. variotii* was 0.027 μm^{-1}. This was significantly different from the dried samples (0.007 μm^{-1}). Again, this trend was similar to the data on cell wall thickness (0.51 ± 0.05 μm) for the wet sample, while the dried *P. variotii* was 0.29 ± 0.05 μm thick.

Table 2
Glucan composition and elemental analyses of *P. variotii* produced on sulphite stillage (SS), glucose (Glc), glycerol (Gly) and cellular *P. variotii* fractions of sample produced on SS using either a continuous or batch fermentation. Rows with different letters are significantly different from each other (p-value <0.05). Values are based on mean ± standard deviation.

Substrate/ method	Continuous Fermentation	Batch Fermentation		Processed SS	
	SS	Glc	Gly	IF	SF
Cell wall glucans (% 'as is basis') [¥]					
Total glucans	10.52 ± 0.38^{c}	16.74 ± 1.18^{b}	21.19 ± 1.27^{a}	17.17 ± 0.84^{x}	2.18 ± 0.69^{y}
β-glucans	10.25 ± 0.38^{c}	14.61 ± 1.09^{b}	20.18 ± 1.26^{a}	16.82 ± 0.84^{x}	1.58 ± 0.67^{y}
α-glucans	0.27 ± 0.01^{c}	2.12 ± 0.12^{a}	1.01 ± 0.02^{b}	0.35 ± 0.02^{x}	0.59 ± 0.02^{y}
Other components (% 'as is basis') [€]					
N	9.15 ± 0.04	8.02 ± 0.0	5.86 ± 0.03	8.4 ± 0.01	7.75 ± 0.56
C	42.72 ± 0.08	44.25 ± 0.01	41.08 ± 0.06	44.2 ± 0.05	35.7 ± 2.39
S	0.57 ± 0.01	0.36 ± 0.03	0.21 ± 0.0	0.5 ± 0.01	1.46 ± 0.03
Crude protein	57.16 ± 0.22	50.13 ± 0.0	36.63 ± 0.18	52.5 ± 0.09	48.41 ± 3.49

[¥] amount of glucans in the cell wall of *P. variotii* analysed with megazyme kit, [€] amount of nitrogen (N), carbon (C) and sulphur (S) in *P. variotii* was analysed using the Vario El Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) in duplicates.

3.3. Expression of immune-related biomarkers in cells stimulated with P. variotii and its fractions

Regarding the gene expression of cytokines, *tnfα* increased at 6, 24 and 48 h for all the stimuli (in HKLs), except for IF at 24 and 48 h (Fig. 3A), while in SLs, its expression was only significant at 24 and 48 h (IF and SF). Thus, the response in SLs was slower when compared to HKLs. The faster response in HKLs was also evident from the steady decline in the expression with time (in the HKLs and vice versa for the SLs) (Fig. 3A).

Furthermore, the expression of *il1β* in HKLs (Fig. 3B) was significantly up-regulated at 6 h (PEK) as well as at 24 and 48 h of exposure for SF (but no changes were observed for IF at any of the time points). None of the compounds caused a notable change in the expression of *il1β* in SLs or *il6* in HKLs (Fig. 3B and C). However, significant changes in *il6* were noted at 24 h in SLs treated with SF, and at 6 and 24 h for SLs treated with PEK (Fig. 3C). There were also no significant differences between stimulated and unstimulated cells for *il10* expression (in HKLs, irrespective of time; Fig. 3D), except for an up-regulation of *il10* in SLs stimulated with SF at 24 h. Relative to *tgfβ*, a duration-dependent expression of this cytokine was observed in HKLs when stimulated with the SF of *P. variotii* (but not in SLs, Fig. 3E).

While no changes in the gene expression of effector molecules such as *inos* and *arg1* were observed in HKLs and SLs (Fig. 3F and 3G), a significant up-regulation of *cath2* (Fig. 3H) was observed at 6 and 24 h post exposure to SF or PEK in HKLs (but with a decrease towards 48h). In SLs, *cath2* was only up-regulated in when cells were stimulated with SF for 24 h. Overall, the results are characteristic of an early response in the HKLs peaking after 6 h with a transient reduction after 48 h of exposure.

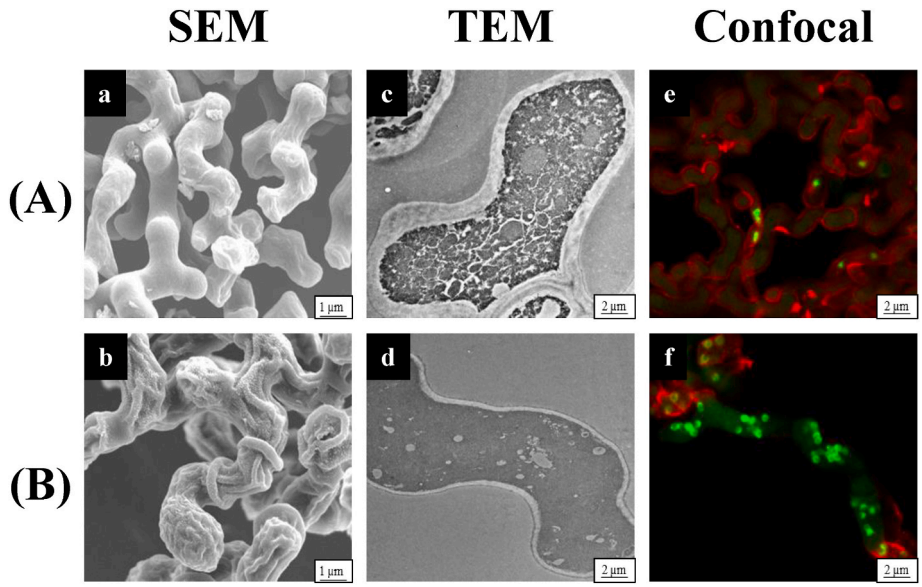

Fig. 1. Cell surface structure of *P. variotii* at different downstream processing. The figure shows scanning electron micrographs (**SEM**: a-b), transmission electron micrograph (**TEM**: c-d), and confocal microscopy of *P. variotii* (e–f). Stained in red (Mannan conA) and green (Sytox G nuclei acid stain). Images were taken on wet cream of *P. variotii* before drying (**A**), and after drying (**B**).

