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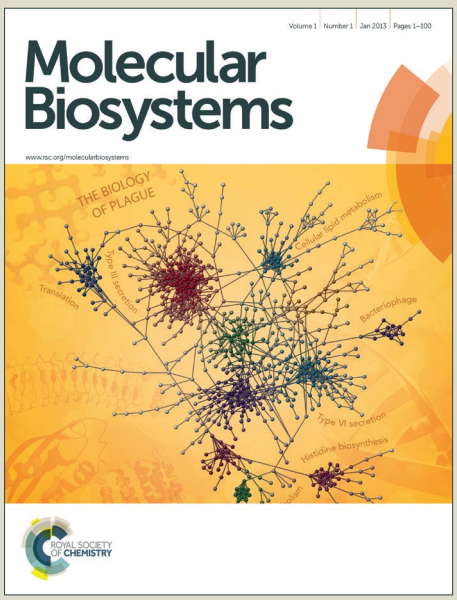

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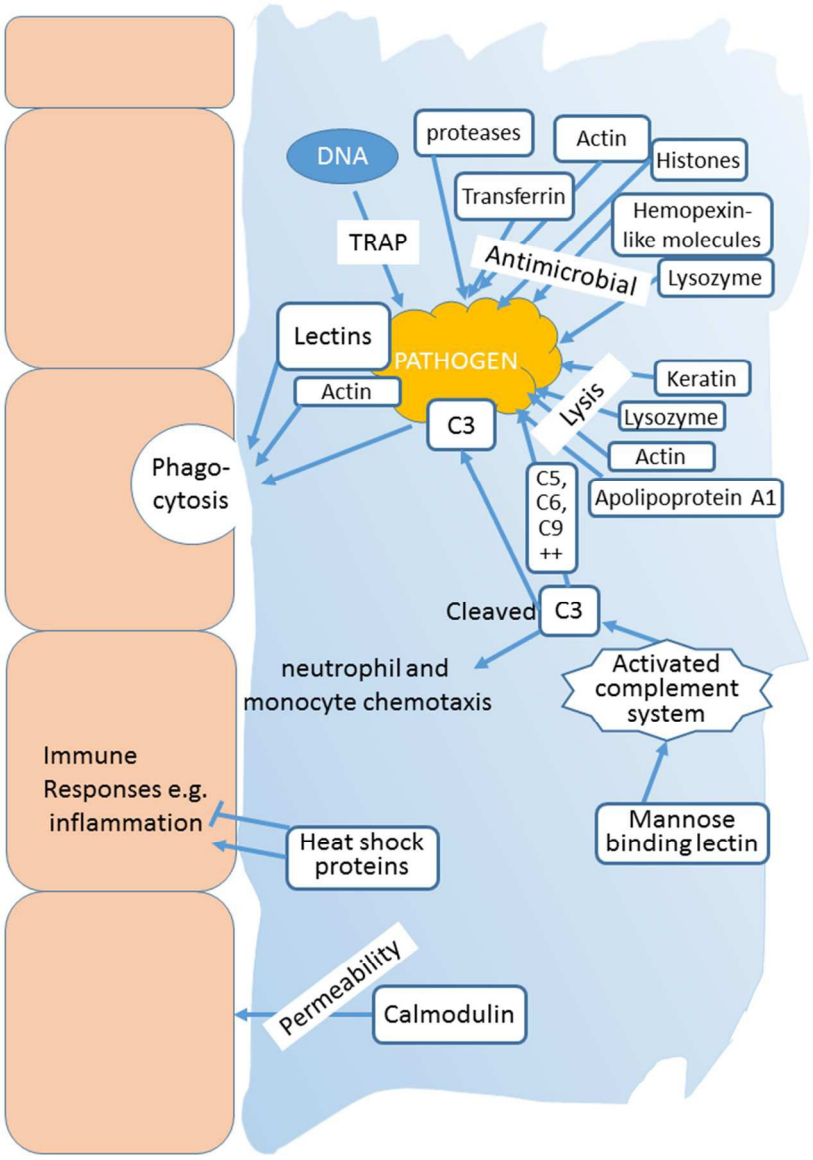

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REVIEW

Immune relevant molecules identified in the skin mucus of fish using -omics technologies

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Abstract

This review will give an overview of immune relevant molecules in fish skin mucus. The skin of fish is continuously exposed to the water environment, and unlike that of terrestrial vertebrates, it is a mucosal surface with a thin epidermis of live cells covered by a mucus layer. The mucosa plays an important role in keeping the homeostasis of the fish and preventing entry of invading pathogens. This review provide an overview of proteins, RNA, DNA, lipids and carbohydrates found in skin mucus of studied species. Proteins such as actin, histones, lectins, lysozyme, mucin, and transferrin have extracellular immune relevant functions, other molecules including complements molecules, heat shock molecules and superoxide dismutase present in mucus show differential expression during pathogen challenge in some species, but their functions in mucus, if any, need to be shown. RNA, DNA, lipids, carbohydrates and metabolites in mucus have been studied to a limited extent in fish, the current knowledge is summarized and knowledge gaps are pointed out.

Introduction

Knowledge on the health and welfare of fish is important for aquaculture production, to be able to manage the fisheries and also in conserving species diversity. The fish mucosal surfaces are of outmost importance to protect the integrity and homeostasis of the body and to prevent entry and colonization of the skin by pathogens. The skin mucosa is continuously exposed to the water in which the fish lives and hence stress and immune defense molecules, cells and networks of interaction between protein and between proteins and cells must be present. The skin mucus is a new focus for biomarker studies aiming to find noninvasive means to monitor fish stress and health status. The study of fish mucus has traditionally been done by focusing on one or a few molecules at the time, however in recent years –omics technologies, in particular proteomics, have been used in more global approaches. The mucus will protect the skin epidermis, where in fish in general few or no keratinized cells are present and the cells are alive throughout. For some fish, however, it has been shown that elevated parts of the skin surface have an outer layer of keratinized dead cells.[1] The skin mucus of fish is rich in proteins and carbohydrates and is a site for a complex network between the host, and commensal and pathogenic bacteria.[2]

The skin mucosa is an essential barrier of fish serving as a protection against the surrounding water with biotic and abiotic factors. The mucosa consist of a cellular and a humoral part. The cellular part, the mucous membrane, are mainly epithelial cells with an underlying connective tissue. In the epidermis of most fish there are three main mucus secreting cell types goblet cells, the sacciform cells and the club cells[3], the skin is a lymphoide tissue with leukocytes such as granulocytes, macrophages and lymphocytes present[4]. The humoral part is the extracellular molecules present in the skin mucus, a viscous layer on the outer surface of the fish.

