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Transcriptomic analysis of turbot (*Scophthalmus maximus*) treated with zymosan a reveals that lncRNAs and inflammation-related genes mediate the protection conferred against *Aeromonas salmonicida*

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ABSTRACT

Aeromonas salmonicida is one of the most harmful pathogens in finfish aquaculture worldwide. Immunostimulants such as β-glucans are used to enhance the immunity of cultured fish. However, their effects on fish physiology are not completely understood. In the present work, we evaluated the effect of a single intraperitoneal (ip) injection of zymosan A on fish survival against *A. salmonicida* infection. A single administration of this compound protected fish against *A. salmonicida* challenge and reduce the bacterial load in the head kidney one week after its administration. Transcriptome analyses of head kidney samples revealed several molecular mechanisms involved in the protection conferred by zymosan A and their regulation by long noncoding RNAs. The transcriptome profile of turbot exposed only to zymosan A was practically unaltered one week after ip injection. However, the administration of this immunostimulant induced significant transcriptomic changes once the fish were in contact with the bacteria and increased the survival of the infected turbot. Our results suggest that the restraint of the infection-induced inflammatory response, the management of apoptotic cell death, cell plasticity and cellular processes involving cytoskeleton dynamics support the protective effects of zymosan A. All this information provides insights on the cellular and molecular mechanisms involved in the protective effects of this widely used immunostimulant.

1. Introduction

Aquaculture is humans' leading source of aquatic food and substantially contributes to global food production. The world's demand for aquatic food is estimated to increase by approximately 13% in the coming years, reaching 204 million tons by 2030 [1]. In this context, aquaculture will cover this demand, taking into consideration that the contribution of capture fisheries to global food supplies is unlikely to experience a significant growth [1]. One of the consistent problems in aquaculture is the management of fish health. Increased fish density results in outbreaks of diseases induced by viruses, bacteria, parasites, and fungi [2]. One of the most important diseases is furunculosis caused by the psychrophilic *Aeromonas salmonicida* subsp. *Salmonicida* [3]. Furunculosis is found in aquaculture worldwide and affects economically relevant fish species such as salmonids (trout and salmon) (*Salvelinus fontinalis, Salmo trutta, Oncorhynchus tshawytscha, O. kisutch* and *O. keta*), sea bass (*Dicentrarchus labrax)* and sea bream (*Sparus aurata*), Atlantic cod (*Gadus morhua*), Senegalese sole (*Solea senegalensis*) (reviewed in [4]) and turbot (*Scophthalmus maximus*) [5]. Fish affected by furunculosis show lethargy, darkened skin, and ulcers on the skin and musculature. The infection in juvenile fish results in rapid septicaemia accompanied by high mortality rates reaching approximately 80% of the stock [3].

Fish affected by bacterial diseases are traditionally treated with antibiotics. However, their use in aquaculture is being limited and often prohibited. Its use contributes to the rise of antimicrobial resistance and water and sediments pollution with harmful effects for animal, human, and ecosystem health [6]. Therefore, alternative strategies are pursued to control bacterial infections, including vaccination and the use of immunostimulants, probiotics and prebiotics [7,8].

Currently, β-glucans extracted from bacteria, algae, fungi and plantae are extensively used in aquaculture because of their immunomodulatory properties (reviewed in [9,10]). Dietary or immersion routes of administration are frequently used since they induce protection against

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bacterial infection and resistance to environmental stress [11–14]. Although the benefits depend on, among others, the molecular structure and source, dose, the duration of the treatment and the route of administration, the effects of β-glucans on the immune system of fish seems beneficial [10–12,15,16]. β-Glucans induce the activation of macrophages after recognition by specific receptors such as *C*-type lectin and promote their phagocytic activity and the reactive oxygen species (ROS) production. The chemotactic response, migration, secretion of interferons and the upregulation of proinflammatory genes (e.g. IL-1, IL-6, TNFa, COX-2) and complement system are also induced (reviewed in [9,10,17–20]). The administration of β-glucans in fish can induce a relatively long-lasting and nonspecific resistance to diseases called the "trained innate immune response" [21]. Trained innate immunity is characterized by the independence of the T- and B-cell activation, the induction of epigenetic changes and the alteration of the metabolic profile. However, Mu et al. [22] analysed the expression abundance of trained immunity related genes at single-cell resolution in turbot (*Scophthalmus maximus*) and revealed that neutrophil displays cardinal features of trained immunity. The administration of glucans by intraperitoneal injection (ip) induces pronounced effects on the fish immune system by diffusing into the nearby immune organs and stimulating immune cells in blood circulation [23].

Transcriptomic analyses have been mainly applied to understand the effect of β-glucans when they are included in the diet of aquatic animals. Qiao et al. [24] described that the introduction of β-glucans in white shrimp (*Litopenaeus vannamei*) for 32 days upregulated the expression of nonspecific immune genes and osmoregulation genes and activated pathways associated with carbohydrate metabolism. In fish, Ji et al. [25] suggested that the protection against *A. salmonicida* in rainbow trout fed for 42 days with β-glucans could be associated with the activation of the complement system, TLRs, the NF-κB signalling pathway and lipid metabolism. Cell cultures have also been used to identify key molecular pathways that are modulated by glucans by transcriptomic analysis. Petit et al. [26] using head kidney macrophages from European common carp (*Cyprinus carpio*) described the activation of the *C*-type lectin signalling pathway as well as the up regulation of several immune related processes such as cytokine-cytokine receptor interactions, apoptosis, NOD-like receptor signalling pathway, and ECM–receptor interaction. The rainbow trout spleen macrophage-like 11 cell line (RTS11 cell line) was also used and responded to the β-glucans [18,20]. Stimulated macrophages can trigger a distinct proinflammatory transcriptomic profile including several key innate immune cytokine genes (IL-1b, IL-8, TNF-a, and serum amyloid A).

Long noncoding RNAs (lncRNAs) are considered important regulators of gene expression in a wide variety of biological processes. They act by binding to the promoter of target genes, by inhibiting RNA polymerase, or by degrading target mRNAs [27]. LncRNAs have been identified in several teleost fish and are able to regulate the expression of immune-related genes during bacterial infections [28,29]. In particular, lncRNAs have been associated with the modulation of the p53, mTOR and Toll-like receptor (TLR) signalling pathways and the regulation of key immune-related genes in Atlantic salmon after *A. salmonicida* infection [30]. Gao et al. [31] reported the participation of lncRNAs in other response processes to this bacterium, such as sugar metabolism, cell adhesion, and neuroactive ligand–receptor interaction, in the black rockfish (*Sebastes schlegelii*). Similar processes were regulated by lncRNAs in turbot (*S. maximus*) after infection with *Vibrio anguillarum* [32]. However, the participation of lncRNAs in the immune modulation induced by zymosan after bacterial infection has yet to be analysed.

In the present work, we used zymosan A from *Saccharomyces cerevisiae* as a source of β-glucan. We studied how a single dose of zymosan protects turbot (*Scophthalmus maximus*) against an *A. salmonicida* infection and evaluated its transcriptomic effect in the protection against infection. Moreover, we explored how lncRNAs participate in the modulation of this protective response. It is important to remind that although zymosan A is mainly constituted by β-glucans, it also contains a variable proportions other molecules such as lipids, proteins, and chitins that can cause a distinct effect of zymosan from purified β-glucans in cell cultures [33]. However *in vivo* experiments in fish revealed that the injection of zymosan A conferred similar and even higher protection than induced by purified β-glucans against bacterial infection [11]. Overall our results help to understand how zymosan modulates cellular and molecular mechanisms involved in the protective effect against bacterial pathogens and complement information obtained with purified β-glucans.

2. Materials and Methods

2.1. Animals

Juvenile turbot (2.5 ± 0.3 g mean) were obtained from a commercial fish farm (Nueva Pescanova S.L., Galicia, Spain). Animals were maintained in 500 L tanks and acclimatized to laboratory conditions for 2 weeks (35 g/L salinity and a 12 L:12 D photoperiod). No clinical signs of diseases were observed during this period. Fish were maintained at 15.5 ± 2 °C with aeration and fed daily with commercial pellet food (LARVIVA-BioMar).

2.2. Bacteria and reagents

The *Aeromonas salmonicida* subsp. *Salmonicida* (strain VT 45.1, kindly provided by Dr ML Lemos; University of Santiago de Compostela, Spain) was used for experimental infections. The bacterium was grown on tryptic soy agar (TSA) plates at 22 °C for 24 h, and a suspension containing 5.3×10^7 colony-forming units (CFUs)/mL was prepared in phosphate-buffered saline (PBS) immediately before inoculation. Zymosan A, as a source of beta 1, 3 glucan, (Sigma–Aldrich, #Z4250) at a final concentration of 5 mg/mL prepared in sterile PBS was used to stimulate the fish.

