View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Aberdeen University Research Archive

Journal Pre-proof

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

Ahmed Attaya, Christopher J. Secombes, Tiehui Wang

PII: S1050-4648(20)30473-3

DOI: https://doi.org/10.1016/j.fsi.2020.06.051

Reference: YFSIM 7141

To appear in: *Fish and Shellfish Immunology*

Received Date: 7 January 2020

Revised Date: 24 June 2020

Accepted Date: 25 June 2020

Please cite this article as: Attaya A, Secombes CJ, Wang T, Effective isolation of GALT cells: Insights into the intestine immune response of rainbow trout (*Oncorhynchus mykiss*) to different bacterin vaccine preparations, *Fish and Shellfish Immunology* (2020), doi: https://doi.org/10.1016/j.fsi.2020.06.051.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Ltd.

Journal Pre-proof

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.

Journal Pre-proof

Journal Pre-proof

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

Ahmed Attaya[a,*, $], Christopher J. Secombes [a,*], Tiehui Wang[a,*]

[a] Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, AB24 2TZ

*Joint corresponding authors

E-mail addresses:
christopher.secombes@abdn.ac.uk
t.h.wang@abdn.ac.uk
ahmed_attaya@uml.edu

$Present address: Department of Biological Sciences, Kennedy College of Sciences, University of Massachusetts, Lowell, MA 01854, USA.

Journal Pre-proof

Abstract

The teleost gut is a multifunction complex structure that plays a pivotal immunological role in homeostasis and the maintenance of health, in addition to digestion of food and/or nutrient absorption. *In vitro* examination of the intestine leucocyte repertoire has the potential to aid our understanding of gut immune competence and allows a rapid screen of host-microorganism interactions in different immunological contexts. To explore this possibility, in the present study we investigated the response of isolated gut leucocytes to 4 bacterins of *Aeromonas salmonicida*, prepared from different strains, combinations and strains grown in different environments, in comparison to a *Yersinia ruckeri* bacterin for which a commercial/effective oral booster vaccine has been developed. To aid this study we also optimised further our method of GALT cell isolation from rainbow trout, so as to avoid mechanical clearance of the intestine contents. This drastically increased the cell yield from ~12 $x10^6$ to ~210 $x10^6$/fish with no change in the percent cell viability over time or presence of transcripts typical of the key leucocyte types needed for the study of immune modulation (i.e. T- and B-cells, dendritic cells and macrophages). A wide array of immune transcripts were modulated by the bacterins, demonstrating the diversity of GALT cell responses to bacterial stimulation. Indeed, the GALT leucocyte responses were sensitive enough to distinguish the different bacterial species, strains and membrane proteins, as seen by distinct kinetics of immune gene expression. However, the response of the GALT cells was often relatively slow and of a low magnitude compared to those of PBL. These results enhance our knowledge of the gut biocapacity and help validate the use of this model for screening of oral vaccine candidates.

Keywords

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

Journal Pre-proof

1. Introduction

The gut is a multifunction complex structure, and its physiological importance stretches beyond food digestion and/or nutrient absorption. It plays a substantial role in health maintenance through the associated innate and adaptive immune systems. Teleost gut is different to that of mammals, in that it lacks organized lymphoid tissues such as lymph nodes, Peyer's patches (PP) and isolated lymphoid follicles. Rather, it has a diffuse gut associated lymphoid tissue (GALT) that is comprised of individual and scattered lamina propria (LP) and intraepithelial (IE) leucocytes [1]. Two populations of B lymphocytes are present in the intestine of rainbow trout, IgT^+ and IgM^+ cells. The IgT secreting lymphocytes are thought of as mammalian IgA^+ B cell counterparts, and account for ~54% of intestinal B cells [2, 3]. B cells are primarily resident in the LP and secrete IgT and IgM into the gut lumen through the polymeric Ig receptor (pIgR) pathway. During infection, B cells migrate to the epithelium where they can execute phagocytic activities [4]. The third type of Ig present in fish, IgD, has not been reported in the intestine or gut secretions to date [2, 5, 6]. The presence of T lymphocytes in teleost intestine has been established with the use of anti-CD3ε, anti-CD8α and anti-CD4-1 antibodies [7-10], and the expression of CD3ε, CD4, CD8, RAG1, TCR and CD28 transcripts [11, 9, 12, 13]. T cells exist in the LP and comprise the main population of the IE lymphocytes (IEL), with $CD8^+$ T cells accounting for ~55% of total gut IELs in rainbow trout [9]. Dendritic cells (DC) have also been reported in rainbow trout and the expression of DC-markers (CD83, DC-LAMP3/CD208, DC-SIGN/CD209 and MHCII) also occurs in the intestine [14, 13]. Anti-CD8α and anti-MHCII monoclonal antibodies allowed the identification of a DC subpopulation in the intestine of rainbow trout that co-expresses CD8α and MHCII ($CD8^+$ DCs), has a high phagocytic capacity, and is presumed to be a regulator of gut immune tolerance [15]. The phagocytic activity of rainbow trout intestinal macrophages has also been described, for instance against latex microspheres, live and inactivated *Aeromonas salmonicida*, and yeast [16-18].

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

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

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

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

Journal Pre-proof

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

2. Materials and methods

2.1 Fish maintenance

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

2.2. Bacterin preparation

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

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

2.3. Optimizing GALT cell isolation

2.3.1. Cell isolation

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

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

Journal Pre-proof

i) Once again, digest the remaining tissue fragments for another 1 h, filter the supernatant and wash with the culture medium then add the cell suspension to S2 (S3),

j) Count and adjust the cell number to 1 x10^7 cell/ml,

k) In a 50 ml tube, layer carefully the cell suspension (5 ml) over a discontinuous Percoll gradient (Sigma) with two densities (75% and 25%, 5 ml+5 ml) and centrifuge at 4 °C for 30 min at 400g,

l) Collect the cells in the intermediate density (ID) and high density (HD) bands, wash 3 times with the cell culture medium to remove the remaining Percoll and resuspend the cells in the culture medium ready for use.

2.3.2. Cell viability

Isolated GALT cells were distributed into 12-well plates at 2x10^6 cells/well and incubated at 20°C for 4 h and 24 h, at which times they were counted using a Neubauer chamber with addition of 0.5% trypan blue. The viabilities were determined by comparison to the respective time 0 h controls.

2.3.3. Marker gene analysis

Isolated GALT leucocytes (2x10^6 cells/well) were incubated at 20°C and harvested at 4 h and 24 h by centrifugation at 400g for 10 min at 4°C. The supernatant was discarded, and TRI reagent added, with pipetting up and down several times. The TRI lysate was stored at -80°C until RNA extraction for marker gene analysis. The genes studied included cell markers for T-lymphocytes (CD3ε, CD4-1, CD8α, CD8β, CD28, CTL4A and TCRα), B-lymphocytes (membrane (m) IgM, secreted (s) IgM, mIgT, sIgT, mIgD and sIgD), dendritic cells (CD83, DC-LAMP3/CD208, DC-SIGN/CD209 and MHCIIβ) and macrophages (MCSFR1a, MCSFR1b, MCSFR2a, MCSFR2b), since these cells are key for adaptive immune responses.

2.4. Stimulation of GALT cells

The GALT cells were distributed into 12-well cell culture plates at 2x10^6 cells/ml. The GALT cells from each fish were stimulated with 100 μg/ml of inactivated bacterin (1) *A. salmonicida* MT423 (MT423), (2) *A. salmonicida* MT423 grown in iron-depleted medium (MT423 (Fe-)), (3) *A. salmonicida* MT004 (MT004), (4) an equal combination of MT423

Journal Pre-proof

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

2.5. RNA extraction, cDNA synthesis and qPCR

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

2.6. Data statistical analysis

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

Journal Pre-proof

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

3. Results and discussion

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

Journal Pre-proof

3.1. Improved methodology boosts the yield of GALT cells

Although the previous optimization of GALT cell isolation increased the average cell yield [13], it is still an expensive process in terms of the time taken and cost compared to cell isolation from other tissues, e.g., the head kidney, spleen and blood [46, 40]. GALT cell isolation requires expensive reagents (e.g., DTT and collagenase IV), 3 pre-/digestion periods and multiple washes to eliminate the associated mucus and reagent residues. In order to boost the effectiveness of GALT cell isolation, i.e., increasing the cell yield within the same cost and time frame, the mechanical disturbance of the intestine contents was excluded and replaced with liquid flushing, using PBS extruded from a syringe against the tissue (hydraulic rinse). This increased the average yield to $210x10^6$ cells/fish (vs $12x10^6$/fish previously) whilst the percent cell viability over time was not different to the previous values, ~93% at 4 h and ~55% at 24 h post-culture [13]. The leucocyte markers examined, for T-cells (CD4-1, CD8 and TCRα), B-cells (mIgM, sIgM, mIgT and sIgT) and dendritic cells (CD83, CD208, CD209 and MHCIIβ) were also expressed by the isolated cells at 4 h and 24 h post-culture (Fig. S1), as seen before [13]. The gene expression levels were mostly lower at 24 h vs 4 h, with a few exceptions (e.g., mIgM, mIgT, mIgD, MCSFR1a/2a/2b). However, compared to the previous method, the marker gene analysis showed a relative increase in the expression of CD4-1 vs CD8 and CD208 vs CD209. In addition, the expression of CD83 and sIgT at 24 h were previously increased compared to values at 4 h but here they were decreased. The present study also demonstrated the expression of the T-cell markers CD28 and CTL4A, B-cell markers mIgD and sIgD, and macrophage cell markers MCSFR1 and MCSFR2 in the isolated cells that were not previously examined (Fig. S1). Regarding the IgD analysis it should be noted that neither IgD^+/IgM^- B cells or IgD protein have been detected in the intestine or gut secretions to date [2, 6]. Whilst teleost B-cells bearing different membrane Igs can correspond to different cell lineages, as fish lack Ig isotype switching [47, 6], nevertheless fish have B-cells that co-express surface IgM and IgD and therefore expression of mIgD and sIgD markers in this study is not a clear indication of an independent B-cell lineage in the GALT cells. Overall, the boost to the average cell yield, with no detrimental effect on viability or the main leucocyte types isolated (as assessed by transcript analysis), is a huge advance in terms of animal welfare, cost and time. Indeed, using the previous method [13] to obtain $210x10^6$ cells would require more than 17 fish, with a similar increase in the reagents needed. These findings suggest that the mechanical treatment of the intestine causes a significant reduction in the number of GALT cells obtained, as was seen by Salinas et al.