Proteins in the skin mucus could arrive there through several routes. The classical delivery of extracellular material is through the secretory pathway where proteins are synthesized by ribosomes on the rough ER and via the Golgi are delivered to the cell membrane (Figure 1)[5]. Alternatively, the proteins are synthesized in the cytosol and delivered by transport routes directly over the cell membrane by transporters or through channels or other non-classical mechanisms.[6, 7] The non-classical secretion by membrane vesicles such as exosomes and microvesicles could deliver other molecules than proteins including RNA (Figure 1). In fish the actual routes of delivery have been studied to little extent, however it might be anticipated that the mode of delivery could be conserved. In terrestrial vertebrates skin mucus is not present, but many of the molecules identified in skin mucus of Atlantic cod[8] were also present in mammalian cervix mucus[9] and a preserved location (i.e. mucus) could indicate that the transport mechanisms are also conserved. An additional source of mucus proteins will be dead cells, lost from the epidermal surface. Delivery through the latter route do not exclude a function in the mucus, as many proteins have additional functions to their better known, classical function(s). Proteins performing more than one function are often called "moonlighting proteins"[10] or "gene sharing" proteins.[11] Histones could serve as an example, with a well-known role in DNA packing and gene regulation for the full-length proteins and another role as antimicrobial molecules for full-length histone[12] and its peptide fragments.[13]

The aim of this review is to provide an overview of immune relevant molecules identified by –omics technologies in skin mucus of naïve fish and fish exposed to stressors including pathogens.

Immune relevant proteins and peptides in skin mucus identified by proteomics techniques

2DE and LC-MS/MS coupled approaches are the most commonly used methods to study the skin mucus proteome of fish, however gel-free proteomics are also used.[14-16] A challenge when working with fish is that for many species the sequence data available are scarce. Homology driven approaches[17] have, however, been used successfully and numerous proteins have been identified. With recent advances in RNA-seq methods, an approach where sequenced mRNA is used for identification of non-model species' proteome is promising[18], also less expensive and faster genomic-sequencing methods are delivering increased number of available sequences.

Gel-free proteomics identifies more proteins than the 2DE and LC-MS/MS approach. In a study of large yellow croaker *Larimichthys crocea*[14] 3209 protein were identified in skin mucus. This accounts for 12% of the protein coding genes of the species. In another gel-free LC-MS/MS study of salmon mucus, 521 proteins were found, only 4 of these were unique to control, non-sea lice infected fish.[15] If we consider that proteins in the mucus could arrive through several routes including that from dead cells, it is not surprising that numerous proteins are present in the mucus. Functional studies are needed to determine whether specific proteins present have a role in mucus or if they are just present as a form of biological waste. Several studies have looked at the differential proteome in fish after immune relevant challenges such as infection, stress and probiotic exposure. [14-16, 19, 20]

A major short-coming of two dimensional gel based techniques is problems with the solubility of hydrophobic proteins. However, in a study of fresh water teleost skin mucus only 3 % of the proteins were found in the detergent soluble fraction, the rest were in the aqueous fraction.[21] This indicate that for fish skin mucus, most proteins are water soluble. The presence of hydrophobic proteins from the cell debris would be expected, if hydrophobic proteins are actively secreted remains to be studied. In a study using both 2D gel coupled with LC-MS/MS as well as gel free analysis the proteins identified were complementary rather than overlapping.[22]

Immune relevant genes are differentially express in the dorsal and ventral part of Atlantic cod skin[23], this suggest that there also could be differences in mucus composition on different parts of the fish surface, this has not yet been addressed as mucus is routinely sampled from the whole fish.

The mucus layer is part of the innate immune system that is present as a first line of defense, and high amounts, evident as strong proteins spots in 2DE, of immune relevant proteins are found in the mucus of naïve fish and stressed or pathogen infected fish. When a protein or peptide binds to a pathogen, the binding can lead to immobilization, inhibition of cell surface binding, stimulation of cellular defense including phagocytosis, killing of the pathogen. Mucus is a chemoattractant to bacteria, bacteria can attach to and feed on mucus, and mucus

can stimulated biofilm formation[24]. Outside the scope of this paper is the use of –omics technology to study the microbiota of the mucosal surface, it is however interesting that a proteomics study of gilthead seabream skin mucus showed that peptides found could be used to identify the bacteria present.[22]

Many proteins with well-established, classical cellular roles are found in mucus, only the ones with possible roles in mucus are mentioned here.

Fish skin mucus contains well established antimicrobial proteins such as lysozyme (Figure 2 and Table 1) found in Atlantic cod[8], Atlantic salmon[15] and European sea bass.[25] Its mucus levels changed in sea lice infected Atlantic salmon[15] and it was upregulated in European sea bream after crowding stress and/or probiotic uptake.[20]

Lectins of different types are found in most studies (Table 1). Lectins are a group of carbohydrate binding proteins. Mannose binding lectin (MBL) can bind to pathogens and activate the complement system (figure 2). MBL were found in Atlantic cod mucus.[8]

Galectins are β-galactoside binding proteins able to bind to[26] and kill[27, 28] bacteria (Figure 1). Galectin-1 proteins were found in Atlantic cod[8], galectin-3 were upregulated in sea lice infected Atlantic salmon[15], several galectins were found in stressed large yellow croaker.[14]

Nattectin, a C-type lectin binding to galactose, was upregulated in Atlantic salmon affected by amoebic gill disease.[16] Fucose binding lectin was found in sea bass mucus.[25] Lectin-like calreticulin induces phagocytosis by microbial binding, it is found in European sea bass[25] and Atlantic cod.[8] In both studies the observed MW in 2DE was higher than the predicted one, this suggest that the protein could be glycosylated as known to explain the high observed MW of human calreticulin in SDS-PAGE.