2.3. Experimental design

Fish were distributed in 50 L tanks (10 fish per tank) and stimulated as follows: two groups of fish were treated once by intraperitoneal (ip) injection (day 0) with 50 μL of a suspension containing 5 mg/mL zymosan A (100 mg/kg of fish), and the other two groups were inoculated with the same volume of PBS. Seven days after stimulation, one group inoculated with zymosan and one group inoculated with PBS were ip injected with 50 μL of a bacterial suspension of *A. salmonicida* containing 5.3×10^7 CFU/mL, and the other two groups were left uninfected and inoculated with 50 μL of PBS. Animals were anaesthetized with MS-222 (50 mg/L) for manipulation and injection.

The effect of zymosan on survival against a bacterial challenge was evaluated in three independent experimental infections. In each trial, two groups of 10 fish were used for each experimental condition. Mortalities were recorded for 10 days after infection. The mean and SD were calculated, and significant differences with a threshold p value < 0.05 were evaluated by Student's t-test using GraphPad Prism software (San Diego; CA; USA).

For transcriptomic analysis, animals were sacrificed with an overdose of MS-222 (500 mg/L) and sampled 24 h after the bacterial challenge. The head kidney from 9 animals in each treatment group was sampled to generate 3 pooled samples per tissue. Additionally, livers from 8 fish (per treatment) were sampled to analyse the abundance of metabolites.

2.4. Analysis of lipid metabolites

The levels of triglycerides and cholesterol were enzymatically determined in the livers extracted from a total of 32 fish (8 fish per treatment). In all determinations, the commercial kits (Spinreact,

Barcelona, Spain) were adapted to a microplate reader format. Individual tissues were homogenized by mechanical disruption in ice-cooled 6% PCA (perchloric acid, 7.5 vol) and neutralized using 1 mol/L potassium bicarbonate. The homogenates were clarified by centrifugation at 16,000×*g* for 4 min, and the supernatants were collected for analysis. The metabolite levels were measured in an iEMS reader MF (Labsystems) at 505 nm, and the concentrations were calculated by using the standards provided in the kits. ANOVA analysis with Tukey's multiple comparison tests were conducted using the GraphPad prism software.

2.5. RNA isolation, transcriptomic analysis and validation

RNA was isolated using the Maxwell 16 LEV robot, according to the instructions for the simple RNA Tissue kit (Promega, Madison, WI; USA). The concentration and purity of the isolated RNA were measured in a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), and the integrity was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A total of 12 libraries were constructed. Sequencing was performed using Illumina HiSeq™ 4000 technology at Macrogen Inc. (Seoul, Republic of Korea).

Results obtained by RNA seq were validated by qPCR using 4 selected genes (Hepcidin-1, IL-1beta, IL-17 and TNF alpha). Specific primers were designed according to qPCR restrictions (Supplementary Fig. 1A). The same RNA samples were used for RNAseq and qPCR experiments. qPCR was performed in a StepOne Plus Real-Time PCR System (Applied Biosystems) using 1 μl of cDNA in a 25-μl reaction following the Power SYBR Green qPCR Master Mix protocol (Applied Biosystems). All reactions were performed as technical triplicates, and an analysis of melting curves was performed in each reaction. The relative expression levels of the genes were normalised using the elongation factor one gene as a housekeeping gene and analysed by the Pfaffl method.

2.6. Bioinformatics approach

Transcriptomic analysis was performed using the QIAGEN CLC Genomics Workbench 21.0 (https://digitalinsights.qiagen.com). Specifically, forward and reverse reads generated by Illumina sequencing of each sample were paired. Raw reads were trimmed to remove adapter sequences and short and low-quality sequences (quality score limit 0.01 = PHRED20). The number of raw and trimmed reads is presented in Supplementary Table 1. Next, the RNA-Seq analyses were performed by mapping the trimmed reads to the reference turbot genome [34], considering maximum hits per read = 10, length fraction = 0.8 and similarity fraction = 0.8. Expression values were set as transcripts per million (TPM). A differential expression analysis test (Robinson and Smyth's Exact Test, which assumes a negative binomial distribution of the data and considers the overdispersion caused by biological variability) was used to compare expression levels in each sample and to identify the differentially expressed genes (DEGs). Transcripts with absolute fold change (FC) values > 2 and FDR (Benjamini and Hochberg's False Discovery Rate) < 0.05 were selected as DEGs. Principal component analysis (PCA) plots and heatmaps were constructed using ClustVist v2.0 and the Morpheus webpage (https://software.broadinstitute.org/morpheus). Common and exclusive DEGs were presented in Venn diagrams using Venny 2.1 software.

To perform general analyses of the biological processes modulated/altered in this transcriptome project, Gene Ontology (GO) enrichment analyses were conducted using OmicsBox v1.3.11 software (https://www.biobam.com/omicsbox). These analyses consisted of Fisher's exact tests using a p-value cut-off of 0.05.

2.7. Identification of long noncoding RNAs (lncRNAs) and their flanking genes

To identify the lncRNAs modulated in this experiment, we first performed a *de novo* assembly of the turbot transcriptome. The assembly settings were set as follows: mismatch cost = 2, deletion cost = 3, insert cost = 3, and a minimum contig length = 200 base pairs. Non annotated contigs longer than 200 bp were retrieved from the *de novo* assembly, and reads were mapped to those contigs to retain only those with an average coverage >50. For the selection of putative lncRNAs, contigs were selected after discarding every possible coding sequence using three filters: potential open reading frame (ORF) prediction, BLASTx against the turbot genome peptide database [34] and coding potential prediction with the Coding Potential Assessment Tool CPAT. RNA-Seq and differential expression analysis of the potential lncRNAs were conducted using the same methodology described above for the coding transcripts.

Moreover, the flanking coding genes of the differentially expressed lncRNAs were retrieved 10,000 bp up- and downstream of each lncRNA. To analyse the correlation between lncRNAs and their flanking genes, a Shapiro–Wilk normality test was performed to select the most appropriate correlation coefficient. Neither of the 2 variables followed a normal distribution. Therefore, we performed a Spearman's correlation analysis by using the selected 25 differentially expressed lncRNAs and their DE flanking genes.

2.8. Bacterial taxonomic profiling and correlation analysis

The taxonomic profiling consisted of mapping all queried reads against a complete database of bacterial genomes. Mapping parameters used to classify read packages in different taxonomic groups included length fraction = 0.5 and similarity fraction = 0.8, as well as a minimum seed length of 30. The software used to perform taxonomic profiling was CLC Microbial Genomics Module 21.1 (Qiagen, Hilden, Germany). The bacterial reference database included 36,027 bacterial assemblies and corresponded to the curated bacteria database included in the CLC Microbial Genomics Module. Moreover, the *Scophthalmus maximus* reference genome [34] was used to remove host-specific reads. We correlated the abundance of Aeromonas taxa with transcriptome expression to identify modulated genes linked to bacterial abundance (genes showing an R^2 value greater than 0.9).

3. Results

3.1. Zymosan a induces protection against A. salmonicida and modify the bacterial load and lipid metabolites after infection

The experimental infection of turbot with *A. salmonicida* induced a rapid increase in cumulative mortality, reaching 95% at 5 days post-infection. The treatment of fish with only one injection of zymosan A one week before the infection induced a significant reduction in cumulative mortality. Only 60% of the fish inoculated with zymosan A died at the end of the experiment. No mortalities were registered in control fish treated with PBS or zymosan An alone (Fig. 1A). The analysis of the bacterial taxonomic profiling using transcriptomic data elucidated the differences in the bacterial load after stimulation. Fish treated with zymosan A before the infection showed a lower bacterial load compared to that of untreated infected fish. The number of reads belonging to Aeromonas in the infected group was 10,303, while only 4781 reads were found in fish treated with zymosan A. The total abundance of reads from other bacterial taxonomic groups such as Cultibacterium, Prevotella and Thioclava was 98, representing a 0.65 % of all the bacterial reads. The distribution of those low represented bacterial groups did not show any significant modification after zymosan A treatment (Fig. 1B).

The production of triglycerides and cholesterol in the liver was significantly increased by infection with *A. salmonicida*. In the absence of infection, the levels of both metabolites in the zymosan A -treated fish were not significantly different from those in the control fish. Interestingly, after infection, glucan treatment significantly decreased the levels of triglycerides and cholesterol (Fig. 1C).