Journal Pre-proof

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

3.2. Responsiveness of GALT leucocytes

3.2.1. Diversity of the immune repertoire

Eighty immune molecules were selected to be examined post-stimulation of the GALT cells that comprise the immune categories that are typically modulated upon bacterial infection, stimulation or vaccination [52, 53, 54, 55, 36]. Sixty of these molecules could be modulated with the *A. salmonicida* and/or *Y. ruckeri* bacterin preparations at 4 h and/or 24 h. The modulated molecules were distributed in all the examined categories, that included the classical pro-inflammatory cytokines - IL-1β1, IL-1β2, IL-1β3, TNFα1, TNFα2, TNFα3, IL-6A, IL-6B (Fig. 1); other inflammatory cytokines - IL-8 (CXCL8), IL-11, IL-17C1, IL-17C2, IL-22, IL-34, CXCL11_L1, type I IFNa (Fig. 2) and IL-20 (Fig. S2B); anti-inflammatory cytokines - nIL-1F, IL-10A, TGF-β1A and TGF-β1B (Fig. 3A-D); suppressors of cytokine signalling - SOCS1A and SOCS3A (Fig. 3E-F); cytokines mainly involved in the adaptive immune response - IFNγ1, IFNγ2, IL-2B, IL-17A/F1A, IL-17A/F2A, IL-17A/F3 (Fig. 4) and IL-4/13B1 (Fig. S4C); IL-12 family subunits - P19, P28A, P35A1, P35A2, P40B1, P40C (Fig. 5), P40B2 and EBI3 (Fig. S2E-F); acute phase proteins (APPs) - COX2, SAA, SAP1 and SAP2 (Fig. 6A-D); antimicrobial peptides (AMPs) - cathelicidin (CATH)1, CATH2, LEAP1, β-defensin 4 (Fig. 6E-H) and β-defensin 1 (Fig. S5B); B cell markers - mIgM, sIgM, sIgT and sIgD (Fig. 7); T cell markers - CD3ε and CD4 (Fig. S6C-D); the transcriptional factor RORγ (Fig. S7C); and chemokine receptors - CXCR3A, CXCR4B, CCR6A, CCR7A, CCR9A and XCR3 (Fig. 8).

Forty-nine of the 60 modulated genes were up-regulated by at least one of the bacterins used and at one or more time points. A few of these genes (SOCS1A, P40B1, P40B2 and sIgT) were also inhibited (relative to the control cells) at the other time point and/or with a different bacterin (s). The remaining 11 modulated genes (TGF-β1A, IL-4/13B1, IL-20, EBI3, sIgM, CD3ε, CD4, RORγ, CXCR4B, CCR9A and XCR3) were (only) inhibited with one or more of the bacterin preparations. Lastly, 20 molecules were not modulated by

Journal Pre-proof

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

It is noteworthy that of the paralogous cytokine genes studied, that have arisen from the third teleost-wide and/or the fourth salmonid-wide whole genome duplication events, some differential modulation was seen in GALT cells. The paralogues of pro-inflammatory cytokines, e.g. IL-1β1-3 [56], IL-6A-B, IL- IL-17A/F1-3 [57], 17C1-2 [58], IFNγ1-2, and TNFα1-3 [59], behaved in a similar way in GALT cells, where they were typically induced after bacterin stimulation. In contrast, the paralogues of the anti-inflammatory cytokines IL-10A-B [60] and TGF-β1A-B [61] and the adaptive cytokines IL-2A-B [45] and IL-4/13A/B1-2 [62], behaved differently, with one induced and the other refractory (as in the case of the IL-2, IL-10 and IL-4/13 paralogues) or one inhibited and the other induced (as in the case of TGF-β1A-B). Differential expression and modulation of paralogous genes has been observed in multiple studies in salmonids [36, 55], and highlights the importance of including all paralogues in an analysis.

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

Journal Pre-proof

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

This panel of bacterin preparations has been used previously to stimulate freshly prepared PBL from rainbow trout in a kinetic study [36]. These PBL were isolated by a hypotonic lysis method, and contain the main immune cell types needed to monitor adaptive immune responses, i.e., T cells, B cells and monocytes/macrophages [40, 64]. The current study aimed to evaluate the intestine immune response relative to that of the PBL, therefore the GALT cells were similarly stimulated, and the expression of selected immune genes was examined at two corresponding time points. Although there was a wide consensus in the response direction with most of the immune molecules studied, the magnitude of the response was generally lower in GALT cells than in PBL, and some responses were not in the same direction. For instance, TNFα2 at 4 h was induced in the GALT leucocytes but inhibited in the PBL, IL-10B was not changed in the GALT cells but induced in the PBL, and RORγ, CD3ε and CD4 were inhibited in the GALT cells but induced in the PBL. Also, some molecules such as IL-2A, IL-4/13, IL-18, IL-21, sIgM, mIgD, T-bet, Foxp3A, Foxp3B, CXCR4B, CCR9A and XCR3 were only induced in the PBL. Lastly, with some molecules, the onset of induction was relatively late in the GALT cells (e.g., 24 h vs 4 h in PBL), as seen with IL-1β3, IL-11, IL-10A, TGF-β1B, SOCS1A, IFNγ1, IFNγ2 and CCR6A.

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

Journal Pre-proof

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

Sixty of the eighty immune genes examined were modulated in GALT cells by bacterin stimulation, with the majority of these genes increased and found higher at 24 h than at 4 h. The genes modulated at 24 h are summarised in Table 1. The expression of most of the pro-inflammatory cytokines (IL-1β1-2, TNFα1-2, IL-8, IL-17C1, IL-22, IL-34 and CXCL11_L1), the α-chains of the IL-12 family (P19, P28A, P35A1), APP (SAA), AMPs (CATH1-2 and LEAP1) and chemokine receptors (CXCR3A and CCR6A) was increased by all the five bacterin preparations at 24 h (Table 1). These data suggest the activation of common inflammatory pathways in GALT cells by different bacterins, as seen in PBL [40].

Despite a common inflammatory response, the *Y. ruckeri* driven response was distinct from *A. salmonicida* bacterins in the GALT leucocytes (Figs. 1-8, Table 1), as seen with PBL [40]. In comparison to *A. salmonicida* bacterins, *Y. ruckeri* induced gene expression was higher at 4 h for IL-6A, IL-8, IL-22, IL-34, IFNa, p19, p35A2, COX2 SAA and CATH1, and in some cases *A. salmonicida* had no effect at this timing. Similarly, at 24 h expression of TNFα3, IL-6A, IL-8, IL-11, IL-22, IL-34, COX2, SAA, CATH1 and CATH2 were all higher relative to the *A. salmonicida* stimulated cells. However, unlike the *A. salmonicida* bacterins, *Y. ruckeri* was unable to induce IFNγ1, IFNγ2 and IL-2B, molecules typically linked to a Th1 type response, SOCS1A, a major suppressor of IFNγ signalling [69], and IL-12 P40C that forms an IL-12 isoform known to induce IL-10 expression [70]. These differences in specific molecules induced with the *Y. ruckeri* bacterin could be used as potential markers for testing oral vaccine candidates; as *Y. ruckeri* can be used as an effective oral booster vaccine [26, 27], however the kinetics of induction might also be (bacterium) species-dependent.

The response induced in GALT cells by the different *A. salmonicida* bacterins also varied. For example, the non-pathogenic *A. salmonicida* MT004 driven response was different to that induced with the pathogenic *A. salmonicida* MT423 bacterin, in that induction of the proinflammatory cytokines IL-1β and TNFα was typically lower at 4 h but at similar levels at 24 h. In addition, induction of P35A1, P28A, P40B1 was lower at 4 h, as was IFNγ1 and IL-2B at 24 h but IL-17C1 and CCR7A were higher at 24 h. These differences might be

Journal Pre-proof

due to the lack of *A. salmonicida* virulence factors in this strain, such as the A-layer [71-73]. The iron depleted form of the *A. salmonicida* strain MT423 (MT423 (Fe-)) bacterin led to a higher induction of IL-17A/F2A, IL-17C1, IL-17C2, β-defensin 4, mIgM, sIgT and CCR6A, but a lower induction of IL-1β1, IL-1β2, IL-P35A1 and P28A, particularly at 24 h. These variations could be due to the production of the iron-regulated outer membrane proteins (IROMPs) that are considered to be potential protective antigens [43, 74], and hints at a better stimulation of mucosal/Th17 type responses. The combination of *A. salmonicida* MT423 (Fe-) with *A. salmonicida* MT004 (MT423 (Fe-) + MT004) has also driven a specific pattern of modulation with some molecules, such as IL-1β1, IL-1β2 and P35A1 at 4 h and CXCL11_L1, β-defensin 4 and mIgM at 24 h where the induction levels were in-between those seen with each bacterin alone. These findings demonstrate that despite the small scale of the GALT cell response compared to PBL, it was possible to distinguish different bacterial species, strains and different antigens. This shows that the intestinal cells are sensitive to potentially small differences in the immunological insults they receive and that this subsequently generates a tailored/specific response. This is in agreement with the conclusion made by Attaya et al. [13] that revealed different pathways are elicited with different stimulants in salmonids gut.