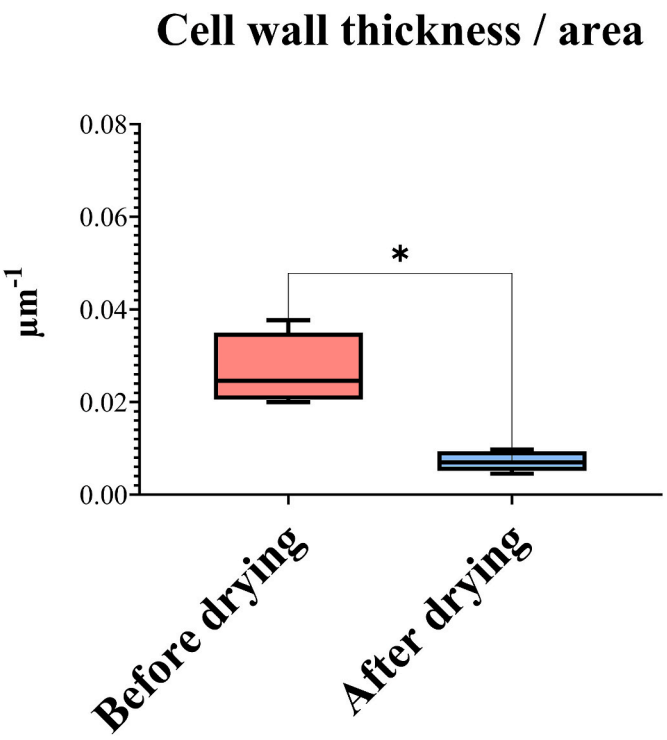

Fig. 2. Ratio of cell wall thickness to total cell area of transmission electron micrographs of *P. variotii* before (red) and after (blue) drying.

The expression of both c-type lectin receptors (*sclra*, *sclrb*) (Fig. 3I and J) only showed a significant down-regulation of *sclrb* in HKLs stimulated with IF after 48 h (compared to cells exposed at 24 h).

3.4. Immune-related biomarkers in cells stimulated with M. viscosa alone or in co-stimulation with P. variotii and its fractions

Tnfα and *il1β* were significantly up-regulated in both HKLs and SLs stimulated with either inactivated *M. viscosa* alone or after a co-stimulation with different preparations of *P. variotii* (Fig. 4A and B). The response in cells from both organs showed a duration-dependent up-regulation with increased transcript levels at 6 h. However, a down-regulation of *tnfα* and *il1β* occurred at 24 h and 48 h in SLs co-stimulated with both IF and *M. viscosa*. Regarding *il6*, a significant up-regulation occurred at 6 h post stimulation with *M. viscosa* alone or co-stimulation with *P. variotii* preparations in HLKs. This was a transient time-dependent expression with no changes at 24h and 48h (Fig. 4C). In addition, the up-regulation of *il6* was observed in cells stimulated with *M. viscosa* and at 48 h post co-stimulation with both PEK and *M. viscosa* showed a late response profile in SLs. The other stimulants did not induce any changes of *il6* in SLs except for a duration-dependent down-regulation in those cells stimulated with *M. viscosa* alone.

Furthermore, a significant down-regulation of *il10* occurred in the HKLs stimulated with *M. viscosa* for 24h and 48h. This cytokine again exhibits a steady decrease in transcripts with increasing duration (Fig. 4D). In SLs, a down-regulation of *il10* was observed in cells induced with *M. viscosa* alone at 24h and 48h, as well as in cells co-stimulated with SF and *M. viscosa* (Fig. 4D). No significant changes in *tgfβ* occurred in HKLs. Although, SLs co-treated with SF and *M. viscosa* showed a down-regulation of *tgfβ* after 48 h, and an up-regulation at 48 h after co-stimulation with PEK and *M. viscosa* (Fig. 4E). Overall, SLs co-stimulated with *M. viscosa* and PEK showed a delayed expression of *il6*, *il10* and *tgfβ*.

Regarding effector molecules, HKLs stimulated with *M. viscosa* alone showed a significant down-regulation of *inos* after 48h, but no change was noted in SLs irrespective of the stimulant or time (Fig. 4F). Moreover, arg*1* was only down-regulated in HKLs co-stimulated with *M. viscosa* and SF (Fig. 4G). Interestingly, the stimulation of HKLs with *M. viscosa* alone resulted in up-regulation of *cath2* at all durations, in addition to 6 h for cells co-stimulated with either IF or SF and *M. viscosa* (Fig. 4H). Again, co-stimulation of HKLs with PEK and *M. viscosa* resulted in the up-regulation of *cath2* at 6 h and 24 h, but with no change in 48h (Fig. 4E). The response in HKLs was prompt, peaking at early hours and declining with increasing duration of exposure. However, in SLs, a late up-regulated transcription level of *cath2* was observed for cells co-stimulated with PEK and *M. viscosa* at 48 h.

