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Circulating miRNAs involved in the immune response of the European seabass (*Dicentrarchus labrax*)

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ABSTRACT

Understanding the immune response in fish through transcriptomic and microRNA (miRNA) profiling may unlock critical insights into disease resistance mechanisms. The objective of the present study was to examine the immune modulation of the European seabass (*Dicentrarchus labrax*) following bacterial infection and vaccination. Therefore, sequencing of circulating miRNA isolated from blood serum and 3′UTR transcriptome sequencing of head kidney was conducted. In the infected fish 19 miRNAs were found to be differentially expressed. This included two novel miRNAs exhibiting high levels in the infected fish. Regarding circulating miRNAs following vaccination, three specific miRNAs have been identified that demonstrated a substantial increase in expression. Two of them, miR-216b and miR-30a-5p, have been documented to possess the capacity to delay the progression of viral infections. 3′UTR sequencing analysis of the infected fish revealed no significant enrichment of down-regulated transcripts. However, there was a significant enrichment of up-regulated transcripts related to ribosome biogenesis and protein processing. In vaccinated fish up-regulated transcripts did not demonstrate substantial enrichment. Down-regulated genes on the other hand were involved in cytoskeleton organization and apoptosis, indicating that cellular disruption might be a potential hindrance to effective immunity. Overall, these results provide first insights into the progression and regulation of host immune responses to pathogen infection and vaccination. Moreover, the detection of in total 13 differential expressed circulating miRNAs, including regulators of critical innate immunity-related genes such as Toll-like receptor 18, suggests a potential for circulating miRNAs to play a significant role in the post-transcriptional control of fish immune defenses.

1. Introduction

Achieving efficient disease prevention is a major challenge in the life cycle of teleosts, particularly in the aquaculture sector [1]. A substantial body of research has been conducted on fish species of economic importance, such as the gilthead seabream (*Sparus aurata*) and the European seabass (*Dicentrarchus labrax*), which also contributed substantially to advancements in research at the molecular level (e.g., Refs. [2–4]). Extensive knowledge (e.g. Genome assembly, whole transcriptomes from lymphoid and non-lymphoid tissues, functional annotation databases) has been built up for both species over the last two decades, and particularly for the European seabass, the immune system has been extensively studied [2,5–8]. Indeed, pioneering works have contributed to elucidating the teleost immune system. These works include, but are not limited to, transcriptome studies, reporting differentially expressed genes (e.g., Refs. [9–11]), as well as small non-coding RNA (sncRNA) patterns for teleost immune response (e.g., Refs. [12–16]).

In the context of teleost immune response, mucosal surfaces constitute the primary line of defense against pathogens. These surfaces, which include skin, gills, gut, and olfactory organ are sites of significant molecular activity that plays a crucial role in innate immunity [17]. In the absence of bone marrow and lymph nodes, the main lymphoid tissues in fish are the thymus, the head kidney, the spleen, gill-associated lymphoid tissue (GIALT) or interbranchial lymphoid tissue (ILT) and the mucosa-associated lymphoid tissue [18]. Among these, the head kidney

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assumes hemopoietic functions and represents the primary immune organ where all leukocyte populations are found, and where antigen processing and formation of IgM and immune memory through melanomacrophage centers occur. The blood on the other hand contains the majority of the leukocyte populations that are recognized in vertebrates, as well as soluble IgM, which constitutes the predominant systemic antibody class in fish.

Along with the high diversity of teleosts, also a broad range of pathogens exists and major losses have been documented in several farmed finfishes. Although to present days extensive research has been conducted to investigate fish immune responses from both physiological and molecular perspectives, reliable non-lethal early detection methods for efficient diagnosis and prognosis in fish are still lacking. The evaluation of expression patterns of marker transcripts, whole transcriptome, or enzymatic activities, as informative as they might be, require fish specimens to be sacrificed. The ultimate challenge in the field appears to be the development of non-lethal and broadly applicable methodologies to investigate potential transcriptomic responses, especially for fish species of commercial interest. The study of circulating miRNAs in teleost fish as potential non-lethal biomarkers is a rapidly emerging field of research. One of the earliest studies examined the association between circulating miRNAs and vitamin K levels, with a focus on their role in reproductive function in the Senegalese sole (*Solea senegalensis*) [19]. Subsequent research investigated the relationship between circulating miRNAs and nutritional and environmental stressors in the rainbow trout (*Oncorhynchus mykiss*), with a view to identifying biomarkers of stress and sex [20,21]. With regard to the European seabass, to the best of our knowledge, only one study has been published that deals with circulating miRNAs. This study reports the association with gender and stress response. The authors identified five potential miRNAs related to the stress response and three related to gender [22]. This finding indicates that only a limited number of circulating miRNAs may function as potential biomarkers. Nonetheless, investigations are still in their nascent stages and have not yet been implemented. In medical research, cell-free-circulating miRNAs were already recognized as important emerging biomarkers in body fluids for various complex human diseases and it has now been established that non-coding RNA molecules exert post-transcriptional control over stability and translation of vast networks of protein-coding genes, during both early embryonic development and in adult cell types [23]. Although miRNA regulation of immune system genes has been extensively studied in higher vertebrates, particularly in humans [24], there is much less information on how miRNAs regulate the immune response in teleost fish. Some studies have identified specific miRNAs with distinct expression patterns following immune challenges i.e. [15,25,26], however, data on miRNA regulation in teleosts, especially in the European seabass, remains limited, particularly regarding the role of circulating miRNAs in immune response regulation. Consequently, exploring teleost circulating miRNAs and the corresponding immune response outcomes holds great potential for providing not only a better understanding of disease pathology but also a foundation for developing non-lethal methods for early disease detection. In the present study, we have examined circulating miRNA in the blood serum of the European seabass along with gene expression patterns in the head kidney after vaccination as well as after infection with *Aeromonas veronii* bv. *Sobria,* an emerging pathogen of the European seabass cultured in the Mediterranean Sea associated with severe losses [27,28]. Identified miRNAs represent the first candidates in European seabass to serve as putative non-lethal biomarkers for disease detection, as early diagnosis of disease provides the opportunity to take immediate action and to prevent the disease from spreading and infecting other organisms.

