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

6

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21 **Abstracts**

22

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

40

41 **Key words:** Rainbow trout *Oncorhynchus mykiss*, Peripheral blood leucocytes, *Aeromonas*
42 *salmonicida*, *Yersinia ruckeri*, Bacterin, Vaccine, Cytokine, Gene expression, Innate immunity,
43 Adaptive immunity

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47 **1. Introduction**

48

49 Fish aquaculture is the world's fastest growing industry within the animal food production sector.
50 However, infectious diseases not only cost up to 20% of production value but also have a significant
51 impact on individual fish health and welfare, and the environment [1-3]. Vaccination is the most
52 appropriate method for controlling disease problems, both from a production and welfare point of
53 view [4-6]. Vaccination plays an important role in large-scale commercial fish farming. It has been a
54 key reason for the success of salmonid cultivation and has reduced the use of antibiotics [7-9]. Almost
55 100% of Atlantic salmon are vaccinated with multivalent vaccines prior to sea transfer in Norway, the
56 largest producer of farmed salmon in the world [10].

57

58 Inactivated whole-cell bacterins constitute the main bacterial vaccines used in aquaculture. The first
59 commercially licensed vaccine for fish (in 1976) was a formalin-killed bacterin delivered by
60 immersion against *Yersinia ruckeri*, the causative agent of enteric redmouth disease (ERM) in
61 salmonids [11-12]. Following the success of this product, vaccines for *vibriosis* were developed.
62 Whilst these vaccines are able to induce protective immunity by bath/dip vaccination, bacterin
63 immersion vaccines against *Aeromonas salmonicida* (the causative agent of furunculosis) were not
64 effective in the field, so oil adjuvant-based bacterin vaccines were developed despite some side effects
65 [7-8, 13]. Iron-regulated outer membrane proteins (IROMPs) of *A. salmonicida* induced under iron-
66 restricted growth conditions were found important protective antigens [14]. Anti-IROMPs antibodies
67 are bactericidal to virulent *A. salmonicida* strains *in vitro* and specific anti-IROMP antibody responses
68 correlate strongly with protection against furunculosis [15]. Thus, bacterins prepared from *A.*
69 *salmonicida* grown under iron-deprived conditions are also good vaccine candidates and led to the
70 non-adjuvanted vaccine against furunculosis AquaVac FNM Plus (Intervet UK Ltd)
71 (http://www.vmd.defra.gov.uk/productinformationdatabase/SPC_Documents/SPC_122154.DOC) that
72 uses both pathogenic and non-pathogenic strains of *A. salmonicida*, i.e. strain MT423 and strain
73 MT004.

74

75 Whilst commercial vaccines are available for many of the major bacterial diseases and a few viral
76 diseases of aquaculture, no commercial vaccines have been produced against fish parasitic infections
77 [4-6] and more vaccines to emerging diseases are waiting to be developed. Furthermore, disease
78 outbreaks do occur on fish farms from time to time even after vaccination with effective commercial
79 vaccines, e.g. the ERM vaccine prepared from motile *Y. ruckeri* lost its efficacy against non-motile
80 strains [16-17]. In Denmark, although the majority of rainbow trout are vaccinated using commercial
81 vaccines against the most serious bacterial pathogens *A. salmonicida* subsp. *salmonicida*, *Yersinia*

82 *ruckeri* and *Vibrio anguillarum*, disease outbreaks requiring treatment with antibiotics still occur [18].
83 These examples highlight the need for continuous improvement of vaccines for long-term protection
84 in aquaculture. The development of fish vaccines requires thorough efficacy and safety testing before
85 they can be marketed. All batches of vaccines must be tested for potency and safety before use. These
86 tests are currently reliant upon mortality testing where fish are exposed to virulent pathogens and
87 relative percent survival is measured, which requires the use of a great number of experimental
88 animals [19-21]. Although a clear-cut approach, it does not unveil the underlying molecular
89 mechanisms of vaccination-mediated protection and is cost- and time-consuming [19-21]. With an
90 increasing focus on the 3 R's (Replacement, Reduction and Refinement), *in vitro* systems using fish
91 immune cells have the potential to replace *in vivo* pathogen exposure for initial screening and testing
92 of novel vaccine candidates/preparations, and for batch potency and safety tests.

93

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

104

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

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114 ## 2. Materials and methods

115

116 ### 2.1. *Bacterin preparation*

117 The *A. salmonicida* subsp. *salmonicida* non-pathogenic strain MT004, and pathogenic strain MT423,
118 and the pathogenic *Y. ruckeri* strain MT3072 used in this study were obtained from the Marine
119 Scotland Science Marine Laboratory, Aberdeen, UK, as described previously [28-29]. The bacteria
120 were inoculated into tryptic soya broth (TSB, Sigma) at 22 ℃ for 18-24 h in a shaking incubator at
121 100 rpm. The MT423 strain was cultured in normal TSB or in TSB supplemented with 100 µm 2,2′-
122 bipyridyl (Sigma, UK) to deplete iron (Fe-) to induce IROMPs expression [14]. After culture, the
123 bacteria were inactivated by addition of formalin solution (Sigma, UK) to the culture media to 1%.
124 The bacteria were then incubated overnight on a slow magnetic stirrer at room temperature. Bacteria
125 were collected by centrifugation and washed three times using phosphate buffered saline (PBS,
126 pH7.4, Sigma, UK). The bacteria were weighed, resuspended in PBS at 10 mg/ml and stored at – 80
127 ℃ ready for use. The bacterins prepared were plated onto TSA plates and incubated for 48 h at 22°C,
128 with no bacterial growth confirming complete inactivation of the bacteria.

129 ### 2.2 PBL preparation and stimulation

130 Rainbow trout (*Oncorhynchus mykiss*) purchased from the Mill of Elrich Trout Fishery
131 (Aberdeenshire, Scotland, UK) were maintained at 14±1°C in a freshwater recirculation aquarium [27,
132 30]. The PBL from four fish were prepared as described previously [25]. Briefly, blood was drawn
133 from the caudal vein using a BD Vacutainer containing lithium heparin (BD, UK). The red blood cells
134 were lysed by combining 4 ml blood and 36 ml ice-cold cell culture grade water (GE Healthcare Life
135 Sciences, UK) for 20 s. Then 4 ml cooled 10x PBS (Sigma, UK) was added to revert the medium to
136 isotonicity. The suspension was left in ice for 5–10 min and filtrated through a 70 µm cell strainer
137 (Greiner Bio One, UK), pelleted by centrifugation (200 g, 5 min), and washed once with incomplete
138 cell culture medium (Leibovitz medium L-15, Life Technologies, UK) supplemented with 100 IU/ml
139 penicillin, 100 µg/ml streptomycin (P/S), and 1% foetal calf serum (FCS, Sigma, UK). The PBL were
140 counted using a Neubauer chamber and 0.5% trypan blue, resuspended in complete cell culture
141 medium (as above except 10% FCS) and distributed in 12-well cell culture plates at 2×10^6 cells/ml, 2
142 ml/well. The PBL from each fish were then stimulated with 100 µg/ml of inactivated bacterin (1) *A.*
143 *salmonicida* MT423 (MT423), (2) *A. salmonicida* MT423 grown in iron-depleted medium (MT423
144 Fe-), (3) *A. salmonicida* MT004 (MT004), (4) an equal combination of MT423 (Fe-) & MT004, (5) *Y.*
145 *ruckeri* MT3072 and PBS as control. The cells were incubated at 20°C for 4 h, 8 h, 24 h and 72 h,
146 harvested in 1.5 ml TRI reagent (Sigma, UK), and stored at –80 °C until RNA extraction.

