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Ahmed Attaya planned and performed the study, analyzed and interpreted the data, and wrote the paper. **Christopher J. Secombes** provided experimental advice, analyzed the data, and wrote the paper. **Tiehui Wang** conceived, analyzed and interpreted the data, and wrote the paper. All authors read and approved the final manuscript.

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Effective isolation of GALT cells: Insights into the intestine immune response of rainbow trout (*Oncorhynchus mykiss*) to different bacterin vaccine preparations

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30 Abstract

31 The teleost gut is a multifunction complex structure that plays a pivotal immunological
32 role in homeostasis and the maintenance of health, in addition to digestion of food and/or
33 nutrient absorption. *In vitro* examination of the intestine leucocyte repertoire has the
34 potential to aid our understanding of gut immune competence and allows a rapid screen of
35 host-microorganism interactions in different immunological contexts. To explore this
36 possibility, in the present study we investigated the response of isolated gut leucocytes to 4
37 bacterins of *Aeromonas salmonicida*, prepared from different strains, combinations and
38 strains grown in different environments, in comparison to a *Yersinia ruckeri* bacterin for
39 which a commercial/effective oral booster vaccine has been developed. To aid this study
40 we also optimised further our method of GALT cell isolation from rainbow trout, so as to
41 avoid mechanical clearance of the intestine contents. This drastically increased the cell
42 yield from $\sim 12 \times 10^6$ to $\sim 210 \times 10^6$ /fish with no change in the percent cell viability over
43 time or presence of transcripts typical of the key leucocyte types needed for the study of
44 immune modulation (i.e. T- and B-cells, dendritic cells and macrophages). A wide array of
45 immune transcripts were modulated by the bacterins, demonstrating the diversity of
46 GALT cell responses to bacterial stimulation. Indeed, the GALT leucocyte responses were
47 sensitive enough to distinguish the different bacterial species, strains and membrane
48 proteins, as seen by distinct kinetics of immune gene expression. However, the response of
49 the GALT cells was often relatively slow and of a low magnitude compared to those of
50 PBL. These results enhance our knowledge of the gut biocapacity and help validate the use
51 of this model for screening of oral vaccine candidates.

52

53 Keywords

54 Rainbow trout; GALT cells; Cell yield; Leucocyte types; *Aeromonas salmonicida*;
55 *Yersinia ruckeri*; Bacterin; Immune modulation; Gene expression

56

57

58 1. Introduction

59 The gut is a multifunction complex structure, and its physiological importance stretches
60 beyond food digestion and/or nutrient absorption. It plays a substantial role in health
61 maintenance through the associated innate and adaptive immune systems. Teleost gut is
62 different to that of mammals, in that it lacks organized lymphoid tissues such as lymph
63 nodes, Peyer's patches (PP) and isolated lymphoid follicles. Rather, it has a diffuse gut
64 associated lymphoid tissue (GALT) that is comprised of individual and scattered lamina
65 propria (LP) and intraepithelial (IE) leucocytes [1]. Two populations of B lymphocytes are
66 present in the intestine of rainbow trout, IgT⁺ and IgM⁺ cells. The IgT secreting
67 lymphocytes are thought of as mammalian IgA⁺ B cell counterparts, and account for ~54%
68 of intestinal B cells [2, 3]. B cells are primarily resident in the LP and secrete IgT and IgM
69 into the gut lumen through the polymeric Ig receptor (pIgR) pathway. During infection, B
70 cells migrate to the epithelium where they can execute phagocytic activities [4]. The third
71 type of Ig present in fish, IgD, has not been reported in the intestine or gut secretions to
72 date [2, 5, 6]. The presence of T lymphocytes in teleost intestine has been established with
73 the use of anti-CD3 ϵ , anti-CD8 α and anti-CD4-1 antibodies [7-10], and the expression of
74 CD3 ϵ , CD4, CD8, RAG1, TCR and CD28 transcripts [11, 9, 12, 13]. T cells exist in the LP
75 and comprise the main population of the IE lymphocytes (IEL), with CD8⁺ T cells
76 accounting for ~55% of total gut IELs in rainbow trout [9]. Dendritic cells (DC) have also
77 been reported in rainbow trout and the expression of DC-markers (CD83, DC-
78 LAMP3/CD208, DC-SIGN/CD209 and MHCII) also occurs in the intestine [14, 13]. Anti-
79 CD8 α and anti-MHCII monoclonal antibodies allowed the identification of a DC
80 subpopulation in the intestine of rainbow trout that co-expresses CD8 α and MHCII (CD8⁺
81 DCs), has a high phagocytic capacity, and is presumed to be a regulator of gut immune
82 tolerance [15]. The phagocytic activity of rainbow trout intestinal macrophages has also
83 been described, for instance against latex microspheres, live and inactivated *Aeromonas*
84 *salmonicida*, and yeast [16-18].

85 The intestinal mucosa is in continuous contact with a broad spectrum of microbiota, i.e.,
86 symbiotic as well as potentially pathogenic and opportunistic microorganisms. Hence
87 breaching the mucosa integrity at any point could result in fish infection, and the intestine
88 is considered to be one of the main portals for pathogen entry [19, 6]. *A. salmonicida* is a
89 Gram-negative bacterium that is the causative agent of furunculosis, an acute systemic
90 disease of trout and salmon associated with high mortality. This bacterium is present in the

91 intestine of several fish species including salmonids and has the ability to translocate
92 across the intestinal epithelium of rainbow trout where it can cause an infection [20-23].
93 After *A. salmonicida* colonizes the intestine of rainbow trout it subsequently disseminates
94 into the fish body, in a pattern similar to that seen with the dissemination of another Gram-
95 negative fish pathogen *Yersinia ruckeri*, the causative agent of enteric redmouth disease
96 (ERM) in salmonids [24]. *Y. ruckeri* colonizes the intestine of rainbow trout 2-4 days prior
97 to systemic infection, indicating the contribution of the intestine to the systemic
98 dissemination of the invading pathogen [25]. The first commercially licensed vaccine for
99 fish (in 1976) was a formalin-killed bacterin of *Y. ruckeri* that was delivered by immersion,
100 although it can also be delivered by injection or orally as a booster [26, 27]. Whilst this
101 vaccine is able to induce protective immunity by mucosal vaccination, bacterin mucosal
102 vaccines against *A. salmonicida* are not effective in the field [28-30], suggesting that
103 specific protective pathways have been elicited by the *Y. ruckeri* bacterin that are not
104 promoted by the *A. salmonicida* bacterin.

105 Gaining a better understanding of the host-microorganism interaction in the intestine
106 would serve as a basis for promoting fish health/welfare and advancing the potential for
107 oral vaccination, feeding programmes and medical diets. Indeed, studies on gut mucosal
108 immunity in response to infection, vaccination, dietary additives and probiotics in fish have
109 increased recently (31-35), where typically whole or segments of the gut were used for
110 transcript analysis. Primary cell culture is a crucial technique in cell and molecular
111 immunology, that provides excellent *in vitro* platform models for studying the cell immune
112 repertoires and potentially the assessment of vaccine candidates [36]. The use of gut
113 leucocytes for this purpose would seem a sensible first step for the *in vitro* testing of oral
114 vaccine candidates. We recently established a method for GALT leucocyte isolation from
115 salmonid intestine [13]. The isolated cells were analysed transcriptionally and deemed to
116 contain the main adaptive immunity cell types (e.g. T and B cells, and dendritic cells). Cell
117 stimulation with bacterial and viral PAMPs, phytohemagglutinin (PHA) and recombinant
118 cytokines revealed the immune competence and specificity of the GALT cells. However,
119 the cell yield was still low compared to that obtained from other tissues, e.g. head kidney,
120 spleen, gills and blood [36-40]. This low yield also prevents study of the cells from
121 different intestine segments separately to explore whether immunological differences exist.

122 In view of the above, this study aimed to: a) Optimize further GALT cell isolation in
123 rainbow trout in an attempt to increase the average cell yield, b) Investigate if immune

124 gene expression in these cells is differentially modulated by a variety of bacterin oral
125 vaccine candidates prepared from *Y. ruckeri* and *A. salmonicida* strains, the latter grown in
126 different conditions, and c) Understand the distinctiveness of the GALT leucocyte immune
127 response relative to the respective peripheral blood leucocyte (PBL) response [36].

128

129

130 **2. Materials and methods**

131 **2.1 Fish maintenance**

132 Rainbow trout (*Oncorhynchus mykiss*) with no history of infection were purchased from
133 the Mill of Elrich Trout Fishery (Aberdeenshire, Scotland, UK) and maintained at $14\pm 1^\circ\text{C}$
134 in 1m-diameter fibreglass tanks with recirculating freshwater within the freshwater
135 aquarium facility at the School of Biological Sciences, University of Aberdeen. At least 10
136 fish from each batch were screened for potential bacterial infection by taking kidney swabs
137 and growing on tryptic soy agar (TSA, Sigma, UK) plates. Fish were fed twice a day with a
138 commercial diet (EWOS) at 2% body weight/day) and acclimatized for at least two weeks
139 prior to the intestine tissue sampling. The experimental procedures were carried out in
140 accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated
141 guidelines, EU Directive 2010/63/EU for animal experiments, and approved by the ethics
142 committee at the University of Aberdeen.