2. Material and methods

All procedures involving fish handling were conducted following the guidelines provided by authorized personnel (FELASA-accredited certification) as well as the "Guidelines for the treatment of animals in behavioral research and teaching" [29], the Ethical Justification for the use and treatment of fishes in research: An update, and the "Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes" [30].

2.1. Fish and experimental conditions

Experiment 1: Healthy European seabass, weighing approximately 250 g were experimentally challenged with *Aeromonas veronii bv. sobria*, strain PDB, as previously described [31]. Briefly, the fish were immersed in a bacterial solution derived from an overnight culture, at a final concentration of 10^5 CFU/mL, for 2.5 h. After exposure, the fish were transferred to 250 L tanks equipped with autonomous filtration systems and monitored for 10 days. In total 10 fish were challenged with the pathogen. Sampling was done at day 3 post challenge when most fish displayed typical clinical signs of the disease including anorexia, jaundice, and hemorrhaging. The fish were anesthetized in MS222 and following blood sampling (n = 4) they were sacrificed in an overdose of the anesthetic. Head kidney of the same individual as of the blood sampling was removed aseptically and placed immediately in RNAlater (Qiagen, Duerren, Germany). Fish were not fed during the challenge period, and anesthesia was achieved using tricaine methanesulfonate (MS222, Sigma-Aldrich Inc., St Louis, MO, USA, #E10521). An equal number of healthy European seabass (*Dicentrarchus labrax*) with comparable weight and not challenged with the pathogen were sampled as control fish from HCMR facilities. The experimental protocol was approved by the competent authority (protocol number 147115, July 17, 2017).

Experiment 2: Fish that were not subjected to any vaccination and/or viral infection and which resulted nodavirus-negative to a PCR screen served as the control experimental group (n = 4, length 24.1 ± 0.68 cm, gutted weight 133 ± 5.08 g). Nodavirus-vaccinated fish received the Pharmaq ALPHA JECT micro® 1Noda vaccine (inactivated Red-spotted Grouper Nervous Necrosis Virus RGNNV ≥0.07 antigenicity units per dose in emulsion for injection, adjuvated with liquid paraffin) by intraperitoneal injection (IP) on December 23rd, 2019 and were sampled on February 4th, 2020 (n = 4, length 25.2 ± 1.30 cm, gutted weight 159.65 ± 10.85 g). Onset of immunity is reported after 466-degree days with the mean water rearing temperature at 19.4 °C. Samples were taken from non-vaccinated and vaccinated individuals.

2.2. Total RNA extraction from fish serum and head kidney

European seabass blood was collected via caudal venipuncture into 2 mL tubes, using 25G needles paying particular attention to avoiding hemolysis. Blood was left undisturbed at room temperature for 4 h and clots were removed by centrifugation at 2000×*g* for 10 min, the supernatant was removed and shock-frozen in liquid nitrogen. Total RNA was extracted from the serum by applying the QIAZol (Qiagen, Germany) extraction protocol. In brief, 5 vol of QIAZol were added to one volume of fish serum and vortexed. After adding an equal volume of chloroform, the upper aqueous phase was collected and 1.5 vol of 100 % EtOH were added and mixed thoroughly by pipetting. Samples, including any precipitate, were transferred into a RNeasy Mini column (miRneasy, Qiagen, Duerren, Germany) followed by washing and precipitation steps as recommended by the manufacturer. The final elution volume was set to 30 μl of RNase-free water and the total RNA was evaluated by RNA picochip (DNAnalyzer, Agilent).

Total RNA was extracted from the head kidneys using the Nucleospin miRNA kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. In brief, the head kidney was disrupted with a mortar and pestle in liquid nitrogen and homogenized in lysis buffer by passing the lysate through a 23-gauge (0.64 mm) needle five times. The quantity of extracted RNA was estimated utilizing a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The

quality of the RNA was additionally assessed through agarose (1 %) gel electrophoresis and capillary electrophoresis using the RNA Pico Bioanalysis chip (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA 95051, USA). Samples with an RNA integrity number value between 8.9 and 9.9 were deemed suitable for library construction.

2.3. sncRNA library construction and sequencing

sncRNA libraries were prepared using the NEBnext multiplex Small RNA Library Preparation kit for Illumina sequencing (New England Biolabs, Ipswich, MA, USA) with 18 ng of isolated serum RNA as starting material. According to the manufacturer's recommendation, size fractioning was carried out by electrophoreses of a polyacrylamide gel (6 % TBE gel, Lonza, Basel, Switzerland) at 4 °C for 1 h. Different multiplex identifier tags provided by NEB were added to each sample. sncRNA libraries were evaluated by DNA high-sensitivity chips (Bioanalyzer, Agilent) and quantified by Qubit (Life Technologies, Carlsbad, CA, USA) measurements. Differentially indexed libraries were pooled at a concentration of 4 nM and single-strand sequenced on an Illumina NextSeq500 sequencing platform at the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, Greece.

2.4. 3′UTR library construction and sequencing

3′UTR libraries were prepared from 500 ng of head kidney total RNA using Lexogen's QuantSeq 3′ mRNA-Seq Library Prep Kit REV for Illumina, according to the manufacturer's instructions. Sequencing (1 × 75 single-end strategy) was conducted on an Illumina NextSeq500 platform at the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, Greece.