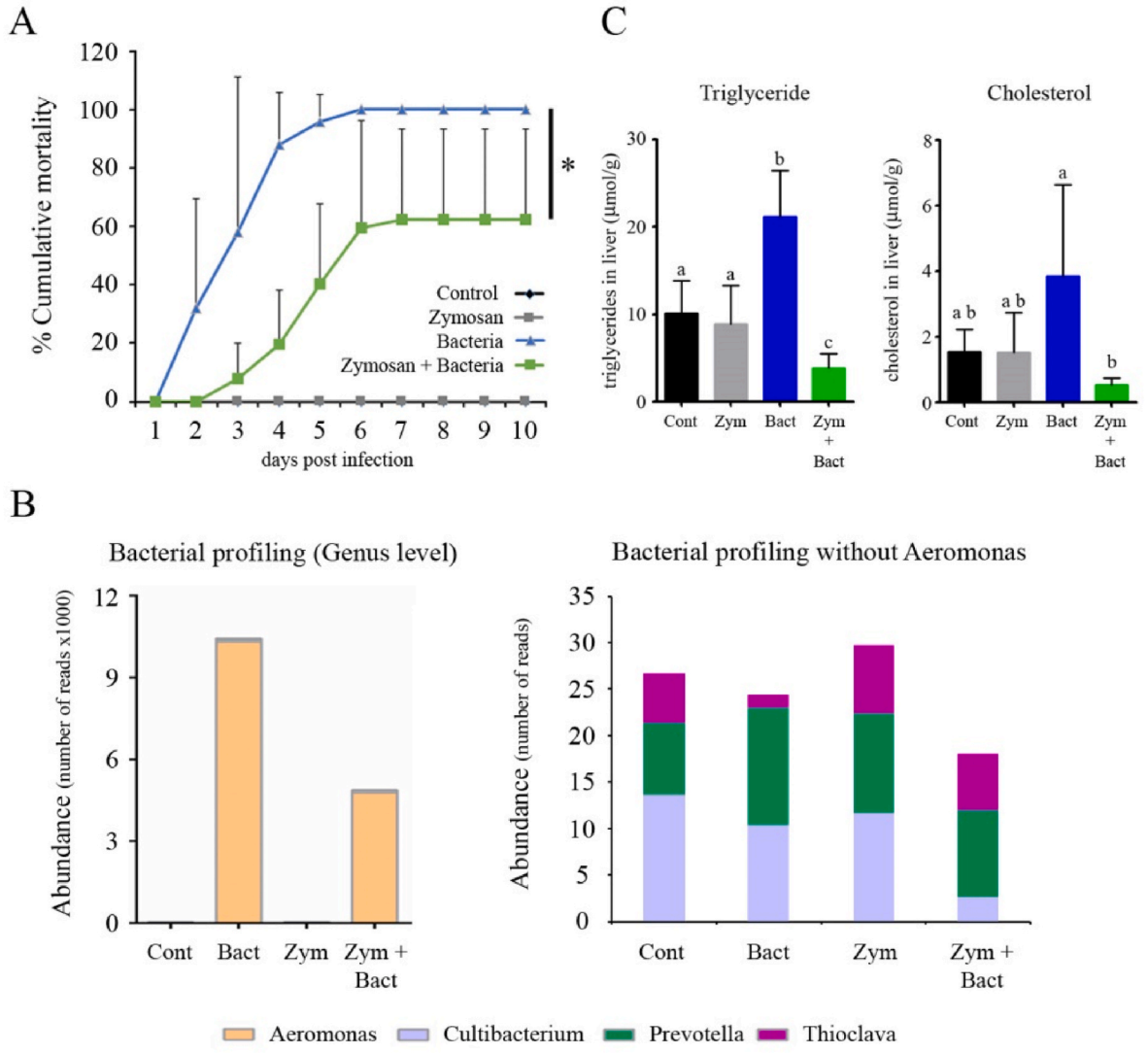


Fig. 1. Protective and metabolic effects mediated by an ip injection of zymosan A in turbot. A) Mortality rates of fish treated with zymosan A after bacterial infection. Graph represents the mean ± SD of three independent experimental infections. Asterisks (*) denote significant differences at $p < 0.05$. B) Bacterial taxonomic profiling at genus level with and without the Aeromonas group. Bar graph showing the absolute abundance of the different bacterial groups in each sample (average abundance of 3 biological replicates). C) Determination of triglyceride and cholesterol levels in the liver. Mean ± SD of tissues extracted from 8 fish. a, b, and c denote significant differences at $p < 0.05$. Groups sharing the same label did not show significant differences.

3.2. Effect of zymosan a treatment on the transcriptomic profile of fish infected with A. salmonicida

The mRNA profiles of the turbot head kidney samples were investigated by RNA-Seq analysis with a total of twelve libraries corresponding to three biological replicates for each experimental group: control group (C1-3), zymosan group (Zym1-3), bacterial group (Bact1-3) and zymosan + bacterial group (ZymBact1-3). The differential expression analyses conducted against the control group revealed that treatment with zymosan A did not induce a significant modification in the gene expression profile (only six genes were significantly upregulated, and one gene was downregulated). The significantly regulated genes and their expression values were ceruloplasmin precursor (47.04), c-type natriuretic peptide 3 (26.23), proepireguli (21.39), serotransferrin (6.58), endonuclease domain-containing 1 protein (6.17), Peptidoglycan recognition protein 2 (5.36) and a myosin-binding protein C (−4.91). (Fig. 2A and Supplementary Table 2). Fish infected with *A. salmonicida* showed the highest number of differentially expressed genes (DEGs) compared to control fish. Pretreatment with zymosan A modified the transcriptomic profile in response to bacterial infection, reducing the number of DEGs by 34% compared to the infected group (Fig. 2A). A complete list of DEGs is included in Supplementary Table 2. Results were validated by qPCR, which confirmed the patterns of up- and down-regulated expression of the four selected genes (hepcidin-1, IL-1 beta, IL-17 and TNF alpha) (Supplementary Fig. 1B).

The principal component analysis revealed that the variable "infection" (PC1) explained 29.1% of the total variation in gene expression registered in all the transcriptomic data (Fig. 2B). The gene expression profiles were divided into two well-defined groups that differed in the exposure to the bacteria (control and zymosan vs. bacteria and zymosan plus bacteria) (Fig. 2B). Those similarities and differences in the gene expression profiles between control and infected fish were also reflected in the hierarchical clustering of the DEGs (Fig. 2C). The Venn diagram revealed that 46.3% of the DEGs (1079 genes) were shared between both infected groups (with and without pretreatment with zymosan A). Infection with *A. salmonicida* induced the highest number of exclusive DEGs (972 DEGs), while 271 genes (11.6%) were exclusively expressed in fish treated with zymosan A prior to infection (Fig. 2D).

