Vaccine-induced modulation of gene expression in turbot peritoneal cells. A microarray approach

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ABSTRACT

We used a microarray approach to examine changes in gene expression in turbot peritoneal cells after injection of the fish with vaccines containing the ciliate parasite *Philasterides dicentrarchi* as antigen and one of the following adjuvants: chitosan-PVMMA microspheres, Freund´s complete adjuvant, aluminium hydroxide gel or Matrix-Q (Isconova, Sweden). We identified 374 genes that were differentially expressed in all groups of fish. Forty-two genes related to tight junctions and focal adhesions and/or actin cytoskeleton were differentially expressed in free peritoneal cells. The profound changes in gene expression related to cell adherence and cytoskeleton may be associated with cell migration and also with the formation of cell-vaccine masses and their attachment to the peritoneal wall. Thirty-five genes related to apoptosis were differentially expressed. Although most of the proteins coded by these genes have a proapoptotic effect, others are antiapoptotic, indicating that both types of signals occur in peritoneal leukocytes of vaccinated fish. Interestingly, many of the genes related to lymphocytes and lymphocyte activity were downregulated in the groups injected with vaccine. We also observed decreased expression of genes related to antigen presentation, suggesting that macrophages (which were abundant in the peritoneal cavity after vaccination) did not express these during the early inflammatory response in the peritoneal cavity. Finally, several genes that participate in the inflammatory response were differentially expressed, and most participated in resolution of inflammation, indicating that an M2 macrophage response is generated in the peritoneal cavity of fish one day post vaccination.

Keywords

Turbot, microarray, vaccines, *Philasterides dicentrarchi*, peritoneal cells, gene expression

1. Introduction

Injection of vaccines is the method that results in the best immune response and protection in fish (Lillehaug, 2014). Although vaccines can be administered via the intramuscular or intraperitoneal route, the latter is the most commonly used. Antigen by itself is usually poorly immunogenic and an adjuvant must be added to enhance the antigenicity (Tafalla at al., 2013). Most studies in fish injected with oil-based vaccines have evaluated the immune response generated (mainly by measuring the serum IgM levels) and the protection induced after experimental challenge. For example, in rainbow trout inoculated with a *Yersinia ruckeri* bacterin and the adjuvant Montanide™ ISA 763 A VG (Seppic), significantly enhanced serum IgM levels, increased numbers of IgM^+ cells in the spleen and significant upregulation of several immune genes in spleen, head kidney and liver were observed (Jaafar et al., 2015). Analysis of the immune response in salmonids suggests simultaneous expression of Th1, Th2, and Treg cytokines after administration of oil-based vaccines, with no clearly predominant response (Mutoloki et al., 2010; Kumari et al., 2013; Jaafar et al., 2015). Although aluminium-containing adjuvants are widely used in mammals, they are less commonly used in fish vaccines, probably because the immune response is narrower than that produced by the more commonly used oil-based adjuvants (Jiao et al., 2010). Very little is known about the immune responses generated by aluminium hydroxide adjuvants in fish, except in relation to the antibody levels generated and the protection induced. Although aluminium hydroxide-based adjuvants are generally considered to prime Th2-type immune responses preferentially, more recent studies in mammals suggest that aluminium hydroxide-based adjuvants can enhance both Th1 and Th2 cellular responses, depending on the vaccination route (He et al., 2015). Several vaccines containing microspheres have been shown to induce activation of innate and adaptive immune responses in fish (Behera and Swain, 2012; Harikrishnan et al., 2012; León-Rodríguez et al., 2012, 2013), although there is no information about how these vaccines affect the activity of peritoneal cells or cells located in lymphoid organs. It is not known what takes place in the peritoneal cavity of fish after administration of a vaccine. Vaccination induces expression of inflammatory genes in lymphoid organs (Frediksen et al., 2011; Dan et al., 2013) and generation of Th1/Th2/Th17 immune responses has been suggested to occur; however, more sensitive tools are required to

help us understand the type of response produced and thus enable more complex analysis of the immune response, comparable to that carried out in mammals.

Vaccines, particularly those containing oil-based adjuvants, can cause important lesions in the peritoneal cavity (Noia et al., 2014). The lesions, which include granulomas as well as adhesions between internal organs or between the organs and the peritoneal wall, vary depending on the adjuvant. Many of the free cells in the peritoneal cavity tend to adhere to the vaccine and thus form masses that become attached to the peritoneal wall. The cell-vaccine masses contain macrophage-like cells and apoptotic cells (Noia et al., 2014). Strong expression of genes related to cell-cell or cell-matrix adhesion and cell-cell junctions and also of genes related to actin cytoskeleton must occur in order to generate cell-vaccine masses. Differentiation, polarization, migration and adhesion processes are affected by the role of the cytoskeleton in innate immunity and cellular self-defence (Mostowy and Shenoy, 2015). In the present study, we used an oligo-microarray containing 43,803 spots and which is rich in genes involved in immunity and reproduction, but also in genes associated with cell adhesion and cytoskeleton (Ribas et al., 2013), to analyse the early cell response in the peritoneal cavity after vaccination. This is the first study of this type carried out in fish.

2. Materials and methods

2.1. Fish

Specimens of turbot *Scophthalmus maximus* (L.), of approximately 30 g body weight, were obtained from a local fish farm. The fish were maintained in 250-L tanks with aerated and recirculated sea water held at 16°C and were fed daily with commercial pellets. Fish were acclimatized to laboratory conditions for two weeks before the experiments began. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain). For all procedures, the fish were anaesthetized with benzocaine hydrochloride (50 mg/l) and killed by pithing.

2.2. Philasterides dicentrarchi: culture and preparation of membrane antigen

Philasterides dicentrarchi (isolate C1) was obtained from ascitic fluid of naturally infected turbot and maintained and cultured in the laboratory, as previously

64 described (Piazzon et al., 2008). The membrane fraction of the ciliate was obtained as
65 described in Budiño et al., (2012). Briefly, the ciliates (1.5×10^7) were resuspended in
66 HEPES buffer containing dibucaine and incubated for 15 min at room temperature (to
67 induce deciliation). After verification (by microscopic examination) that deciliation had
68 occurred, the parasites were washed three times by centrifugation for 5 min at 600×g in
69 PBS at 4°C. The pellet containing the ciliates was then resuspended in PBS with 0.25 M
70 sucrose, disrupted ultrasonically and centrifuged at 8.000 ×g for 5 min. The pellet was
71 dissolved in 3 ml of HEPES buffer and added to a tube containing a sucrose gradient
72 (0.5 M, 1 M and 0.15 M). After centrifugation at 6.000 ×g, the 0.1 M sucrose phase was
73 removed and centrifuged at 11,000 ×g for 20 min. Finally, the pellet was resuspended in
74 HEPES buffer with 0.5% Triton X-114 for 15 h at 0°C, to solubilise the membrane
75 proteins. The suspension was centrifuged at 8.000 ×g for 10 min, and the supernatant
76 was then concentrated by amicon ultrafiltration with 1 kDa nominal molecular weight
77 limit membranes (Millipore). The concentration of protein in the samples was
78 determined by the Bradford method (Bio-Rad Protein Assay kit: Bio-Rad Laboratories,
79 Germany), with BSA (Sigma-Aldrich, Spain) as standard. *Philasterides dicentrarchi*
80 membrane proteins (10 µg) were separated by SDS-PAGE on 10% linear
81 polyacrylamide gels (Fig. S1), as previously described (Budiño et al., 2012).

82 *2.3. Vaccine preparation and administration*

83 Six groups of fish (in duplicate, 114 fish in total) were used. The control group
84 (n= 24) was injected intraperitoneally (i.p.) with 0.1 ml of phosphate-buffered saline
85 (PBS). The remaining groups (n= 18 fish per group) were injected i.p. with 230 µg of
86 antigen in PBS, with 5 mg of microspheres containing 230 µg of covalently linked
87 antigen to their surface in PBS (León-Rodríguez et al., 2012; Noia et al., 2014), or with
88 a mixture of antigen and one of the following adjuvants (1:1, v:v): Freund's complete
89 adjuvant (FCA), aluminium hydroxide gel (Alhydrogel, Sigma) or Matrix-Q (Isconova,
90 Sweden) (5 µg/fish). Four of the fish in each of the duplicate control group and three of
91 the fish in each of the other duplicate groups were then sampled at 1, 3 and 5 days after
92 injection.

93 To determine the effects of vaccination on serum antibody levels, six groups of
94 fish (each n=10) were injected intraperitoneally (ip) on days 0 and 30 with 0.1 ml of
95 PBS, membrane antigen, or antigen and one of the following adjuvants: microspheres,

Matrix-Q, Alhydrogel or Freund's, as indicated above. The water temperature was 17ºC. One month after the second dose, the serum antibody levels were determined in immunized fish. Blood samples for antibody analysis were obtained by caudal vein puncture and were allowed to clot for 16 h at 4^{o}C. The samples were then centrifuged and the serum was collected. The serum antibody levels were determined by double indirect enzyme-linked immunosorbent assay (ELISA), as described by Piazzon et al., (2008).