2.5. Sequencing data processing

Quality control of all reads (mRNA and sncRNA) was achieved using Fastqc v0.10.0 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) prior to and following trimming using Trimmomatic v0.3 [32] to remove residual adapters, low-quality nucleotides (nt). Trimmed reads were imported into CLC Genomics Workbench (v10.1). Putative sncRNAs including all types of sncRNA were extracted, reads were counted accordingly, and the minimum sampling count was set to 10. Putative miRNAs were annotated by mapping sequencing reads against the miRbase (release 21.1) available teleost miRNAs in the following order, *Astatotilapia burtoni, Oryzias latipes, Tetraodon nigroviridis, Fugu rubripes, Danio rerio, Cyprinus carpio, Gadus morhua, Hippoglossus hippoglossus, Paralichthys olivaceus, Ictalurus punctatus, Salmo salar, Petromyzon marinus,* as well as miRNAs from *Gorilla, Homo sapiens,* and *Mus musculus* [33], and against the three-spined stickleback (*Gasterosteus aculeatus,* non-coding RNA Ensembl resource Gasterosteus_aculeatus.BROADS1.ncrna with the default parameters of CLC workbench. Already a decade ago, strong evolutionary relationships have been established between the European seabass and the three-spined stickleback by synteny analysis and collinearity [22,34,35]. Subsequently, variants of the same miRNAs were merged, resulting in a list of "sampled grouped" transcripts with the corresponding read counts. Putative novel miRNAs were annotated by running the mirDeep2 software (mirdeep2-0.1.3) on previously unannotated transcripts with total read counts higher than 500.

3′UTR reads were imported to CLC workbench and mapped onto the European seabass genome v1.0 (GCA_000689215.1). Read counts were obtained and employed for differential gene expression analysis employing a negative binomial generalized linear model using the R DESeq2 package.

2.6. Differential expression analysis

Differential expression (DE) analysis for both miRNA and mRNA was assessed by DESeq2 implemented in SarTools vs 1.2.0 [36] with default parameters. The control group from each experiment was set as reference level respectively. Transcripts with p-adj.< 0.05 or p-value <0.005, and fold change (FC) $>|1|$ were considered as differentially expressed. Heatmaps, volcano plots, as well as PCA analysis, were carried out in R [R Core Team 2017]. Enrichment analysis were carried out applying the gProfiler g:GOSt algorithm and visualization tool.

2.7. Identification of mRNAs targeted by the most abundant, and differentially expressed miRNAs

Putative mRNA targets of the most abundant, differentially expressed, and characterized miRNAs were identified using RNAhybrid, v.2.12 [37], with the energy threshold of the hybridization dynamics set to minimum free energy (mfe) ≤ -20 as well as TargetScan Perl script [38] with customized European seabass 3′UTR. Subsequently, only 3′ UTRs with perfect complementary base-pairing to the miRNA seed region (base pairs 2–7) were taken into consideration. The complete 3′ UTRs of the European seabass were retrieved from the Ensembl database.

3. Results

3.1. sncRNA and mRNA data generation and evaluation

Sequencing of all sncRNA libraries resulted in more than 320 million reads with an average of about 20 million trimmed reads per sample. Plotting the obtained sncRNA read lengths displayed the typical peak at 19–24 nt, which corresponds to the read length of miRNAs.

3′UTR libraries generated about 10 million reads, with an average successful mapping rate onto the European seabass genome of ~75 % (Suppl. Table 1). All sequencing data have been submitted to NCBI under the BioProject accession number PRJNA1174768.

3.2. Gene and miRNA expression analysis in infected fish (experiment 1)

A total of 343 genes were identified as differentially expressed in infected compared to control fish, with 161 being up-regulated and 187 being down-regulated in infected fish (Fig. 1a, Suppl. Table 2). Table 1presents the ten most significant genes being either up or down-regulated. Among the significantly differentially expressed genes, ENSDLAG00005003529 exhibited the highest read abundance and was found to be down-regulated in the head kidney of the infected fish. This is a protein-coding gene encoding for hemoglobin, beta adult 1 (*hba1*) (NCBI accession number XM051389353.1), as per the best NCBI blastn match (e-value = 0.0). The gene with the highest read count being up-regulated in infected fish was found to be the heat shock protein family member 5 (*hspa5*). The transcripts being up- and down-regulated the most in terms of fold change in the infected fish were ENSDLAG00005002533 (uncharacterized protein as per NCBI blastn match, with e-value = 0.0 and accession number XM_051399412.1) and troponin T type 3a (*tnnt3a*) respectively. Fig. 1a illustrates mRNA transcripts, being in the infected fish either up- (negative log2fold change) or down-regulated (positive log2fold change). Enrichment analysis revealed four Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, eight transcription factors and 12 Biological Process (BP) GO terms to be significantly enriched for the genes being down-regulated in infected fish (Fig. 2).

Regarding microRNA expression 19 miRNAs with p-value <0.005 were found and 11 with padj <0.05 (Fig. 1b–Suppl.Table 3). Among the differentially expressed miRNA three miRNAs, i.e. CAJNNU010000002.1, ENSGACT00000029667.1, CAJNNU010000023.1 were found to be novel, not yet described. The latter two are higher expressed in infected fish while CAJNNU010000002.1 has a higher read count abundance in the control fish. It is noteworthy that one of the novel miRNAs (CAJNNU010000023.1, Fig. 3a), exhibit a

