Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*)

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Abbreviations: AFP, antifreeze proteins; APOA1, Apolipoprotein A1; **ASC,** apoptosis-associated speck-like protein containing a caspase recruitment domain; **C3**, complement component 3; **CALM,** calmodulin**; CALR**, calreticulin; **CASP,** caspase; **cDNA,** complementary DNA; **ERp57**,endoplasmic reticulum protein 57; **FBL**, fucose-binding lectin; **H1/4**, histone H1/H4; **IL1B**, interleukin 1-beta; **LEI,** leukocyte elastase inhibitor; **LYZ,** lysozyme; **qPCR**, real-time PCR; **PRDX**, peroxiredoxin; **SOD** superoxide dismutase; **TF**, transferrin; **WAP65,** warm temperature acclimation related 65 kDa protein;

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Abstract

Skin mucus is the first barrier of fish defence. Proteins from skin mucus of European sea bass (*Dicentrarchus labrax*) were identified by 2DE followed by LC-MS/MS. From all the identified proteins in the proteome map, we focus on the proteins associated with several immune pathways in fish. Furthermore, the qPCR transcript levels in skin are shown. Proteins found include apolipoprotein A1, calmodulin, complement C3, fucose-binding lectin, lysozyme and several caspases. To our knowledge, this is the first skin mucus proteome study and further transcriptional profiling of the identified proteins done on this bony fish species. This not only contributes knowledge on the routes involved in mucosal innate immunity, but also establishes a non-invasive technique based on locating immune markers with a potential use for prevention and/or diagnosis of fish diseases.

Statement of significance of the study

The skin mucus is the first defence barrier of teleost fish; together with the skin, it protects the animal from pathogens, potential harmful chemicals and physical factors in the water where it is constantly submerged. This study identifies for the first time the main proteins in the skin mucus of European sea bass (*Dicentrarchus labrax*). In particular, we identify immune relevant proteins. The presence of RNA of the immune relevant genes in skin indicates that the proteins could be synthesised in the skin itself.

1 Introduction

Teleost fish is the largest and most variable vertebrate taxon [1], with 28,644 species recorded [National Center for Biotechnology Information (NCBI) in January 2015]. European sea bass (*D. labrax*, Moronidae, Perciformes, Teleostei) has a strong economic impact as a major aquaculture species. It is one of the most common marine fish in the Mediterranean Sea, in the Eastern Atlantic Sea (from Norway to north of Africa) and in the Black Sea (Ocean Biogeographic Information System, http://www.iobis.org/mapper), whose complete genome has been recently sequenced [2].

Classically, skin mucus of fish is described as a mucin layer of high molecular mass glycoproteins [3], flexible fibres densely coated with short and negatively charged glycans due to carboxyl and sulphate groups [4], which contributes to the observed viscosity. However, functionally, the presence of immune-related proteins in mucus is of great interest since mucus acts as a first defence barrier against different stressors such as bacterial or viral infections. Skin mucus innate and adaptive immune system proteins including immunoglobulins [5], antimicrobial peptides e.g. histones [6], transferrin [7], calmodulin [8] and lysozyme[9], lectins such as galectin [10,11], acute phase proteins and complement components have been studied in different fish species [12].

Recent advances in proteomic research methods have been used for identification and quantification of proteins [13]. Among them, the use of LC-MS/MS after separation by 2DE [14] has been the most common method for protein identification on fish. After protein identification, use of qPCR is an excellent complementary approach to find if local synthesis of the protein is possible, and hence contribute to the study of the biological role of these molecules and their interactions during fish disease [15]. With this background, we aim to identify the proteome map of major skin mucus proteins of European sea bass, trying to find

immune-markers with a non-invasive technique for disease diagnosis in aquaculture. After exhaustive BLAST analysis, transcript levels of immune-related identified proteins were analysed in skin by qPCR. To our knowledge, this is the first time that the proteome map of this important fish species is studied, providing a better knowledge of mucus composition and mucosal immunology through MS-based protein detection.

2 Materials and methods

2.1 Animal care

Specimens of European sea bass (*D. labrax*) (340±35 g body weight) obtained from Culmarex SA (Murcia, Spain), were kept in running (1500 l h^{-1}) seawater aquaria at 28‰ salinity, 22 ºC and a photoperiod of 12 h light: 12 h dark. The Bioethical Committee of the University of Murcia approved the fish handling procedures.

2.2 Mucus and tissues samples

Ten healthy fish were anesthetized with 100 mg l^{-1} tricaine methanesulfonate, MS-222. Mucus was collected from the skin surface by scraping, avoiding contamination with blood, urine and/or faeces. Mucus samples were transferred into 15 ml tubes. Skin tissue samples were collected in TRIzol® reagent (Life Technologies) and stored at -80 ºC until subsequent use.

2.3 Mucus protein purification

Pooled mucus samples were solubilised with 1 mM DTT and 1.5mM EDTA which serves to act as a mild mucolytic agent [16]. Samples were sonicated for 6 seconds twice (1 min cooling between) and centrifuged at 20,000 g for 30 min at 4 ºC. The soluble mucus protein containing supernatants were desalted 3 times (14,000 g, 4 ºC) with 0.2 ml of ice cold proteomic grade water (G Biosciences, MO, USA) using 3 kDa spin filters (VWR, USA). Samples were further purified by 2D clean up kit (Bio-Rad, CA, USA) following the manufacturer's instructions.

2.4 2DE

The resulting pellets were suspended in 2D lysis buffer (BioRad) containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris base, 0.001% bromophenol blue and 50 mM DTT (Sigma-Aldrich) and 0.5% (v/v) Biolytes 3-10 ampholyte (Bio-Rad). The protein content of samples was determined using Qubit protein assay (Life Technologies). Triplicates of 200 µg of protein from each sample were rehydrated in 17 cm 3-10 IPG strips (Bio-Rad) and isoelectrically focused using Protean IEF cell (Bio-Rad). Proteins in the IPG strips were reduced and alkylated for 15 min each in equilibration buffer containing 6M urea (Sigma Aldrich), 0.375 M Tris-HCl pH 8.8 (Bio-Rad), 2% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck) with 0.2% (w/v) DTT (Sigma-Aldrich) and 0.3% (w/v) iodoacetamide (Bio-Rad), respectively. The strips were placed on 12.5% polyacrylamide gels to perform SDS-PAGE [17] on the Protean II xi system (Bio-Rad). Gels were stained overnight with SYPRO Ruby Protein Gel Stain (Life Technologies) following the supplier's protocol, and documented with ChemiDoc^{TM} XRS imaging system (Bio-Rad). Raw pictures were analysed by PDQuest 2D Advanced Software version 8.0.1 (Bio-Rad).