3.3. Conclusion

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

Journal Pre-proof

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

Acknowledgements

Ahmed Attaya's PhD project was funded by the Newton Fund, the British Council, and the National Institute of Oceanography and Fisheries (NIOF), Hurghada, Egypt.

4. References

[1] R.K. Buddington, A. Krogdahl, A.M. Bakke-Mckellep, The intestines of carnivorous fish: Structure and functions and the relations with diet, Act. Phys. Scand. Supp., 638 (1997) 67-80.

[2] Y.A. Zhang, I. Salinas, J. Li, D. Parra, S. Bjork, Z. Xu, S.E. LaPatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol., 11 (2010) 827–835.

[3] D. Gómez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol., 35 (2013) 1729-1739.

[4] J. Li, D.R. Barreda, Y.A. Zhang, H. Boshra, A.E. Gelman, S. LaPatra, L. Tort, J.O. Sunyer, B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities, Nat. Immunol., 7 (2006)1116-1124.

[5] I. Salinas, D. Parra, Fish mucosal immunity: intestine, Muc. Heal. Aquacul., (2015)135-170.

[6] D. Parra, T. Korytář, F. Takizawa, J.O. Sunyer, B cells and their role in the teleost gut, Dev. Com. Immunol., 64 (2016) 150-166.

[7] E.O. Koppang, U. Fischer, L. Moore, M.A. Tranulis, J.M. Dijkstra, B. Köllner, L. Aune, E. Jirillo, I. Hordvik, Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue, J. Anat., 217 (2010) 728–739.

Journal Pre-proof

[8] T. Boardman, C. Warner, F. Ramirez-Gomez, J. Matrisciano, E. Bromage, Characterization of an anti-rainbow trout (*Oncorhynchus mykiss*) CD3ε monoclonal antibody, Vet. Immunol. Immunopathol., 145 (2012) 511–515.

[9] F. Takizawa, J.M. Dijkstra, P. Kotterba, T. Korytář, H. Kock, B. Köllner, B. Jaureguiberry, T. Nakanishi, U. Fischer, The expression of CD8α discriminates distinct T cell subsets in teleost fish, Dev. Com. Immunol., 35 (2011) 752-63.

[10] H. Toda, Y. Saito, T. Koike, F. Takizawa, K. Araki, T. Yabu, T. Somamoto, H. Suetake, Y. Suzuki, M. Ototake, T. Moritomo, T. Nakanishi, Conservation of characteristics and functions of CD4 positive lymphocytes in a teleost fish, Dev. Com. Immunol., 35 (2011) 650–660.

[11] I. Boschi, E. Randelli, F. Buonocore, D. Casani, C. Bernini, A.M. Fausto, G. Scapigliati, Transcription of T cell-related genes in teleost fish, and the European sea bass (*Dicentrarchus labrax*) as a model, Fish Shellfish Immunol., 31 (2011) 655-662.

[12] S. Yoon, A. Attaya, T. Wang, J. Zou, C.J. Secombes, Immune gene profiling of different gut regions and gut associated lymphoid cells from rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol., 53 (2016) 112.

[13] A. Attaya, T. Wang, J. Zou, T. Herath, A. Adams, C.J. Secombes, S. Yoon, Gene expression analysis of isolated salmonid GALT leucocytes in response to PAMPs and recombinant cytokines, Fish Shellfish Immunol., 80 (2018) 426-436.

[14] E. Bassity, T.G. Clark, Functional identification of dendritic cells in the teleost model, rainbow trout (*Oncorhynchus mykiss*), PloS one, 7 (2012) e33196.

[15] I. Soleto, A.G. Granja, R. Simón, E. Morel, P. Díaz-Rosales, C. Tafalla, Identification of CD8α+ dendritic cells in rainbow trout (*Oncorhynchus mykiss*) intestine, Fish Shellfish Immunol., 89 (2019) 309-318.

[16] G.A. Davidson, A.E. Ellis, C.J. Secombes, Cellular responses of leucocytes isolated from the gut of rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish Diseas, 14 (1991) 651-659.

[17] J.L. Balcazar, D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, O. Gironés, J.L. Muzquiz, Immune modulation by probiotic strains: Quantification of phagocytosis of *Aeromonas salmonicida* by leukocytes isolated from gut of rainbow trout

Journal Pre-proof

(*Oncorhynchus mykiss*) using a radiolabelling assay, Com. Immunol. Microbiol. Infect. Diseas, 29 (2006) 335-343.

[18] E. Martin, V. Verlhac-Trichet, C. Legrand-Frossi, J.P. Frippiat, Comparison between intestinal and non-mucosal immune functions of rainbow trout, *Oncorhynchus mykiss*, Fish Shellfish Immunol., 33 (2012) 1258–1268.

[19] J.H.W.M. Rombout, L. Abelli, S. Pichette, G. Scapigliati, V. Kiron, Teleost intestinal immunology, Fish Shellfish Immunol., 31 (2011) 616-626.

[20] M.P. Hiney, J.J. Kilmartin, P.R. Smith, Detection of *Aeromonas salmonicida* in Atlantic salmon with asymptomatic furunculosis infections, Diseas. Aquat. Organ., 19 (1994) 161–167.

[21] E. Ringø, R.E. Olsen, The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr (*Salvelinus alpinus* L.), J. Appl. Microbiol., 86 (1999) 22–28.

[22] F. Jutfelt, R.E. Olsen, J. Glette, E. Ringø, K. Sundell, Translocation of viable *Aeromonas salmonicida* across the intestine of rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish Diseas, 29 (2006) 255–262.

[23] F. Jutfelt, H. Sundh, J. Glette, L. Mellander, B.T. Björnsson, K. Sundell, The involvement of *Aeromonas salmonicida* virulence factors in bacterial translocation across the rainbow trout, *Oncorhynchus mykiss* (Walbaum) intestine, J. Fish Diseas. 31 (2008) 141–151.

[24] S. Bartkova, B. Kokotovic, I. Dalsgaard, Infection routes of *Aeromonas salmonicida* in rainbow trout monitered *in vivo* by real-time bioluminescence imaging, J. Fish Diseas, 40 (2017) 73-82.

[25] J. Mendez, J.A. Guijarro, *In vivo* monitoring of *Yersinia ruckeri* in fish tissues: Progression and virulence gene expression, Env. Microbiol. Rep., 5 (2013)179–185.

[26] G.L. Tebbit, J.D. Erickson, R.B. Van de Water, Development and use of *Yersinia ruckeri* bacterins to control enteric redmouth disease, in international symposium on fish biologies: serodiagnostics and vaccines, Dev. biologic. standard., 49 (1981) 395–401

[27] R.M. Stevenson, Immunization with bacterial antigens: Yersiniosis, Dev. biologic. standard., 90 (1997) 117-24.

571 [28] I. Sommerset, B. Krossøy, E. Biering, P. Frost, Vaccines for fish in aquaculture, Exp.
572 Rev. Vacc., 4 (2005) 89-101.

573 [29] C.M. Press, A. Lillehaug, Vaccination in European salmonid aquaculture: a review of
574 practices and prospects, Brit. Vet. J., (1995)151, 45-69.

575 [30] A.E. Ellis, Immunization with bacterial antigens: Furunculosis, Dev. biologic.
576 standard., 90 (1997) 107-16.

577 [31] C.H. Li, J.B. Xiong, F.F. Ding, J. Chen, Immune and gut bacterial successions of large
578 yellow croaker (*Larimichthys crocea*) during *Pseudomonas plecoglossicida* infection,
579 Fish Shellfish Immunol., 99 (2020) 176-183.

580 [32] N.A. Ballesteros, R. Castro, B. Abos, S.S. Rodríguez Saint-Jean, S.I. Pérez-Prieto,
581 C.Tafalla, The pyloric caeca area is a major site for IgM(+) and IgT(+) B cell
582 recruitment in response to oral vaccination in rainbow trout, PLoS One, 8 (2013)
583 e66118.

584 [33] Y. Li, T.M. Kortner, E.M. Chikwati, H.M. Munang'andu, E.J. Lock, A. Krogdahl, Gut
585 health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black
586 soldier fly (*Hermetia illucens*) larvae meal, Fish Shellfish Immunol., 86 (2019) 1106-
587 1113.

588 [34] H. Noor-Ul, L. Haokun, J. Junyan, Z. Xiaoming, H. Dong, Y. Yunxia, X. Shouqi,
589 Dietary supplementation of *Geotrichum candidum* improves growth, gut
590 microbiota,immune-related gene expression and disease resistance in gibel carp CAS
591 Ⅲ(*Carassius auratus gibelio*), Fish Shellfish Immunol., 99 (2020) 144-153.

