UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

New Insights Into the Skin and Its Mucus in Teleost Fish

Nuevas Perspectivas en el Estudio de la Piel y el Moco de Peces Teleósteos

D. Héctor Cordero Muñoz

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Marinha e Ambiental

A quien corresponda,

La tesis doctoral titulada "New insights into the skin and its mucus in teleost fish", y presentada por Don Héctor Cordero Muñoz, es original y de gran complejidad y de actualidad para el mundo de la acuicultura. La memoria está bien estructurada, con una introducción que describe apropiadamente el estado del arte de la inmunidad de la mucosa en peces y con un enfoque particular en la piel, objetivos bien definidos, 6 capítulos con datos experimentales, y conclusiones generales.
Los experimentos han sido llevados a cabo apropiadamente, con los cuidados necesarios en cuanto a bien estar animal se refiere, y con réplicas y protocolos de muestreo apropiados. Las metodologías por las que se ha optado son pertinentes para la obtención de los datos experimentales de modo a cumplir con los objetivos inicialmente establecidos.
En resumen, la presente memoria es de calidad excepcional, lo cual queda claramente plasmado por la cantidad y versatilidad de los trabajos experimentales. Gran parte de los trabajos se encuentran ya publicados, en revistas internacionales indexadas y de alto impacto. Además, el candidato demuestra una gran capacidad de dedicación y colaboración, ya que ha conseguido publicar otros trabajos de elevada calidad sin comprometer los objetivos de la tesis doctoral.
Por todo ello, recomiendo encarecidamente la presente memoria para optar al grado de Tesis Doctoral con Mención de Doctor Internacional.

Matosinhos, 11 de noviembre de 2016

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A quien corresponda,

La Tesis Doctoral "Nuevas perspectivas en el estudio de la piel y el moco de peces teleósteos" presentada por Sr. Héctor Cordero Muñoz en la Universidad de Murcia, se enfoca en las interacciones piel-mucus como primera barrera de defensa en el pez, combinando estudios histológicos, y ensayos bioquímicos, así como aproximaciones genéticas y proteómicas, proporcionando nuevas perspectivas en la defensa mucosa de la piel y su interacción con diferentes tipos de estrés tales como infecciones virales, alta densidad y heridas crónicas en peces teleósteos.

El doctorando, Sr. Héctor Cordero, ha demostrado un excelente conocimiento de los diferentes tópicos tratados en su Tesis Doctoral, con un fuerte espíritu crítico; y que queda demostrado en el considerable número de artículos en revistas indexadas, reconocidas internacionalmente, de revisión a pares, publicadas durante su doctorando. Para consolidar su espíritu científico, el candidato realizó dos estancias en universidades fuera de España: Universidad de Nuevo Méjico en Estados Unidos y Universidad de Nord en Noruega. Participó activamente en congresos tanto nacionales como internacionales con presentaciones orales y en pósters e, incluso colaborando en la organización de alguno de ellos.

Por todo ello, recomiendo firmemente su candidatura para la obtención de grado de Doctor con Mención Internacional.

En Porto, 11 de noviembre 2016

Patricia Díaz Rosales

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Preface

The present dissertation is submitted as a requirement for the degree of *Philosophiae Doctor* (PhD) at the Faculty of Biology in the University of Murcia (Spain).The different studies compiled in this dissertation represent original research carried out over a period of four years, as part of two projects entitled:

-"Mucosal immunity on Mediterranean farmed fish (gilthead seabream and Senegalese sole). New advances in probiotic-mucosa and pathogen-mucosa interactions" (grant number AGL2011-30381-C03-01).

-"The skin of fish: inflammation, ulceration and immune response against bacteria. Phytotherapy and nanoparticles as possible treatments" (grant number AGL2014-51839-C5-1-R).

The PhD has been also supported by the Spanish Ministry of Economy and Competitiveness (MINECO) with a PhD grant number BES-2012-052742 and two short stay grants (numbers EEBB-I-15-09235 to stay at University of New Mexico in USA and EEBB-1-2016-10533 to stay at Nord University in Norway) as well as by the University of Murcia with a Lifelong Learning Programme Erasmus grant to stay at the University of Nordland (Norway).

The following persons (in alphabetical order) have been partially participants of the present dissertation:

-**Alberto Cuesta**, Lecturer, University of Murcia.

-**Diana Ceballos**, PhD student, University of Murcia.

-**José Meseguer**, Emeritus Professor, University of Murcia.

-**María Ángeles Esteban**, Professor, University of Murcia.

-**Monica F. Brinchmann,** Associate Professor, Nord University.

-**Patricia Morcillo**, PhD, University of Murcia.

Publications related to the present Thesis (7):

Cordero H, Ceballos-Francisco D, Cuesta A, Esteban MA. Dorso-ventral skin characterization of farmed fish gilthead seabream (*Sparus aurata*). 2016, submitted.

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Apoptotic effects of inorganic arsenic exposure on European sea bass (*Dicentrarchus labrax*).
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Cordero H, Morcillo P, Cuesta A, Brinchmann MF, Esteban MA.
Proteome of gilthead seabream (*Sparus aurata*) skin mucus after probiotic feeding and crowding stress.
Oral Communication in the 17th International Conference on Diseases of Fish and Shellfish, Las Palmas, September 7-11, 2015.

Cordero H, Guzmán Villanueva LT, Chaves-Pozo E, Tovar-Ramírez D, Ascencio-Valle F, Cuesta A, Esteban MA.
Comparative ontogenetic development of two marine teleosts, gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*).
Poster Communication in the 13th International Society of Developmental and Comparative Immunology Congress, Murcia, June 28-July 3, 2015.

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Poster Communication in the 9th International Symposium on Viruses of Lower Vertebrates, Málaga, October 1-4, 2014.

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Chapters of book (1)

Cordero H, Esteban MA, Cuesta A. Use of probiotic bacteria against bacterial and viral infections in shellfish and fish aquaculture. In: Sustainable Aquaculture Techniques, Hernández-Vergara, M (Ed.), InTech, *doi: 10.5772/57198*.

Other merits

Organizer of VI Iberian Congress of Ichthyology, Murcia, June 21-24, 2016.

Member of group B of the commission for the Degree in Biology (2016).

Representative of doctoral students in Molecular Biology and Biotechnology from the University of Murcia for 3 years (2014-2016).

Index

Abbreviations

2-DE	two dimensions gel electrophoresis
actb	actin beta
actn	alpha actinin
adh	alcohol dehydrogenase
ADP	adenosine diphosphate
afp	antifreeze protein
agr	anterior gradient protein 2
ANOVA	analysis of variance
Apoa1	apolipoprotein a-1
ARF3	ADP-ribosylation factor 3
Arfgap	ADP-ribosylation factor GTPase-activating protein
ARP	actin-related protein
asc	apoptosis-associated speck-like protein containing a CARD
ASP	agouti-signalling protein
ATP	adenosine triphosphate
ATP5B	ATP synthase subunit beta
BAC	bacterial artificial chromosomes
βc	beta chain
BS-I	*Bandeiraea simplicifolia* agglutinin
c1q	complement component 1 q subcomponent
c3	complement component 3
calm	calmodulin
calr	calreticulin
CAPG	macrophage-capping protein
capza	f-actin-capping protein subunit alpha
CARD	caspase recruitment domain

casp	caspase
CBB	Coomasie brilliant blue
CCs	chemokine/s
cdk7	cyclin-dependent kinase 7
cDNA	complementary deoxyribonucleic acid
CE	capillary electrophoresis
CID	collision-induced dissociation
CS	citrate synthase
CSF(s)	colony stimulating (or stimulatory) factor/s
CSF1	macrophage colony stimulating (or stimulatory) factor
CSF2	granulocyte-macrophage colony stimulating (or stimulatory) factor
CSF3	granulocyte colony stimulating (or stimulatory) factor
csf1r	macrophage colony stimulating factor receptor
clec	c-type lectin
cof2	cofilin 2
ConA	concanavalin A
cotl	coactosin-like protein
CV	coefficient of variation
cypa	cyclophilin A
DIGE	difference gel electrophoresis
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid
ef1a	elongation factor 1-alpha
ELISA	enzyme-linked immunosorbent assay
Enoa	enolase a

Erp57	endoplasmic reticulum protein 57
ESI	electrospray ionization
EST	expressed sequence tag
fbl	fucose-binding lectin
FBS	foetal bovine serum
FCS	foetal calf serum
FDR	false discovery ratio
FT-MS	Fourier transform ion cyclotron
G-CSF	granulocyte colony stimulating (or stimulatory) factor
GALT	gut-associated lymphoid tissue
gapr1	Golgi-associated plant pathogenesis-related protein 1
gdi	GDP-dissociation inhibitor 1
GDP	Guanosine diphosphate
GIALT	gill-associated lymphoid tissue
GM-CSF	granulocyte-macrophage colony stimulating (or stimulatory) factor
gnrh2	progonadoliberin-2
GO	gene ontology
GSN	gelsolin
gsnl	gelsolin-like protein
gst	glutathione s-transferase
H	histone
H2A/B	histone 2A/B
hamp	hepcidin
HBSS	Hank’s balanced salt solution
HEWL	hen egg white lysozyme
HK	head-kidney

HRP	horseradish peroxidase
HSC	haematopoietic stem cells
HSC70	heat shock cognate 71 kDa
ID	identification
idh	NADP-dependent isocitrate dehydrogenase
IP	isoelectric point
IEF	isoelectric focusing
IFN(s)	interferon/s
ighm	immunoglobulin mu heavy chain
ight	Immunoglobulin tau heavy chain
IgM/IgT	Immunoglobulin mu / tau
irf3	interferon regulatory factor 3
IL / ILs	interleukin/s
il1b / IL-1β	interleukin 1 beta
impa	inositol monophosphate 1
IPG	immobilized pH gradient
IT	ion trap
KRT1/2	keratin type I / type II
LC	liquid chromatography
LCD	lymphocystis disease
LCDV	lymphocystis disease virus
LC-MS/MS	liquid chromatography–tandem mass spectrometry
lec	lectin
lei	leucocyte elastase inhibitor
LPS	lipopolysaccharide
LTβ	lymphotoxin beta

lyz	lysozyme
MAF	macrophage-activating factor
MALDI	matrix-assisted laser desorption/ionization
MALT	mucosa-associated lymphoid tissue
mcp	major capsid protein
mhc2a	major histocompatibility complex class 2 alpha
mg	milligram
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
mx	interferon-induced GTP-binding protein mx
m/z	mass-to-charge ratio
NADP	nicotinamide adenine dinucleotide phosphate
NALT	nasal-associated lymphoid tissue
NCBI	national center for biotechnology information
nccrp1	nonspecific cytotoxic cell receptor protein 1
ndk	nucleoside disphosphate kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
nkef	natural killer cell enhancement factor (synonym of prdx, see below)
NO	nitric oxide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PCR	polymerase chain reaction

pdi	protein disulphide isomerase
Pdp11	*probiótico de piel* 11 (skin probiotic 11)
PEBP1	phosphatidylethanolamine-binding protein 1
pfn	profiling
PNA	peanut agglutinin
pNPP	p-nitrophenyl phosphate liquid substrate
prdx	peroxiredoxins
qPCR	quantitative PCR
Q-TOF	quadrupole time-of-flight
RNA	ribonucleic acid
ROS	reactive oxygen species
rps18	ribosomal protein S18
SA	*sociedad anónima*
SALT	skin-associated lymphoid tissue
scgn	secretagogin
scindl	sciderin-like protein
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SH3BGRL	SH3 domain-binding glutamic acid-rich-like protein
sod	superoxide dismutase
SPSS	statistical package for social science
TCA	trichloroacetic acid
tcra	T-cell receptor alpha
tf	transferrin
TGF(s)	transforming growth factor/s
tgfb	transforming growth factor beta

tle	transducin-like enhancer protein 1
TMB	3,3',5,5'- tetramethylbenzidine
TNF(s)	tumor necrosis factor/s
TOF	time-of-flight
tpi	triose phosphate isomerase
tpm	tropomyosin alpha-1 chain
U	units
UEA	*Ulex europaeus* agglutinin
vim	vimentin
wap65	warm temperature acclimation protein 65
WFA	*Wisteria floribunda* agglutinin
WGA	wheat germ agglutinin
WGS	whole-genome sequencing
YWHAZ	14-3-3 protein beta/alpha

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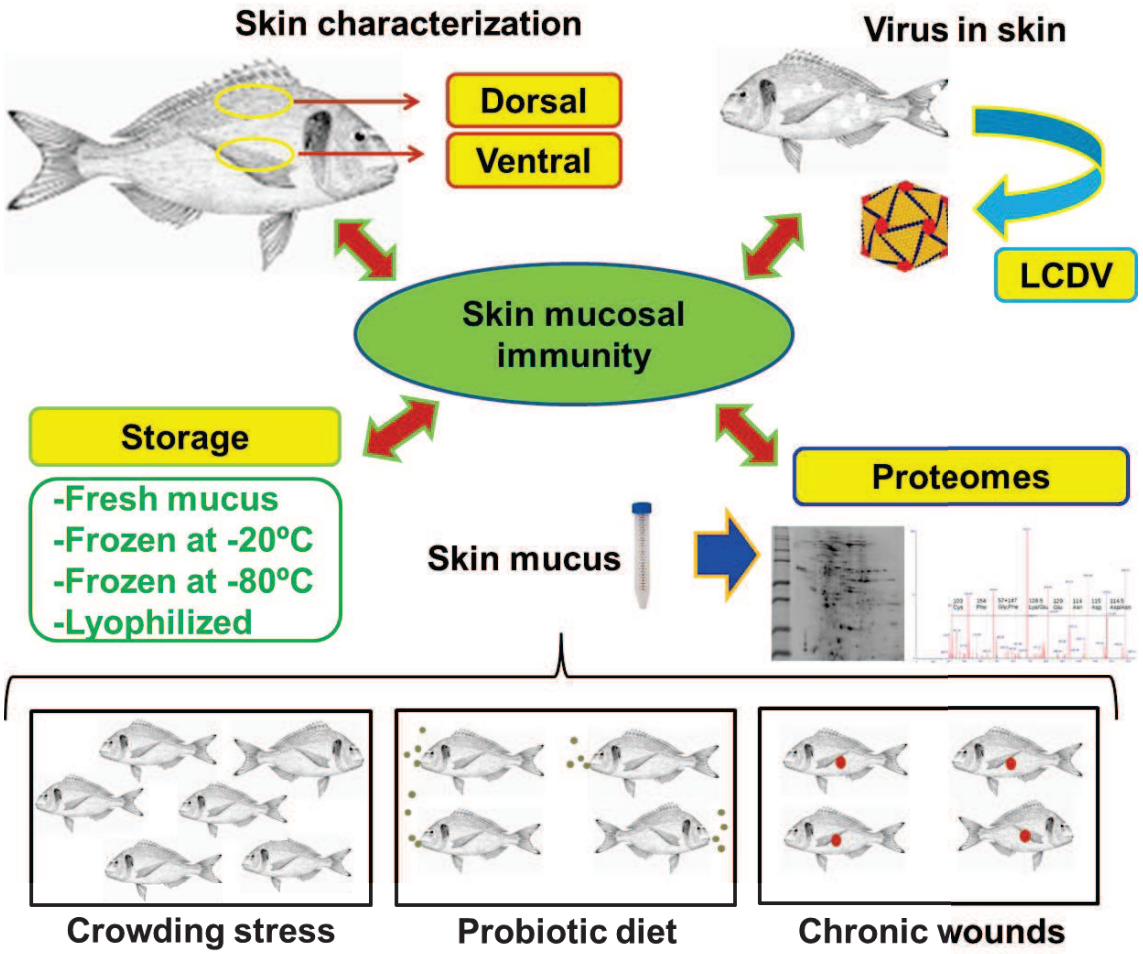
Graphical abstract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Crowding stress
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Summary

The aim of the present Doctoral Thesis is to throw some light in the skin immunity in teleost fish. Thus, this work is divided into 3 parts devoted to: **Part 1,** the skin structure in healthy gilthead seabream (*Sparus aurata*) and its immune response against a viral natural infection with lymphocystis (LCDV); **Part 2**, the best storage conditions of gilthead seabream skin mucus to determine the immunity; **Part 3**, the proteome map of seabream and European sea bass (*Dicentrarchus labrax*) skin mucus and the changes observed in seabream after: dietary probiotics, stress by crowding conditions and experimental skin chronic wounds.

The **Part 1** of the present Doctoral Thesis is divided into two chapters. In the **Chapter I**, our aim was the characterization of gilthead seabream skin by studying the histology, cell isolation and cell cycle, and transcription of cytokines after interaction with the pathogen *Photobacterium damselae* subsp. *piscicida* and the probiotic *Shewanella putrefaciens*, with the aim to compare between the dorsal and the ventral skin. Our results showed a significant increased epidermal thickening in the ventral skin compared to the dorsal skin as well as the staining of some structures such as sensory cells revealed for first time. On the other hand, scanning electron microscopy showed a significant increase in both cell size and area of microridges in cells from the dorsal skin compared to those from the ventral skin. Finally, the gene expression profile of cytokines remained similar in dorsal region, but strongly affected by pathogen/probiotic in ventral region. This study represents an improved knowledge of the skin structure of teleost, concretely, gilthead seabream, through an updated multidisciplinary approach.

In the **Chapter II**, we have studied the immune status of gilthead seabream specimens under a natural outbreak of LCDV. The replication of the virus was demonstrated in the skin of infected fish (target tissue), but not in control fish. The results showed decreased total serum IgM levels and increased innate cellular immune response (peroxidase and respiratory burst activities) of HK leucocytes in LCDV-infected fish, compared to the values obtained in uninfected specimens. In addition, transcription of

antiviral genes (*ifn* and *irf3*) was down-regulated in the skin of LCDV-infected fish as well as genes involved in cellular immunity (*csf1r*, *mhc2a*, *tcra* and *ighm*) that were down-regulated in the skin and HK of infected fish. By contrast, the transcription of *nccrp1* was up-regulated in HK after LCDV infection. These present results show that HK leucocytes are activated to encounter the virus at the sites of replication.

The **Part 2** of the present Doctoral Thesis contains one chapter. In the **Chapter III**, we have evaluated the effects of different storage conditions of skin mucus. Fresh, frozen (immediately after collection at -20ºC and -80ºC) and lyophilized skin mucus samples obtained from the same fish specimens of gilthead seabream were analysed in the assays. The amount of total proteins and sugar residues (determined by lectin binding) present in skin mucus samples fell after both freezing and lyophilization of the samples compared to fresh mucus. While no significant differences were exhibited in the levels of some proteins or enzymes (immunoglobulin M, antiprotease, peroxidase, esterase and alkaline phosphatase) determined in fresh or frozen mucus samples, protease and lysozyme activities were lower in frozen mucus samples than in fresh samples. Lyophilization of the mucus samples drastically decreased the total level of proteins obtained, as well as of protease, peroxidase, lysozyme and alkaline phosphatase activities. The results suggest that freezing skin mucus samples is more suitable than lyophilization if samples are stored before determining enzymatic activities.

The **Part 3** of the present Doctoral Thesis is divided into three chapters. In the **Chapter IV**, we have mapped the proteome from skin mucus of European sea bass by 2-DE followed by LC-MS/MS. From all the identified proteins in the proteome map, we focus on the proteins associated with several immune pathways in fish. Furthermore, the qPCR transcript levels in skin were shown. Proteins found include apolipoprotein A1, calmodulin, complement C3, fucose-binding lectin, lysozyme and several caspases. To our knowledge, this is the first skin mucus proteome study and further transcriptional profiling of the identified proteins done on this bony fish species. This not only

contributes to the knowledge on the routes involved in mucosal innate immunity, but also establishes a non-invasive technique based on locating immune markers with a potential use for prevention and/or diagnosis of fish health.

In the **Chapter V**, we have focused on the skin mucus proteome after dietary probiotic Pdp11 intake in gilthead seabream specimens maintained under normal or overcrowding conditions. 2-DE of skin mucus followed by LC-MS/MS analysis was done for each experimental group and differentially expressed proteins were identified. The results showed differentially expressed proteins especially involved in immune processes, such as lysozyme, complement C3, natural killer cell enhancing factor and nonspecific cytotoxic cell receptor protein 1, whose transcript profiles were also studied by qPCR. A consistency between lysozyme protein levels in the mucus and lysozyme mRNA levels in skin were found.

Finally, in the **Chapter VI**, the changes produced at proteomic level were evaluated (with similar methodology than Chapter V) on experimentally skin chronic wounds in gilthead seabream specimens. Most of the identified proteins were under-expressed in skin mucus of gilthead seabream.

Overall, we have studied first the skin characterization (normal and under viral infection), next the changes produced in the skin mucus due to different storage methods (since there was no unified available criterion) and finally, the proteomic map of skin mucus to search new immune markers and their changes produced in the proteome by dietary probiotics, stress by crowding or by skin chronic wounds.

Introduction

In the field of marine aquaculture, gilthead seabream and European sea bass are two of the most important farmed fish not only in Spain but also for all the Mediterranean Sea. According to fishery statistical datasets released by Food and Agriculture Organisation of the United Nations (FAO, 2016), the world production increased from 77,510 and 58,133 tonnes in 2,002 to 158,389 and 156,500 tonnes in 2014 for gilthead seabream and European sea bass, respectively.

The improved knowledge of the immune system of fish, especially in the species of interest in aquaculture, is one of the primary objectives in research applied to this alimentary industry. Among fish immunology, recent interest focuses on mucosal immunology, due to the fact that mucosal barriers are the first line of defense against any external stressor.

1.1. Overview of the immune system of teleost fish

Teleost fish are the earliest group of vertebrates possessing both innate (also known as nonspecific or natural) and adaptive (also known as specific or acquired) immune system. In teleosts, the equivalent of the bone marrow of mammals is the kidney. The kidney is a primary immune organ and the main hematopoietic organ. It is structured in three parts: head-kidney (HK) (also known as anterior or cephalic kidney), with lymphoid and hematopoietic function; posterior or caudal kidney, with renal function; and the intermediate, which shares both functions. Another primary lymphoid organ is the thymus, which is considered as the main source of mature T lymphocytes (Davis et al., 2002). The main secondary lymphoid tissue is the spleen, which contains B and T lymphocytes (Fänge and Nilsson, 1985; Zapata et al., 1996). In addition, there is a disperse and diffuse mucosa-associated lymphoid tissue (MALT) in the gut, gill and skin (Salinas, 2015) that is also part of the secondary lymphoid tissue.

1.1.1 Innate immunity

By definition, the innate immune system's recognition of non-self is mediated by germline-encoded pattern recognition proteins/receptors that identify molecular

patterns, which are characteristic of microbes (Magnadóttir, 2006). These molecules include polysaccharides, lipopolysaccharide (LPS), peptidoglycans, bacterial DNA and double stranded viral RNA and other molecules not normally found on the surface of multicellular organisms (Medzhitov and Janeway, 1998, 2002). The components of the teleost fish innate immunity are physical barriers (scales, mucosal epithelium, mucus), and humoral and cellular factors (Abbas et al., 2012; Magnadóttir, 2006; Whyte, 2007).

The humoral immunity is played by proteins and glycoproteins with defense functions which are found in serum, mucus and eggs (Salinas et al., 2011; Yano, 1996). Among the humoral factors of the innate immune system the most important is the complement system that consists of a complex cascade of enzymatic glycoproteins, which acts signalling the presence of potential pathogens and contributing to their degradation through chemotaxis and opsonization. The complement system is well developed in fish and includes classical, alternative and lectin pathways (Claire et al., 2002; Sarma and Ward, 2011).

Other important humoral factors are lytic enzymes including hydrolases, such as lysozyme and chitinase, cathepsin and bacteriolytic enzymes (Saurabh and Sahoo, 2008; Yano, 1996), lectins (agglutinins) (Russell and Lumsden, 2005), acute-phase proteins such as haptoglobin (HP), serum amyloid A and P (SAA and SAP), ceruloplasmin (CP), alpha-2-macroglobulin (A2M), fibrinogen, pentraxins (PTX), prothrombin, C-reactive protein (CRP), albumin and transferrin (TF) among others as well as antiproteases (Bayne and Gerwick, 2001).

It is important to mention the presence of many antimicrobial peptides (AMPs), which are low molecular weight peptides that have bactericidal properties against different pathogens (Fernandes et al., 2004; Maier et al., 2008a, 2008b). Among these AMPs there are piscidins (Moon et al., 2007; Noga et al., 2009; Sun et al., 2007), cathelicidins (Bridle et al., 2011; Chang et al., 2005; Li et al., 2015; Lu et al., 2011), NK-lysins (Hirono et al., 2007; Pereiro et al., 2015; Wang et al., 2006), apolipoproteins (Concha

et al., 2004; Villarroel et al., 2007), histones such as H1 and H2B (Valero et al., 2016), histone derived peptides such as histone-like proteins (HLP's), H1, H2B, oncorhyncin II and III (Fernandes et al., 2004, 2003; Noga et al., 2011, 2002), defensins (Casadei et al., 2009; Cuesta et al., 2011; Ruangsri et al., 2013; Zou et al., 2007), and hepcidins (Bao et al., 2005; Chen et al., 2005; Cuesta et al., 2008; Hirono et al., 2005; Kim et al., 2008). Furthermore, some AMPs have been only reported in specific species such as pardaxin in red sea moses sole (*Pardachirus marmoratus*) (Oren and Shai, 1996), epinecidin-1 in orange spotted grouper (*Epinephelus coioides*) (Wang et al., 2010; Yin et al., 2006), gaduscidin in Atlantic cod (*Gadus morhua*) (Browne et al., 2011), pleurocidin in winter flounder (*Pseudopleuronectes americanus*) (Cole et al., 1997), chrysophsin in red sea bream (*Pagrus major*) (Lijima et al., 2003) and dicentracin in European sea bass (*Dicentrarchus labrax*) (Salerno et al., 2007; Valero et al., 2015a).

In the context of the cellular innate immunity, several cell types are involved in fish innate immune response such are non-specific cytotoxic cells (NCCs), which are functionally equivalent to natural killer (NK) cells of mammals, monocytes/macrophages, granulocytes, scavenger endothelial cells, epithelial rodlet cells and mucosal eosinophilic granular cells (EGC) equivalent to mast cells in mammals, as well as the recently discovered B cells with phagocytic capacity in teleosts (Evans et al., 2001; Frøystad et al., 1998; Li et al., 2006; Reite and Evensen, 2006; Seternes et al., 2002).

1.1.2 Adaptive immunity

On the other hand, adaptive immunity with cellular humoral and cellular components first emerged when the earliest vertebrates (*Agnatha*) appeared approximately 500 million years ago (Gómez et al., 2013). There are two main arms in the adaptive immune system of any gnathostome vertebrate: B cells and T cells. The adaptive immune system of teleosts has many similarities with mammals but also some differences such as different immunoglobulins (IgM, IgD and IgT/IgZ in fish against

IgM, IgG, IgA, IgD and IgE in mammals) with less affinity maturation, less response memory of these antibodies and absence of germinal centres and lymph nodes as well as a not true bone marrow (Sunyer, 2013). Both innate and adaptive responses are greatly interconnected and involve a wide array of molecules and pathways such as cytokines.

1.1.3 Cytokines

Due to the importance of these molecules on immunity and to understand some of the results of the present doctoral Thesis, mainly in Chapter I, a small review of cytokines is now presented. Over the term "cytokines" is included a broad group of proteins (mostly glycoproteins) with low molecular weight mainly secreted by immune cells. In general, cytokines can be grouped into interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factors (TGFs), colony stimulatory factors (CSFs) and chemokines (CCs). Cytokines are involved in a wide variety of processes such as mediators of the innate and adaptive immune response, inflammation, cell growth and proliferation, morphogenesis or tissue repairs among others (Abbas et al., 2012).

