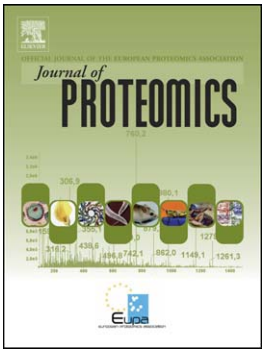

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Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*)

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Keywords: skin mucus; gilthead seabream (*Sparus aurata* L.); teleost; proteome; microbiota

Abbreviations: Apo-A1, apolipoprotein-A1; ENOA, enolase; FABP7, brain-type fatty acid binding protein; FBPA, fructose-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC70, heat-shock cognate 70 kDa protein; HBB, beta globin; LPS, lipopolysaccharide; NACA, nascent polypeptide-associated complex subunit alpha; NDPK, nucleotide diphosphate kinase; PEBP1, phosphatidylethanolamine-binding protein 1; PP2A, serine/threonine-protein phosphatase 2A; PRDX1, peroxiredoxin 1; PRDX2, peroxiredoxin 2; PRDX6, , peroxiredoxin 6; RhoGDI, Rho GDP dissociation inhibitor alpha; SOD, superoxide dismutase; TF, transferrin; TPIS, triose phosphate isomerase; VCP, valosin-containing protein; WAP65, warm temperature acclimation protein 65 kDa;

Highlights

- The skin mucus proteome of farmed *S. aurata* was analyzed for the first time.
- Structural and metabolic proteins are the major functional groups of mucosal proteins.
- Stress response proteins and signal transduction proteins are also present in fish skin mucus.
- Proteomics identified the microbial communities living in the fish mucus layer.

Abstract

Fish skin mucus is the first line of defense against infections and it discriminates between pathogenic and commensal bacterial strains. Mucus composition varies amongst fish species and is influenced by endogenous and exogenous factors. This study describes the first proteome map of the epidermal mucus of farmed gilthead seabream (*Sparus aurata*). We used an integrative proteomic approach by combining a label-free procedure (LC-MS/MS) with the classical 2-DE-PMF-MS/MS methodology. The identified mucosal proteins were clustered in four groups according to their biological functions. Structural proteins (actins, keratins, tubulins, tropomyosin, cofilin-2 and filamin-A) and metabolic proteins (ribosomal proteins, proteasomal subunits, NACA, VCP, histones, NDPK, transferrin, glycolytic enzymes, ATP synthase components, beta-globin, Apo-A1 and FABP7) were the best represented functional categories. We also found proteins involved in stress response (WAP65, HSPC70, Cu,Zn-SOD, and PRDX1 and PRDX2) and signal transduction (PP2A 65 kDa regulatory subunit, 14-3-3 protein beta/alpha, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, RhoGDI and PEBP1). Most of the identified proteins address different aspects of the innate immune response. Additionally, we analyzed bacterial peptides identified in the skin mucus of healthy *S. aurata*. These results revealed that genera belonging to the Lactobacillales order constitute the most abundant microorganism populations in this habitat.

Biological significance

This work shows that proteomic methods can be used to characterize fish skin mucus. Using a coupled approach of LC-MS/MS and a 2-DE-PMF-MS/MS, we have obtained the first comprehensive view of the skin mucosal proteome of *S. aurata,* a fish species that is economically relevant for Mediterranean aquaculture. We identified a panel of proteins involved in a variety of biological functions, particularly in the innate immune response. Furthermore, to our knowledge, this is the first time a proteomic approach has been used to examine the microbiota in the skin mucus of a fish species. Overall, these results support further immunological researches in *S. aurata* and are relevant for the culture of this important fish species.

1. Introduction

The fish skin mucus is mainly produced by goblet cells, which generate mucous granules that release their contents at the cell surface, but other skin cell types also contribute to the mucus layer [1]. The main structural proteins of mucus are high molecular mass (~106 kDa) glycoproteins called mucins [2]. Mucin fibers are long flexible strings that are densely coated with short and negatively charged (carboxyl or sulfate groups) glycans. These glycosylated and highly hydrophilic regions are separated by globular and hydrophobic zones that are stabilized by multiple internal disulfide bonds (Cys-rich domains). The interactions of mucins with each other and with water explain the most apparent properties of this layer, that is, its slipperiness and stickiness [3].

Several vital biological functions have been attributed to the fish mucus, including mechanical and disease protection, respiration, communication, nest building, and particle trapping. Fish skin mucus is the first line of defense against pathogens. Given that it is continuously secreted and shed, pathogens must move 'upstream' through the unstirred layers of mucus on the epithelial surface, so in most cases, sticky mucus prevents the stable colonization of potential infectious microorganisms as well as invasion by metazoan parasites [1, 3]. Mucus also constitutes a biochemical barrier containing enzymes, such as proteases and other antimicrobial proteins, that contribute to the fish innate immunity [4]. Immune molecules in fish mucus include lysozyme [5], immunoglobulin [6], lectins [7], calmodulin [8], interferon [9], galectin [10], histones and ribosomal proteins [11], complement, C-reactive protein, proteolytic enzymes, antimicrobial peptides, and vitellogenin, (reviewed in [12]). Moreover, environmental changes affect the expression of certain genes and proteins in fish skin cells [13].

The role of the mucus layer in fish health is particularly relevant in farmed fish due to the diverse infectious diseases that hinder the development of modern aquaculture. Consequently, the characterization of the mucus from fish skin has been approached from different perspectives, and has focused on fish species of economic interest to aquaculture. Despite the unquestionable potential of having a global vision on the protein composition of fish mucus, only a few studies have addressed this problem by using high throughput techniques. These include a comparison of the mucus and venom of marine catfish (*Cathorops spixii*) [14], the proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) [15] and the study of changes observed in this proteome following infection with the bacteria *Vibrio anguillarum* [16]. Similarly, the epidermal mucus protein composition in Atlantic salmon (*Salmo salar*) after infection with sea lice has been examined [13]. Additionally, the proteome of a cichlid (*Symphysodon aequifasciata*) has been explored to demonstrate parental care [17, 18], and recently, the protein composition of the epidermal mucus of turbot (*Scophthalmus maximus*) living in different temperatures has been analyzed [19]. With the exception of the marine catfish

study [14], these studies constructed the proteome maps by 2-DE followed by MS/MS analysis. This technique limits the identification of proteins to those that reach a relative abundance in samples; the relative abundance required depends on the detection limit of the dye used in gel staining, for example, silver [13, 19], Coomassie brilliant blue [15, 17, 18] or SYPRO Ruby [16]. 2-DE resolution is also hindered by the high mucin content in mucus. The large size of mucins fibers and their high level of glycosylation require specific methodologies to be analyzed [20].

The identification of proteins in non-model organisms typically relies on similarity (rather than identity) between the fragmented peptide sequences and homologous proteins from phylogenetically related species that are available in public databases (reviewed in [21]). As such, homology-driven proteomics is a major tool for characterizing the proteomes of organisms without sequenced genomes [22]. However, the fish skin mucus is not an ordinary tissue in that it would not be expected to contain only proteins synthesized by the fish. Mucus is directly exposed to marine water, which includes suspended particles and a wide variety of microorganisms. Numerous studies indicate that healthy fish possess bacterial populations living on mucus and that the number and taxonomic composition of these bacterial communities generally reflect those of the surrounding water [23]. Therefore, a homology-driven proteomics approach in fish skin mucus could identify a number of proteins from different origins, unless precautions are taken.

In this paper, the proteome of gilthead seabream skin mucus was mapped using two different proteomic methodologies: (i) a gel-free approach based on LC-MS/MS analysis, a well-established technique for mapping the proteomes of complex samples that overcomes the limitations of gel-based proteomics [24], and (ii) a conventional 2-DE experiment followed by PMF and MS/MS coupled identification. Interestingly, the gel-free approach allowed the unambiguous identification of proteins from bacteria living in the mucus samples supporting the potential of proteomic techniques for these studies. The results obtained from both methodologies were integrated to establish the first proteome map for the skin mucus of gilthead seabream which is a major product of marine aquaculture in the Mediterranean area.