2.4. Isolation of peritoneal cells

Fish were bled by caudal vein puncture to prevent the presence of erythrocytes in the peritoneal cavity. The peritoneal cavity was then washed carefully with 5 mL of cold PBS and the cells were counted with a haemocytometer. Some cell samples were also smeared onto glass slides for staining with hemacolor (Merck) or diaminobenzidine (Sigma–Aldrich) (for peroxidase activity) and counterstained with haematoxylin, according to Kiernan (1981). Finally, the cell suspensions were washed twice with cold PBS by centrifugation at 300 x g for 5 min, and the tubes containing the pellets were frozen in liquid nitrogen and held at -80 ^{o}C until RNA extraction.

2.5. RNA extractions and microarray hybridizations

The stimulated cells were pelleted, and total RNA was extracted using the RNAeasy Mini kit (Qiagen) according to the manufacturer's recommendations. All extractions were performed by the same researcher. RNA quality and quantification were evaluated respectively in a Bioanalyzer (Bonsai Technologies) and in a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc.), prior to labelling and hybridization. RNA extracted from peritoneal cells showed high quality parameters and all the samples had a RNA integrity higher than 8.9 and mostly above 9.

A 4 x 44K Agilent oligo-microarray (43,840 spots corresponding to turbot transcripts and 1417 to Agilent controls) (Ribas et al 2013) was used to evaluate the gene expression profiles under the three conditions tested. The microarray platform Agilent-038160 was submitted to the Gene Expression Omnibus (GEO) repository under accession number GPL19564.

Twelve slides were used in the experiment, and samples from three or four groups of fish were included in each slide. One of the fish groups was always a PBS-

injected group, and the others were various groups sampled at the same time as the control group. In total, 42 arrays were analysed. Hybridizations were performed by staff at the Agilent Technology Gene Expression Unit of the Functional Genomics Platform at the University of Santiago de Compostela (USC). Briefly, 50 ng of total RNA was labelled using the Low Input Quick Amp Labeling Kit, One-Color (Cy3) (Agilent Technologies, USA). Copy RNA was hybridized overnight with the corresponding buffers for 17 h at 65°C and washed on the following day. Hybridized slides were scanned in an Agilent G2565B microarray scanner (Agilent Technologies, USA). All work was carried out on consecutive days and by the same researcher. The scanner images were segmented with the Agilent Feature and Agilent software was applied to prevent saturation in the highest intensity range. Agilent Feature Extraction produced the raw data for further pre-processing. The processed signal (gProcessed-Signal) value was the parameter chosen for the absolute hybridization signal, as recommended by Millán et al. (2011).

2.6. Microarray analysis

Microarray processing and filtration were performed as previously described (Millán et al., 2010; Domínguez et al., 2013). The signal was captured and processed in an Agilent scanner (G2565B, Agilent Technologies). The scanner images were segmented with Agilent Feature Extraction Software (v9.5) by applying the GE1-v5_95 protocol, which was used for obtaining raw and processed data. The processed signal (gProcessed-Signal) value was chosen for the statistical analysis. All microarrays were subjected to quality and filtering control before statistical analysis, following the criteria outlined by Millán et al., (2010). The data presented in this publication has been deposited in the NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and is available under accession number GSE82021.

Normality of the log (lognormality) microarray signal (and gene signal) was assumed, and $\log_2$ transformation of the absolute signal or of the ratio treatment/control was therefore used in the statistical analysis. Normalization within each microarray was carried out by the LOESS method, which assumes that most genes in microarrays are not differentially expressed relative to the control. The mean $\log_2$ ratio for each gene was obtained by averaging the values obtained for both replicates per microarray. Two criteria were used simultaneously to identify differentially expressed genes (DEG): i)

159 genes with $\log_2$ ratios ≥ 2 or ≤ -2 for up- and downregulated genes, respectively; and
160 ii) genes that deviated from the null hypothesis (mean $\log_2$ ratio = 0) (t-test at $P < 0.05$,
161 after Bonferroni correction).
162 For comparison of the responses between groups, and to group closely related
163 samples, the groups were clustered using an Agglomerative Hierarchy Method,
164 implemented with the *Cluster* package in R statistical software. The results of
165 hierarchical clustering are presented in a dendrogram. We compared the overall
166 response for all groups, as well as the responses based on the biological functions of
167 each gene (Gene Ontology: GO). We used the Blast2GO software (Conesa et al. 2005)
168 and the AmiGO website http://amigo.geneontology.org/amigo/landing to analyse and
169 study the enriched Gene Ontology (GO) terms in the genes that were differentially
170 expressed between groups. Groups of DEG involved in a biological function were
171 performed for hierarchical cluster, TreeView analyses and generation of heat maps
172 using MultiExperiment Viewer (MeV) 4.9 software. KEGG pathway enrichment was
173 assessed by DAVID Bioinformatics Database (Huang et al., 2009) with *Danio rerio* as
174 background ($P<0.05$). KEGG pathway analysis was performed to determine the
175 involvement of DEG in different biological pathways. The analysis was carried out for
176 all the groups of fish used and for each vaccinated group.

177

178 *2.7. Q-PCR microarray validation*

179 To validate microarray results by Q-PCR, we used a modified version of the
180 random stratified procedure proposed by Mirón et al., (2006), as described by Millán et
181 al., (2011). A set of 16 genes covering the range of variation in fold change (FC) across
182 the different experimental conditions was selected (between -5.06 for C-C motif
183 chemokine 20 in Matrix-Q at day one and 8.49 for claudin 1 in microspheres at day 5).
184 The FCs of these genes in the 24 microarrays were ordered by FC value (16 genes × 42
185 microarrays = 672 cases) and were stratified considering both the width and the
186 abundance of values within each stratum. The gene-specific primers used in Q-PCR
187 assays are shown in Table S1. Sets of FCs were selected at random within each stratum,
188 completing 39 cases out of the 672 possible. Elongation factor 1-alpha was selected
189 amongst the non-regulated genes and was used as housekeeping gene for Q-PCR
190 analysis. The same RNA samples used for microarray hybridizations were used for Q-

PCR. RNA (1 μg) was reverse transcribed into cDNA by using the AffinityScript Multiple Temperature cDNA Synthesis kit according to the supplier's protocol (Agilent Technologies). The Q-PCR analysis was carried out as previously described (Millán et al., 2011). For each gene, the fold change in transcript level was determined by the relative quantitative method (ΔΔCt) (Livak and Schmittgen, 2001).

2.8. Statistical analysis

The results obtained in ELISA (serum antibody levels) were compared by analysis of variance (ANOVA) followed by Duncan's test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Cell dynamics in the peritoneal cavity after vaccination

The four adjuvants induced migration of cells to the peritoneal cavity, with the number increasing on day 1 and then decreasing on days 3 and 5 (Fig. 1). The largest number of peritoneal free cells was found in groups of fish injected with vaccines containing Alhydrogel, FCA or microspheres, while Matrix-Q induced lower cell migration. The number of cells generated by injection of PBS or antigen alone was very low, although the latter induced a higher response. The variations in cell number with time are caused by cell migration to the peritoneal cavity as well as by attachment of free peritoneal cells to the peritoneal wall and migration to lymphomyeloid organs. Attachment of free peritoneal cells, particularly phagocytes, to the visceral and parietal peritoneum and migration of these cells to internal organs occurred very soon after injection, as they were detected after six hours in the spleen and kidney (Folgueira et al., 2015).

3.2. Microarray validation by Q-PCR

A total of 39 cases from 16 randomly selected genes covering most of the FC range in the study were used to validate the microarray data (Table S1). The number of cases analyzed per gene in different microarrays ranged from 2 to 4 due to the random selection approach used. All microarrays were validated using at least 4 genes, up to a maximum of 6 genes with an average of 5 genes/microarray. Elongation factor 1-alpha, the selected housekeeping gene, showed very low variation across the experimental

conditions tested (CV: 0.026). We found a high correlation between Q-PCR and microarray expression values (Pearson ρ coefficient = 0.78; P=0), indicating the consistency of our microarray data, as found in previous studies in turbot (Millán et al., 2010; 2011; Pardo et al., 2012).

3.3. Analysis of differentially expressed genes

In previous studies, we found that the total number of free peritoneal cells in turbot peaked very early after injection of particulate material and that by day 3 most of the cells with phagocytosed material were already attached to the peritoneum or migrated to other body areas (Noia et al., 2014; Folgueira et al., 2015). In the present study, we analyzed this early response (1, 3 and 5 days) in the peritoneal cells by measuring the changes in gene expression. We identified 374 genes that were differentially expressed across all groups of fish injected with vaccine (antigen and adjuvant) or with antigen alone and at the three times selected, relative to PBS injected fish (Fig. S2). Of these genes, 232 were upregulated, 133 were downregulated, and 12 were up- or downregulated, depending on the group or the time selected. The number of differentially expressed genes (DEG) varied depending on the adjuvant used: microspheres (284), Alhydrogel (255), FCA (234) and Matrix-Q (135) (Fig. 2). Only 65 DEG genes were identified in cells from fish injected with antigen alone; 12 of these were upregulated and the others were downregulated, although regulation was usually observed at only one time point.