143

144 **2.2. Bacterin preparation**

145 The bacterins were prepared as described previously by Attaya et al. [36]. The *A.*
146 *salmonicida* subsp. *salmonicida* non-pathogenic strain MT004, and pathogenic strain
147 MT423, and the pathogenic *Y. ruckeri* strain MT3072 used in this study were obtained
148 from the Marine Scotland Science Marine Laboratory, Aberdeen, UK, as described
149 previously [41, 42]. The bacteria were inoculated into tryptic soya broth (TSB, Sigma, UK)
150 at 22°C for 18-24 h in a shaking incubator at 100 rpm. The MT423 strain was cultured in
151 normal TSB or in TSB supplemented with $100\ \mu\text{M}$ 2,2'-bipyridyl (Sigma, UK) to deplete
152 iron (Fe^-) to induce the expression of iron-regulated outer membrane proteins (IROMPs)
153 [43]. After culture, the bacteria were inactivated by addition of formalin (Sigma, UK) to
154 the broth to 1%. The bacteria were then incubated overnight on a slow magnetic stirrer at
155 room temperature. Bacteria were collected by centrifugation and washed three times using

156 phosphate buffered saline (PBS, pH7.4, Sigma, UK). The bacterial pellet was weighed
157 along with a pre-weighed microcentrifuge tube using a microgram balance, resuspended in
158 PBS at 10 mg/ml and stored at -80°C ready for use. The bacterins were also plated onto
159 TSA plates and incubated for 48 h at 22°C, with no bacterial growth confirming complete
160 inactivation of the bacteria.

161

162 **2.3. Optimizing GALT cell isolation**

163 **2.3.1. Cell isolation**

164 The GALT cells were isolated from rainbow trout, average weight 155g, as described
165 previously [13] with a few modifications as follows:

- 166 a) Stop feeding 48 h before use to reduce the gut contents,
- 167 b) Collect the intestine from the pyloric caeca to the hindgut in cold PBS (Sigma, UK),
168 remove the surrounding connective and adipose tissues, dissect the intestine into
169 approx. 1 cm long segments and open the segments longitudinally,
- 170 c) Flush the contents out with a syringe containing PBS, by gently passing PBS over the
171 internal wall of the segments; avoid mechanical flushing (e.g. squeezing or rubbing),
- 172 d) Shake the tissue in 20-25 ml PBS with a wheel shaker at 50 rpm for 10 min, then wash
173 in Petri dishes 3-4 times,
- 174 e) Shake the tissue in 15-20 ml pre-digestion solution (Ca^{2+} & Mg^{2+} free HBSS
175 supplemented with 0.145 mg DL-dithiothreitol (DTT) and 0.37 mg EDTA/ml, Sigma)
176 at 50 rpm for 20 min,
- 177 f) Filter the supernatant through a 100 μm nylon mesh strainer (Greiner), and wash the
178 first cell suspension (S1) 2-3 times with cell culture medium (Leibovitz L-15 medium,
179 Sigma) supplemented with 100 IU penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (P/S, Gibco)
180 and 10% foetal bovine serum (FBS, Sigma), then keep cells at 20°C,
- 181 g) Wash tissue fragments with washing medium (0.05 mg DNase I/ml Ca^{2+} & Mg^{2+} free
182 HBSS, Sigma) supplemented with 5% FBS to remove remaining DTT and shake in 15-
183 20 ml digestion solution (0.37 mg collagenase IV (Sigma)/ml washing medium) at 50
184 rpm for 60 min,
- 185 h) Filter the supernatant through a 100 μm nylon mesh strainer and wash with the culture
186 medium, then add the cell suspension to S1 (S2),

- 187 i) Once again, digest the remaining tissue fragments for another 1 h, filter the supernatant
188 and wash with the culture medium then add the cell suspension to S2 (S3),
189 j) Count and adjust the cell number to 1×10^7 cell/ml,
190 k) In a 50 ml tube, layer carefully the cell suspension (5 ml) over a discontinuous Percoll
191 gradient (Sigma) with two densities (75% and 25%, 5 ml+5 ml) and centrifuge at 4 °C
192 for 30 min at 400g,
193 l) Collect the cells in the intermediate density (ID) and high density (HD) bands, wash 3
194 times with the cell culture medium to remove the remaining Percoll and resuspend the
195 cells in the culture medium ready for use.

196

197 **2.3.2. Cell viability**

198 Isolated GALT cells were distributed into 12-well plates at 2×10^6 cells/well and incubated
199 at 20°C for 4 h and 24 h, at which times they were counted using a Neubauer chamber with
200 addition of 0.5% trypan blue. The viabilities were determined by comparison to the
201 respective time 0 h controls.

202

203 **2.3.3. Marker gene analysis**

204 Isolated GALT leucocytes (2×10^6 cells/well) were incubated at 20°C and harvested at 4 h
205 and 24 h by centrifugation at 400g for 10 min at 4°C. The supernatant was discarded, and
206 TRI reagent added, with pipetting up and down several times. The TRI lysate was stored at
207 -80°C until RNA extraction for marker gene analysis. The genes studied included cell
208 markers for T-lymphocytes (CD3ε, CD4-1, CD8α, CD8β, CD28, CTL4A and TCRα), B-
209 lymphocytes (membrane (m) IgM, secreted (s) IgM, mIgT, sIgT, mIgD and sIgD),
210 dendritic cells (CD83, DC-LAMP3/CD208, DC-SIGN/CD209 and MHCIIβ) and
211 macrophages (MCSFR1a, MCSFR1b, MCSFR2a, MCSFR2b), since these cells are key for
212 adaptive immune responses.

213

214 **2.4. Stimulation of GALT cells**

215 The GALT cells were distributed into 12-well cell culture plates at 2×10^6 cells/ml. The
216 GALT cells from each fish were stimulated with 100 µg/ml of inactivated bacterin (1) *A.*
217 *salmonicida* MT423 (MT423), (2) *A. salmonicida* MT423 grown in iron-depleted medium
218 (MT423 (Fe-)), (3) *A. salmonicida* MT004 (MT004), (4) an equal combination of MT423

219 (Fe-) and MT004 (MT423 (Fe-) + MT004), (5) *Y. ruckeri* MT3072, and (6) PBS as control.
220 The cells were incubated at 20°C for 4 h and 24 h, harvested in 1.5 ml TRI reagent (Sigma,
221 UK), and stored at -80°C until RNA extraction.

222 **2.5. RNA extraction, cDNA synthesis and qPCR**

223 Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) analysis of gene
224 expression were carried out as described by Wang et al. [44, 45]. The TRI lysates were
225 thawed at room temperature and total RNA was prepared as per the manufacturer's
226 instructions. Using Oligo (dT) 28VN (Eurofins), dNTPs and RevertAid Reverse
227 Transcriptase (Thermo Scientific), RNA was reverse transcribed to cDNA. The resultant
228 cDNAs were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, Sigma) and
229 duplicate 10 µl real-time qPCR reactions were run in a LightCycler 480 machine (Roche),
230 using 2x SYBR green (Sigma, UK) qPCR Master Mix made with Immolase DNA
231 Polymerase kit (Bioline). For each gene analysed, all the samples from the same
232 experiment were run in the same 384-well PCR plate. The genes studied included those
233 encoding the main cytokines, chemokines, chemokine receptors, suppressors of cytokine
234 signalling (SOCS), acute phase proteins (APPs), antimicrobial peptides (AMPs), cellular
235 markers, and master transcription factors of T cell responses. The primer sets were
236 designed with at least one primer across an intron, tested to ensure that PCR products could
237 only be amplified from cDNA samples and not from genomic DNA. The PCR efficiency
238 of the primer pairs used was between 1.95 to 2.00. The details of qPCR primers are
239 provided in the supplementary materials (Table S1). The data were analysed using the
240 LightCycler 480 integrated software. The gene expression level for each sample was
241 normalised to that of the housekeeping gene, elongation factor (EF)-1 α , a normalizer used
242 previously [40, 45, 49], and shown to be stable in the current experiment as evidenced by
243 the similar Ct values between samples (Table S2). The gene average expression of each
244 treatment was calculated as a fold change of its average expression in the control cells at
245 the respective time point.

246

247 **2.6. Data statistical analysis**

248 The normalized gene expression data were scaled and log₂ transformed to improve the
249 normality of real-time quantitative PCR measurements before statistical analysis, as
250 described previously [44]. Then, one-way ANOVA was used to detect whether a
251 significant difference was apparent amongst the cell cultures at each time point. The

252 control and bacterin treated cell cultures were aliquoted from the same cell pool that was
253 obtained from a single fish (cognate cell cultures), with each experiment consisting 4
254 replicates of cells from 4 fish. Since the modulation of gene expression in the GALT cells
255 was expected to be relatively small in magnitude, as the gut is in continuous contact with
256 the intestinal microbiome, making the cells tolerant to many types of stimulation, a paired-
257 samples T-test was used as a post-hoc test when a significant difference was detected by
258 one-way ANOVA. The analysis was performed using the IBM SPSS Statistics package
259 26.0 (SPSS Inc., Chicago, Illinois), with differences considered significant at $p \leq 0.05$.

