Full length article

Distinct response of immune gene expression in peripheral blood leucocytes modulated by bacterin vaccine candidates in rainbow trout *Oncorhynchus mykiss*: A potential *in vitro* screening and batch testing system for vaccine development in aquaculture

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A B S T R A C T S

Fish aquaculture is the world's fastest growing food production industry and infectious diseases are a major limiting factor. Vaccination is the most appropriate method for controlling infectious diseases and a key reason for the success of salmonid cultivation and has reduced the use of antibiotics. The development of fish vaccines requires the use of a great number of experimental animals that are challenged with virulent pathogens. *In vitro* cell culture systems have the potential to replace *in vivo* pathogen exposure for initial screening and testing of novel vaccine candidates/preparations, and for batch potency and safety tests. PBL contain major immune cells that enable the detection of both innate and adaptive immune responses *in vitro*. Fish PBL can be easily prepared using a hypotonic method and is the only way to obtain large numbers of immune cells non-lethally. Distinct gene expression profiles of innate and adaptive immunity have been observed between bacterins prepared from different bacterial species, as well as from different strains or culturing conditions of the same bacterial species. Distinct immune pathways are activated by pathogens or vaccines *in vivo* that can be detected in PBL *in vitro*. Immune gene expression in PBL after stimulation with vaccine candidates may shed light on the immune pathways involved that lead to vaccine-mediated protection. This study suggests that PBL are a suitable platform for initial screening of vaccine candidates, for evaluation of vaccine-induced immune responses, and a cheap alternative for potency testing to reduce animal use in aquaculture vaccine development.

1. Introduction

Fish aquaculture is the world's fastest growing industry within the animal food production sector. However, infectious diseases not only cost up to 20% of production value but also have a significant impact on individual fish health and welfare, and the environment [1–3]. Vaccination is the most appropriate method for controlling disease problems, both from a production and welfare point of view [4–6]. Vaccination plays an important role in large-scale commercial fish farming. It has been a key reason for the success of salmonid cultivation and has reduced the use of antibiotics [7–9]. Almost 100% of Atlantic salmon are vaccinated with multivalent vaccines prior to sea transfer in Norway, the largest producer of farmed salmon in the world [10].

Inactivated whole-cell bacterins constitute the main bacterial vaccines used in aquaculture. The first commercially licensed vaccine for

fish (in 1976) was a formalin-killed bacterin delivered by immersion against *Yersinia ruckeri*, the causative agent of enteric redmouth disease (ERM) in salmonids [11,12]. Following the success of this product, vaccines for *vibriosis* were developed. Whilst these vaccines are able to induce protective immunity by bath/dip vaccination, bacterin immersion vaccines against *Aeromonas salmonicida* (the causative agent of furunculosis) were not effective in the field, so oil adjuvant-based bacterin vaccines were developed despite some side effects [7,8,13]. Iron-regulated outer membrane proteins (IROMPs) of *A. salmonicida* induced under iron-restricted growth conditions were found important protective antigens [14]. Anti-IROMPs antibodies are bactericidal to virulent *A. salmonicida* strains *in vitro* and specific anti-IROMP antibody responses correlate strongly with protection against furunculosis [15]. Thus, bacterins prepared from *A. salmonicida* grown under iron-deprived conditions are also good vaccine candidates and led to the non-

adjuvanted vaccine against furunculosis AquaVac FNM Plus (Intervet UK Ltd) (http://www.vmd.defra.gov.uk/productinformationdatabase/SPC_Documents/SPC_122154.DOC) that uses both pathogenic and non-pathogenic strains of *A. salmonicida*, i.e. strain MT423 and strain MT004.

Whilst commercial vaccines are available for many of the major bacterial diseases and a few viral diseases of aquaculture, no commercial vaccines have been produced against fish parasitic infections [4–6] and more vaccines to emerging diseases are waiting to be developed. Furthermore, disease outbreaks do occur on fish farms from time to time even after vaccination with effective commercial vaccines, e.g. the ERM vaccine prepared from motile *Y. ruckeri* lost its efficacy against non-motile strains [16,17]. In Denmark, although the majority of rainbow trout are vaccinated using commercial vaccines against the most serious bacterial pathogens *A. salmonicida* subsp. *salmonicida*, *Yersinia ruckeri* and *Vibrio anguillarum*, disease outbreaks requiring treatment with antibiotics still occur [18]. These examples highlight the need for continuous improvement of vaccines for long-term protection in aquaculture. The development of fish vaccines requires thorough efficacy and safety testing before they can be marketed. All batches of vaccines must be tested for potency and safety before use. These tests are currently reliant upon mortality testing where fish are exposed to virulent pathogens and relative percent survival is measured, which requires the use of a great number of experimental animals [19–21]. Although a clear-cut approach, it does not unveil the underlying molecular mechanisms of vaccination-mediated protection and is cost- and time-consuming [19–21]. With an increasing focus on the 3 R's (Replacement, Reduction and Refinement), *in vitro* systems using fish immune cells have the potential to replace *in vivo* pathogen exposure for initial screening and testing of novel vaccine candidates/preparations, and for batch potency and safety tests.

Fish immune cells can be isolated from major immune organs (e.g. head kidney and spleen), mucosal tissues (e.g. intestine and gills), as well as blood [22–25]. Blood is the only source of leucocytes that can be obtained easily in a non-lethal way and that allows multiple sampling of the same fish to investigate vaccine-mediated immune responses over time. Peripheral blood mononuclear cells (PBMC) have been extensively used in mammalian vaccine development, to evaluate vaccine efficacy and for safety testing [26]. We recently optimised a rapid hypotonic peripheral blood leucocyte (PBL) isolation method in salmonids. As with PBMC isolated by gradient centrifugation, PBL isolated by this hypotonic method contain the major immune cell types needed to monitor adaptive immune responses, such as T cells, B cells, monocytes/macrophages and neutrophils [25]. The PBL prepared can proliferate, phagocytose and respond to stimulation with PAMPs and cytokines [25,27].

The current study aimed to investigate if the expression of immune genes could be differentially modulated in PBL by a variety of bacterin vaccine candidates. The bacterins were prepared from the pathogenic *A. salmonicida* MT423 grown in iron-replete and iron-depleted media, the non-pathogenic MT004, and a formalin inactivated *Y. ruckeri* model vaccine as well. We found that immune genes are differentially modulated in a bacterin- and time-dependent manner. These results are discussed in the context of immune mechanisms of vaccine mediated protection and potential use as surrogate biomarkers of vaccine efficacy and safety.