2.5 LC-MS/MS analysis

The most predominant 100 spots identified after 2D gel analysis were picked, excised and subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 ng/µl trypsin (V511A; Promega) as described elsewhere [18]. Peptide mixtures containing 0.1% formic acid were loaded onto a nano ACQUITY Ultra Performance LC (Waters), containing a 5 µm Symmetry C18 Trap column (180 µm × 20 mm; Waters) in front of a 1.7 µm BEH130 C18 analytical column (100 µm × 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with flow of 0.4 µl min^{-1} eluted to a Q-TOF Ultima mass spectrometer (Micromass/Waters). The samples were run in data dependent tandem mass spectrophotometry (MS/MS) mode. Peak lists from the Protein Lynx Global server software

(version 2.2; Waters) were submitted to the MASCOT search engine (version 2.5.1) against NCBInr with the following parameters: maximum one missed cleavage by trypsin, peptide mass tolerance 100 ppm, MS/MS ion tolerance 0.1 Da, fixed modification carbamidomethylation of cysteine, and variable modification methionine oxidation. Protein hits not satisfying a significant threshold ($p<0.05$) or with low sequence coverage were further searched against Swissprot and vertebrate EST databases, taxonomy Actinopterygii.

2.6 Gene ontology term analysis and protein interaction network

GO term analysis was done according to Uniprot database. The results for immune-relevant proteins are shown in Supplementary table 1. An interaction network of immune-related proteins was generated based on their mammalian orthologues (*Mus musculus*) through BLASTp searches in NCBI database using String v.9.1 (see Supplementary file 1). The predicted protein-protein interaction network was created with confidence score of 0.300 and no more than 10 interactions.

2.7 Primer design

For genes identified by proteome analysis, homologue sequences for each gene from *D. labrax* were retrieved from the NCBInr or EST databases, satisfying the requirement of specificity, and primers designed (Table 1) by OligoPerfect™ Designer (Life Technologies).

2.8 Gene expression analysis

Skin samples (n=3) were extracted with TRIzol® reagent (Life Technologies) following the manufacturer's instructions, quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. In addition, 1 µl of each RNA sample was run on 2% agarose gel to check the integrity. Next, RNA was treated with DNase I (Promega) to remove genomic DNA. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using

SYBR Green PCR Core Reagents (Applied Biosystems) and the $^{2-\Delta\Delta}C_T$ method [19]. Reaction mixtures [containing 10 µl of 2 x SYBR Green supermix, 5 µl of primers (0.6 µM each) and 5 µl of cDNA template] were incubated (10 min, 95ºC), followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. *rps18*, *actb* and *ef1a* were used as reference genes (verified in [20]). Negative controls had no amplification product and control templates showed no primer-dimer formations. All samples were measured in triplicates, expressed as mean ± SEM and analysed with SPSS software v19.0 (SPSS, Chicago, USA).

3 Results and discussion

A draft sequence of the European sea bass (*D. labrax*, ID 13489) genome was recently published [2], however at the time of our study only 2,420 *D. labrax* proteins were registered in the NCBI database, whilst the fully sequenced and well-annotated zebrafish (*Danio rerio*, ID 7955) genome had 81,527 protein entries. Working with species with less-annotated genomes, homology-driven proteomics is the major tool to characterize proteomes [21]. From our own experience working on fish skin mucus [22,23] and with proteomic tools [24,25], 2DE followed by LC-MS/MS provides good resolution and high performance for protein detection and identification.

We have identified a wide range of proteins in skin mucus of *D. labrax* (Figure 1, Tables 2 and 3). These could be remains of dead cells from the skin surface or proteins actively secreted to function in the mucus. Due to the importance of mucus as a barrier of defence, we have focused our attention according to the capacity of these proteins to be candidates as fish health indicators. Furthermore, the transcript levels present in skin of *D. labrax* have been demonstrated (Figure 2).

3.1 Skin mucus proteome as first barrier of defence in *D. labrax*

The proteome of European sea bass skin mucus includes proteins with well-established functions such as several complement components, lectins, proteins involved in apoptosis, inflammation, redox homeostasis, stress as well as antimicrobial activity. The proteins have mainly been studied in cells, tissues and blood, in our results and discussion we focus on these proteins in an extracellular setting.

3.1.1 Proteins identified with pathogen interacting capacity.

We identified several proteins that could interact directly with pathogens and lead to lysis, agglutination, growth inhibition and/or modulation of host cell surfaces binding.

The complement system plays a major role in vertebrate defence against pathogens in the blood as part of both the innate, and adaptive immune systems [26,27]. Upon activation by the surface of pathogens and host factors such as mannose binding lectins, in the innate pathways, or antibodies, in the adaptive pathway, a series of cleavages of complement factors are initiated. An intermediate key factor, C3, can upon cleavage act as a chemoattractant (recruit immune cells), as an opsonin (coat pathogens) to increase phagocytosis, as an agglutinin (coagulate pathogens) or initiate further cleavages resulting in bacterial lysis. C3 was found in different regions in 2DE gels of *D. labrax* skin mucus: two isoforms of around 42 kDa (spots 77 and 83) and another two isoforms of around 85 kDa (spots 101 and 102). We cannot conclude that C3 is cleaved and activated in mucus; however the presence of cleaved C3 could indicate active roles in skin mucus (Figure 3).

C1q and C1q-TNF family members involved in classical complement activation are also present [28] (Figure 3). The present study is restricted to the analysis of the 100 most prominent protein spots in skin mucus (Figure 1), hence it cannot be excluded that antibodies needed for C1q activation or other complement factors are present. It is the first time that C1q proteins are observed in fish skin mucus (spots 36, 38 and 42).

Lectins, carbohydrate binding proteins, have been reported from various tissues of a diversity of fish species and they have also been isolated from skin, mucus, serum, and plasma. Mannose-binding lectins activate the lectin pathway in the complement system (Figure 3), have been identified in Atlantic cod mucus [24], but were not found in our study. Fucose-binding lectin (FBL) recognizes carbohydrates on the surface of potential pathogens, leads to agglutination, immobilization, and opsonization of microbial pathogens, and phagocyte activation [29] (Figure 3). This molecule was identified (spots 13, 14 and 43) in skin mucus of *D. labrax*, and a 34 kDa F-type lectin was previously reported in serum of the same fish species [30]. F-type lectin was not previously reported in fish mucus, however a C-type lectin was reported in skin mucus of a cichlid [31], and galectin in Atlantic cod [24].

Lysozyme (LYZ) has been widely studied in the animal kingdom and identified as a hydrolytic enzyme with the capability to lyse bacteria by cleaving the β-(1,4)-glycosidic bond between N-acetylmuramicacid and N-acetylglucosamine residues of peptidoglycan in the bacteria cell wall (Figure 3). Thus, LYZ is an important component of the vertebrate innate immune system [32]. In the present paper, two isoforms of LYZ have been identified (spots 67 and 70) of around 20 kDa in skin mucus of *D. labrax*. In agreement with our study, two isoforms of LYZ were also identified and characterized in skin mucus of Atlantic cod [24]. Upregulation of lysozyme mRNA in head kidney leukocytes of *D. labrax* after bacterial infection has recently been shown [33], further studies are needed to unravel if LYZ levels change in skin mucus under stress stimulus or pathogen infections.

Apolipoprotein A1 (APOA1) is the major protein component of high density lipoprotein in serum [34]. APOA1 was identified with a MW of around 28 kDa (spots 16 and 33). In Atlantic salmon and Atlantic cod, this protein was identified and over expressed in skin mucus after sea lice and bacterial infection, respectively [35,36]. Moreover, APOA1 in striped bass has demonstrated antibacterial activity *in vitro* [37] and lytic activity has been

shown for APOA1 from channel catfish [38] (Figure 3). The protein-protein interaction network analysis showed that APOA1 has several strong interactions with PDI, WAP65 and TF mammalian orthologues (see Supplementary file 1).