260

261 **3. Results and discussion**

262 The potential to isolate large numbers of GALT cells helps facilitate the examination and
263 quantification of the gut biocapacity relating to studies of fish immunoprophylaxis and
264 dietary supplements. In this study, the GALT cells were isolated from the intestine of
265 rainbow trout using a method that we described previously [13], but now with avoidance of
266 any mechanical processing of the tissue. The average cell yield was calculated from 4 fish
267 and the expression of cell markers typical of the main leucocyte types relevant to adaptive
268 immunity was examined. Lastly, the immune competence of the cells was investigated
269 against bacterins prepared from two different bacterial species. One was the causative
270 agent of enteric redmouth disease (ERM), *Y. ruckeri*, for which an effective (commercial)
271 oral booster vaccine exists [26, 27]. The second was the causative agent of furunculosis,
272 *Aeromonas salmonicida* subsp. *salmonicida*, which has been refractory to successful oral
273 vaccination to date but effective injection (adjuvanted) vaccines exist. Four bacterins of *A.*
274 *salmonicida* were prepared using different strains/combinations and under different growth
275 conditions. Two bacterins were prepared from the *A. salmonicida* non-pathogenic strain
276 MT004 and the pathogenic strain MT423, respectively, that are not effective vaccines
277 against furunculosis [43, 30]. A third bacterin that represents an effective vaccine
278 candidate was from strain MT423 cultured in iron-depleted medium (MT423 (Fe-)) [43].
279 The last bacterin was comprised of equal quantities of the bacterins MT004 and MT423
280 (Fe-), and corresponds an unadjuvanted vaccine that has been used before in the field
281 (AquaVac FNM Plus, Intervet UK Ltd). Their impact on gene expression of a panel of
282 relevant immune molecules (Table S1) in GALT cells was analyzed by RT-qPCR at an
283 early (4 h) and a late (24 h) time point.

284

285 **3.1. Improved methodology boosts the yield of GALT cells**

286 Although the previous optimization of GALT cell isolation increased the average cell yield
287 [13], it is still an expensive process in terms of the time taken and cost compared to cell
288 isolation from other tissues, e.g., the head kidney, spleen and blood [46, 40]. GALT cell
289 isolation requires expensive reagents (e.g., DTT and collagenase IV), 3 pre-/digestion
290 periods and multiple washes to eliminate the associated mucus and reagent residues. In
291 order to boost the effectiveness of GALT cell isolation, i.e., increasing the cell yield within
292 the same cost and time frame, the mechanical disturbance of the intestine contents was
293 excluded and replaced with liquid flushing, using PBS extruded from a syringe against the
294 tissue (hydraulic rinse). This increased the average yield to 210×10^6 cells/fish (vs
295 12×10^6 /fish previously) whilst the percent cell viability over time was not different to the
296 previous values, ~93% at 4 h and ~55% at 24 h post-culture [13]. The leucocyte markers
297 examined, for T-cells (CD4-1, CD8 and TCR α), B-cells (mIgM, sIgM, mIgT and sIgT) and
298 dendritic cells (CD83, CD208, CD209 and MHCII β) were also expressed by the isolated
299 cells at 4 h and 24 h post-culture (Fig. S1), as seen before [13]. The gene expression levels
300 were mostly lower at 24 h vs 4 h, with a few exceptions (e.g., mIgM, mIgT, mIgD,
301 MCSFR1a/2a/2b). However, compared to the previous method, the marker gene analysis
302 showed a relative increase in the expression of CD4-1 vs CD8 and CD208 vs CD209. In
303 addition, the expression of CD83 and sIgT at 24 h were previously increased compared to
304 values at 4 h but here they were decreased. The present study also demonstrated the
305 expression of the T-cell markers CD28 and CTL4A, B-cell markers mIgD and sIgD, and
306 macrophage cell markers MCSFR1 and MCSFR2 in the isolated cells that were not
307 previously examined (Fig. S1). Regarding the IgD analysis it should be noted that neither
308 IgD⁺/IgM⁻ B cells or IgD protein have been detected in the intestine or gut secretions to
309 date [2, 6]. Whilst teleost B-cells bearing different membrane Igs can correspond to
310 different cell lineages, as fish lack Ig isotype switching [47, 6], nevertheless fish have B-
311 cells that co-express surface IgM and IgD and therefore expression of mIgD and sIgD
312 markers in this study is not a clear indication of an independent B-cell lineage in the GALT
313 cells. Overall, the boost to the average cell yield, with no detrimental effect on viability or
314 the main leucocyte types isolated (as assessed by transcript analysis), is a huge advance in
315 terms of animal welfare, cost and time. Indeed, using the previous method [13] to obtain
316 210×10^6 cells would require more than 17 fish, with a similar increase in the reagents
317 needed. These findings suggest that the mechanical treatment of the intestine causes a
318 significant reduction in the number of GALT cells obtained, as was seen by Salinas et al.

319 [48]. This larger cell yield greatly helps *in vitro* experimentation and allows cells to be
320 obtained separately from the pyloric caeca, midgut and hindgut to gain deeper insights into
321 their immune differences and capabilities [49, 50, 51, 6]. It may also allow the possibility
322 of isolating a good number of GALT cells without the need for chemical or enzymatic
323 digestion, and is certainly worth trying in further studies.

324 **3.2. Responsiveness of GALT leucocytes**

325 **3.2.1. Diversity of the immune repertoire**

326 Eighty immune molecules were selected to be examined post-stimulation of the GALT
327 cells that comprise the immune categories that are typically modulated upon bacterial
328 infection, stimulation or vaccination [52, 53, 54, 55, 36]. Sixty of these molecules could be
329 modulated with the *A. salmonicida* and/or *Y. ruckeri* bacterin preparations at 4 h and/or 24
330 h. The modulated molecules were distributed in all the examined categories, that included
331 the classical pro-inflammatory cytokines - IL-1 β 1, IL-1 β 2, IL-1 β 3, TNF α 1, TNF α 2,
332 TNF α 3, IL-6A, IL-6B (Fig. 1); other inflammatory cytokines - IL-8 (CXCL8), IL-11, IL-
333 17C1, IL-17C2, IL-22, IL-34, CXCL11_L1, type I IFN α (Fig. 2) and IL-20 (Fig. S2B);
334 anti-inflammatory cytokines - nIL-1F, IL-10A, TGF- β 1A and TGF- β 1B (Fig. 3A-D);
335 suppressors of cytokine signalling - SOCS1A and SOCS3A (Fig. 3E-F); cytokines mainly
336 involved in the adaptive immune response - IFN γ 1, IFN γ 2, IL-2B, IL-17A/F1A, IL-
337 17A/F2A, IL-17A/F3 (Fig. 4) and IL-4/13B1 (Fig. S4C); IL-12 family subunits - P19,
338 P28A, P35A1, P35A2, P40B1, P40C (Fig. 5), P40B2 and EBI3 (Fig. S2E-F); acute phase
339 proteins (APPs) - COX2, SAA, SAP1 and SAP2 (Fig. 6A-D); antimicrobial peptides
340 (AMPs) - cathelicidin (CATH)1, CATH2, LEAP1, β -defensin 4 (Fig. 6E-H) and β -
341 defensin 1 (Fig. S5B); B cell markers - mIgM, sIgM, sIgT and sIgD (Fig. 7); T cell
342 markers - CD3 ϵ and CD4 (Fig. S6C-D); the transcriptional factor ROR γ (Fig. S7C); and
343 chemokine receptors - CXCR3A, CXCR4B, CCR6A, CCR7A, CCR9A and XCR3 (Fig.
344 8).

345

346 Forty-nine of the 60 modulated genes were up-regulated by at least one of the bacterins
347 used and at one or more time points. A few of these genes (SOCS1A, P40B1, P40B2 and
348 sIgT) were also inhibited (relative to the control cells) at the other time point and/or with a
349 different bacterin (s). The remaining 11 modulated genes (TGF- β 1A, IL-4/13B1, IL-20,
350 EBI3, sIgM, CD3 ϵ , CD4, ROR γ , CXCR4B, CCR9A and XCR3) were (only) inhibited with
351 one or more of the bacterin preparations. Lastly, 20 molecules were not modulated by

352 stimulation with *A. salmonicida* or *Y. ruckeri* bacterins. This group included cytokines (IL-
353 2A, IL-4/13A, IL-4/13B2, IL-10B, IL-12 P35B, IL-18 and IL-21, Figs. S2-4), AMPs
354 (LEAP2, β -defensin 2 and 3, Fig. S5), cellular markers (mIgT, mIgD, CD28 and CTLA4,
355 Fig. S6), master transcription factors (T-bet, GATA3, Foxp3A and Foxp3B) and a
356 chemokine receptor (CXCR4A) (Fig. S7F).

357

358 It is noteworthy that of the paralogous cytokine genes studied, that have arisen from the
359 third teleost-wide and/or the fourth salmonid-wide whole genome duplication events, some
360 differential modulation was seen in GALT cells. The paralogues of pro-inflammatory
361 cytokines, e.g. IL-1 β 1-3 [56], IL-6A-B, IL- IL-17A/F1-3 [57], 17C1-2 [58], IFN γ 1-2, and
362 TNF α 1-3 [59], behaved in a similar way in GALT cells, where they were typically induced
363 after bacterin stimulation. In contrast, the paralogues of the anti-inflammatory cytokines
364 IL-10A-B [60] and TGF- β 1A-B [61] and the adaptive cytokines IL-2A-B [45] and IL-
365 4/13A/B1-2 [62], behaved differently, with one induced and the other refractory (as in the
366 case of the IL-2, IL-10 and IL-4/13 paralogues) or one inhibited and the other induced (as
367 in the case of TGF- β 1A-B). Differential expression and modulation of paralogous genes
368 has been observed in multiple studies in salmonids [36, 55], and highlights the importance
369 of including all paralogues in an analysis.