147 ### 2.3 RNA extraction, cDNA synthesis and qPCR

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148 Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) analysis of gene expression were
149 carried out as described by Wang et al. [27,31]. The TRI lysates were thawed at room temperature and
150 total RNA was prepared as per the manufacturer's instructions. The RNA was reverse transcribed to
151 cDNA using RevertAid Reverse Transcriptase (Thermo Scientific). The resultant cDNAs were diluted
152 in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) (Sigma) ready for use. The qPCR was
153 performed in duplicate using a LightCycler 480 (Roche) system and 2× SYBR green Master Mix
154 made with an Immolase DNA Polymerase kit (Bioline). The genes studied included those encoding
155 the known cytokines, chemokines and chemokine receptors, suppressor of cytokine signalling (SOCS)
156 proteins, acute phase proteins (APPs), antimicrobial peptides (AMPs), cellular markers, and master
157 transcription factors of T cell responses. The primer sets were designed with at least one primer across
158 an intron, tested to ensure that PCR products could only be amplified from cDNA samples and not
159 from genomic DNA, and are detailed in Supplementary Table S1. The data were analysed using the
160 LightCycler 480 integrated software. The gene expression level for each sample was normalised to
161 that of the housekeeping gene, elongation factor (EF)-1α, and expressed as an arbitrary unit (AU)
162 where 1 AU = the average expression in the control cells at 4 h. A fold change for each treatment was
163 also calculated as the average expression divided by that of time matched controls.
164

165 **2.4 Data visualization, clustering and statistical analysis**

166 The fold changes of gene expression were analysed using ClustVis [32] for visualisation of a heatmap
167 and for Principal Component Analysis (PCA). The arbitrary units of each gene were scaled and log2
168 transformed to improve the normality of real-time quantitative PCR measurements before statistical
169 analysis as described previously [31]. A paired-samples T test was used to determine the level of
170 difference between the treatments at each time point using the IBM SPSS Statistics package 25.0
171 (SPSS Inc., Chicago, Illinois), with differences considered significant at $p < 0.05$. The Spearman's rho
172 correlation analysis of gene expression was performed on selected pro-/anti-inflammatory cytokines,
173 adaptive cytokines, IL-12 family and chemokine receptors. The four time points from each bacterin
174 stimulation was combined for this analysis (n=16).
175
176
177

178 **3. Results and discussion**

179

180 In this study, freshly prepared PBL were stimulated for 4 h, 8 h, 24 h and 72 h with five Gram-
181 negative bacterin preparations. Bacterin A was from *Y. ruckeri* that is an effective vaccine against
182 ERM [12]. Bacterins B (MT004) and C (MT423) were from non-pathogenic and pathogenic *A.*
183 *salmonicida* subsp. *salmonicida* strains, respectively, but neither are effective vaccines against
184 furunculosis [13-14]. Bacterin E was from MT423 cultured under iron-depleted condition that
185 represent an effective vaccine candidate [14] and bacterin D was an equal mix of bacterins C and E
186 that represents a vaccine previously used in the field. The expression of 93 immune relevant genes
187 (Table S1) was examined by RT-qPCR and the expression normalised to EF-1α. The expression of 18
188 genes (IL-17A/F1b, IL-17A/F2a, IL-17A/F2b, IL-17A/F3, IL-17D, IL-23P19, IL-27P28B, IL-
189 12P35B, IL-20, SOCS2A, SOCS2B, SOCS3A, SOCS3B, β-defensin 1-3, CC4La and CCR6B, **Table**
190 **S1**) was low or undetectable in control samples at 4 h, and thus were excluded from further analysis.
191 The remaining 75 genes were expressed and modulated in PBL. The relative expression levels across
192 different time points (with statistical analysis) are presented in **Figs. S1-14** and the average fold
193 change at each time point presented in **Table S2**.

194

195 **3.1. Clustering and principal component analysis**

196 In response to bacterin stimulation PBL gene expression changed over time from 4 h to 72 h.
197 Hierarchical clustering analysis of the fold changes across four time points revealed time-dependent
198 gene expression patterns (**Fig. 1**). Three major clusters of genes were apparent. Cluster 1 contained
199 several chemokine receptor genes (CCR6A, CCR7A, CCR7B, CCR9A, CCR9B, CCR4Lc2 and
200 CXCR3B) that were induced/higher at 4 h and 72 h but low at 8 h and 24 h as compared to the
201 unstimulated samples. Cluster 2, mainly consisted of adaptive cytokines, that were gradually induced
202 and peaked at 8 h or 24 h, and remained elevated to 72 h. Cluster 3 genes, including mainly pro- and
203 anti-inflammatory cytokines and APPs, were induced early, peaked at 4 h or 8 h, and returned to
204 resting levels by 24 h to 72 h (**Fig. 1**). The clustering patterns of the different bacterin preparations
205 changed over time but with *Y. ruckeri* always clustering away from the various *A. salmonicida*
206 bacterins at all four time points, suggesting a major effect of bacterial species, as well as different
207 preparations of the same species/strain, on immune gene expression.