Transferrin (TF) belongs to an ancient family found in all metazoans. TF is responsible for the transport and delivery of iron to cells [39]. In addition, it is bacteriostatic by limiting the availability of iron to bacteria (Figure 3). Transferrin, which has been characterized in liver and brain of *D. labrax* [40], was identified in skin mucus (spot 98 and 99) in the present paper with the highest score (see Table 2). Transferrin was previously identified in skin mucus of gilthead seabream [41] and Atlantic cod juveniles [7]. In Atlantic salmon skin mucus, an increased level of a transferrin fragment was found after sea lice infection [35].

Typically, histones and their post-translationally modified forms have roles in chromatin remodelling and gene transcription. However, histones act as damage-associated molecular pattern molecules when they are released into extracellular spaces [42]. In our study, we have identified H1 and H4 in skin mucus of *D. labrax*. It has been reported that H1 has antimicrobial properties (see Figure 3) in Atlantic salmon [43], in skin of rainbow trout [44], and in skin secretions of rainbow trout [45]. H4 has been shown to be antimicrobial (see Figure 3) in humans [46].

The warm temperature acclimation related 65 kDa protein (WAP65) shares high structural similarities with mammalian hemopexins, which can bind iron containing heme serving a protective role against bacterial infections (see Figure 3) in skin mucus by limiting available iron. It has been shown to be involved in temperature acclimation, in immune response, as well as in development in teleost [47]. In our study, two isoforms of this protein (spot 96 and 103) of around 65 kDa and 70 kDa, respectively, were identified in skin mucus of *D. labrax.* This protein was reported in skin mucus of gilthead seabream as well [41].

Calreticulin (CALR), a calcium-binding protein identified in the cell surface of neutrophils as a receptor for C1q [48], also promotes phagocytosis by microbial binding [49]. It has been previously characterized in *D. labrax* [50], and in the present paper, it was identified as a protein of around 60 kDa (spot 72). CALR was also identified in skin mucus of Atlantic cod, with a similar MW [24]. In both cases, the observed MW did not match with the theoretical values of 49.4 (Table 2), suggesting post-translational modifications such as glycosylation, as is known to be the explanation for the observed high molecular weight of human CALR in SDS-PAGE.

3.1.2 Proteins identified with a possible role interacting with or functioning in cells in mucosal surfaces.

Proteins described in 3.1.1, which binds pathogens can in many cases also stimulate phagocytosis (Figure 3). In the mucosal surface of the skin, the mucus will act as a protective barrier to prevent pathogen interaction with the live cells in the surface, should it fail due to e.g. wounds the second line of defence will serve a role, this include the phagocytic cells recruited by chemotaxis. Immune related molecules could have several functions in the mucosal surface (mucus and outer cell layer) such as stimulation of inflammation, chemotaxis and phagocytosis in addition to pathogen binding giving agglutination, lysis or growth inhibition. The surface of the skin also needs to be protected from external stressor, several of the identified proteins could have stress-limiting functions (Figure 3).

Vimentin and beta-actin are multifunctional proteins involved in motility, migration, cell adhesion and phagocytosis, among others. Vimentin was found as two isoforms, spot 76 at 52 kDa, and spot 55 at 25 kDa. Interestingly, in a human monocytic cell line extracellular full length vimentin is a chemoattractant, whilst a cleavage product stimulate phagocytosis (Figure 3) [51]. This is the first time that vimentin is reported in skin mucus, and hence functional studies have not yet been done. Beta-actin, previously found in mucus [24,35,52],

can be fragmented after stress [35]. In agreement with that, in our study we have identified a beta actin of 42 kDa (spot 79), the theoretical MW, another beta-actin of 35 kDa (spot 85), and two isoforms of around 28 kDa (spots 39 and 66). Increased levels of beta-actin and vimentin fragments in skin mucus could be indicators of disease or stress.

In our study, we identified apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC, spot 64) at 28 kDa, matching with the theoretical MW. ASC is an adaptor protein that has a bipartite domain structure, an N-terminal PYRIN domain and a C-terminal caspase activation and recruitment domain [53]. ASC and caspases are important in the cell death pathway, apoptosis. Two caspases (CASP) were identified in skin mucus of *D. labrax*. CASP1 (spot 48), responsible for the production of IL1B proinflammatory cytokine during immune response to microbial pathogens [54], and CASP6 (spot 15), an activator of CASP8 which promotes apoptosis [54,55]. They were identified for the first time in skin mucus. Gene ontology analysis (Supplementary file 1) showed the strongest protein-protein interaction, of the interactions identified, between ASC and CASP1 mammalian orthologues. ASC has a pivotal role in the CASP1-dependent processing of proinflammatory cytokines [56]. Interestingly, ASC and CASP1 have recently, in mouse gut, been identified as key regulators of mucus secretion by exocytosis [57], they are also part of an extracellular oligomeric complex (the NLRP3 inflammasome) which is secreted upon danger signals and is suggested to act to amplify inflammatory response [58].

In our study cyclophilin A was identified as a protein of around 18 kDa (spot 25), it was also found in skin mucus of Atlantic cod [24]. Cyclophilins are a group of highly conserved cytosolic enzymes that have a peptidylprolyl cis/trans isomerase activity and belong to the immunophilin family. Among them, cyclophilin A can be secreted in the extracellular space by inflammatory cells when exposed to stressors and upon cell death [59]. Extracellular cyclophilin A stimulates pro-inflammatory signals in endothelial cells [60]. To our

knowledge this is the first time that cyclophilin A is identified for *D. labrax*, it could be a serious candidate as stress and immune status indicator through *D. labrax* skin mucus analysis.

Several of the identified proteins could protect against external stressor such as physical parameters e.g. temperature and chemical parameters such as oxidation and some could in addition have a role in the immune system.

Peroxiredoxins (PRDX) or natural killer cell enhancing factors (NKEFs) are a family of antioxidant proteins also involved in inflammation and innate immunity (Figure 3) [61,62]. In the present work, NKEF was identified (spot 6) in skin mucus of *D. labrax*. In agreement with our results, NKEF1 and NKEF2 were recently found in skin mucus of gilthead seabream [41]. Furthermore, it has been reported that extracellular NKEF1 act as a "endogenous" danger signal by binding danger signal sensors/receptors [63], suggesting that NKEF may be a stress indicator in skin mucus.

The human thioredoxin superfamily members are thiol oxidoreductases with a role in various cell signalling pathways. ERp57 and protein disulphide isomerase (PDI) are two members of this superfamily, which have a common structure but different multifunctional roles. ERp57 acts on glycosylated substrates in the endoplasmic reticulum through interaction with the lectin-like CALR (spot 72 described in section 3.1.1) [64], and has a critical role in major histocompatibility complex class I assembly [65]. PDI is mainly associated with the protection against reactive oxygen species (ROS) [66], however it also has an extracellular role in regulating flip-flop (transbilayer movement) of phosphatidylserine in the cell membrane[67]. ERp57 was identified as two isoforms (spot 74 and 97) and PDI was identified as a protein of around 57 kDa (spot 104) in skin mucus. Both proteins were previously characterized in *D. labrax* [68].