Heat shock proteins of several types are frequently found in fish skin mucus (Table 1).[14, 15, 22, 25, 29] Extracellular heat shock proteins have been suggested to play a role in fine-tuning inflammation and have both immunostimulatory and immunosuppressive functions depending of the other molecules present (Figure 2).[30]

Apolipoprotein1, a plasma protein, was found in skin mucus of Atlantic cod[8, 31], Atlantic salmon[15, 32],gilthead sea bream[22], sea bass[25], and was upregulated in mucus of *Vibrio anguillarum* infected Atlantic cod[19] and sea lice infected Atlantic salmon (Table 1).[15] Fish apolipoprotein A1 has antibacterial activity[33] and lyse bacteria (Figure 2).[34]

Complement factors were found in mucus in several studies. They are serum proteins, part of the complement system that leads to bacterial lysis, chemotaxis of immune cells, and increased phagocytosis. C3 is a key molecule in the complement pathway, when cleaved the cleavage products lead to chemotaxis and phagocytosis, and start a cascade involving other complement factors eventually leading to bacterial lysis (Figure 2). In sea bass mucus cleaved C3 has been found[25], multiple complement factors were found in this and other studies [14-16, 29, 32] and complement factors were upregulated after stress and probiotic uptake in sea bream[20], and in downregulated in amoebic gill disease affected Atlantic salmon.[16]

Histones are essential chromatin proteins and full length proteins and/or fragments was found in Atlantic salmon[15, 35], gilthead seabream[22], and European sea bass[25]. Full length histones and histone fragments are antimicrobial molecules (Figure 2, Table 1).[12, 13]

Keratins are found in naïve skin mucus of several species including gilthead sea bream[22, 29], Atlantic salmon[15, 32] and Atlantic cod.[8] Keratin was differentially expressed in skin mucus in amoebic gill disease affected Atlantic salmon[16] and after *Vibrio anguillarum* infection in

Atlantic cod[19]. It is a structural protein in fish scales, but an extracellular function is indicated in the fact that a keratin from trout show antibacterial activity by pore formation (Figure 2, Table 1).[16]

β-actin is a structural protein important for phagocytosis and cell motility, it is found in mucus of many species[8, 15, 22, 29, 32] and are differentially expressed in sea lice challenged Atlantic salmon[15, 32] and in overcrowded and probiotic exposed sea bream(Table 1)[20]. The high amount of actin and its fragments in mucus has led to speculations on an immune relevant function in fish mucus.[32] Recently extracellular actin from insects was shown to bind to bacteria and stimulate phagocytosis or to directly kill the bacteria.[36] Insect actin also inhibited *Plasmodium* infection in the gut of mosquitos.[36] These findings suggest that actin could be functionally active in fish skin mucus (Figure 2).

Iron binding proteins will limit bacterial growth by limiting the availability of iron (Figure 2). Several iron binding proteins were found in fish mucus (Table 1). Warm temperature acclimation protein 65 is a homologue to mammalian hemopexin, shown to bind iron containing heme. Warm temperature acclimation protein 65 were found in sea bass[25] and seabream[22], and a hemopexin-like molecule found in Atlantic salmon[32].

Fragments of transferrin, an iron binding protein (Figure 2), were upregulated in skin mucus of sea lice infected Atlantic salmon[32] and are present in naïve Atlantic salmon[15] , sea bass[25] and sea bream[22, 29].

Table 1 shows additional proteins with an possible immune relevance in mucus. Further studies should be conducted to see if the proteins in mucus are functionally active there. To study the differential proteome under stress or infection challenge is important as changes in expression could be related to function. However, since the mucus is a first line of defense proteins with stable expression could be functionally important for immune defense.

It is important to realize that if a spot in a 2DE gel changes intensity, it is not necessarily reflecting changes in the protein level, as post-translational changes can change the pI and/or the apparent MW of the spot. The possibility to observe posttranslational modifications is one of the advantages of using 2DE gels, as isoform changes can be readily detected. In fish there has been a limited focus on this perspective, and in general only changes in spot intensity are reported without further investigation of the molecular changes. In the years to come additional studies in the field of proteomics could shed light on the mucosal protein isoforms and provide additional functional cues. In the previous paragraphs as well as in Table 1 the terms up- and downregulation were used to indicate increase or decrease in spot intensity or peak intensity of an identified protein without considering post-translational modifications.

In many of the proteomics studies there are spots which are not identified, due to the quality of the mass spectrometry data obtained or due to limitations in available genomics and proteomics data. In the latter case, reanalysis of the data, should be performed when more sequences become available in databases.

Mucins, glycoproteins and carbohydrates in skin mucus.