2. Materials and methods

2.1. Bacterin preparation

The *A. salmonicida* subsp. *salmonicida* non-pathogenic strain MT004, and pathogenic strain MT423, and the pathogenic *Y. ruckeri* strain MT3072 used in this study were obtained from the Marine Scotland Science Marine Laboratory, Aberdeen, UK, as described previously [28,29]. The bacteria were inoculated into tryptic soya broth (TSB,

Sigma) at 22 °C for 18–24 h in a shaking incubator at 100 rpm. The MT423 strain was cultured in normal TSB or in TSB supplemented with 100 μM 2,2′-bipyridyl (Sigma, UK) to deplete iron (Fe-) to induce IR-OMPs expression [14]. After culture, the bacteria were inactivated by addition of formalin solution (Sigma, UK) to the culture media to 1%. The bacteria were then incubated overnight on a slow magnetic stirrer at room temperature. Bacteria were collected by centrifugation and washed three times using phosphate buffered saline (PBS, pH7.4, Sigma, UK). The bacteria were weighed, resuspended in PBS at 10 mg/ml and stored at – 80 °C ready for use. The bacterins prepared were plated onto TSA plates and incubated for 48 h at 22 °C, with no bacterial growth confirming complete inactivation of the bacteria.

2.2. PBL preparation and stimulation

Rainbow trout (*Oncorhynchus mykiss*) purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, Scotland, UK) were maintained at 14 ± 1 °C in a freshwater recirculation aquarium [27,30]. The PBL from four fish were prepared as described previously [25]. Briefly, blood was drawn from the caudal vein using a BD Vacutainer containing lithium heparin (BD, UK). The red blood cells were lysed by combining 4 ml blood and 36 ml ice-cold cell culture grade water (GE Healthcare Life Sciences, UK) for 20 s. Then 4 ml cooled 10x PBS (Sigma, UK) was added to revert the medium to isotonicity. The suspension was left in ice for 5–10 min and filtered through a 70 μm cell strainer (Greiner Bio One, UK), pelleted by centrifugation (200 g, 5 min), and washed once with incomplete cell culture medium (Leibovitz medium L-15, Life Technologies, UK) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (P/S), and 1% foetal calf serum (FCS, Sigma, UK). The PBL were counted using a Neubauer chamber and 0.5% trypan blue, resuspended in complete cell culture medium (as above except 10% FCS) and distributed in 12-well cell culture plates at 2×10^6 cells/ml, 2 ml/well. The PBL from each fish were then stimulated with 100 μg/ml of inactivated bacterin (1) *A. salmonicida* MT423 (MT423), (2) *A. salmonicida* MT423 grown in iron-depleted medium (MT423 (Fe-)), (3) *A. salmonicida* MT004 (MT004), (4) an equal combination of MT423 (Fe-) & MT004, (5) *Y. ruckeri* MT3072 and PBS as control. The cells were incubated at 20 °C for 4 h, 8 h, 24 h and 72 h, harvested in 1.5 ml TRI reagent (Sigma, UK), and stored at − 80 °C until RNA extraction.

2.3. RNA extraction, cDNA synthesis and qPCR

Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) analysis of gene expression were carried out as described by Wang et al. [27,31]. The TRI lysates were thawed at room temperature and total RNA was prepared as per the manufacturer's instructions. The RNA was reverse transcribed to cDNA using RevertAid Reverse Transcriptase (Thermo Scientific). The resultant cDNAs were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Sigma) ready for use. The qPCR was performed in duplicate using a LightCycler 480 (Roche) system and $2 \times$ SYBR green Master Mix made with an Immolase DNA Polymerase kit (Bioline). The genes studied included those encoding the known cytokines, chemokines and chemokine receptors, suppressor of cytokine signalling (SOCS) proteins, acute phase proteins (APPs), antimicrobial peptides (AMPs), cellular markers, and master transcription factors of T cell responses. The primer sets were designed with at least one primer across an intron, tested to ensure that PCR products could only be amplified from cDNA samples and not from genomic DNA, and are detailed in Supplementary Table S1. The data were analysed using the LightCycler 480 integrated software. The gene expression level for each sample was normalised to that of the housekeeping gene, elongation factor (EF)-1α, and expressed as an arbitrary unit (AU) where 1 AU = the average expression in the control cells at 4 h. A fold change for each treatment was also calculated as the average expression divided by that of time matched controls.

2.4. Data visualization, clustering and statistical analysis

The fold changes of gene expression were analysed using ClustVis [32] for visualisation of a heatmap and for Principal Component Analysis (PCA). The arbitrary units of each gene were scaled and log2 transformed to improve the normality of real-time quantitative PCR measurements before statistical analysis as described previously [31]. A paired-samples T test was used to determine the level of difference between the treatments at each time point using the IBM SPSS Statistics package 25.0 (SPSS Inc., Chicago, Illinois), with differences considered significant at p < 0.05. The Spearman's rho correlation analysis of gene expression was performed on selected pro-/anti-inflammatory cytokines, adaptive cytokines, IL-12 family and chemokine receptors. The four time points from each bacterin stimulation was combined for this analysis (n = 16).

2.5. Key resources table

Resource	Source	Identifier
Antibody		
Anti-inflammatory		
Chemical		
2,2′- bipyridyl		
EDTA		
FCS		
Fe-		
Formalin		
Iron		
Lithium		
Penicillin		
Phosphate buffered saline		
Streptomycin		
Tris-HCl		
Trypan blue		
Polymerase		
Reverse Transcriptase		

3. Results and discussion

In this study, freshly prepared PBL were stimulated for 4 h, 8 h, 24 h and 72 h with five Gram-negative bacterin preparations. Bacterin A was from *Y. ruckeri* that is an effective vaccine against ERM [12]. Bacterins B (MT004) and C (MT423) were from non-pathogenic and pathogenic *A. salmonicida* subsp. *salmonicida* strains, respectively, but neither are effective vaccines against furunculosis [13,14]. Bacterin E was from MT423 cultured under iron-depleted condition that represent an effective vaccine candidate [14] and bacterin D was an equal mix of bacterins C and E that represents a vaccine previously used in the field. The expression of 93 immune relevant genes (Table S1) was examined by RT-qPCR and the expression normalised to EF-1α. The expression of 18 genes (IL-17A/F1b, IL-17A/F2a, IL-17A/F2b, IL-17A/F3, IL-17D, IL-23P19, IL-27P28B, IL-12P35B, IL-20, SOCS2A, SOCS2B, SOCS3A, SOCS3B, β-defensin 1-3, CCR4La and CCR6B, Table S1) was low or undetectable in control samples at 4 h, and thus were excluded from further analysis. The remaining 75 genes were expressed and modulated in PBL. The relative expression levels across different time points (with statistical analysis) are presented in Figs. S1-14 and the average fold change at each time point presented in Table S2.

3.1. Clustering and principal component analysis

In response to bacterin stimulation PBL gene expression changed over time from 4 h to 72 h. Hierarchical clustering analysis of the fold changes across four time points revealed time-dependent gene expression patterns (Fig. 1). Three major clusters of genes were apparent. Cluster 1 contained several chemokine receptor genes (CCR6A, CCR7A, CCR7B, CCR9A, CCR9B, CCR4Lc2 and CXCR3B) that were induced/

higher at 4 h and 72 h but low at 8 h and 24 h as compared to the unstimulated samples. Cluster 2, mainly consisted of adaptive cytokines, that were gradually induced and peaked at 8 h or 24 h, and remained elevated to 72 h. Cluster 3 genes, including mainly pro- and anti-inflammatory cytokines and APPs, were induced early, peaked at 4 h or 8 h, and returned to resting levels by 24 h–72 h (Fig. 1). The clustering patterns of the different bacterin preparations changed over time but with *Y. ruckeri* always clustering away from the various *A. salmonicida* bacterins at all four time points, suggesting a major effect of bacterial species, as well as different preparations of the same species/strain, on immune gene expression.