208

209 PCA analysis of the fold change of 75 genes at the 4 time points revealed that the first two
210 components accounted for 85% of the variation (**Fig. 2**). The biplot indicates that the bacterin
211 modulated gene expression can be divided into four distinct groups based on time, in agreement with
212 the major effects of stimulation time on gene expression seen in **Fig. 1**. Different bacterin
213 preparations showed distinct effects/variation on gene expression at each time point. At 4 h, the

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214 effects of bacterin preparation were well separated from each other, suggesting this gives the most
215 discriminating power. Thus, the bacterin modulated gene expression was analysed further with a focus
216 at 4 h.
217
218 **3.2. Modulation of the expression of pro- and anti-inflammatory genes by bacterins**
219 Genes involved in the inflammatory response are modulated *in vivo* by vaccination in salmonid
220 lymphoid organs such as head kidney [33] and spleen [30]. Therefore, we examined first in PBL the
221 expression of proinflammatory cytokines (IL-1β [34], TNFα [35], IL-6, IL-11 [36], M17 [37] and IL-
222 34 [38]), chemokines (IL-8 and CXCL11_L1 [39]), anti-inflammatory cytokines (IL-10 [40], TGF-β1
223 [41] and nIL-1Fm [42]), and negative regulators of cytokine signalling (SOCS) [43]. The expression
224 of all these genes could be modulated in PBL by bacterin stimulation (**Figs. S1-3**). The expression of
225 the majority of the genes was rapidly induced at 4 h and peaked at 4 h or 8 h, and this modulation
226 subsided by 24 h or 72 h (**Table S2**). The exception was the down-regulation of TNFα2 and TGFβ1A,
227 that are highly expressed in PBL (as seen by the small ΔCP, **Table S1**). A noticeable difference of
228 gene expression change was the rapid and strong induction of gene expression by *A. salmonicida*
229 preparations, compared to a gradual but lasting increase induced by the *Y. ruckeri* bacterin (e.g. IL-
230 1β1, TNFα3, IL-8, **Figs. S1-2**). Major differences between different paralogues in response to bacterin
231 stimulation were also observed, in terms of magnitude of the response (e.g. IL-1β paralogues),
232 response kinetics (e.g. IL-10 paralogues), and types of response seen (e.g. down-regulation of TNFα2
233 but up-regulation of other TNFα paralogues) (**Figs. S1, S3**). Differential responses of cytokine
234 paralogues have been observed previously [30, 34-35, 40-41] and may indicate neo- or sub-
235 functionalisation.
236
237 At 4 h post stimulation, the expression of IL-1β1-2, TNFα3, IL-6, IL-8 and IL-10A showed a similar
238 pattern, where the highest induction was seen with MT423, followed by MT423(Fe-), MT423 (Fe-) +
239 MT004, MT004, and the lowest (but significant) induction seen with *Y. ruckeri* (**Fig. 3**). This
240 expression pattern, to a lesser extent, was also observed with IL-1β3, TNFα1, IL-11, M17 and nIL-1F.
241 Interestingly, *Y. ruckeri* tended to induce higher levels of the anti-inflammatory gene TGFβ1B and
242 SOCS1A than MT423(Fe-) + MT004 at 4h. These results indicate a stronger inflammatory response
243 induced by *A. salmonicida* strains compared to *Y. ruckeri*, and that the expression of a set of immune
244 genes can distinguish the responses induced in PBL by different bacterin preparations.
245
246 **3.3. Modulation of the expression of genes of adaptive immunity by bacterins.**
247 The expression of signature cytokines for Th1 (IFNγ1-2 [44]), Th2 (IL-4/13A, IL-4/13B1-2, [45]) and
248 Th17 (IL-17A/F1a, [46]) type responses was next studied. Th1 cytokines were induced at 4 h by *A.*
249 *salmonicida* preparations but not by *Y. ruckeri*. However, Th17 and Th2 cytokines (IL-4/13B1-2)
250 were only induced by MT423 at 4 h (**Fig. 4**). The expression of IL-2B [27] and IL-17C1 [47]

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251 followed the pattern of Th1 cytokines. The expression of other cytokines (IL-2A, IL-4/13A, IL-17C2,
252 IL-18, IL-21 and IL-22 [48]) was less responsive. In general, *A. salmonicida* preparations induced a
253 relatively early (at 4 h and/or 8 h) adaptive cytokine expression, and *Y. ruckeri* induced a late (at 8 h
254 or later) cytokine response (**Fig. S4-6**).
255
256 The expression of master transcription factors for Th1 (T-bet), Th2 (GATA3 [49]), Th17 (RORγ
257 [50]), and regulatory T cells (FoxP3A/B [51]) was also examined. At 4 h, T-bet expression was
258 upregulated by MT004 and *Y. ruckeri*, and RORγ expression by *Y. ruckeri* only. A small but
259 significant increase of FoxP3A/B was observed in all stimulated samples, but GATA3 was refractory
260 at 4 h (**Fig. 4**). The effects of bacterin stimulation were small at other time points (**Fig. S6**). The
261 effects of bacterin-stimulation on T cell markers (CD3ε, CD4, CD28 and CTLA4) and B cell markers
262 were also small (**Figs. S7-8**), with a minor increase in the expression of CD3ε and CD4 at 4 h by all
263 bacterins (**Fig. 4**). The large increases of proinflammatory cytokine gene expression and minor
264 changes on adaptive genes at 4 h suggest a rapid PAMP-activated response by bacterins that may
265 trigger later adaptive responses.
266

267 **3.4. Modulation of the expression of the IL-12 family by bacterins.**

268 The expression of subunits of the IL-12 family followed the pattern of the major pro- and anti-
269 inflammatory cytokines, with a rapid induction of the α-subunit of IL-12 (P35A1 and P35A2 [52-53])
270 and IL-27 (P28A, [54]), and the β-subunits of IL-12 (P40B1 and P40B2, [52-53]) at early time points
271 (4 h and 8 h, **Fig. S9**). The exception was P40C where the induced expression peaked at 8 h or 24 h.
272 At 4 h, MT423 was a strong stimulator of IL-12 family expression compared to *Y. ruckeri*, as seen
273 with the major inflammatory and Th1 cytokines (**Fig. 5**). The differential expression of different
274 subunits suggests that different isoforms of IL-12 can be induced by different vaccine candidates,
275 which have the potential to modulate differential Th cell development after vaccination.
276