Mucus is rich in glycoproteins, especially high molecular weight mucins[37], important for viscosity, to trap pathogens and physically protect the skin surface, and to contribute to signaling at the cell surface. Glycoproteins are co-translationally transported into the

endoplasmatic reticulum lumen where they are N-glycosylated and then processing of the N-glycosylation and O-glycosylation takes place in the Golgi apparatus before secretion.[5, 38]

Mucins are generally not identified in the proteomics studies of skin mucus, this is due to the fact that before gel or gel free analysis approaches, the sample preparation include centrifugation and/or filtration that will remove mucins. LC-ESI-MSMS used to characterize Atlantic salmon mucin O-linked glycosylation showed that skin mucin contain shorter and less diverse O-glycans than salmon intestine mucin.[39] The use of LC-MS/MS is advantageous as it allows for analysis of sulfated glycans, this is not possible when matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) or electrospray ionization mass spectrometry (ESI-MS) in the positive-ion mode is used.[40] Atlantic salmon skin mucus show lower levels of sialylation than intestinal mucus, and this is suggested to explain the lower level of *Aeromonas salmonicida* subsp. *Salmonicida* binding observed for skin mucus than for intestinal mucus.[41] In carp skin mucus an early increase and later decrease in glycosylation of mucus proteins were observed when there was an increased bacterial load in the water.[42]

In rainbow trout skin and intestine mucus free amino acids and carbohydrates act as chemoattractants for the pathogen *Vibrio anguillarum*.[43] Gas chromatography-MS (GC-MS), GC and LC-MS/MS were used to identify and quantify the active components. The main chemotactic carbohydrates were fucose, glucose, mannose, and xylose, intestine mucus has higher levels of free amino acids and carbohydrates than skin and this could explain why intestinal mucus is a stronger chemoattractant than skin mucus.[40]

RNA identification in skin mucus

Extracellular RNA (exRNA) is extensively studied in mammals and is actively secreted by cells. ExRNA are found in exosomes, microvesicles (Figure 2), closely associated with proteins or with lipids, free exRNA needs to be hidden to avoid rapidly degradation by RNases. [44] . Salmon head kidney leukocytes secrete exosomes, but their RNA content, if any, was not studied.[45]. If active secretion of exRNA into skin mucus takes place, or if exRNA arrive solely from dead cells is yet to be studied in fish. ExRNA could have functions in mucus, however, even in humans clear extracellular functions of exRNA except signaling between cells are yet to be shown. Interestingly, a role in immune modulation, control of self/non-self and autoimmunity has been suggested.[44] One group of exRNA, microRNA, has been identified in mammalian mucus[46], in fish microRNA studies are scarce[47] and skin mucus microRNA characterization is yet to be done. Non-coding RNAs are interesting as they could serve direct functional roles as e.g. ribozymes and/or function together with their bound carrier lipids or proteins.

The biomarker potential of skin mucus immune related mRNA was explored in a *Flavobacterium columnare* challenge of channel catfish.[48] In the study qPCR was used and immune relevant genes were differentially expressed after the challenge[48], however it is noteworthy that the changes found in skin mucus mRNA were different from those found in

skin.[48] At present additional studies would be needed to see whether mucus mRNA analysis could be used as a noninvasive method to monitor the health status of fish.
The transcriptome of the skin cells has been extensively studied in several species using RT-PCR[23], qPCR[48, 49], microarray[50], and second generation sequencing RNA-seq technology[49]. Studies of the mRNA in skin are often included in proteomics studies of the skin mucus to see if skin mucus proteins are locally produced[8, 19, 20, 25]

DNA identification in skin mucus

In humans, DNA is known to be actively secreted from neutrophils and the extracellular DNA is important in trapping pathogens[51], suggesting an immune role for DNA also in mucus. DNA is present in fish skin mucus, from dead host cells and commensal bacteria or pathogens, if neutrophils or other cell types actively secrete DNA at the skin surface, this would contribute to the viscosity of the mucus.

A mucus sample can be used as a noninvasive method to detect pathogens as shown in a study where *Aeromonas salmonicida* genomic DNA was successfully detected in Atlantic cod mucus by PCR and loop-mediated isothermal amplification albeit with a one log dilution decrease in detection limit.[52] Host DNA in mucus could be used in selective breeding as a noninvasive method for PCR based studies to select disease resistant broodstock. In mantra rays skin mucus samples have been used successfully for genotyping by PCR, however the yields were not, with the present methods, enough to do next generation sequencing studies[53]. Skin mucus has also been used for microsatellite and polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analyses of northern pike and brown trout[54].

Lipids in skin mucus

Shotgun lipidomics has been used successfully to analyze phospholipids in fish viscera.[55] Lipids in fish skin mucus have not been thoroughly studied by lipidomics, however in rainbow trout intestinal mucus free fatty acids, mono- and diglycerides, cholesterol and phospholipids have been analyzed and found to act as chemoattractants towards the pathogenic *Vibrio anguillarum.*[40] Lipids could contribute to mucus viscosity.[56] Intestinal lipid composition analyzed with ESI-MS/MS is different in mice with ulcerative colitis than in healthy individuals, this suggests that mucus lipids could be used as biomarkers and/or that differences in lipid amounts or composition reflect functional role(s) of lipids .[57] Fatty acids and lipids on the skin surface of humans have antimicrobial activity and come from cell debris or are secreted by sebaceous glands[58-60]. The lipid composition of fish skin mucus and the function(s) if any warrants further investigation.

Metabolites in skin mucus

LCMS/MS analysis of the skin mucus of fathead minnow revealed 204 distinct metabolites, 84 were putatively annotated including amino acids, purines, pyrimidines, nucleosides, and nucleotides. Some identified metabolites could be antibacterial such as azelaic acid, N-acetylneuraminic acid and N-acetylglucosamine, and hydroxyisocaproic acid. The study

identified sex differences in the metabolites as well as changes in the metabolite profile after exposure to the contaminant bisphenol A in both males and females.[40] It would be interesting to see whether further studies find changes in the metabolites after pathogen challenge and whether metabolites show antibacterial activity against fish pathogens.

Concluding remarks

The proteome of the skin mucus has been studied in several fish species. Differential characterization of sick vs health fish has also been done for a limited number of diseases and species. In the future, identified proteins should be included in functional studies and controlled infection studies, to test the biomarker potential and immune relevant functions of the proteins. Non-invasive tests for the health and welfare status of fish will be important both for the aquaculture industry and for fish population studies. In the case of DNA, RNA, lipids and carbohydrate less information on their presence and immune relevant function(s) in mucus, if any, is available. There will in the years to come hence be important to continue the study of the mucosal surface of the skin by new and old methods, including the -omics techniques.

Figure legend.