PCA analysis of the fold change of 75 genes at the 4 time points revealed that the first two components accounted for 85% of the variation (Fig. 2). The biplot indicates that the bacterin modulated gene expression can be divided into four distinct groups based on time, in agreement with the major effects of stimulation time on gene expression seen in Fig. 1. Different bacterin preparations showed distinct effects/variation on gene expression at each time point. At 4 h, the effects of bacterin preparation were well separated from each other, suggesting this gives the most discriminating power. Thus, the bacterin modulated gene expression was analysed further with a focus at 4 h.

3.2. Modulation of the expression of pro- and anti-inflammatory genes by bacterins

Genes involved in the inflammatory response are modulated *in vivo* by vaccination in salmonid lymphoid organs such as head kidney [33] and spleen [30]. Therefore, we examined first in PBL the expression of proinflammatory cytokines (IL-1β [34], TNFα [35], IL-6, IL-11 [36], M17 [37] and IL-34 [38]), chemokines (IL-8 and CXCL11_L1 [39]), anti-inflammatory cytokines (IL-10 [40], TGFβ1 [41] and nIL-1Fm [42]), and negative regulators of cytokine signalling (SOCS) [43]. The expression of all these genes could be modulated in PBL by bacterin stimulation (Figs. S1-3). The expression of the majority of the genes was rapidly induced at 4 h and peaked at 4 h or 8 h, and this modulation subsided by 24 h or 72 h (Table S2). The exception was the down-regulation of TNFα2 and TGFβ1A, that are highly expressed in PBL (as seen by the small ΔCP, Table S1). A noticeable difference of gene expression change was the rapid and strong induction of gene expression by *A. salmonicida* preparations, compared to a gradual but lasting increase induced by the *Y. ruckeri* bacterin (e.g. IL-1β1, TNFα3, IL-8, Figs. S1-2). Major differences between different paralogues in response to bacterin stimulation were also observed, in terms of magnitude of the response (e.g. IL-1β paralogues), response kinetics (e.g. IL-10 paralogues), and types of response seen (e.g. down-regulation of TNFα2 but up-regulation of other TNFα paralogues) (Figs. S1, S3). Differential responses of cytokine paralogues have been observed previously [30,34,35,40,41] and may indicate neo- or sub-functionalisation.

At 4 h post stimulation, the expression of IL-1β1-2, TNFα3, IL-6, IL-8 and IL-10A showed a similar pattern, where the highest induction was seen with MT423, followed by MT423 (Fe-), MT423 (Fe-) + MT004, MT004, and the lowest (but significant) induction seen with *Y. ruckeri* (Fig. 3). This expression pattern, to a lesser extent, was also observed with IL-1β3, TNFα1, IL-11, M17 and nIL-1Fm. Interestingly, *Y. ruckeri* tended to induce higher levels of the anti-inflammatory gene TGFβ1B and SOCS1A than MT423 (Fe-) + MT004 at 4 h. These results indicate a stronger inflammatory response induced by *A. salmonicida* strains compared to *Y. ruckeri*, and that the expression of a set of immune genes can distinguish the responses induced in PBL by different bacterin preparations.

3.3. Modulation of the expression of genes of adaptive immunity by bacterins

The expression of signature cytokines for Th1 (IFNγ1-2 [44]), Th2 (IL-4/13A, IL-4/13B1-2 [45]) and Th17 (IL-17A/F1a [46]) type
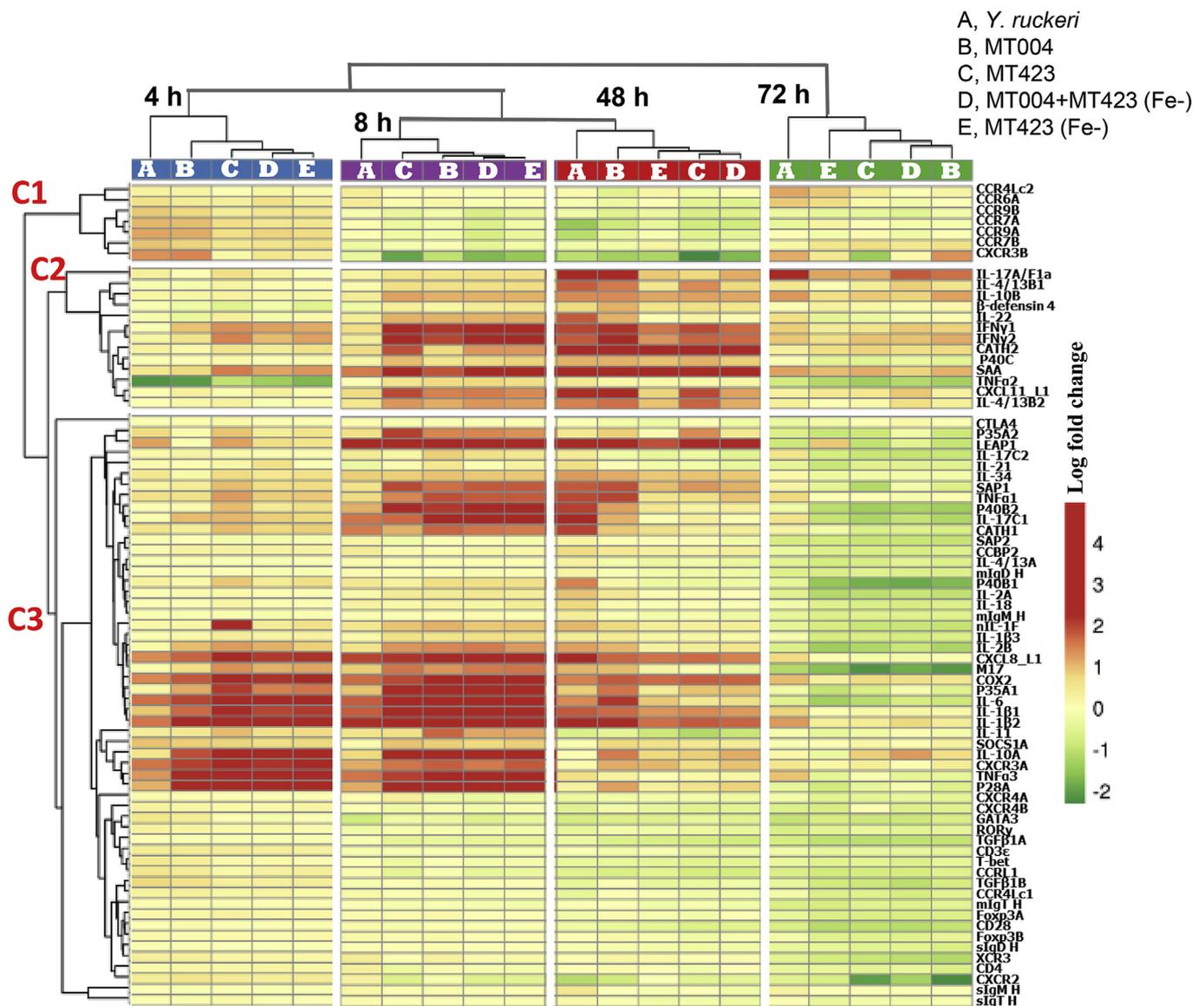
Fig. 1. Hierarchical clustering analysis of immune gene expression modulated by bacterins in PBL. Freshly prepared PBL from four trout were stimulated with formalin inactivated bacterins of *Y. ruckeri* (A)*, A. salmonicida* subsp. *salmonicida* strains MT004 (B) and MT423 (C), MT423 cultured under iron-depleted condition (MT423 (Fe-)) (E), and an equal mixture of MT004 and MT423 (Fe-) (D) for 4 h, 8 h, 24 h and 72 h. The fold change data set from 75 genes (Table S2) was inputted to the CluctVis program for clustering analysis. Three clusters of gene expression patterns, C1, C2 and C3, are apparent.