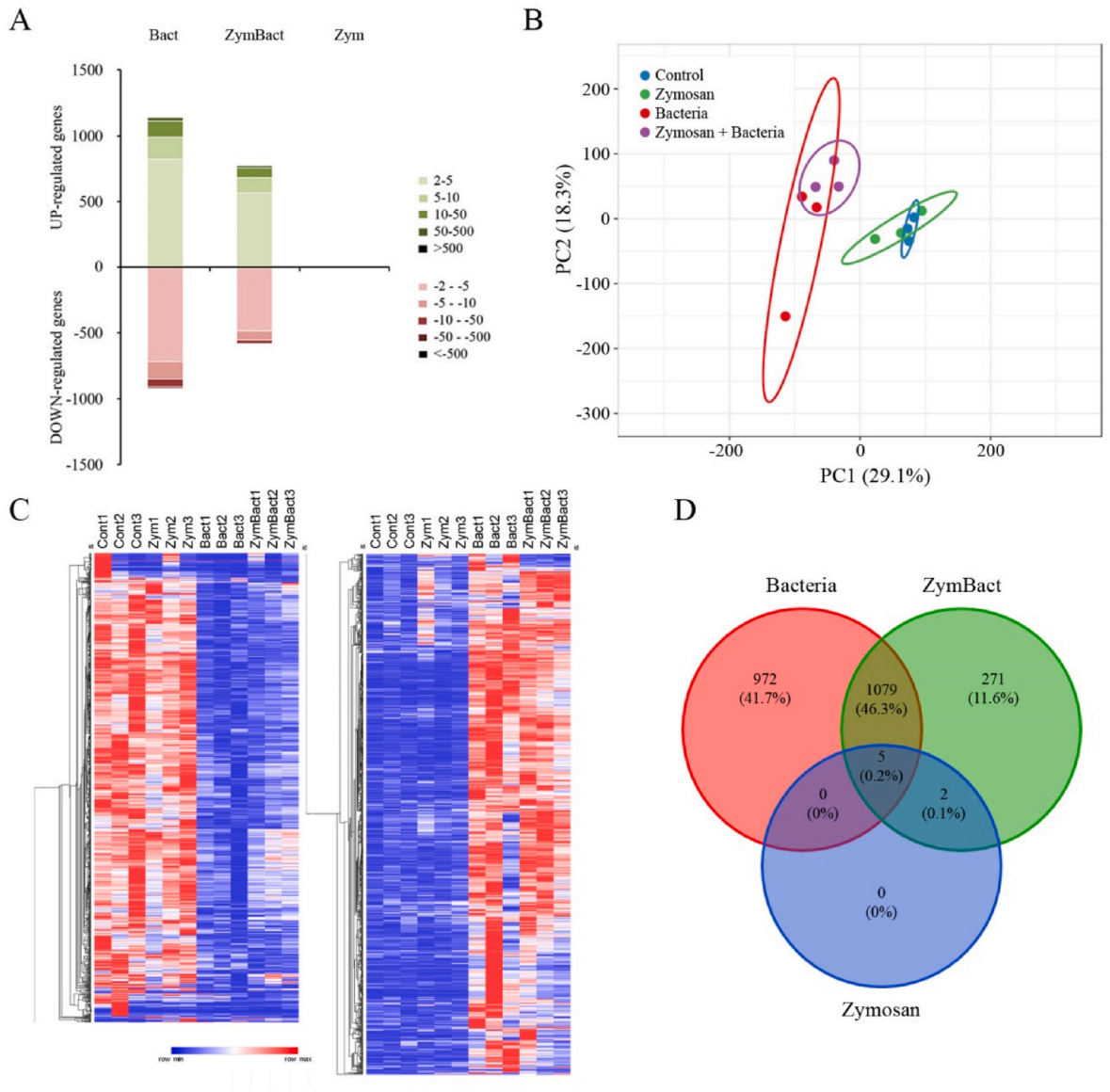


Fig. 2. Comparative transcriptome analyses of head kidney samples from turbot exposed to zymosan A or vehicle (PBS) in the absence or presence of infection. A) Distribution of the number of DEGs in fish after treatment with zymosan A and bacterial infection. The number of significantly modulated genes is subdivided according to the intensity (fold change) and direction of regulation (up or down). B) PCA using gene expression values of all genes obtained in the transcriptome. The factor "infection" explains 29% of the variation observed in gene expression. C) Hierarchical clustering of DEGs in the head kidney of fish in the different experimental groups. D) Venn diagram showing the number of exclusive and shared DEGs between the different treatments.

3.3. Zymosan a suppressed the general gene expression profile induced by the bacteria

To evaluate the effect of zymosan A on the transcriptomic response induced by bacterial infection, we first explored the DEGs shared in both infected groups (Bacteria and ZymBact). Seventy-five percent of all the common genes (702 out of 1079 genes) showed a reduction in their expression value when fish were treated with zymosan A before the infection. The enrichment analysis using these genes revealed that the top 25 biological processes affected by the zymosan A were involved in the response to hormones (oestradiol and progesterone), the activation of the immune response (interleukin-1 and TNF-mediated signalling pathways, regulation of extrinsic apoptotic signalling pathway), the recognition of pathogens (antigen processing and presentation via MHC class I, *C*-type lectin receptor signalling pathway), and the activation of signal transduction pathways (response to cAMP, regulation of canonical Wnt signalling pathway, NF-kappaB transcription factor, regulation of ERK1 and ERK2 cascades) (Fig. 3A). At the gene level, fish treated with zymosan A before infection showed downregulated expression of bacterial receptors (TLR5), proinflammatory cytokines (IL-1B, IL-12, IL-27, *C*–C and *C*-x-C chemokines, TNFR5 and TNFR6), genes related to apoptotic cell death (BH3, Bax, DNA damage-inducible protein, caspases 1, 8 and 10) and signal transduction pathways (importin A5, STAT1, STAT3) (Fig. 3B and Supplementary Table 3). The complete list of the biological processes and the DEGs is included in Supplementary Table 3.

Twenty-five percent of the shared DEGs were upregulated in fish treated with zymosan A before the infection. The enrichment analysis indicated the modulation of similar biological processes related to the immune response (regulation of cytokine production and complement activation), response to hormones (glucocorticoid, oestradiol, oestrogen and progesterone), and the activation of signal transduction pathways (regulation of ERK cascades and PI3K pathways). Interestingly, a high number of biological processes involving cytoskeletal dynamics were enriched after zymosan A treatment (regulation of cell shape, cell

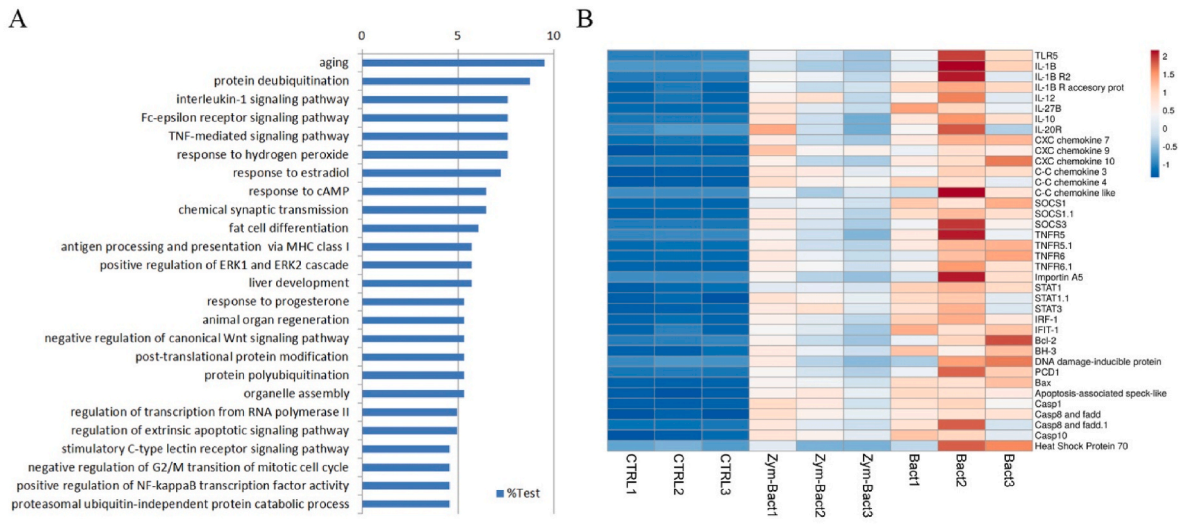


Fig. 3. Analysis of DEGs shared in infected fish with and without previous stimulation with zymosan A (Bacteria and GlucBact groups) and whose expression levels decreased after zymosan A treatment (702 genes). (A) Top 25 biological processes obtained in the enrichment analysis. (B) Hierarchical clustering of selected DEGs in the different experimental groups.

migration, neutrophil degranulation, regulation of phagocytosis, engulfment, actin filament polymerization, and response to magnesium ion) (Fig. 4A). At the gene level, several immune-related genes, such as the complement components C3 and C4 and IL-8, showed higher expression levels in samples from animals treated with zymosan A before infection. Similar upregulated expression was observed in genes involved in cellular processes affecting cytoskeletal activity, such as laminin a3 and a5, semaphorin 4E, myosin 9, calmin, connexin 42, and troponin I (Fig. 4B). The complete list of the biological processes and the DEGs is included in Supplementary Table 3.

3.4. Physiological processes exclusively modulated in the ZymBact group

The effect of the zymosan A was also evaluated in the 271 DEGs that were exclusively detected in the ZymBact group according to the Venn diagram. The treatment of fish with zymosan A prior to infection modulated genes involved in carbohydrate metabolism, such as fructose and sucrose metabolism, and the cellular response to insulin stimulus (Fig. 5A). The DEGs were also enriched in processes related to the physiological activity of the kidney (cellular response to magnesium ion, urate metabolic process) and in the cellular response to leptin stimulus (Fig. 5A).

A list of exclusive DEGs was generated using the analysis of the transcriptome by comparing both infected groups (ZymBact vs. Bact) and by using the Venn diagram. The complete list of DEGs is included in Supplementary Table 4. High expression of genes related to cytoskeletal dynamics, such as myosin, calpain, calponin and plectin, was observed. Moreover, regulatory genes of cation uptake, such as parvoalbumin, solute carriers and cell adhesion molecules, were overexpressed. The metabolism of carbohydrates and lipids was also regulated by the overexpression of the glucokinase-regulator gene and the lysosomal thioesterase PPT2 gene, respectively. Genes with secondary immune