2. Material and methods

2.1. Animals and skin mucus collection

Ten juvenile specimens (125 ± 25 g body weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.) were obtained from a local farm (Murcia, Spain) and were kept in seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water was maintained at 20 ± 2°C with a flow rate of 900 l/h and 28‰ salinity. The photoperiod was 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight per day. Fish were allowed to acclimatize for 15 days before sampling. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

Fish were anesthetized prior to sampling with 100 mg/L MS222 (Sandoz). Skin mucus samples were collected from naïve specimens using the method described by Guardiola *et al* [25] with slight modifications. Briefly, skin mucus was collected by gently scraping the dorso-lateral surface of naïve seabream specimens using a cell scraper while avoiding contamination with blood and urino-genital or intestinal excretions. To obtain a sufficient amount of mucus, equal samples of mucus were pooled (10 fish per pool). The mucus was centrifuged (12,000 *g*, 4°C, 10 min) and immediately stored at -80°C until further analysis.

2.2. Sample preparation

The mucus samples were solubilized by adding stock solutions of DTT, SDS and Tris-HCl, pH 7.6, to reach a final concentration of 40 mM Tris-HCl, 60 mM DTT and 2% SDS. Samples were prepared using a 2-D Clean-Up Kit (GE Healthcare) following the protocol recommended by the manufacturer. Proteins were resuspended in 6 M urea and 200 mM ammonium bicarbonate if the samples were analyzed by LC-MS/MS or in rehydration buffer (8 M urea, 2% CHAPS, 12 µl/ml DeStreak reagent, 2% Pharmalyte 3–10, 0.004% bromophenol blue) if the samples were analyzed by 2-DE.

2.3. LC-MS/MS analysis

All LC-MS/MS analyses were performed at the Proteomic Facility of the University of Córdoba (SCAI, Proteomic Unit). After clean up, the samples were reduced, alkylated and digested with trypsin using standard protocols. All analyses were performed with an Ultimate Plus HPLC System in tandem with a Finnigan LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with a nanoelectrospray ionization interface (nESI). For trapping, a

Zorbax 300 SB-C18 column (Agilent), 5 µm particle size, 300 Å pore size, and 300 µm i.d. x 50 mm length was used at a flow rate of 10 µl/min for 10 min. The trapping column was switched on-line with the separation column, a ProteoPep2 C18 (New Objective), 5 µm particle size, 300 Å pore size, and 150 µm i.d. x 150 mm length. Elution was performed using a 60 min gradient of 5-40% acetonitrile/0.1% formic acid solution at a flow rate of 300 nl/min. MS data (Full Scan) were acquired in the positive ion mode over the 400-1,500 m/z range. MS/MS data were acquired in a data-dependent scan mode, automatically selecting the five most intense ions for fragmentation. The Orbitrap resolution was set at 30,000, and dynamic exclusion was applied during 30-second intervals. Tandem mass spectra were extracted using Thermo Proteome-Discoverer 1.0 software (Thermo Fisher Scientific) and the data were analyzed using the SEQUEST algorithm (Thermo) applying the following search parameters: peptide tolerance, 10 ppm; tolerance for fragment ions, 0.8 Da; b- and y-ion series; fixed modification**,** carbamidomethylation of cysteine; variable modifications, oxidation of methionine; and maximum trypsin missed cleavage sites, 2. Firstly, peptides were searched against the UniProtKB database without taxonomic restriction. Peptide identifications were accepted if they exceeded the filter parameter Xcorr score vs charge state with SequestNode Probability Score (+ 1 = 1.5, + 2 = 2.0, + 3 = 2.25, + 4 = 2.5). In order to avoid false positive determinations derived from natural exposure of mucus to environment, accepted individual peptides were used in a BLAST search against the NCBI non-redundant protein sequence database, which was restricted to the Actinopterygii class. Only proteins that contained the exact full-length sequence of the peptides were considered. Two different skin mucus samples were independently processed.

2.4 Two dimensional gel electrophoresis and MS analysis

To complement this study, the most abundant proteins in the skin mucus of the gilthead seabream were identified by separating proteins by 2-DE. Proteins (200 µg) from skin mucus in rehydration buffer (340 µl) were first separated by isoelectric point in 18 cm, pH 3-10 IPG strips and then by SDS-PAGE as previously described [26]. After electrophoresis, the gels were stained with SYPRO Ruby dye and scanned using a Molecular Imager FX (Bio-Rad). All subsequent procedures were performed at the SCAI, Proteomic Unit, University of Córdoba. A total of 36 of the most abundant spots in 2-DE gels were automatically excised using an Investigator™ ProPic station (Genomic Solutions). Immediately after excision, the gel was rescanned to confirm that the desired protein spots had been accurately obtained. The excised spots were then destained and digested with trypsin using the Investigator™Progest apparatus (Genomic Solution), and digested peptides were placed onto an Opti-TOF® MALDI plate (AB SCIEX) using the Investigator™ProMS apparatus (Genomic Solution). Peptide mixtures were analyzed using a 4700 Proteomics Analyzer (Applied Biosystems) mass spectrometer. Spectra

were obtained using the reflector acquisition mode in the mass range of 700 to 3500 Da, and the eight strongest precursors from the MS scan were isolated and fragmented by CID. Combined MS-MS/MS data were used in Mascot (Version 2.1, Matrix Science, London, UK) to search the NCBInr database, subset Actinopterigyii with the following parameters: parent ion mass tolerance at 100 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine selected as fixed modification, and methionine oxidation as variable modification. The probability score (95% confidence level) was calculated by the software and used as criteria for protein identification.

2.5 Western blot

Protein samples were separated by SDS-PAGE with Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad, Hercules, CA). Ten micrograms of protein sample prepared as described above for 2-DE were loaded onto the gel. After electrophoresis, the gel was activated resulting in UV-induced protein fluorescence. The separated proteins were transferred to a PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The membrane was observed with a ChemiDoc MP imaging system (Bio-Rad) to verify protein transference. The blot was processed using the iBind™ Western System (Life Technology, Carlsbad, CA) according to the manufacturer´s protocol. Rabbit anti-actin polyclonal antibody at a 1:200 dilution (CSA-400, Stressgene Biotechnology, Victoria, Canada), and goat anti-rabbit IgG-Peroxidase antibody at a 1:2000 dilution (A9169, Sigma-Aldrich) were used as the primary and secondary antibodies, respectively. Blots were developed using the ECL-Plus kit (Amersham Biosciences) following the manufacturer´s instructions, and the membrane was imaged at the end of the procedure.

3. Results and Discussion

3.1. Two complementary strategies for mucus proteome analysis

LC-MS/MS yield a total of 99 and 96 different peptides matching proteins from mucus samples 1 and 2, respectively. Both samples shared a total of 50 peptides (Supplementary file 1). As indicated in Material and methods section, individual peptides were used to perform a BLAST search restricted to the Actinopterygii class, the results corresponding to one peptide per identified protein are in Supplementary file 2. The 52 proteins identified and the major parameters of BLAST searches are summarized in Table 1.

A representative gel of 2-DE analysis of mucus samples is shown in Fig. 1, a 2-DE from liver was also included for comparison. Remarkably, most of the *S. aurata* proteins from mucus samples were in the pI 4-6.5 range, while in liver most proteins were in the 4-8.5 range. To exclude a deficient focusing of proteins in the alkaline region of the IPG strips, the experiment was repeated using 3-11NL strips (instead of the 3-10 pH range in Fig. 1) with identical results (data not shown). Moreover, this observation was consistent with several previous studies on different fish species showing that most of the spots in the 2-DE gels were into the area corresponding to pH 4-7 range [13, 15, 16, 19]; thus, we reasoned that the majority of skin mucus proteins were acidic. Thirty of the most intense spots were excised and identified by PMF and MS/MS coupled analysis searching with the Actinopterygii entries in the UniProt database. The identified proteins are shown in Table 2.

Comparison of the proteins identified by the two approaches revealed that few proteins were present in both lists (Table 1 and 2), indicating that the LC-MS/MS and 2-DE-PMF-MS/MS methodologies are complementary, and together they provide a more comprehensive view of the skin mucosal proteome.

Interestingly, the mucus map proteome was very different from the proteomic profiles of other tissues in *S. aurata* [27-32]. However, many of the proteins identified herein have been described as components of the mucus of other fish species. Altogether, these data suggest that these proteins are specific to the mucus proteome of the gilthead seabream and that they are most likely responsible, at least in part, for the biological functions of the skin mucus.

3.2. The *S. aurata* skin mucus contains immune-related proteins

The proteins identified using both proteomic approaches were grouped into four clusters based on their general biological functions (Fig. 2). Two major groups, *structural proteins: cytoskeleton and extracellular* and *metabolism*, collectively represented 92% and 74% of the proteins identified by LC-MS/MS and 2-DE-PMF-MS/MS, respectively. In contrast, the *stress*

response and *signal transduction* functional categories were more equitably represented by each methodology (4% with LC-MS/MS and 10% and 17%, respectively, with 2-DE-PMF-MS/MS). As expected, most proteins in these four groups address different aspects of the immune response. As discussed below, some of them have a recognized function in immunity, and others are indirectly involved or have potential immune roles inferred from the functions of their orthologs in other species.