592 [35] I. Adeshina, M.I. Abubakar, B.E. Ajala, Dietary supplementation with *Lactobacillus*
593 *acidophilus* enhanced the growth, gut morphometry, antioxidant capacity, and the
594 immune response in juveniles of the common carp, *Cyprinus carpio*, Fish Physiol
595 Biochem., (2020).

596 [36] A. Attaya, Y. Jiang, C.J. Secombes, T. Wang, Distinct response of immune gene
597 expression in peripheral blood leucocytes modulated by bacterin vaccine candidates
598 in rainbow trout *Oncorhynchus mykiss*: A potential in vitro screening and batch
599 testing system for vaccine development in aquaculture, Fish Shellfish Immunol., 93
600 (2019) 631-640.

Journal Pre-proof

601 [37] X.J. Zhang, P. Wang, N. Zhang, D.D. Chen, P. Nie, J.L. Li, Y.A. Zhang, B cell
602 functions can be modulated by antimicrobial peptides in rainbow trout *Oncorhynchus*
603 *mykiss*: Novel insights into the innate nature of B cells in fish, Front. Immunol., 8
604 (2017) 388.

605 [38] J. Gu, S. Dai, H. Liu, Q. Cao, S. Yin, K.P. Lai, W.K.F. Tse, C.K.C. Wong, H. Shi,
606 Identification of immune-related genes in gill cells of Japanese eels (*Anguilla*
607 *japonica*) in adaptation to water salinity changes, Fish Shellfish Immunol., 73 (2018)
608 288-296.

609 [39] H.C. Samaï, D. Rioult, A. Bado-Nilles, L. Delahaut, J. Jubréaux, A. Geffard, J.M.
610 Porcher, S. Betoulle, Procedures for leukocytes isolation from lymphoid tissues and
611 consequences on immune endpoints used to evaluate fish immune status: A case
612 study on roach (*Rutilus rutilus*), Fish Shellfish Immunol., 74 (2018) 190-204.

613 [40] Y. Hu, K. Maisey, P.A. Subramani, F. Liu, C. Flores-Kossack, M. Imarai, C.J.
614 Secombes, T. Wang, Characterisation of rainbow trout peripheral blood leucocytes
615 prepared by hypotonic lysis of erythrocytes, and analysis of their phagocytic activity,
616 proliferation and response to PAMPs and proinflammatory cytokines, Dev. Com.
617 Immunol., 88 (2018) 104-113.

618 [41] T. Wang, C.J. Secombes, Complete sequencing and expression of three complement
619 components, C1r, C4 and C1 inhibitor, of the classical activation pathway of the
620 complement system in rainbow trout *Oncorhynchus mykiss*, Immunogenetic, 55
621 (2003) 615-628.

622 [42] N.O. Harun, T. Wang, C.J. Secombes, Gene expression profiling in naïve and
623 vaccinated rainbow trout after *Yersinia ruckeri* infection: Insights into the
624 mechanisms of protection seen in vaccinated fish, Vaccin, 29 (2011) 4388–4399.

625 [43] I. Hirst, A. Ellis, Iron-regulated outer membrane proteins of *Aeromonas salmonicida*
626 are important protective antigens in Atlantic salmon against furunculosis, Fish
627 Shellfish Immunol., 4 (1994) 29–45.

628 [44] T. Wang, P. Diaz-Rosales, M.M. Costa, S. Campbell, M. Snow, B. Collet, S. Martin,
629 C.J. Secombes, Functional characterization of a nonmammalian IL-21: Rainbow
630 trout *Oncorhynchus mykiss* IL-21 upregulates the expression of the Th cell signature
631 cytokines IFN-γ, IL-10, and IL-22, J. Immunol., 186 (2011) 708-721.

Journal Pre-proof

[45] T. Wang, Y. Hu, E. Wangkahart, F. Liu, A. Wang, E. Zahran, K.R. Maisey, M. Liu, Q. Xu, M. Imarai, C.J. Secombes, Interleukin (IL)-2 is a key regulator of T helper 1 and T helper 2 cytokine expression in fish: Functional characterization of two divergent IL2 paralogs in salmonids, Front. Immunol., 9 (2018) 1683.

[46] T. Wang, J.W. Holland, A. Carrington, J. Zou, C.J. Secombes, Molecular and functional characterization of IL-15 in rainbow trout *Oncorhynchus mykiss*: A potent inducer of IFN-γ expression in spleen leukocytes, J. Immunol., 179 (2007) 1475–1488.

[47] F. Ramirez-Gomez, W. Greene, K. Rego, J.D. Hansen, G. Costa, P. Kataria, E.S. Bromage, Discovery and characterization of secretory IgD in rainbow trout: Secretory IgD is produced through a novel splicing mechanism, J. Immunol., 188 (2012) 1341–1349.

[48] I. Salinas, J. Meseguer, M.Á. Esteban, Assessment of different protocols for the isolation and purification of gut associated lymphoid cells from the gilthead seabream (*Sparus aurata* L.), Biologic. Proced. Onlin., 9 (2007) 43-55.

[49] G. Løkka, L. Austbø, K. Falk, E. Bromage, P.G. Fjelldal, T. Hansen, I. Hordvik, E.O. Koppang, Immune parameters in the intestine of wild and reared unvaccinated and vaccinated Atlantic salmon (*Salmo salar* L.), Dev. Com. Immunol., 47 (2014) 6-16.

[50] J.A. Calduch-Giner, A. Sitjà-Bobadilla, J. Pérez-Sánchez, Gene expression profiling reveals functional specialization along the intestinal tract of a carnivorous teleostean fish (*Dicentrarchus labrax*), Front. Physiol., 7 (2016) 359.

[51] C. Tafalla, E. Leal, T. Yamaguchi, U. Fischer, T cell immunity in the teleost digestive tract, Dev. Com. Immunol., 64 (2016) 167-177.

[52] H.Ø. Eggestøl, H.S. Lunde, A. Rønneseth, D. Fredman, K. Petersen, C.K. Mishra, T. Furmanek, D.J. Colquhoun, H.I. Wergeland, G.T. Haugland, Transcriptome-wide mapping of signaling pathways and early immune responses in lumpfish leukocytes upon in vitro bacterial exposure, Sci. Rep., 8 (2018) 5261.

[53] S. Maekawa, P. Wang, S. Chen, Comparative study of immune reaction against bacterial infection from transcriptome analysis, Front. Immunol., 10 (2019) 153.

Journal Pre-proof

[54] H. Lund, A.F. Bakke, I. Sommerset, S. Afanasyev, G. Schriwer, A. Thorisdottir, P. Boysen, A. Krasnov, A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon, Mol. Immunol. 106 (2019) 99-107.

[55] E. Wangkahart, C.J. Secombes, T. Wang, Dissecting the immune pathways stimulated following injection vaccination of rainbow trout (*Oncorhynchus mykiss*) against enteric redmouth disease (ERM), Fish Shellfish Immunol., 85 (2019) 18-30.

[56] M. Husain, S. Bird, R. van Zwieten, C.J. Secombes, T. Wang, Cloning of the IL-1β3 gene and IL-1β4 pseudogene in salmonids uncovers a second type of IL-1β gene in teleost fish, Dev. Comp. Immunol., 38 (2012), 431-446.

[57] T. Wang, Y. Jiang, A. Wang, M. Husain, Q. Xu, C.J. Secombes, Identification of the salmonid IL-17A/F1a/b, IL-17A/F2b, IL-17A/F3 and IL-17N genes and analysis of their expression following in vitro stimulation and infection, Immunogenetics, 67 (2015), 395-412.

[58] T. Wang, S.A. Martin, C.J. Secombes, Two interleukin-17C-like genes exist in rainbow trout *Oncorhynchus mykiss* that are differentially expressed and modulated, Dev. Comp. Immunol., 34 (2010), 491-500.

[59] S. Hong, R. Li, Q. Xu, C.J. Secombes, T. Wang, Two types of TNF-α exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF-α3 in rainbow trout *Oncorhynchus mykiss*, J. Immunol., 191 (2013), 5959-5972.

[60] N.O. Harun, M.M. Costa, C.J. Secombes, T. Wang, Sequencing of a second interleukin-10 gene in rainbow trout *Oncorhynchus mykiss* and comparative investigation of the expression and modulation of the paralogues *in vitro* and *in vivo*, Fish. Shellfish Immunol., 31 (2011), 107-117.

[61] T. Maehr, M.M. Costa, J.L.G. Vecino, S. Wadsworth, S.A.M. Martin, T. Wang, C.J. Secombes, Transforming growth factor-β1b: a second TGF-β1 paralogue in the rainbow trout (*Oncorhynchus mykiss*) that has a lower constitutive expression but is more responsive to immune stimulation, Fish. Shellfish Immunol., 34 (2013), 420-432.

[62] T. Wang, P. Johansson, B. Abós, A. Holt, C. Tafalla, Y. Jiang, A. Wang, Q. Xu, Z. Qi, W. Huang, M.M. Costa, P. Diaz-Rosales, J.W. Holland, C.J. Secombes, First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct

patterns of expression and modulation but overlapping bioactivities, Oncotarget, 7 (2016), 10917-10946.