GO terms were assigned to DEG, which were classified according to their function (Fig. 3). A large number of genes were involved in cell adhesion (between 8.9-14.7% of the total, depending on the group), actin cytoskeleton (4.2-8.1%), cell surface receptors (9.6-12.3%), cell proliferation (11.6-12.3%), immune response (5.9-10.6%), cell death (7.7-10.2) or cell migration (3.8-5.2%). In comparison, the number of DEG involved in the inflammatory response was not particularly high (3.4-4.9%). We estimated the similarity between pairs of adjuvant-treated groups by determining the Pearson's correlation coefficient ρ. We found very strong correlation between the FCA, Alhydrogel and microsphere-treated groups ($\rho>0.98$ for all the combinations). The correlation between Matrix-Q and the other adjuvants was slightly lower, although still very high ($\rho > 0.91$-0.96). KEGG pathway analysis also showed that pathways related with extracellular matrix-receptor interaction, cell adhesion molecules, focal adhesion

and regulation of actin cytoskeleton were among the top regulated ones (Fig.S3), supporting that free peritoneal cells are suffering profound changes related to cell adhesion and actin cytoskeleton.

3.4. Vaccination increased expression of genes involved in cell adhesion and actin cytoskeleton

The adjuvants tested induced differential expression in 42 genes related to cell junctions and focal adhesions and/or actin cytoskeleton organization (Fig. 4). Of these, 33 were upregulated, and the upregulation mainly occurred on day 5, except in the groups injected with microspheres, in which many of those genes were also regulated on days one and three. Claudin 1 (*CLDN1*), claudin 3A (*CLDN3A*), integrin 1-alpha (*ITGA1*), inverted formin (*INF2*), alpha-parvin (*PARVAA*), syndecan 3 (*SDC3*), cell adhesion associated oncogene regulated (*BOC*), receptor-type tyrosine-protein phosphatase kappa (*PTPRK)*, cadherin 2 (*CDH2*) and E-cadherin (*CDH1*) are some of the genes that were upregulated in peritoneal cells of all the groups of fish injected with vaccine (Fig. 4). The increased expression of genes related to cell adhesion was correlated with the formation of groups of peritoneal cells in the peritoneal cavity after vaccination, which was observed in all groups of vaccinated fish at the three times selected (Fig. 5). Claudins are transmembrane proteins that participate in tight junctions between epithelial cells, but they have been reported to participate in tight junctions in other cell types, such as dendritic cells (Rescigno et al., 2001) and macrophages (Van den Bossche et al., 2012a). By using the tight junctions, dendritic cells can pass between epithelial cells without breaking the tight junction barrier. Expression of *CLDN1* occurred in the peritoneal cells of all the groups of fish on days 1, 3 and 5, indicating continuous activation of this gene during several days. We also observed upregulation of *CDH1* on day 5. Although *CDH1* has been associated with maintenance of the epithelial barrier and regulation of epithelial cell function, it can also be express in dendritic cells, Langerhans cells, and macrophages (Van den Bossche et al., 2012b). Other genes involved in focal and adherent adhesions, such as *ITGA1*, *BOC*, *CDH2*, *PTPRK* and *NCAM*, were also upregulated on day 5 in all groups of fish injected with the vaccine, indicating that cell adhesion processes are strongly regulated in the peritoneal cells after vaccination. In previous studies, we have found that some

peritoneal cells of vaccinated fish (especially macrophage-like cells) migrated to lymphoid organs, while others formed cell-vaccine masses in the peritoneal cavity with cells attached to each other and also to the mesothelium (Noia et al., 2014; Folgueira et al., 2015). The generation of tight and adherent junctions may cause the leukocytes to interact with the mesothelium or with other cells during the inflammatory response generated in the peritoneal cavity. Some genes involved in cell adhesion were slightly downregulated, e.g. the B-cell receptor CD22-like gene. This gene, which mediates B-B cell interactions, was downregulated on days one and three in the three groups but not in the group injected with Matrix-Q, probably reflecting the scarce number of B cells in the peritoneal cavity of injected fish, relative to controls.

In addition to the genes involved in cell adhesion, we observed a high level of regulation in genes related to the actin cytoskeleton. Upregulation of several genes involved in the dynamic turnover and restructuring of the actin cytoskeleton, such as inverted formin (*INF2*), parvin alpha (*PARVA*), profilin (*PFN2*), gelsolin (GSN) and others, indicates that this process is highly stimulated in peritoneal cells after injection of the vaccine. Adhesion, polarization and migration occur in leukocytes that migrate in and out of the peritoneum of fish injected with vaccine, and these processes require rearrangement of the actin cytoskeleton. Genes such as *PARVA* link cell adhesion with the actin cytoskeleton and are involved in focal adhesions facilitating association between integrins and actin. The actin cytoskeleton has important functions in leukocyte activities, including cell signalling, adhesion, migration and phagocytosis (Rougerie et al., 2013; Schnoor, 2015), and it is also a sensor of extracellular stimuli and regulator of transcriptional activation of genes involved in other leukocyte activities (Taylor and Halene, 2015). The profound cytoskeletal changes that occurred in free peritoneal cells of stimulated immunized fish may also affect other activities related to cell activation. Apart from migration to lymphoid organs, upregulation of many genes related to cytoskeleton and cell adhesion may also be related to the formation of cell-vaccine masses in the peritoneal cavity and to the attachment of those masses to the peritoneal wall. In previous studies, we have found that all the adjuvants caused some internal adhesions, although these were more apparent for FCA and less evident for microspheres, aluminium hydroxide and Matrix-Q (Noia et al., 2014). However, we did not observe a clear relationship between the levels of gene expression induced by the adjuvants in peritoneal cells and the damage generated in the cavity. As mentioned in

the previous study, other adjuvant properties such as viscosity may have a greater influence on the size of the cell-vaccine masses and the formation of internal adhesions.

3.5. Regulation of apoptosis

Thirty-one genes related to apoptosis were differentially expressed, and six of these were upregulated in all groups, which mainly included genes that were highly expressed. The others included 16 upregulated and 9 downregulated genes in most, although not all, of the groups (Fig. 6). The upregulated genes included 24-dehydrocholesterol reductase (*DHCR24*), caspase 8 (*CASP8*), complement component 6-like (*C6*), transglutaminase 2b (*TG2*), retinoic acid receptor alpha (*RARA*), amyloid beta (A4) precursor-like protein (*APLP1*), PRKC, apoptosis, WT1, regulator (*PAWR*) and adenosine A2a receptor (*ADORA2A*); others were downregulated, including the gene coding for death-associated protein-like 1-A (*DAPL1*). Some of these genes have a proapoptotic effect but others are antiapoptotic, so that both types of signals probably occur in peritoneal leukocytes of vaccinated fish. Genes with a positive effect on apoptosis include *CASP8*, an initiator caspase involved in death receptor-mediated apoptosis stimulated by FasL, TNFα or tumour necrosis factor-related apoptosis-inducing ligand (Mocarski et al., 2011; Monie and Bryant, 2015); *TG2*, a multifunctional gene that has been implicated in apoptotic cell clearance (Nadella et al., 2015); and *RARA*, which has been associated with neutrophil differentiation and apoptosis (Kastner and Chan, 2001). Among the genes related to anti-apoptotic activity, we observed upregulation of those coding for *DHCR24*, a gene that when is overexpressed can protect the cells from apoptosis induced by oxidative stress (Lu et al., 2008), *A4*, which delays the apoptotic rate of human neutrophils (Park et al., 2006), and *ADORA2A*, which induces a delay in spontaneous apoptosis of human neutrophils (Pliyeb et al., 2014). Vaccines containing the same adjuvants as tested in the present study induced generation of numerous apoptotic neutrophils in the peritoneal cavity of turbot (Noia et al., 2014). However, migration of inflammatory neutrophils from the peritoneal cavity to lymphoid organs after injection of an inflammatory stimulus has been described in fish (Chaves-Pozo et al., 2005). The presence of apoptotic and anti-apoptotic stimuli is necessary in order to generate apoptosis in some cells and to maintain the rate of apoptosis at certain levels and to induce cell migration and release of inflammatory cytokines, as described in mammals (Hofman, 2004).