Figure 1. The figure outlines how molecules, especially proteins, could be actively transported to the extracellular space. The classical pathway is from the endoplasmatic curriculum, through Golgi and then to the cell surface. Direct transport could take place over the cell membrane through transporters or protein channels, or by secretion in membrane vesicles.

Figure 2. The figure shows possible extracellular roles for proteins detected in fish skin mucus. Mucosal proteins can interact directly with pathogens (orange) and lead to bacteriostatic or antibacterial effects or lysis of pathogens. Binding to host pathogens can also stimulate phagocytosis, or result in stimulation of chemotaxis to recruit host leukocytes. Extracellular proteins can interact with and modulate the activity of host skin cells.

References

1. A. K. Mittal and M. Whitear, *Cell and tissue research*, 1979, **202**, 213-230.
2. S. Boutin, L. Bernatchez, C. Audet and N. Derôme, *PLoS ONE*, 2013, **8**.
3. G. Zaccone, B. G. Kapoor, S. Fasulo and L. Ainis, in *Advances in Marine Biology*, Academic Press, 2001, vol. Volume 40, pp. 253-348.
4. I. Salinas, Y.-A. Zhang and J. O. Sunyer, *Developmental and comparative immunology*, 2011, **35**, 1346-1365.
5. K. C. Kim, J. I. Rearick, P. Nettesheim and A. M. Jetten, *Journal of Biological Chemistry*, 1985, **260**, 4021-4027.
6. W. Nickel, *European journal of biochemistry / FEBS*, 2003, **270**, 2109-2119.
7. C. E. Chua, Y. S. Lim, M. G. Lee and B. L. Tang, *Journal of cellular physiology*, 2012, **227**, 3722-3730.
8. B. Rajan, J. M. Fernandes, C. M. Caipang, V. Kiron, J. H. Rombout and M. F. Brinchmann, *Fish & shellfish immunology*, 2011, **31**, 224-231.
9. G. Panicker, Y. Ye, D. Wang and E. R. Unger, *Clinical proteomics*, 2010, **6**, 18-28.
10. P. Juszczynski, J. Ouyang, S. Monti, S. J. Rodig, K. Takeyama, J. Abramson, W. Chen, J. L. Kutok, G. A. Rabinovich and M. A. Shipp, *Proceedings of the National Academy of Sciences of the United States of America*, 2007, **104**, 13134-13139.
11. J. Piatigorsky, *Progress in Retinal and Eye Research*, 1998, **17**, 145-174.
12. J. M. O. Fernandes, G. D. Kemp, M. G. Molle and V. J. Smith, *Biochemical Journal*, 2002, **368**, 611-620.
13. T. Lüders, G. A. Birkemo, J. Nissen-Meyer, Ø. Andersen and I. F. Nes, *Antimicrobial Agents and Chemotherapy*, 2005, **49**, 2399-2406.
14. J. Ao, Y. Mu, L.-X. Xiang, D. Fan, M. Feng, S. Zhang, Q. Shi, L.-Y. Zhu, T. Li, Y. Ding, L. Nie, Q. Li, W.-r. Dong, L. Jiang, B. Sun, X. Zhang, M. Li, H.-Q. Zhang, S. Xie, Y. Zhu, X. Jiang, X. Wang, P. Mu, W. Chen, Z. Yue, Z. Wang, J. Wang, J.-Z. Shao and X. Chen, *PLoS Genet*, 2015, **11**, e1005118.
15. F. Provan, L. Jensen, K. Uleberg, E. Larssen, T. Rajalahti, J. Mullins and A. Obach, *Journal of fish diseases*, 2013, **36**, 311-321.
16. V. A. Valdenegro-Vega, P. Crosbie, A. Bridle, M. Leef, R. Wilson and B. F. Nowak, *Fish & shellfish immunology*, 2014, **40**, 69-77.
17. M. Junqueira, V. Spirin, T. S. Balbuena, H. Thomas, I. Adzhubei, S. Sunyaev and A. Shevchenko, *Journal of proteomics*, 2008, **71**, 346-356.
18. V. C. Evans, G. Barker, K. J. Heesom, J. Fan, C. Bessant and D. A. Matthews, *Nature methods*, 2012, **9**, 1207-1211.
19. B. Rajan, J. Lokesh, V. Kiron and M. F. Brinchmann, *BMC veterinary research*, 2013, **9**, 103.
20. H. Cordero, P. Morcillo, A. Cuesta, M. F. Brinchmann and M. A. Esteban, *Journal of proteomics*, 2016, **132**, 41-50.
21. A. K. Nigam, U. Kumari, G. D. Nigam, S. Mittal and A. K. Mittal, *Research in environment and life sciences*, 2012, **5**, 218-222.
22. J. Jurado, C. A. Fuentes-Almagro, F. A. Guardiola, A. Cuesta, M. A. Esteban and M. J. Prieto-Alamo, *Journal of proteomics*, 2015, **120**, 21-34.
23. C. M. Caipang, C. C. Lazado, M. F. Brinchmann, J. H. Rombout and V. Kiron, *Comparative biochemistry and physiology. Part D, Genomics & proteomics*, 2011, **6**, 158-162.
24. S. Benhamed, F. A. Guardiola, M. Mars and M. Á. Esteban, *Veterinary microbiology*, 2014, **171**, 1-12.
25. H. Cordero, M. F. Brinchmann, A. Cuesta, J. Meseguer and M. A. Esteban, *Proteomics*, 2015, **15**, 4007-4020.
26. B. Rajan, V. Kiron, J. M. Fernandes and M. F. Brinchmann, *Developmental and comparative immunology*, 2013, **40**, 83-93.