IFNs are involved in the antiviral processes of cells of vertebrates as the most powerful mechanisms of defense against viral infections (Samuel, 2001). In fish, according to the structural and functional characteristics, there are two different families: Type I and type II IFNs. Type I IFNs are induced by viruses in all nucleated cells, being similar to mammalian IFNα and IFNβ; whilst type II IFNs is specifically produced by activated NK cells and T lymphocytes, being counterparts to mammalian IFNγ (Robertsen, 2006).

ILs are involved in the intercellular regulation of the immune system of vertebrates (Secombes et al., 2011). Interleukin-1 (IL-1) is a family of ILs known to be central mediator of the innate immunity and inflammation (Garlanda et al., 2013), which includes IL-1α, IL-1β, IL-18 and IL-33 among others (Secombes et al., 2011). IL-1β is one of the most popular pro-inflammatory ILs discovered firstly in fish by homology

cloning (Zou et al., 1999), and later also discovered in both European sea bass (G Scapigliati et al., 2001) and gilthead seabream (*Sparus aurata*) (Pelegrín et al., 2004, 2001), showing a pro-inflammatory (positive regulation) profile against fish pathogens and/or pathogen-associated molecular patterns (PAMPs). IL-18, though less known, has been also characterized in fish with a slight pro-inflammatory activity (Zou et al., 2004), but no effects were observed in gilthead seabream against a fish parasite-model (Pérez-Cordón et al., 2014).

In fish, interleukin-6 (IL-6) family includes IL-6 itself, IL-11 and IL-31 (Secombes et al., 2011). IL-6 is other of the most known pro-inflammatory ILs first discovered in fugu (*Takifugu rubripes*) (Steve Bird et al., 2005), and later in both European sea bass (Sepulcre et al., 2007) and gilthead seabream (Castellana et al., 2008), demonstrating its up-regulation after different pathogens/PAMPs mainly in HK leucocytes culture.

In mammals, the IL-2 subfamily includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Secombes et al., 2011), being IL-7 and IL-15 particularly important for the maintenance of T cell memory (Osborne and Abraham, 2010). In fish, the role of IL-7 and IL-15 remains unclear, both of them up-regulated in gut of gilthead seabream after a parasitic infection (Pérez-Cordón et al., 2014).

The IL-10 subfamily is a group of anti-inflammatory cytokines that includes IL-10 itself, IL-22, IL-24 and IL-26 among others (Secombes et al., 2011). In fish, the most studied is IL-10, which was discovered searching in the fugu genome (Zou et al., 2003), and recently reported in gilthead seabream, with an important up-regulation after parasitic infection (Pérez-Cordón et al., 2014). Another less studied ILs include IL-17 subfamily and heterodimeric interleukins such as IL-12, IL-23, IL-27 and IL-35 (Secombes et al., 2011), which remain unknown in fish so far.

TNFs are an expanding superfamily whose members, without exception, exhibit pro-inflammatory activity, in part through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Aggarwal et al., 2012). Among all the

members, TNFα, TNFβ and lymphotoxin beta (LTβ) are, by far, the most studied, including fish (Zou and Secombes, 2016). TNFα is produced by macrophages in response to macrophage-activating factor (MAF) or lipopolysaccharide (LPS) stimulations (Goetz et al., 2004). In zebrafish (*Danio rerio*), TNFα promoted the macrophage survival and restricted the bacterial growth in infected macrophages (Clay et al., 2008). Further, the bacterial-killing activity appears to be accompanied by an increase of reactive oxygen species (ROS) (Roca and Ramakrishnan, 2013). In gilthead seabream, TNFα has been characterized (García-Castillo et al., 2002) and reported to be a powerful activator of endothelial cells but hardly activates phagocytes (Roca et al., 2008).

TGF superfamily includes TGFα (considered a mitogenic polypeptide, not a cytokine) and TGFβ, a pleiotropic cytokine that encodes three isoforms (TGFβ1, TGFβ2, TGFβ3) in mammals (Zou and Secombes, 2016). In fish, there is no much information of this cytokine, however it seems to have an immunosuppressive effect of nitric oxide (NO) production in macrophages according to the functional characterization in goldfish as well as suggest that fish TGFβ is phylogenetically and functionally similar to mammalian TGFβ1 (Haddad et al., 2008).

By its side, CSFs drive the proliferation and differentiation of blood cells from haematopoietic stem cells (HSC) (Zou and Secombes, 2016). In mammals, there are three CSFs (CSF1, CSF2 and CSF3). CSF1, also called macrophage colony stimulatory factor (M-CSF), is a regulator of the mononuclear phagocyte lineage, eliciting its effects on the myeloid lineage by binding to the CSF-1R (CD115) (Zou and Secombes, 2016). Importantly, CSF-1R is a specific marker of gilthead seabream macrophages (Roca et al., 2006). Curiously, IL-34 (not mentioned before) is a relatively new cytokine related to M-CSF, that drives monocyte viability, number and function, and brings about these effects by binding to the CSF-1R (Lin et al., 2008). On the other hand, CSF-2, also known as granulocyte-macrophage CSF (GM-CSF), is possibly an

ancestral gene with relatedness to the βc cytokines that exists in fish although bioactivity studies are lacking (Zou and Secombes, 2016). However, CSF-3, also known as granulocyte-CSF (G-CSF), has been discovered and functionally studied in fish. Although most studies have been performed in zebrafish, where knock-down of the G-CSFR with morpholinos and overexpression of G-CSF by injection of eggs with *in vitro* transcribed G-CSF mRNA has been used to determine G-CSF function (Liongue et al., 2009).

The name CCs comes from the ability to induce the migration of immune cells directly at sites of the infection. In other words, CCs are chemotactic cytokines. CC superfamily includes four subgroups or families: CXC (or α), CC (or β), C (or γ), CX_3C (or δ) (Zou and Secombes, 2016). In mammals, the CXC and the CC families are the two largest, with multiple members in each, whereas the CX_3C and C subgroups only contain one and two members, respectively (Bird and Tafalla, 2015). In fish, this proportion is still maintained and no CX_3C chemokines have ever been reported whereas C chemokines have only been reported thus far in zebrafish (Nomiyama et al., 2008). The repertoire of chemokines, specially subgroup CC, is larger in fish than in mammals, and includes some fish-specific CCs (Bird and Tafalla, 2015). Further, the nomenclature is not unified in fish, finding counterparts with different names in different fish species. Importantly, CXCL8 (also called IL-8) is one of the chemokines for which more functional assays have been performed in fish, with a strong capacity of inducing the neutrophil recruitment in mammals (Bird and Tafalla, 2015). In fish, the chemotactic capacity of both IL-8 paralogues towards neutrophils has been demonstrated in both zebrafish (De Oliveira et al., 2013; Sarris et al., 2012) and carp (Van Der Aa et al., 2010). IL-8 was recently reported in gilthead seabream (Pérez-Cordón et al., 2014), although nothing is still known about the role of this CC in bacterial infections.

1.2 Skin mucosal immunity of teleosts

The main function of the skin of vertebrates is to separate and protect the organism from the environment (Whitear, 1986). In addition to being a mechanical barrier, it represents a metabolically active tissue (Bullock and Roberts, 1974). In fact, fish integument is a multifunctional organ, and its components may have important roles in protection, communication, sensory perception, locomotion, respiration, ion regulation (Marshall and Bellamy, 2010), excretion, and thermal regulation (Elliott, 2000). These functions are possible due to the complexity of skin structure and cell composition (Elliott, 2000). All of these functions (mainly immunity, osmoregulation, respiration, and excretion) are especially significant in fish larvae because the importance of the skin in early developmental stages also relies on the fact that surface to volume ratio is high in early stages and decreases during the development (Varsamos et al., 2005).

Although numerous studies have focused on the histology and cytochemistry of the epidermis of adult teleosts (Bullock and Roberts, 1974; Burkhardt-Holm, 1997; Whitear, 1986, 1970), the structure of the larval skin has been studied only in a few species (Ottesen and Olafsen, 1997; O'Connell, 1981; Roberts et al., 1973). According to the existing data, the larval skin of teleosts is a thin two-cell layer (including mucus/goblet cells and the chloride cells/mitochondrial rich cells/ionocytes) lying on a basal membrane and overlying an extensive haemocoel (Varsamos et al., 2005). In general, the layers of tegument of adult teleosts are the cuticle or mucus layer (with a very complex composition), which have bacteria forming the microbiota, the epidermis (a squamous stratified epithelium with goblet cells) and the dermis that is divided in two layers: the *stratum spongiosum*, which is a loose network of connective tissue, and the innermost layer or *stratum compactum*, which is a dense layer with orthogonal collagen bands (Figure 1) (Elliott, 2000; Hawkes, 1974). The non-keratinized epidermis, 3–15 cells thick, consists entirely of live cells, of which the majority are squamous cells and the minority mucous cells (X. Zhao et al., 2008). The squamous cells are characterized

by numerous desmosomes and associated cytoplasmic filaments (GA Brown and Wellings, 1970) with only minimal quantities of keratin in the cells of the superficial layer, whose cells show microridges that contain mucus and antibacterial substances secreted to the surface from mucous goblet cells located in the intermediate stratum of the epidermis (Mittar and Whitear, 1979). The dermis, separated from epidermis by an acellular basement membrane, is mainly composed of dense connective tissue with a large amount of collagen fibres, although it typically contains relatively little of the connective tissue found in tetrapods (Elliott, 2000; Esteban, 2012). Instead, in most species, it is largely replaced by solid, protective bony scales. Cartilaginous fishes have numerous tooth-like denticles embedded in their skin in place of true scales. Pigment cells are of three types: melanophores, iridophores (guanophores), and lipophores (Roberts et al., 1973). Although melanin is found in the skin of many fish species, the epidermis is often relatively colourless. Instead, the colour of the skin is largely due to chromatophores in the dermis, which, in addition to melanin, may contain guanine or carotenoid pigments (Romer and Parsons, 1977). The hypodermis consists of loosely organized collagen fibres and rich supply of vessels and, as the innermost layer, is closest to the striated muscle underneath the skin. The origins of these skin layers of teleost are still unknown. In this sense, some works have renewed interest in the teleost dermomyotome (Le Guellec et al., 2004), which was initially characterized in the late 19th century. New works are studying the primary myotome morphogenesis, the relationship between the primary myotome and the dermomyotome, as well as the differentiation of axial and appendicular muscles and dermis from the dermomyotome (Stellabotte and Devoto, 2007). Concretely, some of the zebrafish dermomyotome precursors examined recently by lineage labelling were reported to give rise to “dermis” cells, based only on their position (Hollway et al., 2007). As the teleost dermis has not been well characterized in any species, these results must be viewed as preliminary (Stellabotte and Devoto, 2007).

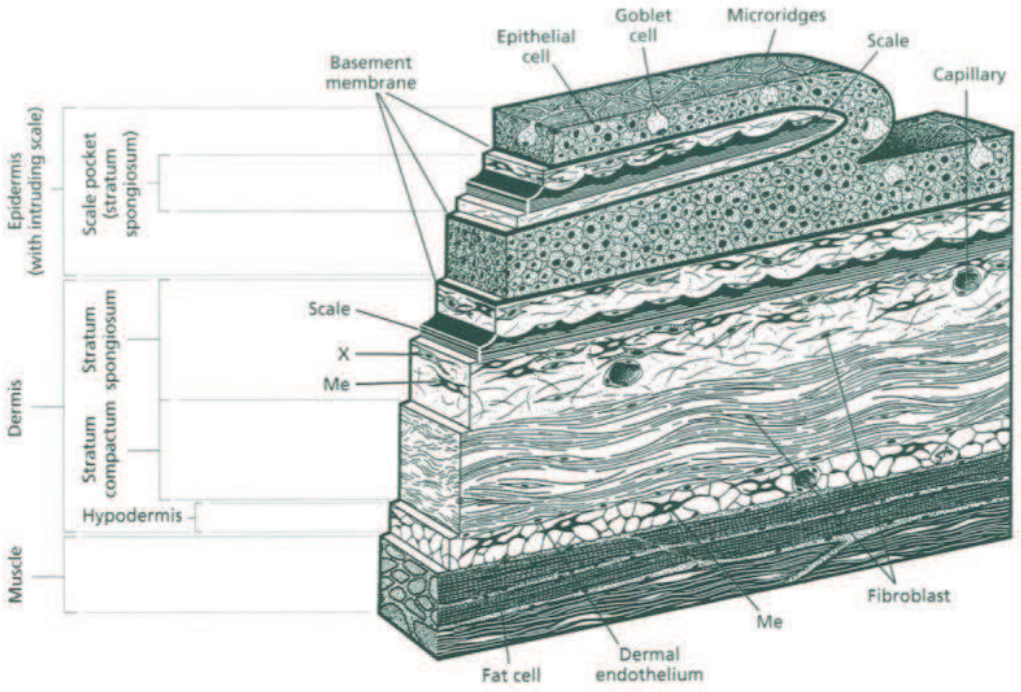


Figure 1. Three dimensional section of the skin of a teleost fish (*Oncorhynchus kisutch*), showing the microscopic structures and some specific structures of the dermis and epidermis, adapted from Elliott, 2000. Me, melanophore; X, santhophore.

Skin mucus is continuously secreted and the thickness of the mucus layer is determined by the balance between the rate of secretion and the rate of degradation and shedding (Cone, 2009). The basic physical structure of skin mucus is composed by a group of glycoproteins called mucins (Fletcher et al., 1976). The mucin fibres that form mucus gel are as long flexible strings densely coated with short glycans, most of which are tipped with a negative charge (carboxyl or sulphate groups) (Cone, 2009). Lipids in mucus secretions, including covalently attached fatty acids, contribute to fibre-fibre interactions that markedly increase the viscoelasticity of the gel (Murty et al., 1984).

Fish represent the most ancient vertebrates with a mucosal adaptive immune system (Salinas et al., 2011), which is generally encompassed by the mucosa-associated lymphoid tissues (MALT). The common canonical features of all teleost MALT are: (i)

the presence of diffuse lymphoid cells with the absence of organized lymphoid structures; (ii) a predominant role for IgT antibodies (the specialized mucosal immunoglobulin class in teleosts) and IgT+ B cells (Xu et al., 2013; Zhang et al., 2010); (iii) the presence of a diverse microbial community and coating of commensals by mucosal Igs. Four different MALT have been characterized in teleost: gill-associated lymphoid tissue (GIALT), gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissues (SALT) or the recently discovered nasal-associated lymphoid tissues (NALT) (Salinas et al., 2011; Tacchi et al., 2014).

As part of the SALT, functionally, skin mucus contains a diversity of humoral factors such as immunoglobulins (Xu et al., 2013), complement components (reviewed by Gómez et al., 2013), antimicrobial peptides (reviewed by Valero et al., 2013, proteases (serin-proteases, aspartic-proteases, cysteine-proteases and metalloproteases) (Rao et al., 1998) as well as protease inhibitors (Bowden et al., 1997), both present in body fluids, including skin mucus. Other enzymes such as esterases and alkaline phosphatases are less known but also important in mucus defences (Guardiola et al., 2014a, 2014b; Ross et al., 2000). However, lysozyme deserves special mention since this enzyme is able to hydrolyse the peptidoglycan present on Gram + and Gram – bacterial cell walls (Grinde, 1989; Saurabh and Sahoo, 2008), a critical process in the skin mucus to protect against foreign pathogens. All this composition in skin mucus determines adhesiveness, viscoelasticity, transport and protective capacity (Gómez et al., 2013). In the last years, international normative for animal handling is addressed to the use of non-invasive techniques [according to the Guidelines of the European Union Council (2010/63/EU)], being the skin mucus a key surface or target for non-invasive studies.

1.3 Omics and their applications in skin mucus of teleosts

The central dogma of molecular biology outlines the flow of information that is stored in genes as DNA, transcribed into RNA, and finally translated into proteins (Crick, 1970).

The ultimate expression of this genetic information modified by environmental factors characterizes the phenotype of an organism. In this context, genomics, transcriptomics and proteomics have arisen in the last decade as promising tools to understand a diversity of behaviours and challenges.

1.3.1 Genomics

On genomics, it has been reported the genome of several teleost fish such as fugu (Aparicio et al., 2002), green spotted puffer (*Tetraodon nigroviridis*) (Jaillon et al., 2004), medaka (*Oryzias latipes*) (Ahsan et al., 2008), Atlantic cod (*Gadus morhua*) (Star et al., 2011), stickleback (*Gasterosteus aculeatus*) (Jones et al., 2012), zebrafish (Howe et al., 2013), five African cichlid fishes including Nile tilapia (*Oreochromis niloticus*) (Brawand et al., 2014), tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2014), yellow croaker (*Larimichthys crocea*) (Ao et al., 2015), Atlantic salmon (*Salmo salar*) (Lien et al., 2016), channel catfish (*Ictalurus punctatus*) (Liu et al., 2016), turbot (*Scophthalmus maximus*) (Figueras et al., 2016), as well as the genome of European sea bass (Tine et al., 2014) but not the genome of gilthead seabream. Despite of other methods such as bacterial artificial chromosomes (BAC), most of the mentioned genomes were sequenced using whole-genome sequencing (WGS).

1.3.2 Transcriptomics

On transcriptomics, it has been also a huge increase of interest in fish applications as demonstrated the search of "Fish Transcriptomes" in PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) with only 13 entries from 1,950 to 2,004 against 1,320 entries from 2,005-2,016. Classic methods of differential expression sequencing such as microarrays based on hybridization principle (Schena et al., 1995) or expressed sequence tag (EST) sequencing based on Sanger sequencing method (Sanger et al., 1977) have been shift by high-throughput next generation sequencing (NGS), mostly RNA-seq (Mortazavi et al., 2008; Nagalakshmi et al., 2006). Despite of microarrays or EST sequencing are still used in many cases, there are several

advantages (summarized in Table 1) of using RNA-seq over the other technologies such as less costs of production, less required RNA amount, less background noise, high performance, more range of quantification levels of gene expression, ability to distinguish isoforms and allelic expression (Wang et al., 2009).

Table 1. Advantages of RNA-seq technology compared with microarray and EST sequencing technologies. This table was summarized from Wilhelm and Landry (2009) and Wang et al., (2009).

Technology	Microarray	EST sequencing	RNA-seq
Cost for mapping	High	High	Low
Required RNA amount	High	High	Low
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Throughput	High	Low	High
Reliance on genomic sequence	Yes	No	Sometimes
Background noise	High	Low	Low
Resolution	From several to 100bp	Single base	Single base
Simultaneously map transcribed regions	Yes	No	Yes
Dynamic range	Less than 1,000-fold	No practical	>8,000-fold
Distinguish different isoforms?	Limited	Yes	Yes
Distinguish allelic expression	Limited	Yes	Yes

1.3.3 Proteomics

Proteomics covers: (i) a structural proteomics that analyses the structure of proteins by techniques such as X-ray crystallography or nuclear magnetic resonance (NMR); (ii) an expression proteomics that help to identify the main proteins found in a particular sample/group or to know the differentially expressed proteins; and (iii) an interaction

proteomics that help to determine the protein functions using affinity purification or two hybrid system technologies among others.

Focusing on expression proteomics, several key steps are necessary to carry out a suitable study including protein isolation, protein separation, staining/labelling, identification and annotation (Figure 2).

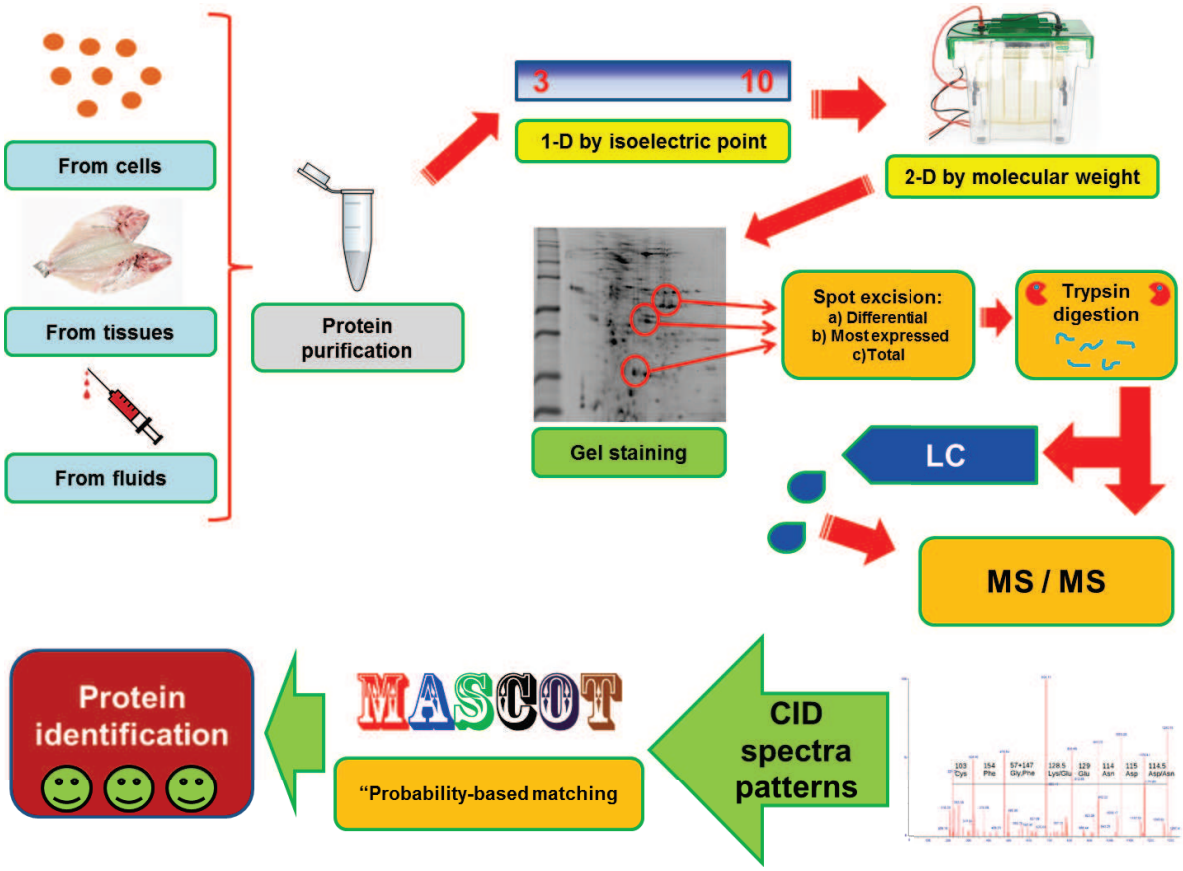


Figure 2. Summarized generic MS-based experiment in proteomics.

The most common method for protein separation is two dimensional electrophoresis gel (2-DE). This method is based on separating the proteins first by isoelectric point or pH, usually in commercial immobilized pH gradient (IPG) strips, whose length can be 7cm, 11cm, 17cm, 18cm or 24cm. The general range of pH is 3-10, other narrow range gradients can be used for specific goals such as 3-6, 4-7, 5-8 or 7-10. After running this first dimension, the second dimension is based on the molecular weight of the proteins,

usually through of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

To label or stain the peptides/proteins, most popular (post-electrophoretic) staining are (i) Coomasie Brilliant Blue (CBB) (Groth et al., 1963; Meyer and Lamberts, 1965) followed later by (ii) colloidal CBB (which reduces the background), but the sensitivity in terms of limits of detection is significantly lower than other methods such as silver stains [which have divided into (iii) silver diamine (alkaline) and (iv) silver nitrate (acidic)], (v) zinc-imidazole (Ortiz et al., 1992) and (vi) copper (both negative staining) or (vii) SYPRO Ruby stain, which is an end-point stain compatible with mass spectrometry (MS) fluorescence technique (Berggren et al., 2000). In recent years it has emerged an alternative fluorescent method called difference gel electrophoresis (DIGE), a pre-electrophoretic labelling (Marouga et al., 2005; Ünlü et al., 1997). Anecdotally, a recent report described a “spicy SDS-PAGE” with curcumin (Kurien et al., 2012), an environmental-friendly stain but quite unpopular so far.

After protein separation, the digestion of individual proteins (usually with trypsin) is highly recommendable to get accurate data, obtaining a peptide mixture prior to identification of proteins through MS. By definition, a mass spectrometer consists of an ion source, a mass analyser that measures the mass-to-charge ratio (*m/z*) of the ionized analytes, and a detector that registers the number of ions at each *m/z* value. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [or its variation surface-enhanced laser desorption/ionization (SELDI)] are the two techniques most commonly used to volatize and ionize the proteins or peptides for MS analysis (Fenn et al., 1989; Karas and Hillenkamp, 1988). ESI is coupled to liquid-based separated tool such as liquid chromatography (LC) or capillary electrophoresis (CE) whilst MALDI sublimates and ionizes the samples out of a dry matrix via laser pulses (Aebersold and Mann, 2003). After that, there are four basic types of mass analysers: the ion trap (IT), time-of-flight (TOF), quadrupole and Fourier transform ion

cyclotron (FT-MS) analysers. Today, most studies are working with the combination of this technology as a complementary protein identification approach.

The proteins can be identified using mass mapping [known as peptide-map fingerprinting (PMF)] or using peptide collision-induced dissociation (CID) spectra, whose protein identification is more clear-cut than those achieved by mass mapping because, in addition to the peptide mass, the peak pattern in the CID spectrum also provides information about peptide sequence. But this information is not readily convertible into a full, unambiguous peptide sequence. Thus, the CID spectra are scanned against comprehensive protein sequence databases using one of a number of different algorithms, each with its strengths and weaknesses. Mainly, three methods are used to scan the CID spectra: "peptide sequence tag" approach using 'Peptide Search' software (which is no longer available) (Mann and Wilm, 1994), "cross-correlation" method using mainly SEQUEST (Eng et al., 1994) and 'probability based matching' approach using mainly MASCOT search engine (Perkins et al., 1999), probably the last one the most used. In this approach, the calculated fragments from peptide sequences in the database are compared with observed peaks; and from this comparison a score is calculated which reflects the statistical significance of the match between the spectrum and the sequences significance of the match contained in a database, including the false discovery ratio (FDR).

In each of these methods of matching, the identified peptides are compiled into a protein 'hit list', which is the output of a typical proteomic experiment. Since protein identifications rely on matches with sequence databases, high-throughput proteomics is currently restricted largely to those species for which comprehensive sequence databases are available; fortunately the recent increase of genome availability in farmed fish species are enabling this task.

The basic information of the proteins found is: the spot identification in the gel, the name of the matched protein, the species in which these peptides were found, both

theoretical and real IP and MW, the coverage (number of amino-acids regarding the total known sequence), the score statistically significant (number of amino-acids) and the level of significance (p-value or E-value). Additionally, if a comparative approach is done, the study should include also the fold-change and the level of significance for this change.

Objectives

The present Doctoral Thesis tries to improve our knowledge of fish skin and mucus with special focus on immunity.

The specific objectives are:

1. Characterization of the skin of gilthead seabream, including morphology, cell cycle and gene expression analyses, differentiating dorsal and ventral skin areas.

2. Determination of the immune response of gilthead seabream faced by a natural outbreak of lymphocystis disease virus.

3. A search for the best storage conditions of gilthead seabream skin mucus to evaluate its humoral immune activities.

4. Characterization of the skin mucus proteome map of European sea bass to search for new molecules involved in mucosal immunology.