3.6. Genes associated with lymphocyte activity and lymphocyte migration are downregulated

Interestingly, many of the genes associated with lymphocytes and lymphocyte activity were downregulated in the groups injected with vaccines containing microspheres, FCA and Alhydrogel (Fig. 7). The downregulation affected a smaller number of genes in the group injected with vaccine containing Matrix-Q. Some of the downregulated genes included chemokines such as CC chemokine (C-C motif) 20/Macrophage Inflammatory Protein-3 (*CCL20*), which plays an important role in the constitutive trafficking of leukocytes, including immature dendritic cells and T and B lymphocytes (Zhao et al., 2014), and chemokine (C-C motif) ligand 4 /macrophage inflammatory protein-1β (*CCL4*), which participates in the migration of immune cells, and blockage of this chemokine reduced T-cell migration to lymph nodes after vaccination (Castellino et al., 2006). Fish studies have shown that *CCL4* attracts leukocytes, induces an inflammatory response and drives lymphocyte differentiation in the Th1 pathway (Hsu et al., 2013). Other downregulated genes included those coding for chemokine (C-C motif) receptor 7 (*CCR7*) and chemokine (C-C motif) receptor 9 (*CCR9*), both of which are expressed in lymphocytes in mammals and fish (Kim, 2005; Castro et al., 2014); transcription factor 7 (T-Cell Specific, HMG-Box) and T-cell receptor beta, which are expressed in T cells. We also observe a decrease in the expression of genes coding for proteins expressed in B cells, such as B-cell CLL/lymphoma 11Ab, immunoglobulin heavy variable 4-31, immunoglobulin gamma 2A chain, lymphocyte antigen 6 complex, locus G6F, B-cell receptor CD22, immunoglobulin mu heavy chain, and B-cell antigen receptor complex-associated protein alpha chain. These results indicate that genes related to B and T lymphocyte activity and migration were inhibited in peritoneal cells after injection of vaccine and contrast with those found in the spleen of Asian sea bass, where up-regulation of genes related to lymphocytes was observed on days one and seven after vaccination (Jiang et al., 2014). It has been shown that the peritoneal cavity of vaccinated turbot has low levels of lymphocytes in comparison with other cell types (Folgueira et al., 2015). The decrease in the production of chemokines that attract lymphocyte would decrease the lymphocyte population and because there are few lymphocytes we found very low expression of lymphocyte markers. We have also found important differences between vaccines containing Matrix-Q and the vaccines containing the other adjuvants. Matrix-Q

only induced downregulation of 4 genes (*CCR7, TCF7, CCL4, CCL20*) and at a much lower level than the other adjuvants, indicating that this adjuvant has a lower inhibitory effect on lymphocyte activity and migration than the others.

In fish injected with vaccines containing microspheres, FCA or Alhydrogel, we observed decreased expression of genes related to antigen presentation, such as MHC class II antigen alpha chain (*MHCIIa*) and gamma-interferon-inducible lysosomal thiol reductase (*GILT*) (Fig. 7). *GILT* is constitutively expressed in antigen presenting cells in mammals and catalyzes disulphide bond reduction that may be important for generation of class II epitopes (Phan et al., 2000). *MHCII* is also expressed in antigen presenting cells, such as dendritic cells, macrophages and B cells. The number of peritoneal macrophages in the cavity was high at the times used in the present and a previous study (Folgueira et al., 2015). However, these macrophages did not appear to express these genes during this early inflammatory response in the peritoneal cavity, suggesting that many of the free macrophages were $MHCII^-$. Most of the inflammatory macrophages during acute peritonitis in mice were $MHCII^-$, while all the migratory macrophages in lymph nodes were $MHCII^+$ (Gautier et al., 2013). Something similar may occur in fish, in which MHCII expression may increase during migration to lymphoid organs or in areas of cell-vaccine masses.

3.7. Regulation of the inflammatory response

Several genes that participate in the inflammatory response were differentially expressed. Some of them had a positive effect on induction of inflammation, although the majority participate in their resolution. Expression of interleukin 6 receptor (*IL6R*) increased on day one, except in the group injected with Matrix-Q (Fig. 8). *IL6* exhibits pro-inflammatory and anti-inflammatory properties, plays a central role in host defence against infection and tissue injury, and differentially regulates the expression of inflammatory chemokines including interleukin 8 (*CXCL8/IL8*) and chemokine (C-C motif) ligand 2 as well as neutrophil migration to inflamed sites. However, soluble IL-6R-mediated signalling is an important intermediary in the resolution of inflammation and supports the transition between the early predominantly neutrophilic stage of an infection and the more sustained mononuclear cell influx (Hurst et al., 2001). Very few proinflammatory cytokines were upregulated in peritoneal cells of vaccinated turbot. *CXCL8* was upregulated in the groups injected with aluminium or with matrix-Q, and

tumour necrosis factor alpha was upregulated in one group and only at one time point. The TNF receptor-associated factor 4 was upregulated on day 5 in the groups injected with FCA or with microspheres. However, several genes associated with resolution of the inflammatory response were regulated. This is the case with *CDH1*, a gene that was upregulated in all groups, which has been associated with M2 macrophage activation in mammals, inducing suppression of the inflammatory response (Van den Bossche et al., 2012b). Similarly, upregulation of *CLDN1* expression occurred in the peritoneal cells of all the groups of fish on days 1, 3 and 5, indicating continuous activation of this gene over several days. *CLDN1* expression has also been associated with TGF-β activated M2 macrophages (Van den Bossche et al., 2012a). We also observed upregulation of *ADORA2A* in the cells of all three groups injected with vaccine and annexin A1 (*ANXA1*) in the group injected with microspheres. Adenosine signalling promotes the induction of M2 activation via adenosin receptors (Ferrante et al., 2013). ANXA1 is a protein with potent anti-inflammatory activity (Perretti and D´Acquisto, 2009). Another sign of M2 polarisation is the upregulation of several scavenger receptors able to bind a diverse array of endogenous and foreign molecules (Peiser and Gordon, 2001), and we observed upregulation of scavenger receptor class B, an LDL receptor, in all groups of fish injected with vaccine and of mannose receptor in some groups. Finally, we also observed upregulation of peroxisome proliferator-activated receptor gamma (*PPARG*) also occurred in all groups on day one. The *PPARG* ligand has been shown to exert potent anti-inflammatory effects in mammals, promoting apoptosis of neutrophils and reducing macrophage inflammatory activation while enhancing phagocytosis. In addition, *PPARG* is the main member of the *PPAR* family in promoting M2 macrophages (Croasdell et al., 2015).

We also observed downregulation of genes related to antigen presentation such as *MCHIIa*, and of molecules related to antigen processing, such as *GILT* (Fig. 7). Antigen presentation is crucial for the generation of protective T-cell responses against pathogens and for later T cell-dependent activation of the B cells and for antibody production. Decreased antigen presentation and an overall suppressive response has also been associated with the M2 macrophage response (Moser, 2003), and certain cytokines characteristic of M2 response can also downregulate *MHCII* expression and antigen presentation (de Waal Malefyt et al., 1991). M2 macrophages can be further divided into several phenotypes based on gene expression profiles in mammals, and these phenotypes seem to be present in fish (Hodgkinson et al., 2015). The macrophage states

449 can be induced by several cytokines, such as IL4 or IL13, or the macrophages
450 themselves can produce certain cytokines such as IL10. Unfortunately, those cytokines
451 were not included in the microarray and therefore it was not possible to determine their
452 expression levels. It is therefore difficult to suggest which subsets of M2 macrophages
453 dominate in this response. Nonetheless, analysis of the microarray suggests that the
454 resolution of inflammatory reaction had already started at one day after injection in all
455 the groups of vaccinated turbot, and that this effect was more marked in the group
456 injected with microspheres and was less evident in the group injected with Matrix-Q.
457 Resolution of acute inflammation involves the cessation of PMN trafficking, pro-
458 inflammatory mediator catabolism, apoptosis and phagocytosis, collectively allowing
459 stromal cells and parenchymal tissues of the injured site to resume their normal
460 physiological function. Resolution of inflammation has been observed to be initiated
461 within 6 hours in mammalian models of acute self-limited inflammatory response to
462 carrageenan and zymosan (Bannenberg et al., 2005). Our results indicate that this
463 process can also be activated early on in fish.

464 *3.8. Comparison of the gene expression responses between groups*

465 We compared the different vaccinated groups, in order to relate the changes in
466 the peritoneal cells to the administration of a particular adjuvant. The overall response
467 in the groups injected with antigen alone was similar at the three times tested and was
468 also similar to the response on days 1 and 3 post injection in groups injected with
469 antigen plus Matrix-Q. The responses in the other groups were more dependent on the
470 time after infection than on the adjuvant administered (Fig. 9). On day 5, all the
471 adjuvanted vaccines generated a similar response, although the groups injected with
472 microspheres and FCA were more closely related than the groups injected with
473 Alhydrogel and Matrix-Q. Interestingly, this pattern of response was similar when
474 groups were compared in relation to genes whose expression is related to cytoskeleton
475 reorganization, cell adhesion, apoptosis, angiogenesis or cell migration (Figs. S3 and
476 S4). With the exception of Matrix-Q, the response to which was more similar to the
477 response to antigen alone on days 1 and 3, the associations found in the other groups
478 were primarily related to the time since injection and secondarily to the adjuvant used.
479 The transcriptomic response induced by antigen alone was very low compared with that
480 induced by antigen and adjuvant, and very similar to that induced by PBS, indicating

481 that the response generated is more closely related to the adjuvant used than to the
482 antigen.