370

371 Some of the molecules that could not be modulated in this study, such as IL-4/13A, IL-
372 4/13B2 and IL-21, have been induced in the intestine of rainbow trout following oral
373 administration of *A. salmonicida* MT004 + MT423 bacterin conjugated with nanocarriers
374 for 5-10 days [63]. The IL-4/13B1 cytokine was induced in rainbow trout and Atlantic
375 salmon (*Salmo salar*) GALT cells stimulated with PHA and poly I:C, and recombinant IL-
376 21 and IL-2B cytokines [12, 13], but consistent with the present study was also inhibited
377 with the bacterial PAMP lipopolysaccharide (LPS) in Atlantic salmon GALT cells. This
378 suggests that some of the molecules that were unresponsive to stimulation in this study
379 might be modulated under different conditions or at later time points, and that the direction
380 of the modulation is reliant on the stimulant type. Taken together, the responsiveness of
381 this wide array of molecules that cross several immune categories demonstrates the diverse
382 immune repertoire of isolated GALT cells.

383

384 **3.2.2. Distinctiveness of the immune response between GALT cells and PBL**

385 This panel of bacterin preparations has been used previously to stimulate freshly prepared
386 PBL from rainbow trout in a kinetic study [36]. These PBL were isolated by a hypotonic
387 lysis method, and contain the main immune cell types needed to monitor adaptive immune
388 responses, i.e., T cells, B cells and monocytes/macrophages [40, 64]. The current study
389 aimed to evaluate the intestine immune response relative to that of the PBL, therefore the
390 GALT cells were similarly stimulated, and the expression of selected immune genes was
391 examined at two corresponding time points. Although there was a wide consensus in the
392 response direction with most of the immune molecules studied, the magnitude of the
393 response was generally lower in GALT cells than in PBL, and some responses were not in
394 the same direction. For instance, TNF α 2 at 4 h was induced in the GALT leucocytes but
395 inhibited in the PBL, IL-10B was not changed in the GALT cells but induced in the PBL,
396 and ROR γ , CD3 ϵ and CD4 were inhibited in the GALT cells but induced in the PBL. Also,
397 some molecules such as IL-2A, IL-4/13, IL-18, IL-21, sIgM, mIgD, T-bet, Foxp3A,
398 Foxp3B, CXCR4B, CCR9A and XCR3 were only induced in the PBL. Lastly, with some
399 molecules, the onset of induction was relatively late in the GALT cells (e.g., 24 h vs 4 h in
400 PBL), as seen with IL-1 β 3, IL-11, IL-10A, TGF- β 1B, SOCS1A, IFN γ 1, IFN γ 2 and
401 CCR6A.

402

403 The variation in the molecules modulated, and response onset, direction and magnitude
404 could be attributed to the composition of different cell populations/types present in the
405 GALT leucocytes vs PBL. For example, both IgM- and IgT-expressing B cells are present,
406 but the abundance is different between GALT cells and PBL [65]. On the other hand, the
407 same type of leucocytes in the gut environment may have developed into an immuno-
408 tolerogenic phenotype, as a consequence of the continuous contact of the mucosal surface
409 with the microbial community in the intestine that is dominated by *Aeromonadaceae* and
410 *Enterobacteriaceae* bacteria [66, 67, 68, 3, 6]. Hence the distinct response of the immune
411 molecules in the GALT cells may indicate a level of molecule sub-functionalisation that is
412 suited to the intestine non-sterile environment. These distinct immune responses suggest
413 that GALT cells could be a more appropriate model for mucosal immune studies and
414 mucosal vaccine development.

415

416 3.2.3. Sensitivity of the detection of immune responses in GALT cells

417 Sixty of the eighty immune genes examined were modulated in GALT cells by bacterin
418 stimulation, with the majority of these genes increased and found higher at 24 h than at 4 h.
419 The genes modulated at 24 h are summarised in Table 1. The expression of most of the
420 pro-inflammatory cytokines (IL-1 β 1-2, TNF α 1-2, IL-8, IL-17C1, IL-22, IL-34 and
421 CXCL11_L1), the α -chains of the IL-12 family (P19, P28A, P35A1), APP (SAA), AMPs
422 (CATH1-2 and LEAP1) and chemokine receptors (CXCR3A and CCR6A) was increased
423 by all the five bacterin preparations at 24 h (Table 1). These data suggest the activation of
424 common inflammatory pathways in GALT cells by different bacterins, as seen in PBL
425 [40].

426

427 Despite a common inflammatory response, the *Y. ruckeri* driven response was distinct from
428 *A. salmonicida* bacterins in the GALT leucocytes (Figs. 1-8, Table 1), as seen with PBL
429 [40]. In comparison to *A. salmonicida* bacterins, *Y. ruckeri* induced gene expression was
430 higher at 4 h for IL-6A, IL-8, IL-22, IL-34, IFN α , p19, p35A2, COX2 SAA and CATH1,
431 and in some cases *A. salmonicida* had no effect at this timing. Similarly, at 24 h expression
432 of TNF α 3, IL-6A, IL-8, IL-11, IL-22, IL-34, COX2, SAA, CATH1 and CATH2 were all
433 higher relative to the *A. salmonicida* stimulated cells. However, unlike the *A. salmonicida*
434 bacterins, *Y. ruckeri* was unable to induce IFN γ 1, IFN γ 2 and IL-2B, molecules typically
435 linked to a Th1 type response, SOCS1A, a major suppressor of IFN γ signalling [69], and
436 IL-12 P40C that forms an IL-12 isoform known to induce IL-10 expression [70]. These
437 differences in specific molecules induced with the *Y. ruckeri* bacterin could be used as
438 potential markers for testing oral vaccine candidates; as *Y. ruckeri* can be used as an
439 effective oral booster vaccine [26, 27], however the kinetics of induction might also be
440 (bacterium) species-dependent.

441

442 The response induced in GALT cells by the different *A. salmonicida* bacterins also varied.
443 For example, the non-pathogenic *A. salmonicida* MT004 driven response was different to
444 that induced with the pathogenic *A. salmonicida* MT423 bacterin, in that induction of the
445 proinflammatory cytokines IL-1 β and TNF α was typically lower at 4 h but at similar levels
446 at 24 h. In addition, induction of P35A1, P28A, P40B1 was lower at 4 h, as was IFN γ 1 and
447 IL-2B at 24 h but IL-17C1 and CCR7A were higher at 24 h. These differences might be

448 due to the lack of *A. salmonicida* virulence factors in this strain, such as the A-layer [71-
449 73]. The iron depleted form of the *A. salmonicida* strain MT423 (MT423 (Fe-)) bacterin
450 led to a higher induction of IL-17A/F2A, IL-17C1, IL-17C2, β -defensin 4, mIgM, sIgT and
451 CCR6A, but a lower induction of IL-1 β 1, IL-1 β 2, IL-P35A1 and P28A, particularly at 24
452 h. These variations could be due to the production of the iron-regulated outer membrane
453 proteins (IROMPs) that are considered to be potential protective antigens [43, 74], and
454 hints at a better stimulation of mucosal/Th17 type responses. The combination of *A.*
455 *salmonicida* MT423 (Fe-) with *A. salmonicida* MT004 (MT423 (Fe-) + MT004) has also
456 driven a specific pattern of modulation with some molecules, such as IL-1 β 1, IL-1 β 2 and
457 P35A1 at 4 h and CXCL11_L1, β -defensin 4 and mIgM at 24 h where the induction levels
458 were in-between those seen with each bacterin alone. These findings demonstrate that
459 despite the small scale of the GALT cell response compared to PBL, it was possible to
460 distinguish different bacterial species, strains and different antigens. This shows that the
461 intestinal cells are sensitive to potentially small differences in the immunological insults
462 they receive and that this subsequently generates a tailored/specific response. This is in
463 agreement with the conclusion made by Attaya et al. [13] that revealed different pathways
464 are elicited with different stimulants in salmonids gut.

465

466 **3.3. Conclusion**

467 The current study optimized further a method of salmonid GALT cell isolation, increasing
468 the average cell yield by more than 17 fold. This will reduce fish use, save money and
469 time, and enable the potential *in vitro* experimentation on leucocytes obtained from the
470 pyloric caeca, midgut and hindgut separately from the same fish. Stimulation of the GALT
471 cells with *Y. ruckeri* and 4 bacterin preparations of *A. salmonicida* revealed that the GALT
472 cell immune repertoire is diverse but distinct although often relatively slow and of a lower
473 magnitude compared to PBL [36], perhaps as a consequence of the tolerogenic nature of
474 the gut environment. The GALT cell response was sensitive enough to distinguish the
475 different bacterial species, strains and membrane proteins present by producing specific
476 immune profiles. The *Y. ruckeri* bacterin drove a range of exclusive changes in the GALT
477 leucocytes that could have value as potential biomarkers for the evaluation of oral vaccine
478 candidates. These results, that show differential expression of immune molecules to
479 different stimulants, will aid future research into *in vitro* assays using GALT cells to

480 evaluate oral vaccine candidates, establish efficacious oral vaccination strategies, and
481 progress fish feeding regimes.

482

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489 **4. References**

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728 vaccination with an *Aeromonas salmonicida* vaccine containing iron-regulated outer
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731

732

733 **Figure and table legends**

734

735 **Table 1. Differential modulation of immune gene expression by bacterin in GALT**
736 **cells.** Rainbow trout GALT cells were stimulated with bacterins prepared from *Yersinia*
737 *ruckeri* strain MT3072 and *Aeromonas salmonicida* strains MT423, MT004, MT423
738 grown under iron-depleted conditions (Fe-), and a mixture of MT423 (Fe-) and MT004.
739 The expression of immune genes was analysed by RT-qPCR. The up- (↑) or down- (↓)
740 regulation of genes by a bacterin at 24 h post stimulation is summarised. Dash (-) indicates
741 no significant change relative to unstimulated control.