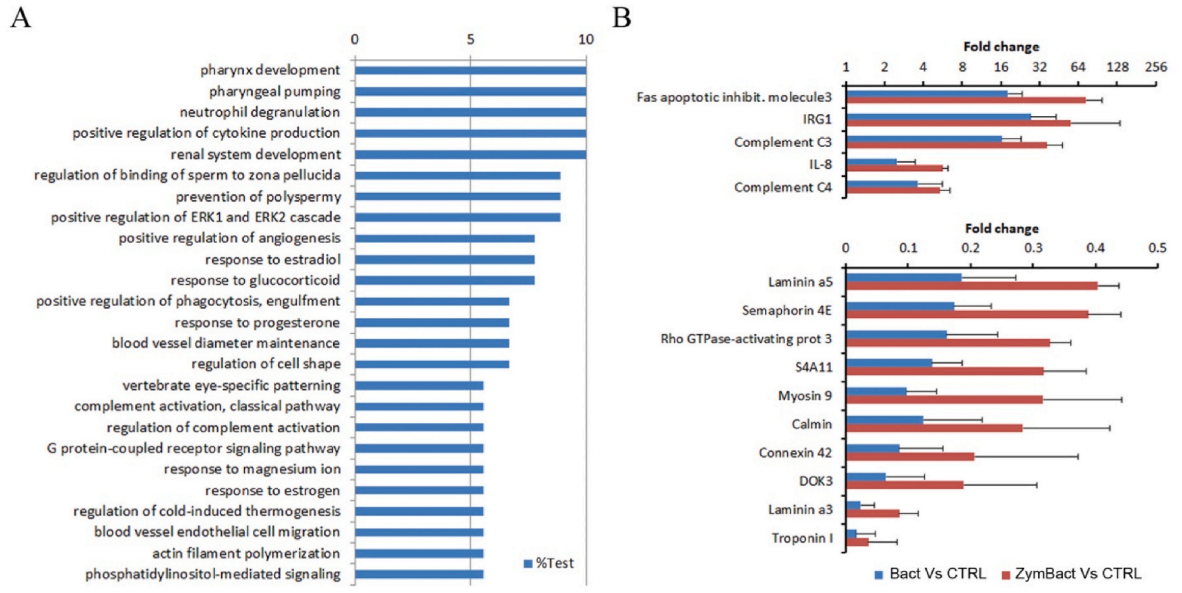
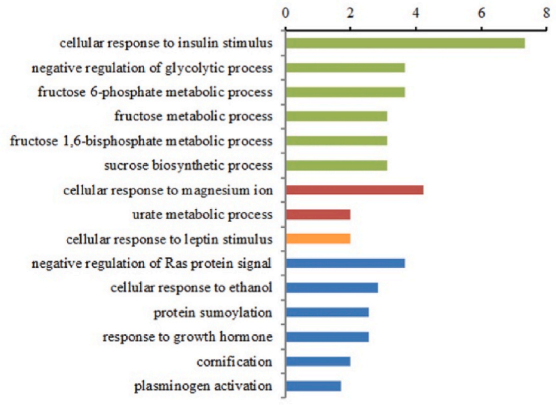


Fig. 4. Analysis of DEGs shared between the Bacteria and ZymBact groups whose expression levels increased after zymosan A treatment (377 genes). (A) Top 25 biological processes obtained in the enrichment analysis. (B) Expression levels of selected DEGs in the different experimental groups. Fold changes represent the mean ± SD of the three biological replicates.

A



B

Venn analysis		ZymBact Vs Bact	
UP modulated genes			
Annotation	**Fold change**	**Annotation**	**Fold change**
Rhodopsin kinase GRK1	18.96	Myosin 2	440.30
Toxin 3ftx-oxy5-like	15.86	Myosin fast skeletal muscle	205.33
Glucokinase regulator	9.62	Myosin fast skeletal muscle	178.79
Calpain-3	6.27	Myosin 13	178.22
L-amino-acid oxidase	5.94	Myosin fast skeletal muscle	135.17
tRNA-splicing endonuclease Sen15	5.93	Myosin fast skeletal muscle	103.13
Fibrinogen beta chain	5.47	Myosin fast skeletal muscle	83.46
Plectin	5.11	Myosin fast skeletal muscle	51.71
Ras-related protein Rab-9A	4.39	Myosin fast skeletal muscle	41.15
Cell adhesion molecule 4	4.19	Myosin fast skeletal muscle	39.26
Cell adhesion molecule regulated by oncogene	4.14	Myosin fast skeletal muscle	35.60
Lysosomal thioesterase PPT2	4.11	Myosin 7	33.22
Mucin-19	4.10	Myosin fast skeletal muscle	28.59
Latent TGF beta-binding protein	4.05	Solute carrier family 12 member 3	25.82
Calponin-1	4.01	Parvalbumin	25.58
DOWN modulated genes			
Annotation	**Fold change**	**Annotation**	**Fold change**
Fish-egg lectin	-36.17	Elastin	-8.14
Cell adhesion molecule 2	-28.05	Rieske domain-containing protein	-8.06
Synaptic vesicle glycoprotein 2B	-19.96	Xylose-NADP dehydrogenas	-7.61
Neural cell adhesion molecule L1	-15.92	Alpha-type embriyonic hemoglobin	-5.87
GDNF family receptor alpha-2	-14.90	KN motif containing protein 2	-5.73
Netrin-G2	-12.49	Granulocyte colony-stimulating factor	-5.27
Creatine kinase S	-12.48	Hemoglobin subunit beta-2	-5.18
Uncharacterized protein	-11.32	Endothelin-converting enzyme 1	-5.04
Myosin light chain kinase 4	-10.53	Heat shock protein 70	-4.94
Long-chain-fatty-acid--CoA ligase	-9.85	Hemoglobin beta chain	-4.88
NTP diphosphohydrolase 3	-8.67	Hemoglobin subunit alpha-D	-4.73
C-type lectin domain family 11	-8.56	Regulator of g-protein signaling 14	-4.66
Short transient receptor potential channel 4	-8.25	Hemoglobin subunit alpha-1	-4.58
Anoctamin-8	-7.80	Lymphocyte antigen 6g	-4.52
Voltage-dependent Ca- channel alpha-1D	-7.65	Uncharacterized protein	-4.46

Fig. 5. Comparative analysis of the exclusive DEGs between Bacteria and ZymBact groups. (A) Top 15 biological processes obtained in the enrichment analysis of the exclusive DEGs (271 genes) in fish treated with zymosan A prior to the bacterial challenge. (B). List of exclusive DEGs identified in the transcriptomic analysis by comparing both infected groups (ZymBact vs. Bact) and by using Venn diagram analysis.

functions, such as L-amino-acid oxidase, were also upregulated (Fig. 5B). Downregulated genes were also involved in similar physiological pathways. The cytoskeleton and vesicle trafficking were modulated by the low expression of genes such as cell adhesion molecule 2, myosin light chain kinase 4, and KN motif containing protein 2. The immune response seemed to be repressed, as suggested by the observed low expression of fish-egg lectin, elastin, granulocyte colony-stimulating factor, HSP70, and lymphocyte antigen 6 g. Seven downregulated genes were related to the production of ATP and the metabolism of carbohydrates and lipids: creatine kinase S, NTP diphosphohydrolase 3, Rieske domain-containing protein, xylose-NADP dehydrogenase, *C*-type lectin domain family 11, and long-chain-fatty-acid-CoA ligase (Fig. 5B). Interestingly, haemoglobin genes and genes related to the function and maintenance of the nervous system (synaptic vesicle glycoprotein 2B, neural cell adhesion molecule L1, GDNF family receptor alpha-2, netrin-G2, regulator of G protein signalling 14) were also downregulated (Fig. 5B).

3.5. Identification of lncRNAs and their flanking DEGs

To determine if the DEGs potentially involved in the protective effects of zymosan A were potentially regulated by lncRNAs, we conducted identification and differential expression analysis of the turbot lncRNA transcriptome. Potential lncRNAs were identified following the workflow previously described in the Materials and Methods section and are summarized in Fig. 6A. After the whole process, 8842 potential lncRNAs were detected. A total of 980 lncRNAs were modulated in infected fish, while only 517 potential lncRNAs were differentially expressed in fish treated with zymosan A before the infection (ZymBact group). Treatment with zymosan A by themselves did not modulate lncRNAs in the animals that were not infected (Fig. 6B). Venn analysis revealed a cluster of 123 lncRNAs that were exclusively modulated in fish treated with zymosan A prior to infection (Fig. 6C).

By mapping these 123 exclusive lncRNAs on the turbot genome, we identified and recovered the expression values (TPMs) of their neighbouring coding genes that were 10,000 bp up- and downstream of each lncRNA. A heatmap showing the TPM values of the DE lncRNAs and the DEGs located nearby them in the comparison ZymBact vs. control is shown in Fig. 6D. A strong correlation was found between the 25 lncRNAs and their neighbouring DEGs (Fig. 6D). Among these 25 lncRNAs, only one showed a negative correlation with its flanking DEG. Spearman's correlation analysis indicated a positive and significant correlation ($R = 0.86$; $p = 4.5e\text{-}9$) (Fig. 6E).

Genes that were potentially regulated by the lncRNAs were involved in six main biological processes (Table 1). A downregulation of genes related to cytoskeletal dynamics and vesicle trafficking (myosin-binding proteins, Rho GTPase-activating proteins, integrin alpha-8 gene and the dachshund homolog 1 gene) and genes related to the function of the nervous system (neurexophilin-2-like, homer protein homolog 2-like, ephrin type-b receptor 1, myelin-associated glycoprotein, px domain-containing protein 1) was found. Moreover, drug-metabolizing genes (udp glucuronosyltransferase 1 and cytochrome p450 2j2) and genes for carbohydrate metabolism (phosphorylase b kinase regulatory and glycogen debranching enzyme) also appeared to be downregulated (Table 1). Genes related to the immune response and inflammatory processes such as regulator of g-protein signalling gene, the NOD-like receptor family pyrin domain containing 6 (NLRP6), the cell surface OX2 glycoprotein (CD200) and the secretory phospholipase A2 (sPLA2) were also modulated. Genes involved in the epigenetic modification of DNA including the structural maintenance of chromosomes flexible hinge domain-containing protein gene and the histone-lysine *N*-methyltransferase set-1 gene were overexpressed (Table 1).