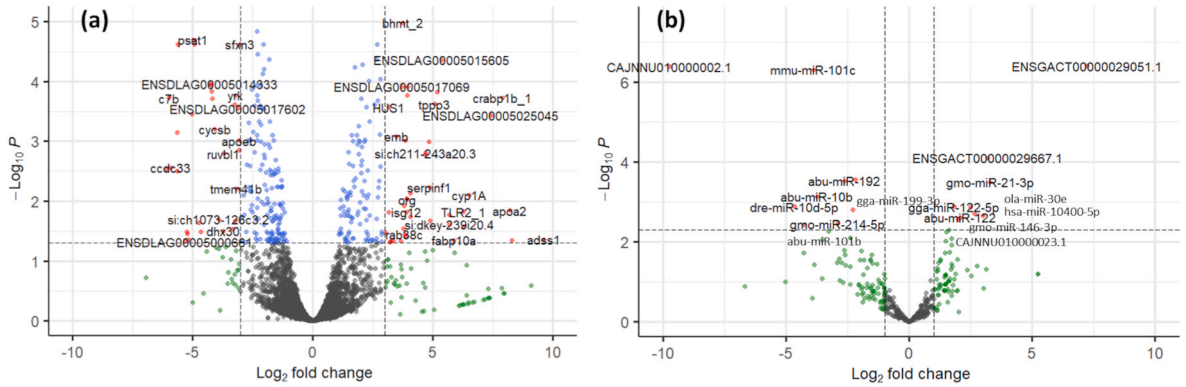


Fig. 1. (a) mRNA volcano plot of infected and control fish. Dots represent all genes tested for differential expression. Grey dots: not significantly differentially expressed. Green dots: Log2FC greater than 3 but not significant (padj. > 0.05). Blue dots: Log2FC less than 3 but padj. < 0.05. Red dots: Log2FC greater than 3 and padj. < 0.05. Positive log2fold change means down-regulation and negative log2fold change indicates up-regulation in infected fish.(b) sncRNA volcano plot of infected and control fish. Dots represent all sncRNA tested for differential expression. Grey dots: not significantly differentially expressed. Green dots: Log2FC greater than 1 but not significant (pval. > 0.005). Red dots: Log2FC greater than 1 and pval. < 0.005.

Table 1
List of 10 most up or down-regulated genes of experiment 1 as per statistical support (padj). The $\log_2$ fold changes of the up regulated genes in the infected fish are in bold.

Id	DL Inf mean	DL C mean	log2 fold change	padj	Encoded protein annotation
ENSDLAG 00005018951	1628	12	**−6.585**	6.31E-30	Folate gamma-glutamyl hydrolase, involved in glutamine and tetrahydrofolylpolyglutamate metabolic processes
ENSDLAG 00005005393	787	12	**−5.492**	1.01E-19	CD209 antigen-like protein C, probable pathogen-recognition receptor, may recognize high mannose N-linked oligosaccharides in a variety of pathogen antigens
ENSDLAG 00005002533	394	2	**−6.887**	2.92E-14	Uncharacterized
steap4_1	500	19	**−4.199**	2.41E-13	STEAP4 Metalloreductase, resides in the golgi apparatus, reduces both Fe (3+) to Fe (2+) and Cu(2+) to Cu(1+), using NAD (+) as acceptor
g0s2	4	124	5.491	3.24E-13	G0/G1 Switch Regulatory Protein 2, involved in extrinsic apoptotic signaling pathway and positive regulation of extrinsic apoptotic signaling pathway
ENSDLAG 00005004712	352	25	**−3.298**	5.60E-11	CD209 antigen-like protein C
si:dkeyp-75h12.2	75	335	2.715	8.51E-10	Testis associated actin remodelling kinase 1b, predicted to have protein serine/threonine kinase activity, involved in actin cytoskeleton organization and positive regulation of stress fiber assembly
FSTL1	11	106	3.882	8.51E-10	Follistatin-like 1b, predicted to have calcium ion binding activity, involved in various physiological processes such as regulation of the immune response, cell proliferation and differentiation
AIF1	539	31	**−3.511**	9.80E-10	Allograft Inflammatory Factor 1, induced by cytokines and interferon and may promote macrophage activation and growth of vascular smooth muscle cells and T-lymphocytes
ENSDLAG 00005015228	19	250	4.326	1.59E-09	Interferon alpha-inducible protein 27-like protein 2A, involved in several processes, including defense response to symbiont, protein K48-linked ubiquitination and pyroptotic inflammatory response

significant increase in expression in the infected fish, with an average read count of approximately 2000. RNAhybrid analysis, employing the ENSEMBL European seabass 3′UTR and setting a threshold mfe value of −30, identified 643 genes that were putatively regulated by CAJNNU010000023.1. Of these, 38 were found to be associated with the immune response.

Among the differentially expressed genes in the head kidney of the present study, 6 genes were identified as the putative targets of CAJNNU010000023.1 with four (sash3, pipk1ba, pcdh18a, nexn) being down-regulated in infected fish (Fig. 3b). and two (stard3, ruvbl1) being up-regulated.

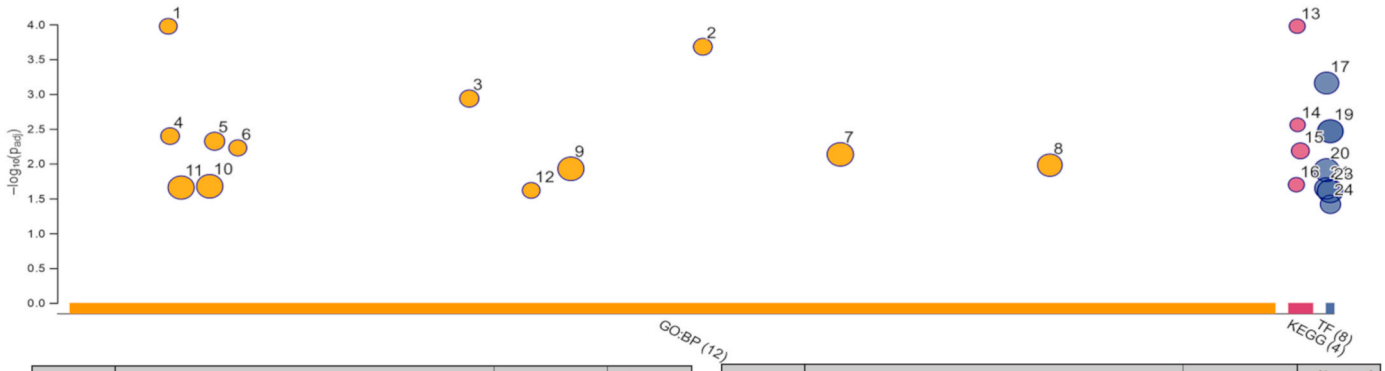
3.3. Gene and miRNA expression analysis in vaccinated fish (experiment 2)

A total of 473 genes were identified as differentially expressed between vaccinated and control fish. Of these, 175 were up-regulated and 298 were down-regulated in vaccinated fish (Fig. 4, Suppl. Table 2). Among the significantly differentially expressed genes (Table 2), ferritin, heavy polypeptide 1a (fth1a) exhibited the highest copy number and was found to be down-regulated in the head kidney of the vaccinated fish. The transcript with the highest fold change and being up-regulated in vaccinated fish was myeloid-specific peroxidase (mpx). Enrichment analysis revealed two KEGG pathways and 6 Biological process GO terms to be significantly enriched for the genes being down-regulated in vaccinated fish (Fig. 5).