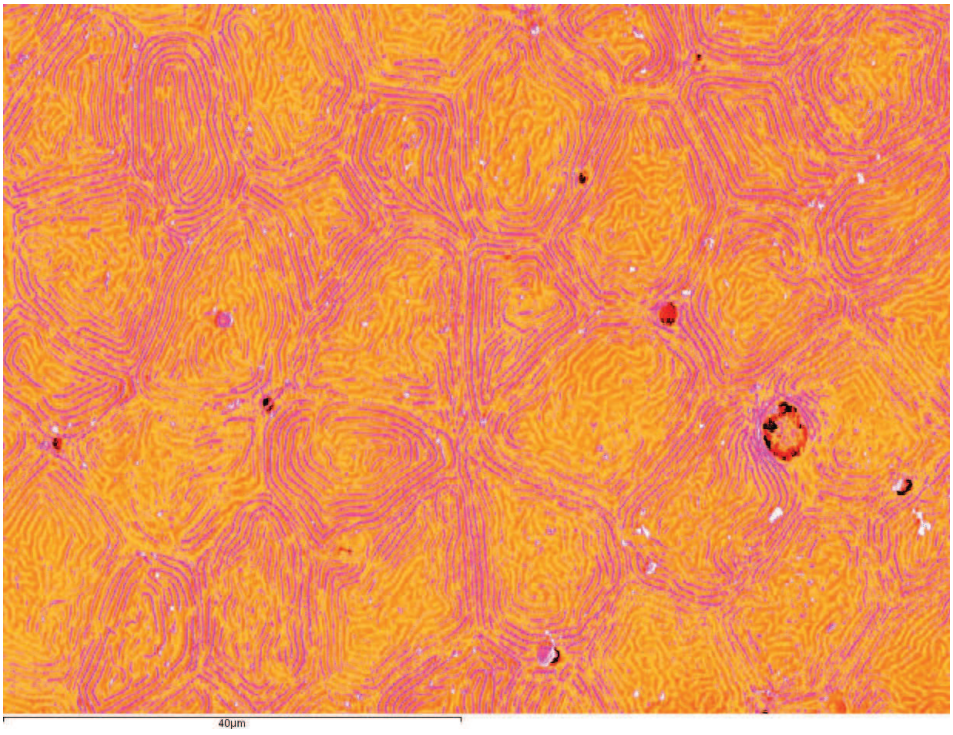
5. Analysis of the changes produced by dietary probiotics, stress by crowding or chronic skin wounds in the skin mucus proteome map of gilthead seabream.

Experimental chapters

Chapter 1

Characterization of gilthead seabream skin:

dorso-ventral differentiation



Abstract

The aim of this study was to characterize gilthead seabream skin by isolating cells and studying the cell cycle by flow cytometry, to study the skin histology by light and scanning electron microscopy and the transcription level of nine cytokine genes by RT-PCR. Furthermore, the results obtained from samples taken from the dorsal and the ventral parts of the specimens are compared. No differences were observed in the cell cycle of cells isolated from the dorsal and ventral zones of the skin. However, the epidermis thickness of the ventral skin was higher than that of the dorsal skin, as demonstrated by image analysis using light microscopy. Furthermore, scanning electron microscopy pointed to a greater cell size and area of microridges in the apical part of the dorsal epidermal cells compared with ventral skin epidermal cells. Finally, gene expression profile of cytokines remained similar in dorsal region, but strongly affected by pathogen/probiotic in ventral region. This study represents an improved knowledge of the skin structure of teleost, concretely, gilthead seabream, through an updated multidisciplinary approach.

1. Introduction

Teleost skin, in sharp contrast to mammals, is a non-keratinised tegument, with living active-cells. Epidermis is the outermost layer of cells, which comes from ectodermal origin and is responsible for secreting the skin mucus through goblet cells (Kanitakis, 2002). The exterior surface of superficial epithelial cells has special structures called microridges, whose functions are poorly understood (Hawkes, 1974). Epidermis is separated from the next inner layer (dermis) by an acellular basement membrane. Dermis is thicker, vascularised, derived from the mesoderm and can be divided into two clear sublayers: (i) the called *stratum spongiosum* with vascular and neural components where scales are embedded and most of the chromatophores are located; and (ii) the called *stratum compactum* which mostly consists of a dense matrix of collagen fibers (Elliott, 2000). Last internal layer, hypodermis, is mainly (but not exclusively) a reservoir fat layer comprised by adipose cells that links the skin with the underlying muscle. In gilthead seabream, the study of the skin cells is very scarce and old-fashioned, most of them with techniques of classical microscopy in gilthead seabream (Ferrer et al., 1999; Sarasquete et al., 1998).

Regarding the properties of the different regions of the fish skin, few studies have taken in consideration the differences among dorsal and ventral parts. One of them evaluated the role of agouti-signalling protein (ASP) in the different dorsal-ventral pattern of skin pigmentation of fish (Cerdá-Reverter et al., 2005). At transcription level, only one study from isolated skin cells revealed changes after probiotic-pathogen interaction in Atlantic cod (Lazado and Caipang, 2014). However, no further studies have been carried out in the skin differentiation.

Concretely, the intensive fish farming triggers the appearance of recurrent diseases such as vibriosis (Austin and Zhang, 2006) or photobacteriosis (previously known as pasteurellosis) (Remuzgo-Martínez et al., 2014), which, in many cases, causes high mortalities and economic losses. To overcome the impact of these outbreaks in the fish

health, probiotics are an effective tool against pathogens since they are able to improve the immune system, produce inhibitory compounds and inhibit pathogen colonization by competitive exclusion (Nayak, 2010; Newaj-Fyzul et al., 2014; Ninawe and Selvin, 2009; Verschuere et al., 2000). *Shewanella putrefaciens* strain Pdp11 is a probiotic isolated from skin of gilthead seabream (Chabrillón et al., 2005b). Importantly, previous studies demonstrate that Pdp11 inhibits the attachment to skin mucus and competitive exclusion properties against *Photobacterium damselae* subsp. *piscicida* (Chabrillón et al., 2005b) and *Vibrio harveyi* (Chabrillón et al., 2005a) *in vitro.*

Produced at the site of entry of a pathogen, cytokines drive inflammatory signals that regulate the capacity of resident and newly arrived phagocytes to destroy the invading pathogen (Wang and Secombes, 2013). Furthermore, it has been reported that cytokines are modulated by probiotics (Panigrahi et al., 2007; Román et al., 2013), including Pdp11 (Cordero et al., 2016d). To the best of our knowledge, although the skin is the first barrier of defense there are few studies about the direct effect of probiotics on this fish mucosa.

The aim of this work was the characterization of the dorsal-ventral skin through the isolation of the dorsal and ventral skin cells, the histological analysis by optic and electronic microscopy, measuring thickness of epidermal layer, cell area and the area of microridges, as well as studying the gene expression profile of nine cytokines in dorsal and ventral skin with the pathogen *P. damselae* subsp. *piscidida* and the probiotic *S. putrefaciens* Pdp11 in the teleost gilthead seabream.

2. Materials and methods

2.1 Animal maintenance and collection

Specimens of the hermaphroditic protandrous teleost gilthead seabream obtained from a local farm in Murcia (Spain) were kept in re-circulating seawater aquaria (250 l) with a flow rate of 900 l h^{-1} in the Marine Fish Facility at the University of Murcia and allowed to acclimatize for 2 weeks. The temperature and salinity were 22 ± 2ºC and 28‰,

respectively. The photoperiod was of 12 h light: 12 h dark. Commercial diet (Skretting, Spain) was administrated at rate of 2% body weight day^{-1}. Fish were anesthetized with 100 mg l^{-1} MS222 prior to sampling the skin in each trial. Mucus was gently removed by a cell scraper before sampling the skin as detailed in the following sections.

2.2. Bacterial culture and *in vitro* skin exposure

The fish pathogen *P. damselae* subsp. *piscicida* and the skin isolated probiotic *S. putrefaciens* Pdp11 (Chabrillón et al., 2005b) were grown in agar plates for 24 h and, afterwards, one colony of each of them was inoculated in tubes containing 5 ml of tryptic soy broth (TSB; Sigma-Aldrich) supplemented with 1.5% NaCl (TSBs). After 24 h of shaking incubation at 22ºC and 200 rpm, the bacterial concentrations were measured and adjusted at 10^6 bacteria ml^{-1}.

Skin samples (1 cm diameter) from the dorsal and ventral regions of the middle of the body (above and below of lateral line) were obtained from each fish in sterility with a biopsy punch and the muscle was removed by dissection. Samples were washed in PBS supplemented with 100 I.U. ml^{-1} penicillin (Life Technologies) and 100 µg ml^{-1} streptomycin (Life Technologies). Samples were incubated in RPMI culture medium (Life Technologies) supplemented with 0.35% NaCl, SBF 10% (Life Technologies), HEPES 20 mM (Gibco), epidermal growth factor 100 µg ml^{-1} (EGF; Life Technologies) and glutamine 2 mM (Life Technologies). Skin samples were incubated for 2 h in flat-bottomed 12-well plates without bacteria (control samples) or with 10^3 cfu ml^{-1} of the pathogen bacterial suspension, with 10^3 cfu ml^{-1} of the probiotic suspension or with a combination of both bacteria. Afterwards, samples were placed in TRIzol® reagent (Life Technologies) and stored at -80ºC for later RNA extraction.

2.3 Isolation of cells

Dorsal and ventral skin (n = 4-8 fish specimens) was cut into small pieces (~ 0.5 cm^2) removing previously the muscle and washing with ice-cold phosphate buffered saline

(PBS) supplemented with antibiotics (100 I.U. ml^{-1} penicillin and 100 µg ml^{-1} streptomycin). Then, skin fragments were separately (without mixing skin from different specimens) disposed in keratinocytes medium (Sigma-Aldrich) supplemented with 5 mg ml^{-1} of dispase (Gibco), 5% foetal bovine serum (FBS; Gibco), 10 ng ml^{-1} epidermal growth factor (EGF; Life Technologies), and the aforementioned antibiotics. Tissue fragments were incubated on ice with shaking for 3 h. Disaggregated cells and fragments were separated by a 100 µm cell strainer and previous steps were repeated once with the fragments. Finally, all the cell suspensions were combined, centrifuged at 400 g for 10 min, and cells resuspended finally with the same medium without dispase. Cell concentration and viability was determined in Neubauer's chambers and by the trypan blue test, respectively.

2.4 Cytocentrigation

The dorsal and ventral isolated skin cells were adjusted to 10^5 cell ml^{-1} and cytocentrifuged at 800 g for 5 min at 4ºC. Next, the cells were fixed in methanol for 10 min and stained with Giemsa (Merck) at 10% (v/v) for 60 min, washed with tap water and mounted with DPX (Merck). The slides were then examined under a light microscope (Leica DM6000B) and images were obtained with a digital camera (Leica DFC280) and processed by Leica Application Suite V2.5.0. software.

2.5 Cell cycle analysis

Freshly isolated skin cells were resuspended in 200 µl of PBS and 1 ml of a 70% ethanol solution was added dropwise while stirring. After 30 min of incubation, cells were washed and resuspended in 800 µl of PBS. Finally, 100 µl of RNAse (1 mg ml^{-1}; Thermo Fisher Scientific) and 100 µl of propidium iodide (PI, 400 µg ml^{-1}; Sigma-Aldrich) were added and incubated at 25ºC for 30 min in the dark. Dorsal and ventral cells were acquired by a FACScalibur flow cytometer and the cell cycle analysis was performed on 10,000 events using the ModFit LT™ software (Verity Software House). The experiment was performed twice with duplicated samples.

2.6 Histology analysis

Dorsal and ventral skin (n = 4-8 fish specimens) was dissected into pieces (~ 0.5 cm^2), washed in PBS and fixed in 4% paraformaldehyde for 24 h. Next, samples were decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) solution for 24 h, dehydrated in increased concentrations of ethanol (Merck) (70% for 24 h, 80% for 30 min, 96% for 1 h, and 100% for 1 h three times), then washed for 1 h three times in isoamyl acetate, and finally paraffin embedded. Slides containing sections of 5 μm were stained with hematoxylin-eosin (H&E; Merck), periodic acid–Schiff (PAS; Merck) or trichrome of Mallory (Merck) according to the manufacturer's instructions. Sections were studied under a light microscope and images taken. The quantitative analysis of epidermal thickness was performed with Leica QWin software (Leica Microsystems Ltd.).

2.7 Electron microscopy

For scanning electron microscopy (SEM), skin from dorsal and ventral zones (n = 4-8 fish specimens) were washed, fixed according to McDowell and Trump (1976) for 7 h, placed in washing buffer containing 0.2 M cacodilate buffer with 8% saccharose (Merck) and post-fixed later in 1% OsO_4 for 1 h and 30 min. Afterwards, samples were dehydrated in acetone (from 30% to 100%, 20 min each), critical-point dried, sputter coated with gold and examined with a Jeol JSM-6100 scanning electron microscope. Images were acquired with the software INCA Suite V4.09 (Oxford Instruments). The quantitative analysis of the cell area and microridge area was performed with Leica QWin software.

2.8 Gene expression analysis

For RNA, dorsal and ventral pieces of skin (n = 4) in TRIzol® reagent were processed as indicated by the manufacturer's instructions in order to extract the total RNA from skin. RNA present in samples was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. The RNA was then treated with

DNase I (Promega) to remove genomic DNA contamination. To check the RNA quality, an agarose gel was run with all the samples. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript IV reverse transcriptase (Life Technologies) with an oligo-dT18 primer according to the manufacturer's instructions.

The transcription analysis was carried out by real-time PCR (qPCR). The expression of selected genes was analysed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), which was performed as described elsewhere (Cordero et al., 2015a). Specificity of reactions was analysed using similar samples without cDNA as negative controls. All qPCR reactions were carried out in duplicate and quantification cycle (Ct) values of target genes were converted into relative quantities using reference genes. So for each mRNA, gene expression was corrected by both the elongation factor 1α (*ef1a*) and ribosomal protein S18 (*rps18*) RNA content in each sample. The efficiency was calculated as described elsewhere (Cordero et al., 2016c). The results are expressed as mean ± SEM. The primers used in the present study are listed in Table 2.

2.9 Statistical analysis

Data were statistically analysed by Student t-test (or ANOVA in the case of gene expression) to determine differences between the dorsal and the ventral skin parts using Statistical Package for Social Science (SPSS for Windows; v19) and differences were considered statistically significant when $p < 0.05$.

Table 2. Primers used to qPCR study.

Gene	GenBank	Efficiency (%)	Sequences
ef1a	AF184170	109.1	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA:
rps18	AM490061	100.5	F: CGAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC
il1b	AJ277166	104.8	F: GCGAGCAGAGGCACTTAGTC R: GGTAGGTCGCCATGTTCAGT
tnfa	AJ413189	108.4	F: TCGTTCAGAGTCTCCTGCAG R: TCGCGCTACTCAGAGTCCATG
il6	AM749958	111.4	F: AGGCAGGAGTTTGAAGCTGA R: ATGCTGAAGTTGGTGGAAGG
il7	JX976618	103.9	F: GATCTGGAAAACACCGGAGA R: TGGACGTGCAGTTCTGTAGC
il8	AM765841	110.5	F: GCCACTCTGAAGAGGACAGG R: TTTGGTTGTCTTTGGTCGAA
il10	FG261948	103.4	F: CTCACATGCAGTCCATCCAG R: TGTGATGTCAAACGGTTGCT
tgfb	AF424703	130.0	F: GCATGTGGCAGAGATGAAGA R: TTCAGCATGATACGGCAGAG
il15	JX976625	111.3	F: CTACTGGACCGGGATCAATG R: TCGTCTATGATCTGCGCAAC
il18	JX976626	121.0	F: TTGAGGGGTTGTCCTGTTTC R: AGTTTTTACCCCAGCCCTGT

3. Results and discussion

3.1 Skin cells were isolated revealing no changes in cell cycle analysis

Skin is probably the toughest organ of bony fish since it is the responsible of keeping the fish integrity (among other functions). For this reason, the protocols to isolate fish skin cells are scarce and skin cells very difficult to isolate, so only one study attempted to do so with success (Lazado and Caipang, 2014). They showed that for Atlantic salmon skin cells disaggregation, trypsin was less efficient in terms of cell number and viability than dispase, the enzyme used in the present research. In our study, skin cells from dorsal and ventral regions were successfully isolated in gilthead seabream (Figures 3A, 3B) but showed very different morphological characteristics. Thus, most cells are roughly round with a small and eccentric nucleus. Interestingly, the staining pattern show great differences resulting in eosinophilic or light eosinophilic cytoplasm in the epithelial cells of dorsal skin whilst very clear to non-stained in the ventral area. Strikingly, seabream skin cells have a 10-15 µm in size whilst the Atlantic salmon skin

isolated cells were of 200-300 µm in size (Lazado and Caipang, 2014) but shared the round morphology and eccentric nucleus. In addition, and since epidermal cells of fish skin are actively replicating we evaluated the cell cycle (**Figures 3C, 3D**). In the dorsal skin, the results showed 76.9% of cells in G1/G0 phase and 23.1% in G2/M phase, whilst in the ventral skin the percentages were 83.8% and 16.2% in G1/G0 and G2/M phases, respectively, finding no significant differences in the cell cycle between skin areas. Moreover, apoptosis is more remarkable in ventral cells than in dorsal cells suggesting that they are more susceptible to the isolation procedure and perhaps to injury. To our knowledge this is the first time that cell cycle is studied among the regions of the skin. However, the application of the study the cell cycle in skin cells open new possibilities to study the cell regulation in common fish skin infections such as lymphocystis (Cordero et al., 2016a) or vibriosis (Tapia-Paniagua et al., 2014), a field with potential applications in aquaculture. In this sense, apoptosis of salmon isolated skin cells was increased upon *in vitro* exposure to *Vibrio anguillarum* (Lazado and Caipang, 2014).

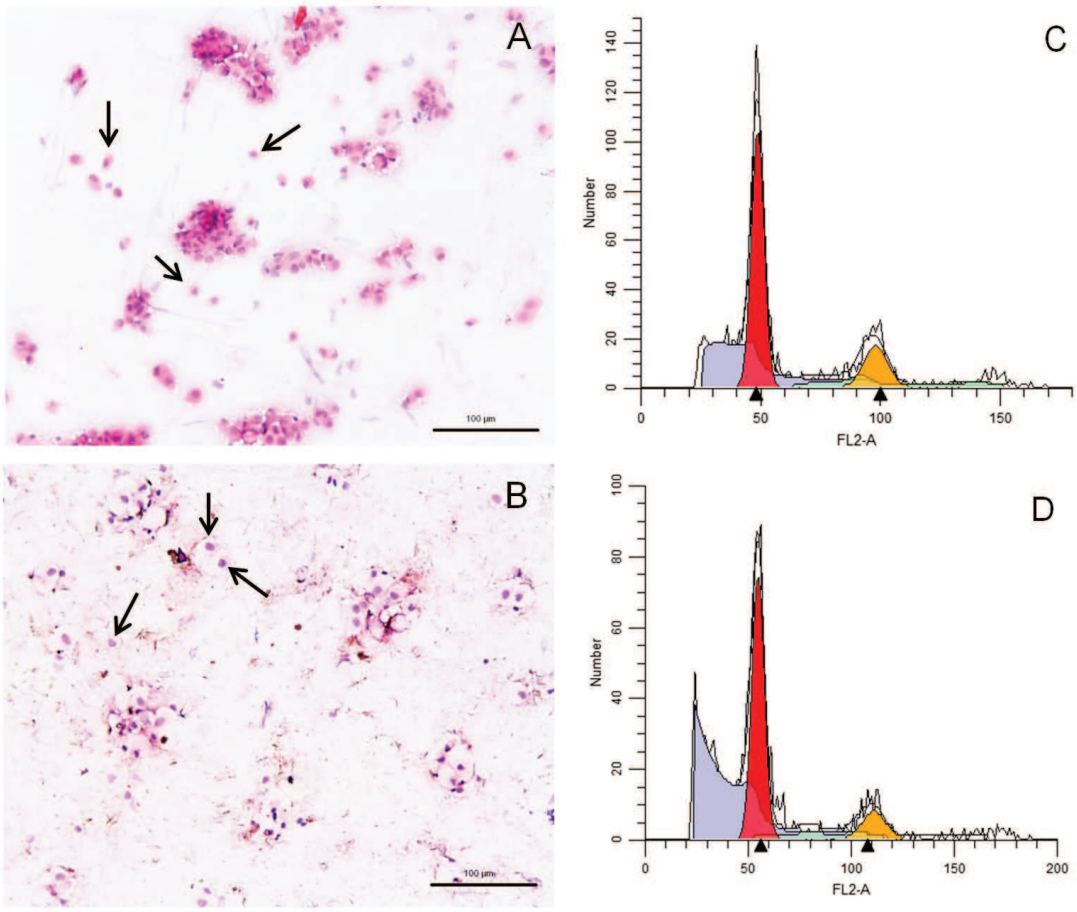


Figure 3. Representative images of freshly isolated skin cells from dorsal (A) and ventral (B) regions stained with haemotoxylin-eosin after cytocentrifugation. Cell cycle analysis of isolated dorsal (C) and ventral (D) skin cells of gilthead seabream by flow cytometry showing cells in G0/G1 (red) and G2/M (yellow) phases as well as apoptotic cells (blue).

3.2 Light microscopy showed different thickness in dorsal and ventral skin

Histological studies are a powerful tool used for tissue analysis since many years ago. In the past, some articles studied the epidermal thickness, which seem to be influenced mostly by seasonal changes and/or sex (Burton and Burton, 1989; Pickering, 1977) but neither of them could state conclusively. In our results, light microscopic analysis showed a significant lower epidermal layer thickness in the dorsal skin compared with the ventral skin layer (**Figures 4, 5**). These differences in the skin of gilthead seabream were confirmed by the image analysis (**Figure 4C**). The staining pattern of epithelial

cells, however, showed very little differences in both skin locations (**Figures 4, 5**). Goblet cells are embedded in the epidermal layer but predominant in the outer part of the epidermis (**Figure 5C**). In the epidermal layer, and externally, we have described a type of "sensory cells" whose function is still unknown (**Figure 5B**). Chromatophores are mainly located in the upper layer of the dermis (*stratum spongiosum*) and in the ventral area (**Figures 4A, 5A**). Chromatophores are quite well dated to be mainly in the *stratum spongiosum* in both scaled and non-scaled skin (Elliott, 2000). Goblet cells are also reported in most of the teleost (Whitear, 1986), showing variations due to skin infections (Marel et al., 2010) as well as in different parts of the body (Burton and Burton, 1989; Pickering, 1977). Regarding epidermal thickness in the different regions of the fish body, there is no recent information, and only a review indicated that in benthic species the epidermal layer of ventral skin is often thicker (Bullock and Roberts, 1974). Again, these differences in the epidermal thickness can be in relation with the susceptibility of pathogens to colonize or cause skin lesions or ulcers in some regions of the skin rather in others.

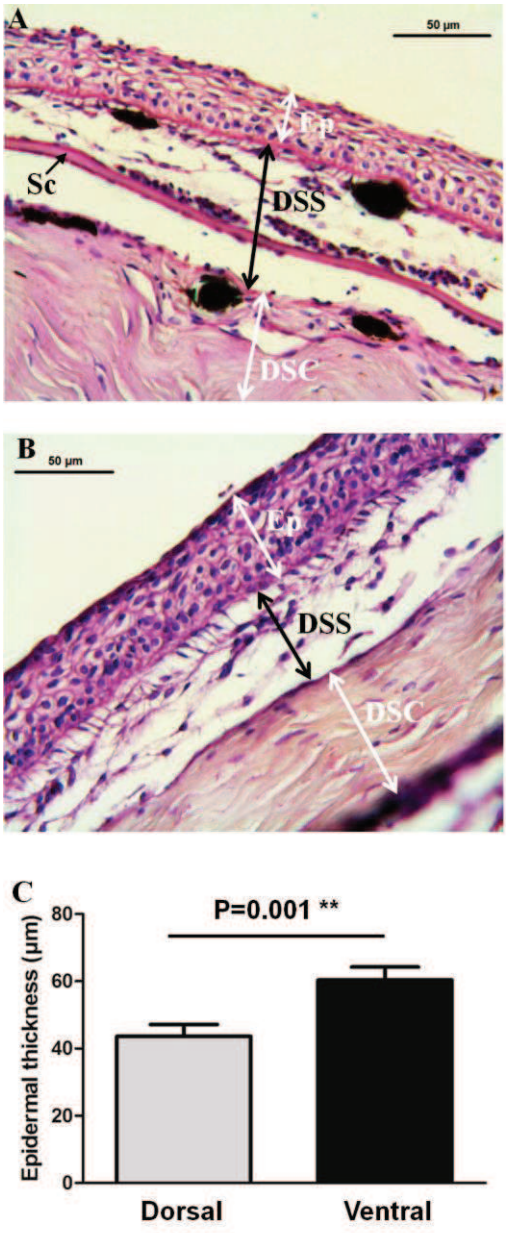


Figure 4. Epidermis thickness analysis. Representative images of dorsal (A) and ventral (B) skin from gilthead seabream stained with PAS. C. Analysis of epidermis layer thickness (µm) from dorsal and ventral skin. Bars represent the mean ± SE (n = 10). P value resulting from the Student t-test comparing both groups is indicated. DSC, dermis *stratum compactum*; DSS, dermis *stratum spongiosum*; Ep, epidermis; Sc, scale.

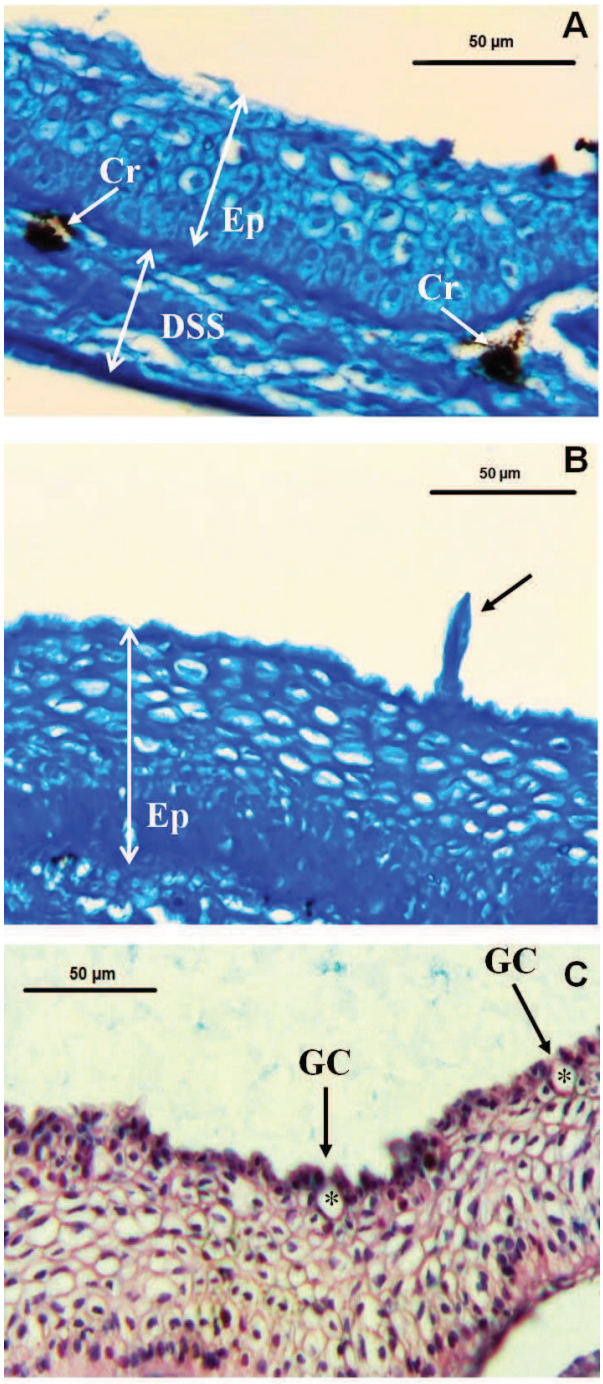


Figure 5. Specific structures of skin. Representative images of dorsal (A) and ventral (B) skin from gilthead seabream stained with trichrome of Mallory detailing "sensory cells" (black arrow) and chromatophores (white arrows) as well as goblet cells (asterisks) in ventral regions stained with PAS (C). Cr, chromatophores; DSS, dermis *stratum spongiosum*; Ep, epidermis; GC, goblet cells.