3.2.1 Structural proteins

This group includes several isoforms of actins, keratins and tubulins and also tropomyosin4-2, cofilin-2 and filamin-A-like protein. We found β-actin using both, the LC-MS/MS (Table 1) and 2-DE-PMF-MS/MS (Table 2) approaches, and the 2-DE spot intensities (Fig. 1) indicated that this is one of the most abundant proteins in the gilthead seabream skin mucus. β-actin is a significant component of Atlantic salmon mucus and its high level suggests that its presence is not simply due to contamination by ruptured cells but rather has a discrete role in mucus structure [13]. It has been previously reported that increased fragmentation of mucus actin correlates to stress situations, and the protein fragments generated by protease activity could trigger or prime an immune response [33] similar to the nitric oxide response in goldfish (*Carassius auratus*) macrophages, which is induced by transferrin cleavage products [34]. Comparison of the expected and observed molecular weights from the matched ortholog proteins in Table 2 (approximately 42 kDa) suggested that β-actin remained mostly intact in agreement with the healthy and non-stressed status of the fish used in this study. Western blot analysis confirmed that β-actin was not fragmented in the mucus samples (Fig. 3).

We identified keratins type I and type II by LC-MS/MS (Table 1) and 2-DE-PMF-MS/MS (Table 2), and these proteins were some of the most abundant proteins in the *S. aurata* mucus proteome (Fig. 1). Although human keratin is a frequent contaminant in proteomic analyses, our data clearly showed the presence of fish keratin (Supplementary file 3). Only three out of fourteen peptides showed a perfect match to human sequences, indicating that *S. aurata* skin mucus samples were not contaminated. The presence of keratins or their fragments in fish skin mucus is not surprising [13, 15, 16, 19], according to the well known structural role of these proteins but different functions of keratins in skin mucus have been suggested. Interestingly, a pore-forming glycoprotein with substantial homology to trout type II cytokeratin is present in the skin mucus of rainbow trout (*Oncorhynchus mykiss*). The pore-forming properties of this glycoprotein correlate well with strong antibacterial activity [35]. This immune related function might explain the high levels of keratins in *S. aurata* skin mucus (Fig. 1).

Both, α- and β-tubulins were also identified by LC-MS/MS (Table 1). In addition to their structural function, a role in phagocytic activity has been discussed to justify the increased levels of β-tubulin protein in Atlantic cod skin mucus after infection with *V. anguillarum* [16]. This phenomenon was explained by the up-regulation of the *tubb2* gene in the mucosal cells associated with phagocytic processes.

The 2-DE-PMF-MS/MS experiment (Table 2) demonstrated that tropomyosin is present in the *S. aurata* epithelial mucus, and the molecular weight and pI of the spot matched the expected values for the full-length protein. Tropomyosin has been found previously in the skin mucus of Atlantic cod [15] and *Cathorops spixii* (a common catfish on the Brazilian coast) [14]. To our knowledge tropomyosin has not been directly tied to immunity, although it has been suggested that the down-regulation of tropomyosin in patients with ulcerative colitis may decrease immune functions [36]. Moreover, this protein may have antigenic properties [37, 38].

We identified filamin and cofilin in *S. aurata* skin mucus by LC-MS/MS (Table 1) and 2-DE PMF-MS/MS (Table 2), respectively. Cofilin has been previously reported in fish mucus [15], but to our knowledge, this is the first report describing filamin in this specific extracellular location. Both structural proteins have been associated with immunity at least in mammals and insects. Cofilins are components of a co-stimulatory signaling pathway in human T cells[39], and filamins interact with the cytoplasmic domain of Toll receptors [40], but their roles in fish immunity has not been investigated so far.

3.2.2 Metabolic proteins

This group includes proteins involved in pathways related to the central metabolism of biomolecules (proteins, carbohydrates, lipids and nucleic acids) and also proteins related to metal and energy metabolism.

We identified eight different ribosomal proteins (L7A, L8, L11, L19, L24, S3, S7 and Sa-like) by LC-MS/MS (Table 1). These proteins have not been reported before in fish skin mucus, but many other ribosomal proteins have been identified in the epithelial mucus of different fish species. For instance, a peptide derived from the S30 ribosomal protein is present in skin secretions from rainbow trout [41], and L40, L36A, L35 and a protein similar to ribosomal protein P2 are found in the epidermal mucus of Atlantic cod [11, 16]. These ribosomal proteins or their fragments have been shown to have antimicrobial properties, although the specific mechanisms of action are not known [42]. Similarly, ribosomal proteins in the human colonic mucus seem to be involved in the host defense against microorganisms [42]. Hence, we speculate that the ribosomal proteins identified in the gilthead seabream skin mucus have a similar role.

The 20S protease proteasome complex is well represented in the *S. aurata* skin mucus. We found the beta 6 subunit by LC-MS/MS (Table 1), and five spots were identified as proteasome subunits (four alpha and one beta type) with the conventional 2-DE-PMF-MS/MS approach (Table 2). These results are in agreement with a previous study that identified proteasome subunit alpha type 3 and 7 in Atlantic cod skin mucus [15]. Furthermore, the proteasome 26S subunit levels are significantly increased in the skin mucus of this fish in response to *V. anguillarum* infection [16], and the proteasome subunit alpha type 4 is up-regulated in the oral mucus of mouthbrooding tilapia fish (*Orechromis* spp.) [43]. Although the role of the proteasome subunits in mucus-rich organs has not been experimentally determined, the authors of this last study speculated that proteasome-mediated degradation of phosphorylated complexes might contribute to the release of the NF-κβ, a transcription factor that activates the expression of several genes essential for mucus production ([43] and the references therein).

Nascent polypeptide-associated complex subunit alpha (NACA) and valosin-containing protein (VCP) are involved in protein metabolism, and we identified them in *S. aurata* skin mucus by LC-MS/MS (Table 1). NACA and VCP have not been reported in fish skin mucus, and their functions in cell secretions are unknown. In mammals, NACA is involved in innate immune signaling; because NACA binds DNA, it may sense DNA in the cytoplasm and transduce signals to α-taxilin, which is required for HSV-induced type I interferon production [44]. VCP has an important role in antiviral immunity based on its energy-dependent, ubiquitin-selective segregase and unfoldase activity. VCP is an essential cofactor in the antibody-dependent intracellular neutralization of virus, which is mediated by tripartite motif-containing 21, a cytosolic IgG receptor [45].

We identified proteins involved in DNA metabolism, including histones and nucleotide diphosphate kinase (NDPK). The presence of H4 and A2A-like histones in skin mucus of *S. aurata* (Table 1) agreed with previous studies that found these histones in the mucus of Atlantic salmon [46] and Atlantic cod [11]. These proteins could have implications in gilthead seabream immunity since it is known that some derived peptides from histones have antimicrobial activity. Furthermore, the N-terminal segments of catfish H2A are induced in the epidermal mucus upon stimulation, while expression of this histone is suppressed during the early stages of stress and reduced in the absence of disease (reviewed in [12]). The identification of NDPK by both, the LC-MS/MS and 2-DE-PMF-MS/MS approaches in the skin mucus of *S. aurata* confirmed previous data in the mucus of discus fish (*Symphysodon* spp.) [18] and Atlantic cod [15, 16]. To our knowledge NDPK has not been linked to immunity in fish. However, this association has been established in other species such as *Chlamys farreri,* where NDPK transcripts increase in hemocytes after bacterial challenge, and the protein is up-regulated in hemolymph [47], *Litopenaeus vannamei*, where NDPK acts as a defense-related enzyme

involved in the anti-viral innate immune response [48] or even in mammalian NDPK-B is required for the activation of human CD4 T lymphocytes [49].

Two spots in the 2-DE gels were identified as transferrin (TF). The difference in the pI of these two isoforms was less than 0.1. In mammals, this protein has sites for *O*- and *N*-glycosylation [50] and the diverse grade of glycosylation of these sites could explain the differences in pI. TF in fish skin mucus was first reported in Atlantic salmon [46]. TF is also observed in salmon skin mucus, where TF is cleaved during sea lice infection. As discussed above, during infection, increased levels of fragmented TF may activate the nitrous oxide response in salmon macrophages, to counteract sea lice infection [13]. In addition, TF is part of the innate defense system against bacteria due to its iron chelating affinity [51]. Overall, these data could explain the presence of TF in the skin mucus of *S. aurata*.