Regarding microRNA expression only 5 miRNAs with p-value <0.005 were found and only 2 had significant statistical support (padj <0.05). The two miRNAs with padj <0.05 were uncharacterized and down-regulated in vaccinated fish (CAJNNU010000012.1 and CAJNNU010000003.1) but with low read counts (<50). The remaining three were miR-216b, miR-30a-5p, and miR-455b, all up-regulated in vaccinated fish (Suppl. Table 3).

4. Discussion

The role of circulating miRNAs in teleost fish is of great importance for a highly coordinated and efficient immune response [19], as they serve as pivotal regulators, modulating a plethora of immune system functions and signaling pathways in response to pathogenic infections [39]. Recent studies have highlighted the involvement of miRNAs in critical processes such as cytokine signaling, antigen presentation, and



Source	Term name	Term id	adjusted p-value
GO:BP	protein folding	GO:0006457	0.0001
GO:BP	protein maturation	GO:0051604	0.0002
GO:BP	ncRNA metabolic process	GO:0034660	0.0012
GO:BP	amino acid metabolic process	GO:0006520	0.0040
GO:BP	macromolecule catabolic process	GO:0009057	0.0048
GO:BP	regulation of catabolic process	GO:0009894	0.0060
GO:BP	organic substance metabolic process	GO:0071704	0.0074
GO:BP	organonitrogen compound metabolic process	GO:1901564	0.0105
GO:BP	primary metabolic process	GO:0044238	0.0119
GO:BP	metabolic process	GO:0008152	0.0211
GO:BP	nitrogen compound metabolic process	GO:0006807	0.0220
GO:BP	ribosome biogenesis	GO:0042254	0.0242

Source	Term name	Term id	adjusted p-value
KEGG	Ribosome biogenesis in eukaryotes	KEGG:03008	0.0001
KEGG	Proteasome	KEGG:03050	0.0028
KEGG	Protein processing in endoplasmic reticulum	KEGG:04141	0.0065
KEGG	Biosynthesis of amino acids	KEGG:01230	0.0200
TF	Factor: C-Myc; motif: NGCCACGTGNN; match cl	TF:M07601_1	0.0007
TF	Factor: c-Myc:Max; motif: NNNNNNNCACGTGN	TF:M00615	0.0034
TF	Factor: c-Myc:Max; motif: NNNNNNNCACGTGN	TF:M00615_1	0.0034
TF	Factor: C-Myc; motif: NGCCACGTGNN	TF:M07601	0.0124
TF	Factor: c-Myc; motif: KACCACGTGSYY; match cla	TF:M01154_1	0.0226
TF	Factor: c-Myc:Max; motif: NNACCACGTGGTNN	TF:M00118	0.0248
TF	Factor: c-Myc:Max; motif: NNACCACGTGGTNN;	TF:M00118_1	0.0248
TF	Factor: c-Myc:Max; motif: GCCAYGYGSN; match	TF:M00322_1	0.0385

Fig. 2. Enrichment analysis conducted on significantly up-regulated genes (log2FC > 1, padj <0.05) in infected fish. Graphs were obtained applying the gProfiler g:GOSt algorithm and visualization. The dots indicate the Gene Ontologies categories as well as KEGG terms that passed the p-adjustment threshold value and therefore appeared to be significantly enriched within the set of differentially expressed genes.

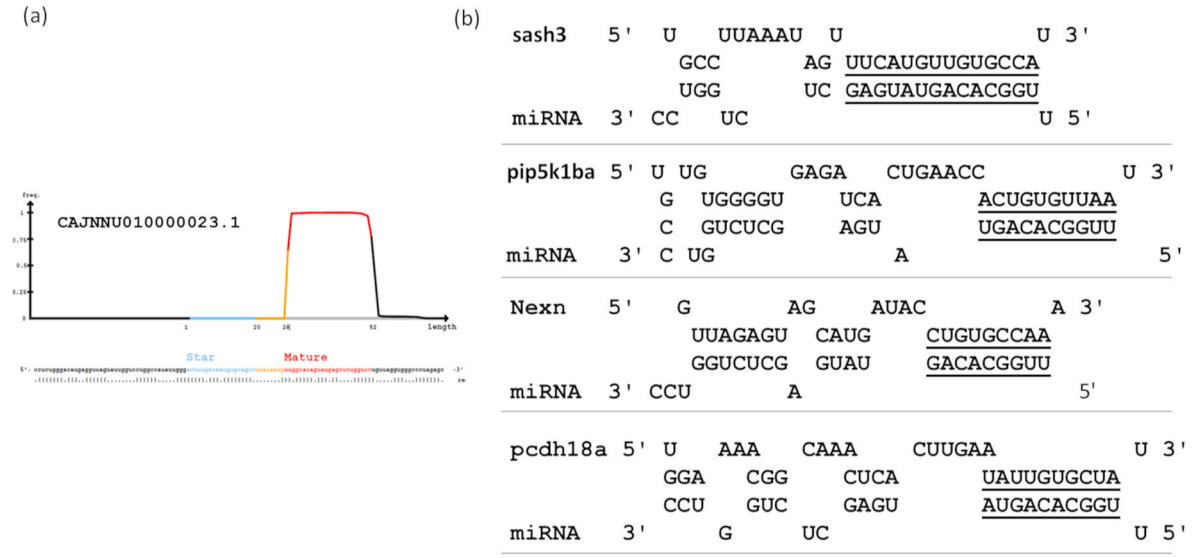


Fig. 3. (a) Structure of the putative novel miRNA CAJNNU010000023.1 obtained by running the mirDeep2 software (mirdeep2-0.1.3). The seed region is underlined, the Mature sequence is highlighted in red and the star is in blue. (b) Hybridization of CAJNNU010000023.1 with 8mer alignment of the seed region (by RNAhybrid-2.1.2) to four targets found to be down-regulated in infected fish.