3.3 SEM study revealed different cell size and area of microridges in dorsal and ventral skin

Our study showed the dorsal and ventral skin of gilthead seabream by SEM (Figure 6). Both regions showed the microridges, the sensory cells and the capacity to secrete mucus. Interestingly, the cell area (Figure 7A) and the area of microridges (Figure 7B) in the dorsal region are larger than in the ventral region. There is no previous information about the differences in cell size or the area of microridges, only a previous work that describes preliminary data of microridges in wound healing (Rai et al., 2012); however, it would be interesting to investigate their alteration since they seem to be involved not only in mechanical protection but also in the capacity to hold the skin mucus (Hawkes, 1974), and therefore in a better mucosal immune barrier of fish.

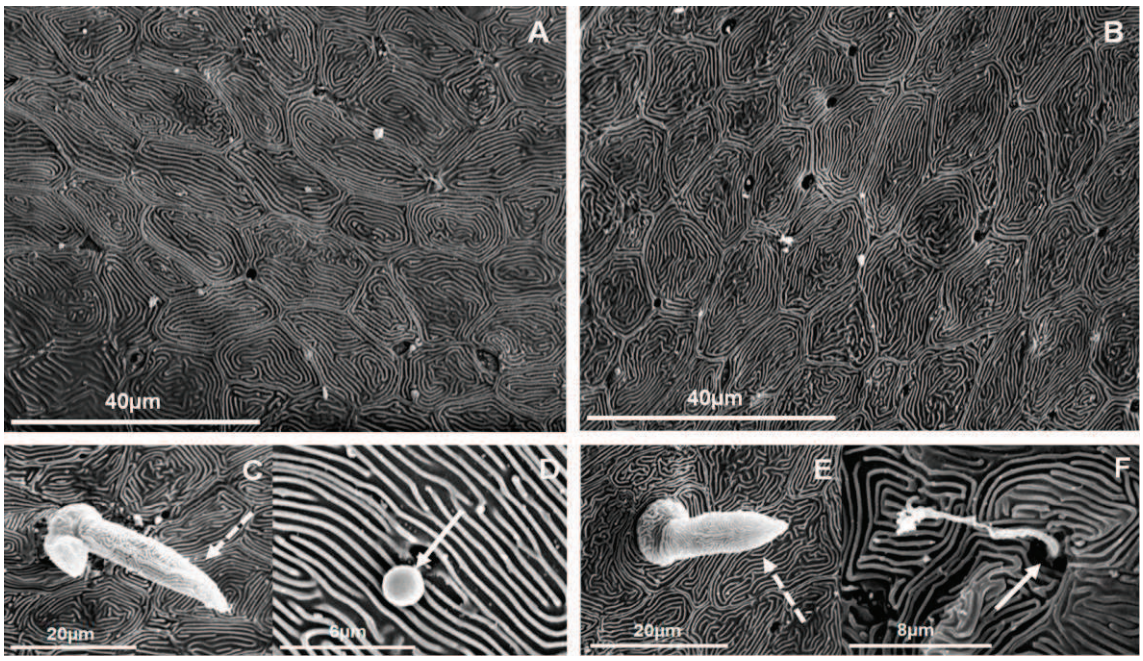


Figure 6. Analysis of fish skin by scanning electron microscopy (SEM). Representative images of dorsal (A, C, D) and ventral (B, E, F) skin from gilthead seabream by SEM. Skin structures such as "sensory cells" (discontinuous arrows) (C, E) and skin mucus secretions (continuous arrows) (D, F) are detailed.

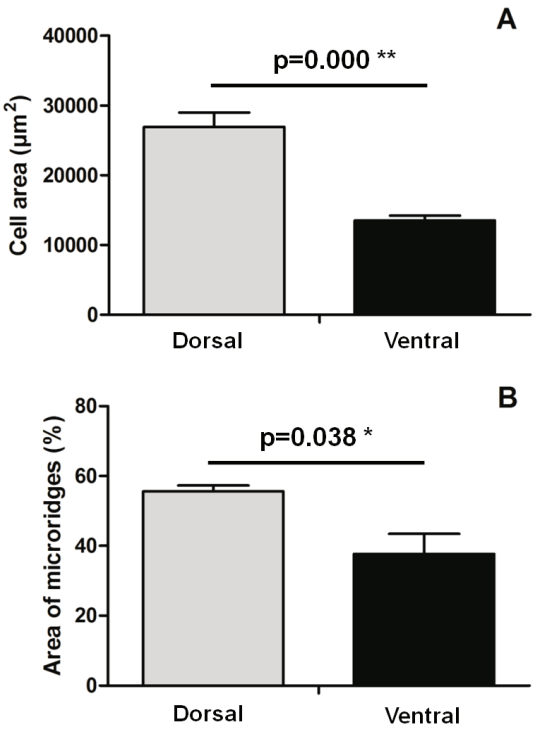


Figure 7. Analysis of cell area and area of microridges. Cell area (A) expressed in µm2, and area of microridges (B) expressed as percentage (%), in the dorsal and ventral skin of gilthead seabream determined by analysis of SEM images. Bars represent the mean ± SE (n = 10). P value resulting from the Student t-test comparing both groups is indicated.

3.4 *In vitro* incubation with the pathogen and the probiotics revealed a different susceptibility of dorso-ventral skin expression profile

The nine cytokines analyzed were constitutively expressed in both studied skin zones (dorsal and ventral) although at different transcript levels. Regarding total gene expression levels, the highest values were found for *il8* and the lowest for *il7*, in both dorsal and ventral skin zones. In addition, the expression of each target gene was generally higher in dorsal skin samples than the values obtained for ventral skin samples (Figure 8).

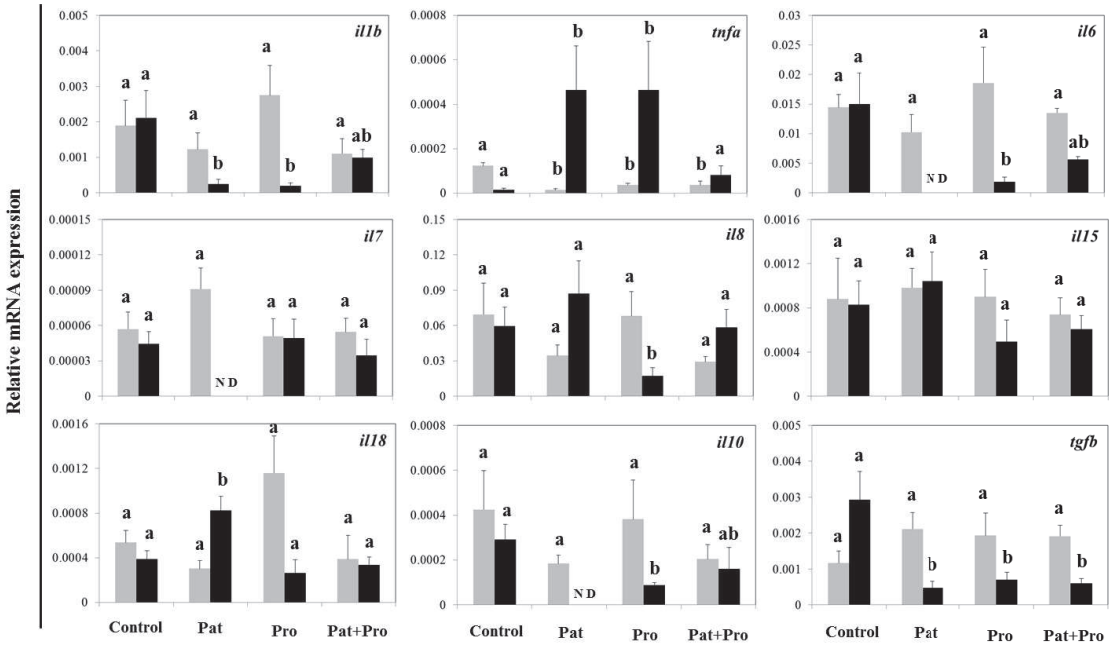


Figure 8. *In vitro* gene expression patterns of nine cytokines (*il1b*, *tnfa*, *il6*, *il7*, *il8*, *il15*, *il18*, *il10* and *tgfb*) after *P. damselae* subsp. *piscicida* and *S. putrefaciens* Pdp11 exposure single or combined in dorsal (gray bars) and ventral (black bars) skin of gilthead seabream (*S. aurata*). Details of each cytokine are showed in **Table 2**. Statistically significant differences among unexposed and exposed groups were denoted with different letters when $p<0.05$.

After incubation of dorsal skin samples with pathogen, probiotic bacteria or with pathogen and probiotic bacteria no statistically significant differences were observed in the transcript levels of *il1b*, *il6*, *il7*, *il8*, *il15*, *il18 il10* and *tgfb* genes while the *tnfa* gene expression was significantly down-regulated, compared to the control dorsal skin samples (**Figure 8**).

Regarding the ventral skin samples, a high variability in the gene expression levels was detected. No significant differences were detected in the *il7* and *il15* transcript levels after incubation of skin samples with bacteria, although in the case of *il7*, the gene expression levels were undetected (ND) after pathogen exposure. The expression patterns of *il6* and *il10* were very similar with significant decreases of the values recorded in skin samples incubated with probiotics and ND levels after pathogen

exposures. However, those samples incubated with both pathogen and probiotic showed a transcription levels very similar to those recorded on control skin samples. Otherwise, the present findings showed a significant down-regulation of *il1b* transcript levels after skin incubation with pathogen or probiotic but not with combination of both bacteria. A similar pattern was observed in *tgfb* gene expression profile, except for samples incubated with pathogen and probiotic which showed also a down-regulation profile compared to the control samples. Finally, expression of *il18* and *tnfa* genes was statistically significant increase in skin samples incubated with the pathogenic bacterial cells although the values recorded for samples incubated with both pathogen and probiotics were very similar to those found for control skin samples (Figure 8).

In our study, we have developed a suitable protocol to isolate gilthead seabream skin cells and analyse the cell cycle as well as characterized the skin by histological (changes of epidermal thickness, cell area and the area of microridges) and transcriptomic analysis, making comparisons between dorsal and ventral in relation probiotic-pathogen interaction.

These techniques represent useful tools for use in future studies of skin alterations and biology, while the study has also provided new insights into the skin structure of teleosts.

Chapter 2

Gilthead seabream immune response under a natural lymphocystis virus (LCDV) outbreak

Abstract

The immune status of gilthead seabream specimens under a natural outbreak of LCDV was studied. The replication of the virus was demonstrated in LCDV-infected fish, but not in control fish. The results showed decreased total serum IgM levels and increased innate cellular immune response (peroxidase and respiratory burst activities) of HK leucocytes in LCDV-infected fish, compared to the values obtained in uninfected specimens. In addition, transcription of antiviral genes (*ifn* and *irf3*) was down-regulated in the skin of LCDV positive fish as well as genes involved in cellular immunity (*csf1r*, *mhc2a*, *tcra* and *ighm*) that were down-regulated in skin and HK of infected fish. By contrast, the transcription of *nccrp1* was up-regulated in HK after LCDV infection. These present results show that HK leucocytes are activated to encounter the virus at the sites of replication.

1. Introduction

Lymphocystis disease virus (LCDV) is a double stranded DNA virus that belongs to the family *Iridoviridae* and genus *Lymphocystivirus*. LCDV is the causative agent of lymphocystis disease, which primarily affects fibroblasts in the dermal connective tissue, causing papilloma-like hypertrophy or nodular masses (Samalecos, 1986). Each nodule is an individual infected host cell called a lymphocystis cell or lymphocyst (González de Canales et al., 1996). LCDV is usually self-limiting, and their lesions are spontaneously solved and the host recovers within a few days or weeks (Paperna et al., 1982). LCDV has been reported to occur in over 100 different fish species in freshwater and saltwater worldwide (Tidona and Darai, 1997). It is in farmed fish species where the outbreaks of LCDV may cause economic losses in the aquaculture industry. LCDV is one of the commonest viral diseases of gilthead seabream, an important commercial species in the Mediterranean area. For this reason, most of the available information has focused on the detection and transmission of this virus in this fish species (Cano et al., 2013, 2009, 2006), while there is little information about fish immunity against LCDV.

Regarding the innate immunity, interferon (IFN), complement and cell-mediated cytotoxic activities are the most important immune responses fighting the virus (Ellis, 1998). Thus, at gene level, the implication of several immune-related genes in the fish defense against LCDV, mainly related to the IFN response, has been corroborated (Fernández-Trujillo et al., 2013; Hu et al., 2013; Lu et al., 2014). Unfortunately, only one study has showed that in Japanese flounder (*Paralichthys olivaceus*) affected by LCDV the dietary administration of probiotics increased the activities of complement, lysozyme, phagocytosis and production of reactive oxygen species (ROS) in the blood, which was concomitant with increased resistance to the infection (Harikrishnan et al., 2010a). However, these authors did not compare the same innate immune responses between unaffected and LCDV-affected fish, and therefore, the alterations produced by

the LCDV infection still remain unknown. Interestingly, one study has evaluated the morphological changes that the virus causes after a natural outbreak at the infection site, the skin (Dezfuli et al., 2012). They have shown that acidophilic granulocytes (AG) (the most important phagocytic cells in seabream and functional counterparts to neutrophils), but not mast cells, are mobilized to the infection site and activated to produce interleukin-1 beta (IL-1β). Similarly, the proliferation of macrophages and epithelioid cells around lymphocystis cells has been previously described as an immune response to LCDV in other fish species (Colorni and Diamant, 1995; Roberts, 1976; Sheng et al., 2007). In seabream infected with other fish viruses, the HK leucocyte innate immune parameters of phagocytosis, respiratory burst or cell-mediated cytotoxicity are activated by viral haemorrhagic septicaemia virus (VHSV) and nodavirus (NNV) (Chaves-Pozo et al., 2012; Esteban et al., 2008). These data indicate the importance of the innate immune response upon virus infection, and scarce available data suggest that LCDV activates the innate immunity both local and systemic.

On the other hand, more studies regarding immunity against LCDV have focused on adaptive immunity and efficacy of vaccines but, unfortunately, they have only focused on Japanese flounder. Thus, several works have shown the protection conferred in flounder by vaccines against LCDV towards the generation of specific antibodies (Tian and Yu, 2011; Tian et al., 2008a, 2008b). In addition, a study demonstrated that this acquired immunity, determined as immunoglobulin (IgM)-positive cells and circulating IgM levels, was optimal at 21°C and depressed at not natural temperatures for flounders (Xu et al., 2011).

Taken into account all these considerations, the aim of this work was to study the humoral and cellular innate immune responses of gilthead seabream specimens suffering a natural LCDV outbreak, as well as the changes in the expression of some immune-related genes in HK (as the main hematopoietic organ) and in the skin (the

target tissue of LCDV). The results will be useful to understand the effect of this viral infection upon some of the major immune system parameters, to better design preventive or curative future treatments.

2. Materials and methods

2.1 Animals and natural outbreak

Specimens of gilthead seabream obtained from a local farm (Murcia, Spain) (average weight 41.6 ± 3.6 g and average length 14.2 ± 0.4 cm) were kept in re-circulating seawater aquaria (900 l) in the Marine Fish Facilities at the University of Murcia. Aquaria were provided with a flow water of 900 l h^{-1}, 28‰ salinity, 20 ± 2ºC temperature and a photoperiod of 12 h light: 12 h dark. Fish were fed daily at 2% rate of the fish biomass per day with a commercial diet (Skretting). After arrival, during the acclimation process, a natural outbreak of LCDV emerged and fish with clear symptoms of the disease (white nodules, individually or in clusters distributed by the body surface) were sampled. Another group of fish without any symptoms were also sampled at the same time as controls. No mortality was detected in both groups (infected and non-infected).

2.2. Sample collection

Fish (n = 4) were anesthetized with 100 mg l^{-1} MS222 prior to sampling. Blood samples were collected from the caudal vein with an insulin syringe, allowed to clot at 4°C for 4 h and after centrifugation (9,500 g, 5 min) the serum was individually collected and stored at -80°C until use. Fish were also dissected to obtain skin (around the lesions belonging to ventral area) and HK fragments that were stored in TRIzol® reagent (Life Technologies) at -80ºC for later RNA isolation. For leucocyte isolation, HK fragments were transferred to 8 ml sRPMI-1640 culture medium (RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs], 2% (v/v) foetal calf serum (FCS, Gibco), 10 IU ml^{-1} heparin (Sigma-Aldrich), 100 IU ml^{-1} penicillin (Gibco) and 100 µg ml^{-}

[1] streptomycin (Gibco). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100µm), washed twice in sRPMI (400 g, 10 min), counted (Z2 Coulter Particle Counter) and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was determined by the trypan blue exclusion test (Freshney, 1987).

2.3. Humoral and cellular immune activities

Total serum immunoglobulin M (IgM) levels were analysed using the enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Cuesta et al., 2004). Thus, 100 µl per well of 1/100 fold diluted serum were placed in flat-bottomed 96-well plates in triplicate and the protein coating was performed by overnight incubation at 4°C with 200 µl carbonate–bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.05% Tween 20 (PBT, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBT, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer; Sigma-Aldrich). After exhaustive rinsing with PBT, the plates were developed using 100 µl 0.42 mM 3,3,5,5-tetramethylbenzidine hydrochloride (TMB; Sigma-Aldrich) solution, prepared daily in distilled water containing 0.01% H_2O_2 (Merck). The reaction was allowed to proceed for 10 min and stopped by the addition of 50µl 2M H_2SO_4 and the plates were read at 450 nm in a plate reader (BMG, Fluostar Omega). Negative controls consisted of samples without serum or without primary antibody by triplicate, whose OD values were subtracted for each sample value.

The serum alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets according to Ortuño et al., 1998. Briefly, 100 µl of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) (Sigma-Aldrich) containing

Mg^{+2} and EGTA were mixed with 100 µl of serially diluted serum to give final serum concentrations ranging from 10% to 0.078% (v/v). After incubation (90 min, 22°C), the samples were centrifuged (400 g, 5 min, 4ºC) to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader. The values of maximum (100%) and minimum haemolysis were obtained by adding 100 µl of distilled water or HBSS to 100 µl samples of SRBC, respectively. The volume yielding 50% haemolysis was determined and used for calculating the complement activity of the sample (ACH_{50}) as follows:

ACH_{50} value (units/ml) = 1/K x (reciprocal of the serum dilution) x 0.5.

Where K is the amount of serum (ml) giving 50% lysis and 0.5 is the correction factor since this assay was performed on half scale of the original method.

Peroxidase activity in HK leucocytes and serum was measured according to Quade and Roth, 1997. To determine leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich) and, after centrifugation (400 g, 10 min, 22ºC), supernatant was transferred to a fresh 96-well plate containing 100µl from a prepared solution with 10 mM TMB and 0.015% of H_2O_2. For serum, 15 µl of serum samples were diluted with 135 µl of HBSS without Ca_{+2} or Mg^{+2} in flat-bottomed 96-well plates and substrates added. In both cases, colour-change reaction was stopped after 2 min by adding 50 µl of 2M sulphuric acid and the optical density was read at 450 nm in a plate reader. Samples without leucocytes or serum were used as negative controls.

The respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method using phorbol myristate acetate (PMA; Sigma-Aldrich) and luminol (Sigma-Aldrich) (Bayne and Levy, 1991). The plates were shaken and immediately read in a plate reader for 1 h at 2 min intervals. The kinetic of the reactions was analysed and the maximum slope of each curve was calculated.

2.4. RNA extraction

Total RNA was isolated from TRIzol® reagent frozen samples following the manufacturer instructions. RNA was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with random primers (Life Technologies).

2.5. Detection of virus replication

For the detection of virus replication, skin samples from each experimental group (n = 4) were run by nested PCR. Amplification was performed in 20 µl samples containing 2.5 µl of PCR reaction buffer (10×, Life Technologies), 2 µl forward and reverse primers (F3 and R1 and F1 and R2, respectively) for major capsid protein (*mcp*) from LCDV (10 mM each), 0.5 µl dNTP mix (2.5 mM each), 1.25 µl MgCl2 (50 mM), 0.25µl Taq DNA polymerase (5 U/l, Life Technologies), 15.25 µl DNase/RNase-free distilled water (Life Technologies) and 2 µl cDNA template (corresponding to 10 ng of RNA in the first PCR and 10-fold diluted cDNA from the first PCR products in the second PCR). PCR reactions were performed in a MasterCycler Gradient PCR: 94ºC for 5 min, 35 cycles of 94ºC for 45 s, 60ºC for 45 s, 72ºC for 45 s, and followed by 72ºC for 10 min. The PCR products were separated in a 1.2% agarose (Pronadisa) gel containing 0.01% (v/v) of Red Safe® (Life Technologies) and visualised under UV light. The expression of beta-actin (*actb*) was used as endogenous PCR control in each sample.

2.6. Gene expression analysis

The evaluation of immune-related genes transcription was carried out by real-time PCR (qPCR). The expression of selected genes was analysed with the $^{2-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001), which was performed as described elsewhere (Cordero et al., 2015a). The primers with references are showed in **Table 3**. Specificity of reactions was analysed using similar samples without cDNA as negative controls. All qPCR reactions

were carried out in duplicate and quantification cycle (Ct) values of target genes were converted into relative quantities. Normalization factors were calculated as the geometric mean of relative quantities of reference genes *ef1a*, *actb*, and *rps18* using bestkeeper® algorithm (Pfaffl et al., 2004). Standard curves were performed for each reference and target gene, and in all cases the efficiency was over 90%. Data are expressed as fold change (mean ± SEM), obtained by dividing each sample value by the mean control values.

2.7. Statistical analysis

Data were statistically analysed by Student t-test to determine differences between LCDV infected and control (non-infected) groups using Statistical Package for Social Science (SPSS for Windows; v20) and differences were considered statistically significant when $p<0.05$.

Table 3. Oligonucleotides used for gene expression analysis.

Gene	GenBank	Temperature (ºC)	Sequence (5´→3´)
ef1a	AF184170	60ºC	F:TGTCATCAAGGCTGTTGAGC R:GCACACTTCTTGTTGCTGGA
actb	X89920	60ºC	F:GGCACCACACCTTCTACAATG R:GTGGTGGTGAAGCTGTAGCC
rps18	AM490061	60ºC	F:CGAAAGCATTTGCCAAGAAT R:AGTTGGCACCGTTTATGGTC
ifn	FM882244	60ºC	F: ATGGGAGGAGAACACAGTGG R:GGCTGGACAGTCTCTGGAAG
irf3	AM956899	60ºC	F:TCAGAATGCCCCAAGAGATT R:AGAGTCTCCGCCTTCAGATG
mx	FJ490556	60ºC	F:AAGAGGAGGACGAGGAGGAG R:CATCCCAGATCCTGGTCAGT
mhc2a	DQ019401	60ºC	F:CTGGACCAAGAACGGAAAGA R:CATCCCAGATCCTGGTCAGT
csf1r	AM050293	60ºC	F:ACGTCTGGTCCTATGGCATC R:AGTCTGGTTGGGACATCTGG
ighm	AM493677	60ºC	F:CAGCCTCGAGAAGTGGAAAC R:GAGGTTGACCAGGTTGGTGT
tcra	AY751745	60ºC	F:GCCGAACCGGACTACTACAA R:GTATGGTCGGCTGTTCCATT
hamp	CB184616	60ºC	F:GCCATCGTGCTCACCTTTAT R:CTGTTGCCATACCCCATCTT
il1b	AJ277166	60ºC	F:GCGAGCAGAGGCACTTAGTC R:GGTAGGTCGCCATGTTCAGT
nccrp1	AY651258	60ºC	F:ACTTCCTGCACCGACTCAAG R:TAGGAGCTGGTTTTGGTTGG
mcp	L63545	60ºC	F1:CGCTTGCATAGGCTTCTTCT F3:CAATTGGATCTGCTGCCACT R1:ACGGCGACTTATGGGTACAA R2:AGTTCAAACTGCGCCAAAAC

3. Results

Visual evidences of LCDV (white nodules) were observed in all the specimens from the infected group while they were not observed in any specimen from the control group. In addition, nested PCR results showed a band of around 182 bp in the skin of naturally-infected gilthead seabream confirming the presence of replicating LCDV (**Figure 9**). This band never appeared in fish samples from control group (uninfected).

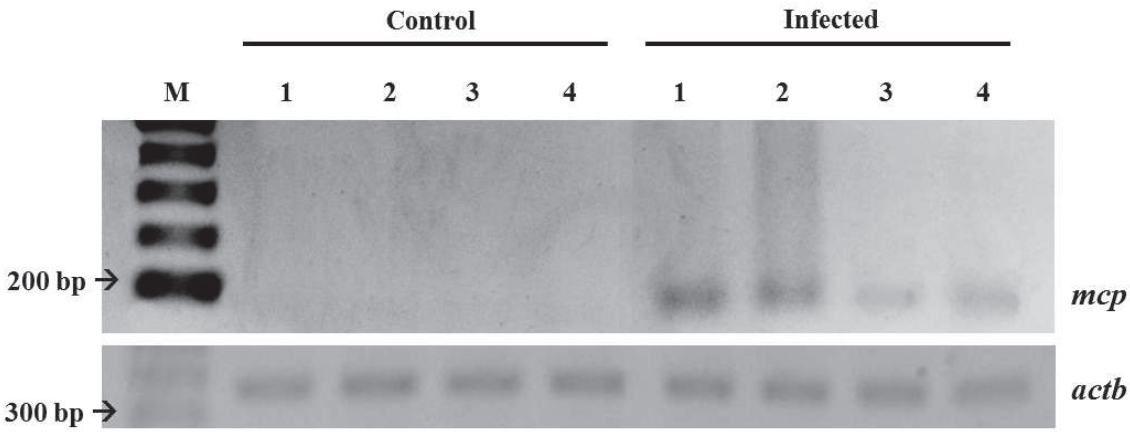


Figure 9. Amplification products of major capsid protein (*mcp*) from LCDV in individual skin samples (n = 4) from LCDV-infected and non-infected specimens of *S. aurata* by nested PCR using the primers (shown in **Table 3**) F3 and R1 and F1 and R2, respectively. Beta-actin was used as a PCR control. 1-4, individual skin samples from naturally-infected and control (non-infected) specimens; M, 100 bp marker.

Regarding the seabream immunity, specimens under natural LCDV infection showed a statistically significant decrease of total serum IgM levels (**Figure 10A**) although alternative complement and serum peroxidase activities (**Figures 10B, 10C**) were not altered, compared to the values found in non-infected fish.