27. G.-H. Cha, Y. Liu, T. Peng, M.-Z. Huang, C.-Y. Xie, Y.-C. Xiao and W.-N. Wang, *Molecular immunology*, 2015, **67**, 325-340.
28. S. R. Stowell, C. M. Arthur, R. McBride, O. Berger, N. Razi, J. Heimburg-Molinaro, L. C. Rodrigues, J. P. Gourdine, A. J. Noll, S. von Gunten, D. F. Smith, Y. A. Knirel, J. C. Paulson and R. D. Cummings, *Nature chemical biology*, 2014, DOI: 10.1038/nchembio.1525.
29. I. Sanahuja and A. Ibarz, *Fish & shellfish immunology*, 2015, **46**, 426-435.
30. A. G. Pockley, M. Muthana and S. K. Calderwood, *Trends in biochemical sciences*, 2008, **33**, 71-79.
31. R. H. Easy, E. A. Trippel, M. D. B. Burt and D. K. Cone, *Journal of Fish Biology*, 2012, **81**, 2059-2063.
32. R. H. Easy and N. W. Ross, *Comparative biochemistry and physiology. Part D, Genomics & proteomics*, 2009, **4**, 159-167.
33. L. D. Johnston, G. Brown, D. Gauthier, K. Reece, H. Kator and P. Van Veld, *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology*, 2008, **151**, 167-175.
34. J. W. Pridgeon and P. H. Klesius, *Fish & shellfish immunology*, 2013, **35**, 1129-1137.
35. I. L. Uttakleiv Ræder, S. M. Paulsen, A. O. Smalås and N. P. Willassen, *Microbial Pathogenesis*, 2007, **42**, 36-45.
36. S. L. Sandiford, Y. Dong, A. Pike, B. J. Blumberg, A. C. Bahia and G. Dimopoulos, *PLoS Pathog*, 2015, **11**, e1004631.
37. K. L. Shephard, *Reviews in Fish Biology and Fisheries*, **4**, 401-429.
38. M. Neutra and C. P. Leblond, *The Journal of Cell Biology*, 1966, **30**, 119-136.
39. C. Jin, J. T. Padra, K. Sundell, H. Sundh, N. G. Karlsson and S. K. Linden, *Journal of proteome research*, 2015, **14**, 3239-3251.
40. D. R. Ekman, D. M. Skelton, J. M. Davis, D. L. Villeneuve, J. E. Cavallin, A. Schroeder, K. M. Jensen, G. T. Ankley and T. W. Collette, *Environmental Science & Technology*, 2015, **49**, 3091-3100.
41. J. T. Padra, H. Sundh, C. Jin, N. G. Karlsson, K. Sundell and S. K. Lindén, *Infection and Immunity*, 2014, **82**, 5235-5245.
42. M. van der Marel, N. Caspari, H. Neuhaus, W. Meyer, M. L. Enss and D. Steinhagen, *J Fish Dis*, 2010, **33**, 431-439.
43. R. O'Toole, S. Lundberg, S.-Å. Fredriksson, A. Jansson, B. Nilsson and H. Wolf-Watz, *Journal of Bacteriology*, 1999, **181**, 4308-4317.
44. J. G. Patton, J. L. Franklin, A. M. Weaver, K. Vickers, B. Zhang, R. J. Coffey, K. M. Ansel, R. Blelloch, A. Goga, B. Huang, N. L'Etoille, R. L. Raffai, C. P. Lai, A. M. Krichevsky, B. Mateescu, V. J. Greiner, C. Hunter, O. Voinnet and M. T. McManus, *2015*, 2015.
45. D. B. Iliev, S. M. Jorgensen, M. Rode, A. Krasnov, I. Harneshaug and J. B. Jorgensen, *Developmental and comparative immunology*, 2010, **34**, 29-41.
46. G. Wu, G. Yang, R. Zhang, G. Xu, L. Zhang, W. Wen, J. Lu, J. Liu and Y. Yu, *Allergy, asthma & immunology research*, 2015, **7**, 449-457.
47. T. T. Bizuayehu and I. Babiak, *Genome Biology and Evolution*, 2014, **6**, 1911-1937.
48. Y. Ren, H. Zhao, B. Su, E. Peatman and C. Li, *Fish & shellfish immunology*, 2015, **46**, 537-542.
49. L. Liu, C. Li, B. Su, B. H. Beck and E. Peatman, *PLoS ONE*, 2013, **8**, e74581.
50. C. Li, B. Beck, B. Su, J. Terhune and E. Peatman, *Fish & shellfish immunology*, 2013, **34**, 920-928.
51. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky, *Science*, 2004, **303**, 1532-1535.
52. A. Kulkarni, C. M. A. Caipang, M. F. Brinchmann, K. Korsnes and V. Kiron, *Journal of Rapid Methods & Automation in Microbiology*, 2009, **17**, 476-489.
53. T. Kashiwagi, E. A. Maxwell, A. D. Marshall and A. B. Christensen, *PeerJ*, 2015, **3**, e1188.
54. L. Livia, P. Antonella, L. Hovirag, N. Mauro and F. Panara, *Molecular Ecology Notes*, 2006, **6**, 257-260.