742

743 **Figure 1. Modulation of the expression of classic pro-inflammatory cytokine genes in**
744 **GALT cells.** GALT cells isolated from the intestine of rainbow trout were stimulated for 4
745 h and 24 h with bacterins prepared from *Yersinia ruckeri* strain MT3072 and *Aeromonas*
746 *salmonicida* strains MT423, MT004, MT423 grown under iron-depleted conditions (Fe-),
747 and a mixture of MT423 (Fe-) and MT004. The gene expression of IL-1β1 (A), IL-1β2
748 (B), IL-1β3 (C), TNFα1 (D), TNFα2 (E), TNFα3 (F), IL-6A (G) and IL-6B (H) was
749 examined by RT-qPCR as described in Materials and Methods. Bars are mean fold changes
750 + SEM of 4 fish. Letters over the bars at the same time point indicate a significant
751 difference ($p \leq 0.05$) was found by one-way ANOVA, and are the results of a post-hoc
752 paired samples T-test. Bars having the same letter are not different statistically.

753

754 **Figure 2. Modulation of the expression of other pro-inflammatory cytokine genes in**
755 **GALT cells.** Rainbow trout GALT cells were stimulated with different bacterin
756 preparations for 4 h and 24 h, and the expression of pro-inflammatory cytokines IL-8 (A),
757 IL-11 (B), IL-17C1 (C), IL-17C2 (D), IL-22 (E), IL-34 (F), CXCL11_L1 (G) and IFN α (H)
758 was examined as described in **Figure 1**. Bars are mean fold changes + SEM of 4 fish.
759 Letters over the bars at the same time point indicate a significant difference ($p \leq 0.05$) was
760 found by one-way ANOVA, and are the results of a post-hoc paired samples T-test. Bars
761 having the same letter are not different statistically.

762

763 **Figure 3. Modulation of the expression of anti-inflammatory genes in GALT cells.**
764 Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and
765 24 h, and the expression of anti-inflammatory cytokines IL-1Fm (A), IL-10A (B), TGF-
766 β 1A (C) and TGF- β 1B (D), and the suppressors of cytokine signalling SOCS1A (E) and
767 SOC3A (F) was examined as described in **Figure 1**. Bars are mean fold changes + SEM of
768 4 fish. Letters over the bars at the same time point indicate a significant difference ($p \leq$
769 0.05) was found by one-way ANOVA, and are the results of a post-hoc paired samples T-
770 test. Bars having the same letter are not different statistically.

771

772 **Figure 4. Modulation of the expression of adaptive cytokine genes in GALT cells.**
773 Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and
774 24 h, and the expression of adaptive cytokines IFN γ 1 (A), IFN γ 2 (B), IL-2B (C), IL-
775 17A/F1A (D), IL-17A/F2A (E), and IL-17A/F3 (F) was examined as described in **Figure**
776 **1**. Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point
777 indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the
778 results of a post-hoc paired samples T-test. Bars having the same letter are not different
779 statistically.

780

781 **Figure 5. Modulation of the expression of IL-12 family cytokine genes in GALT cells.**
782 Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and
783 24 h, and the expression of IL-12 family cytokines IL-23 P19 (A), IL-27 P28A (B), and IL-
784 12 P35A1 (C), p35A2 (D), P40B1 (E) and P40C (F) was examined as described in **Figure**
785 **1**. Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point

786 indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the
787 results of a post-hoc paired samples T-test. Bars having the same letter are not different
788 statistically.

789

790 **Figure 6. Modulation of the expression of genes encoding acute phase proteins and**
791 **antimicrobial peptides in GALT cells.** Rainbow trout GALT cells were stimulated with
792 different bacterin preparations for 4 h and 24 h, and the expression of acute phase proteins
793 COX2 (A), SAA (B), SAP1 (C) and SAP2 (D), and antimicrobial peptide genes CATH1
794 (E), CATH2 (F), LEAP1 (G), and β -defensin 4 (H) was examined as described in **Figure**
795 **1.** Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point
796 indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the
797 results of a post-hoc paired samples T-test. Bars having the same letter are not different
798 statistically.

799

800 **Figure 7. Modulation of the expression of immunoglobulin genes in GALT cells.**
801 Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and
802 24 h, and the expression of immunoglobulin genes mIgM (A), sIgM (B), sIgT (C) and sIgD
803 (D) was examined as described in **Figure 1.** Bars are mean fold changes + SEM of 4 fish.
804 Letters over the bars at the same time point indicate a significant difference ($p \leq 0.05$) was
805 found by one-way ANOVA, and are the results of a post-hoc paired samples T-test. Bars
806 having the same letter are not different statistically.

807

808 **Figure 8. Modulation of the expression of chemokine receptor genes in GALT cells.**
809 Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and
810 24 h, and the expression of chemokine receptor genes CXCR3A (A), CXCR4B (B),
811 CCR6A (C), CCR7A (D), CCR9A (E) and XCR3 (F) was examined as described in **Figure**
812 **1.** Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point
813 indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the
814 results of a post-hoc paired samples T-test. Bars having the same letter are not different
815 statistically.

816

Table 1. Differential modulation of immune gene expression by bacterin in GALT cells. Rainbow trout GALT cells were stimulated with bacterins prepared from *Yersinia ruckeri* strain MT3072 and *Aeromonas salmonicida* strains MT423, MT004, MT423 grown under iron-depleted conditions (Fe-), and a mixture of MT423 (Fe-) and MT004. The expression of immune genes was analysed by RT-qPCR. The up- (↑) or down- (↓) regulation of genes by a bacterin at 24 h post stimulation is summarised. Dash (-) indicates no significant change relative to unstimulated control.

Group	Gene	<i>Aeromonas salmonicida</i>				<i>Yersinia ruckeri</i>
		MT423	MT423 (Fe-) + MT004	MT423 (Fe-)	MT004	MT3072
Pro-inflammatory cytokines	IL-1β1	↑	↑	↑	↑	↑
	IL-1β2	↑	↑	↑	↑	↑
	IL-1β3	-	↑	-	↑	-
	TNFα1	↑	↑	↑	↑	↑
	TNFα2	↑	↑	↑	↑	↑
	TNFα3	↑	-	↑	↑	↑
	IL-6A	↑	↑	↑	-	↑
	IL-6B	-	-	-	↑	↑
	IL-8	↑	↑	↑	↑	↑
	IL-11	-	-	-	-	↑
	IL-17C1	↑	↑	↑	↑	↑
	IL-17C2	-	↑	↑	↑	-
	IL-22	↑	↑	↑	↑	↑
	IL-34	↑	↑	↑	↑	↑
	CXCL11_L1	↑	↑	↑	↑	↑
Anti-inflammatory Cytokines and SOCS	IFNα	↑	↑	↑	↑	-
	IL-10A	↑	↑	-	-	-
	TGF-β1B	↑	-	-	-	-
	SOCS1A	↑	↑	↑	↑	-
Adaptive immunity cytokines	SOCS3A	↑	↑	↑	↑	-
	IFNγ1	↑	↑	↑	↑	-
	IFNγ2	↑	-	-	-	-
	IL-2B	↑	-	-	-	-
	IL-17A/F1A	-	-	-	-	↑
IL-12 family cytokines	IL-17A/F3	-	-	↑	↑	-
	P19	↑	↑	↑	↑	↑
	P28A	↑	↑	↑	↑	↑
	P35A1	↑	↑	↑	↑	↑
	P35A2	-	-	-	-	↑
	P40B1	↑	↑	↑	↑	↑
	P40B2	-	-	-	-	↑
Acute phase proteins (APPs)	P40C	↑	↑	↑	↑	-
	COX2	-	-	-	-	↑
	SAA	↑	↑	↑	↑	↑
Antimicrobial peptides (AMPs)	SAP1	-	-	-	↑	-
	CATH1	↑	↑	↑	↑	↑
	CATH2	↑	↑	↑	↑	↑
	LEAP1	↑	↑	↑	↑	↑
Immunoglobulins (Igs)	β-defensin 4	-	↑	↑	-	↑
	mIgM	-	-	↑	-	-
	sIgT	-	↑	↑	-	-
Chemokine receptors	sIgD	-	↑	↑	↑	↑
	CXCR3A	↑	↑	↑	↑	↑
	CCR6A	↑	↑	↑	↑	↑
	CCR7A	-	-	-	↑	-
	XCR3	-	-	-	↓	↓