Superoxide dismutase (SOD) is an enzyme that catalyses the reaction of anion superoxide (O_2^-) into hydrogen peroxide (H_2O_2) at the extracellular matrix [69], protecting the tissue against oxidative stress by regulating various ROS and reactive nitrogen species molecules [70]. In our study, SOD Zn/Cu (spot 12) was identified in skin mucus of *D. labrax* with a MW around 16 kDa. Recently, this protein was also reported in skin mucus of fish with a similar p*I* and MW [41]. Moreover, in another study it was observed that antigen-dependent activation of T lymphocytes significantly increased extracellular SOD-1 levels [71]. In mice, deletion of the extracellular SOD, lead to lung injury at ambient air due to increased levels of superoxide [72], indicating that extracellular SOD could be important for cellular integrity also in the mucosal surface of the fish skin.

Glutathione S-transferases (GST) are the superfamily of phase II detoxification enzymes that play crucial roles in cellular defence. In our study, two GSTs were identified (spots 46 and 49), at 31 kDa and 26 kDa, respectively. It was previously reported in Atlantic cod skin mucus [24], and differentially expressed after infection [36]. In rat it has been suggested to play a role in detoxification of electrophilic compounds the small intestine mucus [73] (Figure 3).

Serpins are a homologous family of proteins with diverse functions in processes such as blood coagulation, fibrinolysis, programmed cell death, development and inflammation [74]. Leukocyte elastase inhibitor (LEI) belongs to this family and function to limit and fine tune protease activity to limit host damage (Figure 3) during inflammation and apoptosis [75] and during pathogen destruction by host through proteolytic activity. In our study, LEI (spot 29) has been identified in skin mucus of *D. labrax* as a protein of 34 kDa. In another report, it was identified in numerous spots in Atlantic cod skin mucus [24], one of them with similar *pI* and MW than in the present paper.

Heat-shock proteins (HSP) are part of a superfamily of stress proteins, highly conserved across species, often classified based on their molecular weight. HSP70 is constitutively expressed but can be induced to higher levels by stressors such heat, pathogens, heavy metals. It can be cytosolic [76], or extracellular [77]. HSP70 can function both as an inhibitor or stimulator of inflammation, it has been suggested that the mode of activation, location and/or co-molecules present will influence the function [77] (Figure 3). In our study, HSP70 (spot 91) was identified at the expected 70 kDa. *hsp70* is induced after heavy metal exposition and hypothermia in skin of common carp [78]. Moreover, a HSC70 was reported in skin mucus of gilthead seabream [41].

Antifreeze proteins (AFP) are a group of small proteins with a carbohydrate domain typically secreted by the liver into the blood in teleosts. However, it has become clear that AFP isoforms are produced in the epidermis (skin, scales, fin, and gills) and may serve as a first line of defence against ice propagation into the fish [79] (Figure 3). In our study, AFP was identified as a protein of around 10 kDa (spot 17). Little information is available about its structure and function in *D. labrax*, it may not only be needed to be able to live in different parts of Europe (see introduction), but may through its carbohydrate-binding domain interact with bacteria (Figure 3).

Calmodulin (CALM) is a calcium-binding messenger protein involved in apoptosis, inflammation and immune response [80]. In our study, this protein was identified in two isoforms (spots 1 and 2) with different sizes; suggesting post-translational modifications in skin mucus (see Figure 1). CALM was previously found in fish mucus of tilapia, catfish and rainbow trout, increased secretion of CALM was found when there were decreased calcium concentrations in the water (Figure 3), and a role in control of cell membrane permeability in the epithelium has been suggested [8].

Keratins are intermediate filaments that form heteropolymeric filaments containing type I and type II keratins, they have a physical protective role in skin, and are also involved in cell proliferation and apoptosis [81], both filament types were found in skin mucus of *D. labrax*. BLASTp analysis against human keratins was carried out to discard any human contamination (data not shown), the peptides identified were non-human. Keratins have been reported in skin mucus from gilthead seabream [41] and Atlantic cod [24]. Further studies are needed in fish to see if keratin could have a role in fish mucus.

Other proteins identified are involved in other cellular processes (see Table 2) and have at present an unlikely role in skin mucus, and were therefore discarded as candidates for fish disease diagnosis. Their presence could be due to natural sloughing of cells in mucus, rather than active secretion and their extracellular function, if any, remains unknown.

3.2. Gene expression profile in *D. labrax* skin

The selected immune-related gene expression profiles showed a transcript for every target in *D. labrax* skin (Figure 2), for each gene, including reference genes, a single peak in each melt curve was observed (not shown). To our knowledge, despite of being involved in many immune-related processes, this is the first time that most of the immune relevant transcripts such as *c3*, *c1q*, *afp*, *fbl*, *lyz*, *calm*, *calr*, *erp57*, *pdi*, *apoa1*, *tf*, *lei*, *gst*, *cypa*, *asc*, *casp1*, *casp6*, *sod and wap65a and wap65b* are shown in skin from *D. labrax*, although *hsp70* and *nkef* gene expression have been previously reported [62,82]. In other teleosts, *tf* was found in skin of Atlantic salmon [35] and gilthead seabream [83] and *cypa* was found in Atlantic cod [24] while *lyz* and *c1q* were present in channel catfish [84]. The presence of transcripts in skin indicates that the protein products of these transcripts could be synthesised in the skin itself. Hence, this could be considered a starting point to study immune-related processes in skin, especially against microbial infections.

4 Concluding remarks

This study establishes the first proteome map of European sea bass (D. labrax) skin mucus. We identified proteins with known or suspected stress response or immune function, of which several were not previously described in fish skin mucus. We found proteins that could interact directly with pathogens, as well as proteins with a potential role in interacting with or functioning in the cellular surface of the skin. The results support the idea that intricate local signalling networks are present in the mucosal surface of fish skin. Further studies are needed to establish if any of these proteins could be used as immune- or stress markers.

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Conflict of interest:

The authors declare no competing financial interest.

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Figure 1. *D. labrax* skin mucus 2DE map. Two hundred µg of proteins were loaded on 17 cm, 3-10 nonlinear IPG strips. Second dimension was a 12.5 % polyacrylamide vertical gel. Red circles and numbers show analysed protein spots.

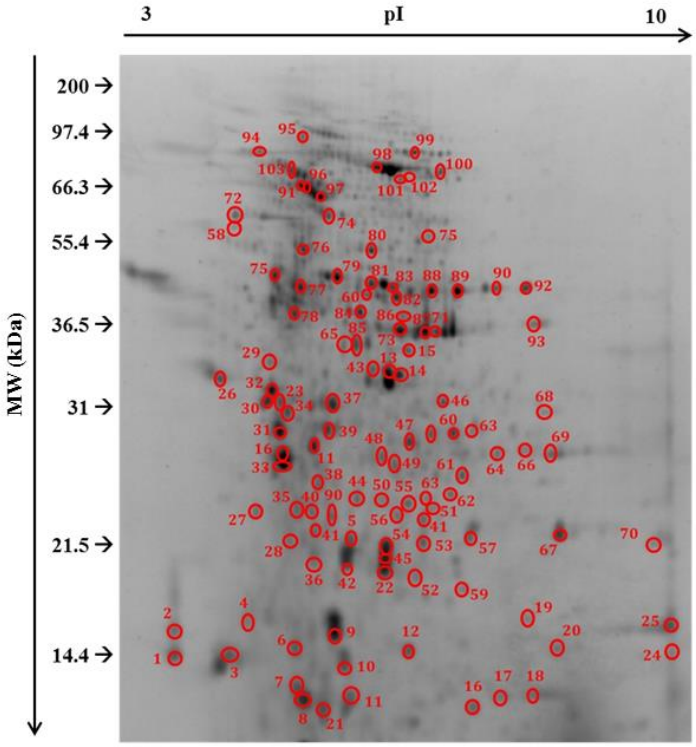

Figure 2. RNA expression levels in skin for the identified immune-related molecules from *D. labrax* mucus, relative to the reference genes *rps18*, *actb* and *ef1a*. n=3; and three replicates of analysis.