277 **3.5. Modulation of the expression of chemokine receptors by bacterins.**

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

287 CCR4Lc2 and CCRL1 [56], compared to MT423 at 4 h (**Fig. 6**). In contrast *A. salmonicida*
288 preparations induced a higher level of CXCR3A and CCBP2 [57] transcripts than *Y. ruckeri* (**Fig. 6**).
289
290 Chemokine receptors are present on all leucocytes and their expression can be differentially
291 modulated to position the right immune cells in the right place [58]. The adaptive immune response is
292 initiated in the secondary lymphoid organs with the arrival of antigen or mature dendritic cells (DCs).
293 The antigen-loaded DCs and naive T cells must be co-localized to allow the rare antigen-specific
294 naive T cells to scan and interact with the DCs. The co-localization of immune cells is promoted by
295 chemokine receptor-mediated migration [58-59]. Of note, mammalian CXCR3, CCR4 and CCR6 are
296 important for Th1, Th2 and Th17 type adaptive immunity, respectively; CCR7 regulates the migration
297 of DCs, B cells and T cells; and CCR9 regulates GALT development and homing of T cells to gut
298 [58]. The differential ability of vaccine candidates to modulate early chemokine receptor expression
299 may provide novel insights into the immune pathways activated by vaccination. Interestingly, the *Y.*
300 *ruckeri* bacterin that is an effective vaccine induces a stronger expression of CXCR3B, CCR4Lc2,
301 CCR7, and CCR9, compared to the other vaccine candidates studied. This information could be a
302 starting point to further dissect the immune pathways activated by vaccination that provide protection.
303
304 **3.6. Modulation of the expression of APP and AMP genes by bacterins.**
305 Lastly, the expression of several APP and AMP genes that are known to be induced *in vivo*
306 by vaccination and bacterial infection was studied [30, 33]. COX2 expression was rapidly
307 induced at 4 h and followed the expression pattern of the other inflammatory genes (**Fig.**
308 **S13**). The induced expression of SAA, SAP1, CATH1, CATH2 and LEAP1/hepcidin was
309 relatively low at 4 h, peaking at 8 h or 24 h (**Figs. S13-14**). Such expression kinetics are in
310 line with the *in vivo* observation that early induction of proinflammatory cytokines by
311 vaccines and PAMPs induces APPs and AMPs [30, 60]. Bacterin stimulation had only minor
312 effects on the expression of SAP2 and β-defensin 4. At 4 h, MT423 was more potent at
313 induction of SAA, SAP1, COX-2 and CATH1 than *Y. ruckeri* (**Fig. 7**).
314
315 **3.7. PBL immune gene expression mimics the *in vivo* response to vaccination**
316 The expression of major pro- and anti-inflammatory genes (IL-1, IL-6, IL-8, TNFα, IL-10 etc.), acute
317 phase protein genes (SAA, SAP1 and COX-2), antimicrobial peptide genes (CATH1, CATH2 and
318 LEAP1), as well as adaptive cytokine genes (IFNγ, IL-17A/F and IL-4/13) are increased in PBL by
319 bacterin vaccine candidates in this study. Their expression is also increased *in vivo* by injection
320 vaccination in rainbow trout [18, 30, 61], Atlantic salmon [33, 62] and other fish species [63-65].
321 However it should be noted that some of the vaccines used *in vivo* include adjuvants that may

322 contribute to the increased gene expression observed. However, *Y. ruckeri* bacterin modulated gene
323 expression in rainbow trout PBL is similar to the spleen response to injected unadjuvanted vaccine
324 [30], suggesting that *in vitro* PBL immune gene expression does indeed largely mimic the immune
325 response to vaccination *in vivo*.
326
327 Despite the similarity, a direct analogy or generalisation of the PBL immune response to vaccine
328 candidates *in vitro* to the response *in vivo* should be cautious. The *in vivo* vaccine responses are
329 usually studied in internal organs, such as spleen, head kidney or liver that are away from the primary
330 injection/vaccination site. The response in these internal organs depends on the degree of immune
331 activation at the vaccination site, the trafficking of immune cells (especially antigen presenting cells)
332 between tissues and the vaccination site, and the cellular composition of each organ. Consequently,
333 immune gene expression in response to vaccination can be different in different tissues [29, 30, 65],
334 and can even show fish species-specificity [66]. For example, genes associated with T and B
335 lymphocyte activity and migration, such as CCR7 and CCR9, are decreased in peritoneal cells after
336 intraperitoneal injection vaccination [63]. This decreased gene expression may be a consequence of
337 different phenomena, that include a rapid influx of myeloid cells to the peritoneal cavity after
338 vaccination [67], or exit of leucocytes expressing high levels of CCR7 and CCR9 that home to
339 secondary lymphoid tissues to initiate adaptive immune responses, or the actions of both.
340
341 **3.8. Correlation analysis of gene expression**
342 To further exploit the differential power of immune gene expression in PBL after bacterin stimulation,
343 the Spearman rank ordered correlation analysis was performed on selected genes that showed large
344 responses. A good positive correlation was observed within groups of pro-/anti-inflammatory
345 cytokines (IL-1β, IL-6, IL-8, TNFα3 and IL-10), adaptive cytokines (IFNγ1-2, IL-4/13B1-2, IL-
346 17A/F1a and IL-22), IL-12 family cytokines (IL-12 P35A1 and P40B2, and IL-27P28A) and
347 chemokine receptor genes (CXCR3A, CCR7B and CCR9A) after stimulation with each bacterin
348 preparation as detailed in Tables S3-7. However, the correlations between different groups were
349 complex and largely stimulation-dependent. For example, in ERM stimulated samples, the expression
350 of IL-1β1 (and to a large extent IL-8 and IL-10A) was found to be positively correlated with the
351 expression of T cell growth factor (IL-2B), Th1 (IFNγ1-2), Th2 (IL-4/13B1-2), Th22 (IL-22), and IL-
352 12 (P35A1 and P40B2) family cytokines, but negatively correlated to chemokine receptors (CCR7B
353 and CCR9A), and there was no correlation with Th17 (IL-17A/F1a) and Treg (TGFβ1A-B) cytokines
354 (Table 1). These correlations were lost in most cases when stimulated with *A. salmonicida* bacterins.
355 Notably, positive correlations were found between pro-/anti-inflammatory cytokines (IL-1β1, IL-6,
356 IL-8, TNFα3 and IL-10A) and Treg cytokines, and chemokine receptors after *A. salmonicida* bacterin
357 stimulation (Table 1).
358

359 A coordinated activation of proinflammatory genes and adaptive cytokines with a negative-correlation
360 to chemokine receptor expression may retain immune cells at the injection site after efficacious ERM
361 vaccination. Although the interpretation of the correlations will be context dependent and needs
362 further investigation, these complex correlations of gene expression further demonstrate the power of
363 PBL gene expression to distinguish differential responses to stimulation with vaccine candidates.
364

365 **3.9. The value of PBL responses for vaccine candidate screening and vaccine potency testing**

366 In contrast to vaccine-induced immune responses *in vivo*, that occur in immune organs usually after
367 immune cell trafficking, the PBL immune cell composition is relatively constant and the response to
368 vaccine components is direct. This may allow analysis of subtle changes in gene expression in
369 response to vaccine antigens to be detectable in PBL. For instance, a small but significant rapid
370 induction of chemokine receptor expression (e.g. CCR7 and CCR9) was observed in PBL after
371 bacterin stimulation in this study, that may be undetectable or even decreased as seen in peritoneal
372 cells *in vivo* after vaccination [53]. Unlike cell lines that consist of a pure cell population, PBL
373 prepared by the hypotonic method [25] contain multiple immune cells seen *in vivo*, that allow both
374 innate and adaptive immune responses to be analysed. PBL are the only leucocytes in fish that can be
375 prepared non-lethally, allowing the same fish to be sampled multiple times. The distinct gene
376 expression profiles of innate and adaptive immunity in PBL after bacterin stimulation observed in this
377 study suggest that PBL are a suitable platform for initial screening of vaccine candidates and for
378 evaluation of vaccine-induced immune responses. The PBL system may also be used to evaluate some
379 adjuvant responses (e.g. PAMPs and cytokines) which could be simply added to the cells with the
380 antigens.
381