483 *3.9. Comparison between gene expression in peritoneal cells and serum antibody levels*

484 In parallel with the microarray experiment, we evaluated the antibody responses
485 generated in turbot after vaccination with the same antigens and adjuvants used in the
486 gene expression experiment. The microarray analysis showed that Alhydrogel, Freund's
487 and microspheres induced a very similar cell migration and gene expression response in
488 free peritoneal cells. However, the antibody response generated by Freund's was much
489 higher than that induced by the other adjuvants (Fig. 10). Fish injected with Matrix-Q,
490 Alhydrogel or microspheres showed similar antibody levels, although the former was
491 less inflammatory. There are several possible reasons explaining these discrepancies.
492 The immune response generated by vaccines is more closely related to the events that
493 take place in the cell-vaccine masses and in the immune organs rather than in free
494 peritoneal cells. Other effects, such as the depot effect, are also probably important in
495 generating antibody production, as shown in mammals (Batista-Duharte et al., 2013).

496 *3.10. Conclusions*

497 The study findings provide new information about the early gene expression
498 responses in free peritoneal cells of vaccinated turbot. Although antigen alone had little
499 effect on cell migration and gene expression levels, in comparison with PBS injected
500 fish, the four adjuvants tested (Alhydrogel®, FCA, microspheres and Matrix-Q®)
501 strongly induced activation of genes involved in cell adhesion and actin cytoskeleton,
502 induced pro- and anti-apoptotic signals, downregulated genes related to lymphocyte
503 migration and activity and also increased the expression of a number of genes involved
504 in resolution of the inflammatory response. Matrix-Q induced lower cell migration and
505 gene expression levels than the other three adjuvants, which generated very similar
506 responses. Interestingly, we also observed decreased expression of genes involved in
507 antigen presentation, indicating that this process does not occur in free peritoneal cells.
508 Finally, we did not observe any relationship between gene expression in peritoneal cells
509 and serum antibody levels in vaccinated fish. The latter is probably more closely related
510 to the events occurring in the cell-vaccine masses generated in the peritoneal cavity and

511 in lymphoid organs. However, further research is necessary in order to clarify the
512 relationship.

513

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720

721 **Figures**

722

723 **Fig.1.** Number of free cells in the peritoneal cavity of fish injected with PBS,
724 antigen or with vaccines containing antigen and different adjuvants, on days 0, 1, 3 and
725 5 post injection. Results are presented as means ± S.E.M; n=6/group, with the exception
726 of the PBS and control 0h groups (n=8).

727 **Fig. 2.** Venn diagram showing the total number of upregulated (red) and
728 downregulated (green) genes at the three sampling times in the groups injected with
729 antigen and adjuvant, in comparison with PBS injected groups.

730 **Fig. 3.** Pie charts showing the proportion (%) of DEG by biological function in
731 peritoneal cells from fish injected with a vaccine containing antigen and one of the four
732 adjuvants tested. Genes can be differentially expressed at one or more sampling times.

733 **Fig. 4**. Heat map displaying hierarchical clustering of DEG involved in cell
734 adhesion and cytoskeleton. Data are from fish injected with antigen (Ant) or with
735 antigen plus adjuvant (Alhydrogel [Al], Freund's [FCA], Matrix-Q [MQ] or
736 microspheres [Mic]), on days 1, 3 and 5 after injection. Results are expressed as mean
737 expression ratio (log2- of different groups vs PBS). Red indicates increased gene
738 expression levels; green indicates decreased levels.

739 **Fig. 5**. Peritoneal cells of fish vaccinated with antigen and Freund's adjuvant at
740 1 (A), 3 (B) and 5 (C) days after injection. Groups of neutrophils (N) or neutrophils and
741 macrophage-like cells (M) were observed in the peritoneal cavity after injection. Some
742 cells are joined by cytoplasmic extensions (blue arrow). A, Hemacolor; B, C.
743 Peroxidase staining. Bar, 10 µm.

744 **Fig. 6**. Heat map displaying hierarchical clustering of DEG involved in
745 apoptosis. Data are from fish injected with antigen (Ant), or with antigen plus adjuvant
746 (Alhydrogel [Al], Freund's [FCA], Matrix-Q [MQ] or microspheres [Mic]), on days 1,
747 3 and 5 after injection. Results are expressed as mean expression ratio (log2- of
748 different groups vs PBS). Different colours indicate upregulation (red) or
749 downregulation (green).

750 **Fig. 7**. Heat map displaying hierarchical clustering of DEG related to
751 lymphocyte migration and lymphocyte activity. Data are from fish injected with antigen
752 (Ant), or with antigen plus adjuvant (Alhydrogel [Al], Freund's [FCA], Matrix-Q [MQ]
753 or microspheres [Mic]), on days 1, 3 and 5 after injection. Results are expressed as

754 mean expression ratio (log2- of different groups vs PBS). Different colours indicate
755 upregulation (red) or downregulation (green).
756 **Fig. 8**. Heat map displaying hierarchical clustering of DEG related to the
757 inflammatory response. Data are from fish injected with antigen (Ant), or with antigen
758 plus adjuvant (Alhydrogel [Al], Freund's [FCA], Matrix-Q [MQ] or microspheres
759 [Mic]), on days 1, 3 and 5 after injection. Results are expressed as mean expression
760 ratio (log2- of different groups vs PBS). Different colours indicate upregulation (red) or
761 downregulation (green).
762 **Fig. 9.** Dendrogram showing the cluster hierarchy for fish injected with antigen
763 or antigen plus adjuvant, taking into account all the DEG. Antigen (ANTIG), Matrix-Q
764 (MAT-Q), Freund's adjuvant (FCA), microspheres (MICROS), Alhydrogel (ALUM);
765 on days 1, 3 and 5 post injection.
766 **Fig. 10**. Serum antibody levels, determined by ELISA, generated by turbot
767 injected with PBS, *Philasterides dicentrarchi* membrane antigen (Ant) or antigen plus
768 microspheres (Mic), Alhydrogel (AL), Matrix-Q (MQ) or Freund's adjuvant (FCA).
769 The results are expressed in chemiluminiscence units and values shown are means ±
770 standard error (n=10 fish per group). Groups indicated by different letters (a–d) differ
771 significantly ($p < 0.05$).
772
773 **Supplementary figures**
774 **Fig. S1**. Silver-stained SDS-PAGE gel of *P. dicentrarchi* membrane proteins run
775 under non-reducing conditions. Lane M, molecular markers in kDa.
776 **Fig. S2.** Heat map displaying hierarchical clustering of all DEG. Data are from
777 fish injected with antigen (Ant), or with antigen plus adjuvant (Alhydrogel [Al],
778 Freund's [FCA], Matrix-Q [MQ] or microspheres [Mic]), on days 1, 3 and 5 after
779 injection. Results are expressed as mean expression ratio (log2- of different groups vs
780 PBS). Different colours indicate upregulation (red) or downregulation (green).
781 **Fig. S3**. List of pathways with DEG based on Kyoto Encyclopedia of Genes and
782 Genomes (KEGG) analysis. Results are shown for DEG in all groups of vaccinated fish.
783 **Fig. S4.** Dendrogram showing the cluster hierarchy for fish injected with antigen
784 or antigen and adjuvant, taking into account all DEG related to cytoskeleton
785 organization. Antigen (ANTIG), Matrix-Q (MAT-Q), Freund's adjuvant (FCA),
786 microspheres (MICROS), Alhydrogel (ALUM), on days 1, 3 and 5.

Fig. S5. Dendrogram showing the cluster hierarchy for fish injected with antigen or antigen and adjuvant, taking into account all DEG related to apoptosis. Antigen (ANTIG), Matrix-Q (MAT-Q), Freund's adjuvant (FCA), microspheres (MICROS), Alhydrogel (ALUM), on days 1, 3 and 5 post injection.

Supplementary table

Table S1. Gene-specific primers used in Q-PCR assays.