the regulation of inflammatory responses, underscoring their significance in both innate and adaptive immunity within these aquatic species [15,40]. The present study aimed to detect circulating miRNAs that may play a role in immune response. Additionally, it examined the gene expression patterns in the head kidney o the European seabass, an aquaculture-relevant species that has gradually but consistently been established since 1994 as a marine model species in immunology [7]. Therefore, circulating blood miRNAs were identified, along with comparisons of the integrated transcriptome response in the head kidney. The 3′UTR RNA-Seq method was selected, as this method has been shown to be more accurate and at a lower cost. Particularly, at lower read depths, a focus on the 3′ end results in greater stability of differential gene expression assessments [41], and since the entire European seabass genome is available, it was possible to proceed with 3′ UTR sequencing. On average, more than 10 million reads were obtained for each biological condition. The latter ensures an appropriate representation of genes containing putative isoforms. In terms of miRNA sequencing, an average of 20 million reads were obtained for each

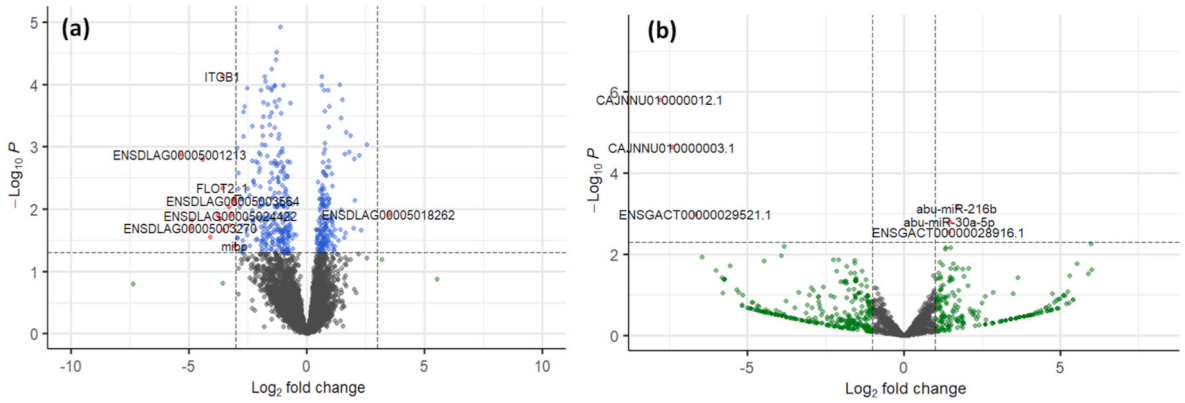


Fig. 4. (a) mRNA volcano plot of vaccinated fish. Dots represent all genes tested for differential expression. Grey dots: not significantly differentially expressed. Green dots: Log2FC greater than 3 but not significant (padj. > 0.05). Blue dots: Log2FC less than 3 but padj. < 0.05. Red dots: Log2FC greater than 3 and padj. < 0.05. (b) sncRNA volcano plot of vaccinated fish. Dots represent all sncRNA tested for differential expression. Grey dots: not significantly differentially expressed. Green dots: Log2FC greater than 1 but not significant (pval. > 0.005). Red dots: Log2FC greater than 1 and pval. < 0.005.

sample. In order to achieve differential expression (with high fold changes), a typical recommendation is to have between 1 and 5 million mapped reads per sample [42]. Conversely, for the discovery of novel miRNAs, more extensive sequencing is necessary, with 5–10 million reads per sample recommended [43]. Accordingly, the present study addresses the aforementioned requirements for novel miRNA discovery as well as for differential expression assessments.

No significant gene set enrichment resulted from 3′UTR analysis of down-regulated transcripts in infected fish and up-regulated transcripts in vaccinated fish had been detected. Instead, up-regulated transcripts in infected fish revealed an enrichment of 12 GO Biological process terms with the majority related to protein processing and ribosome biogenesis, along with enriched motives of transcription factor c-Myc and four KEGG pathways being again involved in the ribosome biogenesis and diverse protein processes. Similarly, in the East Asian fourfinger threadfin fish (*Eleutheronema tetradactylum*) head-kidney-specific differentially expressed genes (DEGs) were also enriched in the KEGG pathway "Ribosome biogenesis in eukaryotes" [44]. In that context, *Streptococcus iniae*, a pathogen frequently isolated from the brains and head kidneys of infected fish, presented a relevant case; the authors hypothesized that the up-regulation of genes related to ribosome biogenesis in the head kidney following infection may suppress immune defense mechanisms, potentially facilitating a higher bacterial load of *S. iniae* in the head kidney. This could suggest a potential trade-off between ribosome production and immune system efficiency, where increased ribosome biogenesis might compromise the fish's ability to mount an effective immune response. It is noteworthy that the relationship between ribosome biogenesis and innate immunity has also been the subject of investigation in several other studies (e.g. Refs. [45, 46]). Ribosomes are paramount to a multitude of processes ensuring homeostasis in organisms [47]. While ribosomal biogenesis is effectively essential for protein production, and therefore cell growth, proliferation, and animal development, increasing evidence is accumulating as to the consequences of both deregulated over-production and haploinsufficiency of ribosomal biogenesis to tumorigenesis [48]. The growth-promoting factor C-Myc is one of the oncoproteins displaying regulatory roles in ribosomal biogenesis [49] and induces ribosome biogenesis by stimulating *Pol*I, Pol II, and Pol III transcription [50]. Studies have further revealed that rRNA accumulation and/or nucleolar activity regulate double-stranded (ds) DNA-sensing, which in turn restricts virus reproduction and regulates inflammation [45]. Given the considerable energy expenditure associated with ribosome formation, it is maybe unsurprising that this process is integrated into vital cellular responses that determine survival, including proliferation, lifespan, and cellular immunity.