responses was next studied. Th1 cytokines were induced at 4 h by A. *salmonicida* preparations but not by *Y. ruckeri*. However, Th17 and Th2 cytokines (IL-4/13B1-2) were only induced by MT423 at 4 h (Fig. 4). The expression of IL-2B [27] and IL-17C1 [47] followed the pattern of Th1 cytokines. The expression of other cytokines (IL-2A, IL-4/13A, IL-17C2, IL-18, IL-21 and IL-22 [48]) was less responsive. In general, A. *salmonicida* preparations induced a relatively early (at 4 h and/or 8 h) adaptive cytokine expression, and *Y. ruckeri* induced a late (at 8 h or later) cytokine response (Figs. S4-6).

The expression of master transcription factors for Th1 (T-bet), Th2 (GATA3 [49]), Th17 (RORγ [50]), and regulatory T cells (FoxP3A/B [51]) was also examined. At 4 h, T-bet expression was upregulated by MT004 and *Y. ruckeri*, and RORγ expression by *Y. ruckeri* only. A small but significant increase of FoxP3A/B was observed in all stimulated samples, but GATA3 was refractory at 4 h (Fig. 4). The effects of bacterin stimulation were small at other time points (Fig. S6). The effects of bacterin-stimulation on T cell markers (CD3ε, CD4, CD28 and CTLA4) and B cell markers were also small (Figs. S7-8), with a minor increase in the expression of CD3ε and CD4 at 4 h by all bacterins (Fig. 4). The

large increases of proinflammatory cytokine gene expression and minor changes on adaptive genes at 4 h suggest a rapid PAMP-activated response by bacterins that may trigger later adaptive responses.

3.4. Modulation of the expression of the IL-12 family by bacterins

The expression of subunits of the IL-12 family followed the pattern of the major pro- and anti-inflammatory cytokines, with a rapid induction of the α-subunit of IL-12 (P35A1 and P35A2 [52,53]) and IL-27 (P28A [54]), and the β-subunits of IL-12 (P40B1 and P40B2 [52,53]) at early time points (4 h and 8 h, Fig. S9). The exception was P40C where the induced expression peaked at 8 h or 24 h. At 4 h, MT423 was a strong stimulator of IL-12 family expression compared to *Y. ruckeri*, as seen with the major inflammatory and Th1 cytokines (Fig. 5). The differential expression of different subunits suggests that different isoforms of IL-12 can be induced by different vaccine candidates, which have the potential to modulate differential Th cell development after vaccination.
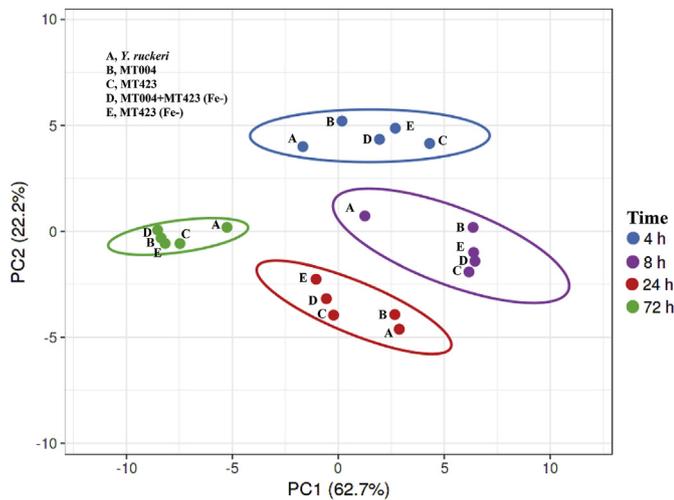
Fig. 2. Principal component analysis of immune gene expression modulated by bacterins in PBL. Freshly prepared PBL from four trout were stimulated with formalin inactivated bacterins of *Y. ruckeri* (A), *A. salmonicida* subsp. *salmonicida* strains MT004 (B) and MT423 (C), MT423 cultured under iron-depleted condition (MT423 (Fe-)) (E), and an equal mixture of MT004 and MT423 (Fe-) (D) for 4 h, 8 h, 24 h and 72 h. The fold change data set from 75 genes (Table S2) was inputted to the CluctVis program for principal component analysis.

3.5. Modulation of the expression of chemokine receptors by bacterins

The expression of chemokine receptor CXCR3A [55] was low but highly induced by bacterin stimulation in PBL, and followed the pattern of the major inflammatory and Th1 cytokines (Fig. S10). The changes of expression of other chemokine receptors were small and showed a decrease in expression at late time points (24 h and 48 h) for several receptors, e.g. CXCR2, CXCR3B, CXCR4A, CXCR4B, CCR9A and CCR9B (Figs. S10-11), perhaps due to a decrease of the receptor expressing cells during *in vitro* culture [27]. A small transient induction at 4 h was seen with the majority of chemokine receptors except CXCR4A (Figs. S11-12). The expression of CXCR2, CXCR4B, XCR3, CCR6A and CCR4Lc1 was induced by all the bacterin preparations with similar levels. However, *Y. ruckeri* bacterin induced a higher expression level of CXCR3B, CCR7A, CCR7B, CCR9A, CCR9B, CCR4Lc2 and CCRL1 [56], compared to MT423 at 4 h (Fig. 6). In contrast *A. salmonicida* preparations induced a higher level of CXCR3A and CCBP2 [57] transcripts

than *Y. ruckeri* (Fig. 6).