Figure 1

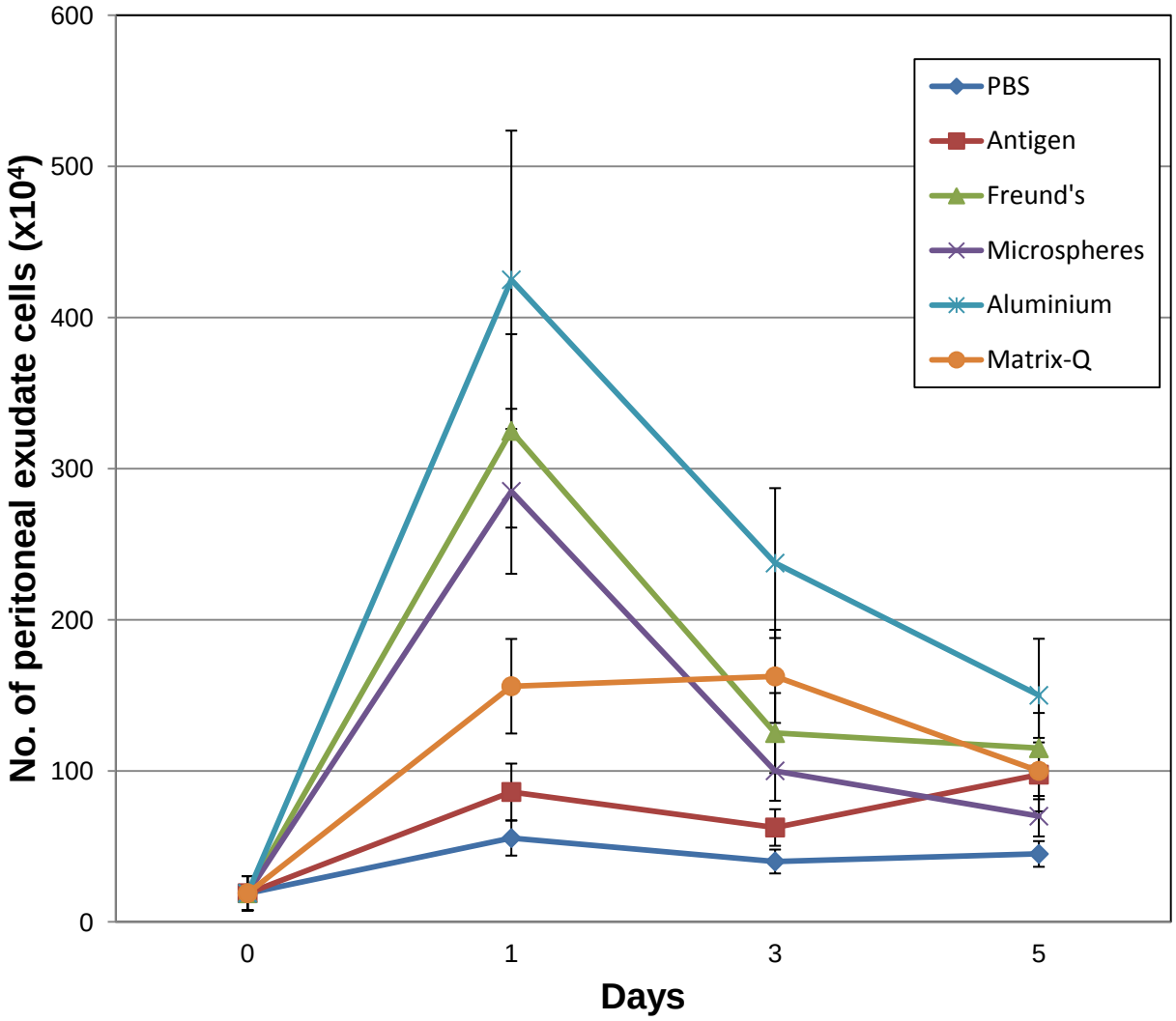

Fig. 1

Figure 2

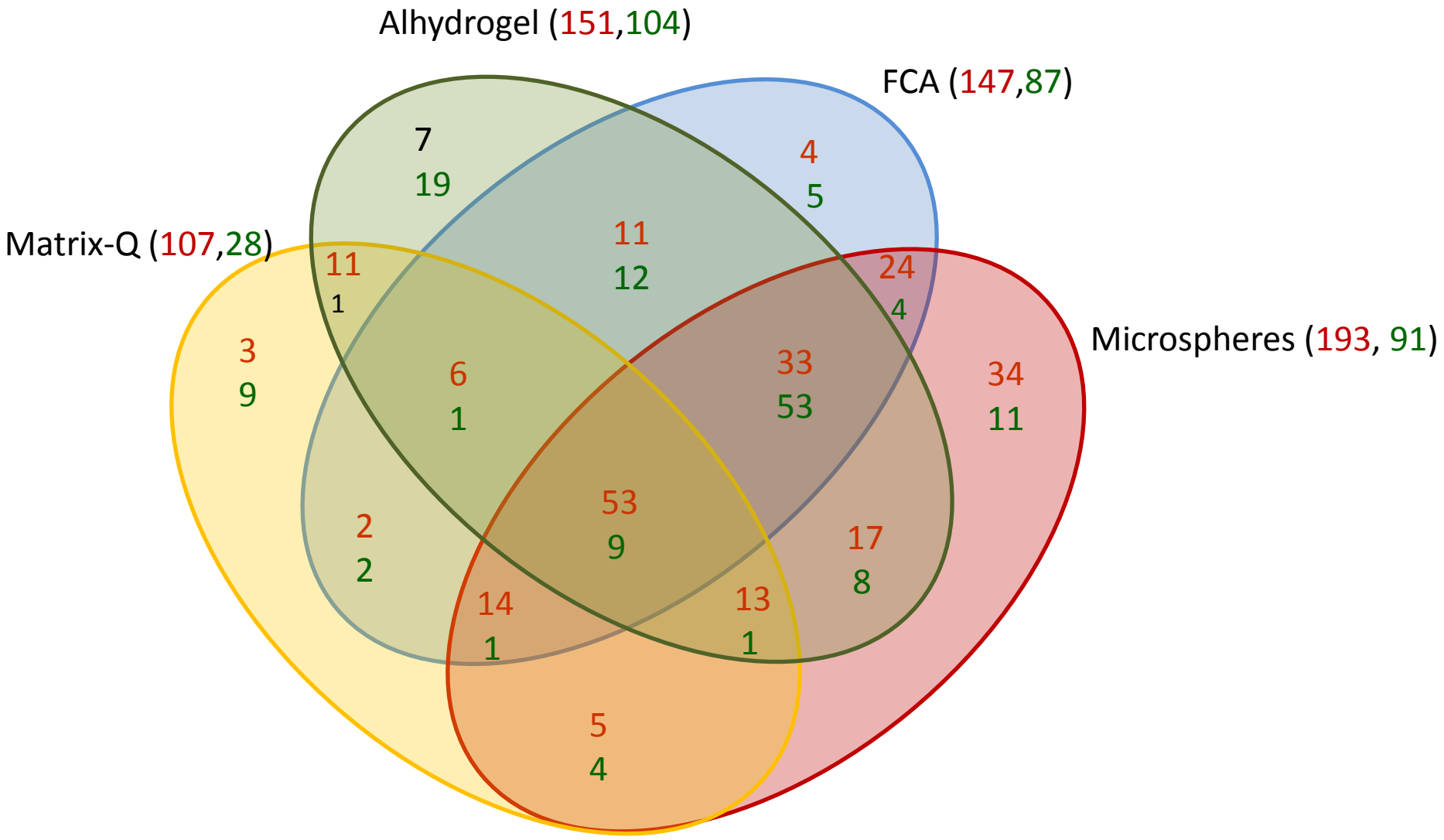

Fig. 2

Figure 3

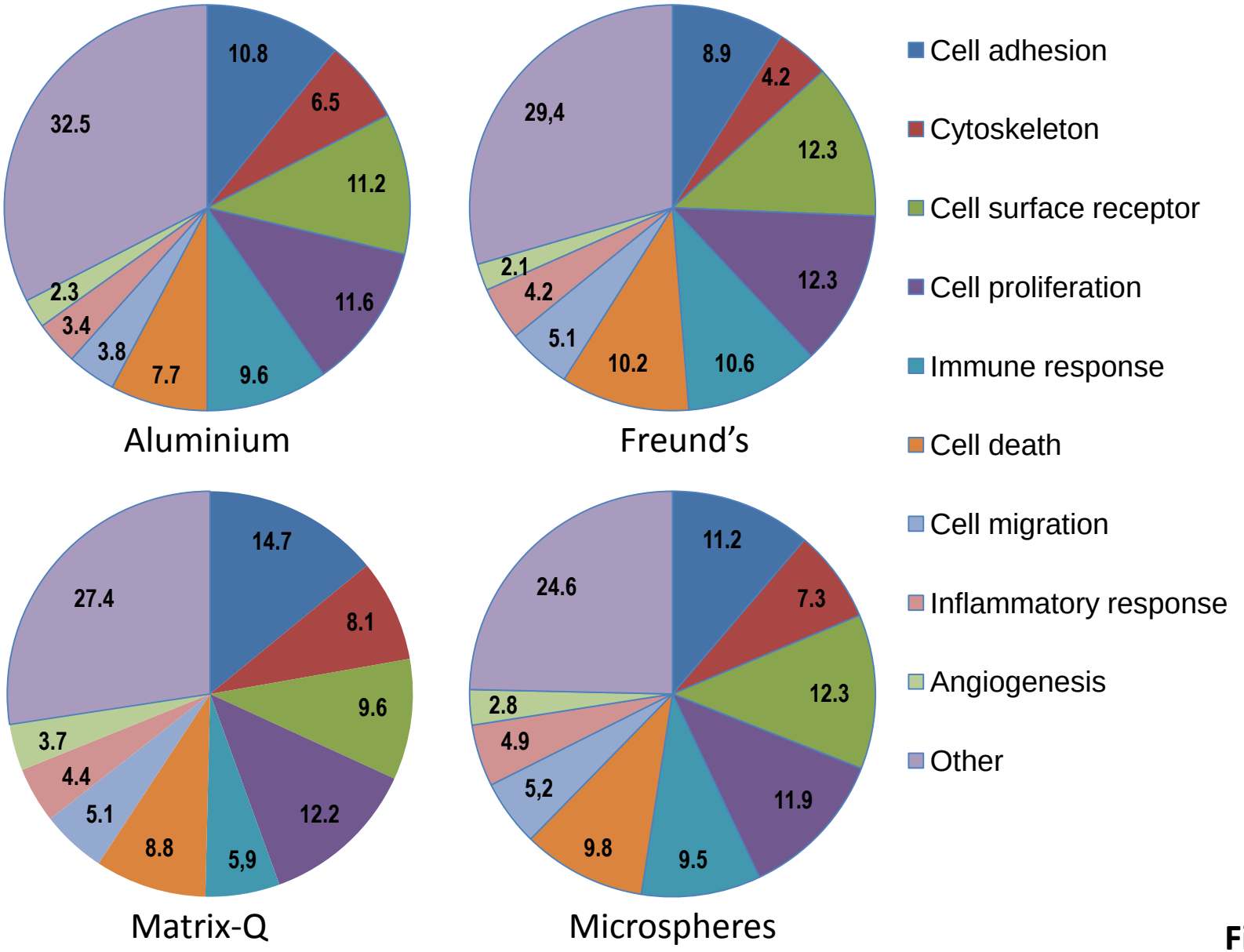

Fig. 3

Figure 4

Click here to download high resolution image