In vaccinated fish, 18 down-regulated genes were significantly enriched in the Biological Process GO terms mainly related to cytoskeleton biogenesis, organization, and polymerization, as well as two KEGG pathways, namely "Regulation of actin cytoskeleton" and "Apoptosis". Similarly, in turbot (*Scophthalmus maximus*), genes associated with cell adhesion and the cytoskeleton, as well as apoptosis, were observed to exhibit differential expression following the IP administration of vaccines containing *Philasterides dicentrarchi* antigen differing in terms of adjuvants [51]. In that case, intense cell traffic to and from the peritoneal cavity, including free cells, cells attached to the mesothelium, and others migrating to lymphoid organs was observed, but, with regards to transcriptional patterns, the authors reported a generally strong up-regulation of genes underlying the above processes and pathways in vaccinated fish. Additionally, while both, the "Regulation of actin cytoskeleton" and "Apoptosis" KEGG pathways were identified as significantly enriched in the aforementioned and in the present study, the mapping genes differed. The disparate transcriptomic findings may be attributed to the distinct adjuvants employed. In turbot chitosan-PVMMA microspheres, Freunds' complete adjuvant, aluminum hydroxide gel or Matrix-Q (Isconova, Sweden) were used as adjuvants, while in the present study the vaccine's adjuvant was liquid paraffin (mineral oil). The lesions in the peritoneal cavity, including granulomas and adhesions between organs or the organs and the peritoneal wall, possibly caused by vaccines [52], may vary depending on the adjuvant. Moreover, a significant proportion of the free cells present within the peritoneal cavity demonstrate an affinity for the vaccine, leading to the formation of masses that become attached to the peritoneal wall. Consequently, the generation of cell-vaccine masses necessitates the robust expression of genes associated with cell-cell or cell-matrix adhesion and cell-cell junctions, as well as those involved in the actin cytoskeleton. The cytoskeleton plays a pivotal role in regulating differentiation, polarization, migration, and adhesion processes, which are essential for innate immunity and cellular self-defense [53]. Actin dynamics are essential for the activation of immune cells, particularly T cells and macrophages. Hence, the down-regulation of pathways involved in actin regulation may impair these cells' ability to form immunological synapses and properly engage with antigens. This could result in a weaker activation signal, potentially leading to a reduction in the efficacy of the vaccine. The down-regulation of the "Regulation of Actin Cytoskeleton" pathway after vaccination therefore suggests a potential compromise in the teleost immune response, affecting essential processes such as cell migration, activation, cytokine secretion, and intercellular interactions. An understanding of these changes can facilitate the optimization of vaccination strategies for enhanced immune outcomes in teleosts.

With regards to the identified circulating microRNAs undergoing regulation in infected fish, a total of 19 microRNAs exhibited differential

Table 2
List of 10 most up or down-regulated genes of experiment 2 as per statistical support (padj). The log_2 fold changes of the up regulated genes in the vaccinated fish are in bold.

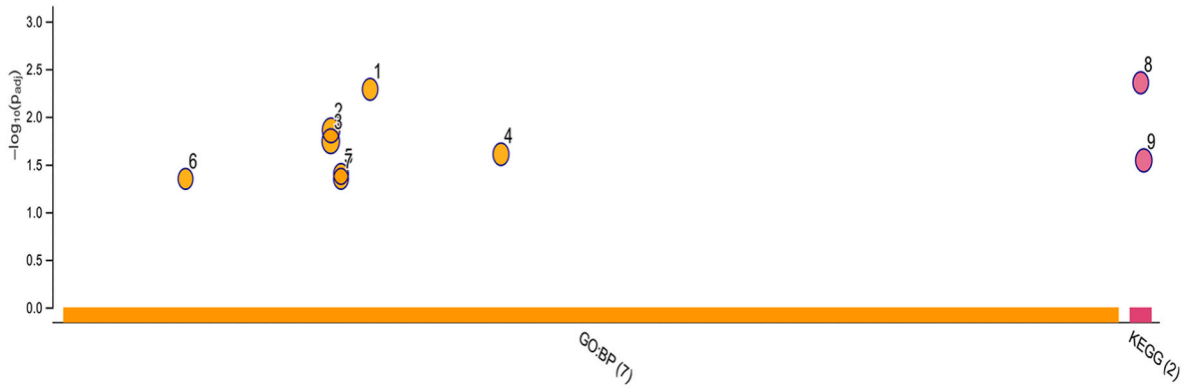
Id	CPBL Mean	VPBL Mean	log_2 fold change	padj	Encoded protein annotation
btg1	414	94	−2.1	3.52E-11	BTG Anti-Proliferation Factor 1, interacts with several nuclear receptors, and functions as a coactivator of cell differentiation
HSPA8_2	331	86	−1.941	8.224E-11	Heat Shock Protein Family A (Hsp70) Member 8, functions as a chaperone and binds to nascent polypeptides to facilitate correct folding
ENSDLAG00005019812	127	15	−3.071	2.02E-10	Ubiquitin A-52 residue ribosomal protein fusion product 1, involved in protein catabolic processes
fth1a	30284	3013	−3.271	2.50E-08	Ferritin Heavy Chain 1, contributes to storage of iron in a soluble and nontoxic state
atg3	107	37	−1.54	3.44E-08	Autophagy-Related Protein 3, ubiquitin-like-conjugating enzyme, a component of ubiquitination-like systems involved in autophagy
atp5mc1	62	165	**1.431**	3.44E-08	ATP Synthase Membrane Subunit C Locus 1, a subunit of mitochondrial ATP synthase that catalyzes ATP synthesis
rps24	652	218	−1.579	5.97E-08	Ribosomal Protein S24, a component of the 40S subunit
kdm5ba	122	46	−1.392	5.97E-08	Lysine Demethylase 5B, demethylates lysine 4 of histone H3 for transcriptional repression, and may also play a role in DNA repair
si:dkey-56i24.1	618	1257	**1.066**	1.02E-07	Glycosyltransferase family 92, transfers glycosyl groups
spns3	372	705	**0.912**	3.11E-07	SPNS lysolipid transporter 3, sphingosine-1-phosphate, predicted to be involved in sphingolipid transport and transmembrane transport