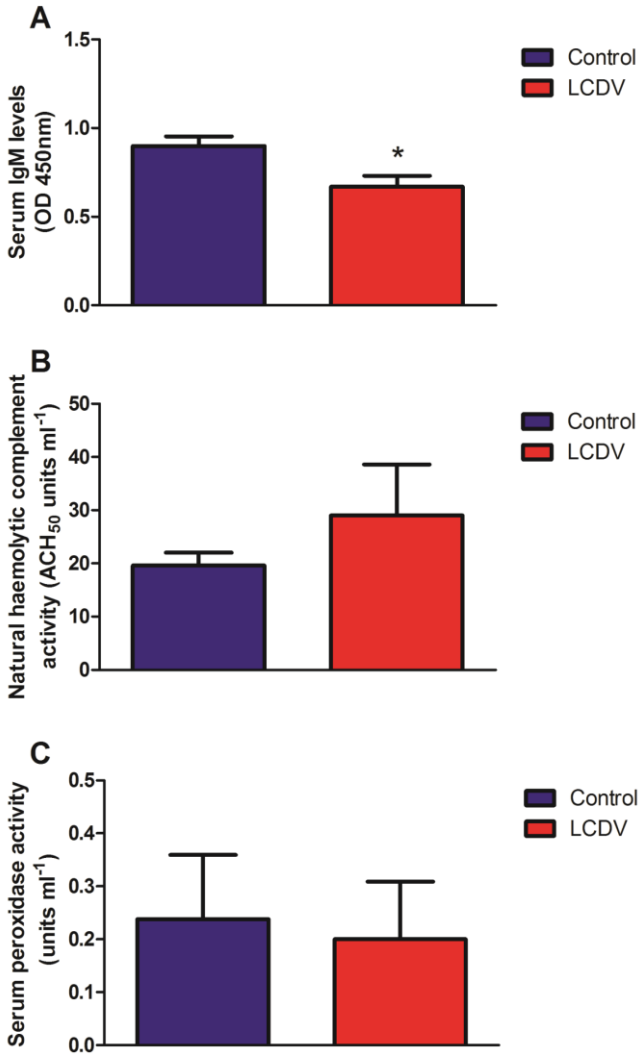


Figure 10. Humoral immune response of *S. aurata* non-infected (blue bars) and LCDV naturally-infected (red bars) specimens. Serum total IgM levels (A), alternative complement (B) and peroxidase (C) activities. Bars represent the mean ± SEM (n = 4). Asterisks denote significant differences when $p<0.05$.

By contrast, LCDV infection triggered a statistically significant increase of HK leucocyte peroxidase and respiratory burst activities, compared to the values recorded for control fish (control group) (**Figures 11A, 11B**).

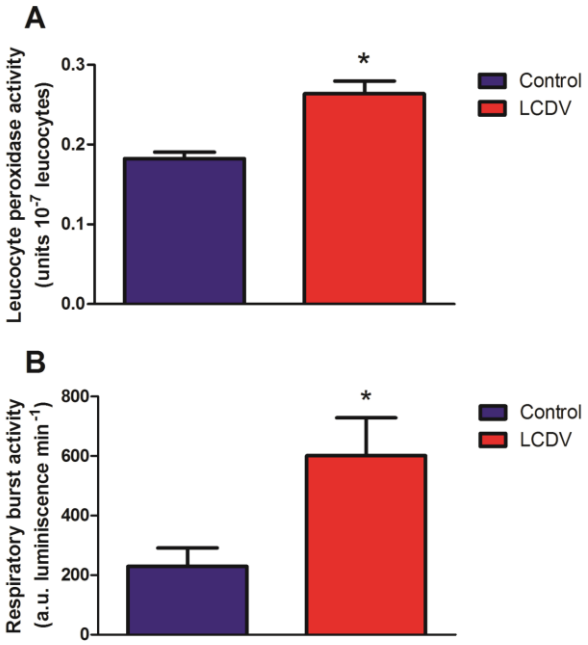


Figure 11. Cellular immune response of *S. aurata* non-infected (blue bars) and LCDV naturally-infected (red bars) specimens. Peroxidase (A) and respiratory burst (B) activities of head-kidney leucocytes. Bars represent the mean ± SEM (n = 4). Asterisks denote significant differences when $p<0.05$.

In addition, related to the antiviral response, the expression of type I interferon (*ifn*), interferon regulatory factor 3 (*irf3*) and interferon-induced GTP-binding protein Mx (*mx*) was tested. While *ifn* and *irf3* transcription was significantly down-regulated, *mx* did not show significant differences ($p = 0.084$) in the skin from LCDV-naturally infected fish compared to the expression levels found in the skin from non-infected fish (**Figure 12**). However, the transcription of these same genes was not regulated in the HK by the LCDV infection. Our results also showed a statistically significant down-regulation of macrophage colony stimulating factor receptor (*csf1r*), major histocompatibility complex class 2 alpha (*mhc2a*), T-cell receptor alpha (*tcra*) and immunoglobulin mu heavy chain (*ighm*) transcription in both skin and HK from LCDV-infected fish (**Figure 12**). Finally, the expression of hepcidin (*hamp*) and interleukin-1 beta (*il1b*) was not affected by the LCDV-infection while the expression of the nonspecific cytotoxic cell receptor protein 1

(*nccrp1*), the main marker of NCC cells, was up-regulated in HK but not in skin from LCDV-naturally infected fish, respect to the values recorded for control (non-infected) fish (**Figure 12**).

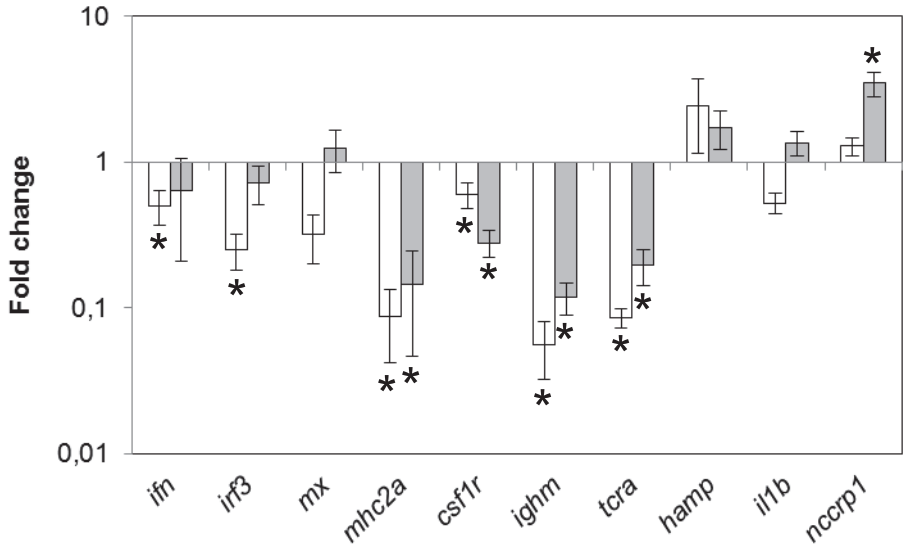


Figure 12. Gene expression profile in skin (white bars) and head-kidney (grey bars) from *S. aurata* naturally-infected with LCDV in comparison to non-infected specimens. Bars represent the mean ± SEM (n = 4). Asterisks denote significant differences when $p<0.05$.

4. Discussion

LCDV is one of the most common viruses in gilthead seabream, which is macroscopically characterized by white nodules on the body and fins. Due to the difficulty for controlling the virus, scarce studies with LCDV have been carried out with experimental infections (Harikrishnan et al., 2010a, 2010b; Iwakiri et al., 2014; Jang et al., 2011), including gilthead seabream (Cano et al., 2013; Garcia-Rosado et al., 2004; Kvitt et al., 2008), and only a few have analysed the impact of the disease in natural infections (Dezfuli et al., 2012; Paperna et al., 1982). Furthermore, very little is known about the immunity of gilthead seabream in response to LCDV infection. No significant effects were observed on the humoral immune parameters of LCDV infected seabream

except for total IgM levels, respect to the values of control fish. However, statistically significant increases were observed in the cellular immune parameters of LCDV-naturally infected seabream specimens, respect to the activities recorded in control fish. A very similar pattern in the immune response was also observed in gilthead seabream specimens infected with nodavirus or viral haemorrhagic septicaemia virus (Chaves-Pozo et al., 2012; Esteban et al., 2008; Valero et al., 2015c). This is interesting since the three virus assayed could replicate into the host but failed to produce mortality. Thus, these results show that HK leucocytes are activated to encounter, and probably to clear, the virus at the sites of replication, but further studies deserve to be addressed to confirm this last assumption.

The immune response against viral infections can use several mechanisms such as interferon (IFN), complement system or cytotoxic cells (Ellis, 2001). For RNA virus recognition, there are three classes of pattern-recognition receptors (PRRs), which are designated retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Takeuchi and Akira, 2009). Similar mechanisms have been suggested for DNA viruses, becoming a hot topic in virology for several years now. Thus, trying to unravel the immune response against LCDV, three key genes involved in the interferon pathway were investigated. We found that LCDV-infected fish had diminished expression of antiviral genes (*ifn*, *irf3*, and *mx*) at the nodules site. The mechanisms for inhibiting the expression of *ifn* and *irf3*, two main components of RLRs in IFN type I pathway, would need to be investigated. The *mx* gene, which encodes an IFN-inducible protein, shows antiviral activity against a wide range of virus including LCDV (Fernández-Trujillo et al., 2013). Interestingly, and in agreement to our results, the expression of *mx* in a seabream cell line (SAF-1) was unaltered after *in vitro* LCDV infection (Fernández-Trujillo et al., 2013).

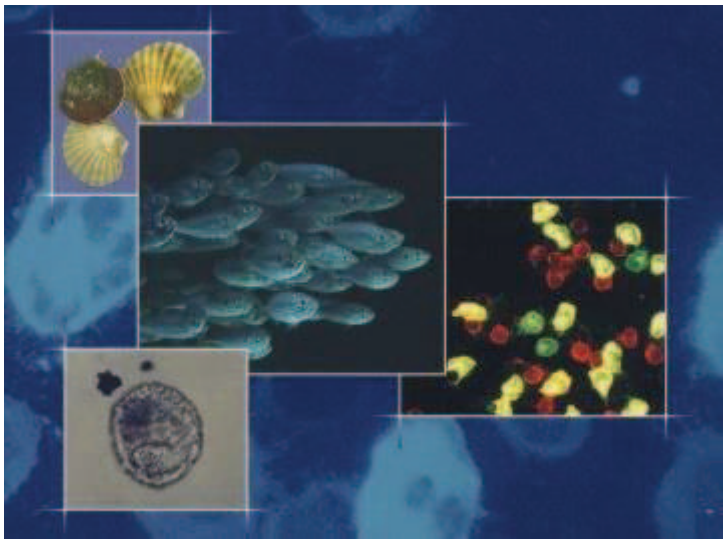
We also investigated some genes to understand the involvement of gilthead seabream leucocytes in the immune response against LCDV. In this sense, antigen presenting cells (APCs) play a critical role on this pathway (Cummings et al., 2007), mainly macrophages and B cells in fish, which have *csf1r* and *igm* genes as key receptors in the immune response. It was demonstrated that LCDV promotes the infiltration of acidophilic granulocytes (AG) to the detriment of macrophages in skin from naturally infected specimens (Dezfuli et al., 2012). Taken this into account, the present paper may confirm the crosstalk between AG and macrophages through *csf1r* down-regulation in the skin of LCDV infected fish. As a link between innate and adaptive immune response, our findings showed that *mhc2a* (a major expert in pathogen recognition on the surface complex of APCs) as well as *tcra* and *ighm* (the main receptors for T and B cells, respectively) are all of them strongly inhibited in LCDV infected specimens, suggesting an impairment of a lack of local adaptive immune response. According to a previous study demonstrating a down-regulation of *mhc2* in APCs after infectious haemorrhagic necrosis virus (IHNV) infection (Hansen and LaPatra, 2002), our results suggest a decreased APC activity during LCDV infection that would negatively affect to the onset and potency of the acquired immunity. In fact, this is supported by the concomitant decreased T and B cell markers. Regarding other immune-related genes, *hamp* and *il1b* transcription did not show changes in infected specimens, respect to the values recorded in control fish; *hamp* is an antimicrobial peptide that typically has been shown to be up-regulated after bacterial infection (Armitage et al., 2011; Cuesta et al., 2008). However, in agreement with this study, gilthead seabream *hamp* is unchanged after nodavirus infection in the target-tissue (Valero et al., 2015a) though, in contrast, the number of piscidin-positive cells was increased in the skin of naturally LCDV-infected seabream (Dezfuli et al., 2012). On the other hand, the pro-inflammatory cytokine IL-1β, which is mainly produced by AGs in the seabream, remained unaltered after LCDV infection. Interestingly, these authors

(Dezfuli et al., 2012) also reported that the number of AGs surrounding lymphocystis was increased but most of them were not immunostained for IL-1β suggesting a degranulation process, though our data suggest that the gene transcription is not regulated locally and therefore it could be possible that the protein is not produced instead of being degranulated, a hypothesis that merits deeper characterization. Similarly, it has been also reported that the expression of *il1b* did not change after VHSV infection (Tafalla et al., 2005). NCCs (nonspecific cytotoxic cells) are known to be the most important cytotoxic cell effectors in innate immune response of teleost fish, including gilthead seabream (Cuesta et al., 2005). In agreement to the present data, it was reported that *nccrp1* is up-regulated after nodavirus infection in HK of gilthead seabream (Chaves-Pozo et al., 2012), suggesting an increase in the leucocyte killing of virus-infected cells. More studies are needed to determine if *nccrp1* is playing a major role in LCDV since it is the only one of all analysed genes that is up-regulated after LCDV infection in gilthead seabream and points to the importance of the cytotoxic cells in clearing the LCDV-infected cells.

In summary, the present paper is a starting point to describe new insights about the innate and adaptive immune response to LCDV in gilthead seabream, promoting an innate cytotoxic cell response and laying the groundwork for future preventive strategies and treatments.

Chapter 3

Optimization of the skin mucus storage conditions to assess the humoral immune activities



Abstract

Skin mucus is increasingly used as a source for determining immunity-related proteins and enzymes. However, the ability to accurately measure some activities may be modified by inadequate handling and storage of the samples. This study aims to measure the effect of freezing and lyophilization at the time of collection on such activities. Fresh, frozen (immediately after collection at -20ºC and -80ºC) and lyophilized skin mucus samples obtained from the same fish specimens of gilthead seabream were analysed in the assays. The amount of total proteins and sugar residues (determined by lectin binding) present in skin mucus samples fell after both freezing and lyophilization of the samples. While no significant differences were exhibited in the levels of some proteins or enzymes (immunoglobulin M, antiprotease, peroxidase, esterase and alkaline phosphatase) determined in fresh or frozen mucus samples, protease and lysozyme activities were lower in frozen mucus samples than in fresh samples. Lyophilization of the mucus samples drastically decreased the total level of proteins obtained, as well as of protease, peroxidase, lysozyme and alkaline phosphatase activities. The results suggest that freezing skin mucus samples is more suitable than lyophilization if samples are stored before determining enzymatic activities.

1. Introduction

Skin mucus has become a hot topic in recent years in research. According to the NCBI database PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), “skin mucus of fish” has 425 entries in May 2,016, of which more than half (228 entries) are from 2,006 onwards. Skin mucus is studied for its capacity to adhere to fish pathogens (Benhamed et al., 2014; Bordas et al., 1998; Chabrillón et al., 2005b) and also as a valuable source for the purification and/or identification of several immune molecules such as antimicrobial peptides (Bergsson et al., 2005; Cho et al., 2002; Rakers et al., 2013), lectins (Suzuki et al., 2003; Vasta et al., 2011) and immunoglobulins (Ig) (Salinas et al., 2011; Xu et al., 2013). Beside the recent proteomic mapping of skin mucus to find new molecules which may be involved in mucosal immunity (Cordero et al., 2015a; Jurado et al., 2015; Rajan et al., 2011; Sanahuja and Ibarz, 2015), most available research results concern the changes that take place in humoral immune activities, such as IgM, lysozyme or alkaline phosphatase in the skin mucus of multiple fish species (Guardiola et al., 2014a; Subramanian et al., 2007). Furthermore, changes in these and other humoral immune activities are typically studied in relation with immunostimulants and/or host resistance to infection (Cordero et al., 2015b; Dawood et al., 2016; Du et al., 2015; Fast et al., 2002; Guardiola et al., 2014b; Muñoz et al., 2007; Reyes-Becerril et al., 2015; Siwicki et al., 1994).

There is also a considerable amount of information available on how humoral immune activities are influenced by seasonal variations such as photoperiod and/or temperature (Bowden et al., 2004; Cheng et al., 2009; Esteban et al., 2006; Jung et al., 2012; Valero et al., 2014). But curiously there are no studies on the effects of storage conditions on the humoral immune activities of fish skin mucus. The aim of this paper therefore was to study the changes that take place in different humoral activities (including total protein levels, specific lectin binding, IgM, protease, antiprotease,

peroxidase, lysozyme, esterase and alkaline phosphatase) and lectin binding in gilthead seabream fish mucus after storage following freezing or lyophilization.

2. Materials and methods

2.1 Animal maintenance

Sixty specimens of the hermaphroditic protandrous teleost gilthead seabream obtained from a local farm in Murcia (Spain) were kept in re-circulating seawater aquaria (500 l) with a flow rate of 900 l h^{-1} in the Marine Fish Facility at the University of Murcia. The temperature and salinity were 22 ± 2ºC and 28‰, respectively and a photoperiod of 12 h light:12 h dark was followed. A commercial diet (Optibream D4, Skretting) was administered at a rate of 2% body weight day^{-1}.

2.2 Mucus collection

Fish were anesthetized prior to sampling with 100 mg l^{-1} MS222 (Sandoz). Skin mucus was gently scraped off from the skin surface, avoiding blood, urine and feces during collection (Palaksha et al., 2008).

2.3 Experimental design

Three pooled samples from 20 different fish were used in this study. Samples were later divided into four groups: fresh mucus, which was analysed immediately, mucus frozen at -20ºC, mucus frozen at -80ºC and lyophilized mucus. Frozen and lyophilized mucus samples were stored for one month before being analysed. For the analysis of lyophilized samples, they were first dissolved in Milli-Q water by vortexing for 10 min before the supernatant was used.

2.4 Total protein levels

The total protein concentration was estimated using the Coomassie Brilliant Blue G-250 method (Bradford, 1976) with Bradford reagent (Sigma-Aldrich). Briefly, 5 µl of skin mucus samples in triplicate were incubated with 250 µl of Bradford reagent in flat-bottomed 96-well plates (Nunc), and similar volumes of bovine serum albumin (BSA, Sigma-Aldrich) serial dilutions were used as standard. After incubating for 10 min at

room temperature and in darkness, the absorbance was read in a plate reader (BMG, Fluostar Omega) at 550 nm. The total protein concentration was expressed as mg ml^{-1}.

2.5 Specific lectin binding levels

Specific lectin binding to skin mucus was determined by lectin ELISA. Briefly, skin mucus was dissolved 1:4 in 50 mM carbonate-bicarbonate buffer (pH 9.6) and mucus samples were placed in flat-bottomed 96-well plates in triplicate and coated before leaving overnight at 4ºC. Samples were rinsed three times with phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.05% Tween 20 (PBT, pH 7.3), blocked for 2 h at room temperature with blocking buffer (3% BSA in PBS-T) and rinsed again. Next, samples were incubated for 1 h with 2 µg per well of biotinylated lectin (Table 4), washed and incubated with streptavidin-HRP (1:1,000; Life Technologies) for 1 h. After exhaustive rinsing with PBS-T, the samples were developed using 100µl of a 0.42 mM solution of 3,3',5,5'- tetramethylbenzidine (TMB, Sigma-Aldrich), prepared daily in Milli-Q water containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min, stopped by the addition of 50 µl of 2 M H_2SO_4 and the plates were read at 450 nm in a plate reader. Negative controls consisted of samples without skin mucus or without lectins, whose optical density (OD) values were subtracted from each sample value.

Table 4. Information related to each lectin used in ELISA.

Name	Symbol	Species of lectin source	Affinity for
***Bandeiraea simplicifolia* agglutinin**	BS-I	*Bandeiraea simplicifolia*	α-D-galactose (primary) N-acetyl-α-D-galactosamine (secondary)
Peanut agglutinin	PNA	*Arachis hypogaea*	β-D-galactose
***Ulex europaeus* agglutinin I**	UEA-I	*Ulex europaeus*	L-fucose
Concanavalin A	ConA	*Canavalia ensiformis*	α-D-mannose α-D-glucose
***Wisteria floribunda* agglutinin**	WFA	*Wisteria floribunda*	N-acetyl-D-galactosamine
Wheat germ agglutinin	WGA	*Triticum vulgaris*	N-acetyl-β-D-glucosamine N-acetylneuraminic acid

2.6 Total Immunoglobulin M levels

Total skin mucus IgM levels were analysed using the ELISA method (Cuesta et al., 2004) with some modifications. Thus, 100 µl per well of 1:4 fold diluted mucus were placed in flat-bottomed 96-well plates and the proteins were coated by overnight incubation at 4ºC with 200 µl carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.05% Tween 20 (PBT, pH 7.3), the plates were blocked for 1 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBT, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer). After exhaustive rinsing with PBT, the plates were developed using 100 µl of 0.42 mM TMB solution, prepared daily in Milli-Q water containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2 M H_2SO_4 and the plates were read at 450 nm in a plate reader. Negative controls consisted of triplicate samples without skin mucus or without primary antibody, whose OD values were subtracted for each sample value.

2.7 Protease activity

Protease activity was quantified using the azocasein hydrolysis assay (Ross et al., 2000). Briefly, an equal volume of skin mucus was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma-Aldrich) for 19 h at 25ºC in triplicate. The reaction was stopped by adding 4.6% trichloroacetic acid (TCA, Sigma-Aldrich) and the mixture centrifuged at 13,000 g, for 5 min. The supernatants were transferred to a 96-well plate containing 100 µl $well^{-1}$ of 0.5 N NaOH. Then, the OD was read at 450 nm using a plate reader. Skin mucus was replaced by trypsin solution (5

mg ml^{-1}) as positive control (100% of protease activity), or by buffer, as negative control (0% activity).

2.8 Antiprotease activity

Total antiprotease activity was determined as indicated by the capacity of skin mucus to inhibit trypsin activity (Hanif et al., 2004). Aliquots of 10 µl of mucus samples were incubated with 10 µl of standard trypsin solution (5 mg ml^{-1}; Sigma-Aldrich) for 10 min at 25ºC in Eppendorf tubes in triplicates. Then, 100 mM of ammonium bicarbonate buffer containing 0.7% azocasein were added, and incubated for 2 h at 25ºC. The reaction was stopped by the addition of 4.6% TCA, incubated for 30 min at 25ºC, and then centrifuged at 13,000 g for 5 min. The supernatants (100 µl) were transferred to a 96-well plate containing 100 µl $well^{-1}$ of 1 N sodium hydroxide (NaOH, Sigma-Aldrich). The OD was read at 450 nm using a plate reader. For a positive (100%) control, PBS replaced the skin mucus, and for a negative control, PBS replaced both skin mucus and trypsin. The antiprotease activity was expressed in terms of percentage of trypsin inhibition according to the formula:

% Trypsin inhibition = Trypsin OD - Sample OD/Trypsin OD x 100.

2.9 Peroxidase activity

Peroxidase activity in skin mucus was measured according to Quade and Roth (1997) with some modifications. Thus, skin mucus samples were dissolved 1:4 in Hank's balanced salt solution (HBSS) without Ca^{+2} or Mg^{+2} in triplicates and dispensed into flat-bottomed 96-well plates. Then, 20 mM 3,3´,5,5´-tetramethylbenzidine (TMB; Sigma-Aldrich) and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µl 2 M H_2SO_4 and the optical density was read at 450 nm in a plate reader. Standard samples without skin mucus were used as blanks. One unit was defined as the amount producing an absorbance change of 1 and the activity was expressed as U mg^{-1} mucus proteins.

2.10 Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. (1965) with some modifications. Equal volumes of skin mucus samples and PBS (pH 6.2) were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 µl of freeze-dried *Micrococcus lysodeikticus* (0.3 mg ml^{-1}, Sigma-Aldrich) were added as lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22ºC in the plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Roche) and the results expressed as U mg^{-1} mucus proteins.

2.11 Esterase activity

Esterase activity was determined as described elsewhere (Ross et al., 2000) with some modifications. Skin mucus samples were incubated with an equal volume of 0.4 mM p-nitrophenyl myristate substrate, previously heated at 65ºC for 10 min and then cooled (Fluka) in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, Sigma-Aldrich). The OD was measured at 15 min intervals over a period of 3 h at 405 nm in a plate reader. The initial reaction rate was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product in 1 min and the activity was expressed as U mg^{-1} mucus proteins.

2.12 Alkaline phosphatase activity

Alkaline phosphatase activity was measured by incubating an equal volume of skin mucus samples with 4 mM p-nitrophenyl phosphate liquid substrate (pNPP, Sigma-Aldrich) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8) as described elsewhere (Ross et al., 2000). The OD and activity were determined as described in section 2.11.

2.13 Statistical analysis

The results are expressed as mean ± SEM. Data were statistically analysed by one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test to determine differences among groups. All the statistical analyses were conducted using Statistical Package for Social Science (SPSS for Windows; v19.0, USA) and differences were considered statistically significant when $p<0.05$.

3. Results

3.1 The concentration of total proteins is highly susceptible to change after lyophilization

The present study revealed that skin mucus stored at -20ºC or -80ºC showed very similar protein levels compared to fresh mucus. However, the storage of lyophilized skin mucus produced a significant decrease in the protein concentration compared with the rest of the mucus samples (Figure 13).

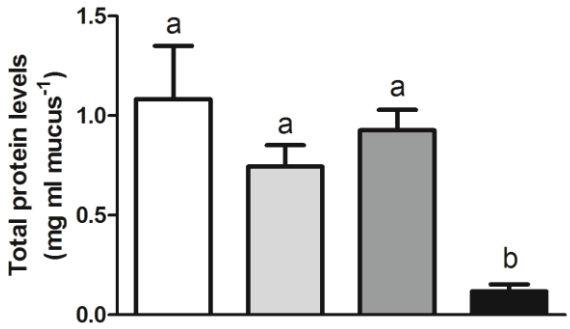


Figure 13. Total protein concentration expressed as mg ml^{-1} in skin mucus of gilthead seabream stored under different conditions. Bars represent mean ± SEM (n = 3 pools) of fresh (white), -20ºC (light grey), -80ºC (dark grey) or lyophilized (black) stored skin mucus. Samples were statistically analysed by ANOVA followed by Tukey post-hoc test. Significant differences are denoted with different letters when $p<0.05$.