[63] A. Attaya, K. Veenstra, M.D. Welsh, S. Yoon, C.J. Secombes, *In vitro* evaluation of novel (nanoparticle) oral delivery systems allow selection of gut immunomodulatory formulations, Vet. Immunol. Immunopathol. (In press)

[64] E. Sasaki, H. Momose, Y. Hiradate, K.J. Ishii, T. Mizukami, I. Hamaguchi, *In vitro* marker gene expression analyses in human peripheral blood mononuclear cells: A tool to assess safety of influenza vaccines in humans, J. Immunotoxicol., 15 (2018) 53-62.

[65] Z. Xu, F. Takizawa, D. Parra, D. Gómez, L. von Gersdorff Jørgensen, S.E. LaPatra, J.O. Sunyer. Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods. Nat Commun., 7 (2016) 10728.

[66] I. Huber, B. Spanggaard, K. Appel, L. Rossen, T. Nielsen, L. Gram, Phylogenetic analysis and *in situ* identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum), J. Appl. Microbiol., 96 (2004)117–132.

[67] D.H. Kim, J. Brunt, B. Austin, Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*), J. Appl. Microbiol. 102 (2007) 1654–1664.

[68] S.K. Nayak, Role of gastrointestinal microbiota in fish, Aquacul. Resear., 41 (2010) 1553–1573.

[69] B. Wang, E. Wangkahart, C.J. Secombes, T. Wang, Insights into the evolution of the suppressors of cytokine signaling (SOCS) gene family in vertebrates. Mol Biol Evol., 36 (2019) 393-411.

[70] T. Wang, M. Husain, S. Hong, J.W. Holland, Differential expression, modulation and bioactivity of distinct fish IL-12 isoforms: implication towards the evolution of Th1-like immune responses, Eur. J. Immunol., 44 (2014) 1541-1551.

[71] R.A. Garduño, A.R. Moore, G. Olivier, A.L. Lizama, E. Garduño, W.W. Kay, Host cell invasion and intracellular residence by *Aeromonas salmonicida*: Role of the S-layer, Cana. J. Microbiol., 46 (2000) 660–668.

[72] V. Lund, J.A. Arnesen, D. Coucheron, K. Modalsli, C. Syvertsen, The *Aeromonas salmonicida* A-layer protein is an important protective antigen in oil-adjuvanted vaccines, Fish Shellfish Immunol., 15 (2003) 367–372.

[73] S. Dallaire-Dufresne, K.H. Tanaka, M.V. Trudel, A. Lafaille, S.J. Charette, Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis, Vet. Microbiol., 169 (2014) 1–7.

[74] I. Bricknell, J. King, T. Bowden, A. Ellis, Duration of protective antibodies, and the correlation with protection in Atlantic salmon (*Salmo salar* L.), following vaccination with an *Aeromonas salmonicida* vaccine containing iron-regulated outer membrane proteins and secretory polysaccharide, Fish Shellfish Immunol., 9 (1999) 139-151.

Figure and table legends

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.

Figure 1. Modulation of the expression of classic pro-inflammatory cytokine genes in GALT cells. GALT cells isolated from the intestine of rainbow trout were stimulated for 4 h and 24 h 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 gene expression of IL-1β1 (A), IL-1β2 (B), IL-1β3 (C), TNFα1 (D), TNFα2 (E), TNFα3 (F), IL-6A (G) and IL-6B (H) was examined by RT-qPCR as described in Materials and Methods. Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the results of a post-hoc paired samples T-test. Bars having the same letter are not different statistically.

Journal Pre-proof

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

Figure 3. Modulation of the expression of anti-inflammatory genes in GALT cells. Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and 24 h, and the expression of anti-inflammatory cytokines IL-1Fm (A), IL-10A (B), TGF-β1A (C) and TGF-β1B (D), and the suppressors of cytokine signalling SOCS1A (E) and SOC3A (F) was examined as described in **Figure 1**. Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the results of a post-hoc paired samples T-test. Bars having the same letter are not different statistically.

Figure 4. Modulation of the expression of adaptive cytokine genes in GALT cells. Rainbow trout GALT cells were stimulated with different bacterin preparations for 4 h and 24 h, and the expression of adaptive cytokines IFNγ1 (A), IFNγ2 (B), IL-2B (C), IL-17A/F1A (D), IL-17A/F2A (E), and IL-17A/F3 (F) was examined as described in **Figure 1**. Bars are mean fold changes + SEM of 4 fish. Letters over the bars at the same time point indicate a significant difference ($p \leq 0.05$) was found by one-way ANOVA, and are the results of a post-hoc paired samples T-test. Bars having the same letter are not different statistically.

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

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

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

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

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

Journal Pre-proof

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	*Aeromonas salmonicida*				*Yersinia ruckeri*
		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	↑	↑	↑	↑	↑
	IFNa	↑	↑	↑	↑	–
Anti-inflammatory Cytokines and SOCS	IL-10A	↑	↑	–	–	–
	TGF-β1B	↑	–	–	–	–
	SOCS1A	↑	↑	↑	↑	–
	SOCS3A	↑	↑	↑	↑	–
Adaptive immunity cytokines	IFNγ1	↑	↑	↑	↑	–
	IFNγ2	↑	–	–	–	–
	IL-2B	↑	–	–	–	–
	IL-17A/F1A	–	–	–	–	↑
	IL-17A/F3	–	–	↑	↑	–
IL-12 family cytokines	P19	↑	↑	↑	↑	↑
	P28A	↑	↑	↑	↑	↑
	P35A1	↑	↑	↑	↑	↑
	P35A2	–	–	–	–	↑
	P40B1	↑	↑	↑	↑	↑
	P40B2	–	–	–	–	↑
	P40C	↑	↑	↑	↑	–
Acute phase proteins (APPs)	COX2	–	–	–	–	↑
	SAA	↑	↑	↑	↑	↑
	SAP1	–	–	–	↑	–
Antimicrobial peptides (AMPs)	CATH1	↑	↑	↑	↑	↑
	CATH2	↑	↑	↑	↑	↑
	LEAP1	↑	↑	↑	↑	↑
	β-defensin 4	–	↑	↑	–	↑
Immunoglobulins (Igs)	mIgM	–	–	↑	–	–
	sIgT	–	↑	↑	–	–
	sIgD	–	↑	↑	↑	↑
Chemokine receptors	CXCR3A	↑	↑	↑	↑	↑
	CCR6A	↑	↑	↑	↑	↑
	CCR7A	–	–	–	↑	–
	XCR3	–	–	–	↓	↓

Figure 1

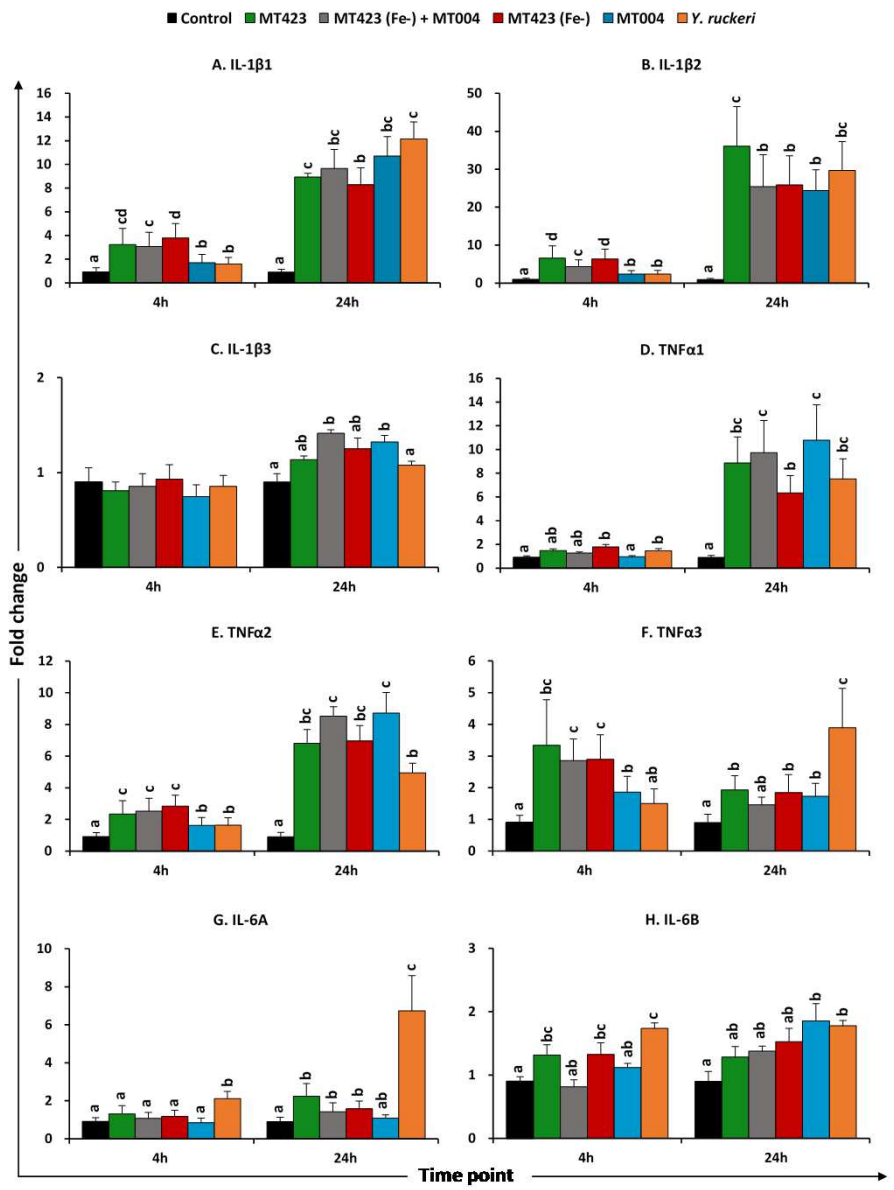

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. IL-1β1
B. IL-1β2
C. IL-1β3
D. TNFα1
E. TNFα2
F. TNFα3
G. IL-6A
H. IL-6B
4h
24h
Fold change
Time point