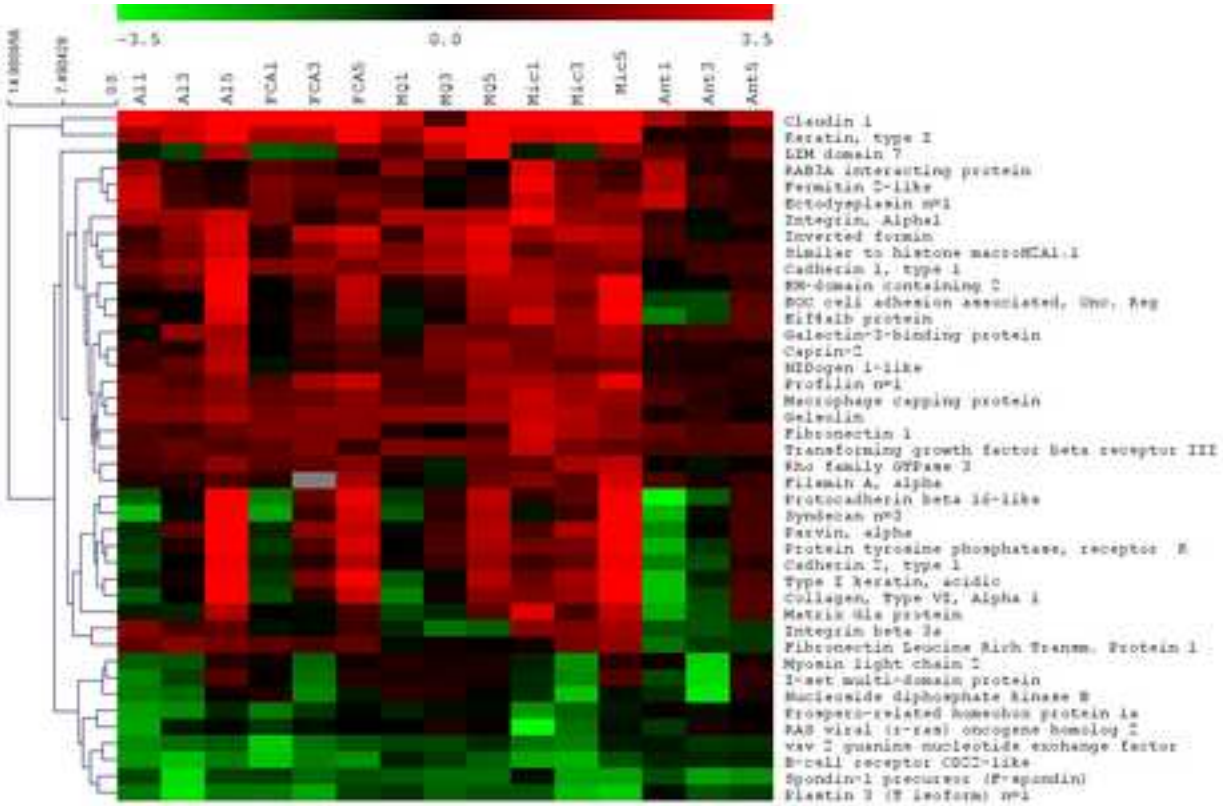

Figure 5

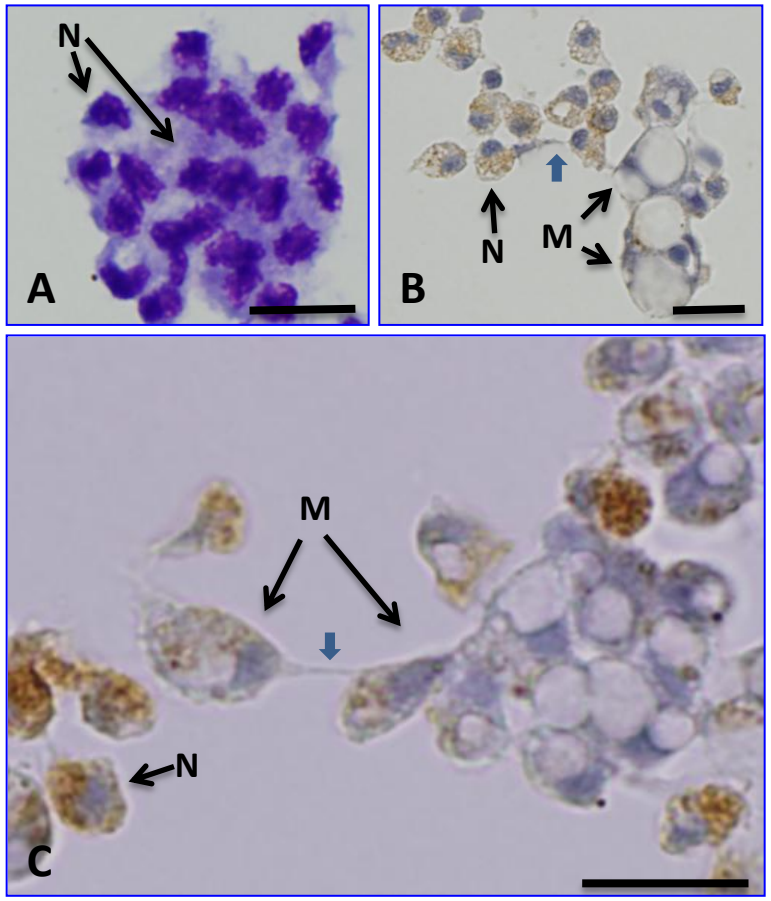

Fig. 5

Figure 6

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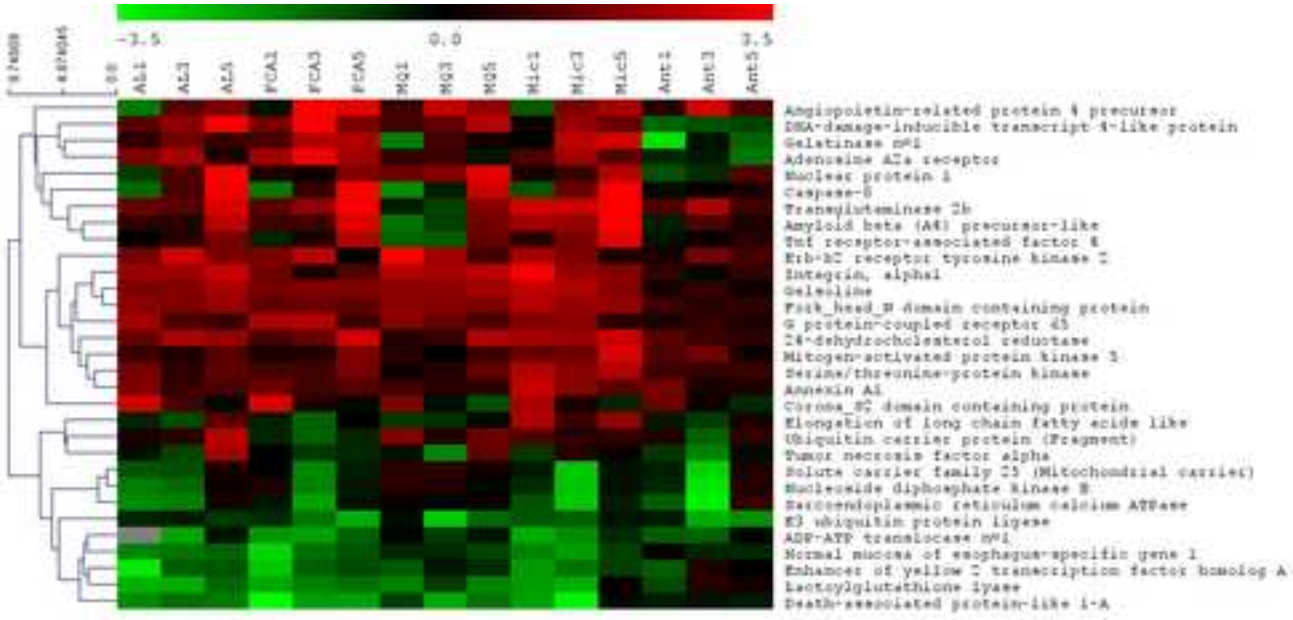