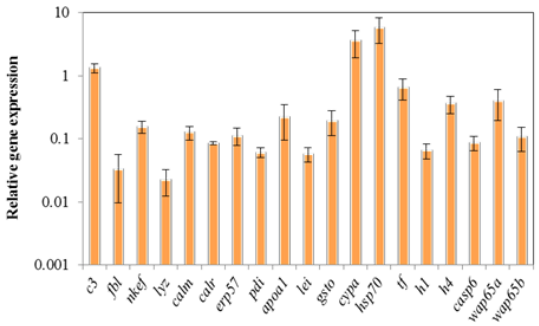

Figure 3. The figure shows some of the proteins identified from the skin mucus of *D.labrax* and their possible stress or immune related roles in the mucus and mucosal surface. Arrows point to the process or molecule influenced; lines with a perpendicular end indicate inhibition of a process. The abbreviations are found in table 2 and details of the biological processes are described in the results and discussion section.

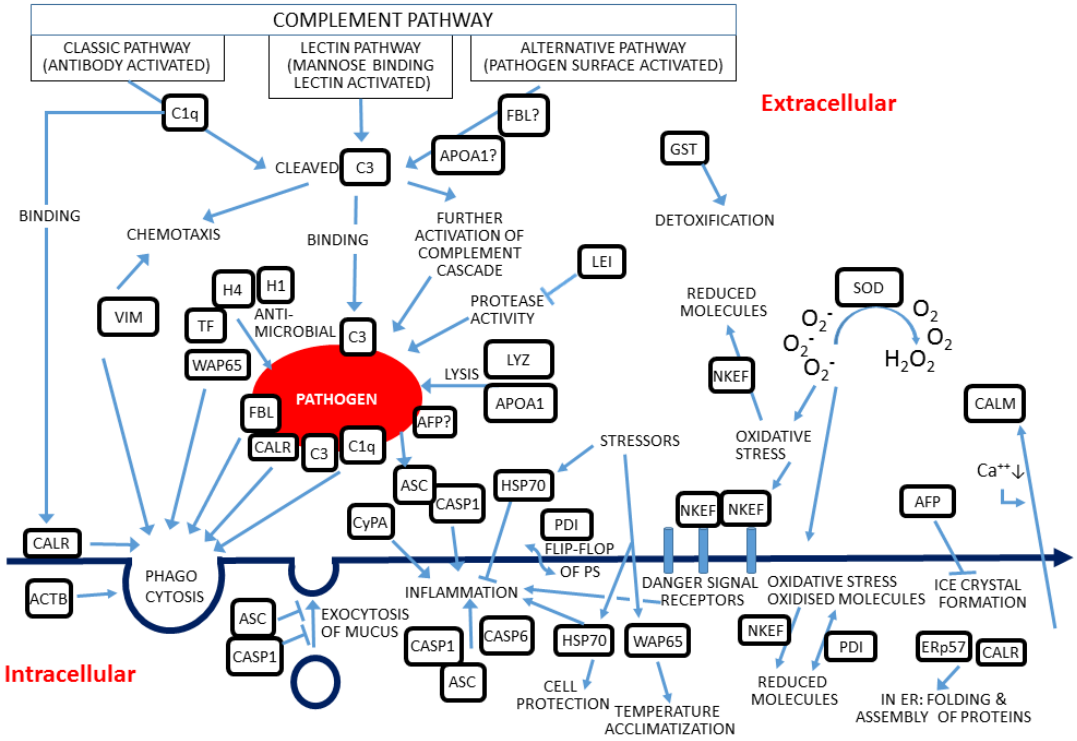

Table 1. Primers used in qPCR study.

Gene[a)]	Symbol [b)]	ACC number [c)]	Sequence (5' →3')	AS [d)]
elongation factor 1 alpha	*ef1a*	AJ866727	F: CGTTGGCTTCAACATCAAGA R: GAAGTTGTCTGCTCCCTTGG	99
beta-actin	*actb*	AJ537421	F: TCCCTGGAGAAGAGCTACGA R: AGGAAGGAAGGCTGGAAAAG	98
ribosomalprotein s18	*rps18*	AY831388	F: TTCCTTTGATCGCTCTTAACG R: TCTGATAAATGCACGCATCC	102
complementcomponent 3	*c3*	FK942306	F: TGAGAGGAGAGCTGGAGAGC R: GTTGTCGATGTTGCCCTTCT	103
c1q tumor necrosis factor-related protein 3	*c1q*	FM002850	F: ACACCAACACCACGCTGATA R: CCGGTAATCCGGGTGTAGTA	118
antifreeze protein	*afp*	FN565768	F: GGCTGACAATGAATGGGTTT R: AGCCAACACGTGTACCATCA	113
fucosebindinglectin	*fbl*	EU877448	F: TGCCTACAGCGCTATTGATG R: CTCCAGCAGGTCAACTCTCC	106
natural killer cell enhancement factor	*nkef*	FM024824	F: CACTGAGATCGTGGCTTTCA R: TGTGTTGATCCATGCCAAGT	112
g-type lysozyme	*lyz*	FN667957	F: TTGCAGCTCATTCCAGTTTG R: TGTCCTGCTGAGATGTTTGC	116
calmodulin	*calm*	FL487943	F: ATTGACTTCCCGGAGTTCCT R: TTGTCAAATACCCGGAAAGC	95
calreticulin	*calr*	JX235975	F: CATCAAATGCAAGGATGACG R: AGCCAGACTCCACCTTCTCA	104
endoplasmic reticulum protein 57	*erp57*	JX891474	F: CCCACATGACAGACGACAAC R: CATCACCCTGTTCCTCCAGT	119
protein disulphide isomerase	*pdi*	JX891476	F: AGAGAGCATCACCGCATTCT R: GGGTTTTGTCCCAGTCTTCA	95
apolipoprotein A1	*apoa1*	CV186176	F: GGCAGTCATCGATAAGCACA R: CTTTACCTTCAGGGCATCCA	106
leukocyte elastase inhibitor	*lei*	FM018382	F: TGTACGGGGAGCAGTCCTAC R: GAGCCTAGCTGCGTCTGAGT	116
glutathione S-transferase	*gst*	FM027169	F: CTGCTTCCCTCCTCTCCTTT R: TCCCTGTGGGGATCTTGTAG	97
cyclophilin A	*cypa*	FM026623	F: GGGGAGAAGTTTGCTGATGA R: AGTTTTAGCCGTGCAGAGGA	120
heat-shock protein 70KDa	*hsp70*	AY423555	F: CTGCTAAGAATGGCCTGGAG R: CTCGTTGCACTTGTCCAGAA	119
transferrin	*tf*	FJ197144	F: CGCTTCATCTACTGCCATCA R: CGTCAGCACCCATACTGTTG	92
caspase-1	*casp1*	DQ198376	F: CCAGATCGTGGGTGTTTTCT R: TCTTCAAAGCGTTGCATGAC	110
caspase-6	*casp6*	AM988220	F: ACAAGTGCAACAGCCTTGTG R: CAGCTCACTGTCCACAGCAT	110
apoptosis-associated speck-like protein containing a CARD	*asc*	FM020581	F: GATCAACAGAGCGAGCAACA R: AGTGGTACGCAGAGCCCTAA	103
superoxide dismutase Zn/Cu	*sod*	FJ860004	F: TGTTGGAGACCTGGGAGATG R: ATTGGGCCTGTGAGAGTGAG	90
warm temperature acclimation protein 65-1	*wap65a*	BK006867	F: TCCGCTTTATGGAGCACTTT R: GCCTCTTTGGGGTATCTTCC	97
warm temperature acclimation protein 65-2	*wap65b*	BK006868	F: AGGAGGTGACCAATGGAGTG R: TGTAGTGAGCCGCTGCTTTA	102