Four glycolytic enzymes were found in the skin mucus of gilthead seabream. Fructose-bisphosphate aldolase (FBPA), triose phosphate isomerase (TPIS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and enolase (ENOA). All of these proteins have been found in the mucus of other fish [15, 16, 18]. While glycolysis is not known to occur in mucus, extracellular glycolysis has been proposed in other biological fluids, for example, the peritoneal lavage fluid [52] and insect spermatophore [53]. Moreover, the intermediates and products of extracellular glycolysis may have intracellular signaling actions [54].

We also found the F1 complex alpha and beta subunits in *S. aurata* mucus (Table 1). To our knowledge, these subunits have not been reported in skin fish secretions. While the function of these proteins in mucus is unknown, recent studies show that cell surface ATP synthases are expressed on normal human cells and that these enzymes may be implicated in different processes including innate immunity and intracellular pH regulation [55].

LC-MS/MS (Table 1) also revealed beta globin (HBB), a subunit of the α2β2 hemoglobin tetramer, in the mucus of gilthead seabream. This protein was not previously reported in fish mucus, and consequently, its function in this location remains undisclosed. However, this protein could serve the function suggested for other metal-binding proteins in mucus (e.g., lactoferrin and transferring) to create low-iron environments that limit microbial pathogenesis. It is known that this activity damages the lipopolysaccharide (LPS) of the Gram-negative outer membrane altering its permeability [51], and there is also evidence for globin-LPS binding [56] supporting a role for mucosal globins in fish immunity.

We found two proteins related to lipids metabolism in skin secretions of *S. aurata*, apolipoprotein-A1 (Apo-A1) in mucus samples analyzed by LC-MS/MS (Table 1) and brain-type fatty acid binding protein (FABP7) in samples analized by 2-DE-PMF-MS/MS (Table 2). Apo-A1 protein of *S. aurata* is mainly expressed in liver and to a lower extent in intestine [57]

but it has also been found in skin secretions of other fish species. In this line, Apo-A1 is overexpressed in the skin mucus of infected Atlantic cod [15] and Atlantic salmon [13, 16]. In carp (*Cyprinus carpio*) it has antibacterial effects *in vitro* [58]. All these findings support a role for mucosal Apo-A1 in fish immunity, but the mechanism has not been elucidated. To our knowledge, this is the first time that FABP7 has been identified in fish skin secretions, although a member of the family specific to adipocytes is present in *S. salar* mucus [15].

3.2.3 Stress response proteins

We found five proteins related to stress response in the mucus of *S. aurata*: warm temperature acclimation protein 65 kDa (WAP65), heat-shock cognate 70 kDa protein (HSC70), peroxiredoxin 1 and 2 (PRDX1 and PRDX2) and Cu/Zn superoxide dismutase (Cu/Zn-SOD). HSC70 and SOD were detected by both LC-MS/MS and 2-DE, while WAP65, PRDX1 and PRDX2 were only detected by 2-DE. With the exception of WAP65 [14], these proteins have not been reported in fish mucus, but Rajan *et al* identified a different peroxiredoxin, PRDX6, in Atlantic cod skin mucus [16]. In mammals, SOD and PRDX1 and PRDX2 are secreted in fluids and are involved in inflammation, immunity and tissue repair [59, 60]. Nevertheless, the role of these antioxidant proteins in skin mucosal needs clarification.

WAP65 and HSC70 proteins are both involved in the response to temperature changes. WAP65 expression is a natural physiological adaptation to warm temperature in teleost fish, although WAP65 may also have other functions including immune system modulation [61]. WAP65 protein has a high affinity for heme, and given that iron is a key element in bacterial infections, the role of this protein could be involved in mucus innate immunity. WAP65 is found in *C. spixii* skin mucus and has inflammatory action [14]. HSC70 is constitutively expressed in non-stressed cells and only mildly induced during stress. This protein forms a stable complex with HSP70 upon stress and plays specific roles in many cellular processes including innate immunity. HSC70 protein is well characterized in mammals and may be secreted under specific conditions [62]. In fish, the HSC70 mRNA is ubiquitously expressed in several species [63, 64], but so far, the protein has not been found in fish skin secretions.

3.2.4 Signal transduction proteins

Five proteins belonging to this group were identified, the beta isoform of the 65 kDa regulatory subunit A of serine/threonine-protein phosphatase 2A (PP2A), two members of the 14-3-3 family (14-3-3 protein beta/alpha-1-like and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide like), Rho GDP dissociation inhibitor alpha (RhoGDI), and phosphatidylethanolamine-binding protein 1 (PEBP1). To our knowledge, only

the 65 kDa regulatory subunit A of PP2A and 14-3-3 proteins have been previously reported in fish skin mucus [15, 16], but their role in fish mucus is unknown. The spot intensity of the 65 kDa regulatory subunit A of PP2A increases 2.3-fold upon natural infection of Atlantic cod with *V. anguillarum*, suggesting a role in the immunity [16].

3.3 A proteomic approach to the bacterial population of *S. aurata* skin mucus

Thirty-six of the peptides derived from LC-MS/MS did not completely match any database sequence when the search was restricted to the Actinopterygii class. Five peptides contain sequences from more or less specific taxonomic group, i.e., the genus *Caenorhabditis* (EEVSAVDEIIKDK) or kingdom Viridiplantae (HVVFGQVVEGLDVVK). Organisms in these taxa could live in the tanks where gilthead seabream is farmed and thus, become incorporated into fish the skin mucus. The most remarkable finding was that thirty-one of the peptides exclusively matched bacterial or archaeal sequences (Table 3). When these peptides were used to perform a BLAST search against the Actinopterygii database, the score was low, and the E-value did not indicate a statistically significant match. For comparison, the result of a similar unrestricted search is shown in Supplementary file 4. Because we used healthy animals, we hypothesize that certain species of bacteria, or closely related ones, live commensally in the gilthead seabream epidermal mucus. The peptides, the most closely related organisms, and the database search parameters are summarized in Table 3. The represented taxonomic groups are organized on a phylogenetic tree in Fig. 4.

Some peptides matched only one genus, indicating that species in these genera are most likely living in the fish mucus. We found peptides from the genera *Streptococcus*, *Lactobacillus*, *Mycoplasma*, *Arthobacter*, *Clostridium*, and *Pelobacter*, and one peptide matching only the Achaea species *Metanocella arvoryzae*. Notably, 15 peptides matched only *Streptococcus* species and eight more matched *Streptococcus* and other Lactobacillales species. In general, the Lactobacillales order was the most abundant population of bacteria in this particular habitat. Two different peptides matched only Enterobacteriaceae family strains, signifying that this group of bacteria is also present in the skin mucus of healthy *S. aurata*. The remaining three peptides were less informative because they matched species of several bacterial phyla; these matches most likely occurred in a conserved region of the protein.

To date, different methods have been used to study fish-associated bacteria, including dilution and spread-plating, microscopic and automated direct epifluorescent filter techniques [23]. Additionally, there are molecular techniques including 16S rRNA gene sequencing [65] and microplate hybridization [66], but to our knowledge, this study is the first to apply a proteomic approach.

The skin surfaces of fish contain a relatively low bacterial load compared with other tissues such as the digestive tract, and these bacteria are only loosely associated with fish skin. The bacterial population of skin reaches 10^2 to 10^4 bacteria/cm^2, but this community is larger in polluted waters [23]. Most bacteriological studies have focused on changes in the microbiota in response to infections or pathogenicity, but there is limited information about the microbiota on the healthy fish epidermis and skin mucus. By terminal restriction fragment length polymorphism (tRFLP) analysis of the 16S rRNA gene, Smith *et al* [67] studied skin mucus bacterial communities of whiting in comparison to the surrounding water. They found that the water community was the most diverse, with only a small number of shared water-mucus phylotypes present. This result was dissimilar to another study reviewed by Austin *et al* [23], which described many similarities between the bacterial populations in fish and water. Lactic acid bacteria, notably carnobacteria, are common on/in fish, particularly in the digestive tract; *Lactococcus*, *Lactobacillus*, *Aerococcus*-like bacteria, *Leuconostoc*, *Pediococcus*, *Streptococcus*,*Vagococcus*, and *Weissella* are part of the normal microflora [68]. Typical bacteria in seawater have been recovered from the surface of marine fish and include strains of genera *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Caulobacter*, *Flexibacter*, *Escherichia*, *Hyphomicrobium*, *Vibrio*, *Photobacterium*, *Prosthecomicrobium* and *Pseudomonas* [69] [23].