Chemokine receptors are present on all leucocytes and their expression can be differentially modulated to position the right immune cells in the right place [58]. The adaptive immune response is initiated in the secondary lymphoid organs with the arrival of antigen or mature dendritic cells (DCs). The antigen-loaded DCs and naive T cells must be co-localized to allow the rare antigen-specific naive T cells to scan and interact with the DCs. The co-localization of immune cells is promoted by chemokine receptor-mediated migration [58,59]. Of note, mammalian CXCR3, CCR4 and CCR6 are important for Th1, Th2 and Th17 type adaptive immunity, respectively; CCR7 regulates the migration of DCs, B cells and T cells; and CCR9 regulates GALT development and homing of T cells to gut [58]. The differential ability of vaccine candidates to modulate early chemokine receptor expression may provide novel insights into the immune pathways activated by vaccination. Interestingly, the *Y. ruckeri* bacterin that is an effective vaccine induces a stronger expression of CXCR3B, CCR4Lc2, CCR7, and CCR9, compared to the other vaccine candidates studied. This information could be a starting point to further dissect the immune pathways activated by vaccination that provide protection.

### 3.6. Modulation of the expression of APP and AMP genes by bacterins

Lastly, the expression of several APP and AMP genes that are known to be induced *in vivo* by vaccination and bacterial infection was studied [30,33]. COX2 expression was rapidly induced at 4 h and followed the expression pattern of the other inflammatory genes (Fig. S13). The induced expression of SAA, SAP1, CATH1, CATH2 and LEAP1/hepcidin was relatively low at 4 h, peaking at 8 h or 24 h (Figs. S13 and 14). Such expression kinetics are in line with the *in vivo* observation that early induction of proinflammatory cytokines by vaccines and PAMPs induces APPs and AMPs [30,60]. Bacterin stimulation had only minor effects on the expression of SAP2 and β-defensin 4. At 4 h, MT423 was more potent at induction of SAA, SAP1, COX-2 and CATH1 than *Y. ruckeri* (Fig. 7).

### 3.7. PBL immune gene expression mimics the in vivo response to vaccination

The expression of major pro- and anti-inflammatory genes (IL-1β, IL-6, IL-8, TNFα, IL-10 etc.), acute phase protein genes (SAA, SAP1 and COX-2), antimicrobial peptide genes (CATH1, CATH2 and LEAP1), as well as adaptive cytokine genes (IFNγ, IL-17A/F and IL-4/13) are increased in PBL by bacterin vaccine candidates in this study. Their expression is also increased *in vivo* by injection vaccination in rainbow
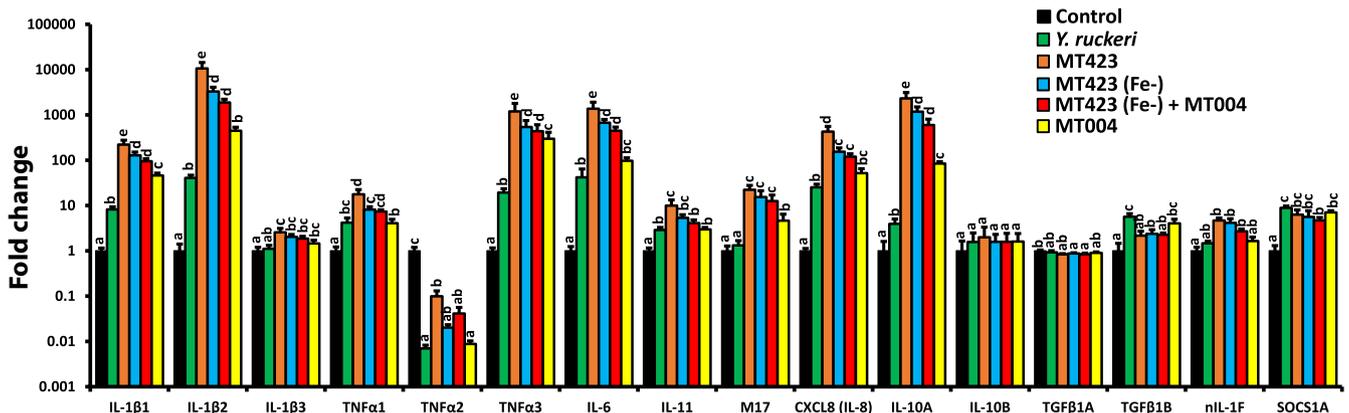
Fig. 3. Modulation of PBL expression of pro- and anti-inflammatory genes by bacterins. Rainbow trout PBL were stimulated with bacterins of *Y. ruckeri*, *A. salmonicida* strains MT423 and MT004, MT423 grown under iron-depleted conditions (Fe-), and a mixture of MT423 (Fe-) and MT004 for 4 h, 8 h, 24 h and 72 h. The average (+SEM, N = 4) gene expression was quantified by RT-qPCR. The expression level of each sample was normalised with that of EF-1α, and expressed as arbitrary units where one unit equals the average expression level in the control samples at 4 h. The fold change of expression at 4 h is shown with the full range of times studied in Figs. S1-3. Different letters over the bars indicate significant differences (p < 0.05, paired samples T test).
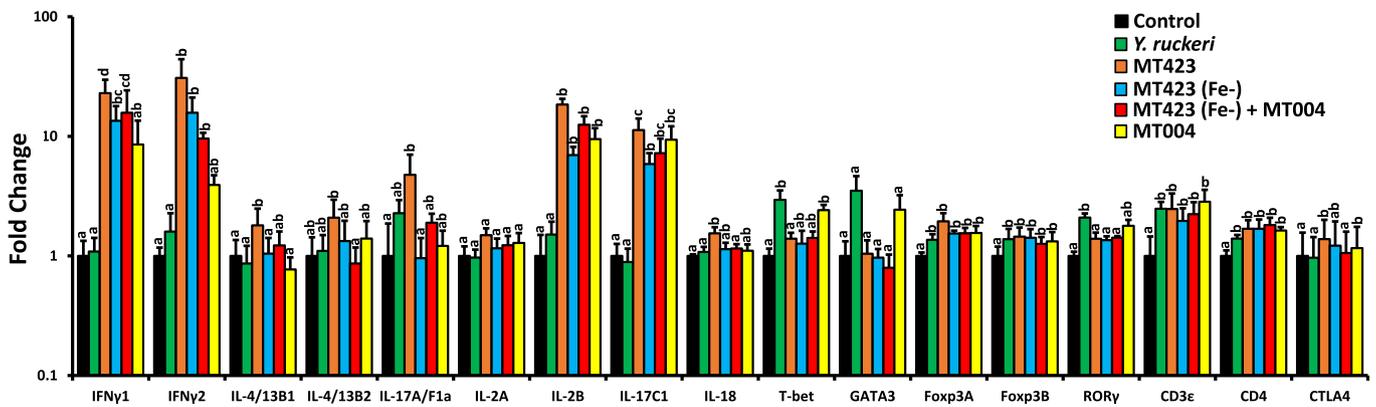
Fig. 4. Modulation of PBL expression of cytokines and markers of adaptive immunity by bacterins. Rainbow trout PBL were stimulated with bacterins and gene expression was quantified as described in Fig. 3. The fold change of gene expression at 4 h is shown with the full range of times studied in Figs. S4-7. Different letters over the bars indicate significant differences (p < 0.05, paired samples T test).
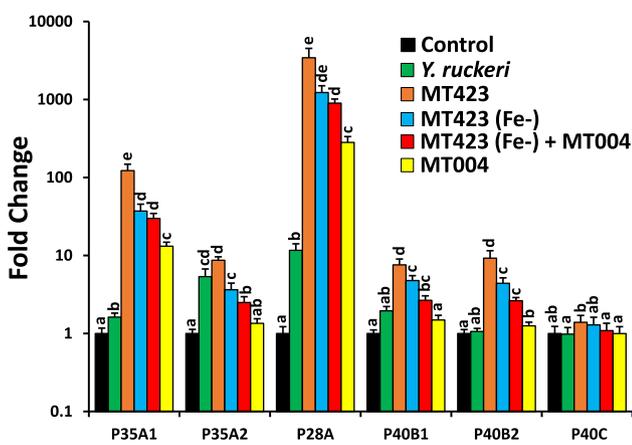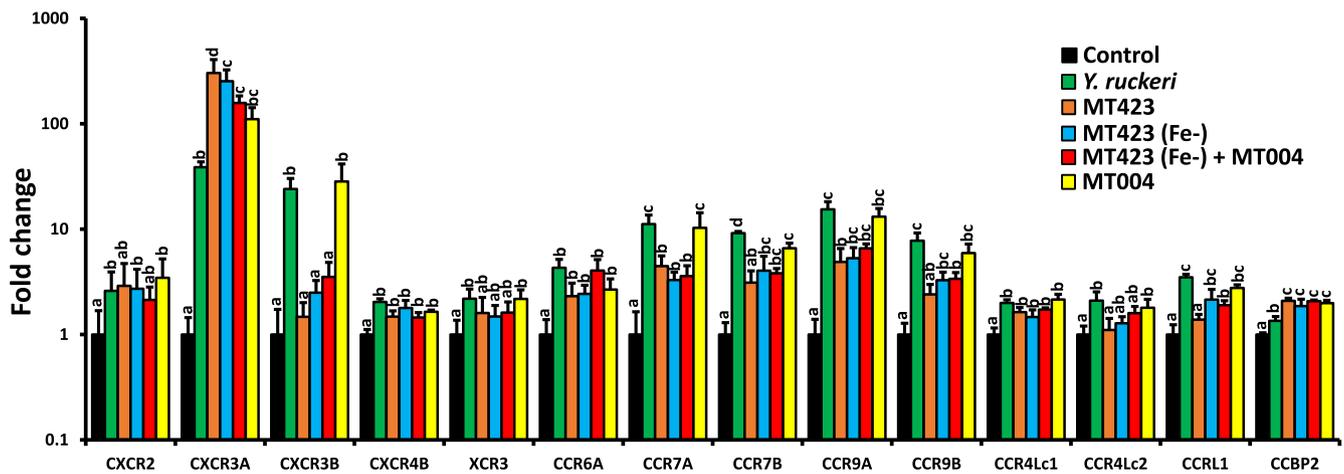