HKLs co-stimulated with the SF and *M. viscosa* resulted in an up-regulation of *sclra* (at 6 h and 24 h, Fig. 4I). Again, a significant duration-dependent decrease in the transcript levels of *sclra* was observed in cells stimulated with either IF and *M. viscosa* or PEK and

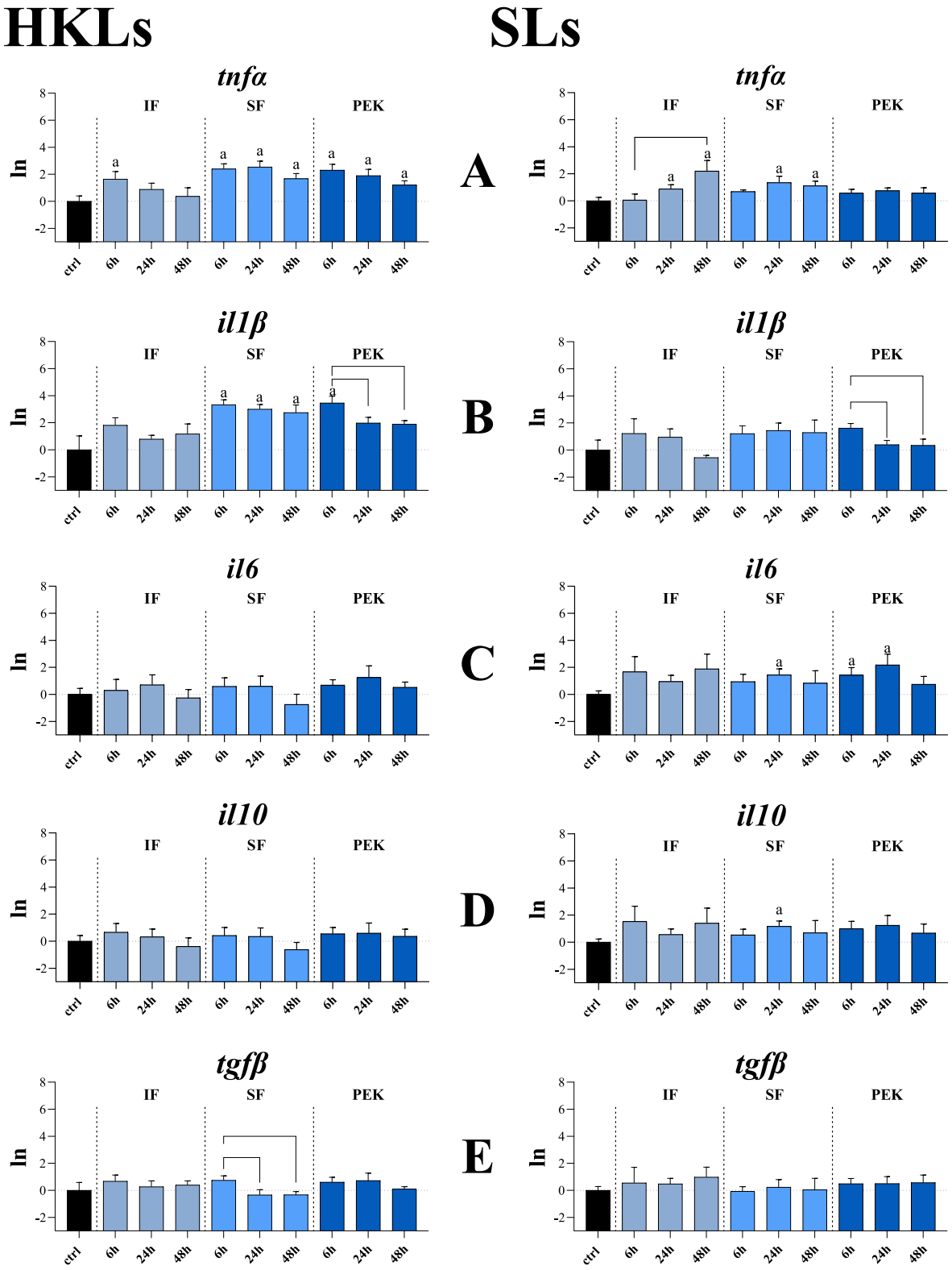

Fig. 3. Gene expression of immune biomarkers (in Ln) in primary cultures of Atlantic salmon (HKLs: left panel and SLs: right panel) stimulated with *P. variotii*: insoluble fraction (IF), soluble fraction (SF) and UV inactivated (PEK). Cytokines (3A, 3B, 3C, 3D, 3E). Effector molecules (3F, 3G, 3H). Receptors (3I, 3J). Bars annotated with **a** indicates significant difference ($p < 0.05$) between that treatment and the control (ctrl). Bars connected with lines are significantly different from each other ($p < 0.05$). Bars in black (non-stimulated control group), sky blue (stimulated with the insoluble fraction), light blue (stimulated with soluble fraction) and dark blue (stimulated with UV inactivated *P. variotii*).