4. Conclusions

Because infectious diseases severely limit intensive aquaculture, the role of fish skin mucus in the defense against infections is of great economic interest. In this study, the protein composition of gilthead seabream skin mucus was defined for the first time using proteomic techniques. Structural and metabolic proteins are predominant, although proteins involved in the stress response and signal transduction are also represented. Some of these proteins have been previously identified in the skin mucus of different fish species, but others are novel to skin mucus, for example, filamin, several ribosomal and proteasome subunits, nascent polypeptide-associated complex subunit alpha, valosin, subunits of the ATP synthase complex, globin, heat-shock cognate 70k Da protein, peroxiredoxins 1 and 2, Cu/Zn superoxide dismutase, Rho GDP dissociation inhibitor alpha and phosphatidylethanolamine-binding protein 1. We inferred the potential immune functions of skin mucus proteins from their orthologs in other species, but their actual functions should be investigated to obtain a comprehensive understanding of the mucus layer. Additionally, peptides from bacteria and archaea were unambiguously identified in fish skin mucus. The analysis of these peptides allowed us to design a map of microbial communities living in healthy farmed gilthead seabream. To our knowledge, this is the first proteomic approach to defining the microorganisms living in the fish skin surface. Proteomic techniques are thus very useful tools that may drive additional microbiological studies.

Acknowledgements

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Legends of Figures.

Fig. 1 – Representative 2-DE gel of *S. aurata* skin mucus proteome (left) and liver (right). The UniProt ID of the proteins identified by PMF-MS/MS is in bold next to the spot number.

Fig. 2 – Functional distribution of the proteins identified by both LC-MS/MS and 2DE-PMF-MS/MS. Functional annotations retrieved from UniProt were used to manually place the proteins into these four general groups.

Fig. 3 – Western blot showing intact β-actin in *S aurata* skin mucus. SDS-PAGE was performed as described in the Materials and methods section. Lane 1: five microliters of molecular weight marker (Precision Plus Protein All Blue Standards, Bio-Rad). Lane 2: Ten micrograms of *S. aurata* skin mucus proteins. The left panel corresponds to the total protein in the transferred membrane. Molecular weight markers, which were prestained to direct monitoring electrophoresis and transfer efficiency, are refractory to UV activation and appear as a negative fluorescence signal. The right panel shows the same membrane after antibody hybridization and contains a unique band corresponding in size to β-actin. No fragmentation of this protein was observed. The actin immunoreactive band overlaps one of the two major bands observed in the left panel, according to the abundance of this protein observed in 2-DE gels (Fig. 1).

Fig. 4 – Phylogenetic tree of microorganisms matching peptides found in healthy *S. aurata* skin mucus. A filled circle on the right of the figure next to the genus name means that one peptide exclusively matched this genus. An open circle indicates that a peptide matched this genus, but other genus sequences had the same score.