3.6. Analysis of the correlation between bacterial load and gene expression

An alternative method to analyse the effect of zymosan A on the transcriptomic response against Aeromonas was performed by correlating the bacterial load with gene expression. A total of 1654 genes showing expression values correlated with the abundance of Aeromonas in each sample were identified (Table 2, Supplementary Table 5).

The genes with expression correlated with the bacterial load showed biological functions similar to those previously described in Fig. 3 and Supplementary Table 2 identified by using the classical transcriptomic approach. The positively correlated genes were involved in the immune response against bacterial infection, such as receptors, regulatory molecules and effector genes. Moreover, apoptosis-related genes (BCL-2, dna damage-inducible transcript, tnfaip3-interacting protein 2) were similarly correlated (Table 2). In contrast, most of the 30 top genes that

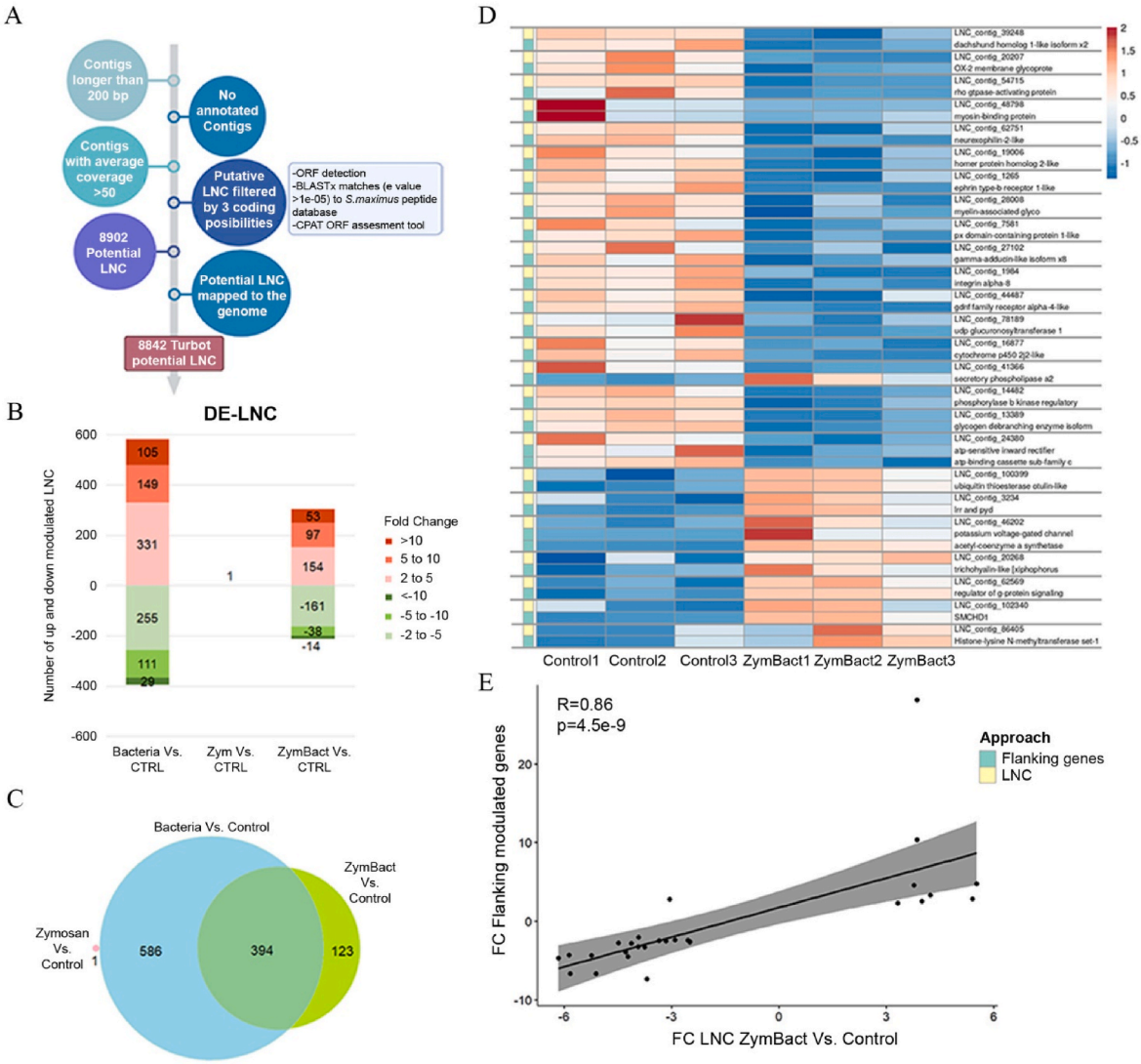


Fig. 6. Analysis of long noncoding RNAs. A) Workflow for the detection and analysis of lncRNAs. B) Number of differentially expressed lncRNAs in the three experimental groups (Bacteria, Zymosan and Zymosan-Bacteria) compared to the Control group. Their distribution based on the expression level was also presented. C) Venn diagram showing the shared and exclusively modulated lncRNAs in each group. D) Heatmap showing the expression of the modulated lncRNAs and their flanking genes (expression values represented as TPMs). E) Spearman's correlation analysis between the 25 modulated lncRNAs and their flanking DEGs.

were negatively correlated with the bacterial load were not identified by the differential expression analysis and were involved in vesicle trafficking pathways (lysosomal-trafficking regulator, exocyst complex component 3-like), chemotactic activity (IL-16), cell proliferation and apoptosis (palmitoyltransferase, mastermind-like protein 3-like), and general regulation of gene expression and transcription (transducin-like enhancer protein) (Table 2).

4. Discussion

The *in vivo* administration of β-glucans and their effect to improve the protection against several bacterial pathogens including Aeromonas is being studied in several fish species [9,14,16,17]. Our data confirmed that intraperitoneal (ip) injection was an effective method to administer β-glucans, since a single dose of zymosan A applied seven days before the infection decreased the mortality rates of turbot up to 40% against pathogenic *Aeromonas salmonicida*. This procedure (ip injection of a single dose of zymosan A) has also been reported to result in substantial protection against the microsporidian *Loma salmonae* in rainbow trout [35], *Aeromonas hydrophyla* in zebrafish [16], *Vibrio damsela* and *Aeromonas salmonicida* in turbot [11,15], and *Edwardsiella ictaluri* and *E. piscicida* in channel catfish [36]. Interestingly, we found that the treatment with zymosan decreased the abundance of Aeromonas, in turbot's microbiome suggesting that the physiological processes modulated by the treatment were able to limit the bacteria growth. This ability of glucans to decrease the bacterial burden was described in other *in vivo* models, such as in mice infected with *Mycobacterium bovis* [37]. However, we cannot exclude a direct inhibition of bacterial growth by zymosan since β-glucans exhibit potential antibacterial activity against a broad spectrum of bacteria [38]. Interestingly, the zymosan treatment did not impact on the other low represented bacterial groups (Cultibacterium, Prevotella and Thioclava).

Our experimental infections in turbot resulted in 100% mortality and triggered transcriptomic activation of several biological processes, consistent with previous publications [4,11,39]. Understanding the biological processes modulated during *A. salmonicida* infection in turbot could help us evaluate the effect of zymosan A. Interestingly, we did not detect significant changes in the transcriptome of fish treated with zymosan A. Only 7 coding genes and 1 lncRNA were differentially expressed compared with controls, indicating that the animals returned to baseline transcriptomic conditions at least seven days after zymosan administration in our experimental design. Injection of zymosan A

Table 1
Genes potentially modulated by lncRNAs.