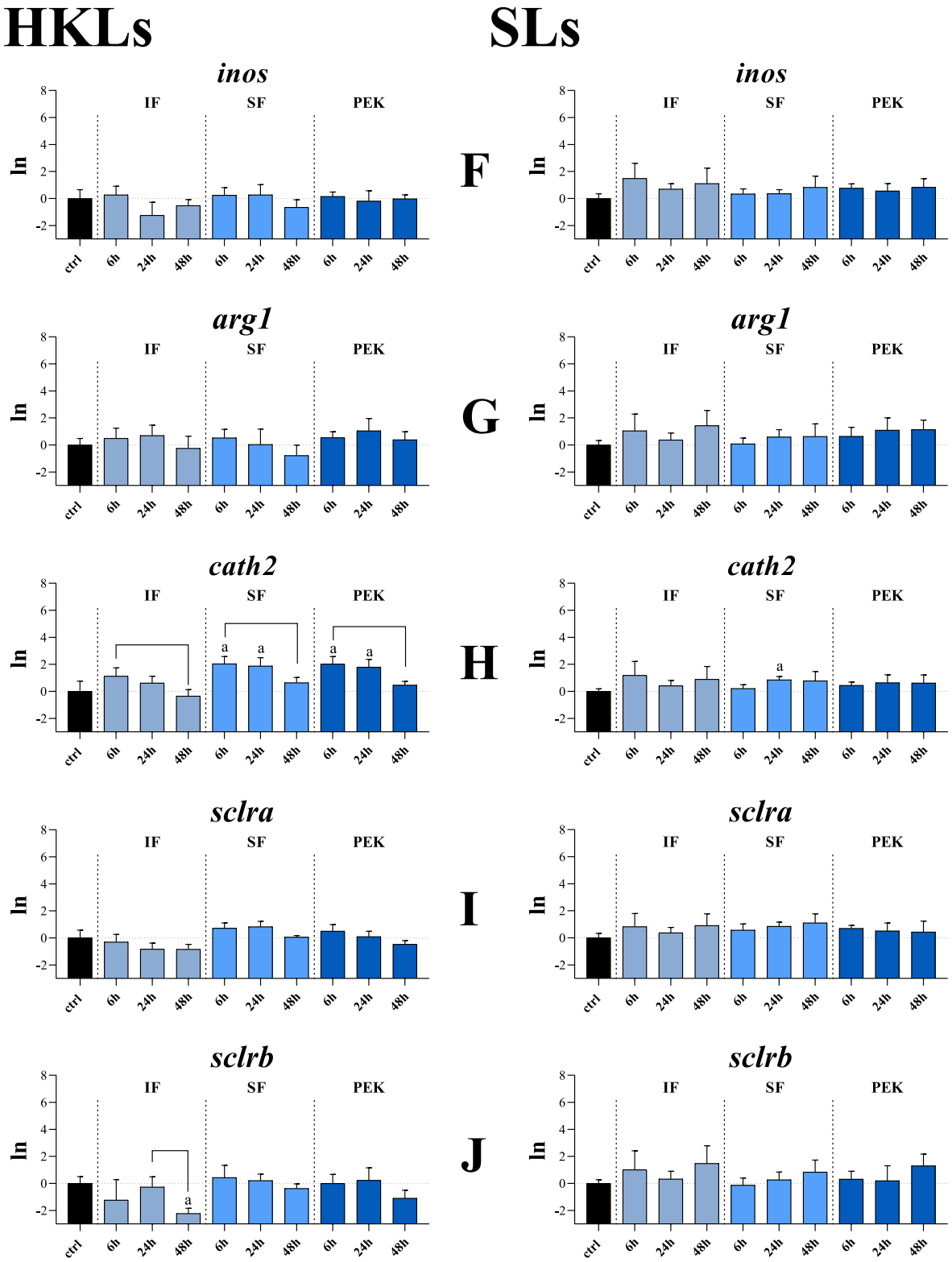

Fig. 3. (*continued*).

M. viscosa. HKLs and SLs stimulated with *M. viscosa* alone showed an up-regulation of *sclra* at 24h depicting a U-shaped model for the SLs. Both co-stimulation with SF and *M. viscosa* or PEK and *M. viscosa* showed a significant up-regulation at 48h (Fig. 4I) in SLs. No change in the expression of *sclrb* occurred in any SLs (irrespective of the stimuli and duration, Fig. 4J). Nevertheless, in HKLs, an up-regulation of *sclrb* occurred at 24 h for cells stimulated with *M. viscosa* alone and in cells co-stimulated with *M. viscosa* and the SF. These cells along with those treated with both PEK and *M. viscosa* showed an up-regulation at 24 h and a decline in the transcripts after 48 h (Fig. 4J).