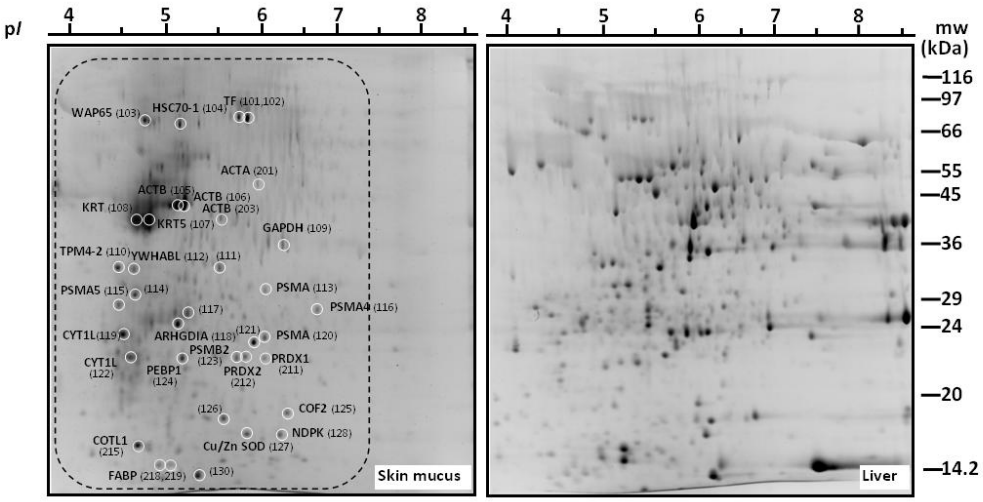

Figure 1

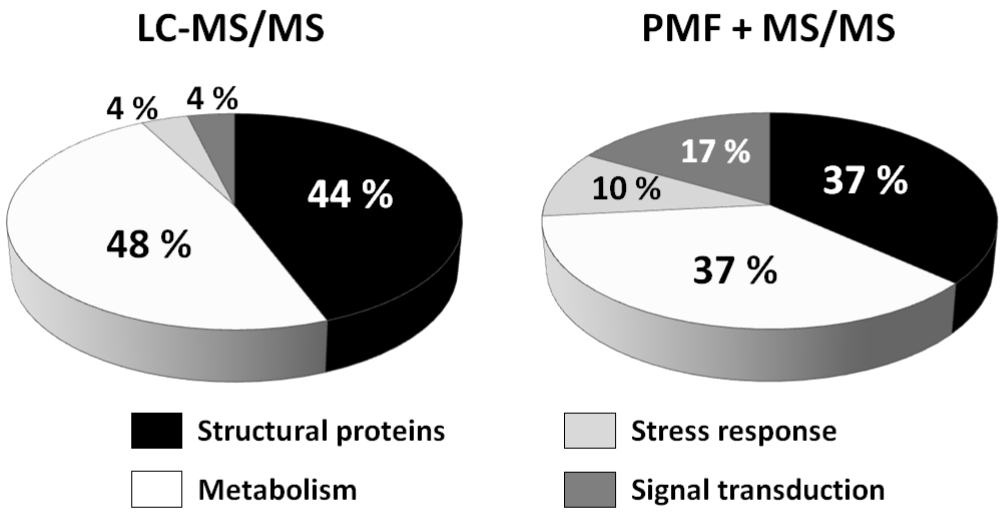

Figure 2

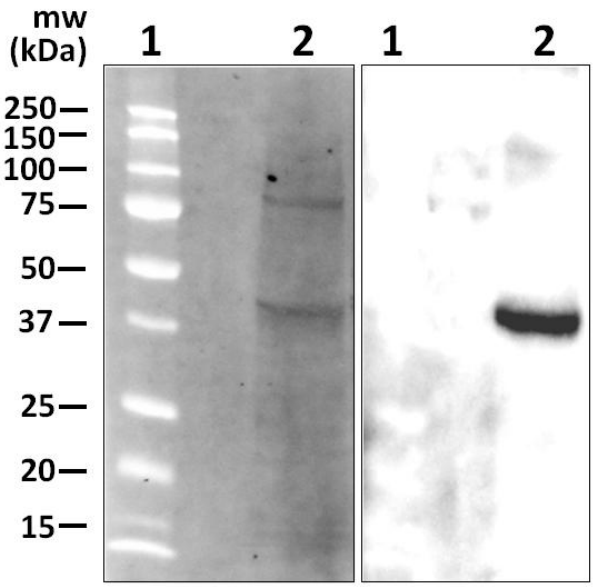

Figure 3

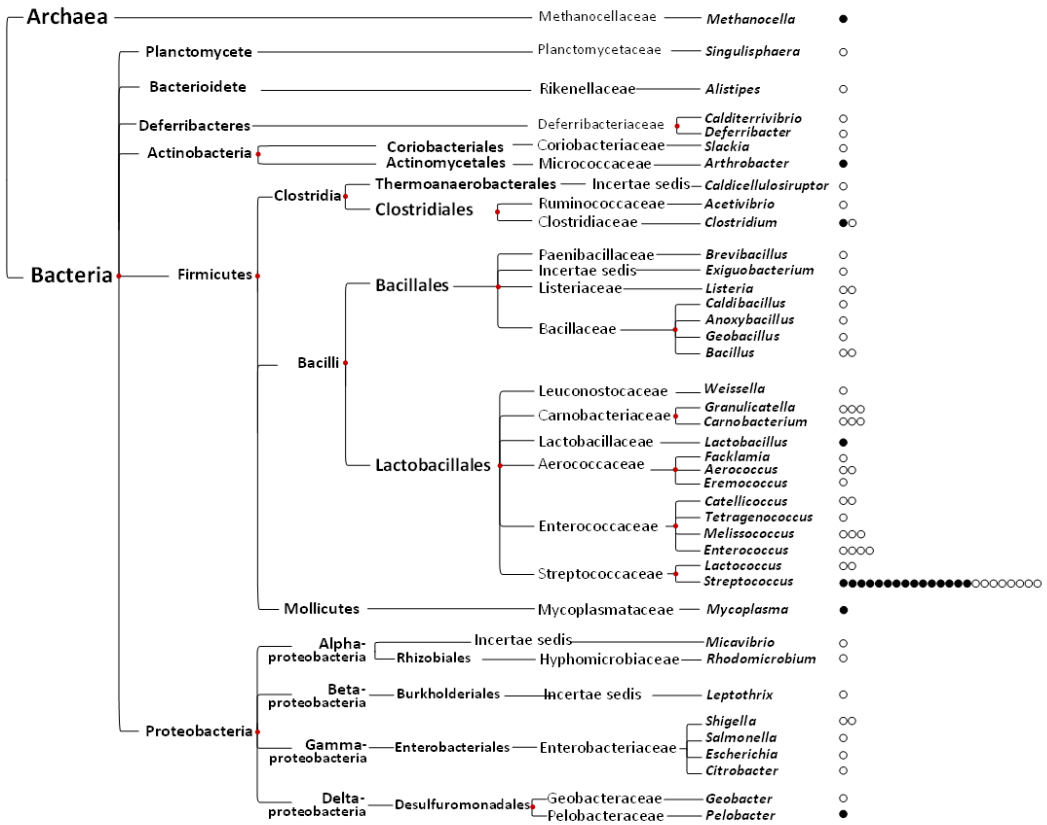

Figure 4

Graphical abstract

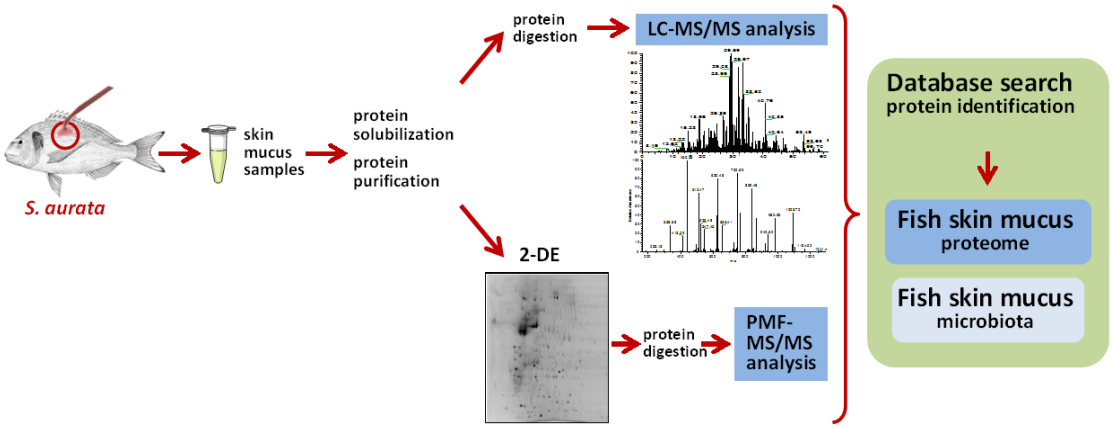

Table 1 - Proteins in the skin mucus of *S. aurata* identified by tandem MS. MS/MS-derived peptide sequence data were used for a BLAST analysis in which the search was restricted to the class Actinopterygii. Proteins shown match completely with the sequence peptide.

Protein[(a)]	Sequence ID[(a)]	UniProt[(a)]	PM[(b)]	Sample[(c)]	Score[(d)]	Expect [(e)]
Structural proteins: cytoskeleton and extracellular						
Alpha-actin, partial [Deltistes luxatus]	gb|AEO79977.1|	H6DA56	1, 1	1, 2	75.3	2.0E-16
Skeletal alpha-actin [Sparus aurata]	gb|AAF22646.1|	Q9PTJ5	1, 1	1, 2	60.4	2.0E-11
Actin-related protein 3 [Perca flavescens]	gb|ADX97138.1|	F1C778	1	1	48.1	4.0E-08
Beta-actin [Sparus aurata]	gb|AAK63074.1|	Q90Z11	7, 7	1, 2	99.0	7.0E-16
Beta actin isoform 2a, partial [Sparus aurata]	gb|AFA25665.1|	H6UWY4	1, 1	1, 2	96.5	2.0E-23
B-actin [Pagrus major]	dbj|BAA89429.1|	Q9PTU4	1	1	84.6	7.0E-20
Beta-actin, partial [Oreochromis niloticus]	gb|ABK20357.1|	A0FKD6	1	2	33.3	3.0E-03
Keratin, type I cytoskeletal 13 [Oncorhynchus mykiss]	ref|NP_001117848.1|	Q8JFQ6	1	2	42.2	2.0E-05
Keratin, type I cytoskeletal 13-like [Oreochromis niloticus]	ref|XP_003442600.1|	N.E.	1	1	39.2	2.0E-04
Type I keratin-like protein [Sparus aurata]	gb|ACN62548.1|	C0LMQ3	1, 1	1, 2	53.7	4.0E-09
Type I keratin isoform 1 [Solea senegalensis]	dbj|BAF56913.1|	A4UYK3	2, 3	1, 2	43.9	5.0E-06
PREDICTED: keratin, type I cytoskeletal 13-like [Maylandia zebra]	ref|XP_004556558.1|	N.E.	1	2	37.1	7.0E-04
Keratin, type II cytoskeletal 8 [Epinephelus coioides]	gb|AEG78360.1|	F6KMI6	1, 1	1, 2	43.5	7.0E-06
Keratin type II [Epinephelus coioides]	gb|AEG78338.1|	F6KMG4	1, 1	1, 2	52.8	6.0E-09
Type II keratin E3, partial [Gillichthys seta]	gb|ACO57583.1|	C1J0K3	1, 1	1, 2	51.5	2.0E-08
Type II keratin E3-like protein [Sparus aurata]	gb|AAT44423.1|	Q4QY72	1	2	34.6	4.0E-03
Keratin [Poecilia reticulata]	gb|AAD47884.1|AF172645_1	Q9PW53	1	1	30.8	6.9E-02
Keratin 18 [Epinephelus coioides]	gb|ACE06742.1|	B3GPH2	1	2	52.4	1.0E-10
Alpha-tubulin [Sparus aurata]	gb|AAP89018.1|	Q7T1F8	2	1	59.2	7.0E-11
Beta tubulin [Chionodraco rastrospinosus]	gb|AAG15329.1|AF255955_1	Q9DFS7	1, 2	1, 2	57.1.5	3.0E-10
Uncharacterized protein LOC767806 [Danio rerio]	ref|NP_001070241.1|	Q08CC8	1	1	57.1	3.0E-10
PREDICTED: collagen alpha-1(I) chain-like isoform X1 [Maylandia zebra]	ref|XP_004572575.1|	N.E.	1	1	73.6	2.0E-15
Filamin-A-like [Oreochromis niloticus]	ref|XP_003454305.1|	N.E.	1	1	60.9	2.0E-11
Metabolism						
Protein metabolism						
Ribosomal protein L8 [Sander lucioperca]	gb|AEE81293.1|	F6KH17	1, 1	1, 2	52.8	7.0E-09
Ribosomal protein L11 [Perca flavescens]	gb|ABW06869.1|	A8HTH7	1	1	46.4	7.0E-07
40S ribosomal protein Sa-like protein [Sparus aurata]	gb|AAT44424.1|	Q4QY71	1,1	1, 2	56.2	6.0E-10
40S ribosomal protein S7 [Oncorhynchus mykiss]	ref|NP_001117902.1|	Q2YHL9	1	1	71.5	6.0E-15
40S ribosomal protein S3 [Salmo salar]	gb|ACI67536.1|	B5X9L6	1	2	44.8	3.0E-06
60S ribosomal protein L7A [Siniperca chuatsi]	gb|AAY79207.1|	Q2KL19	1, 1	1, 2	51.1	2.0E-08
60S ribosomal protein L24 [Gillichthys mirabilis]	gb|AAG13295.1|AF266175_1	Q9DFQ7	1	2	42.2	2.0E-05
60S ribosomal protein L19 [Epinephelus coioides]	gb|ADG29150.1|	D6PVQ5	1	2	59.2	5.0E-11
20S proteasome beta 6 subunit [Pagrus major]	gb|AAP20145.1|	Q6Y267	1	1	64.3	1.0E-12
NAC alpha, partial [Oryzias melastigma]	gb|AEB71553.1|	I1SSG5	1	2	48.6	2.0E-07
Valosin containing protein [Oncorhynchus mykiss]	ref|NP_001117982.1|	Q1M179	1, 1	1, 2	57.1	4.0E-10
DNA metabolism						
Histone H2A-like [Oreochromis niloticus]	ref|XP_003451178.1|	N.E.	2, 2	1, 2	71.9	9.0E-16
Histone h2a.x [Perca flavescens]	gb|ADX97213.1|	F1C7F3	2, 1	1, 2	90.1	2.0E-21
Histone H4-like [Oreochromis niloticus]	ref|XP_003460383.1|	P62796	2, 1	1, 2	41.4	3.0E-05
Nucleoside diphosphate kinase [Sparus aurata]	gb|ACF75416.1|	B5APB7	1, 1	1, 2	56.6	3.0E-10
Carbohydrate metabolism						
Alpha-1 enolase-1 [Salmo trutta]	gb|AAG16310.1|	N.E.	2, 1	1, 2	56.6	3.0E-12
Enolase [Epinephelus bruneus]	gb|AEB31337.1|	F5BZS7	1	2	49.8	7.0E-08
Fructose-bisphosphate aldolase [Epinephelus coioides]	gb|ACL98138.1|	B9V3W3	1	1	44.8	2.0E-08
Glyceraldehyde 3-phosphate dehydrogenase [Pagrus major]	dbj|BAB62812.1|	Q90WD9	3, 1	1, 2	81.2	4.0E-18
Triose phosphate isomerase [Polypterus ornatipinnis]	dbj|BAD17930.1|	Q76BC6	1	2	51.5	2.0E-08
Triose phosphate isomerase [Amia calva]	dbj|BAD17915.1|	Q76BE1	1	2	43.1	1.0E-05
Energy metabolism						
ATP synthase subunit alpha, mitochondrial precursor [Psetta maxima]	emb|CAY56619.1|	C4QUY7	1	2	70.6	5.0E-15
ATP synthase beta-subunit [Pagrus major]	dbj|BAF37105.1|	A0PA13	1, 1	1, 2	61.7	7.0E-12
Beta globin [Sparus aurata]	gb|ABE28021.1|	Q1PCB2	1, 1	1, 2	43.1	9.0E-06
Lipid metabolism						
Apolipoprotein A-I [Sparus aurata]	sp|O42175.1|APOA1_SPAAU	O42175	3, 4	1, 2	74.4	6.0E-18
Stress response						
Heat shock cognate 70 kDa [Carassius auratus]	dbj|BAC67185.1|	Q801X8	2, 2	1, 2	71.9	7.0E-15
Superoxide dismutase [Cu-Zn] [Xiphias gladius]	sp|P03946.2|SODC_XIPGL	P03946	1	1	53.7	3.0E-11
Signal transduction						
PREDICTED: 14-3-3 protein beta/alpha-1-like [Oryzias latipes]	ref|XP_004070571.1|	H2M383	1, 1	1, 2	46.0	1.0E-08
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform [Salmo salar]	gb|ACN58639.1|	C0PUA0	1	1	57.5	2.0E-10