a) Gene names according to UniProt database (http://www.uniprot.org/).
b) Gene symbol according to zebrafish nomenclature (http://zfin.org/).
c) Accession number of each gene sequence from NCBI database (http://www.ncbi.nlm.nih.gov/).
d) Amplicon size (bp).

Table 2. Identified proteins from *D. labrax* skin mucus grouped into biological groups.

SN[a)]	Protein	Symbol[b)]	Species	ID / Database[c)]	*pI* / MW[d)]	S / C[e)]	U/T[f)]
	Structural proteins						
39	Actin beta	ACTB	*Dicentrarchus labrax*	CAD60932 / NCBI	5.29/42.1	51/2	1/1
66	Actin, cytoplasmic 1	ACTB	*Ctenopharyngodon idella*	P83751 / Sprot	5.30/42.1	20/4	1/1
79	Actin, cytoplasmic 1	ACTB	*Oreochromis mossambicus*	P68143 / Sprot	5.30/42.1	856/40	7/16
85	Actin, cytoplasmic 1	ACTB	*Oreochromis mossambicus*	P68143 / Sprot	5.30/42.1	266/20	7/7
95	Alpha actinin-4 isoform X1	ACTN	*Stegastes partitus*	XP_008281276 / NCBI	4.90/103.2	578/14	1/7
6	Coactosin-likeprotein-like	COTL1	*Oryzias latipes*	XP_004069874 / NCBI	4.83/16.2	141/19	1/2
5	Cofilin-2	COF2	*Dicentrarchus labrax*	FM006818 / EST	8.38/28.2	167 / 16	5/5
84	F-actin-capping protein subunit alpha-2	CAPZA2	*Salmo salar*	ACN58682 / NCBI	5.84/32.3	131/11	1/2
65	F-actin-capping protein subunit alpha-1	CAPZA1	*Dicentrarchus labrax*	CBN80762 / NCBI	5.42/32.8	80/14	2/2
60	Gelsolin-like	GSNL1	*Oreochromis mossambicus*	ABE98236 / NCBI	5.96/42.9	66/5	3/3
89	Gelsolin-like	GSNL1	*Stegastes partitus*	XP_008276815 / NCBI	6.54/79.8	159/6	1/4
92	Gelsolin-like	GSNL1	*Xiphophorus maculatus*	XP_005802408 / NCBI	6.28/79.6	241/7	6/6
100	Gelsolin-like	GSNL1	*Oreochromis mossambicus*	ABE98236 / NCBI	5.96/42.9	87/5	2/2
7	Keratin type II cytoskeletal 8-like	KRT8	*Maylandia zebra*	XP_004545214 / NCBI	5.03/62.2	104/5	1/3
26	Keratin, type I cytoskeletal 17-like	KRT17	*Stegastes partitus*	XP_008298721 / NCBI	5.22/48.3	128/3	3/3
90	Keratin, type II cytoskeletal 8-like	KRT8	*Stegastes partitus*	XP_008303627 / NCBI	5.97/50.3	82/7	2/2
93	Keratin, type I cytoskeletal 13-like	KRT13	*Lepisosteus oculatus*	XP_006638395 / NCBI	5.05/49.7	63/2	1/6
47	Microfibril-associatedglycoprotein 4	MFAP4	*Dicentrarchus labrax*	FM019963 / NCBI	5.88/30.0	95/12	2/2
3	Myosin light polypeptide 6	MYL6	*Anoplopoma fimbria*	ACQ58516 / NCBI	4.41/17.1	61/17	2/2
88	Scinderin-likeprotein	SCINL	*Paralichthys olivaceus*	AFQ38973 / NCBI	6.54/80	120/4	1/3
28	Type II keratin E3-like protein	KRT	*Sparus aurata*	AAT44423/ NCBI	4.89/38.6	71/6	1/2
78	Type II keratin E3	KRT	*Oncorhynchus mykiss*	NP_001123458 / NCBI	5.32/55.3	509/20	1/12
63	Tropomyosin alpha-1 chain	TPM1	*Liza aurata*	P84335 / Sprot	4.69/32.8	24/4	1/1
55	Vimentin	VIM	*Cynoglossus semilaevis*	XP_008332705 / NCBI	5.26/52.8	47/3	1/2
76	Vimentin	VIM	*Cyprinus carpio*	1807305A / NCBI	5.07/52.6	49/3	1/2
18	Profilin	PFN1	*Dicentrarchus labrax*	FM000924 / EST	7.74/23.5	280/16	4/4
	Protein metabolism						
54	40S ribosomalprotein S18	40S	*Ictalurus punctatus*	Q90YQ5 / Sprot	10.99/17.7	19/5	1/1
41	60S ribosomalprotein L15	60S	*Carassius auratus*	Q7T3N9 / Sprot	11.53/24.1	13/3	1/1
87	Alcohol dehydrogenase	ADH	*Salmo salar*	ACN10195 / NCBI	6.32/37.2	99/6	1/2
24	Anterior gradient protein 2 homolog	AGR2	*Maylandia zebra*	XP_004561006 / NCBI	8.87/19.1	136/22	2/4
22	Cyclin-dependent kinase 7	CDK7	*Carassius auratus*	P51953 / Sprot	8.98/38.6	19/4	1/1
82	Elogantion factor 1-alpha	EF1A	*Oryzias latipes*	Q9YIC0 / Sprot	9.23/50.6	24/6	1/1
9	Golgi-associated plant pathogenesis-related protein 1-like	GAPR1	*Maylandia zebra*	XP_004576580 / NCBI	5.38/18.7	62/6	1/1
45	Progonadoliberin-2	GNRH2	*Clarias gariepinus*	P43306 / Sprot	9.27/10	19/10	1/1
34	Secretagogin	SCGN	*Astyanax mexicanus*	XP_007256889	5/31.7	117/8	2/2
8	SH3 domain-binding glutamic acid-	SH3BGRL	*Osmerus mordax*	ACO10145 /	4.78/13.1	68/21	2/2