3.5. Principal component analysis

In HKLs, the three largest contributors to PC1 are *il6*, *tnfα*, *il10,* while

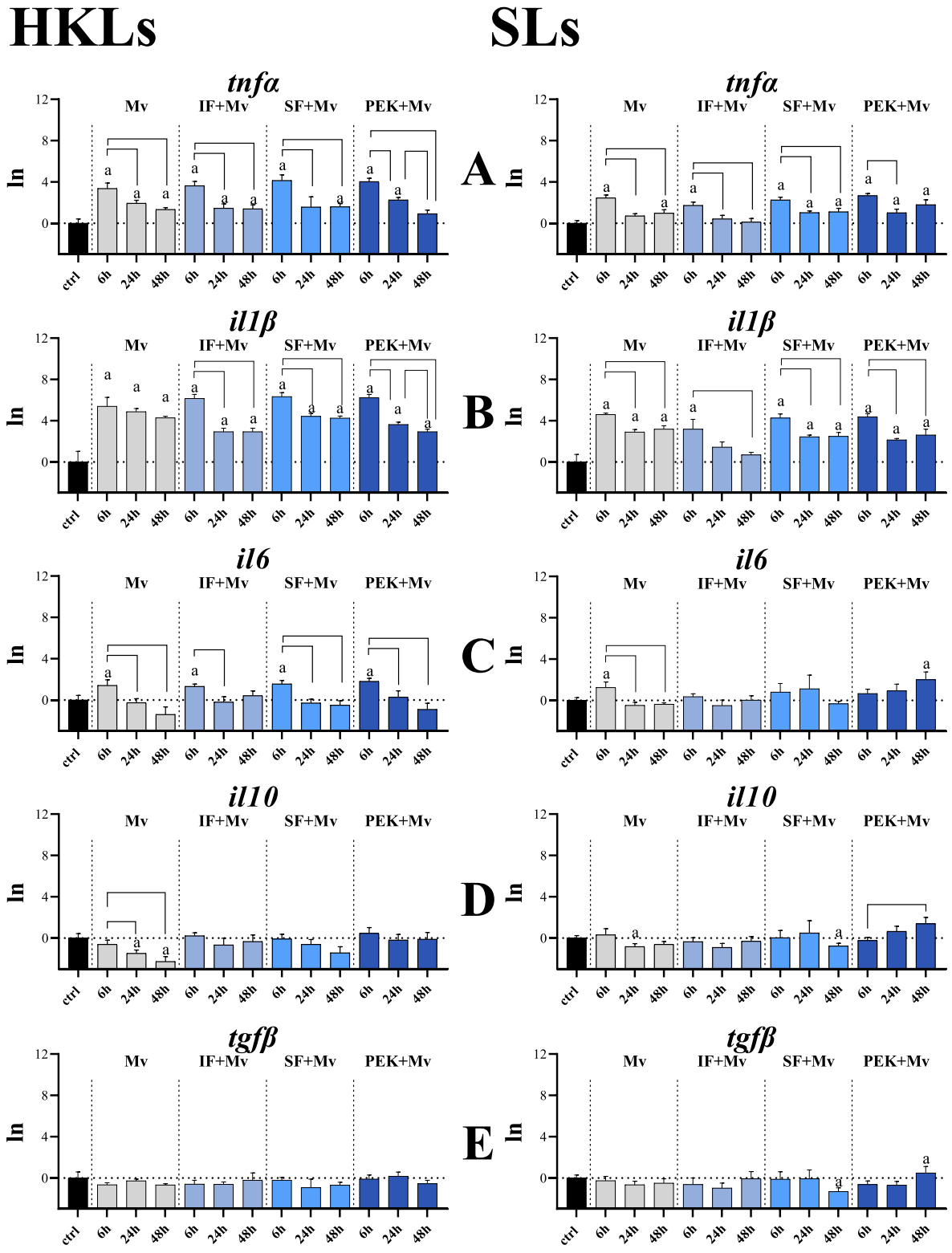

Fig. 4. Gene expression of immune biomarkers (in Ln) in primary cultures of Atlantic salmon (HKLs: left panel and SLs: right panel) stimulated with *M. viscosa* alone (Mv) and in co-stimulation with insoluble fraction (IF), soluble fraction (SF) and UV inactivated (PEK) *P. variotii*. Cytokines (4A, 4B, 4C, 4D, 4E). Effector molecules (4F, 4G, 4H). Receptors (4I, 4J). Bars annotated with **a** indicates significant difference ($p < 0.05$) between that treatment and the control (ctrl). Bars connected with lines are significantly different from each other ($p < 0.05$). Bars in black (non-stimulated control group), light grey (stimulated with only *M. viscosa*), sky blue (co-stimulated with the insoluble fraction and *M. viscosa*), light blue (co-stimulated with soluble fraction and *M. viscosa*) and dark blue (co-stimulated with UV inactivated *P. variotii* and *M. viscosa*).