expression, comprising two novel microRNAs that have not yet been characterized. The miRNA differential expression in vaccinated fish amounted to a total of five miRNAs, two of which were novel. While innate immune pathways triggered by viral or bacterial infections viruses and extracellular bacteria are generally considered to be distinct, some mechanisms were demonstrated to be triggered by both pathogen types [54] our data did not highlight this, as there were no common differentially expressed miRNAs between infected and vaccinated fish. It is reasonable to expect that the anticipated number of differentially expressed circulating microRNAs may remain low when comparing the two conditions. A study of rainbow trout, for example, investigated the circulating miRNAs between dissolved oxygen (DO) levels (normoxia and hypoxia) and diet composition (low-carbohydrate (LC) or high-carbohydrate (HC)). In total, 252 circulating miRNAs were identified, of which only four were differentially expressed (with threshold criteria p-value <0.05 and log2 fold change >0.58) between the control fish and the treated ones [20]. It can thus be concluded that the current findings have significant scientific value. A further focus of the present study has been the microRNAs with higher expression levels, as well as those with no or low expression levels in the control or treated fish, respectively. Markedly one of the novel miRNAs (CAJNNU010000023.1, Fig. 3a) has been found to be a putative regulator of 38 genes belonging to immune-related categories. Among them *toll like receptor 18* (*tlr18)*, an extensively expressed fish-specific TLR belonging to the TLR1 family (Suppl.Table 5). TLRs are essential pattern recognition receptors (PPRs) that can identify conserved pathogen-associated molecular patterns (PAMPs). Moreover, it has also been reported that TLR18 is up-regulated in isolated peripheral blood lymphocytes of yellow catfish after stimulation with lipopolysaccharide (LPS), peptidoglycan (PGN), and polyinosinic:polycytidylic acid (poly (I:C)) [55].

CAJNNU010000023.1 has further been detected to hybridize (seed region, 8mer) to four genes significantly down-regulated in infected fish (Fig. 3b). One of the four genes, sash3 has been recently described in humans to play a significant role in T cell proliferation, activation and survival. The loss of function in human has been associated with immunodeficiency and may negatively affect T-cell function [56]. The available evidence on the role of sash3 in teleosts is currently limited. However, its evolutionary conservation suggests that it may also be involved in analogous immune processes in fish. The highest expressed gene was ENSDLAG000000329, annotated via NCBI as hemoglobin, beta adult 1 (*hbba1*) and down-regulated in the infected fish revealed 8mer hybridization with miR-30e-3p which was found to be up-regulated after infection (p-value <0.005). In *Megalobrama amblycephala*, studies have demonstrated a reduction in the expression levels of Hbβ (as well as Hbα) following infection. This could suggest a potential correlation between haemoglobin function and the physiological responses to pathogens. The observed down-regulation may also be associated with damage to red blood cells or haematopoietic organs during infections [57].

Concerning miRNAs being up-regulated in vaccinated fish of particular interest are the miR-216b and miR-30a-5p. The former was reported to have the potential to exert an inhibitory effect on the expression of viral genes. It is also possible that this miRNA may target host genes that are involved in antiviral signalling pathways [15]. As for the latter miRNA, miR-30a-5p much less information is available. Yet in the miiuy croaker (*Miichthys miiuy*) miR-30a-5p was down-regulated after poly (I:C) stimulation [58], even though, its mature sequence matches that of miR-30e-5p, which has been documented to be up-regulated in mammals in response to viral infections [59].

Overall, these findings, obtained for one of the most relevant marine aquaculture species in Europe that is becoming a marine model species in the field of comparative immunobiology, contribute to a deeper understanding of the molecular basis of immune responses in teleosts. A



Source	Term name	Term id	Adjusted p value
GO:BP	regulation of cellular component size	GO:0032535	0.0052
GO:BP	actin cytoskeleton organization	GO:0030036	0.0139
GO:BP	actin filament-based process	GO:0030029	0.0181
GO:BP	regulation of cellular component biogenesis	GO:0044087	0.0247
GO:BP	regulation of actin filament polymerization	GO:0030833	0.0398
GO:BP	regulation of actin polymerization or depolymerization	GO:0008064	0.0447
GO:BP	regulation of actin filament length	GO:0030832	0.0447
KEGG	Apoptosis	KEGG:04210	0.0044
KEGG	Regulation of actin cytoskeleton	KEGG:04810	0.0287

Fig. 5. Enrichment analysis of genes being significantly down-regulated (log2fold >1, padj <0.05) in vaccinated fish. Enrichment analysis were carried out applying the gProfiler g:GOSt algorithm and visualization tool. The dots indicate the Gene Ontologies categories as well as KEGG terms that passed the p-adjustment threshold value and therefore appeared to be significantly enriched within the set of differentially expressed genes.

more thorough understanding of host immune regulation against bacterial infection or vaccination against NNV was achieved by elucidating the signatures and possible interactions between transcriptional and post-transcriptional regulatory mechanisms, paving the way to the development of more effective vaccination strategies aimed at improving disease resistance in aquaculture. Future efforts may be devoted to elucidating the affected pathways and, most importantly, their implications for fish health management.

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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.2025.110232.

Data availability

Data will be made available on request.

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