55. Q. Shen, Y. Wang, L. Gong, R. Guo, W. Dong and H.-Y. Cheung, *Journal of Agricultural and Food Chemistry*, 2012, **60**, 9384-9393.
56. R. W. Lewis, *Lipids*, 1970, **5**, 947-949.
57. A. Braun, I. Treede, D. Gotthardt, A. Tietje, A. Zahn, R. Ruhwald, U. Schoenfeld, T. Welsch, P. Kienle, G. Erben, W. D. Lehmann, J. Fuellekrug, W. Stremmel and R. Ehehalt, *Inflammatory bowel diseases*, 2009, **15**, 1705-1720.
58. D. R. Drake, K. A. Brogden, D. V. Dawson and P. W. Wertz, *Journal of Lipid Research*, 2008, **49**, 4-11.
59. D. J. Bibel, R. Aly and H. R. Shinefield, *The Journal of investigative dermatology*, 1992, **98**, 269-273.
60. D. J. Bibel, S. J. Miller, B. E. Brown, B. B. Pandey, P. M. Elias, H. R. Shinefield and R. Aly, *The Journal of investigative dermatology*, 1989, **92**, 632-638.
61. C. Li, R. Wang, B. Su, Y. Luo, J. Terhune, B. Beck and E. Peatman, *Developmental & Comparative Immunology*, 2013, **39**, 447-455.
62. M. Ai-Jun, H. Zhi-hui and W. Xin-An, *Fish physiology and biochemistry*, 2013, **39**, 1411-1418.
63. T. M. Tadiso, A. Krasnov, S. Skugor, S. Afanasyev, I. Hordvik and F. Nilsen, *BMC Genomics*, 2011, **12**, 1-17.
64. A. D. Ramos, K. Conceicao, P. I. Silva, Jr., M. Richardson, C. Lima and M. Lopes-Ferreira, *Toxicon : official journal of the International Society on Toxinology*, 2012, **59**, 651-665.
65. G. Bergsson, B. Agerberth, H. Jornvall and G. H. Gudmundsson, *The FEBS journal*, 2005, **272**, 4960-4969.
66. J. Liu, J. D. Farmer, W. S. Lane, J. Friedman, I. Weissman and S. L. Schreiber, *Cell*, 1991, **66**, 807-815.
67. M. I. Concha, V. J. Smith, K. Castro, A. Bastias, A. Romero and R. J. Amthauer, *European journal of biochemistry / FEBS*, 2004, **271**, 2984-2990.
68. V. Molle, S. Campagna, Y. Bessin, N. Ebran, N. Saint and G. Molle, *Biochem J*, 2008, **411**, 33-40.
69. T. Ishii, E. Warabi and T. Yanagawa, *Journal of clinical biochemistry and nutrition*, 2012, **50**, 91-105.
70. M. Hirayama, A. Kobiyama, S. Kinoshita and S. Watabe, *The Journal of experimental biology*, 2004, **207**, 1387-1398.

436 Table 1. Immune relevant proteins identified in skin mucus of naïve fish.

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Protein family	Identified protein	Immune function, upregulation (↑) or downregulation (↓) of protein (P) in skin mucus or mRNA (R) in infected or stressed fish
Mucin	Mucin 5AC, B	↑R *Aeromonas hydrophila* infection of channel catfish skin[61]
Lectin		
	C-type lectin[20, 32]	↑P in skin mucus after sea lice infection of Atlantic salmon.[32] ↑P in gilthead seabream skin mucus after overcrowding stress[20]
	Fucose binding lectin[25]	Bacteria agglutination, hemaglutination, opsonizing ↑P in gilthead seabream skin mucus after probiotic administration.[20] ↑P in gilthead seabream skin mucus after overcrowding stress.[20]
	Galectin 1[8]	Bacteria agglutination, hemaglutination.[26]
	Galectin 3[15]	↑P in Atlantic salmon skin mucus after se lice infection[15] ↑R *Aeromonas hydrophila* infection of channel catfish skin.[61]
	Mannose binding lectin[8] And mannose binding lily-type (puffer) lectin[62]	↑R *Aeromonas hydrophila* infection of channel catfish skin.[61] ↓R *Aeromonas hydrophila* infected blue catfish skin [50] ↑P in gilthead seabream skin mucus of turbot at high temperatures.[62]
Protease inhibitors	Leukocyte elastase inhibitor/serine leukoproteinase inhibitor (serpins)[8, 25]	↑P in gilthead seabream skin mucus after probiotic administration.[20] ↓P in gilthead seabream skin mucus after overcrowding stress.[20]
Complement		
	C1q and family members[15, 25, 29]	Complement activation ↓R first days after infection of Atlantic salmon skin by salmon louse.[63] ↑R *Aeromonas hydrophila* infection of channel catfish skin[61] ↑R *Aeromonas hydrophila* infected blue catfish skin.[50]
	C3[15, 16, 25, 32]	Chemoatraction, opsoninisation, agglutination, activation of complement cascade to lyse bacteria. ↑P in gilthead seabream skin mucus after probiotic administration[20] ↑P in gilthead seabream skin mucus after

		overcrowding stress.[20]
	C5[15]	
	C6[15]	↓R *Aeromonas hydrophila* infection of channel catfish skin.[61] ↓R *Aeromonas hydrophila* infected blue catfish skin. [50]
	C9[16]	↓P in gilthead seabream skin mucus of Atlantic salmon affected by amoebic gill disease[16]
	Complement factor B[15]	
Histone		
	Histone H1[15, 25, 35]	
	Histone 2A[15, 22, 64]	Histone 2A Antibacterial[12] ↓R *Aeromonas hydrophila* infected blue catfish skin [50]
	Histone 2B[15] and H2B-like	Antibacterial activity in skin mucus[65] ↓↑R *Aeromonas hydrophila* infection of channel catfish skin[61] ↑R *Aeromonas hydrophila* infected blue catfish skin [50]
	Histone 3[15, 64]	
	Histone 4[15, 22, 25, 35]	
Immunophilin	FK506 binding protein[8, 15]	In complex with the immune suppressor FK506 FK506BP12 in humans blocks calcineurin needed for T-cell signalling[66]. ↓R *Aeromonas hydrophila* infection of channel catfish skin[61]
	cyclophilin[8]	In complex with the immune suppressor ciclosporin cyclophilin blocks calcineurin needed for T-cell signalling[66]
Heat shock protein		
	Hsc70[15, 22]	↓R *Aeromonas hydrophila* infected blue catfish skin.[50] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15]
	HSp60 mitochondrial (?)	↑R Aeromonas hydrophila infection of channel catfish skin[61]
	HSP70[25, 29]	
	Heat shock protein HSP 90 alpha & beta[15, 35]	↑R Aeromonas hydrophila infection of channel catfish skin[61] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15]
Other proteins		
	14-3-3 protein family[8, 15, 20, 22, 25, 29]	↑P in gilthead seabream skin mucus after probiotic administration.[20] ↑P in gilthead seabream skin mucus after overcrowding stress.[20] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15]