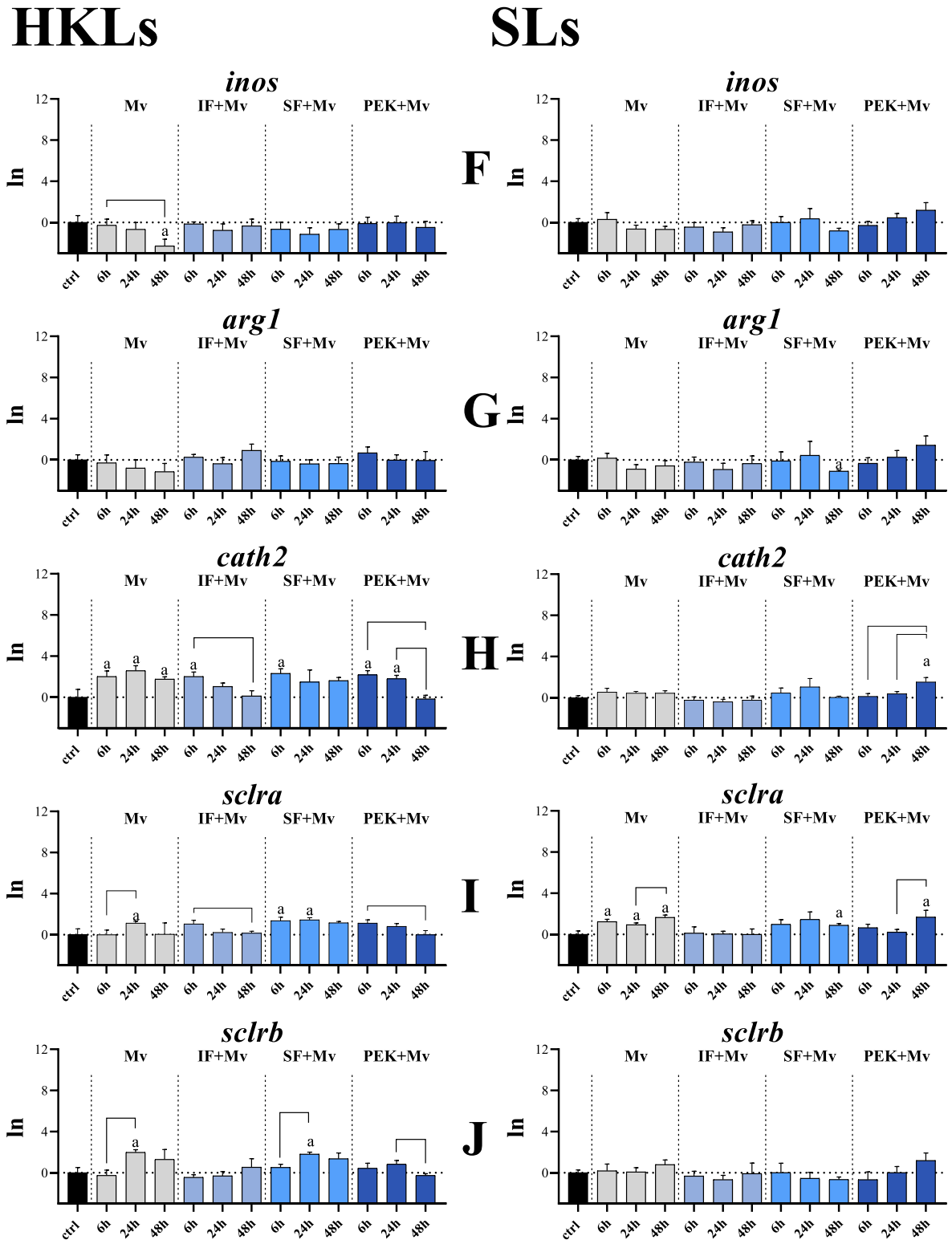

Fig. 4. (*continued*).

il1β, *cath2* and *sclra* are the largest contributors to PC2. The first two principal components (in parenthesis) explained 77.6% of the total variability in the dataset (Table 3). Overall, *tnfα* and *il6* contributed most to the two principal components (Fig. 5). No observable distinct clusters based on the different stimuli were formed. However, there was a positive correlation between *il1β*, *tnfa*, *cath2* and *sclra*. These biomarkers were negatively correlated with *inos*, *il10*, arg*1*, *tgfβ*, *il6* and *sclrb* (which are in turn correlated with each other).

4. Discussion

In microbial organisms such as fungi, an important moment to extract β-glucans is during the stationary phase since growth and protein synthesis slow down with accompanied increase in cell wall volume [1].

Table 3
Loading matrix of the first two principal components related to gene expression in head kidney leucocytes of Atlantic salmon stimulated with *P. variotii*, its fractions and *M. viscosa*.

Gene	PC1 (61.7%)	PC2 (15.9%)
tnfα	0.35	0.33
il1β	0.15	0.57
il6	0.36	−0.28
inos	0.34	−0.06
cath2	0.30	0.37
il10	0.35	−0.11
arg*1*	0.34	−0.23
tgfβ	0.34	−0.27
sclra	0.30	0.34
sclrb	0.28	−0.31

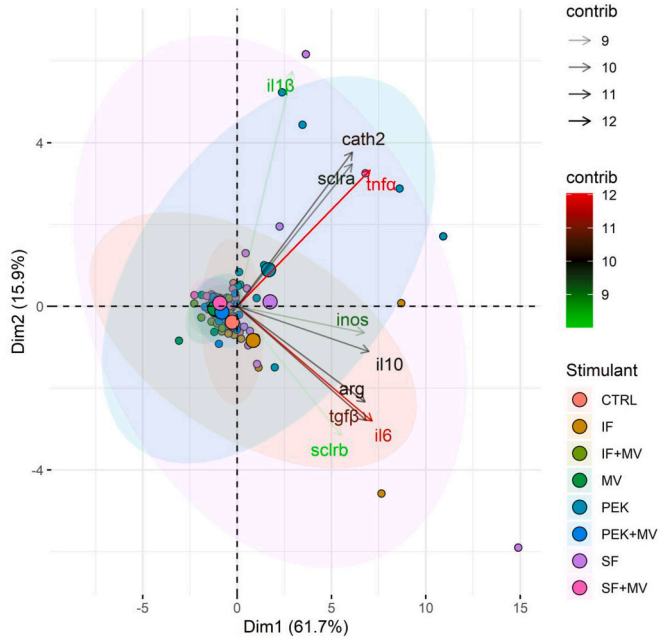

Fig. 5. Principal component analysis of gene expression in stimulated and unstimulated HKLs of Atlantic salmon. The biplot shows the PCA scores of the explanatory variables as vectors and their contribution (contrib) to the principal components (colour scale) and individual points (coloured circles) of each stimulant [unstimulated (red), stimulated with insoluble fraction; IF (orange), IF + MV (green), *M. viscosa*; MV (deep green), UV inactivated *P. variotii*; PEK (turquoise), PEK + MV (blue), soluble fraction; SF (purple), SF + MV (magenta)]. The arrows and labels show the direction of the component loading of each gene studied. Vectors pointing in the same direction are positively correlated variables, while those pointing in opposite directions are negatively correlated. Colour concentrated ellipsoids shows observations grouped by stimulants.

In our study, *P. variotii* was cultured by continuous fermentation, hence, it was harvested during the exponential growth phase when the cells undergo active cell division. This was evident considering the high crude protein content, which contrasts with the samples cultured using fed-batch fermentation.

Higher concentration of glycerol constitutes a stress factor and inhibits the growth of fungi such as *C. utilis* [34]. Thus, aside the differences in the culture methods used for fermentation, the high concentration of glycerol could alter these polysaccharides in favour of β-glucans in *P. variotii* in the present study compared with the glucose substrate. Again, the presence of other macromolecules such as proteins and lipids could inhibit the access to glycosidic bonds between glucose molecules, hence the determination of β-glucans. Moreover, the complexity of the forestry biomass substrate and the fact that the cell wall from *P. variotii* was not purified prior to the β-glucans analysis, could have also inhibited the proportion of glucans quantified in this study. This could also explain the high β-glucan content recorded in the partly refined IF, where some soluble components, such as proteins, nucleic acids from the fungi were extracted in SF, leaving a more concentrated cell wall matrix.