[(a)] Protein name, sequence ID and UniProt database ID of the record with the highest score retrieved by BLAST. N.E.: No entry in UniProt.
[(b)] PM: Number of MS/MS derived peptides from samples 1, and/or 2 that match exactly the protein sequence.
[(c)] Sample(s) in which the protein has been identified.
[(d)] Maximum score obtained in BLAST analysis by a peptide matching this protein
[(e)] Number of times we would expect to obtain an equal or higher score by chance.

Table 2 - Proteins identified by coupled PMF and MS/MS

SN[(a)]	Protein[(b)]	Organism[(b)]	UniProt ID[(b)]	Symbol[(c)]	Score[(d)]	Expect[(e)]	PM[(f)]	PF[(f)]	SC %[(f)]	Mass (kDa)	p*I*
	Structural proteins: cytoskeleton and extracellular										
201	Alpha-actin 4	*Rachycentron canadum*	E9L834	ACTA	572	2.4E-52	11	5	41	42.3	5.22
203	Beta actin	*Acipenser transmontanus*	B6E4I1	ACTB	279	4.9E-23	10	5	37	42.1	5.30
105	Beta-actin	*Tetraodon nigroviridis*	Q4SMI4	ACTB	508	6.1E-46	12	8	43	42.9	5.57
106	Beta-actin (Fragment)	*Gobio gobio*	G8A4Z9	ACTB	397	7.7E-35	7	5	45	30.0	5.33
119	keratin, type I cytoskeletal 13-like	*Oreochromis niloticus*	I3JS53	CYT1L	229	4.9E-18	7	3	11	49.0	5.68
122	Type I cytokeratin, enveloping layer, like	*Oreochromis niloticus*	I3JS53	CYT1L	224	1.5E-17	11	3	17	49.0	5.68
107	Type II cytokeratin	*Danio rerio*	Q9PUB5	KRT5	462	2.4E-41	18	4	32	58.5	5.34
108	Type II keratin E3 (Fragment)	*Gillichthys mirabilis*	C1J0K	KRT	52	2.4E-00	3	2	11	34.1	4.83
215	Coactosin-like protein	*Tetraodon nigroviridis*	Q4SKB8	COTL1	89	4.5E-01	3	2	16	16.2	4.92
125	Cofilin-2	*Tetraodon nigroviridis*	Q4RP95	COF2	229	4.9E-18	4	3	18	18.8	6.82
110	Tropomyosin4-2	*Takifugu rubripes*	Q805C2	TPM4-2	93	1.8E-04	7	1	30	28.4	4.58
	Metabolism										
	Protein metabolism										
113	Proteasome subunit alpha type	*Oryzias latipes*	H2L6P7	PSMA	283	1.9E-23	9	3	43	29.7	6.07
115	Proteasome subunit alpha type	*Tetraodon nigroviridis*	Q4SRB7	PSMA5	407	7.7E-36	9	5	41	26.5	4.74
116	Proteasome subunit alpha type	*Gasterosteus aculeatus*	G3PZP3	PSMA4	353	1.9E-30	7	4	32	29.5	5.34
120	Proteasome subunit alpha type	*Oreochromis niloticus*	I3JJY5	PSMA	704	1.5E-65	9	7	52	25.9	5.99
123	Proteasome subunit beta type	*Danio rerio*	Q6DHI9	PMSB2	242	2.4E-19	6	2	27	22.7	6.1
	Other metabolism pathways										
218	Brain-type fatty acid binding protein	*Epinephelus coioides*	A8HG12	FABP	96	1.0E-04	3	2	25	14.9	6.17
219	Brain-type fatty acid binding protein	*Epinephelus coioides*	A8HG12	FABP	118	6.1E-07	3	2	25	14.9	6.17
109	Glyceraldehyde-3-phosphate dehydrogenase	*Pagrus major*	Q90WD9	GAPDH	114	1.5E-06	12	1	40	36.4	6.36
128	Nucleoside diphosphate kinase	*Sparus aurata*	B5APB7	NDPK	236	9.7E-19	4	3	26	17.1	6.42
101	Transferrin	*Sparus aurata*	F2YLA1	TF	720	3.9E-67	26	6	40	76.1	5.93
102	Transferrin	*Sparus aurata*	F2YLA1	TF	491	3.1E-44	14	6	30	76.1	5.93
	Stress response										
211	Peroxiredoxin 1	*Sparua aurata*	G0T332	PRDX1	119	4.9E-07	5	2	29	22.1	6.30
212	Peroxiredoxin 2	*Sparus aurata*	G0T333	PRDX2	232	2.4E-18	6	4	38	21.9	5.79
104	Stress protein HSC70-1	*Seriola quinqueradiata*	B6F133	HSC70-1	1020	3.9E-97	25	7	37	71.4	5.23
127	Superoxide dismutase [Cu-Zn] (Fragment)	*Sparus aurata*	Q571Q7	Cu/Zn SOD	89	5.2E-04	2	2	28	6970	5.41
103	Warm temperature acclimation-related 65 kDa protein	*Sparus aurata*	C0L788	WAP65	394	1.5E-34	20	6	41	49.7	5.41
	Signal transduction										

124	Phosphatidylethanolamine-binding protein 1	*Takifugu rubripes*	H2UXL0	PEBP1	172	2.4E-12	3	2	13	21.1	5.65
118	Rho GDP dissociation inhibitor (GDI) alpha	*Danio rerio*	Q6P3J2	ARHGDIA	268	6.1E-22	3	2	20	23.1	5
112	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide like	*Gasterosteus aculeatus*	G3NHX0	YWHABL	69	5.4E-02	6	1	21	29.6	4.65

[a] Spot number in reference 2-DE gel

[b] Protein name, organism and UniProt ID of the first hit returned by Mascot search, except for spots 105, 119, 122, 125, 112, 124, 215 in which the first in the list was an unidentified protein. In these cases, the protein name that is shown is the first identified protein after a BLAST search performed in the UniProt page.