Figure 2

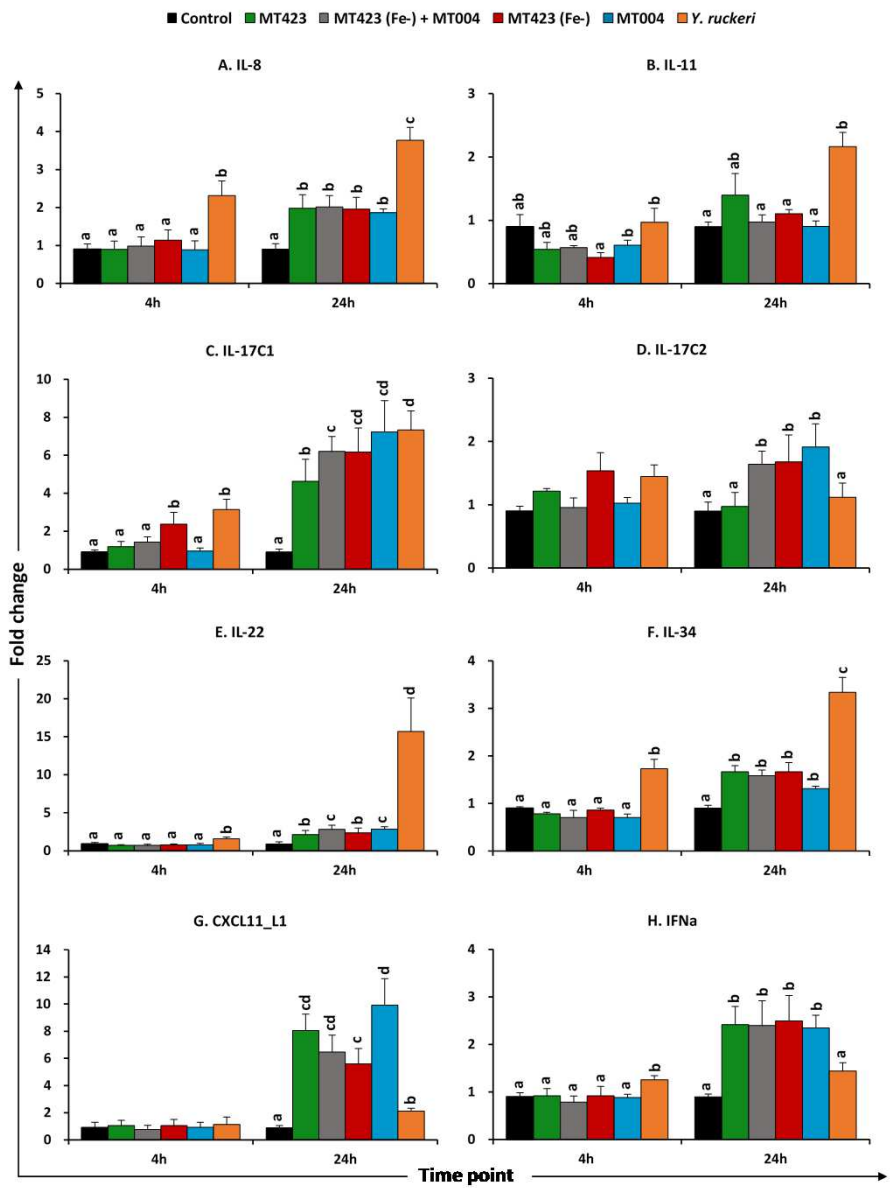

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. IL-8
B. IL-11
C. IL-17C1
D. IL-17C2
E. IL-22
F. IL-34
G. CXCL11_L1
H. IFNa
4h
24h
Fold change
Time point

Figure 3

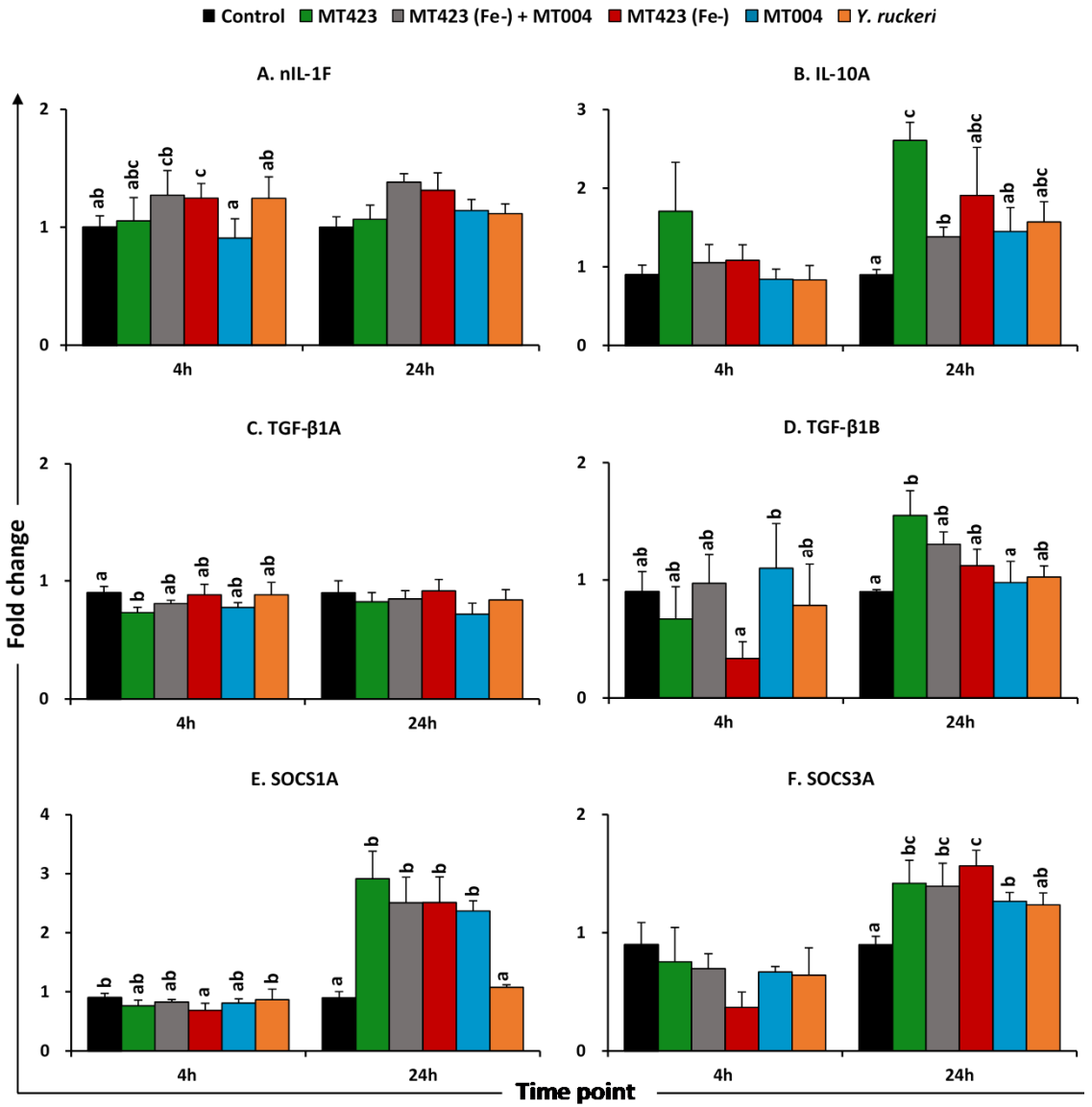

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. nIL-1F
B. IL-10A
C. TGF-β1A
D. TGF-β1B
E. SOCS1A
F. SOCS3A
Fold change
Time point
4h
24h