LNC	Neighbor gene	Correlation value	LNC modulation (FC)	Gene modulation (FC)
lnc_contig_39248	dachshund homolog 1	0.9423	−6.16	−4.66
lnc_contig_20207	CD200	0.9719	−5.86	−4.32
lnc_contig_54715	rho gtpase-activating protein	0.9038	−5.83	−6.65
lnc_contig_48798	myosin-binding protein	0.9915	−5.23	−4.35
lnc_contig_62751	neurexophilin-2-like	0.8982	−5.11	−6.66
lnc_contig_19006	homer protein homolog 2-like	0.9554	−4.28	−3.90
lnc_contig_1265	ephrin type-b receptor 1	0.9463	−4.21	−4.48
lnc_contig_28008	myelin-associated glycoprotein	0.9889	−4.13	−2.81
lnc_contig_7581	px domain-containing protein 1	0.7453	−3.93	−3.23
lnc_contig_27102	gamma-adducin	0.6249	−3.92	−2.04
lnc_contig_1984	integrin alpha-8	0.9574	−3.75	−3.29
lnc_contig_44487	gdnf family receptor alpha-4	0.8657	−3.68	−7.34
lnc_contig_78189	udp glucuronosyltransferase 1	0.9271	−3.35	−2.47
lnc_contig_16877	cytochrome p450 2j2	0.9495	−3.16	−2.50
lnc_contig_41366	secretory phospholipase a2	−0.7525	−3.05	2.81
lnc_contig_14482	phosphorylase b kinase regulatory	0.9523	−2.91	−2.38
lnc_contig_13389	glycogen debranching enzyme	0.9765	−2.54	−2.42
lnc_contig_24380	atp-sensitive inward rectifier	0.6529	−2.49	−2.57
lnc_contig_24380	atp-binding cassette sub-family c	0.6529	−2.49	−2.67
lnc_contig_100399	ubiquitin thioesterase otulin	0.9521	3.32	2.32
lnc_contig_3234	NLRP6	0.9318	3.77	4.56
lnc_contig_46202	potassium voltage-gated channel	0.9401	3.85	28.14
	acetyl-coenzyme a synthetase	0.9401	3.85	10.36
lnc_contig_20268	trichohyalin-like	0.7359	3.99	2.52
lnc_contig_62569	regulator of g-protein signalling	0.9771	4.23	3.33
lnc_contig_102340	Structural maintenance of chromosomes flexible hinge domain-containing protein	0.8871	5.40	2.81
lnc_contig_86405	Histone-lysine *N*-methyltransferase set-1	0.9867	5.53	4.78

altered the transcriptomic response elicited by bacterial challenge. After a second exposure of fish to bacteria, the modulatory effect of zymosan A was detected. This suggests a possible adaptation of the response to bacterial infection, including the development of trained immunity, metabolic reprogramming, and epigenetic changes, as previously described in other species [9,11,14,21].

4.1. Control of the immune response

As expected, the transcriptomic data showed an activation of multiple pathways related to the immune system after bacterial infection [4,11,39]. Our results suggest that zymosan A can decrease the inflammatory response induced by Aeromonas. The production of pro- and anti-inflammatory interleukins (IL-1B, IL-12, IL-27, and IL-10) elicited by the bacteria as well as other important proinflammatory molecules, such as TNFa, chemokines and other mediators (SOCS1 and 3, importin A5, STAT1, and STAT3), decreased significantly in zymosan treated fish, underlining the role of glucans in controlling inflammation. A similar reduced inflammatory response to *A. salmonicida* infection was described in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements [40]. In this context, the β-glucans can induce a weakening of the innate immune system in fish at high dose and long stimulatory period [14,17]. Toll-like receptor 5 (TLR5) is needed for the recognition of Aeromonas flagellins [41]. Its down regulation could influence the production of downstream chemokines and cytokines since β-glucans are able to supress TLR-mediated NF-κB activation and diminish the production of cytokines during chronic inflammatory conditions [42,43]. Cell adhesion molecules 2 and 4 facilitate the contact between host cells and bacteria [44]. They were also significantly modulated and may hamper bacterial infection and subsequent growth in the host tissues.

Zymosan A could also affect the recruitment and activation of immune cells. The analysis of genes exclusively modulated in the ZymBact group revealed a reduction in the expression of lymphocyte antigen 6 g (Ly6G), and inhibition of Ly6G has been shown to decrease the number of neutrophils recruited to the site of infection [45]. Moreover, a reduction in the expression of granulocyte colony-stimulating factor could influence the recruitment and activity of leukocytes during bacterial infection [46]. LncRNAs could be involved in the regulation of those processes since two out of the 25 DE selected lncRNAs were associated with the upregulation of the "regulator of g-protein signalling pathway", which inhibits the proinflammatory response during bacterial infection [47], and the reduction in the levels of the cell surface OX2 glycoprotein (CD200), which could imply a restriction of macrophage activation [48].

Effector molecules involved in the degradation of bacterial membranes, such as secretory phospholipase A2 (sPLA2) and complement proteins, were significantly upregulated, in agreement with previous publications [49,50]. The upregulated expression of complements C3 and C4 could also be involved in the enhanced resistance to *A. salmonicida,* as previously demonstrated in resistant families of Atlantic salmon challenged with the same bacterial pathogen [51].

Interestingly, clear downregulation of several haemoglobin genes was found in the transcriptomic analysis. Bacterial infection can induce tissue damage in the kidney, affecting the production of erythropoietin and haemoglobin. Alternatively, turbot could modify the production of haemoglobin to reduce its availability for the bacterial pathogen [52] and to control inflammation since circulating cell-free haemoglobin is a potent proinflammatory mediator associated with excessive production of nitric oxide and free oxygen radicals [53]. The biological importance of this reduction in the protection conferred by zymosan A is not clear and should be studied in more detail.

Based on all of these results, we suggest that zymosan A appear to maintain the immune system at homeostatic levels to reduce the adverse effects of an uncontrolled inflammatory response, as previously suggested in fish and mammals [9,54].

4.2. Regulation of apoptotic cell death

A. salmonicida infection modulates apoptosis in turbot. This has also been described in other fish species [4,39,55]. Apoptotic cell death can be considered a side effect of the host immune response but can also be induced by the pathogen itself to ensure its survival and propagation [56]. The treatment of fish with zymosan A could regulate this process as previously described [55]. Differential apoptotic effects of β-glucans have been reported in teleost immune cells based on the source of the stimulant, the structure and the dose [57,58]. Among the DEGs shared in

Table 2
Positive and negative correlated genes with abundance of Aeromonas (top 30). Pearson correlation coefficient was over 0.999. Genes that were not differentially expressed in the transcriptomic analysis were marked in red. Genes exclusively expressed in the infected fish (Bacteria group) were highlighted in blue.

Positive correlation			Negative correlation		
Genus/RNAseq	**Annotation**	**Pearson**	**Genus/RNAseq**	**Annotation**	**Pearson**
SMAX5B009655	non-syndromic hearing impairment	0.99999	SMAX5B017954	filensin-like isoform x1	-0.99997
SMAX5B010625	apoptosis facilitator bcl-2-like	0.99998	SMAX5B017193	exocyst complex component 3-like	-0.99995
SMAX5B013304	tumor necrosis factor receptor	0.99998	SMAX5B016805	lysosomal-trafficking regulator	-0.99994
SMAX5B011764	uncharacterized protein	0.99998	SMAX5B009770	serine threonine-protein	-0.99993
SMAX5B015139	atrial natriuretic peptide receptor	0.99996	SMAX5B020017	pro-interleukin-16-like isoform x1	-0.99992
SMAX5B011490	tnfaip3-interacting protein 2-like	0.99991	SMAX5B010986	dedicator of cytokinesis protein	-0.99989
SMAX5B015715	receptor-transporting protein	0.99991	SMAX5B021224	two pore calcium channel protein 2	-0.99988
SMAX5B009382	fos-related antigen 2-like isoform	0.99986	SMAX5B018339	krueppel-like factor 7-like	-0.99986
SMAX5B010056	leptin a	0.99983	SMAX5B015103	sorting nexin-30-like isoform x1	-0.99985
SMAX5B005182	mitochondrial import inner membrane	0.99982	SMAX5B019787	mastermind-like protein 3-like	-0.99985
SMAX5B020299	sulfotransferase family cytosolic	0.99979	SMAX5B003485	serine threonine-protein kinase	-0.9998
SMAX5B019136	dna damage-inducible transcript	0.99977	SMAX5B002885	srsf protein kinase 2-like isoform	-0.99978
SMAX5B012349	protein mis18-alpha	0.99975	SMAX5B009478	uncharacterized protein	-0.99976
SMAX5B001696	girdin-like isoform x1	0.99971	SMAX5B011860	phosphatidylserine synthase 1	-0.99973
SMAX5B004505	endophilin-a1-like isoform x1	0.99971	SMAX5B000323	protein quaking-a-like isoform x3	-0.99971
SMAX5B022065	c-x-c motif chemokine 10-like	0.99965	SMAX5B011829	neurobeachin-like protein 2-like	-0.9997
SMAX5B021451	cc chemokine	0.99963	SMAX5B015696	nuclear transcription factor y	-0.99964
SMAX5B003934	rho-related gtp-binding protein	0.99963	SMAX5B006380	trio and f-actin-binding	-0.99962
SMAX5B007877	pi-plc x domain-containing protein	0.99960	SMAX5B014998	intraflagellar transport protein	-0.9996
SMAX5B001800	transcription elongation factor b	0.99959	SMAX5B003385	probable palmitoyltransferase	-0.9996
SMAX5B004934	transcription factor jun-b-like	0.99955	SMAX5B019438	protein enabled homolog isoform x2	-0.99959
SMAX5B016744	uncharacterized protein	0.99954	SMAX5B016538	transducin-like enhancer protein	-0.99958
SMAX5B004353	traf-interacting protein with fha	0.99949	SMAX5B015838	engulfment and cell motility	-0.99958
SMAX5B008336	nf-kappa-b inhibitor alpha	0.99946	SMAX5B010541	membrane-associated guanylate	-0.99954
SMAX5B010075	potassium voltage-gated channel	0.99937	SMAX5B018387	low-density lipoprotein	-0.99953
SMAX5B007487	importin subunit alpha-5-like	0.99931	SMAX5B003515	upstream stimulatory factor 2-like	-0.99951
SMAX5B007818	-	0.99930	SMAX5B000325	protein o-mannosyl-transferase	-0.99947
SMAX5B000078	cyclic amp-dependent transcription	0.99929	SMAX5B000445	-	-0.99946
SMAX5B014363	interleukin 10 precursor	0.99928	SMAX5B010516	sesquipedalian-1 [alligator	-0.99944
SMAX5B008422	chordin-like isoform x2	0.99925	SMAX5B010860	transcription factor 7-like	-0.99941

infected fish, we found upregulated expression of Fas apoptotic inhibitory molecule 3 (FAIM3) in fish treated with zymosan A. FAIM3 has been shown to protect against apoptosis by blocking the activation of the initiator caspase 8 [59]. Moreover, FAIM3 also regulates the proinflammatory functions of dendritic and T-regulatory cells to maintain the homeostasis of the innate immune system [60]. This secondary activity of FAIM3 could also participate in the ability of zymosan A to control the immune response, as we described above.