Fig. 5. Modulation of PBL expression of IL-12 cytokine family by bacterins. Rainbow trout PBL were stimulated with bacterins and gene expression was quantified as described in Fig. 3. The fold change of gene expression at 4 h is shown with the full range of times studied in Fig. S9. Different letters over the bars indicate significant differences (p < 0.05, paired samples T test).
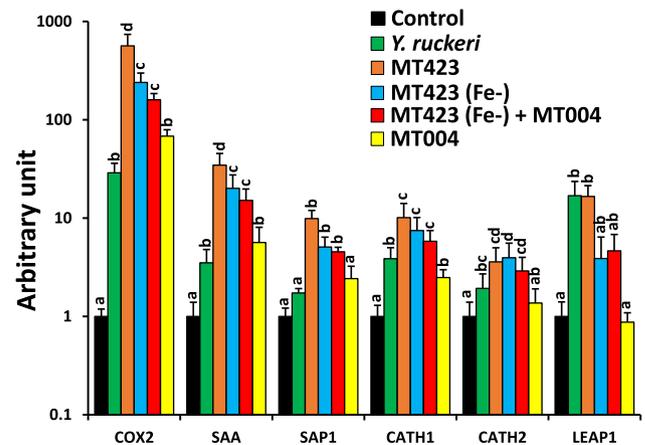
Fig. 7. Modulation of PBL expression of acute phase protein and antimicrobial peptide genes by bacterins. Rainbow trout PBL were stimulated with bacterins and gene expression was quantified as described in Fig. 3. The fold change of gene expression at 4 h is shown with the full range of times studied in Figs. S13 and 14. Different letters over the bars indicate significant differences (p < 0.05, paired samples T test).

Fig. 6. Modulation of PBL expression of chemokine receptor genes by bacterins. Rainbow trout PBL were stimulated with bacterins and gene expression was quantified as described in Fig. 3. The fold change of gene expression at 4 h is shown with the full range of times studied in Figs. S10-12. Different letters over the bars indicate significant differences (p < 0.05, paired samples T test).

trout [18,30,61], Atlantic salmon [33,62] and other fish species [63–65]. However it should be noted that some of the vaccines used *in vivo* include adjuvants that may contribute to the increased gene expression observed. However, *Y. ruckeri* bacterin modulated gene expression in rainbow trout PBL is similar to the spleen response to injected unadjuvanted vaccine [30], suggesting that *in vitro* PBL immune gene expression does indeed largely mimic the immune response to vaccination *in vivo*.

Despite the similarity, a direct analogy or generalisation of the PBL immune response to vaccine candidates *in vitro* to the response *in vivo* should be cautious. The *in vivo* vaccine responses are usually studied in internal organs, such as spleen, head kidney or liver that are away from the primary injection/vaccination site. The response in these internal organs depends on the degree of immune activation at the vaccination site, the trafficking of immune cells (especially antigen presenting cells) between tissues and the vaccination site, and the cellular composition of each organ. Consequently, immune gene expression in response to vaccination can be different in different tissues [29,30,65], and can even show fish species-specificity [66]. For example, genes associated with T and B lymphocyte activity and migration, such as CCR7 and CCR9, are decreased in peritoneal cells after intraperitoneal injection vaccination [63]. This decreased gene expression may be a consequence of different phenomena, that include a rapid influx of myeloid cells to the peritoneal cavity after vaccination [67], or exit of leucocytes expressing high levels of CCR7 and CCR9 that home to secondary lymphoid tissues to initiate adaptive immune responses, or the actions of both.