3.2 Lectin binding levels usually decrease after skin mucus storage

The carbohydrate specificity binding of six different lectins was evaluated in the skin mucus samples. The highest lectin-binding capacity (except for WGA lectin) of all the

studied samples was found in fresh skin mucus (Figure 14). In the case of BS-I, PNA, UEA-I and WFA lectins, the highest degree of binding was found in fresh mucus samples, followed by samples stored at -80ºC, lyophilized samples and those stored at -20ºC in that order (Figure 14). Interestingly, only small differences in ConA and WGA lectin binding were found between the different samples and, generally speaking, the lowest values were recorded in mucus samples stored at -20ºC (Figure 14)

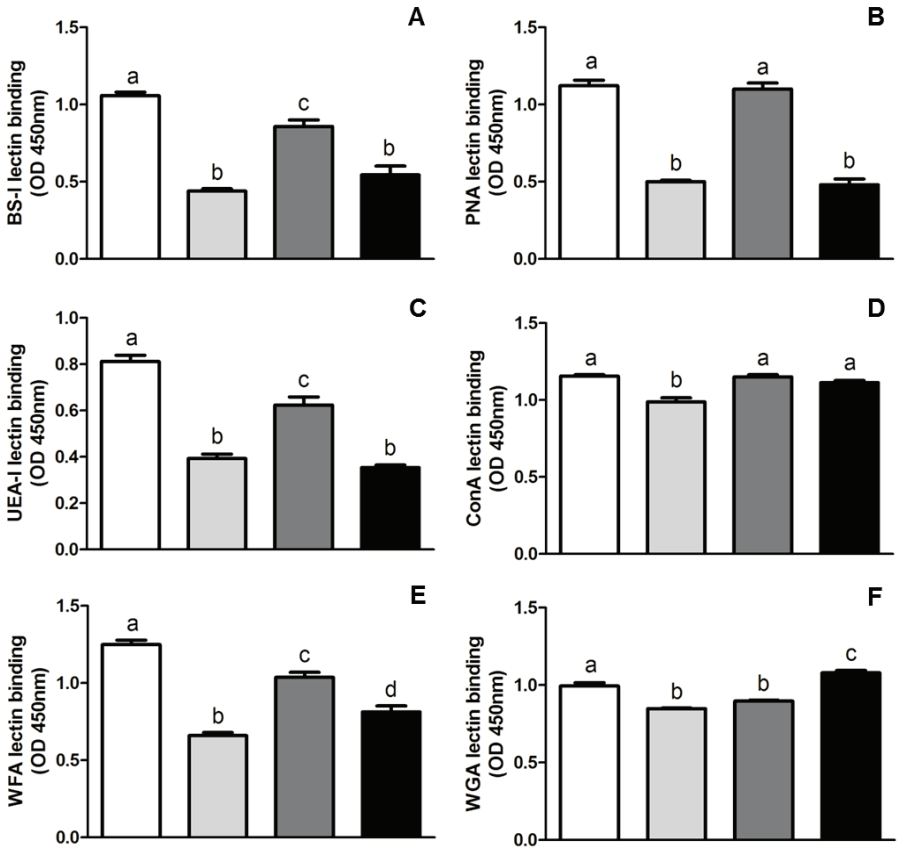


Figure 14. Levels (OD 450nm) of specific lectin binding (BS-I, A; PNA, B; UEA-I, C; ConA, D; WFA, E; WGA, F) measured by ELISA in skin mucus of gilthead seabream stored under different conditions. Bars represent mean ± SEM (n = 3 pools) of fresh (white), -20ºC (light grey), -80ºC (dark grey) or lyophilized (black) stored skin mucus. Samples were statistically analysed by ANOVA followed by Tukey post-hoc test. Significant differences are denoted with different letters when $p<0.05$. See Table 4 for lectin specificity.

3.3 Total IgM levels in skin mucus samples remained similar in the different storage conditions

In the field of fish immunology, the study of total IgM levels is a fundamental parameter in order to evaluate the adaptive response against pathogens. Our results demonstrated no significant changes in IgM levels in the different storage conditions compared with the levels detected in fresh skin mucus samples (Figure 15).

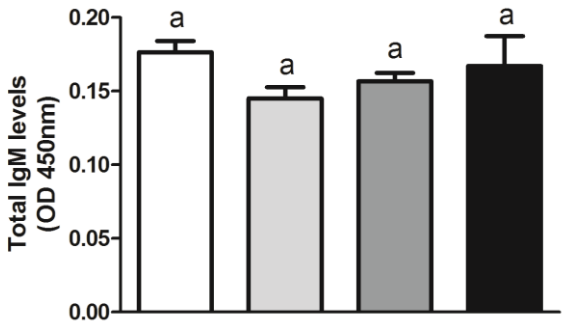


Figure 15. Total IgM levels (OD 450nm) in skin mucus of gilthead seabream stored under different conditions. Bars represent mean ± SEM (n = 3 pools) of fresh (white), -20ºC (light grey), -80ºC (dark grey) or lyophilized (black) stored skin mucus. Samples were statistically analysed by ANOVA followed by Tukey post-hoc test, and significant differences were denoted with different letters when $p<0.05$.

3.4 Enzymatic activities decrease after lyophilization

As regards the enzymatic activities of proteins related to the immune response (Figure 16), protease, lysozyme and alkaline phosphatase activities followed a very similar pattern in all the storage conditions. For these enzymes, fresh mucus always showed the highest activity, followed by -20ºC frozen samples. The lowest lysozyme and alkaline phosphatase activities were found in lyophilized samples, while the lowest protease activity was found in -80ºC frozen samples (Figure 16).

Interestingly, neither antiprotease nor esterase activities were significantly altered by the storage conditions. Strikingly, peroxidase activity was not affected by frozen storage compared to fresh mucus but was sharply reduced by lyophilization (Figure 16).

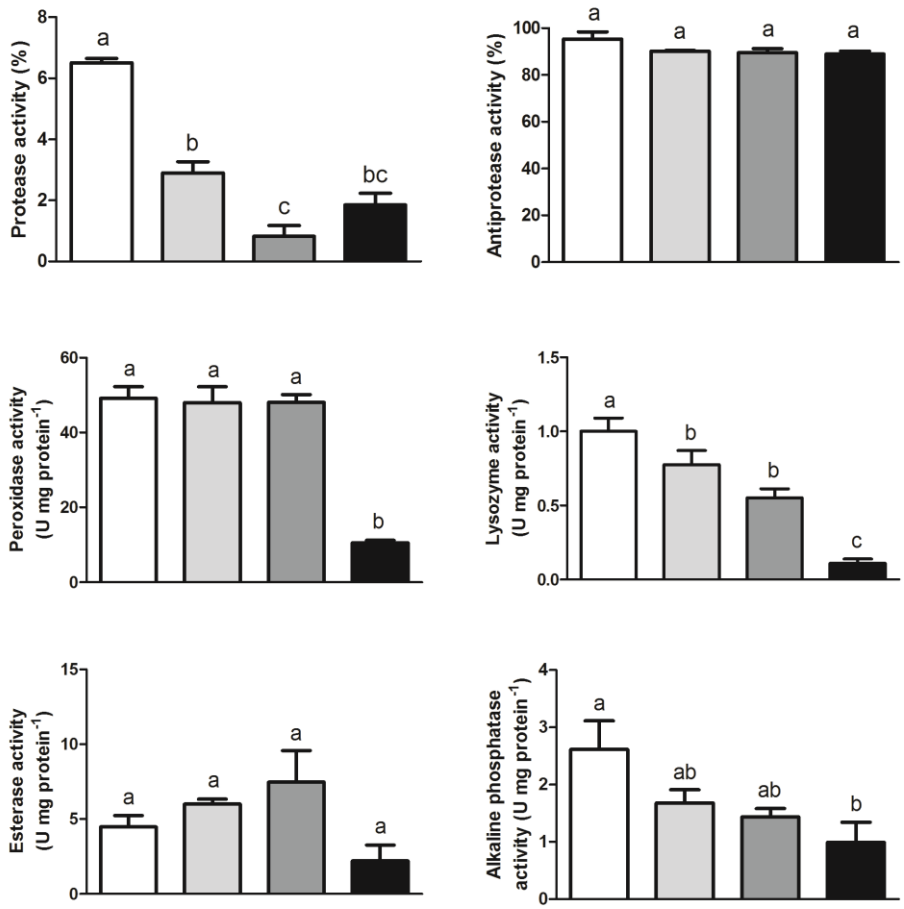


Figure 16. Protease (A) and antiprotease (B) activities, expressed in percentage (%), and peroxidase (C), lysozyme (D), esterase (E) and alkaline phosphatase (F) activities, expressed as U mg^{-1}, in skin mucus of gilthead seabream stored under different conditions. Bars represent mean ± SEM (n = 3 pools) of fresh (white), -20ºC (light grey), -80ºC (dark grey) or lyophilized (black) stored skin mucus. Samples were statistically analysed by ANOVA followed by Tukey post-hoc test, and significant differences were denoted with different letters when $p<0.05$.

4. Discussion

Innate defense is the first barrier against pathogens and among them, mainly humoral immune activities have been broadly studied in fish immunology (Magnadóttir, 2006; Ye et al., 2013). Usually changes in these parameters are evaluated after challenges such as infection (Collet, 2014; Parisi et al., 2015; Reyes-Becerril et al., 2015), environmental/management stress (Chebaani et al., 2014; Cordero et al., 2016d) or

environmental (abiotic) factors (Esteban et al., 2006; Jung et al., 2012; Nakanishi, 1986; Valero et al., 2014). First studies focused on fish immunity studied in blood but, at present, there is a great interest in study fish mucus as a non-invasive technique. Curiously, there is no consensus concerning the most suitable conditions for storing mucus for later analysis even though this might be important. Thus, some authors have stored fish skin mucus frozen at -20ºC (Chabrillón et al., 2006, 2005a, 2005b; Wold and Selset, 1978), or at -80ºC (Cordero et al., 2015a; Jung et al., 2012; Jurado et al., 2015; Rajan et al., 2011; Sanahuja and Ibarz, 2015), while others have used lyophilized skin mucus samples in their studies (Ebran et al., 2000; Guardiola et al., 2014b; Nigam et al., 2012). Here, for the first time, we analyse the changes in humoral immune activity levels in skin mucus due to the storage conditions.

Since many humoral immune activity levels are expressed as units per mg of protein (or equivalent), we first evaluated the total protein levels in mucus samples. Surprisingly, those levels were the most strongly affected by lyophilization. The significant decrease in total protein levels in lyophilized skin mucus with respect to the values recorded in fresh or frozen mucus samples might be explained by the fact that fibrous proteins, which are mostly structural, are insoluble in water. Supporting this idea, skin mucus is reported to have a matrix of gel-forming macroproteins called mucins (Shephard, 1994). In addition, other structural proteins such as keratins have been recently found in the skin mucus of several fish (Cordero et al., 2015a; Jurado et al., 2015; Rajan et al., 2011). On the other hand, frozen storage (at -20ºC or -80ºC) did not modify the total protein levels, and both temperatures can be considered suitable for storing samples to evaluate this parameter when fresh skin mucus samples are unavailable.

IgM is the main immunoglobulin responsible for teleost mucosal adaptive immunity after IgT. IgM has been evaluated in a huge diversity of fish species and experimental conditions, the present study revealed that total IgM levels were unaffected by the

storage conditions. While there are no studies on fish, it has been reported that human serum IgM levels (also tested by ELISA) did not change after storage at -20ºC storage (Tran et al., 2006). Finally, it would be interesting to study the levels of IgT, which seems to play an active role in mucosal immunology (Salinas et al., 2011; Zhang et al., 2010), but, unfortunately, there are no available antibodies for seabream.

Lectins are a diverse group of carbohydrate-binding proteins. Glycosylation is the primary cause of heterogeneity in proteins (Dwek, 1998). In mammals, it is well-established that the glycosylation patterns are important in many immune processes such as immune-cell differentiation, apoptosis or both innate and adaptive immunity (Marth and Grewal, 2008; Rudd et al., 2001). In general terms, glycobiology is less understood in fish than in mammals. In particular, the glycosylation of fish mucus has received little attention (Gómez et al., 2013) but important changes have been demonstrated after bacterial or parasitic infections in the glycosylation pattern of skin and gut mucus, respectively (Estensoro et al., 2013; Marel et al., 2010). In the present work, the specific lectin biding levels were affected by the storage conditions, especially by freezing at -20ºC and lyophilization, although, curiously, the highest levels of WGA lectin binding were found in lyophilized samples. In addition, according to our findings concerning IgM levels, the glycosylation of monoclonal antibodies did not change after frozen storage (Zheng et al., 2014). Overall, if working with fresh mucus is not possible, freezing at -80ºC seems to be the most suitable method for conserving glycoproteins in skin mucus.

Proteases are a group of proteins responsible for the hydrolysis of peptide bonds. By contrast, antiprotease has the capacity to inhibit the proteases, which are present in skin mucus in this case. A correct balance between protease and antiprotease activities is needed to preserve the correct functionality of any system. In addition, an imbalance of both activities has been reported in disease conditions (Greene and McElvaney, 2009), including skin inflammatory disorders (Streit et al., 1995). Whilst

protease activity levels were strongly affected by both temperatures (-20ºC or -80ºC) and lyophilization, surprisingly, antiprotease activity levels in skin mucus were unaffected by any storage condition compared with the values recorded for fresh mucus samples. Based on the diversity of proteases, some of them seem to be more influenced by storage conditions than antiproteases, which form a more limited group of proteins. Further protein characterizations are necessary to confirm these suggestions.

Peroxidase is an important enzyme that catalyses the anion superoxide produced by other immune defense pathways, such as respiratory burst, to produce chloride acid. Among the little information available about peroxidases affected by storage, a decrease of the glutathione peroxidase levels under various storage conditions has been described in swine plasma (Zhang et al., 1986). However, the same enzyme was also reported to vary widely among different animal species (Davidson et al., 1990). In our experiment, skin mucus peroxidase was not affected by either level of frozen storage, but was strongly affected by lyophilization, which produced significantly lower levels. Thus, we suggest using fresh or frozen skin mucus rather than lyophilized to obtain high levels of peroxidase.

Lysozyme is one of the most studied enzymes because of its bactericidal role, hydrolysing β-(1, 4)-linked glycoside bonds of bacterial cell wall peptidoglycans (Magnadóttir, 2006), and further acting as opsonin as well as activating the complement system and phagocytes (Grinde, 1989; Jolles and Jolles, 1984). In the present study, the levels of lysozyme were decreased by both lyophilization and freezing. In serum of fish there is no consensus on this issue. Thus, a previous study has reported a significant decrease in lysozyme levels following storage at -26ºC for 3 weeks (Hutchinson and Manning, 1996), whilst in another study reported levels were unaltered by storage at -20ºC for the same period (Lange et al., 2001). New studies are clearly needed on the storage of samples to study this important molecule for immunity.

Esterase is a global activity responsible for the hydrolysis of esters. It has been demonstrated that an activated-esterase is involved in human phagocytosis (Musson and Becker, 1977) and neutrophil chemotaxis in rabbit (Ward and Becker, 1970). Recently, higher levels of esterase have been reported in skin mucus than in serum in the gilthead seabream (Guardiola et al., 2014b). Here, we demonstrate that esterase activity levels were unaffected by any of the skin mucus storage conditions assayed. However, since skin mucus immunity is trendy, the chemotactic role of esterase in skin mucus is entirely unknown and deserves further investigations.

Alkaline phosphatases are a general group of enzymes involved in dephosphorylation. Alkaline phosphatase activity has been reported as a stress indicator in skin mucus (Ross et al., 2000). Here, we demonstrate that alkaline phosphatase levels are strongly affected by lyophilization, but not by frozen storage. In agreement with our results, the alkaline phosphatase activity in frozen human serum was similar to that obtained in fresh serum, but when serum was lyophilized the activity was significantly lower (Brojer and Moss, 1971). Thus, freezing (rather than lyophilization) is a suitable storage method to obtain reliable levels of alkaline phosphatase activity in skin mucus.

Taking all together, it can be concluded that storage at -20ºC or -80ºC decreases all the analysed lectin binding levels (except PNA and ConA in the case of -80ºC), and protease and lysozyme activity levels in skin mucus. Furthermore, lyophilization decreases the levels of most of the activities, including total protein levels, four specific lectin-binding levels (BS-I, PNA, UEA-I, WFA), protease, lysozyme, peroxidase and alkaline phosphatase in the skin mucus of gilthead seabream. Finally, total IgM levels and both antiprotease and esterase activity levels were similar in all the storage conditions assayed. These findings could represent a step forward in the study of humoral immune activity in fish and provide a useful tool for establishing the basis for selecting the most suitable storage conditions for future studies on different humoral immune activities in skin mucus.

Chapter 4

Skin mucus proteome map of European sea bass

Abstract

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

1. Introduction

Teleost fish are the largest and most variable vertebrate taxon with 28,644 species recorded [National Center for Biotechnology Information (NCBI) in January 2,015]. European sea bass (*D. labrax*, *Moronidae*, *Perciformes*, *Teleostei*) has a strong economic impact as a major aquaculture species. It is one of the most common marine fish in the Mediterranean Sea, in the Eastern Atlantic Sea (from Norway to north of Africa) and in the Black Sea (Ocean Biogeographic Information System, http://www.iobis.org/mapper), whose complete genome has been recently sequenced (Tine et al., 2014).

Classically, skin mucus of fish is described as a mucin layer of high molecular mass glycoproteins (Shephard, 1994), flexible fibres densely coated with short and negatively charged glycans due to carboxyl and sulphate groups (Cone, 2009), which contributes to the observed viscosity. However, functionally, the presence of immune-related proteins in mucus is of great interest since mucus acts as a first defense barrier against different stressors such as bacterial or viral infections. Skin mucus innate and adaptive immune system proteins including immunoglobulins (Xu et al., 2013), antimicrobial peptides e.g. histones (Lüders et al., 2005), transferrin (Easy et al., 2012), calmodulin (Flik et al., 1984) and lysozyme (Ourth, 1980), lectins such as galectin (Rajan et al., 2013a; Tasumi et al., 2004), acute phase proteins and complement components have been studied in different fish species (Esteban, 2012).

Recent advances in proteomic research methods have been used for identification and quantification of proteins (Gstaiger and Aebersold, 2009). Among them, the use of LC-MS/MS after separation by 2-DE (Delahunty and Yates, 2005) has been the most common method for protein identification on fish. After protein identification, qPCR is an excellent complementary approach to find if local synthesis of the protein is possible, and hence contribute to the study of the biological role of these molecules and their interactions during fish diseases (Forné et al., 2010). With this background,

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

2. Materials and methods

2.1 Animal care

Specimens of European sea bass (*D. labrax*) (340 ± 35g body weight) obtained from Culmarex SA (Murcia, Spain), were kept in running (1,500 l h^{-1}) seawater aquaria at 28‰ salinity, 22ºC and a photoperiod of 12 h light: 12 h dark. The Bioethical Committee of the University of Murcia approved the fish handling procedures.

2.2 Mucus and tissues samples

Ten apparently healthy fish were anesthetized with 100 mg l^{-1} MS222. Mucus was collected from the skin surface by scraping, avoiding contamination with blood, urine and/or faeces. Mucus samples were transferred into 15 ml tubes. Skin tissue samples were collected in TRIzol® reagent (Life Technologies) and stored at -80ºC until subsequent use.

2.3 Mucus protein purification

Pooled mucus samples were solubilised with 1mM DTT and 1.5mM EDTA which serves to act as a mild mucolytic agent (Reddy et al., 2004). Samples were sonicated for 6 seconds twice (1 min cooling between) and centrifuged at 20,000 g for 30 min at 4ºC. The soluble mucus protein containing supernatants were desalted 3 times (14,000 g, 4ºC) with 0.2 ml of ice cold proteomic grade water (G Biosciences) using 3 kDa spin filters (VWR, USA). Samples were further purified by 2-D clean up kit (Bio-Rad) following the manufacturer's instructions.

2.4 2-DE

The resulting pellets were suspended in 2-D lysis buffer (BioRad) containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris base, 0.001% bromophenol blue and 50mM DTT (Sigma-Aldrich) and 0.5% (v/v) Biolytes 3-10 ampholyte (Bio-Rad). The protein content of samples was determined using Qubit protein assay (Life Technologies). Triplicates of 200 µg of proteins from each sample were rehydrated in 17 cm 3-10 IPG strips (Bio-Rad) and isoelectrically focused using Protean IEF cell (Bio-Rad). The IPG strips were reduced and alkylated for 15 min each in equilibration buffer containing 6M urea (Sigma-Aldrich), 0.375 M Tris-HCl pH 8.8 (Bio-Rad), 2% (w/v) SDS (Sigma-Aldrich), 20% (v/v) Glycerol (Merck) with 0.2% (w/v) DTT (Sigma-Aldrich) and 0.3% (w/v) iodoacetamide (Bio-Rad), respectively. The strips were loaded on 12.5% polyacrylamide gels to perform SDS-PAGE (Laemmli, 1970) on PROTEAN II xi system (Bio-Rad). Gels were stained overnight with SYPRO® Ruby Protein Gel Stain (Life Technologies) following the supplier's protocol, and pictures taken with ChemiDoc™ XRS imaging system (Bio-Rad). Raw pictures were analysed by PDQuest 2-D Advanced Software version 8.0.1 (Bio-Rad).

2.5 LC-MS/MS analysis

The most predominant 100 spots identified after 2-DE gel analysis were picked, excised and subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 ng μl^{-1} trypsin (V511A; Promega) as described elsewhere (Shevchenko et al., 1996). Peptide mixtures containing 0.1% formic acid were loaded onto a nano ACQUITY Ultra Performance LC (Waters), containing a 5 µm Symmetry C18 Trap column (180 µm × 20 mm; Waters) in front of a 1.7 µm BEH130 C18 analytical column (100 µm × 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with flow of 0.4 µl min^{-1} eluted to a Q-TOF Ultima mass spectrometer (Micromass/Waters). The samples were run in data dependent tandem mass spectrometry (MS/MS) mode. Peak lists from the Protein Lynx Global server software

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

2.6 Primer design

For genes identified by proteome analysis, homologue sequences for each gene from *D. labrax* were retrieved from the NCBInr or EST databases, satisfying the requirement of specificity, and primers designed (**Table 5**) by OligoPerfect™ Designer (Life Technologies).

Table 5. Primers used in qPCR study.

Gene	GenBank	Sequence (5' →3')	Amplicon
ef1a	AJ866727	F: CGTTGGCTTCAACATCAAGA R: GAAGTTGTCTGCTCCCTTGG	99
actb	AJ537421	F: TCCCTGGAGAAGAGCTACGA R: AGGAAGGAAGGCTGGAAAAG	98
rps18	AY831388	F: TTCCTTTGATCGCTCTTAACG R: TCTGATAAATGCACGCATCC	102
c3	FK942306	F: TGAGAGGAGAGCTGGAGAGC R: GTTGTCGATGTTGCCCTTCT	103
c1q	FM002850	F: ACACCAACACCACGCTGATA R: CCGGTAATCCGGGTGTAGTA	118
afp	FN565768	F: GGCTGACAATGAATGGGTTT R: AGCCAACACGTGTACCATCA	113
fbl	EU877448	F: TGCCTACAGCGCTATTGATG R: CTCCAGCAGGTCAACTCTCC	106
nkef	FM024824	F: CACTGAGATCGTGGCTTTCA R: TGTGTTGATCCATGCCAAGT	112
lyz	FN667957	F: TTGCAGCTCATTCCAGTTTG R: TGTCCTGCTGAGATGTTTGC	116
calm	FL487943	F: ATTGACTTCCCGGAGTTCCT R: TTGTCAAATACCCGGAAAGC	95
calr	JX235975	F: CATCAAATGCAAGGATGACG R: AGCCAGACTCCACCTTCTCA	104
erp57	JX891474	F: CCCACATGACAGACGACAAC R: CATCACCCTGTTCCTCCAGT	119
pdi	JX891476	F: AGAGAGCATCACCGCATTCT R: GGGTTTTGTCCCAGTCTTCA	95
apoa1	CV186176	F: GGCAGTCATCGATAAGCACA R: CTTTACCTTCAGGGCATCCA	106
lei	FM018382	F: TGTACGGGGAGCAGTCCTAC R: GAGCCTAGCTGCGTCTGAGT	116
gst	FM027169	F: CTGCTTCCCTCCTCTCCTTT R: TCCCTGTGGGGATCTTGTAG	97
cypa	FM026623	F: GGGGAGAAGTTTGCTGATGA R: AGTTTTAGCCGTGCAGAGGA	120
hsp70	AY423555	F: CTGCTAAGAATGGCCTGGAG R: CTCGTTGCACTTGTCCAGAA	119
tf	FJ197144	F: CGCTTCATCTACTGCCATCA R: CGTCAGCACCCATACTGTTG	92
casp1	DQ198376	F: CCAGATCGTGGGTGTTTTCT R: TCTTCAAAGCGTTGCATGAC	110
casp6	AM988220	F: ACAAGTGCAACAGCCTTGTG R: CAGCTCACTGTCCACAGCAT	110
asc	FM020581	F: GATCAACAGAGCGAGCAACA R: AGTGGTACGCAGAGCCCTAA	103
sod	FJ860004	F: TGTTGGAGACCTGGGAGATG R: ATTGGGCCTGTGAGAGTGAG	90
wap65a	BK006867	F: TCCGCTTTATGGAGCACTTT R: GCCTCTTTGGGGTATCTTCC	97
wap65b	BK006868	F: AGGAGGTGACCAATGGAGTG R: TGTAGTGAGCCGCTGCTTTA	102

2.7 Gene expression analysis

Skin samples (n = 3) were extracted with TRIzol® reagent (Life Technologies) following the manufacturer's instructions, quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. In addition, 1µl of each RNA sample was run on 2% agarose gel to check the integrity. Next, RNA was treated with DNase I (Promega) to remove genomic DNA. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Reaction mixtures [containing 10 µl of 2 x SYBR Green supermix, 5 µl of primers (0.6 µM each) and 5 µl of cDNA template] were incubated (10 min, 95ºC), followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. *Rps18*, *actb* and *ef1a* were used as reference genes [verified in (Mitter et al., 2009)]. Negative controls had no amplification product and control templates showed no primer-dimer formations. All samples were measured in triplicates, expressed as mean ± SEM and analysed with SPSS software v19.0 (SPSS, Chicago, USA).

3. Results and discussion

A draft sequence of the European sea bass (*D. labrax*, ID 13489) genome was recently published (Tine et al., 2014), however at the time of our study only 2,420 *D. labrax* proteins were registered in the NCBI database, whilst the fully sequenced and well-annotated zebrafish (*D. rerio*, ID 7955) genome had 81,527 protein entries. Working with species with less-annotated genomes, homology-driven proteomics is the major tool to characterize proteomes (Junqueira et al., 2008). From our own experience working on fish skin mucus (Cordero et al., 2016b; Guardiola et al., 2014a, 2014b) and with proteomic tools (Kulkarni et al., 2014; Rajan et al., 2013b, 2011), 2-DE followed by

LC-MS/MS provides good resolution and high performance for protein detection and identification.

We have identified a wide range of proteins in skin mucus of *D. labrax* (**Figure 17, Table 6**).