Alpha glucans are major constituents of filamentous fungi, although their precise biological functions remain to be fully elucidated. Nevertheless, in *Aspergillus nidulans*, α-glucans serve as storage polysaccharides due to their early accumulation during culture and their usage in carbon-limiting conditions [35]. Another study showed that α-(1,3) glucans in *Aspergillus fumigatus* contribute to their pathogenesis by concealing their molecular patterns while they evade the host defence mechanism [36]. In our study, we detected more α-glucans in *P. variotii* when cultured on glucose compared to glycerol and the other substrates. Interestingly, the α-(1,3) glucans in the cell wall of *A. nidulans* depends on the concentration of glucose in the culture media [35]. The polymer disappears completely when external glucose supply is depleted with accompanied sharp increase in α-(1,3) glucanase titres. This implies that substrate limitation may have played a significant role in the observed disparity of α-(1,3) glucan content in *P. variotii* when cultured on different substrates. For instance, between the glucose and glycerol substrates, considering that more nitrogen and α-glucans, but less β-glucan levels were quantified in the glucose cultured *P. variotii* (compared to glycerol substrate). This suggests carbon limitation in the case of the glycerol substrate.

The result of the microscopy indicates that drying has a profound effect on *P. variotii,* characterized by reduction of the cell wall thickness and withdrawn cellular surface. The decreased thickness of the mannan layer in the dried *P. variotii* may also indicate that drying affects the abundance of this polysaccharide. This change in the ultrastructure of *P. variotii* could influence the digestibility and nutrient availability to salmonids when included in their diets.

The current study uncovered that all the three preparations of *P. variotii* induced immunomodulation in both HKLs and SLs in a time-dependent manner, but with a more pronounced response in the HKLs. In principle, the adherent mononuclear fraction used in the present study are to a larger extent, monocytes which can differentiate to macrophages. In the studies of Smith et al. [37,38], morphological analysis of Atlantic salmon HKLs primary cultures showed predominantly monocyte-like cells on the first day, which subsequently differentiated into macrophage-like cells over five days with differential gene expression towards macrophage activation and differentiation. Immune cells are endowed with pattern recognition receptors (PRRS) such as toll-like receptors (TLRs), c-type lectin receptors (CLRs) which recognize MAMPs such as β-glucans. In rainbow trout, CLR (CLE4T) has been reported to be highly expressed in monocytes/macrophages [39]. In addition, the study of Petit et al. [40] also described genes encoding c-type lectin family 4 member C (*CLEC4C*) and salmon C-type lectin receptor A (*SCLRA*) as possible β-glucan receptors. Three c-type lectin receptors (*sclra*, *sclrb*, *sclrc*) and complement receptor 3 (*cr3*) have been described as β-glucan candidate receptors based on their recognition of the polysaccharide in the intestine of Atlantic salmon intubated with MacroGard® [41]. In addition, these receptors have been described to be expressed in the salmon macrophage-like cell lines (SHK-1) [42]. The two salmon CLRs (*sclra*, *sclrb*) evaluated in our current study did not show any transcriptional changes following the stimulation with *P. variotii* and its fractions except the down-regulation of *sclrb* by the IF of *P. variotii*. The immunostimulatory properties of β-glucan in salmonids is characterized by the up-regulation of pro- and anti-inflammatory genes like *tnfα*, *il1β*, *il6, il10* and *cox-2* [8,43,44] associated with the M1 and M2 pathways [45]. However, in our study, the up-regulation of *tnfα*, *il1β*, *il6*, and *il10* suggest that *P. variotii* induces an M1/M2-like response in Atlantic salmon leucocytes, despite the lack of change in the gene levels of the receptors. Only cells stimulated with MAMPs of *M. viscosa* or in combination with *P. variotii* showed elevated transcripts of these

receptors. This finding corroborates the study of Soanes et al. [42], which observed an up-regulation of these receptors in different Atlantic salmon tissues following infection with *Aeromonas salmonicida* or LPS stimulation in SHK-1 cells. Thus, this suggests that the up-regulation of these receptors may be primarily driven by the MAMPs from *M. viscosa* rather than the β-glucan content of *P. variotii*.

Il1β and *tnfa* are proximal pro-inflammatory cytokines involved in several immune signalling pathways, including the activation of *il6* transcription. Our study detected the up-regulation of *tnfa* in both HKLs and SLs, as well as elevated *il1β* levels in HKLs. This is in line with previous studies [25,46,47], also reporting an up-regulation of these cytokines in response to β-glucans (in salmonids). Therefore, the results suggest that observed immunological responses induced by *P. variotii* may be driven by its β-glucan content, and that *P. variotii* induces immune activation and coordinates cellular communication in the HKLs and SLs of Atlantic salmon. The results obtained here are also in line with earlier reports that β-glucans of *P. variotii* [17] and *Paecilomyces lilacinus* [18] have induced pro-inflammatory response in murine peritoneal macrophages and RAW 264.7 cell line respectively.

Moreover, regarding effector molecules, the antimicrobial peptide *cath2* has as antibacterial functions, which can be involved in wound healing, phagocytosis and other innate immune mechanisms [48]. In addition, *cath2* is an immunomodulator of both pro-inflammatory and anti-inflammatory responses [57] that has been documented in RTgutGC cells exposed to a commercial β-glucan (Zymosan®) [49]. This may be an underlying reason for the up-regulation of *tnfa* and *il1β* in the present study. Again, the increased expression of *cath2* and cytokines suggest that *P. variotii* modulate the immune response of Atlantic salmon HKLs.