3.8. Correlation analysis of gene expression

To further exploit the differential power of immune gene expression in PBL after bacterin stimulation, the Spearman rank ordered correlation analysis was performed on selected genes that showed large responses. A good positive correlation was observed within groups of pro-/anti-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα3 and IL-10), adaptive cytokines (IFNγ1-2, IL-4/13B1-2, IL-17A/F1a and IL-22), IL-12 family cytokines (IL-12 P35A1 and P40B2, and IL-27P28A) and chemokine receptor genes (CXCR3A, CCR7B and CCR9A) after stimulation with each bacterin preparation as detailed in Tables S3–7. However, the correlations between different groups were complex and largely stimulation-dependent. For example, in *Y. ruckeri* stimulated samples, the expression of IL-1β1 (and to a large extent IL-8 and IL-10A) was found to be positively correlated with the expression of T cell growth factor (IL-2B), Th1 (IFNγ1-2), Th2 (IL-4/13B1-2), Th22 (IL-22), and IL-12 (P35A1 and P40B2) family cytokines, but negatively correlated to chemokine receptors (CCR7B and CCR9A), and there was no correlation with Th17 (IL-17A/F1a) and Treg (TGFβ1A-B) cytokines (Table 1). These correlations were lost in most cases when stimulated with *A. salmonicida* bacterins. Notably, positive correlations were found between pro-/anti-inflammatory cytokines (IL-1β1, IL-6, IL-8, TNFα3 and IL-10A) and Treg cytokines, and chemokine receptors after *A. salmonicida* bacterin stimulation (Table 1).

A coordinated activation of proinflammatory genes and adaptive cytokines with a negative-correlation to chemokine receptor expression may retain immune cells at the injection site after efficacious ERM vaccination. Although the interpretation of the correlations will be context dependent and needs further investigation, these complex correlations of gene expression further demonstrate the power of PBL gene expression to distinguish differential responses to stimulation with vaccine candidates.

3.9. The value of PBL responses for vaccine candidate screening and vaccine potency testing

In contrast to vaccine-induced immune responses *in vivo*, that occur in immune organs usually after immune cell trafficking, the PBL

immune cell composition is relatively constant and the response to vaccine components is direct. This may allow analysis of subtle changes in gene expression in response to vaccine antigens to be detectable in PBL. For instance, a small but significant rapid induction of chemokine receptor expression (e.g. CCR7 and CCR9) was observed in PBL after bacterin stimulation in this study, that may be undetectable or even decreased as seen in peritoneal cells *in vivo* after vaccination [53]. Unlike cell lines that consist of a pure cell population, PBL prepared by the hypotonic method [25] contain multiple immune cells seen *in vivo*, that allow both innate and adaptive immune responses to be analysed. PBL are the only leucocytes in fish that can be prepared non-lethally, allowing the same fish to be sampled multiple times. The distinct gene expression profiles of innate and adaptive immunity in PBL after bacterin stimulation observed in this study suggest that PBL are a suitable platform for initial screening of vaccine candidates and for evaluation of vaccine-induced immune responses. The PBL system may also be used to evaluate some adjuvant responses (e.g. PAMPs and cytokines) which could be simply added to the cells with the antigens.

Although a bacterin dose-dependent response was not performed in this study, a trend to intermediate gene expression of many genes in MT423 (Fe-) + MT004 stimulated cells, compared to MT423 (Fe-) and MT004 used individually, was apparent (Figs. 3–7). These three groups contain the same total amount of bacteria but the first group has half each of the last two, suggesting a sensitive dose-response effect on PBL gene expression. As seen in mammals [68], vaccine-mediated protection in fish is also vaccine dose-dependent [10,69]. When the correlates of vaccine dose-response profiles of PBL gene expression and *in vivo* protection have been established, PBL will potentially be a cheap alternative for potency tests to reduce animal use [12].

PBL may also be used to investigate immunological memory, which is a feature of adaptive immune responses to vaccination. Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens due to the existence of antigen-specific memory T/B cells. The PBL isolated from vaccinated fish should contain memory T cells and B cells that are expected to respond differently compared to PBL from naïve fish when cultured *in vitro* in the presence of antigens. However, due to the complexity of PBL and the rarity of memory cells, pre-isolation of T/B cells, and homogenous dendritic cells/antigen presenting cells for presenting antigens may have to be applied.

3.10. PBL immune gene expression may help shed light on the protective mechanisms induced by vaccination

Distinct immune gene expression profiles in PBL have been induced in this study by bacterin preparations including those reflecting effective vaccines, such as *Y. ruckeri* and MT423 (Fe-), and non-effective bacterin candidates. Bacterial species have major effects on PBL gene expression with bacterin preparations from *A. salmonicida* behaving in a more similar way in comparison to *Y. ruckeri*. *A. salmonicida* preparations induce a rapid upregulation of proinflammatory genes whilst the kinetics of inflammatory gene expression was slower and peaked later with *Y. ruckeri*. These effects have also been observed *in vivo* with live bacterial infection. For example, the expression of IL-1β, TNFα and IL-10 peaked at 6–12 h after *A. salmonicida* infection in Atlantic salmon [70], whilst in *Y. ruckeri* infected rainbow trout the peak occurred after 1–3 days [29,71]. Although a direct comparison between the two pathogens in terms of dose, kinetics and immune genes examined in the same fish species is lacking, the current PBL response and *in vivo* data in salmonids suggest that distinct immune pathways are activated by each pathogen *in vivo* that can be mimicked *in vitro* by bacterin stimulation of PBL.

It is interesting to note that whilst the *Y. ruckeri* bacterin is an effective vaccine it induces a weak and relatively slow induction of proinflammatory genes in PBL. In addition, the bacterin from *A. salmonicida* grown under iron-depleted conditions has a lower capacity to

Table 1

Spearman's rho correlation coefficient (R) and the 2-tailed significance (* = p < 0.05, ** = p < 0.01) between expression levels of selected pro-/anti-inflammatory cytokines and adaptive cytokines, IL-12 family and chemokine receptors in PBL stimulated with ERM, MT423 (Fe-), MT004, MT423 (Fe-) + MT004 and MT423 as described in Fig. 1. The numbers (R) in red indicate a significant and positive Spearman rank ordered correlation, whilst those in blue indicate a negative correlation.