Figure 7

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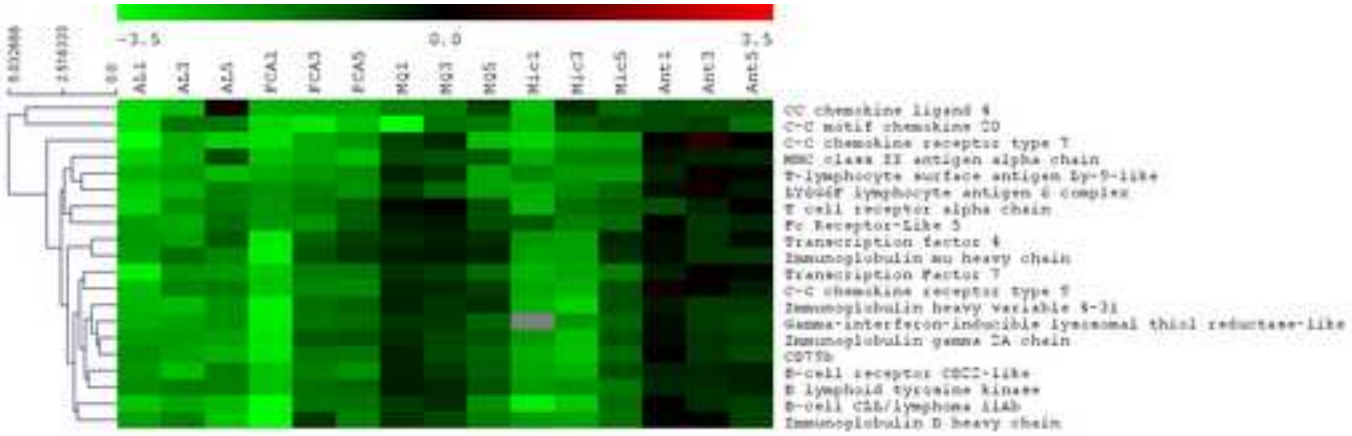

Figure 8

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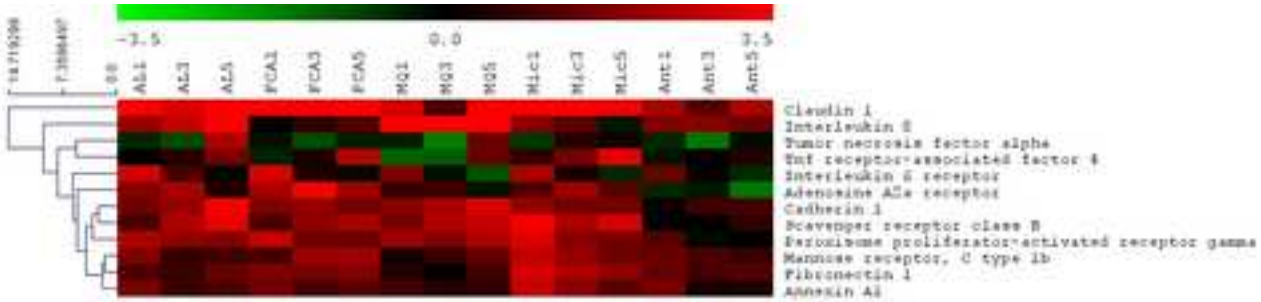


Figure 9

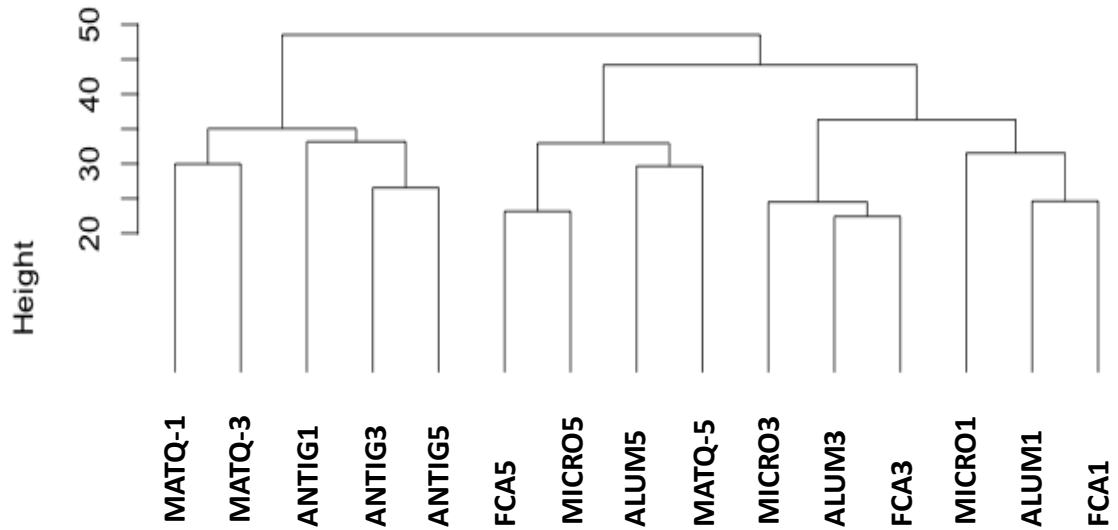
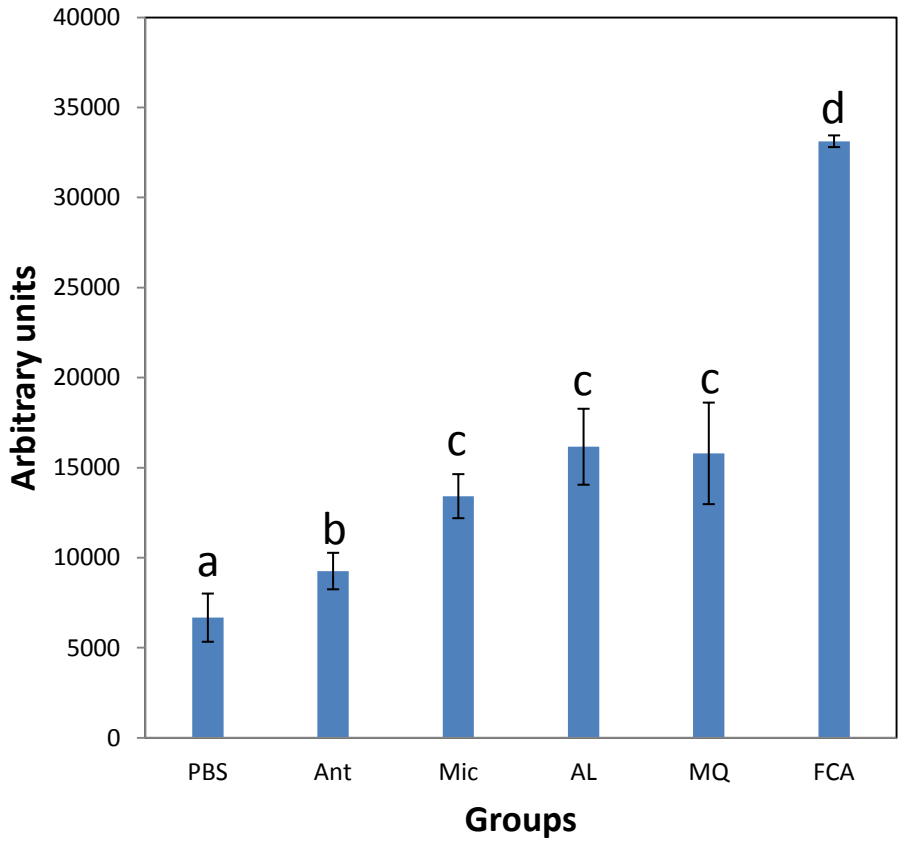


Fig. 9

Figure 10


40000
35000
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25000
20000
15000
10000
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0
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b
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d
PBS
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Mic
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MQ
FCA
Groups