Figure 1

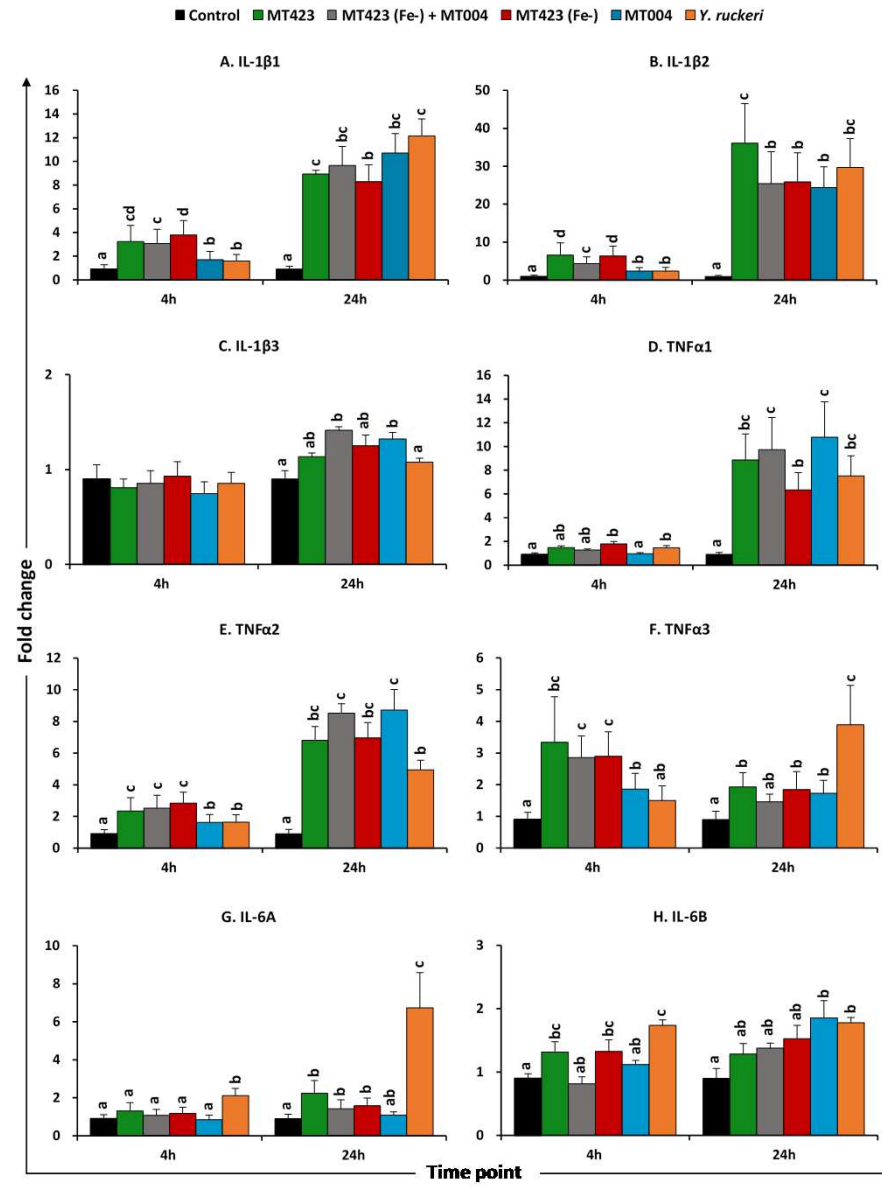


Figure 2

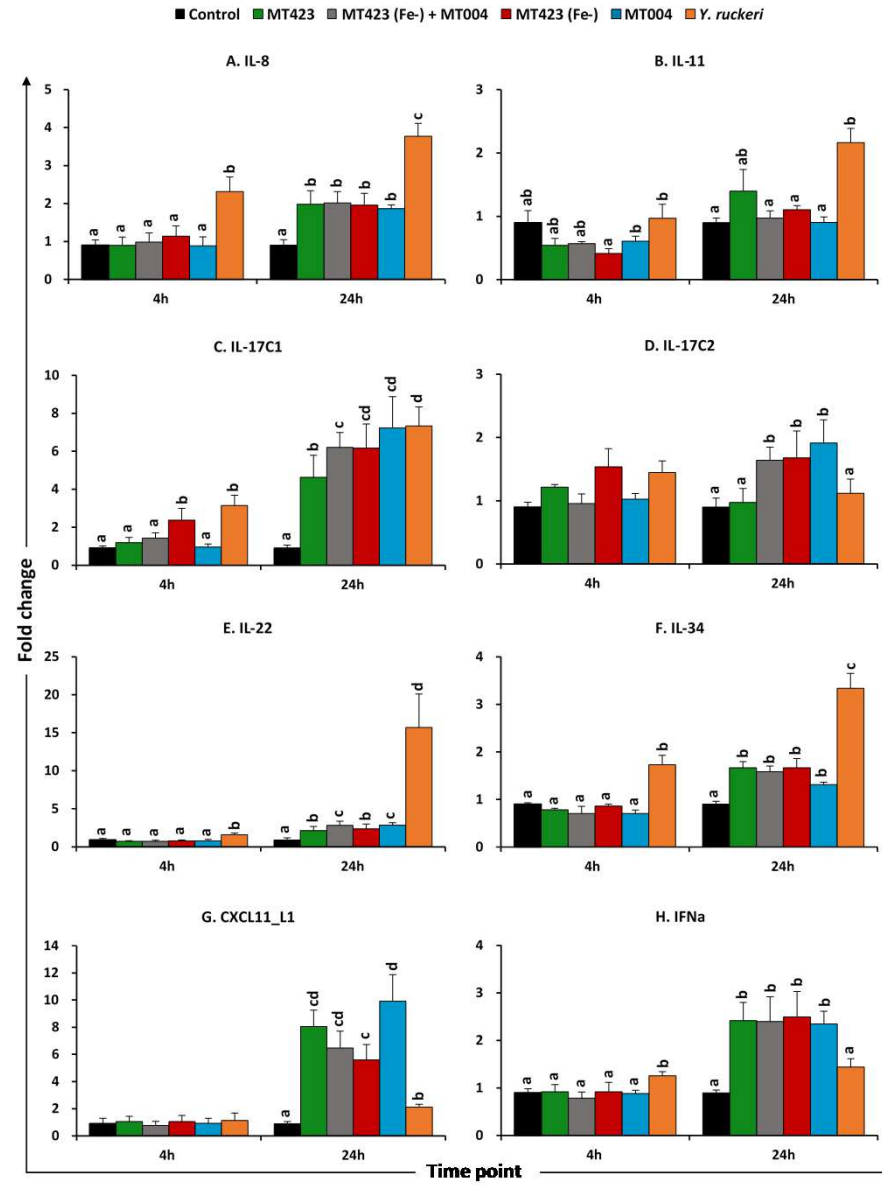


Figure 3

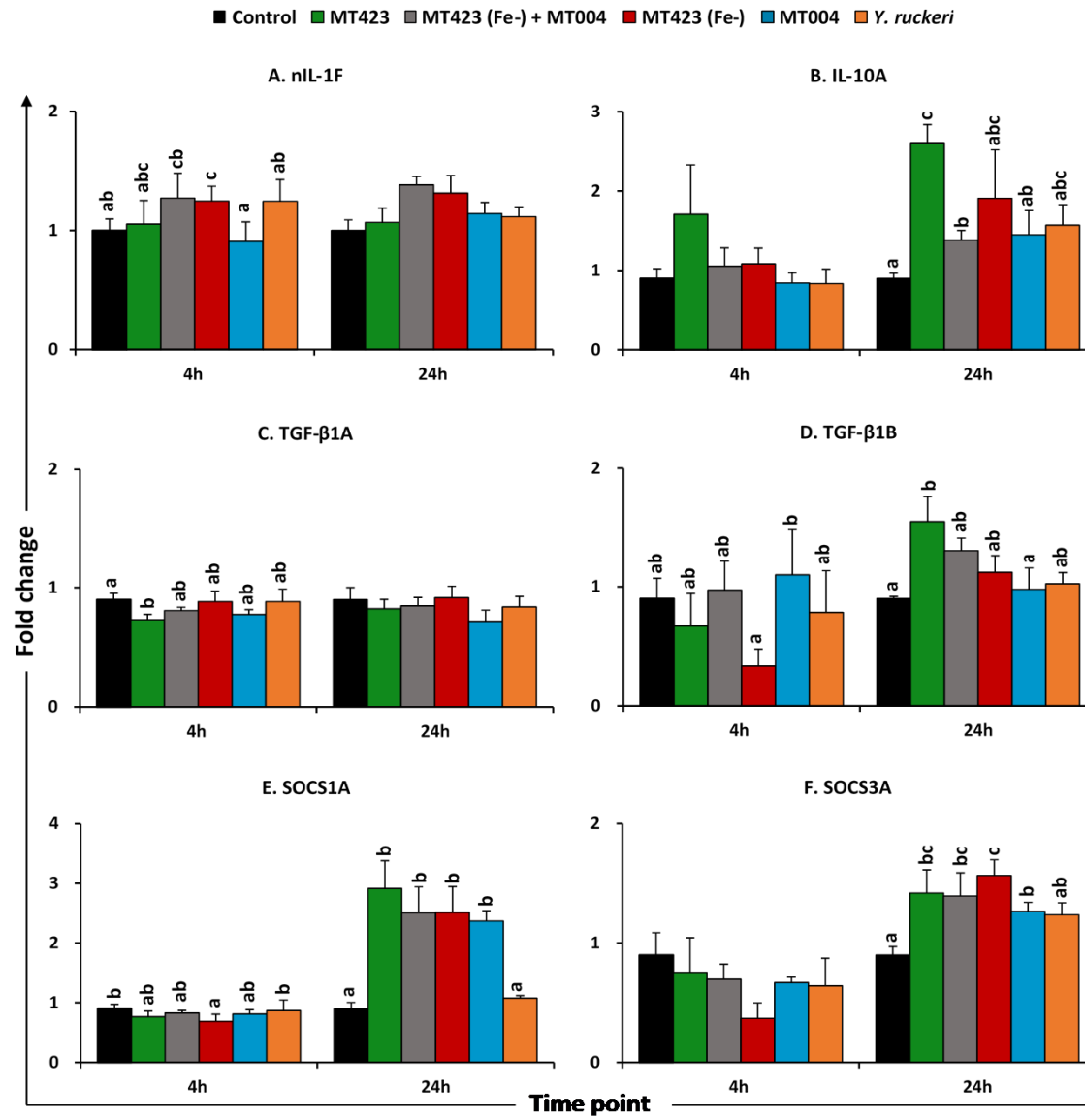


Figure 4

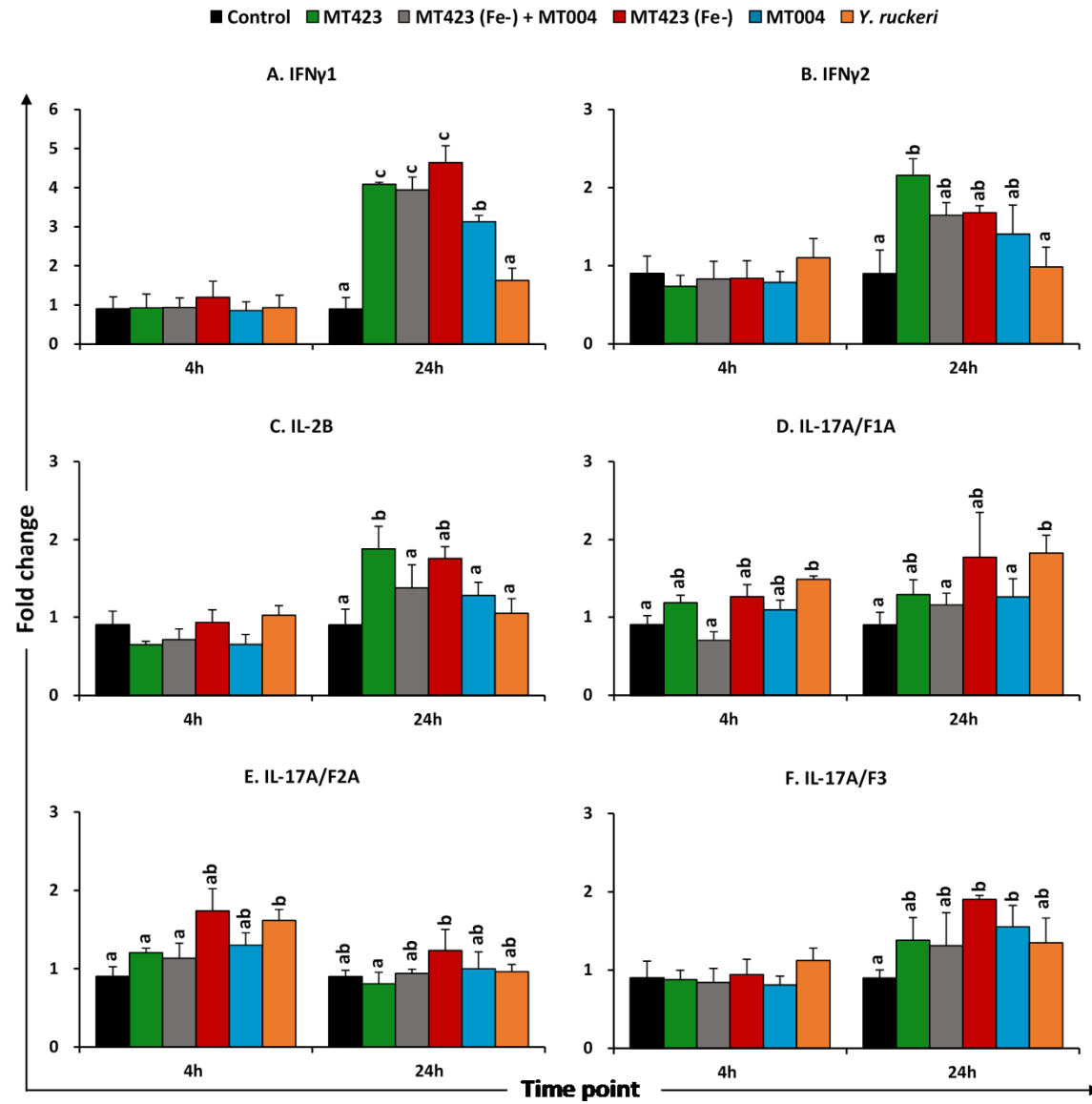


Figure 5

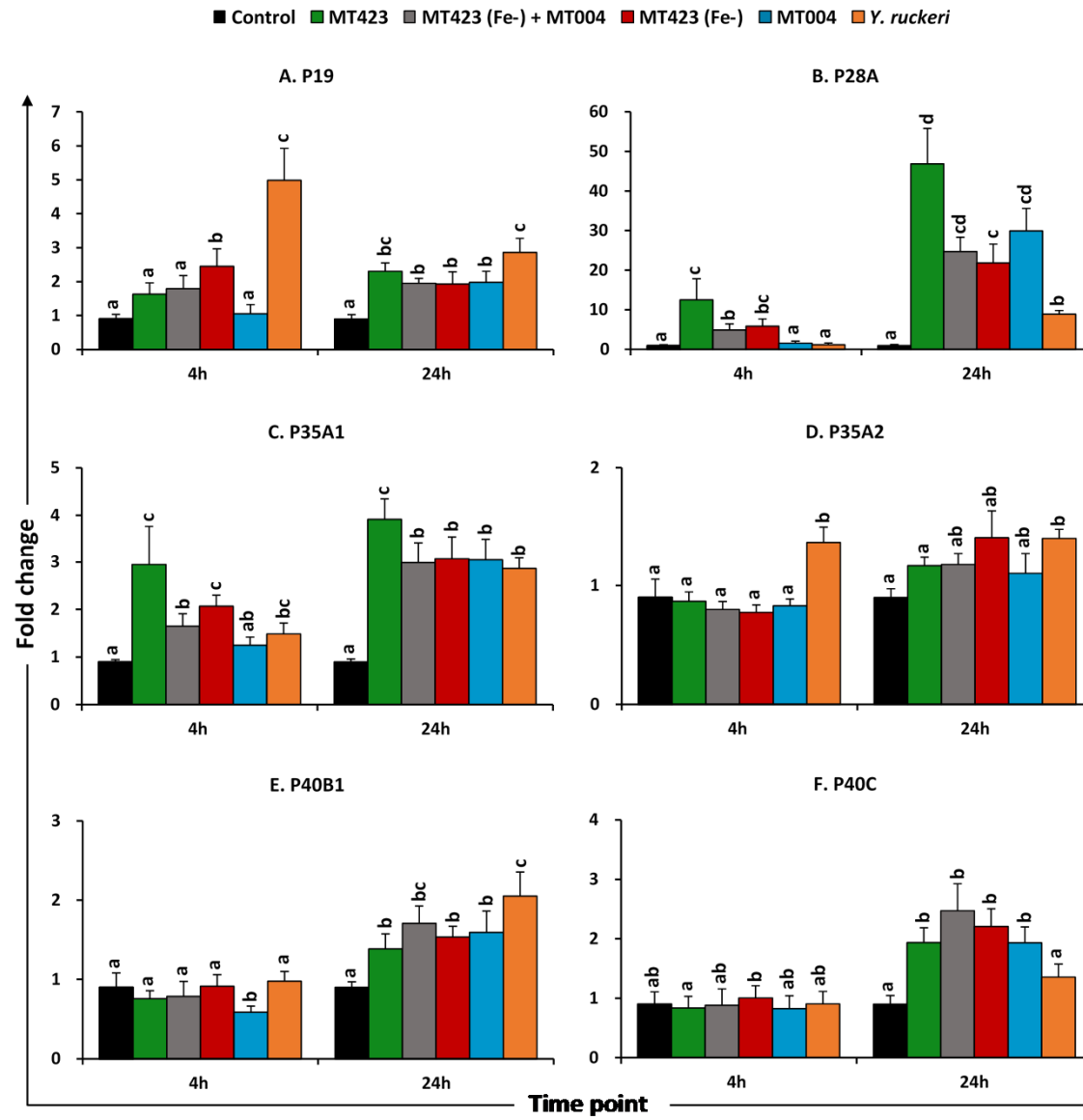


Figure 6

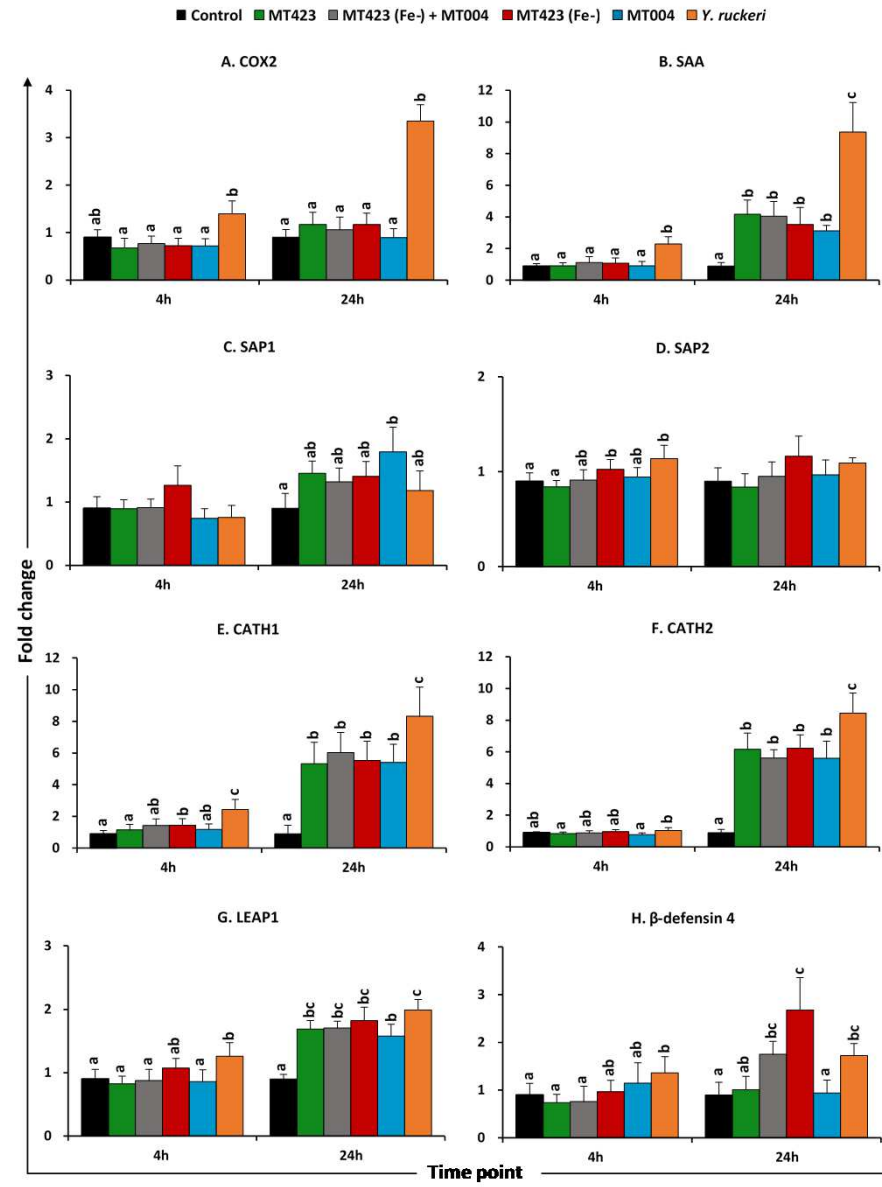


Figure 7