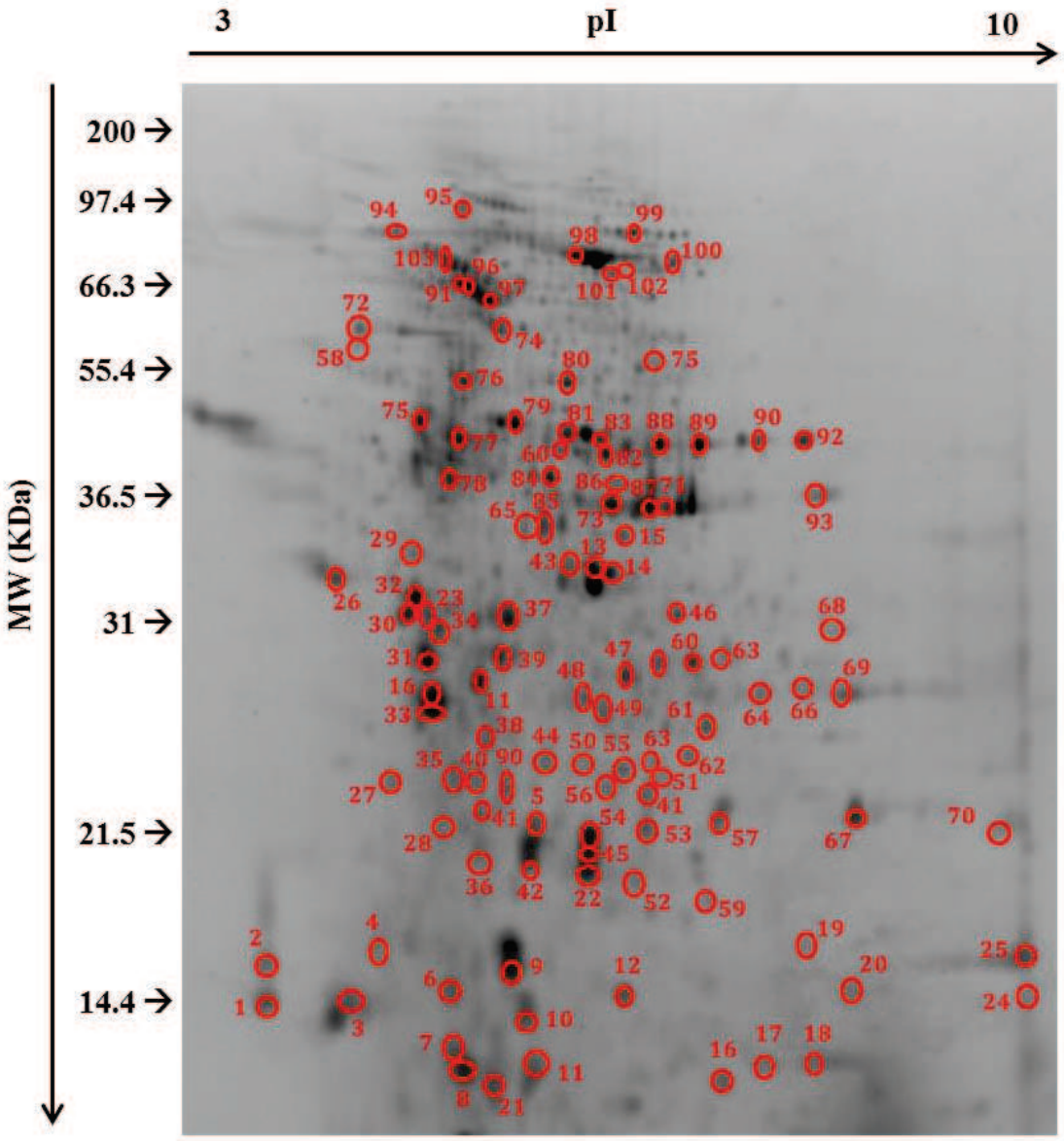


Figure 17. *D. labrax* skin mucus 2-DE map. Two hundred µg of proteins were loaded on 17 cm, 3-10 nonlinear IPG strips. Second dimension was a 12.5% polyacrylamide vertical gel. Red circles and numbers show analysed protein spots.

Table 6. Identified proteins from *D. labrax* skin mucus grouped into biological groups.

Spot no	Protein	Species	Accession no	*pI* / MW[d)]	S / C[e)]	U/T[f]
Structural						
39	ACTB	*Dicentrarchus labrax*	CAD60932	5.29/42.1	51/2	1/1
66	ACTB	*Ctenopharyngodon idella*	P83751	5.30/42.1	20/4	1/1
79	ACTB	*Oreochromis mossambicus*	P68143	5.30/42.1	856/40	7/16
85	ACTB	*Oreochromis mossambicus*	P68143	5.30/42.1	266/20	7/7
95	ACTN	*Stegastes partitus*	XP_008281276	4.90/103.2	578/14	1/7
6	COTL1	*Oryzias latipes*	XP_004069874	4.83/16.2	141/19	1/2
5	COF2	*Dicentrarchus labrax*	FM006818	8.38/28.2	167 / 16	5/5
84	CAPZA2	*Salmo salar*	ACN58682	5.84/32.3	131/11	1/2
65	CAPZA1	*Dicentrarchus labrax*	CBN80762	5.42/32.8	80/14	2/2
60	GSNL1	*Oreochromis mossambicus*	ABE98236	5.96/42.9	66/5	3/3
89	GSNL1	*Stegastes partitus*	XP_008276815	6.54/79.8	159/6	1/4
92	GSNL1	*Xiphophorus maculatus*	XP_005802408	6.28/79.6	241/7	6/6
100	GSNL1	*Oreochromis mossambicus*	ABE98236	5.96/42.9	87/5	2/2
7	KRT8	*Maylandia zebra*	XP_004545214	5.03/62.2	104/5	1/3
26	KRT17	*Stegastes partitus*	XP_008298721	5.22/48.3	128/3	3/3
90	KRT8	*Stegastes partitus*	XP_008303627	5.97/50.3	82/7	2/2
93	KRT13	*Lepisosteus oculatus*	XP_006638395	5.05/49.7	63/2	1/6
47	MFAP4	*Dicentrarchus labrax*	FM019963	5.88/30.0	95/12	2/2
3	MYL6	*Anoplopoma fimbria*	ACQ58516	4.41/17.1	61/17	2/2
88	SCINL	*Paralichthys olivaceus*	AFQ38973	6.54/80	120/4	1/3
28	KRT	*Sparus aurata*	AAT44423	4.89/38.6	71/6	1/2
78	KRT	*Oncorhynchus mykiss*	NP_001123458	5.32/55.3	509/20	1/12
63	TPM1	*Liza aurata*	P84335	4.69/32.8	24/4	1/1
55	VIM	*Cynoglossus semilaevis*	XP_008332705	5.26/52.8	47/3	1/2
76	VIM	*Cyprinus carpio*	1807305[a]	5.07/52.6	49/3	1/2
18	PFN1	*Dicentrarchus labrax*	FM000924	7.74/23.5	280/16	4/4
Protein metabolism						
54	40S	*Ictalurus punctatus*	Q90YQ5	10.99/17.7	19/5	1/1
41	60S	*Carassius auratus*	Q7T3N9	11.53/24.1	13/3	1/1
87	ADH	*Salmo salar*	ACN10195	6.32/37.2	99/6	1/2
24	AGR2	*Maylandia zebra*	XP_004561006	8.87/19.1	136/22	2/4
22	CDK7	*Carassius auratus*	P51953	8.98/38.6	19/4	1/1
82	EF1A	*Oryzias latipes*	Q9YIC0	9.23/50.6	24/6	1/1
9	GAPR1	*Maylandia zebra*	XP_004576580	5.38/18.7	62/6	1/1
45	GNRH2	*Clarias gariepinus*	P43306	9.27/10	19/10	1/1
34	SCGN	*Astyanax mexicanus*	XP_007256889	5/31.7	117/8	2/2
8	SH3BGRL	*Osmerus mordax*	ACO10145	4.78/13.1	68/21	2/2
27	TCTP	*Dicentrarchus labrax*	FM000425	5.91/31.8	73/11	3/3
Carbohydrate metabolism						
59	DCTD	*Dicentrarchus labrax*	FM019776	8.60/31.7	67/3	1/1
75	ENOA	*Acipenser baerii*	ABF60006	5.98/47.5	125/10	3/3
58	ALDOB	*Sparus aurata*	P53447	8.43/40.2	17/4	1/1
50	GAPDH	*Oncorhynchus mykiss*	O42259	6.37/36.6	18/5	1/1
86	IUNH	*Maylandia zebra*	XP_004575422	6.88/35.4	63/5	2/2
11	IMPA	*Dicentrarchus labrax*	CBN82127	5.47/28.9	403/29	4/6
20	NDK	*Siniperca chuatsi*	AAY79301	5.86/13	107/37	2/4
44	PEBP	*Ictalurus punctatus*	NP_001187975	6.82/21.2	92/13	3/3
4	INPPL1	*Sparus aurata*	FM148029	4.54/13.2	54/8	1/1
71	TALDO	*Oryzias latipes*	XP_004066906	6.69/37.8	163/9	3/3

68	TPI1B	*Oreochromis niloticus*	XP_003450633	6.9/26.9	151/14	3/3
69	TPI1B	*Oryzias latipes*	BAD17901	6.14/23	182/24	1/4
RNA/DNA metabolism						
51	HNRNPA0	*Salmo salar*	ACI67551	9.1/29.4	119/6	1/1
61	HMX3B	*Oryzias latipes*	Q90XN9	6.42/32.6	13/2	1/1
35	SEPSIP	*Oreochromis niloticus*	XP_003439510	4.15/31.1	93/8	2/2
94	RBP12	*Stegastes partitus*	XP_008296875	9.24/95.5	45/1	1/1
52	TLE1	*Oryzias latipes*	XP_004072378	8.08/69.5	45/1	1/1
73	H1	*Astyanax fasciatus*	AEC13086	11.05/20.8	46/5	1/1
81	H4	*Oncorhynchus mykiss*	P62797	11.36/113.6	25/11	1/1
Signal transduction						
23	14-3-3	*Poecilia Formosa*	XP_007563007	5.02/36.9	153/10	3/3
30	14-3-3	*Astyanax mexicanus*	XP_007230880	4.65/28	178/15	2/4
31	14-3-3	*Xiphophorus maculatus*	XP_005805709	4.62/27.7	291/21	3/6
32	14-3-3	*Stegastes partitus*	XP_008291071	4.74/30	116/10	1/2
21	S100A6	*Anoplopoma fimbria*	ACQ58920	5.08/13	74/11	1/1
80	GDI2	*Astyanax mexicanus*	XP_007252464	5.6/50.8	368/16	2/6
19	GDI1	*Dicentrarchus labrax*	FM018448	5.36/32.4	88/11	1/2
37	GDI1	*Oryzias latipes*	XP_004071582	5.01/23.5	178/16	4/4
Immune-related						
17	AFP	*Dicentrarchus labrax*	FN565768	6.47/21.8	88/14	2/2
16	APOA1	*Morone saxatilis*	ACH90227	4.75/20.6	300/31	4/7
33	APOA1	*Morone saxatilis*	ACH90229	5.09/16.1	434/35	5/8
64	ASC	*Dicentrarchus labrax*	FM020581	5.85/28.5	196/11	2/2
1	CALM	*Electrophorus electricus*	P02594	4.6/16.8	96/7	2/2
2	CALM	*Electrophorus electricus*	P02594	4.09/16.8	118/11	1/1
72	CALR	*Dicentrarchus labrax*	AGI60286	4.37/49.4	258/17	2/5
48	CASP1	*Dicentrarchus labrax*	AM984268	8.57/24.5	293/32	5/5
15	CASP6	*Cynoglossus semilaevis*	XP_008315389	6.02/34.5	84/5	1/1
36	C1Q	*Dicentrarchus labrax*	FM002850	8.64/19.4	100/15	1/3
38	C1Q	*Dicentrarchus labrax*	FM000708	5.77/25.4	143/9	2/2
42	C1Q	*Dicentrarchus labrax*	FL487070	8.03/20.2	278/45	2/7
77	C3	*Solea senegalensis*	ACR20030	6.04/6.5	69/17	1/1
83	C3	*Epinephelus coioides*	ADU33222	6.07/186.2	62/1	2/2
102	C3	*Larimichthys crocea*	AHZ41228	6.15/186.8	108/2	2/3
101	C3	*Sparus aurata*	ADM13620	8.08/186.9	67/1	1/2
25	CyPA	*Gadus morhua*	AEK21703	8.51/18	48/5	1/1
74	PDIA3	*Dicentrarchus labrax*	AGI60170	5.39/56.3	256/18	5/7
97	PDIA3	*Dicentrarchus labrax*	AGI60170	5.39/56.3	122/8	4/4
13	FBL	*Dicentrarchus labrax*	ACF94293	6.08/34.8	317/29	6/6
14	FBL	*Dicentrarchus labrax*	ACF94293	6.08/34.8	212/13	5/5
43	FBL	*Morone chrysops*	ABB29990	6.21/34.7	115/7	2/2
46	GST	*Anoplopoma fimbria*	ACQ58017	7.01/27.8	47/3	1/1
49	GST	*Takifugu obscurus*	ABV24049	5.47/26.4	124/11	1/3
91	HSP70	*Dicentrarchus labrax*	AAR01102	5.31/71.6	52/5	2/2
29	LEI	*Dicentrarchus labrax*	CBN81773	4.9/44.7	205/14	5/5
67	LYZ	*Paralichthys olivaceus*	Q90VZ3	8.69/21.4	86/14	2/2
70	LYZ	*Dicentrarchus labrax*	CBJ56263	8.53/20.4	58/9	1/1
6	NKEF	*Anoplopoma fimbria*	ACQ58049	6.3/22.2	212/18	3/4
104	PDI1	*Dicentrarchus labrax*	AGI60172	4.54/57.2	323/11	6/7
12	SOD	*Dicentrarchus labrax*	FM000596	6.18/22.0	99/6	1/1
98	TF	*Dicentrarchus labrax*	ACN80997	5.93/76	1103/38	21/23
99	TF	*Dicentrarchux labrax*	ACN80997	5.93/76	69/2	1/1
96	WAP65A	*Dicentrarchus labrax*	DAA12503	5.45/49.7	423/26	1/11
103	WAP65B	*Dicentrarchus labrax*	DAA12504	5.47/49.3	948/50	19/20

These proteins could be remains of dead cells from the skin surface or proteins actively secreted to function in the mucus. Due to the importance of mucus as a barrier of defense, we have focused our attention according to the capacity of these proteins to be candidates as welfare indicators. Furthermore, the transcript levels present in skin of *D. labrax* have been demonstrated (Figure 18).

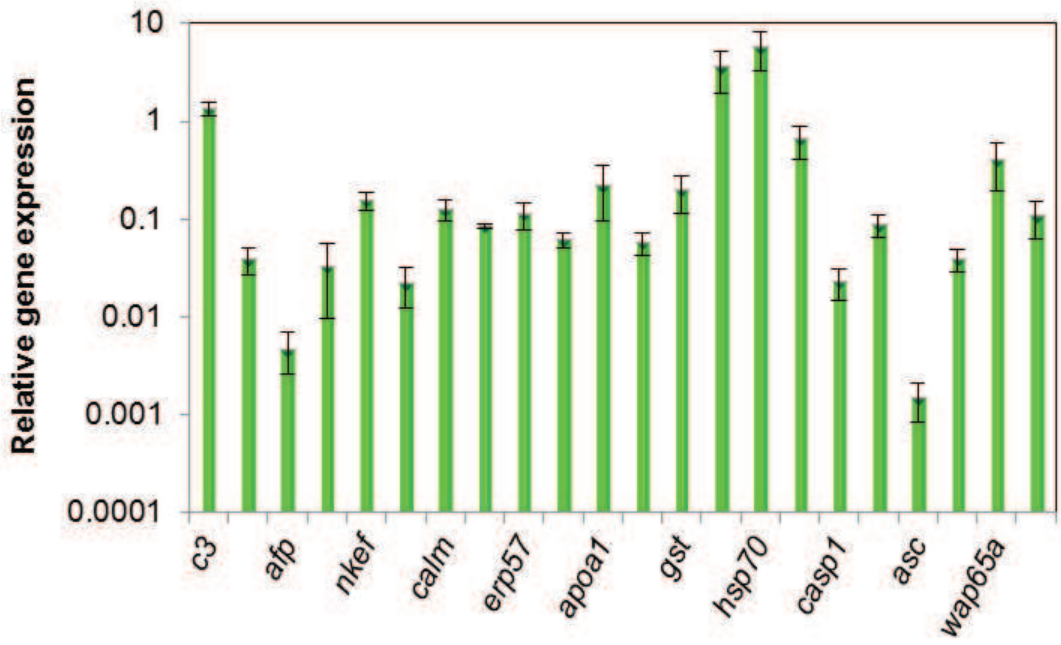


Figure 18. RNA expression levels in skin for the identified immune-related molecules from *D. labrax* mucus, relative to the reference genes *rps18*, *actb* and *ef1a*. n = 3; and three replicates of analysis.

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

The proteome of European sea bass skin mucus includes proteins with well-established functions such as several complement components, lectins, proteins involved in apoptosis, inflammation, redox homeostasis, stress as well as antimicrobial activity. The classical functions of many of these proteins take place in cells or body fluids inside organisms, in our results and discussion we will focus on these proteins in an extracellular setting as far as such activity is found in the literature.

3.1.1 Proteins identified with pathogen interacting capacity

We identified several proteins that could interact directly with pathogens, pathogen interacting proteins could among others lyse pathogens, agglutinate them, inhibit their growth or inhibit or stimulate binding to host cell surfaces.

The complement system plays a major role in vertebrate defense against pathogens in the blood as a part of both the innate, and adaptive immune systems (Walport, 2001a, 2001b). Upon activation by the surface of pathogens and host factors such as mannose binding lectins, in the innate pathways, or antibodies, in the adaptive pathway, a series of cleavages of complement factors are initiated. An intermediate key factor is C3, which upon cleavage can act as a chemoattractant (recruitment of immune cells), lead to phagocytosis by opsonization (coating of pathogen), produce agglutination (clustering of pathogens) or initiate further cleavages leading to lysis of bacteria. C3 was found in different regions in 2-DE gels of *D. labrax* skin mucus: two isoforms of around 42 kDa (spots 77 and 83) and another two isoforms of around 85 kDa (spots 101 and 102). We cannot conclude that C3 is cleaved and activated in mucus; however the presence of cleaved C3 could indicate active roles in skin mucus (Figure 19).

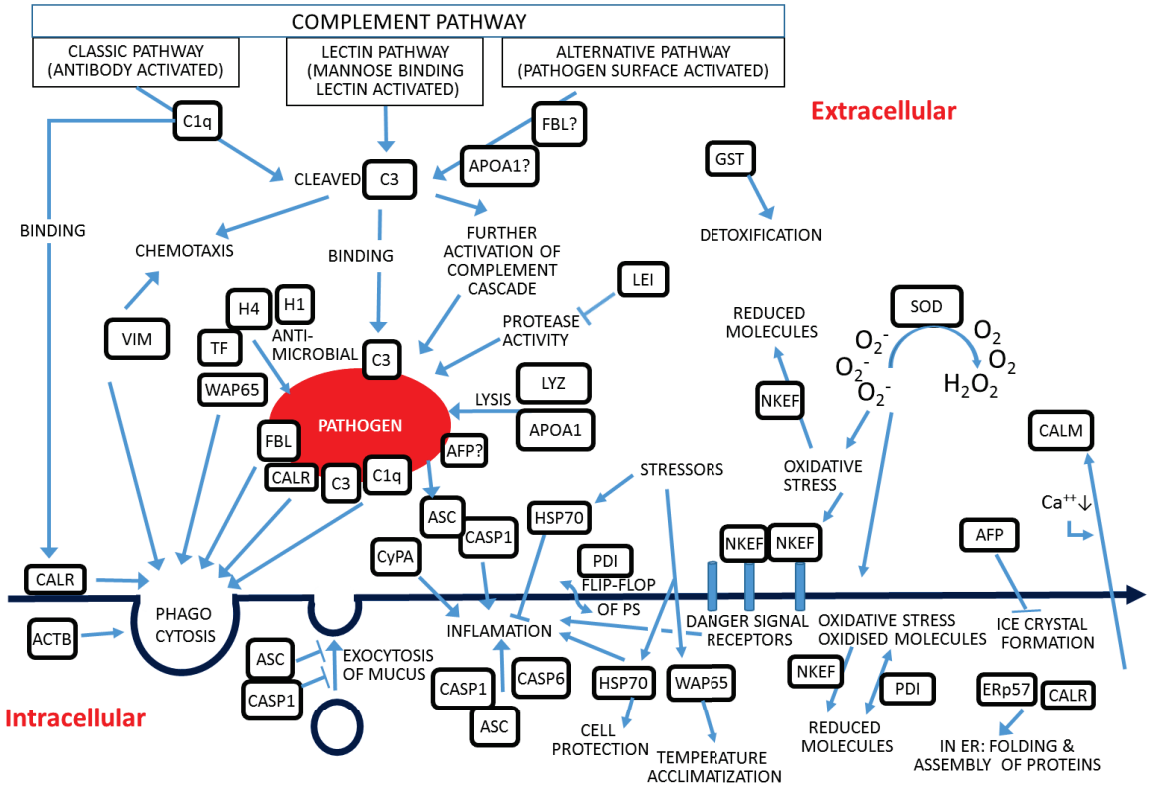


Figure 19. The figure shows some of the proteins identified from the skin mucus of *D.labrax* and their possible stress or immune related roles in the mucus and mucosal surface. Arrows point to the process or molecule influenced; lines with a perpendicular end indicate inhibition of a process. The abbreviations are found in **Table 6** and details of the biological processes are described in the results and discussion section.

C1q and C1q-TNF family members involved in classical complement activation are also present in skin mucus (Kishore et al., 2004) (**Figure 19**). The present study is restricted to the analysis of the 100 strongest protein spots in skin mucus (**Figure 19**), hence it cannot be excluded that also antibodies needed for C1q activation or other complement factors are present. It is the first time that C1q proteins have been observed in fish skin mucus (spots 36, 38 and 42).

Lectins, carbohydrate binding proteins, have been reported from various tissues of a diversity of fish species and they have also been isolated from skin, mucus, serum, and plasma. Mannose-binding lectins activate the lectin pathway in the complement system

(Figure 19), have been identified in Atlantic cod mucus (Rajan et al., 2011), but were not found in our study. Fucose-binding lectin (FBL) recognizes carbohydrates on the surface of potential pathogens, leads to agglutination, immobilization, and opsonization of microbial pathogens, and phagocyte activation (Russell and Lumsden, 2005) (Figure 19). This molecule was identified (spots 13, 14 and 43) in skin mucus of *D. labrax*, and a 34 kDa F-type lectin was previously reported in serum of the same fish species (Salerno et al., 2009). F-type lectin is not previously reported in fish mucus, however a C-type lectin was reported in skin mucus of a cichlid (Chong et al., 2006), and galectin in Atlantic cod (Rajan et al., 2011).

Lysozyme (LYZ) has been widely studied in the animal kingdom and identified as a hydrolytic enzyme with the capability to lyse bacteria by cleaving the β-(1,4)-glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues of peptidoglycan in the bacteria cell wall (Figure 19). Thus, LYZ is an important component of the vertebrate innate immune system (Saurabh and Sahoo, 2008). In the present paper, two isoforms of LYZ have been identified (spots 67 and 70) of around 20 kDa in skin mucus of *D. labrax*. In agreement with our study, two isoforms of LYZ were also identified and characterized in skin mucus of Atlantic cod (Rajan et al., 2011). Up-regulation of lysozyme mRNA in HK leucocytes of *D. labrax* after bacterial infection has recently been shown (Buonocore et al., 2014), further studies are needed to unravel if there are modulations of LYZ levels in skin mucus under stress stimulus or pathogen infections.

Apolipoprotein A1 (APOA1) is the major protein component of high density lipoprotein in serum (Breslow et al., 1982). APOA1 was identified with a MW of around 28 kDa (spots 16 and 33). In Atlantic salmon and Atlantic cod, this protein was identified and over-expressed in skin mucus after sea lice and bacterial infection, respectively (Easy and Ross, 2009; Rajan et al., 2013b). Moreover, striped bass APOA1 has

demonstrated antibacterial activity *in vitro* (Johnston et al., 2008) and lytic activity has been shown for APOA1 from channel catfish (Pridgeon and Klesius, 2013) (Figure 19). Transferrin (TF) belongs to an ancient family found in all metazoans. TF is responsible for the transport and delivery of iron to cells (Dautry-Varsat et al., 1983). In addition, it is bacteriostatic by limiting the availability of iron to bacteria (Figure 19). Transferrin, which has been characterized in liver and brain of *D. labrax* (Neves et al., 2009), was identified in skin mucus (spot 98 and 99) in the present paper with the highest score (see Table 6). Transferrin was previously identified in skin mucus of gilthead seabream (Jurado et al., 2015) and Atlantic cod juveniles (Easy et al., 2012). In Atlantic salmon, a transferrin fragment was upregulated after sea lice infection (Easy and Ross, 2009).

Typically, histones and their post-translationally modified forms have roles in chromatin remodelling and gene transcription. However, histones act as damage-associated molecular pattern molecules when they are released into extracellular spaces (Chen et al., 2014). In our study, we have identified H1 and H4 in skin mucus of *D. labrax*. It has been reported that H1 has antimicrobial properties (see Figure 19) in Atlantic salmon (Richards et al., 2001), in skin of rainbow trout (*Oncorhynchus mykiss*) (Noga et al., 2011), and in skin secretions of rainbow trout (Fernandes et al., 2004). H4 has been shown to be antimicrobial (see Figure 19) in humans (Lee et al., 2009).

The warm temperature acclimation related 65 kDa protein (WAP65) shares high structural similarities with mammalian hemopexins, which can bind iron containing heme serving a protective role against bacterial infections (see Figure 19) in skin mucus by limiting available iron. It has been shown to be involved in temperature acclimation, in immune response, as well as in development in teleost (Sha et al., 2008). In our study, two isoforms of this protein (spot 96 and 103) of around 65 kDa and 70 kDa, respectively, were identified in skin mucus of *D. labrax*. This protein was reported in skin mucus of gilthead seabream as well (Jurado et al., 2015).

Calreticulin, a calcium-binding protein identified in the cell surface of neutrophils as a receptor for C1q (Ghiran et al., 2003), also promotes phagocytosis by microbial binding (Liu et al., 2013). It has been previously characterized in *D. labrax* (Pinto et al., 2013), and in the present paper, it was identified as a protein of around 60 kDa (spot 72). Calreticulin was also identified in skin mucus of Atlantic cod, with a similar MW (Rajan et al., 2011). In both cases, the observed MW did not match with the theoretical values of 49.4 (Table 6), suggesting post-translational modifications such as glycosylation, as is known to be the explanation for the observed high molecular weight of human calreticulin in SDS-PAGE.

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

Proteins described in 3.1.1 which binds to pathogens can in many cases also stimulate phagocytosis (Figure 19). In the mucosal surface of the skin, the mucus will act as a protective barrier to prevent pathogen interaction with the live cells in the surface, should it fail due to e.g. wounds the second line of defense will serve a role, this include the phagocytic cells recruited by chemotaxis. Immune-related molecules could have several functions in the mucosal surface (mucus and outer cell layer) such as stimulation of inflammation, chemotaxis and phagocytosis in addition to pathogen binding giving agglutination, lysis or growth inhibition. The surface of the skin also needs to be protected from external stressors and several of the identified proteins could have stress-limiting functions (Figure 19).

Vimentin and beta-actin are multifunctional proteins involved in motility, migration, cell adhesion and phagocytosis, among others. Vimentin was found as two isoforms, spot 76 at 52 kDa, and spot 55 at 25 kDa. Interestingly, it has been shown for human monocytic cell lines that extracellular full length vimentin is a chemoattractant, whilst a cleavage product stimulates phagocytosis (Starr et al., 2012) (Figure 19). This is the first time that vimentin is reported in skin mucus, and hence functional studies has not

yet been done. Beta-actin, which has been previously found in mucus (Easy and Ross, 2009; Provan et al., 2013; Rajan et al., 2011), can be fragmented after stress (Easy and Ross, 2009). In agreement with that, in our study we have identified a beta actin of 42 kDa (spot 79), which is correlated with the theoretical MW, another beta-actin of 35 kDa (spot 85), and two isoforms of around 28 kDa (spots 39 and 66). Increased levels of beta-actin and vimentin fragments in skin mucus could be indicators of disease or stress.