Aeromonas spp. Induce apoptosis by extrinsic and intrinsic pathways [61]. Interestingly, zymosan A seem to affect the apoptotic process by modulating both pathways. We observed a reduced expression of the initiator caspases 8 and 10 that could interfere with the consequent propagation of the apoptotic signal to downstream executioner caspases (extrinsic pathway) or modify the cleavage of the Bcl-2 family proteins and release of cytochrome *c* from the mitochondria (intrinsic pathway). This last pathway also seemed to be affected since the expression of the proapoptotic molecules BH3, Bax, and DNA damage-inducible proteins were downregulated by zymosan A treatment.

4.3. Modulation of metabolism

Aeromonas salmonicida affects the metabolism of carbohydrates and lipids during infection in fish [4,11]. Librán-Pérez et al. [4] described that the treatment of fish with β-glucans modulated the expression of key metabolic genes to balance the effect of *A. salmonicida* in turbot, resulting in similar or even significantly lower levels of several metabolites (ATP, glucose and lactate) compared to those observed in non-infected fish. Our results were in line with this previous work. The metabolism of carbohydrates was reduced, as suggested by the upregulated expression of the glucokinase-regulator gene, which specifically inhibits some glucose-metabolizing enzymes [62], and the downregulation of the glycogen debranching enzyme and the phosphorylase b kinase (PhK), resulting in a reduction in the breakdown of glycogen and glycogenolysis, respectively [63]. The detection of DE lncRNAs close to the glycogen debranching enzyme and the PhK gene suggests that the metabolism of carbohydrates could be regulated by those lncRNAs.

Lipid metabolism was also influenced by glucans, as previously suggested by Librán-Pérez et al. [11]. The downregulated expression of the long-chain-fatty-acid-CoA ligase gene could explain the reduction in triglycerides and cholesterol in the liver of infected fish, as was observed in our *in vivo* experiments. This ability of glucans to reduce cholesterol levels has already been described in several animal models and humans [64]. All of these results support a decrease in lipid metabolism and beta oxidation to reduce the increased levels induced by the infection.

Additionally, the high expression of the L-amino-acid oxidase gene could contribute to the protection conferred by zymosan A in turbot, since L-amino acid oxidases have strong antimicrobial and selective cytotoxic activities against gram-positive and gram-negative bacteria [65].

4.4. Cytoskeleton dynamics and remodelling

The organization of the cytoskeleton and the extracellular matrix is manipulated by *A. salmonicida* during infection [66]. The rearrangement of the host cytoskeleton triggers numerous processes that facilitate bacterial invasion, including alteration of vesicular trafficking and actin-dependent bacterial movement, evasion of autophagy/inflammasome degradation and blockage of phagocytosis, among other processes [67]. We observed that treatment with zymosan A prior to infection with the bacteria induced a generalized modulation of genes related to cytoskeleton dynamics, cell movement (locomotion and phagocytosis) and intracellular and extracellular vesicle trafficking. Our analysis revealed the upregulation of several myosins [2,7,13] in fish treated with zymosan A. Myosin genes are activated or inhibited by bacteria since they are essential for the infectious cycle [67]. Other upregulated genes were plectin and semaphorin 4E, both of which play important roles in cytoskeleton network organization, regulation of cell shape, adhesion and motility [68,69]. A second group of upregulated genes was related to the muscular system and muscle contraction. This group includes a variety of myosins and key genes for the activation of striated (troponin and calpain-3) and smooth muscle (calponin). These results show that muscle activity and structural organization could be increased by the treatment with zymosan A increasing the protection of fish against the bacteria, as described by Castro et al. [70].

All of these processes could be regulated by lncRNAs since four out of the 25 selected DE lncRNAs were associated with genes involved in the control of cytoskeleton dynamics. For example, the inhibition of the negative regulator Rho GTPase-activating proteins (RhoGAPs) suggests the activation of the major Rho protein pathways involved in cytoskeletal dynamics, cell polarity, cell migration, differentiation, cell-cycle progression, vesicle trafficking and cytokinesis [71]. Moreover, the downregulation of Gamma-adducin and the Dachshund homolog 1 (DACH1) could also promote cytoskeleton rearrangement since they have a critical role in the assembly of the actin network, affecting cell locomotion, migration and proliferation [72].

4.5. Coordinated activity of the nervous system

Specific regulation of cellular processes involving the maintenance and activity of the nervous system was observed after treatment with zymosan A. We detected downregulated expression of genes involved in the control, migration, proliferation and differentiation of neurons in the nervous system, such as the neural cell adhesion molecule L1 and the Glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-2 [73]. Genes involved in cell communication by synaptic processes (synaptic vesicle glycoprotein 2B, the netrin-G2 protein, neurexophilin-2-like, homer protein homolog 2-like, ephrin b receptor 1, and myelin-associated glycoprotein) were also downregulated and were under the control of DE lncRNAs. It is important to remember that those processes involve the plasticity of synaptic cells, vesicle trafficking and exocytosis under the control of the cytoskeleton [74]. Although the nervous system can control immune responses through neuroendocrine interactions in fish [75], the importance of those gene modifications in the protection conferred by zymosan A is not known.

5. Conclusions

The main effects observed one week after the intraperitoneal injection of zymosan A in juvenile turbot are summarized in Fig. 7. The administration of zymosan A in turbot induced significant physiological and cellular changes that could induce protection against *A. salmonicida*. The modulation of metabolism and the immune response balanced the increase in metabolite levels and inflammation induced by the bacteria. The management of cell plasticity and cellular processes involving cytoskeleton dynamics, including synaptic communication, seems to be essential for the protection conferred by zymosan A. However further functional experiments are needed to confirm the participation of those physiological processes. Also it is important to remind that there may be other mechanisms not identified by the techniques used in this study. We described for the first time the participation of lncRNAs modulated by zymosan A in the regulation and coordination of all of those processes, suggesting that they are essential for the protective effect of zymosan A against *A. salmonicida* infection.

Ethics statement

All animal studies were performed in accordance with the principles of care and use of laboratory animals following the EU Directive 2010/

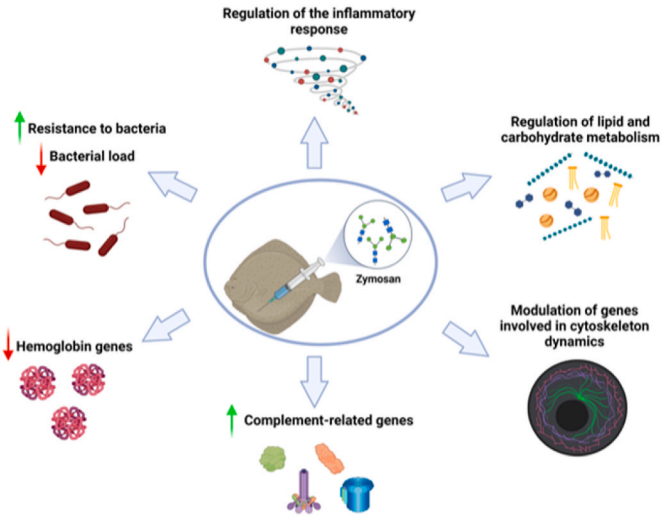


Fig. 7. Summary of some of the main effects observed one week after the intraperitoneal injection of zymosan A in juvenile turbot. Figure created with BioRender.com.

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CRediT authorship contribution statement

Alejandro Romero: Investigation, Methodology, Validation, Formal analysis, Writing – original draft. Writing – review & editing. **Magalí Rey-Campos:** Investigation, Methodology, Validation, Formal analysis, Writing – original draft. **Patricia Pereiro:** Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing – original draft. **Marta Librán-Pérez:** Investigation, Methodology. **Antonio Figueras:** Formal analysis, Writing – review & editing. **Beatriz Novoa:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109456.

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