Figure 4

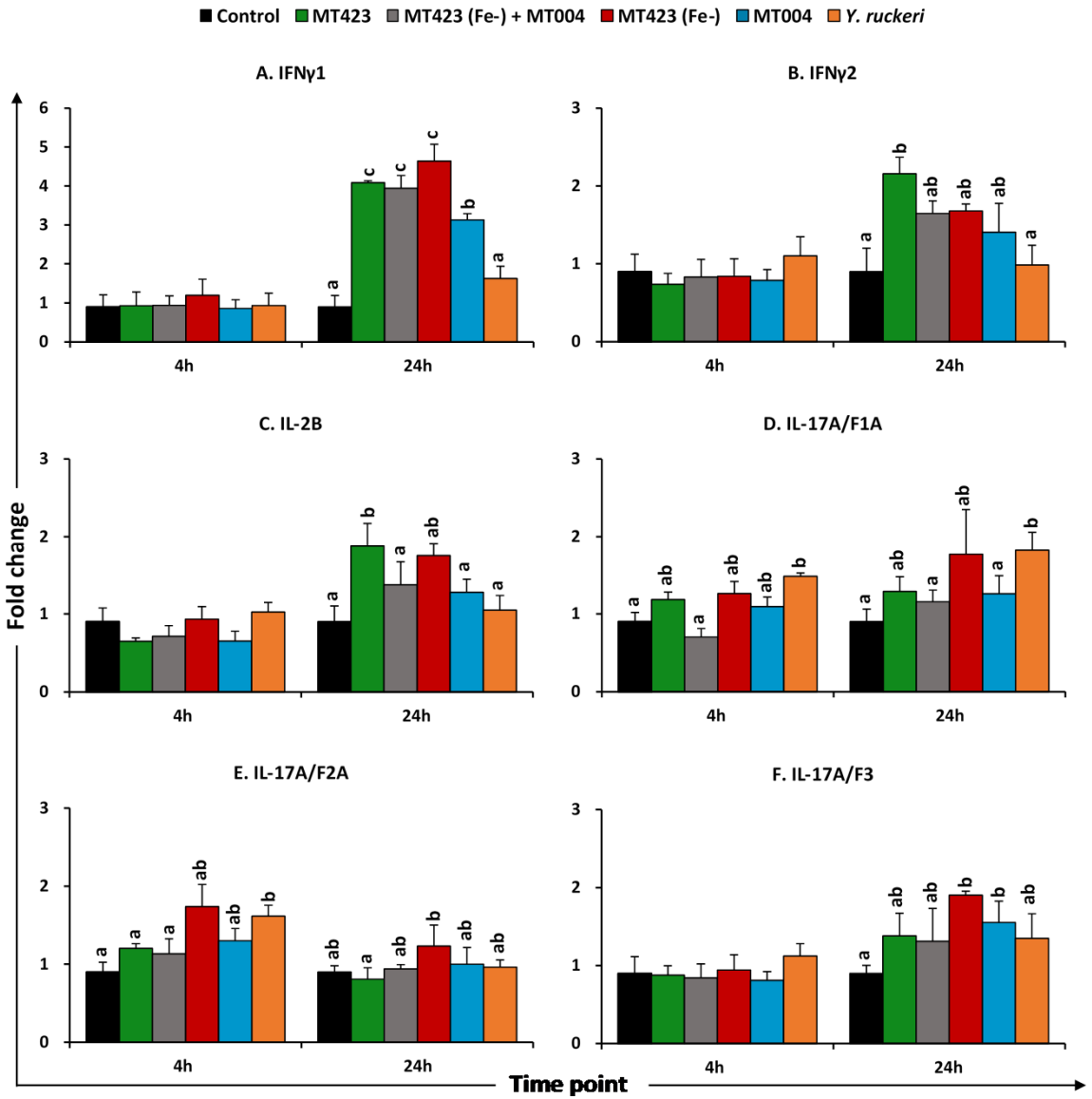

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. IFNγ1
B. IFNγ2
C. IL-2B
D. IL-17A/F1A
E. IL-17A/F2A
F. IL-17A/F3
Fold change
Time point
4h
24h

Figure 5

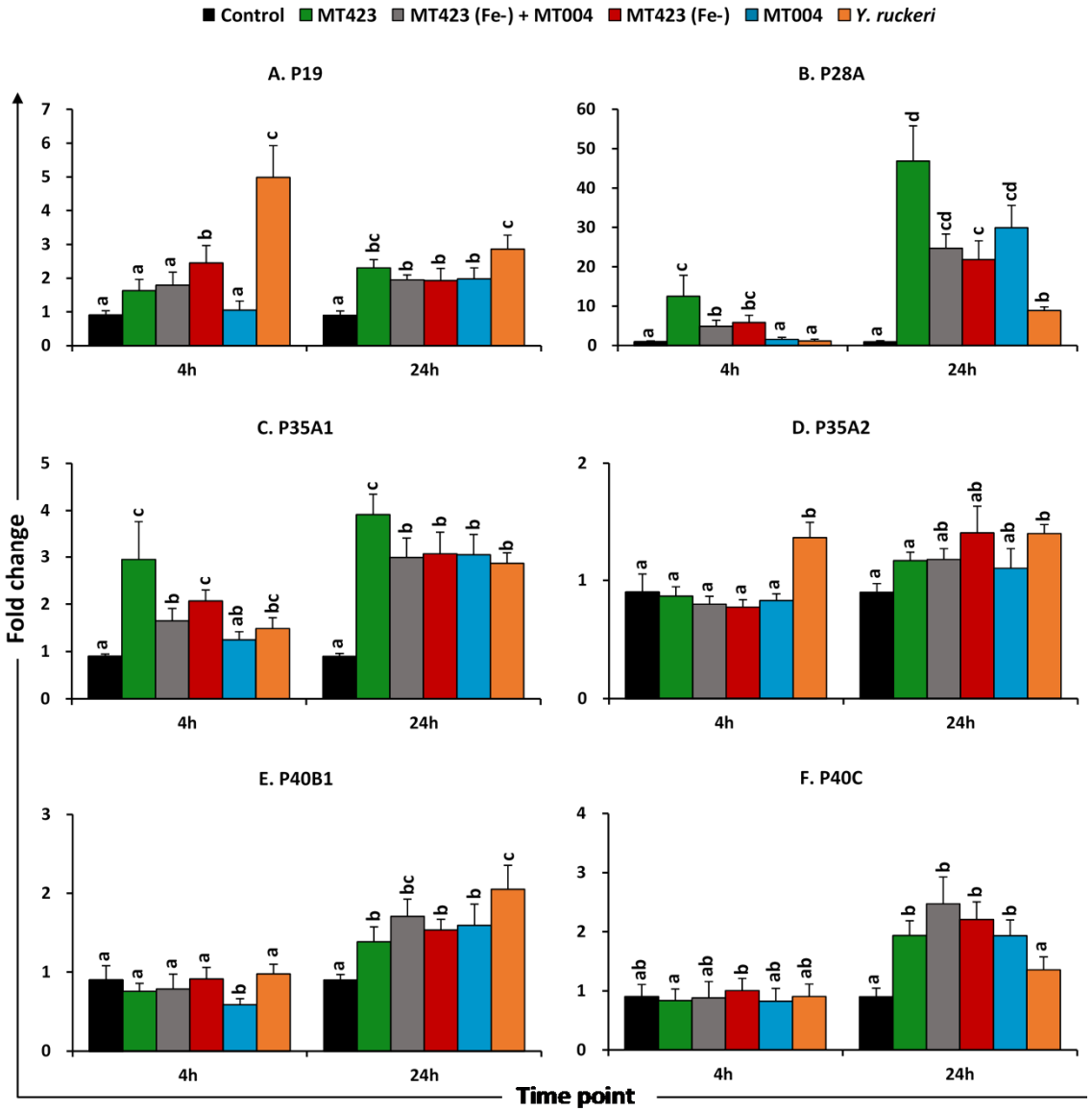

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. P19
B. P28A
C. P35A1
D. P35A2
E. P40B1
F. P40C
Fold change
Time point
4h
24h

Figure 6

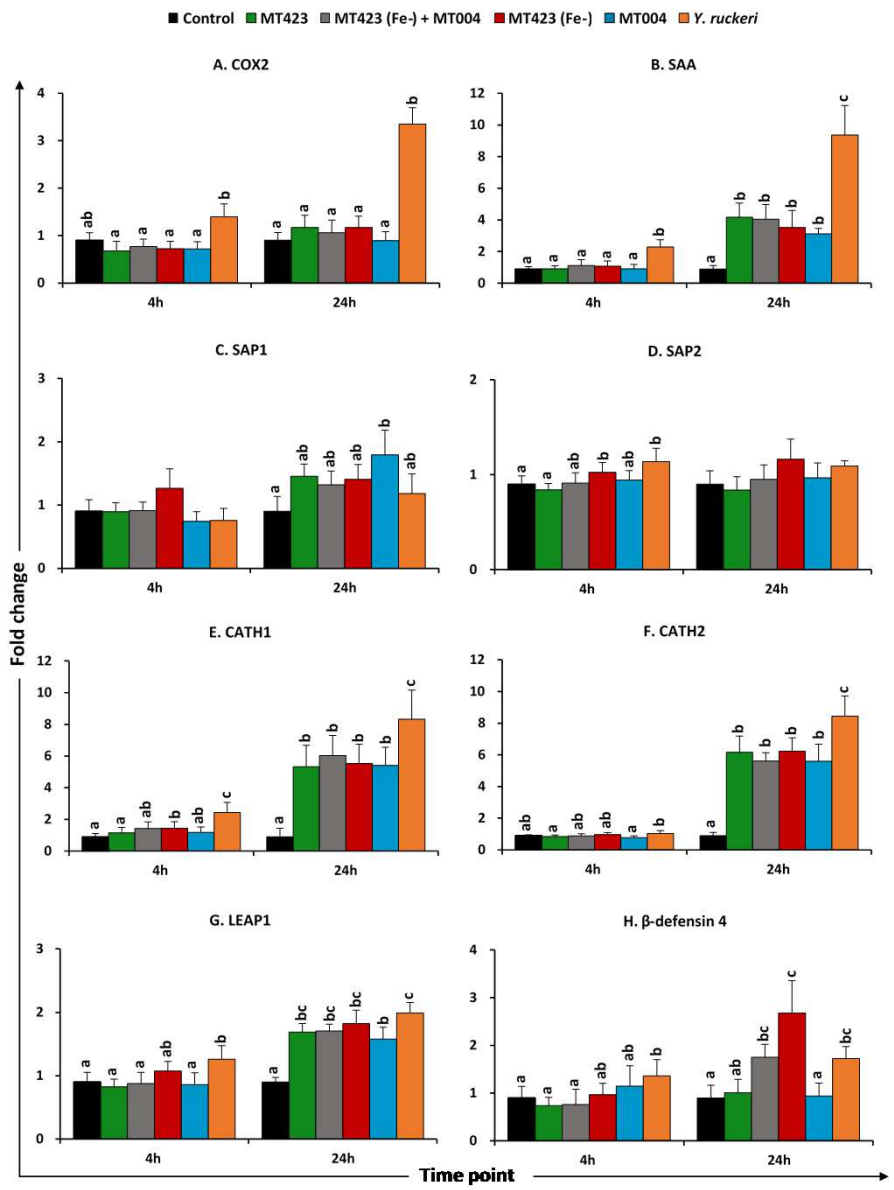

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. COX2
B. SAA
C. SAP1
D. SAP2
E. CATH1
F. CATH2
G. LEAP1
H. β-defensin 4
Fold change
Time point
4h
24h