	rich-like protein			NCBI			
27	Translationally-controlled tumor protein	TCTP	*Dicentrarchus labrax*	FM000425 / EST	5.91/31.8	73/11	3/3
	Carbohydrate metabolism						
59	Deoxycytidylate deaminase	DCTD	*Dicentrarchus labrax*	FM019776 / NCBI	8.60/31.7	67/3	1/1
75	Enolase A	ENOA	*Acipenser baerii*	ABF60006 / NCBI	5.98/47.5	125/10	3/3
58	Fructose-bisphosphatealdolase B	ALDOB	*Sparus aurata*	P53447 / Sprot	8.43/40.2	17/4	1/1
50	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	*Oncorhynchus mykiss*	O42259 / Sprot	6.37/36.6	18/5	1/1
86	Inosine-uridine preferring nucleoside hydrolase-like	IUNH	*Maylandia zebra*	XP_004575422 / NCBI	6.88/35.4	63/5	2/2
11	Inositol monophosphatase	IMPA	*Dicentrarchus labrax*	CBN82127 / NCBI	5.47/28.9	403/29	4/6
20	Nucleoside diphosphate kinase	NDK	*Siniperca chuatsi*	AAY79301 / NCBI	5.86/13	107/37	2/4
44	Phosphatidylethanolamine binding protein	PEBP	*Ictalurus punctatus*	NP_001187975 / NCBI	6.82/21.2	92/13	3/3
4	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2B	INPPL1	*Sparus aurata*	FM148029 / NCBI	4.54/13.2	54/8	1/1
71	Transaldolase-like	TALDO	*Oryzias latipes*	XP_004066906 / NCBI	6.69/37.8	163/9	3/3
68	Triosephosphate isomerase B	TPI1B	*Oreochromis niloticus*	XP_003450633 / NCBI	6.9/26.9	151/14	3/3
69	Triosephosphate isomerase B	TPI1B	*Oryzias latipes*	BAD17901 / NCBI	6.14/23	182/24	1/4
	RNA/DNA metabolism						
51	Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	*Salmo salar*	ACI67551 / NCBI	9.1/29.4	119/6	1/1
61	Homeobox protein HMX3-B	HMX3B	*Oryzias latipes*	Q90XN9 / Sprot	6.42/32.6	13/2	1/1
35	Protein SET-like	SEPSIP	*Oreochromis niloticus*	XP_003439510 / NCBI	4.15/31.1	93/8	2/2
94	RNA-bindingprotein 12	RBP12	*Stegastes partitus*	XP_008296875 / NCBI	9.24/95.5	45/1	1/1
52	Transducin-like enhancer protein 1	TLE1	*Oryzias latipes*	XP_004072378 / NCBI	8.08/69.5	45/1	1/1
73	Histone H1	H1	*Astyanax fasciatus*	AEC13086 / NCBI	11.05/20.8	46/5	1/1
81	Histone H4	H4	*Oncorhynchus mykiss*	P62797 / Sprot	11.36/113.6	25/11	1/1
	Signal transduction						
23	14-3-3 protein epsilon-like isoform X1	14-3-3	*Poecilia formosa*	XP_007563007 / NCBI	5.02/36.9	153/10	3/3
30	14-3-3 protein beta/alpha-A-like	14-3-3	*Astyanax mexicanus*	XP_007230880 / NCBI	4.65/28	178/15	2/4
31	14-3-3 protein beta/alpha-1-like	14-3-3	*Xiphophorus maculatus*	XP_005805709 / NCBI	4.62/27.7	291/21	3/6
32	14-3-3 protein epsilon-like isoform X1	14-3-3	*Stegastes partitus*	XP_008291071 / NCBI	4.74/30	116/10	1/2
21	S100-A6	S100A6	*Anoplopoma fimbria*	ACQ58920 / NCBI	5.08/13	74/11	1/1
80	Rab GDP dissociation inhibitor beta-like	GDI2	*Astyanax mexicanus*	XP_007252464 / NCBI	5.6/50.8	368/16	2/6
19	Rho GDP-dissociation inhibitor 1	GDI1	*Dicentrarchus labrax*	FM018448 / EST	5.36/32.4	88/11	1/2
37	Rho GDP-dissociation inhibitor 1-like	GDI1	*Oryzias latipes*	XP_004071582 / NCBI	5.01/23.5	178/16	4/4
	Immune-related proteins						
17	Antifreeze protein	AFP	*Dicentrarchus labrax*	FN565768 / EST	6.47/21.8	88/14	2/2
16	Apolipoprotein A-1	APOA1	*Morone saxatilis*	ACH90227 / NCBI	4.75/20.6	300/31	4/7
33	Apolipoprotein A-1	APOA1	*Morone saxatilis*	ACH90229 / NCBI	5.09/16.1	434/35	5/8
64	Apoptosis-associated speck-like protein containing a CARD	ASC	*Dicentrarchus labrax*	FM020581 / EST	5.85/28.5	196/11	2/2
1	Calmodulin	CALM	*Electrophorus electricus*	P02594 / Sprot	4.6/16.8	96/7	2/2
2	Calmodulin	CALM	*Electrophorus electricus*	P02594 / Sprot	4.09/16.8	118/11	1/1