[c] Protein symbol as UniProtKB/Swiss-Prot database.

[d] MOWSE score based on MS data. Protein scores greater than 68 are significant ($p<0.05$). SN 108 have a score below 68 but two fragmented peptides from this spot match with high score with this protein.

[e] Number of times we would expect to obtain an equal or higher score by chance.

[f] PM: Number of non redundant matching peptides. PF: Number of fragmented peptides matching the protein. SC: % of sequence coverage.

Table 3 – LC-MS/MS-derived peptide sequences from skin mucus of *S. aurata* matching exclusively bacterial or archaeal sequences.

	Peptide sequence[a]	Protein[b]	Organism[c]	Score[b]	E value[b]	Accession[b]
1	DLLSEYDFPGDDLPVIQGSALK	elongation factor Tu	*Streptococcus* (144)	72.3	2E-13	ACX81418.1
2	DLLSEYDFPGDDIPVIQGSALK	elongation factor Tu	*Streptococcus* (33)	72.7	3E-13	ABW24197.1
3	AVVELAGVADITSK	30S ribosomal protein S5	*Streptococcus* (12)	43.9	4E-04	WP_019319452.1
4	MLADLAVNDAVAFTALADAAK	50S ribosomal protein L20	*Streptococcus* (3)	66.4	1E-11	WP_001841176.1
5	ELADAAVSTIEIER	30S ribosomal protein S3, partial	*Streptococcus* (35)	46.0	7E-05	WP_008291857.1
6	AAAELELISGQKPLITK	50S ribosomal protein L5	*Streptococcus* (5)	54.1	2E-07	WP_018367195.1
7	YPEFAQLEGQLK	6-phosphofructokinase	*Streptococcus* (89)	42.2	1E-03	WP_001831447.1
8	ITDFLSANAEVIAR	adenylosuccinate synthetase	*Streptococcus* (115)	46.4	2E-05	WP_006150535.1
9	VVFGENIGTTVSNNIEEKE	uridylate kinase	*Streptococcus* (23)	63.0	2E-10	WP_008275990.1
10	LVVLYDSNDINLDGETK	transketolase, partial	*Streptococcus* (162)	57.1	2E-08	AGG36758.1
11	LSQETSVYVTGIVK	asparaginyl-tRNA synthetase	*Streptococcus* (86)	46.0	9E-05	YP_003879782.1
12	GGAVGDQYVTVNVVTPTGLNDR	chaperone protein DnaJ	*Streptococcus* (98)	70.6	6E-13	WP_001808904.1
13	GVYLNEDGSVNLSK	pyruvate formate lyase, partial	*Streptococcus* (180)	46.0	8E-05	AFN66486.1
14	ISQALEVAEPGVTNR	selenide, water dikinase	*Streptococcus* (96)	49.0	7E-06	WP_001852559.1
15	LFLEEEGLQSR	pyruvate oxidase	*Streptococcus* (105)	38.0	2E-02	ETJ01702.1
16	TGAQVAGPIPLPTER	30S ribosomal protein S10	*Streptococcus* and *Aerococcus* (9)	49.0	7E-06	WP_000649303.1
17	FDATVEVAYNLNIDVK	50S ribosomal protein L1, partial	*Streptococcus and Lactococcus* (64)	54.5	2E-07	WP_019785915.1
18	IEDQLGEVAEYR	alpha-enolase	*Streptococcus* and *Catellicoccus marimammalium* (96)	42.2	8E-04	ADQ38428.1
19	LADAAVSTIEIER	30S ribosomal protein S3, partial	*Streptococcus, Enterococcus, and Melissococcus* (53)	42.6	1E-03	WP_008291857.1
20	ADIDYAWEEADTTYGK		*Streptococcus, Listeria, Bacillus, Lactococcus, Enterococcus, Tetragenococcus, Carnobacterium, and Granulicatella* (126)	56.6	3E-09	WP_008288328.1
21	VLLGLSGGVDSSVVGVLLQK	GMP synthase, partial	*Streptococcus, Enterococcus, Weissella, Granulicatella, Carnobacterium, Melissococcus, and Aerococcus* (286)	60.4	2E-09	ETJ01807.1
22	SGETEDSTIADIAVATNAGQIK	alpha-enolase	*Streptococcus, Listeria, Bacillus, Geobacillus, Exiguobacterium, Singulisphaera, Leptothrix, Brevibacillus, Caldibacillus, Catellicoccus, Anoxybacillus, Facklamia, Carnobacterium, Melissococcus, Granulicatella,, Enterococcus, Acetivibrio, Eremococcus, Micavibrio, Rhodomicrobium, and Alistipes* (244)	68.9	6E-13	ADQ38428.1
23	IWPTEDALKR	phosphoribosylaminoimidazolecarboxamide formyltransferase	*Clostridium* (6)	37.1	6E-02	WP_017210864.1
24	TDLVHAVYDEIPDVLRDLGVSEVHGVLMDLGVSSLQLDERER	S-adenosyl-methyltransferase MraW	*Arthrobacter* sp. (1)	85.5	7E-19	YP_831056.1
25	NMITGASQADAAILVcAAPDGVMQQTK	elongation factor 1-alpha	*Methanocella arvoryzae* (1)	88.0	3E-18	YP_684657.1
26	EDDDIWDVLEDVIK	DNA-directed RNA polymerase subunit beta	*Lactobacillus* (26)	51.1	2E-06	WP_021355686.1
27	FEQFVAEGAYLDALR	glycyl-tRNA ligase subunit beta	*Pelobacter carbinolicus* (1)	51.1	2E-06	YP_006716293.1
28	MLEAVLCDYQTSQILQLTDAMVR	DNA polymerase III, delta subunit	*Mycoplasma* (3)	79.5	2E-15	WP_014574988.1
29	ADMLQQECEALLVDFPDQEKELR	lipoate-protein ligase A domain protein, partial	*Escherichia coli* and *Shigella* (78)	79.5	4E-17	WP_001342437.1
30	STLIRCVNLLERPTEGSVQVGGQELTTLSESELTK	methionine ABC transporter ATP-binding protein	*Salmonella enterica, Citrobacter youngae, Citrobacter freundii, Shigella flexneri* (Family Enterobacteriaceae) (31)	70.5	1E-14	WP_001570617.1
31	ANVLIFPDLDAGNIAYK	phosphate acetyltransferase	*Geobacter, Caldicellulosiruptor* and *Deferribacter desulfuricans, Calditerrivibrio nitroreducens, Slackia heliotrinireducens and Clostridium thermocellum* (21)	57.1	3E-08	YP_003497026.1

[a] Peptide sequences derived from MS/MS data of *S. aurata* skin mucus (samples 1 and/or 2, see Supplementary file 1).

[b] First hit retrieved by BLAST search of the peptide sequence against non-redundant protein sequences database (100% identity). Protein name, score, E value, and accession number of the first hit are shown.

[c] Organism having sequences matching 100% with peptide sequence (in parentheses is the number of hits showing the highest score). When only genus is reported indicates that several species of the genus satisfy the condition.

Biological significance

This work shows that proteomic methods can be used to characterize fish skin mucus. Using a coupled approach of LC-MS/MS and a 2-DE-PMF-MS/MS, we have obtained the first comprehensive view of the skin mucosal proteome of *S. aurata*, a fish species that is economically relevant for Mediterranean aquaculture. We identified a panel of proteins involved in a variety of biological functions, particularly in the innate immune response. Furthermore, to our knowledge, this is the first time a proteomic approach has been used to examine the microbiota in the skin mucus of a fish species. Overall, these results support further immunological researches in *S. aurata* and are relevant for the culture of this important fish species.

Highlights

- The skin mucus proteome of farmed *S. aurata* was analyzed for the first time.
- Structural and metabolic proteins are the major functional groups of mucosal proteins.
- Stress response proteins and signal transduction proteins are also present in fish skin mucus.
- Proteomics identified the microbial communities living in the fish mucus layer.