	Actin[8, 15, 22, 25, 29, 32, 35, 64]	↓P in gilthead seabream skin mucus after overcrowding stress.[20] ↑P cleaved actin during sea lice infection of Atlantic salmon[32] ↑P in gilthead seabream skin mucus after probiotic administration.[20] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15]
	Apolipoprotein A1 and preproapolipoprotein [8, 15, 22, 25, 31, 32]	Bactericidal and/or bacteriostatic activity shown for carp apolipoproteins.[67] ↑P in gilthead seabream skin mucus after probiotic administration.[20] ↑P in gilthead seabream skin mucus after overcrowding stress.[20] ↑P in skin mucus of *Vibrio anigiullarium* infected Atlantic cod [19]
	Calpain small subunit-1[19]	↑P *Vibrio anigiullarium* infected Atlantic cod [19]
	Cold inducible RNA-binding protein [15, 19]	↑P *Vibrio anigiullarium* infected Atlantic cod [19]
	Keratin[8, 15, 16, 22, 25, 29, 32, 62, 64]	Rainbow trout keratin is shown to have pore-forming avtivity[68]. ↑P in skin mucus of Atlantic salmon affected by amoebic gill disease[16] ↑P in skin mucus of turbot at high temperatures[62] ↑P in gilthead seabream skin mucus after overcrowding stress.[20]
	Lysozyme[8, 15, 25]	↑P in gilthead seabream skin mucus after probiotic administration.[20] ↑P in gilthead seabream skin mucus after overcrowding stress.[20] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15] ↓R early stages of infection of Atlantic salmon skin by salmon louse.[63] ↑R *Aeromonas hydrophila* infection of channel catfish skin[61] ↓R (lysozyme like 2) *Aeromonas hydrophila* infected blue catfish skin [50]
	Perforin-1-like	↑R *Aeromonas hydrophila* infection of channel catfish skin[61]
	Peroxiredoxin 1[15, 22], 2[22, 29], 5[15], 6[15, 19]	Inflammation and innate immunity[69] ↓P in gilthead seabream skin mucus after overcrowding stress.[20] ↓R *Aeromonas hydrophila* infected blue catfish skin.[50]
	Profilin 2[15, 19, 29]	↑P *Vibrio anigiullarium* infected Atlantic cod [19] ↓P in gilthead seabream skin mucus after

		overcrowding stress.[20] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15]
	Ribosomal proteins[15, 16, 19, 22, 25, 29, 65]	Antimicrobial activity in skin mucus[65] ↑P in skin mucus of Atlantic salmon affected by amoebic gill disease[16] Protein(s) differentially expressed in skin mucus of sea lice infected Atlantic salmon[15]
	Superoxid dismutase[15, 22, 25, 29]	↑R Aeromonas hydrophila infection of channel catfish skin[61]
	Transferrin[15, 22, 29, 31, 32, 35]	By chelating iron, it limits bacterial growth. ↑P cleaved transferrin during sea lice infection of Atlantic Salmon[32]
	Warm temperature acclimation-related 65 kDa protein[22, 29, 64], Hemopexin-like protein[15, 32]	Inflammatory action[64] Some forms bind heme[70]

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443 Figure 1

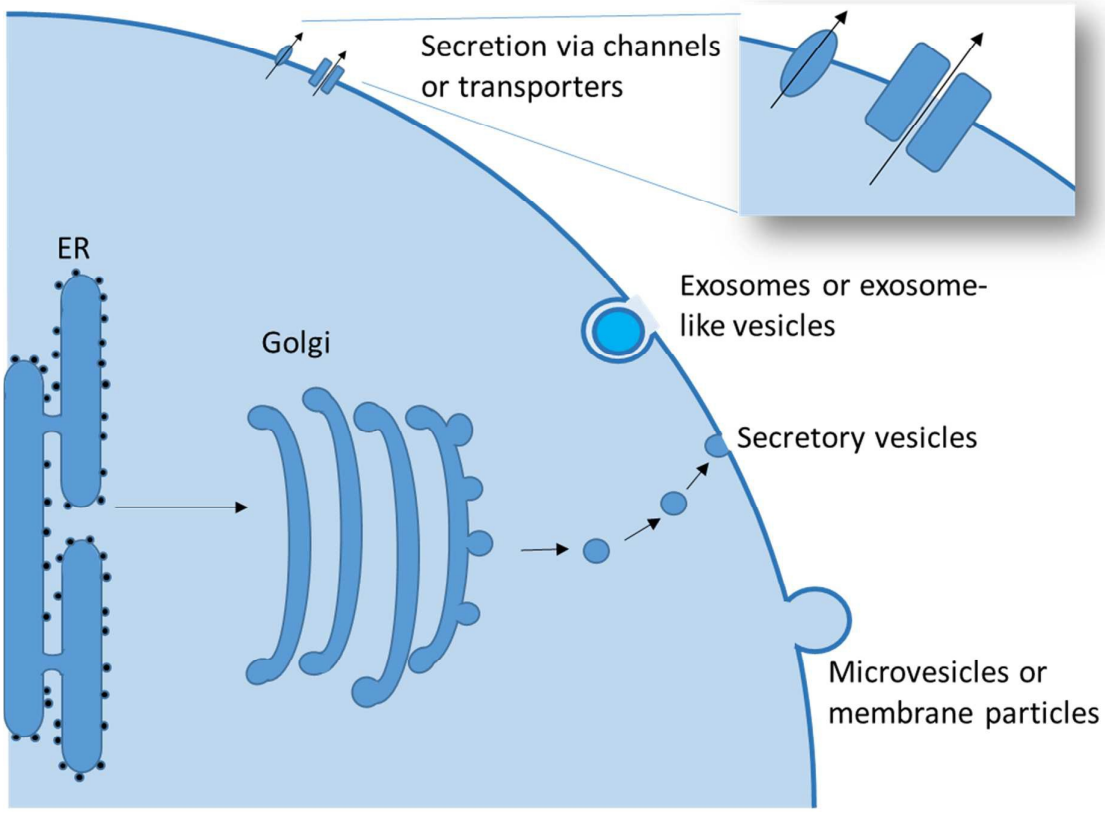

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Figure 2