72	Calreticulin precursor	CALR	*Dicentrarchus labrax*	AGI60286 / NCBI	4.37/49.4	258/17	2/5
48	Caspase-1	CASP1	*Dicentrarchus labrax*	AM984268 / EST	8.57/24.5	293/32	5/5
15	Caspase-6	CASP6	*Cynoglossus semilaevis*	XP_008315389 / NCBI	6.02/34.5	84/5	1/1
36	C1q family protein	C1Q	*Dicentrarchus labrax*	FM002850 / EST	8.64/19.4	100/15	1/3
38	C1q-like protein	C1Q	*Dicentrarchus labrax*	FM000708 / EST	5.77/25.4	143/9	2/2
42	C1q tumor necrosis factor-related protein 3-like	C1Q	*Dicentrarchus labrax*	FL487070 / EST	8.03/20.2	278/45	2/7
77	Complement component 3	C3	*Solea senegalensis*	ACR20030 / NCBI	6.04/6.5	69/17	1/1
83	Complement component 3	C3	*Epinephelus coioides*	ADU33222 / NCBI	6.07/186.2	62/1	2/2
102	Complement component 3	C3	*Larimichthys crocea*	AHZ41228 / NCBI	6.15/186.8	108/2	2/3
101	Complement component 3	C3	*Sparus aurata*	ADM13620 / NCBI	8.08/186.9	67/1	1/2
25	Cyclophilin A	CyPA	*Gadus morhua*	AEK21703 / NCBI	8.51/18	48/5	1/1
74	Endoplasmic reticulum p57	PDIA3	*Dicentrarchus labrax*	AGI60170 / NCBI	5.39/56.3	256/18	5/7
97	Endoplasmic reticulum p57	PDIA3	*Dicentrarchus labrax*	AGI60170 / NCBI	5.39/56.3	122/8	4/4
13	Fucose binding lectin	FBL	*Dicentrarchus labrax*	ACF94293 / NCBI	6.08/34.8	317/29	6/6
14	Fucose binding lectin	FBL	*Dicentrarchus labrax*	ACF94293 / NCBI	6.08/34.8	212/13	5/5
43	Fucose binding protein precursor	FBL	*Morone chrysops*	ABB29990 / NCBI	6.21/34.7	115/7	2/2
46	Glutathione S-transferase omega-1	GST	*Anoplopoma fimbria*	ACQ58017 / NCBI	7.01/27.8	47/3	1/1
49	Glutathione S-transferase mu	GST	*Takifugu obscurus*	ABV24049 / NCBI	5.47/26.4	124/11	1/3
91	Heat shock protein 70 kDa	HSP70	*Dicentrarchus labrax*	AAR01102 / NCBI	5.31/71.6	52/5	2/2
29	Leukocyte elastase inhibitor	LEI	*Dicentrarchus labrax*	CBN81773 / NCBI	4.9/44.7	205/14	5/5
67	Lysozyme	LYZ	*Paralichthys olivaceus*	Q90VZ3 / NCBI	8.69/21.4	86/14	2/2
70	Lysozyme g protein	LYZ	*Dicentrarchus labrax*	CBJ56263 / NCBI	8.53/20.4	58/9	1/1
6	Natural killer cell enhancing factor	NKEF	*Anoplopoma fimbria*	ACQ58049 / NCBI	6.3/22.2	212/18	3/4
104	Protein disulphide isomerase precursor	PDI1	*Dicentrarchus labrax*	AGI60172 / NCBI	4.54/57.2	323/11	6/7
12	Superoxide dismutase Cu/Zn	SOD	*Dicentrarchus labrax*	FM000596 / EST	6.18/22.0	99/6	1/1
98	Transferrin	TF	*Dicentrarchus labrax*	ACN80997 / NCBI	5.93/76	1103/38	21/23
99	Transferrin	TF	*Dicentrarchus labrax*	ACN80997 / NCBI	5.93/76	69/2	1/1
96	Warm temperature acclimation protein 65-1	WAP65A	*Dicentrarchus labrax*	DAA12503 / NCBI	5.45/49.7	423/26	1/11
103	Warm temperature acclimation protein 65-2	WAP65B	*Dicentrarchus labrax*	DAA12504 / NCBI	5.47/49.3	948/50	19/20

a) SN: spot number in reference 2DE gel.
b) Protein symbol according to Uniprot database.
c) Identification or accession number according to the corresponding database.
d) Theoretical *pI* and mass (kDa) for each identified protein.
e) Total protein score (S) and percentage of coverage (C) for each identified protein.
f) Total unique peptides (U) against total matched peptides (T).

Table 3. Identified peptide sequences after MASCOT analysis for the immune-related proteins.

Immune-related protein	Identified peptide sequences
Complement component 3	YEMDTVLSER / SVPFIIIPMK / AILHNYSPDPITVR
C1q-tnf-related protein	TNIGNAYNPLTGIFTAPVK /ITGCAANSGMNIAVMKDGVNMFAVK /DGVNMFAVK /DNQQMHSSASNGMTLALEQGDQLSVTLWTGNSIFDHGR
Antifreeze protein	FFPTATTWSEAER / ACLALGANLASVHSR
Fucose binding lectin	EMNPWWR / APTGENLALQGK / FLTLCEVEVYGYR / VDLLEPYIVTSIIITNR / VGVISHIPAGISHTFSFTER / CAVITSIPASATTEFQCNGMDGR / IGDSLDNNGNNNQR / YGSVEIDELGK / LYTLAMTDPDAPSRK
Natural killer cell enhancement factor	LAPDFTAK / EDEGIAYR / QITINDLPVGR / TISTDYGVLKEDEGIAYR
G-type lysozyme	VGGSCGIDPALIAAIISR / GGIAAYNMGDK
Calmodulin	DTDSEEEIR / MKDTDSEEEIR / EAFSLFDKDGDGTITTK
Calreticulin	EQFLDGDGWK / SGTIFDNLLISNDVK / FDNIGVLGLDLWQVK / FYGDAEADKGLQTSQDAR / FEPFSNEGKPVVIQFTIK
Endoplasmic reticulum protein 57	TADGIVSFLK / FLQDYFDGK / DGEETGPYDGPR / SEPIPESNDGPVK
Protein disulphide isomerase	ALAPEYAK / VIDYNGER / MDSTANEIEAVK / VDATEETELAQDYGVR / GNQLPLVIEFTEQTAPK
Apolipoprotein a1	ITPLAEEVK / TALMPIVESVR / LQEIFEAIAASITK / TLMDPILTEYYAK / LQEIFEAIAASITKN / AKLQEIFEAIAASITK / LEDMYNQVK / TLQGAVSPMTDSVVSTISDATAEFR
Leukocyte elastase inhibitor	DLSMLIFLPK / EIEDDTTGLEK / EDAPYALSLANR / DLLAEGVVDNMTR / HYDAELESVDFK
Glutathione s-transferase omega 1	LLPSSPFGK
Cyclophilin a	VFFDITIDGANAGR / FADENFQLK
Heat-shock protein 70KDa	SINPDEAVAYGAAVQAAILSGDK / FELTGIPPAPR
Transferrin	APAIVCVK / DNTIDCIIAIK / EPYYDYAGAFQCLVEDAGEVAFVK/ ANYELLCK / VPAHAVVTR / DQQMADLILK / LVALPPNTDSFLYLGAEYMSIVR / EQTPAASSTAIK / EADAMAVDGGQVYTAGK / CGLVPVMVEQYDEAK / CANPGEASSYYAVAVVK / NSGVTWDTLK / TAGWNIPMGHIHSITNDCDFTK / FFSSGCAPGADPSSSFCTQCAGSGK / YYGYAGAFR / CLVEGAGDVAFIK / FGSTGSDPTFR / LFQSESGK / QCSGSTPDLEK
Caspase-1	MGDIPPIQVSK / VTAQDGVPAQER / GHEASSILIAALR / IINDEEMQSATTK / ISSGAEGPGVPTEDRVPTEDR
Caspase-6	AACTENLTETDAFIR
Apoptosis-associated speck-like protein containing a CARD	LLADTLEDLSVEDLDKFR /VISAEIYDTIR
Superoxide dismutase Zn/Cu	MLTLTGPLSIIGR
Warm temperature acclimation protein 65-1	LEDGYPK / ADTIENAFK / DDHLFLYK / VSLEVFGCDH / GQNIYDVELK / FSDDSDHVER / GLEMDAVAVNEEGIPYFFK / MHYEDNPTDHDHMFFFLNNK / ELHSEVDAVFTYEDHLYMIKDDHLFLYK
Warm temperature acclimation protein 65-2	IDAITTDDAGK / ILYVDLTATPR / EELGVEGR / DGLHAFPITR / ILYVDLTATPR / TYFFAGPIYMR / AAAHYTLIEGYPK / GECMADSVLFFK / GYHGSAQPSNEHFK / MHNIDNPDKHDHIYFFLDDK / AVTQNLPLPLDGIDAALCNAK / AVTQNLPLPLDGIDAALCNAK / VDAAFVCPDDNTVHIIQGQR