382 Although a bacterin dose-dependent response was not performed in this study, a trend to intermediate
383 gene expression of many genes in MT423 (Fe-) + MT004 stimulated cells, compared to MT423 (Fe-)
384 and MT004 used individually, was apparent (Figs. 3-7). These three groups contain the same total
385 amount of bacteria but the first group has half each of the last two, suggesting a sensitive dose-
386 response effect on PBL gene expression. As seen in mammals [68], vaccine-mediated protection in
387 fish is also vaccine dose-dependent [10, 69]. When the correlates of vaccine dose-response profiles of
388 PBL gene expression and *in vivo* protection have been established, PBL will potentially be a cheap
389 alternative for potency tests to reduce animal use [12].
390

391 PBL may also be used to investigate immunological memory, which is a feature of adaptive immune
392 responses to vaccination. Immunological memory is the ability of the immune system to respond more
393 rapidly and effectively to pathogens due to the existence of antigen-specific memory T/B cells. The
394 PBL isolated from vaccinated fish should contain memory T cells and B cells that are expected to
395 respond differently compared to PBL from naïve fish when cultured *in vitro* in the presence of

396 antigens. However, due to the complexity of PBL and the rarity of memory cells, pre-isolation of T/B
397 cells, and homogenous dendritic cells/antigen presenting cells for presenting antigens may have to be
398 applied.
399

400 **3.10. PBL immune gene expression may help shed light on the protective mechanisms induced**
401 **by vaccination**

402 Distinct immune gene expression profiles in PBL have been induced in this study by bacterin
403 preparations including those reflecting effective vaccines, such as *Y. ruckeri* and MT423 (Fe-), and
404 non-effective bacterin candidates. Bacterial species have major effects on PBL gene expression with
405 bacterin preparations from *A. salmonicida* behaving in a more similar way in comparison to *Y. ruckeri*.
406 *A. salmonicida* preparations induce a rapid upregulation of proinflammatory genes whilst the kinetics
407 of inflammatory gene expression was slower and peaked later with *Y. ruckeri*. These effects have also
408 been observed *in vivo* with live bacterial infection. For example, the expression of IL-1β, TNFα and
409 IL-10 peaked at 6-12 h after *A. salmonicida* infection in Atlantic salmon [70], whilst in *Y. ruckeri*
410 infected rainbow trout the peak occurred after 1-3 days [29, 71]. Although a direct comparison
411 between the two pathogens in terms of dose, kinetics and immune genes examined in the same fish
412 species is lacking, the current PBL response and *in vivo* data in salmonids suggest that distinct
413 immune pathways are activated by each pathogen *in vivo* that can be mimicked *in vitro* by bacterin
414 stimulation of PBL.
415

416 It is interesting to note that whilst the *Y. ruckeri* bacterin is an effective vaccine it induces a weak and
417 relatively slow induction of proinflammatory genes in PBL. In addition, the bacterin from *A.*
418 *salmonicida* grown under iron-depleted conditions has a lower capacity to induce pro- and anti-
419 inflammatory gene expression at 4 h than the MT423 bacterin but also represents an effective vaccine
420 candidate. This suggests that inflammatory potential doesn't correlate to protective efficacy of a
421 vaccine candidate. The *Y. ruckeri* bacterin induces an early expression of master transcription factors
422 for Th1, Th2 and Th17 (T-bet, GATA3 and RORγ) cells as well as chemokine receptors (CXCR3B,
423 CCR6, CCR7, CCR9 and CCR4Lc2) at 4 h, suggesting the activation of Th1, Th2 and Th17 type
424 adaptive responses that may be essential for this vaccine mediated immunity. Both MT423 (Fe-) and
425 *Y. ruckeri* bacterins induce higher levels of CCR4Lc2 and CCR6A at 72 h, with their mammalian
426 orthologues important for Th2 and Th17 type adaptive immunity [58]. A Th2 and Th17 biased
427 response was observed in Atlantic salmon vaccinated with an oil-adjuvanted *A. salmonicida* bacterin
428 [72]. The induction of a Th2 type response is in agreement with the fact that antibody titre correlates
429 with vaccine-mediated protection against infection with *A. salmonicida* [10] and *Y. ruckeri* [69]. Thus,
430 immune gene expression in PBL after stimulation with vaccine candidates may shed light on the
431 immune pathways involved that lead to protection.
432

433 **3.11. Conclusions**

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

444

445

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447

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666 **6. Figure legend**

667

668 **Fig. 1. Hierarchical clustering analysis of immune gene expression modulated by bacterins in**
669 **PBL.** Freshly prepared PBL from four trout were stimulated with formalin inactivated bacterins of *Y.*
670 *ruckeri* (A), *A. salmonicida* subsp. *salmonicida* strains MT004 (B) and MT423 (C), MT423 cultured
671 under iron-depleted condition (MT423 (Fe-)) (E), and an equal mixture of MT004 and MT423 (Fe-)
672 (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
673 CluctVis program for clustering analysis. Three clusters of gene expression patterns, C1, C2 and C3,
674 are apparent.

675

676 **Fig. 2. Principal component analysis of immune gene expression modulated by bacterins in**
677 **PBL.** Freshly prepared PBL from four trout were stimulated with formalin inactivated bacterins of *Y.*
678 *ruckeri* (A), *A. salmonicida* subsp. *salmonicida* strains MT004 (B) and MT423 (C), MT423 cultured
679 under iron-depleted condition (MT423 (Fe-)) (E), and an equal mixture of MT004 and MT423 (Fe-)
680 (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
681 CluctVis program for principal component analysis.

682

683 **Fig. 3. Modulation of PBL expression of pro- and anti-inflammatory genes by bacterins.**
684 Rainbow trout PBL were stimulated with bacterins of *Y. ruckeri*, *A. salmonicida* strains
685 MT423 and MT004, MT423 grown under iron-depleted conditions (Fe-), and a mixture of
686 MT423 (Fe-) and MT004 for 4 h, 8 h, 24 h and 72 h. The average (+SEM, N=4) gene
687 expression was quantified by RT-qPCR. The expression level of each sample was normalised
688 with that of EF-1α, and expressed as arbitrary units where one unit equals the average
689 expression level in the control samples at 4 h. The fold change of expression at 4 h is shown
690 with the full range of times studied in **Figs. S1-3**. Different letters over the bars indicate
691 significant differences ($p<0.05$, paired samples T test).

692

693 **Fig. 4. Modulation of PBL expression of cytokines and markers of adaptive immunity**
694 **by bacterins.** Rainbow trout PBL were stimulated with bacterins and gene expression was
695 quantified as described in **Fig. 3**. The fold change of gene expression at 4 h is shown with the
696 full range of times studied in **Figs. S4-7**. Different letters over the bars indicate significant
697 differences ($p<0.05$, paired samples T test).