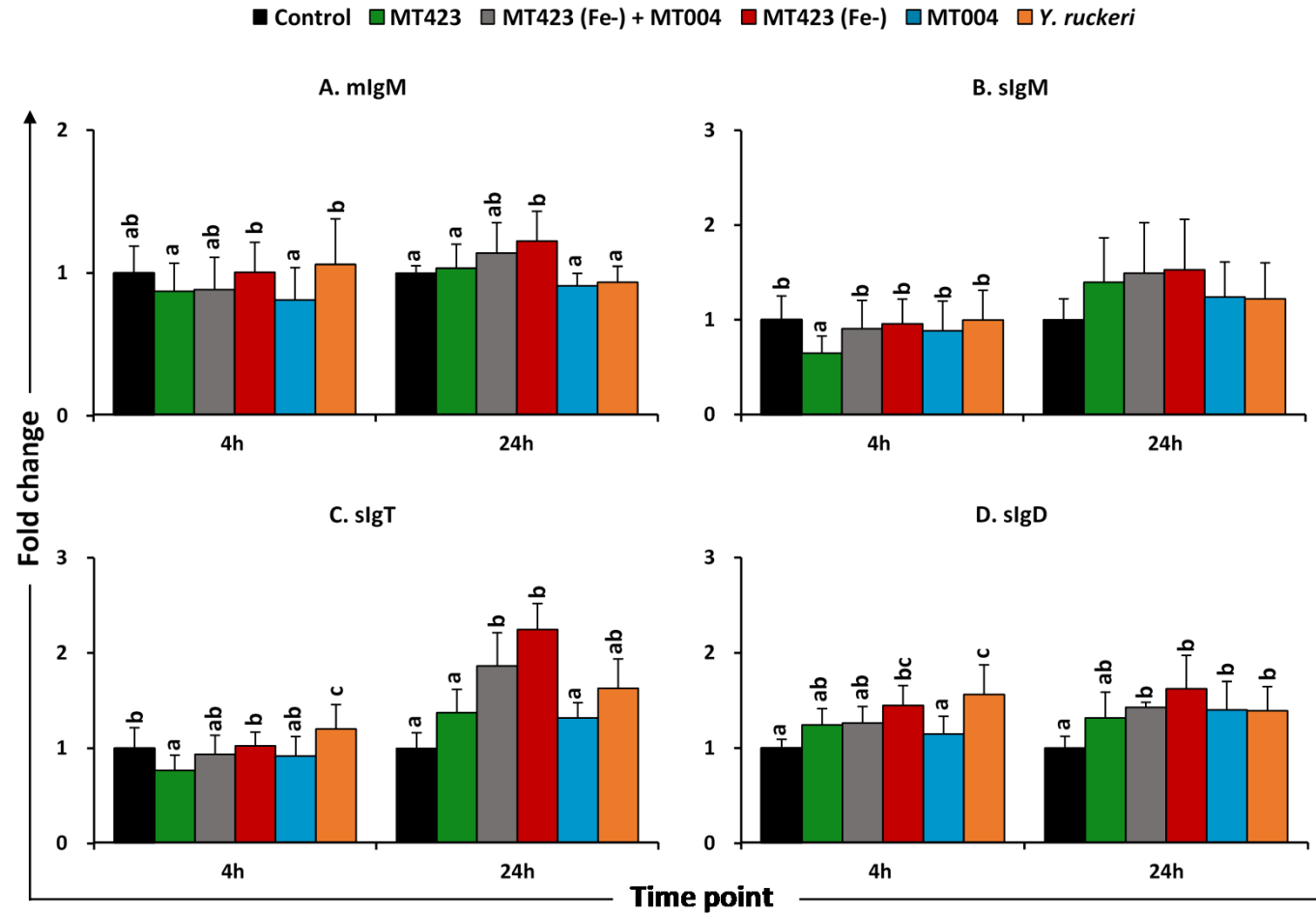
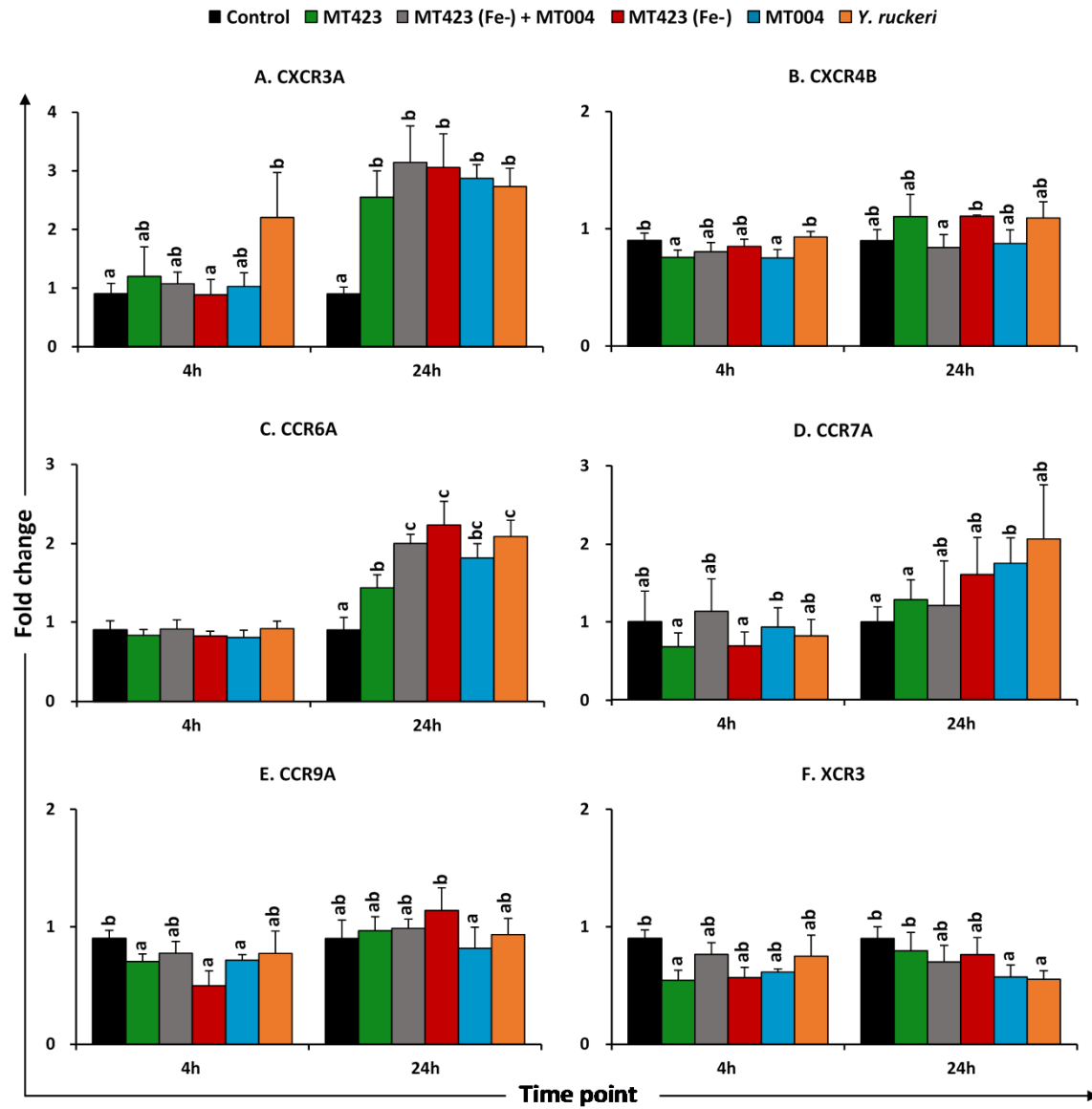


Figure 8



Highlights

- Further optimization of the GALT leucocyte isolation procedure boosted the average cell yield more than 17 fold.
- Transcripts for markers of T- and B-lymphocytes, dendritic cells and macrophages were detectable in cells isolated with the optimized method.
- The GALT cell immune responsiveness to bacterins is diverse and distinct but relatively slow and of a lower magnitude compared to PBL.
- The *Y. ruckeri* bacterin elicited a range of exclusive changes in the GALT cells that could have value as biomarkers to evaluate oral vaccine candidates.