	IL-1β1	IL-6	IL-8	CXCL11_L1	TNFα3	IL-10A	IL-2B	IFNγ1	IFNγ2	IL-4/13B1	IL-4/13B2	IL-17A1a	IL-22	TGFβ1A	TGFβ1B	P35A1	P28A	P40B2	CXCR3A	CCR7B	CCR9A
IL-1β1																					
ERM		.648**	.924**	.799**	.625**	.864**	.873**	.799**	.620*	.667**	.680**	.304	.589*	.218	-.156	.848**	.234	.883**	-.365	-.776**	-.792**
MT423 (Fe-)		.982**	.641**	-.015	.890**	.921**	.152	.477	.433	.082	.121	.109	.447	.432	.632**	.976**	.974**	.391	.876**	.653**	.715**
MT004		.974**	.762**	.262	.715**	.735**	.675**	.168	.171	-.076	.038	.065	.212	.088	.526*	.947**	.782**	.690**	.589*	.135	.109
MT423(Fe-)+MT004		.962**	.809**	.100	.915**	.767**	.376	.297	.168	-.026	.021	.306	.235	.609*	.509*	.947**	.962**	.518*	.741**	.240	.482
MT423		.918**	.647**	-0.259	.938**	.798**	-.371	.185	.368	-.391	-.338	.096	.226	.633**	.571*	.950**	.947**	.432	.876**	.774**	.824**
IL-6																					
ERM	.648**		.385	.235	.188	.632**	.321	.135	-.003	.068	.047	-.308	.077	.499*	-.447	.694**	.653**	.432	-.182	-.386	-.379
MT423 (Fe-)	.982**		.621*	-.035	.887**	.941**	.075	.452	.419	.072	.129	.112	.429	.476	.597*	.982**	.962**	.406	.856**	.632**	.712**
MT004	.974**		.724**	.235	.679**	.788**	.661**	.135	.156	-.109	.038	.079	.188	.062	.515*	.953**	.776**	.674**	.620*	.132	.126
MT423(Fe-)+MT004	.962**		.706**	.047	.900**	.702**	.318	.184	.085	-.118	-.024	.188	.132	.647**	.336	.944**	.976**	.459	.697**	.208	.468
MT423	.918**		.438	-.174	.847**	.758**	-.218	.185	.259	-.488	-.359	-.066	.029	.481	.541*	.953**	.885**	.424	.829**	.768**	.765**
IL-8																					
ERM	.924**	.385		.921**	.656**	.738**	.962**	.906**	.768**	.835**	.859**	.480	.764**	.160	-.018	.762**	-.035	.953**	-.412	-.808**	-.812**
MT423 (Fe-)	.641**	.621*		.700**	.571*	.541*	.539*	.908**	.790**	.648**	.729**	.646**	.726**	-.038	.826**	.594*	.547	.885**	.447	.229	.097
MT004	.762**	.724**		.671**	.429	.829**	.868**	.674**	.617*	.379	.424	.450	.691**	-.206	.647**	.744**	.429	.915**	.165	-.332	-.362
MT423(Fe-)+MT004	.809**	.706**		.547*	.662**	.790**	.650**	.620*	.521*	.382	.491	.535*	.571*	.209	.668**	.788**	.703**	.826**	.441	-.090	.100
MT423	.647**	.438		.112	.691**	.327	-.266	.279	.403	.226	.162	.549*	.609*	.745**	.644**	.556*	.662**	.468	.538*	.350	.512*
CXCL11_L1																					
ERM	.799**	.235	.921**		.515*	.668**	.944**	.868**	.771**	.771**	.812**	.514*	.784**	.146	-.129	.700**	-.124	.891**	-.515*	-.815**	-.806**
MT423 (Fe-)	-.015	-.035	.700**		-.025	-.124	.508*	.681**	.637**	.796**	.862**	.558*	.550*	-.538*	.503*	-.021	-.100	.856**	-.168	-.279	-.494
MT004	.262	.235	.671**		-.200	.582*	.746**	.838**	.805**	.768**	.876**	.821**	.815**	-.441	.344	.206	-.250	.745**	-.487	-.703**	-.726**
MT423(Fe-)+MT004	.100	.047	.547*		-.015	.322	.585*	.681**	.724**	.679**	.826**	.553*	.582*	-.374	.458	.159	-.009	.794**	-.174	-.496	-.412
MT423	-.259	-.174	.112		-.315	-.255	.609*	.659**	.497	.738**	.897**	.400	.438	-.316	.474	-.144	-.297	.550*	-.432	-.447	-.553*
TNFα3																					
ERM	.625**	.188	.656**	.515*		0.476	.568*	.553*	.315	.382	.429	.360	.258	.166	.182	.506*	.153	.618*	.112	-.434	-.441
MT423 (Fe-)	.890**	.887**	.571*	-.025		.827**	-.172	.543*	.608*	.175	.130	.151	.537*	.618*	.686**	.893**	.936**	.380	.918**	.779**	.806**
MT004	.715**	.679**	.429	-.200		.209	.166	-.079	-.106	-.188	-.282	-.318	-.079	.532*	.582*	.721**	.944**	.389	.877**	.615*	.615*
MT423(Fe-)+MT004	.915**	.900**	.662**	-.015		.545*	.062	.322	.191	.009	-.053	.268	.209	.782**	.511*	.821**	.912**	.485	.874**	.464	.712**
MT423	.938**	.847**	.691**	-.315		.726**	-.536*	.197	.368	-.300	-.306	.218	.297	.824**	.547	.871**	.929**	.435	.906**	.821**	.894**
IL-10A																					
ERM	.864**	.632**	.738**	.668**	0.476		.768**	.638**	.494	.512*	.503*	.178	.403	.149	-.326	.765**	.194	.753**	-.379	-.730**	-.759**
MT423 (Fe-)	.921**	.941**	.541*	-.124	.827**		.025	.383	.359	.029	.094	.050	.356	.482	.585*	.897**	.915**	.303	.838**	.671**	.715**
MT004	.735**	.788**	.829**	.582*	.209		.858**	.529*	.501*	.150	.329	.465	.541*	-.347	.379	.753**	.344	.730**	.116	-.452	-.423
MT423(Fe-)+MT004	.767**	.702**	.790**	.322	.545*		.561*	.323	.275	.050	.213	.312	.316	.018	.547*	.796**	.727**	.475	.372	-.194	.035
MT423	.798**	.758**	.327	-.255	.726**		-.281	.365	.512*	-.330	-.275	.167	.266	.338	.543*	.768**	.759**	.506*	.756**	.755**	.681**

induce pro- and anti-inflammatory gene expression at 4 h than the MT423 bacterin but also represents an effective vaccine candidate. This suggests that inflammatory potential doesn't correlate to protective efficacy of a vaccine candidate. The *Y. ruckeri* bacterin induces an early expression of master transcription factors for Th1, Th2 and Th17 (T-bet, GATA3 and RORγ) cells as well as chemokine receptors (CXCR3B, CCR6, CCR7, CCR9 and CCR4Lc2) at 4 h, suggesting the activation of Th1, Th2 and Th17 type adaptive responses that may be essential for this vaccine mediated immunity. Both MT423 (Fe-) and *Y. ruckeri* bacterins induce higher levels of CCR4Lc2 and CCR6A at 72 h, with their mammalian orthologues important for Th2 and Th17 type adaptive immunity [58]. A Th2 and Th17 biased response was observed in Atlantic salmon vaccinated with an oil-adjuvanted *A. salmonicida* bacterin [72]. The induction of a Th2 type response is in agreement with the fact that antibody titre correlates with vaccine-mediated protection against infection with *A. salmonicida* [10] and *Y. ruckeri* [69]. Thus, immune gene expression in PBL after stimulation with vaccine candidates may shed light on the immune pathways involved that lead to protection.

3.11. Conclusions

PBL can be easily prepared non-lethally and contain major immune cells that enable the detection of both innate and adaptive immune responses. Distinct gene expression profiles of innate and adaptive immunity have been observed between bacterins prepared from different bacterial species, as well as from different strains or culturing conditions of the same bacterial species. Hence immune pathways activated by pathogens or vaccines *in vivo* can also be detected in PBL *in vitro*. Immune gene expression in PBL after stimulation with vaccine candidates may shed light on the immune pathways involved in vaccine-mediated protection in fish. Taken as a whole, this study suggests that PBL are a suitable platform for initial screening of vaccine candidates, for evaluation of vaccine-induced immune responses, and represent a cheap alternative for potency testing to reduce animal use in aquaculture vaccine development.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2019.08.002.

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