698

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699 **Fig. 5. Modulation of PBL expression of IL-12 cytokine family by bacterins.** Rainbow
700 trout PBL were stimulated with bacterins and gene expression was quantified as described in
701 **Fig. 3**. The fold change of gene expression at 4 h is shown with the full range of times
702 studied in **Fig. S9**. Different letters over the bars indicate significant differences ($p<0.05$,
703 paired samples T test).

704

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

710

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

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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**

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Figure 1

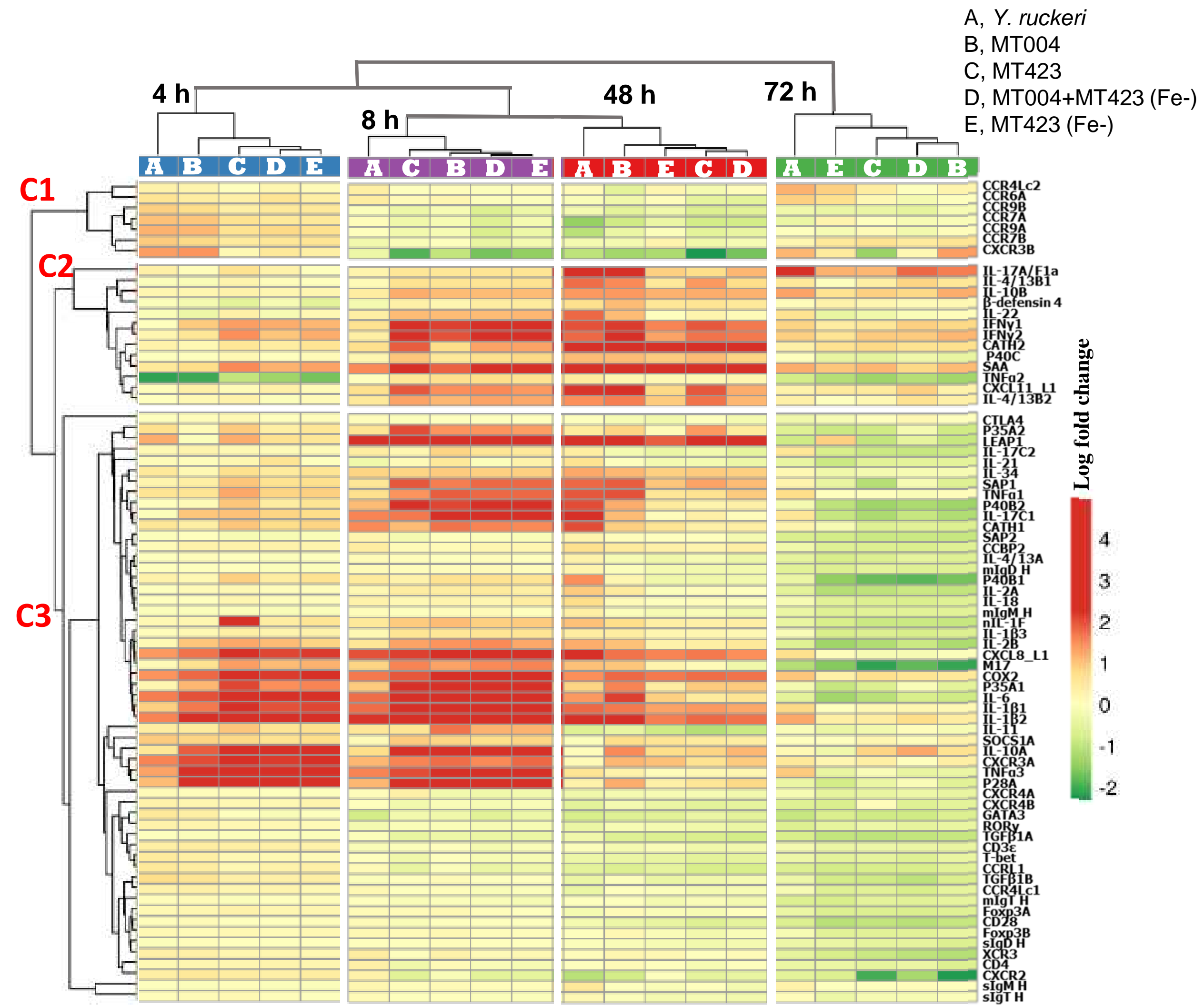

Figure 2

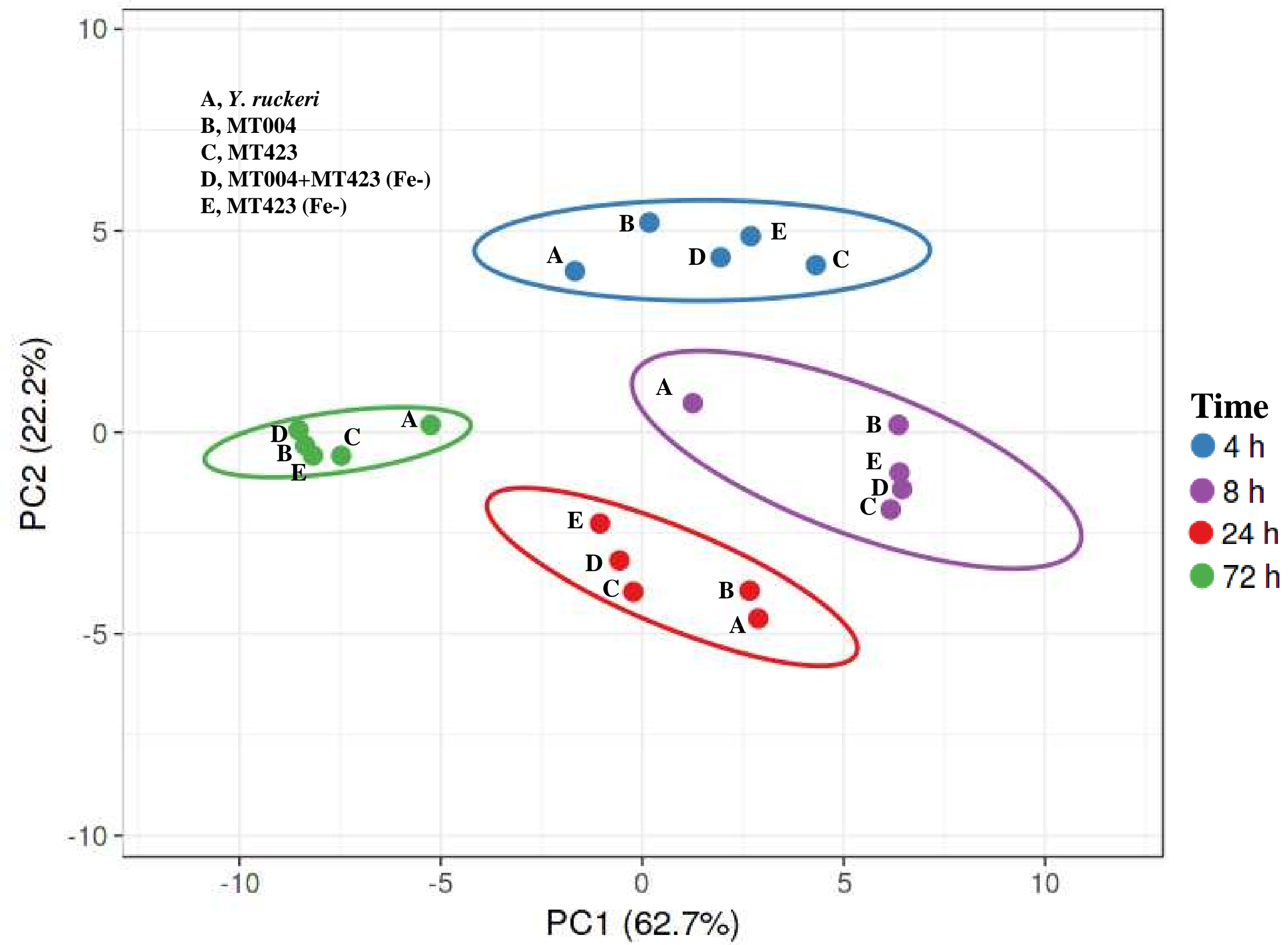

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Figure 3

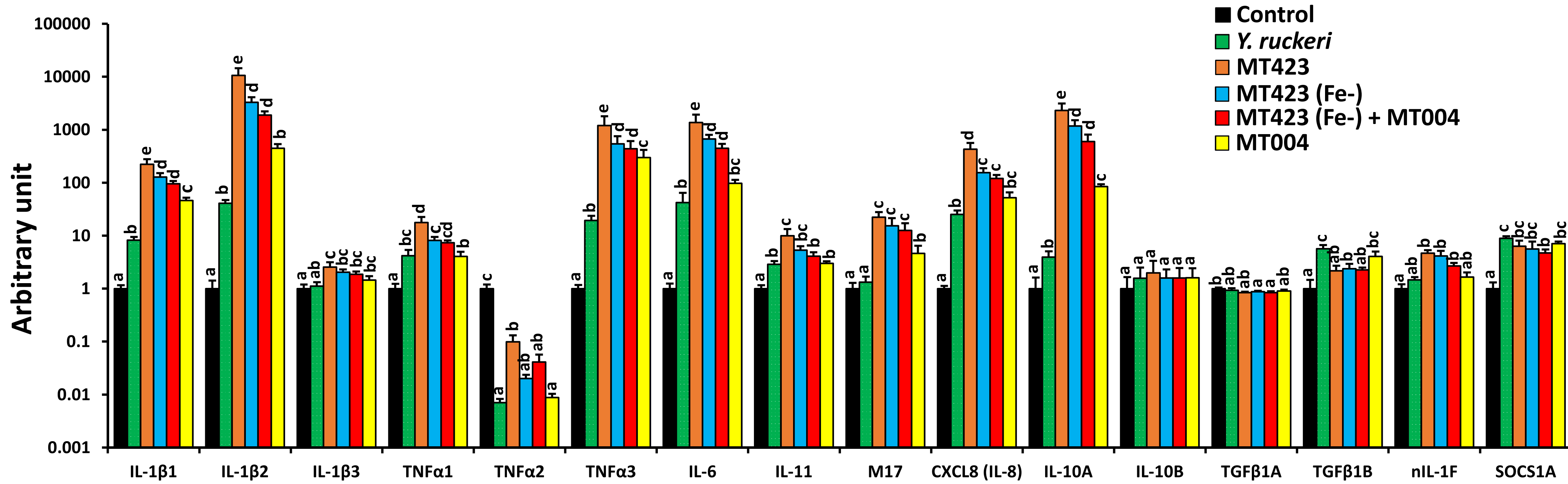

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Figure 4

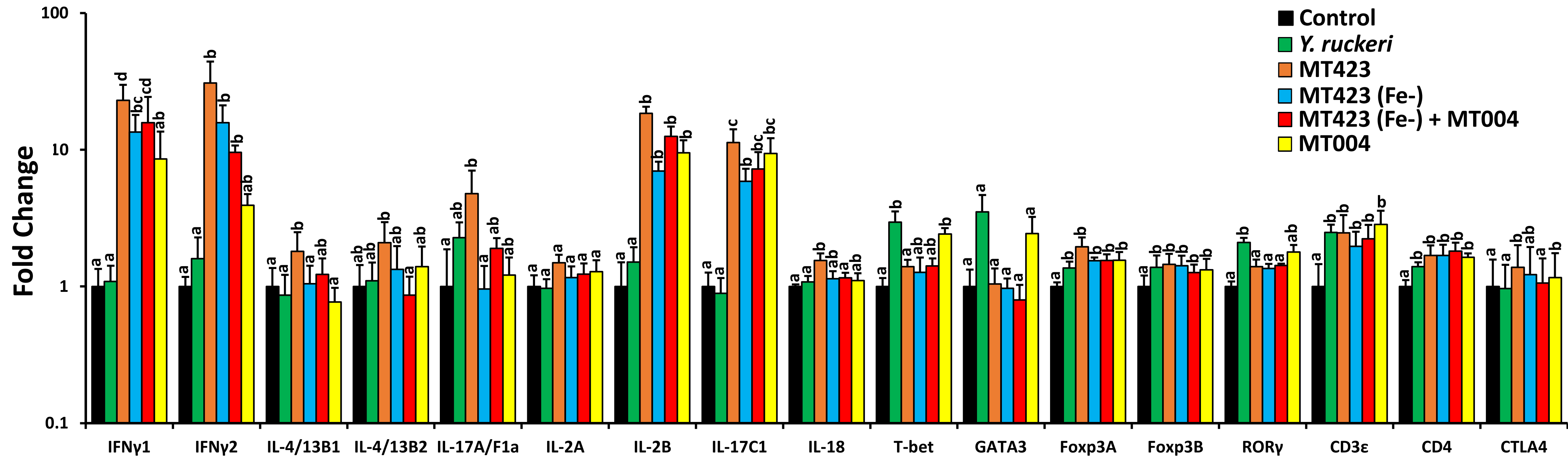


Figure 5

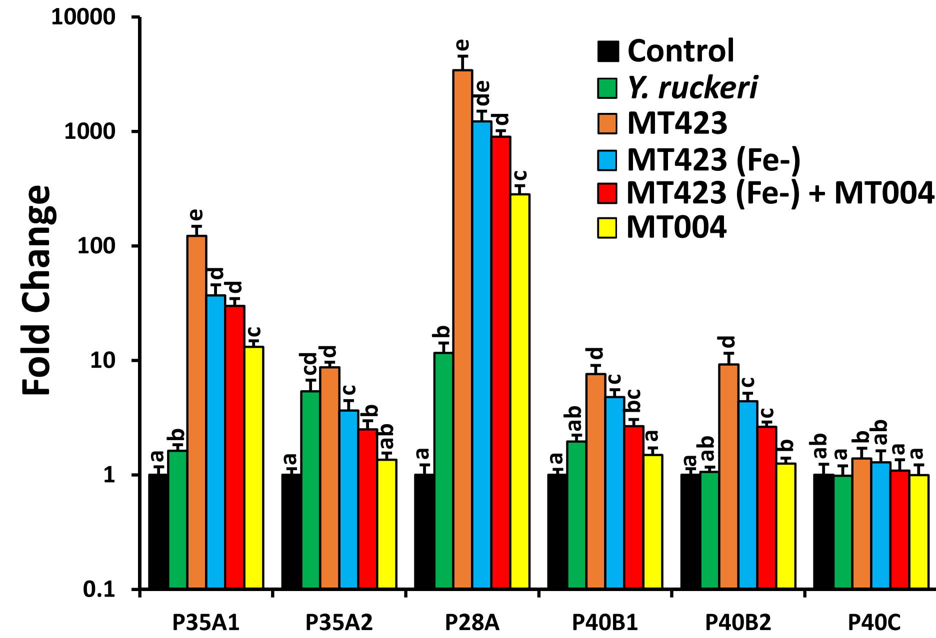


Figure 6

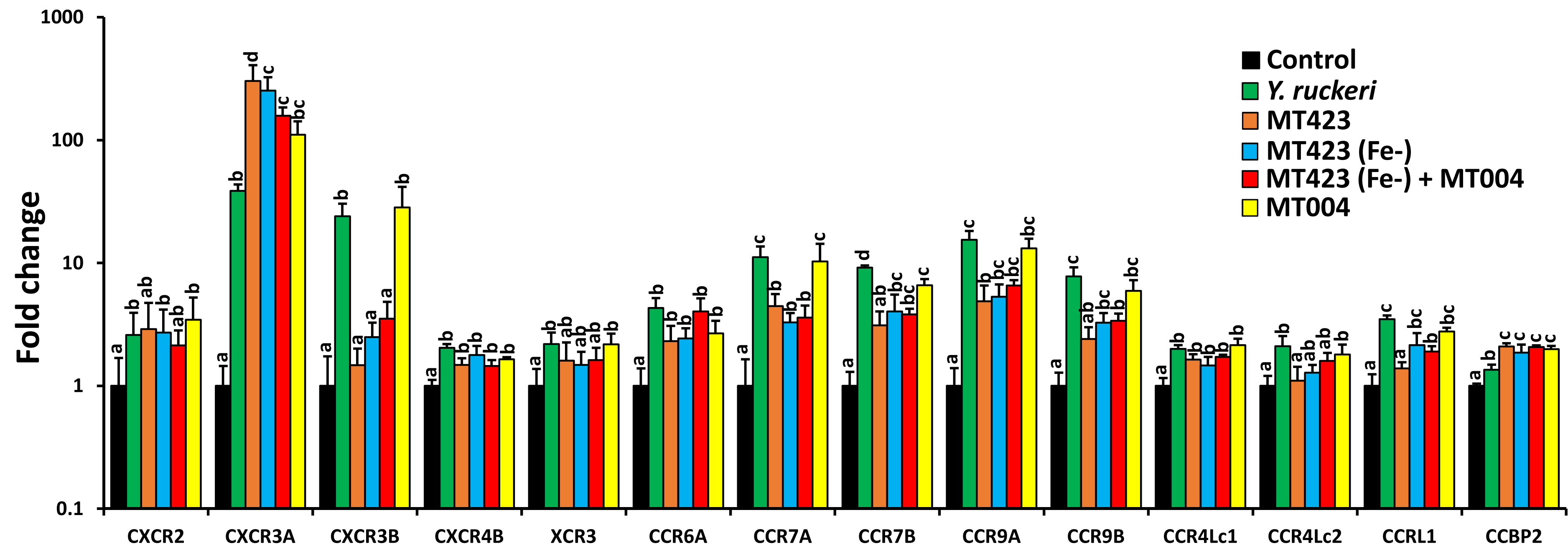
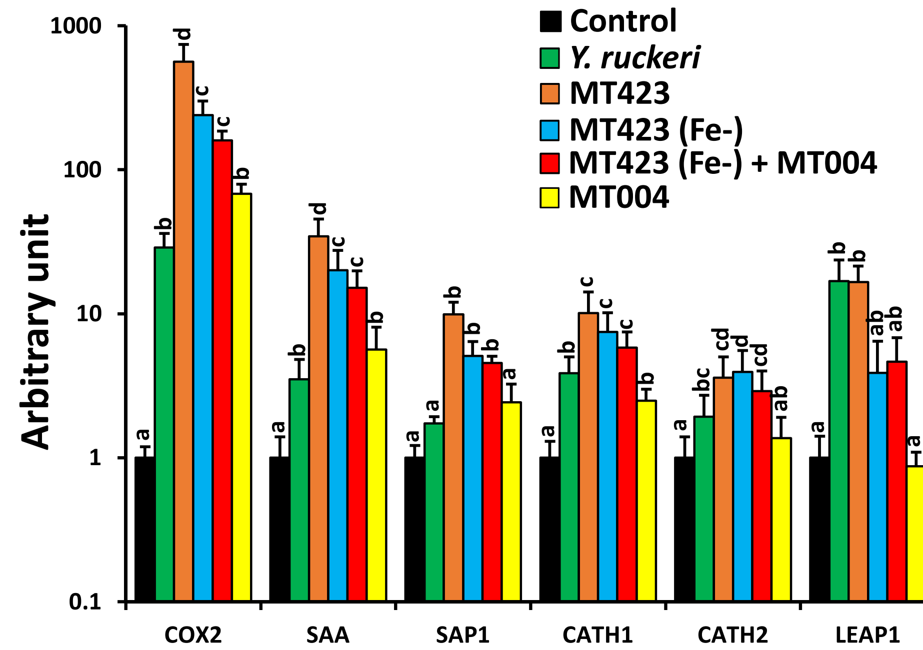


Figure 7



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Highlights

1. PBL contain major immune cells to allow the detection of innate and adaptive immune responses.
2. Distinct immune gene expression profiles are activated in PBL by bacterin vaccine candidates.
3. A rapid 4 h stimulation gives the most discriminating power of the effects of bacterin preparations.
4. Immune gene expression in PBL may shed light on the mechanisms of vaccine-mediated protection.
5. PBL are a suitable *in vitro* platform to reduce animal use in aquaculture vaccine development.

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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		
ProteinPeptide		
Polymerase		

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proteins		
Reverse Transcriptase		

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