The spleen of teleost fish has long been considered a secondary lymphoid organ responsible for antigen presentation process since it contains a high lymphocyte population that plays a key role in the coordination of adaptive immunity [50] along with antigen presenting cells (APCs), hence enhance communication between innate and adaptive immune system [32]. Head kidney, on the other hand, has a strong phagocytic activity, antigen retention and processing [50,51], making it an important organ for isolating systemic pathogens during fish pathological studies [52]. In the present study, the immunological response to *P. variotii* observed in the SLs contradict the findings from Ordás et al. [8], who observed that RTS11 was highly responsive to β-glucans. This organ response discrepancy has also been demonstrated *in vivo* by Douxfils et al. [43], where the authors reported that a stronger immune reaction was provoked in the spleen than the head kidney of rainbow trout fed β-glucan (at different doses). Nevertheless, our study further supports the conclusions made by Douxfils et al. [43], that the response to β-glucan is both species and organ-specific and depends on the dosage.

Besides β-glucans, fungal cell walls also contain other molecules, such as chitin and chitosan, which are known to induce immune responses in fish and enhance their resistance to various stressors [53–55]. For instance, while Sakai [55] found that injecting rainbow trout with chitin improved their resistance against *Vibrio anguillarum* infection, Cuesta et al. [56] observed that chitin particles enhanced the innate immune response of gilthead seabream, and mannan oligosaccharides improved gut health and immune responses of salmonids, making them more resistant to fish pathogens [9]. Also, interesting from the confocal staining was the presence of nucleic acids, which could be as high as 10% of dry matter of *P. variotii*. Nucleotides have been reported to have immunostimulatory effects and improves disease resistance in salmonids [4]. Thus, these molecules may have acted in unison with β-glucans to modulate the immune response in HKLs and SLs (as was also observed in our study). Quantitatively, IF contains more β-glucans. Previous reports indicated that particulate β-glucans are more immunologically reactive than their soluble counterparts [8,57]. However, these reports differ from the observations made in our current study, since the SF induces pronounced immunological reactions. This suggest that SF may contain more nucleic acids due to their soluble nature hence up-regulating more immune related biomarkers than IF.

Furthermore, in conformity to previous studies at *in vitro* [24] and *in vivo* level [44], our data showed that *M. viscosa* up-regulated pro-inflammatory cytokines (e.g., *tnfa, Il1β*, *il6*), and down-regulated *il10* in a time-dependent manner in salmon HKLs and SLs. It is documented that stimulation of salmonid cell lines with lipopolysaccharides (LPS) from gram-negative bacteria can induce up-regulation of these genes [42,47,58]. However, the down-regulation of *il10* in cells stimulated with *M. viscosa* contradicts the findings of Chettri et al. [47] and Lulijwa et al. [58] since they observed an up-regulation of this cytokine upon stimulation of salmonid leucocytes with LPS. Nevertheless, besides LPS, other bacterial MAMPs such as the flagellin (from flagella), peptidoglycan (from capsule), or antigens from bacterial fimbria [59] could have contributed to increased gene expression in response to *M. viscosa*.

To evaluate the potential of *P. variotii* to modulate pathogen induced inflammatory response in salmonids, we co-stimulated both HKLs and SLs with inactivated *M. viscosa* and *P. variotii* or its fractions. The co-stimulation effect was more pronounced in SLs (particularly those cells stimulated with *M. viscosa* and the UV inactivated *P. variotii*). For instance, the up-regulation of several genes (e.g., *il6* and *cath2*) after 48h was observed in SLs co-stimulated with *M. viscosa* and inactivated *P. variotii*, but not in cells stimulated with *M. viscosa* alone. These results suggest that the presence of *P. variotii* could reinforce the induction of an M1-profile and antimicrobial responses in SLs after 48h stimulation, which then could also be controlled by the up-regulation of *tgfβ* since it has a regulatory role to countervail the mounted immune response to ensure homeostasis and to resolve inflammation [60]. This is a necessary immune phenomenon to prevent tissue damage caused by prolonged inflammatory response induced by pathogens.

Finally, we built a PCA biplot to determine if the different stimuli resulted in an exclusive cluster and in the correlation between the explanatory variables. The failure of the stimuli to form distinct clusters may be characteristic of the lack of compositional differences in the stimuli or the variability in individual response of the cells from the fish used in the current study.

5. Conclusion

The present study showed that the fermentation conditions and down-stream processing of *P. variotii* have a significant effect on the glucan content, mannan layer and the cell wall ultrastructure. Moreover, intact *P. variotii* and fractions induced gene expression related to a pro-inflammatory and antimicrobial immune response in Atlantic salmon leucocytes. The stimulation with *M. viscosa* alone (both in HKLs and SLs) also up-regulated genes related to pro-inflammatory response and cell signalling while suppressing regulatory responses. Interestingly, SLs co-stimulated with the UV inactivated *P. variotii* and *M. viscosa* showed a response profile related to immune homeostasis after 48 h of induction compared with those cells stimulated with *M. viscosa* alone. Thus, the response induced by *P. variotii* may help to prime and regulate the immune function in cells from Atlantic salmon. Taken together, *P. variotii* is a potential candidate as feed ingredient with promising health benefits that are relevant to be explored for farmed salmonids.

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Declaration of interest

The authors declare that they have no commercial or financial relationships that could be considered a potential conflict of interest.

CRediT authorship contribution statement

Dominic Duncan Mensah: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Byron Morales-Lange:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Margareth Øverland:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Kartik Baruah:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Liv Torunn Mydland:** Conceptualization, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition, All authors are aware and agree to changes made to the document for resubmission.

Data availability

Data will be made available on request.

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