Figure 7

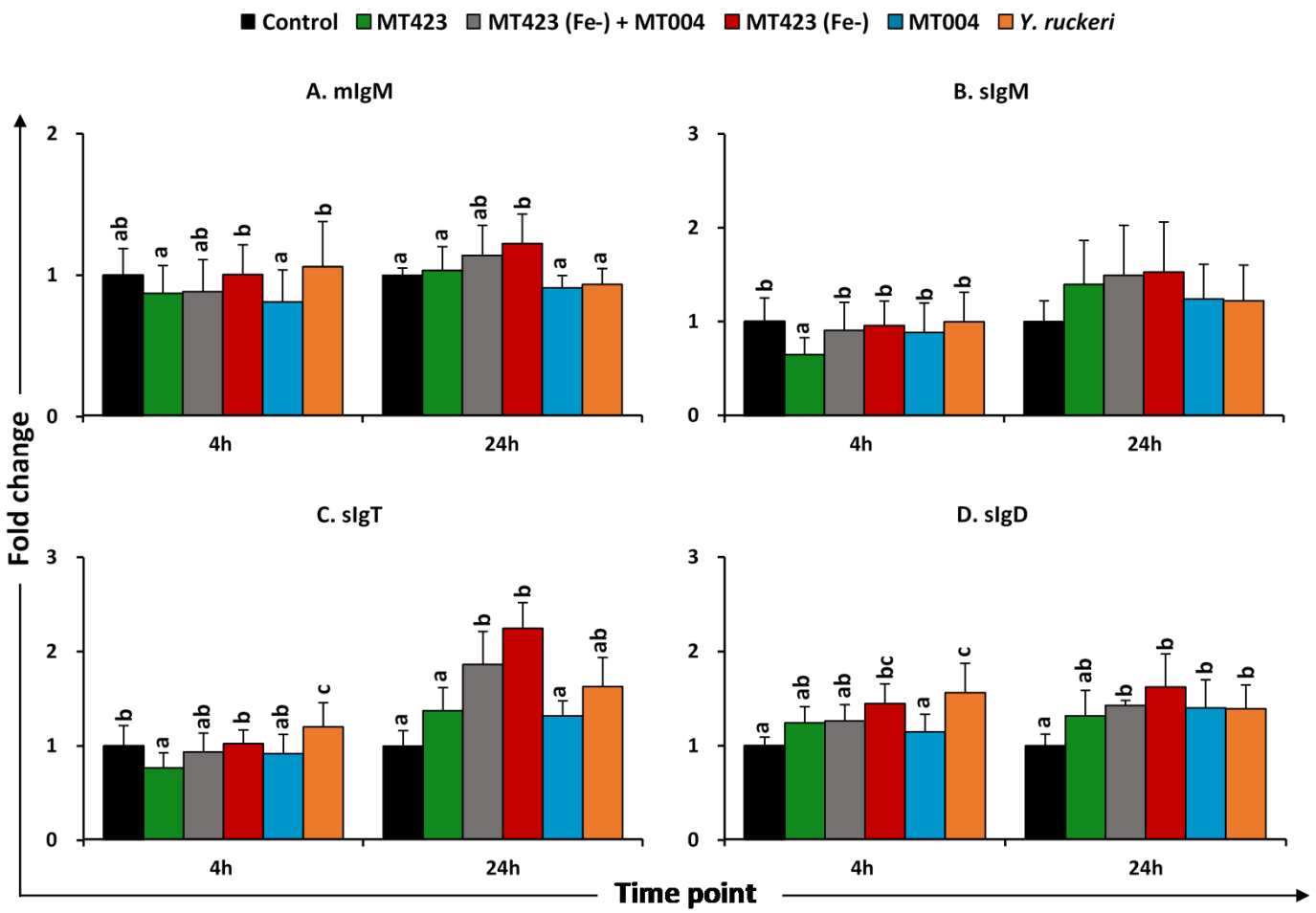
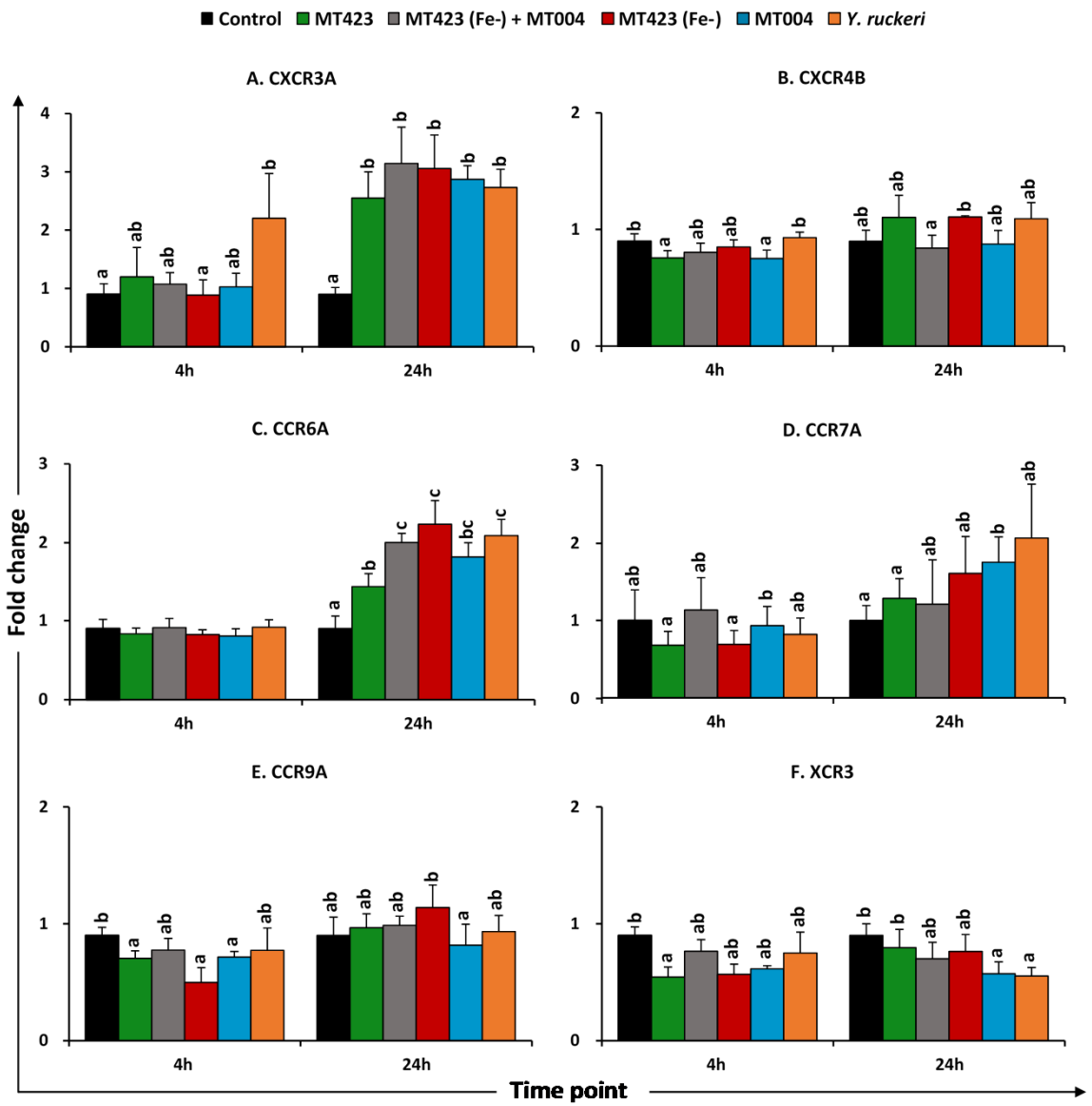

Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. mIgM
B. sIgM
C. sIgT
D. sIgD
Fold change
Time point
4h
24h

Figure 8


Control
MT423
MT423 (Fe-) + MT004
MT423 (Fe-)
MT004
Y. ruckeri
A. CXCR3A
B. CXCR4B
C. CCR6A
D. CCR7A
E. CCR9A
F. XCR3
Fold change
Time point
4h
24h

Journal Pre-proof

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.

Journal Pre-proof