In our study, we identified apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC, spot 64) at 28 kDa, matching with the theoretical MW. ASC is an adaptor protein that has a bipartite domain structure, an N-terminal PYRIN domain and a C-terminal caspase activation and recruitment domain (CARD) (Ayukawa et al., 1999). ASC and caspases are important in the cell death pathway by apoptosis. Two caspases were identified in skin mucus of *D. labrax*. CASP1 (spot 48), responsible for the production of IL-1β pro-inflammatory cytokine during immune response to microbial pathogens (Creagh et al., 2003), and CASP6 (spot 15), an activator of CASP8 which promotes apoptosis (Cowling and Downward, 2002; Creagh et al., 2003). They were identified for the first time in fish skin mucus. ASC has a pivotal role in the caspase-1 (CASP1)-dependent processing of pro-inflammatory cytokines (Liu et al., 2013). Interestingly, ASC and CASP1 have recently, in mouse gut, been identified as key regulators of mucus secretion by exocytosis (Wlodarska et al., 2014), they are also part of an extracellular oligomeric complex (the NLRP3 inflammasome) which is secreted upon danger signals and is suggested to act to amplify inflammatory response (Baroja-Mazo et al., 2014).

In our study cyclophilin A was identified as a protein of around 18 kDa (spot 25), it was also found in skin mucus of Atlantic cod (Rajan et al., 2011). Cyclophilins are a group of highly conserved cytosolic enzymes that have a peptidylprolyl cis/trans isomerase activity and belong to the immunophilin family. Among them, cyclophilin A can be

secreted in the extracellular space by inflammatory cells when exposed to stressors and upon cell death (Nigro et al., 2013). Extracellular cyclophilin A stimulates pro-inflammatory signals in endothelial cells (Jin et al., 2004). To our knowledge this is the first time that cyclophilin A is identified for *D. labrax*, it could be a serious candidate as stress and immune status indicator through *D. labrax* skin mucus analysis.

The skin as a first barrier against the external environment would have to protect itself against variations in physical parameters e.g. temperature and chemical parameters such as oxidation. Several of the identified proteins could protect against external stressor and some could in addition have a role in the immune system. Peroxiredoxins (PRDX) or natural killer cell enhancing factors (NKEFs) are a family of antioxidant proteins also involved in inflammation and innate immunity (Figure 19) (Esteban et al., 2013; Ishii et al., 2012). In the present work, NKEF was identified (spot 6) in skin mucus of *D. labrax*. In agreement with our results, NKEF1 and NKEF2 were recently found in skin mucus of gilthead seabream (Jurado et al., 2015). Furthermore, it has been reported that extracellular NKEF1 act as a "endogenous" danger signal by binding danger signal sensors/receptors (Riddell et al., 2010), suggesting that NKEF may be a stress indicator in skin mucus.

The human thioredoxin superfamily members are thiol oxidoreductases with a role in various cell signalling pathways. ERp57 and protein disulphide isomerase (PDI) are two members of this superfamily, which have a common structure but different multifunctional roles. ERp57 acts on glycosylated substrates in the endoplasmic reticulum through interaction with the lectin-like calreticulin (spot 72 described in section 3.1.1) (Zapun et al., 1998), and has a critical role in major histocompatibility complex class I assembly (Wearsch and Cresswell, 2008). PDI is mainly associated with the protection against reactive oxygen species (ROS) (Laurindo et al., 2012), however it also has an extracellular role in regulating flip-flop of phosphatidylserine in the cell membrane (Popescu et al., 2011). ERp57 was identified as two isoforms (spot

74 and 97) and PDI was identified as a protein of around 57 kDa (spot 104) in skin mucus. Both proteins were previously characterized in *D. labrax* (Pinto et al., 2013).

Superoxide dismutase (SOD) is an enzyme that catalyses the reaction of anion superoxide (O^{2-}) into hydrogen peroxide (H_2O_2) at the extracellular matrix (Marklund, 1982), protecting the tissue against oxidative stress by regulating various ROS and reactive nitrogen species (RNS) molecules (Break et al., 2012). In our study, SOD Zn/Cu (spot 12) was identified in skin mucus of *D. labrax* with a MW around 16 kDa. Recently, this protein was also reported in skin mucus of fish with a similar pI and MW (Jurado et al., 2015). Moreover, in another study it was observed that antigen-dependent activation of T lymphocytes significantly increased extracellular SOD-1 levels (Terrazzano et al., 2014). In mice, deletion of extracellular SOD lead to lung injury at ambient air due to increased levels of superoxide (Gongora et al., 2008), indicating that extracellular SOD could be important for cellular integrity also in the mucosal surface of the fish skin.

Glutathione S-transferases are the superfamily of phase II detoxification enzymes that play crucial roles in cellular defense. In our study, two glutathione S-transferases were identified (spots 46 and 49), at 31 kDa and 26 kDa, respectively. It was previously reported in Atlantic cod skin mucus (Rajan et al., 2011), and differentially expressed after infection (Rajan et al., 2013b). In rat it has been suggested to play a role in detoxification of electrophilic compounds in rat small intestine mucus (Samiec et al., 2000) (**Figure 19**).

Serpins are a homologous family of proteins with diverse functions in processes such as blood coagulation, fibrinolysis, programmed cell death, development and inflammation (Van Gent et al., 2003). Leucocyte elastase inhibitor belongs to this family and function to limit and fine tune protease activity to limit host damage (**Figure 19**) during inflammation and apoptosis (Bird, 1999) and during pathogen destruction by host through proteolytic activity. In our study, leucocyte elastase inhibitor (spot 29) has

been identified in skin mucus of *D. labrax* as a protein of 34 kDa. In another report, it was identified in numerous spots in Atlantic cod skin mucus (Rajan et al., 2011), one of them with similar pI and MW than in the present paper.

Heat-shock proteins are part of a superfamily of stress proteins, highly conserved across species, often classified based on their molecular weight. HSP70 is constitutively expressed but can be induced to higher levels by stressors such heat, pathogens, heavy metals, it can be cytosolic (Yamashita et al., 2004), or extracellular (Pockley et al., 2008). HSP70 can function both as an inhibitor or stimulator of inflammation, it has been suggested that the mode of activation, location and/or co-molecules present will influence the function (Pockley et al., 2008) (**Figure 19**). In our study, HSP70 (spot 91) was identified at the expected 70 kDa. It has been found that *hsp70* transcription is induced after heavy metal exposition and hypothermia in skin of common carp (Ferencz et al., 2012). Moreover, a HSC70 was reported in skin mucus of gilthead seabream (Jurado et al., 2015).

Antifreeze proteins (AFP) are a group of small proteins with a carbohydrate domain typically secreted by the liver into the blood in teleosts. However, it has become clear that AFP isoforms are produced in the epidermis (skin, scales, fin, and gills) and may serve as a first line of defense against ice propagation into the fish (Fletcher et al., 2001) (**Figure 19**). In our study, AFP was identified as a protein of around 10 kDa (spot 17). Little information is available about its structure and function in *D. labrax*, it may not only be needed to be able to live in different parts of Europe (see introduction of this chapter), but may through its carbohydrate-binding domain interact with bacteria (**Figure 19**).

Calmodulin (CALM) is a calcium-binding messenger protein involved in apoptosis, inflammation and immune response (Tidow and Nissen, 2013). In our study, this protein was identified in two isoforms (spots 1 and 2) with different sizes; suggesting post-translational modifications in skin mucus (see **Figure 19**). CALM was previously

found in fish mucus of tilapia, catfish and rainbow trout, increased secretion of CALM was found when there were decreased calcium concentrations in the water (Figure 19), and a role in control of cell membrane permeability in the epithelium has been suggested (Flik et al., 1984).

Keratins are intermediate filaments that form heteropolymeric filaments containing type I and type II keratins, they have a physical protective role in skin, and are also involved in cell proliferation and apoptosis (Marceau et al., 2001), both filament types were found in skin mucus of *D. labrax*. BLASTp analysis against human keratins was carried out to discard any human contamination (data not shown), the peptides identified were non-human. Keratins have been reported in skin mucus from gilthead seabream (Jurado et al., 2015) and Atlantic cod (Rajan et al., 2011). Further studies are needed in fish to see if keratin could have a role in fish mucus.

Other proteins identified are involved in other cellular processes (see Table 6) and have at present an unlikely role in skin mucus, and were therefore discarded as candidates for fish disease diagnosis. Their presence could be due to natural sloughing of cells in mucus, rather than active secretion and their extracellular function, if any, remains unknown.

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

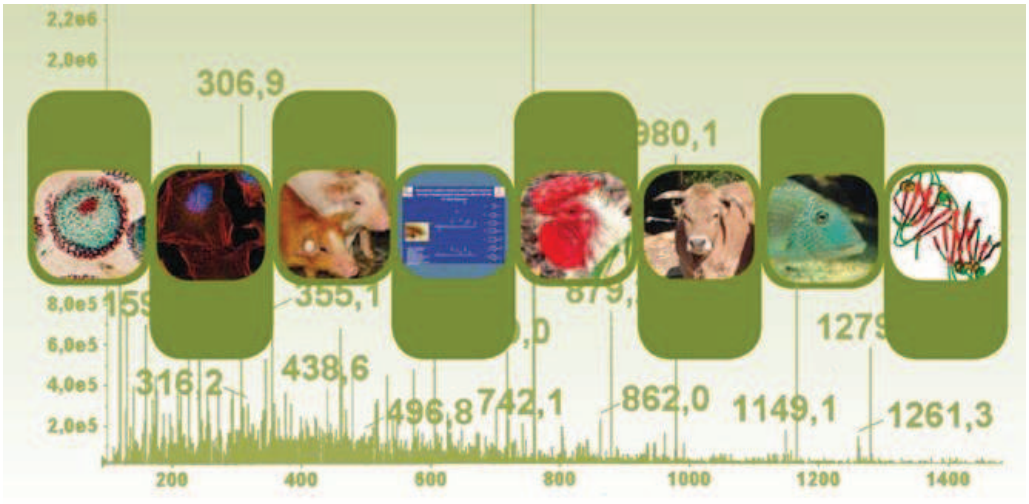
The selected immune-related gene expression profiles showed a transcript for every target in *D. labrax* skin (Figure 18), for each gene, including reference genes, a single peak in each melt curve was observed (not shown). To our knowledge, despite of being involved in many immune-related processes, this is the first time that most of the immune-relevant transcripts such as *c3*, *c1q*, *afp*, *fbl*, *lyz*, *calm*, *calr*, *erp57*, *pdi*, *apoa1*, *tf*, *lei*, *gst*, *cypa*, *asc*, *casp1*, *casp6*, *sod* and *wap65a* and *wap65b* are shown in skin from *D. labrax*, although *hsp70* and *nkef* gene expression have been previously reported (Esteban et al., 2013; Poltronieri et al., 2007). Comparing with proteins found in other teleosts, *tf* was found in skin of Atlantic salmon (Easy and Ross, 2009) and

gilthead seabream (Cordero et al., 2014b) and *cypa* was found in Atlantic cod (Rajan et al., 2011) while *lyz* and *c1q* was present in channel catfish (Liu et al., 2013). The presence of transcripts in skin indicates that the protein products of these transcripts could be synthesised in the skin itself. Hence, this could be considered a starting point to study many immune-related processes in skin, especially against microbial infections. The present results confirm the idea that intricate local signalling networks are present at and in the fish skin. Further studies are needed to corroborate if any of these proteins could be used as immune- or stress markers or how these networks are involved in the mucosal stress and immune response against different pathogens.

This study represents the first proteome map from *D. labrax* skin mucus. The results demonstrated in the skin mucus of European sea bass the presence of many proteins with known or suspected stress management or immune function; most of the identified proteins are not previously described in fish skin mucus. We found proteins that could interact directly with pathogens as well as proteins with a potential role in interacting with or functioning in the cellular surface.

Chapter 5

Differential proteome profile of skin mucus of gilthead seabream after probiotic intake and/or overcrowding stress



Abstract

High density stocking causes stress and increases the impact of diseases leading to economic losses. Probiotics could represent a solution to prevent diseases through several mechanisms such as improving the immune status and/or mucosal microbiota or competing with pathogens. The probiotic *S. putrefaciens*, also known as Pdp11, was firstly isolated from the skin of healthy gilthead seabream. Our study focuses on the skin mucus proteome after dietary probiotic Pdp11 intake in fish maintained under normal or overcrowding conditions. 2-DE of skin mucus followed by LC-MS/MS analysis was done for each experimental group and differentially expressed proteins were identified. The results showed differentially expressed proteins especially involved in immune processes, such as lysozyme, complement C3, natural killer cell enhancing factor and nonspecific cytotoxic cell receptor protein 1, whose transcript profiles were studied by qPCR. A consistency between lysozyme protein levels in the mucus and lysozyme mRNA levels in skin were found. Further research is necessary to unravel the implications of skin mucosal immunity on fish welfare and disease.

1. Introduction

Due to intensive fish farming, large number of diseases could emerge, commonly in relation to stress problems, i.e. overcrowding (Wedemeyer, 1997). In the recent years, to avoid or diminish both fish diseases and/or stress problems, probiotics have arisen as promising tools. Probiotics may act as sources of nutrients, improving fish health by exclusion competition with pathogenic bacteria and modulating immune parameters (Balcázar et al., 2006; Cordero et al., 2014a; Nayak, 2010), and consequently prevent stress problems and pathogen infections in fish. In this sense, *Shewanella putrefaciens*, also known as Pdp11, is a gilthead seabream probiotic isolated from the skin of healthy specimens, which was tested as potential probiotic (Chabrillón et al., 2005a, 2005b), getting some benefits after dietary intake such as an increase of survival against vibriosis (Tapia-Paniagua et al., 2014) and stimulation of systemic immune parameters (Cordero et al., 2015b; Díaz-Rosales et al., 2009; Guzmán-Villanueva et al., 2014).

Recent advances in proteomics research methods have been used for identification and quantification of proteins (Gstaiger and Aebersold, 2009). These methodologies have been successfully used to evaluate the proteome in fish after administration of dietary supplements or under stress factors (Cordeiro et al., 2012; Ibarz et al., 2010; Rufino-Palomares et al., 2011). However, most of them are based on liver proteomes. Nowadays, the characterization of mucosal surfaces are taking importance, mainly from the immunological point of view, since skin mucus is the first barrier of defense in fish (Shephard, 1994) and can be studied with non-invasive techniques. Thus, many molecules involved in immunity have been reported in skin mucus (Esteban, 2012). Due to this, in the last years, several skin/epidermal mucus proteomes have been studied in fish, including European sea bass (Cordero et al., 2015a) and gilthead seabream (Jurado et al., 2015; Sanahuja and Ibarz, 2015). For example, it has been observed changes in protein composition after infection (Provan et al., 2013; Rajan et

al., 2013b), handling stress (Easy and Ross, 2010) or parental care (Chong et al., 2006). However, so far, very little is known about the regulatory mechanisms of dietary probiotics and/or overcrowding stress conditions at the proteomic level in the fish skin mucus.

The aim of this work was to study the differentially expressed proteins present in skin mucus after probiotic feeding under overcrowding stress, identified using 2-DE followed by LC-MS/MS, and next study the changes of transcript levels of four of these molecules (*c3*, *nkefb*, *nccrp1* and *lyz*) in skin of gilthead seabream, which will contribute to a better understanding of changes in mucosal immunity as well as checking if probiotic Pdp11 could improve fish health of stressed fish.

2. Materials and methods

2.1 Animal care

Forty specimens of gilthead seabream (104.2 ± 7.4 and 116.2 ± 5.1 g of initial and final mean body weight, respectively), obtained from a local farm (Murcia, Spain), were kept in running seawater aquaria (flow water 900 l h^{-1}) at 28‰ salinity, 22ºC and a photoperiod of 12 h light: 12 h dark. All the fish handling procedures were approved by Bioethical Committee of the University of Murcia.

2.2 Probiotic diet

Bacteria cells of *S. putrefaciens* were grown in tryptic soy broth (TSB; Sigma-Aldrich) agar plates for 24 h at 25ºC after which one colony was inoculated in tubes containing 5 ml of TSB supplemented with 1.5% NaCl (TSBs). After 24 h of incubation at 22ºC and 200 rpm, the number of probiotic bacteria present per millilitre of TSB was measured by using a Particle Counter (Beckman Coulter). Bacteria were washed twice in phosphate buffer saline (PBS) and added with water into a crushed commercial diet (Optibream D4, Skretting) to a final concentration of 10^8 cfu g^{-1}, mixed and re-pelleted again. The same process without adding the probiotic was used for the control diets.

2.3 Experimental design

Fish were weighted and measured in order to calculate diet and density ratios, and divided into 4 different tanks. Two groups received the commercial diet and the other two a Pdp11-supplemented diet at a rate of 1% biomass once per day. A tank of each dietary group was maintained at a density of 5 kg m^{-3}, considered low density, while the other was maintained at a density of 20 kg m^{-3}, considered high density or overcrowding condition for gilthead seabream (Montero et al., 1999). Fish were maintained under these experimental circumstances for 15 and 30 days until sampling.

2.4 Mucus and tissues samples

Five fish per group were anesthetized with 100 mg l^{-1} MS222 prior to sampling. Mucus was gently scraped off from the skin surface, avoiding blood, urine and faeces during collection (Palaksha et al., 2008). Mucus was transferred into tubes of 15 ml and stored at -80ºC until use. Skin tissue was collected in QIAzol lysis reagent (Qiagen) and stored at -80ºC for subsequent RNA extraction.

2.5 Mucus protein purification

Pooled mucus samples were obtained from five individual fish. These pooled samples in triplicate for each group were solubilised with 1 mM DTT and 1.5 mM EDTA, which serves to act as a mild mucolytic agent (Reddy et al., 2004). Next, after two rounds of sonication for 6 seconds followed by cooling for 1 min, samples were centrifuged at 20,000 g for 30 min at 4ºC. The supernatant containing the soluble mucus proteins was desalted with proteomic grade water (G Biosciences) using centrifugal filters of 3 kDa (VWR) by spinning 3 times at 14,000 g at 4ºC with 0.2 ml of ice cold water each time. The dialysed protein solution was further purified by 2-D clean-up kit (Bio-Rad) following the manufacturer's instructions.

2.6 2-DE

The samples obtained after the 2-D clean-up process were suspended in 2 D lysis buffer (Bio-Rad) containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris

base, 0.001% bromophenol blue and 50 mM DTT (Sigma-Aldrich) and 0.5% (v/v) Biolytes 3-10 ampholyte (Bio-Rad). The protein content of solubilised samples was estimated using Qubit protein assay (Life Technologies). Two hundred µg proteins for each sample were rehydrated in 17 cm 3-10 IPG strips (Bio-Rad) and isoelectric focusing (IEF) was carried out using protean IEF cell (Bio-Rad). After IEF, the electro-focused IPG strips were reduced and alkylated for 15 min each in equilibration buffer containing 6 M urea (Sigma-Aldrich), 0.375 M Tris-HCl pH 8.8 (Bio-Rad), 2% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck) with 0.2% (w/v) DTT (Sigma-Aldrich) or 0.3% (w/v) iodoacetamide (Bio-Rad), respectively. The equilibrated strips were loaded on 12.5% polyacrylamide gels to perform SDS-PAGE (Laemmli, 1970), running on PROTEAN II system (Bio-Rad). The gels were stained overnight with SYPRO® Ruby Protein Gel Stain (Life Technologies) according to the supplier's protocol. Gel image documentation was carried out using ChemiDoc™ XRS imaging system (Bio-Rad). Raw pictures were analysed using PDQuest Advanced software version 8.0.1 (Bio-Rad) including detection of spots, normalization using local regression, spot matching and differential expression analysis. The coefficient of variation (CV) intra-pools was also analysed for each spot of interest in each treatment group and showed in **Table 7**. Protein spots were considered as differentially expressed when expression level was at least 1.5-fold different compared to the control group and when the differences were detected as significant at $p<0.01$ by two tailed Student's t-test.

2.7 LC-MS/MS analysis

Spots from SYPRO-stained gilthead seabream skin mucus 2-DE gels (n = 3) were picked, excised and subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 ng μl^{-1} trypsin (V511A; Promega) as described elsewhere (Shevchenko et al., 1996). Peptide mixtures containing 0.1% formic acid were loaded onto a nanoACQUITY UltraPerformance LC (Waters), containing a 5 µm Symmetry C18 Trap column (180 µm × 20 mm; Waters) in front of a 1.7 µm BEH130 C18 analytical column

(100 µm × 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with a flow of 0.4 µl min^{-1} eluted to a Q-TOF Ultima mass spectrometer (Micromass/Waters). The samples were run in data dependent tandem mass spectrometry (MC/MC) mode. Peak lists were generated from MS/MS by the ProteinLynx Global server software (version 2.2; Waters) and submitted to MASCOT search engine (version 2.5.1) and searched against NCBInr with the following parameters: maximum one missed cleavage by trypsin, peptide mass tolerance 100 ppm, MS/MS ion tolerance set to 0.1 Da, carbamidomethylation of cysteine selected as fixed modification and methionine oxidation as variable modification. Protein hits not satisfying a significance threshold ($p<0.05$) or with low sequence coverage were further searched against Swissprot and vertebrate EST (expressed sequence tags) databases, taxonomy *Actinopterygii*.

2.8 Primer design

Primers were designed by OligoPerfect™ Designer (Life Technologies) from *S. aurata* sequences which are available in NCBInr database. Details regarding oligonucleotide primers and their attributes are given in Table 7.

Table 7. Information of primers used for qPCR study.

Gene & GenBank	Amplicon size	R^2	Efficiency (%)	Sequence (5' → 3')
c3 **CX734936**	147	0.978	109	F: ATAGACAAAGCGGTGGCCTA R: GTGGGACCTCTCTGTGGAAA
nkefb **GQ252680**	99	0.998	96	F: CAAGCAGTAAATGTGAAGGTC R: GATTGGACGCCATGAGATAC
lyz **AM749959**	280	0.986	95	F: CCAGGGCTGGAAATCAACTA R: CCAACATCAACACCTGCAAC
nccrp1 **AY651258**	100	0.995	93	F: ACTTCCTGCACCGACTCAAG R: TAGGAGCTGGTTTTGGTTGG
ef1a **AF184170**	115	0.987	113	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA
rps18 **AM490061**	109	0.937	117	F: CGAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC

2.9 Gene expression analysis

The mRNA levels corresponding to four differentially expressed immune-related proteins in the skin of the experimental fish were analysed by real-time PCR (qPCR). RNA was extracted from 50 mg of skin from five specimens of gilthead seabream for each treatment group and control group using QIAzol lysis reagent method (Qiagen) as described elsewhere (Lokesh et al., 2012). Quality of total RNA was checked on 1% agarose gel, followed by the quantification using the Qubit® RNA assay kit and Qubit® 2.0 fluorometer (Life Technologies). The complementary DNA (cDNA) was synthetised from 1 µg of RNA using QuantiTec Reverse Transcription Kit (Qiagen). Ten times diluted cDNA was used to conduct qPCR on ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as described elsewhere (Cordero et al., 2015a) and the $^{2-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). Each plate subjected to qPCR contained a negative control for cDNA template (water) as well as a control for reverse transcription. No amplification product was observed in negative controls and neither primer-dimer formation nor secondary structures were observed in any case. Additionally, a three-fold serially diluted cDNA (pooled) was included for each plate of each gene to evaluate the efficiency of qPCR reaction based on standard curve method, using the formulae $E = 10^{(-1/\text{slope}) - 1} \times 100$. All qPCR reactions were carried out in duplicate and quantification cycle (Ct) values of each gene (target) were converted into relative quantities. Normalization factors were calculated as the geometric mean of relative quantities of reference genes elongation factor 1 alpha (*ef1a*) and ribosomal protein S18 (*rps18*) using the BestKeeper© algorithm (Pfaffl et al., 2004).

The qPCR data of target genes (*nkefb* and *nccrp1*) were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test post-hoc analysis. Normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. A non-parametric Kruskal–Wallis H test was used

when data did not meet parametric assumptions, concretely for *c3* and *lyz* genes. Data are expressed as relative gene expression of each target gene (mean ± SEM). Statistical tests were performed using SPSS software v19.0 (SPSS) and the differences of means were considered significant at $p<0.05$.

3. Results and discussion

In our experience working with proteomic tools (Kulkarni et al., 2014; Rajan et al., 2013b, 2011) and taking into account previous proteomic studies (Chong et al., 2006; Cordeiro et al., 2012; Ibarz et al., 2010; Rufino-Palomares et al., 2011), 2-DE followed by coupled tandem LC-MS/MS provides good resolution for comparatives studies. Furthermore, the development of fluorescent dyes from SYPRO group get great performance (in terms of linear range and limit of detection) (Berggren et al., 2000), compared with other classical dyes such as silver or Coomassie brilliant blue.

A range from 431 to 452 spots were detected in total. Next, an exhaustive analysis, adjusting and optimizing the quantification of spots, and discarding spots that were not consistent, was carried out. The present study shows several differentially expressed proteins (through 22 identified spots) in the skin mucus from gilthead seabream specimens (**Tables 8, 9**): fed with commercial diet and non-stressed (control, **Figure 20A**), fed with probiotic and non-stressed (**Figure 20B**), fed commercial diet and stressed by overcrowding (**Figure 20C**), fed probiotic diet and stressed by overcrowding (**Figure 20D**).

Table 8. Details of the differentially expressed protein spots in skin mucus of *S. aurata* after dietary probiotic administration and/or overcrowding stress. Theoretical isoelectric point (pI) and molecular weight (MW) in kDa; total score (S) and coverage (C) in %; total matched peptides (Mp); total unique peptides (Up).

Spot	Protein name	Organism / Accession no	pI/MW	S/C	M_p/U_p
S1	C-type lectin	*S. aurata* / CB177017	7.2/28.8	59/3	1/1
S2	Leukocyte elastase inhibitor	*S. aurata* / FM146914	8.8/28.3	80/12	2/2
S3	Nonspecific cytotoxic cell receptor protein-1	*S. aurata* / AAT66406	5.0/26.6	59/12	2/2
S4	Apolipoprotein A1	*S. aurata* / O42175	5.2/29.6	50/13	3/3
S5	Profilin	*S. aurata* / FM146227	9.6/21.3	337/46	7/7
S6	Inositol monophosphate	*Oreochromis mossambicus* / AFY10067	5.3/31.2	284/30	7/1
S7	Beta actin	*O. mossambicus* / P68143	5.3/42.1	176/15	5/5
S8	F-type lectin	*Oplegnathus fasciatus* / BAK38714	5.7/31.0	144/8	2/2
S9	Triose phosphate isomerase A	*Danio rerio* / Q1MTI4	4.9/29.2	33/5	1/1
S10	PREDICTED: aldose reductase-like	*Haplochromis burtoni* / XP_005915666	6.2/36.0	201/13	6/5
S11	Peroxiredoxin 2	*Oncorhynchus mykiss* / Q91191	7.0/22.3	46/5	1/1
S12	Complement c3	*S. aurata* / ADM13620	8.1/187	138/2	5/5
S13	Complement c3	*S. aurata* / ADM13620	8.1/187	110/1	3/3
S14	Keratin, type I cytoskeletal 50 kDa	*Carassius auratus* / Q90303	5.1/49.7	34/6	2/2
S15	Lysozyme	*S. aurata* / CAO78618	6.9/20.3	217/31	4/4
S16	ADP-ribosylation factor GTPase-activating protein	*Carassius auratus* / AM930069	9.4/23.6	60/9	1/1
S17	Glutathione S-transferase	*S. aurata* / AAQ56182	8.5/24.8	120/11	3/3
S18	NADP-dependent isocitrate dehydrogenase	*S. aurata* / AGU38793	7.2/38.1	79/10	3/3
S19	PREDICTED: peroxiredoxin-6-like	*Astyanax mexicanus* / XP_007259536	5.8/24.8	59/4	1/1
S20	Glutathione S-transferase	*S. aurata* / AFV39802	6.9/25.5	286/31	6/4
S21	Beta actin	*Morone saxatilis* / AAA53024	5.1/31.7	92/15	3/3
S22	14-3-3 protein	*S. aurata* / AM957903	4.6/26.9	114/9	2/2

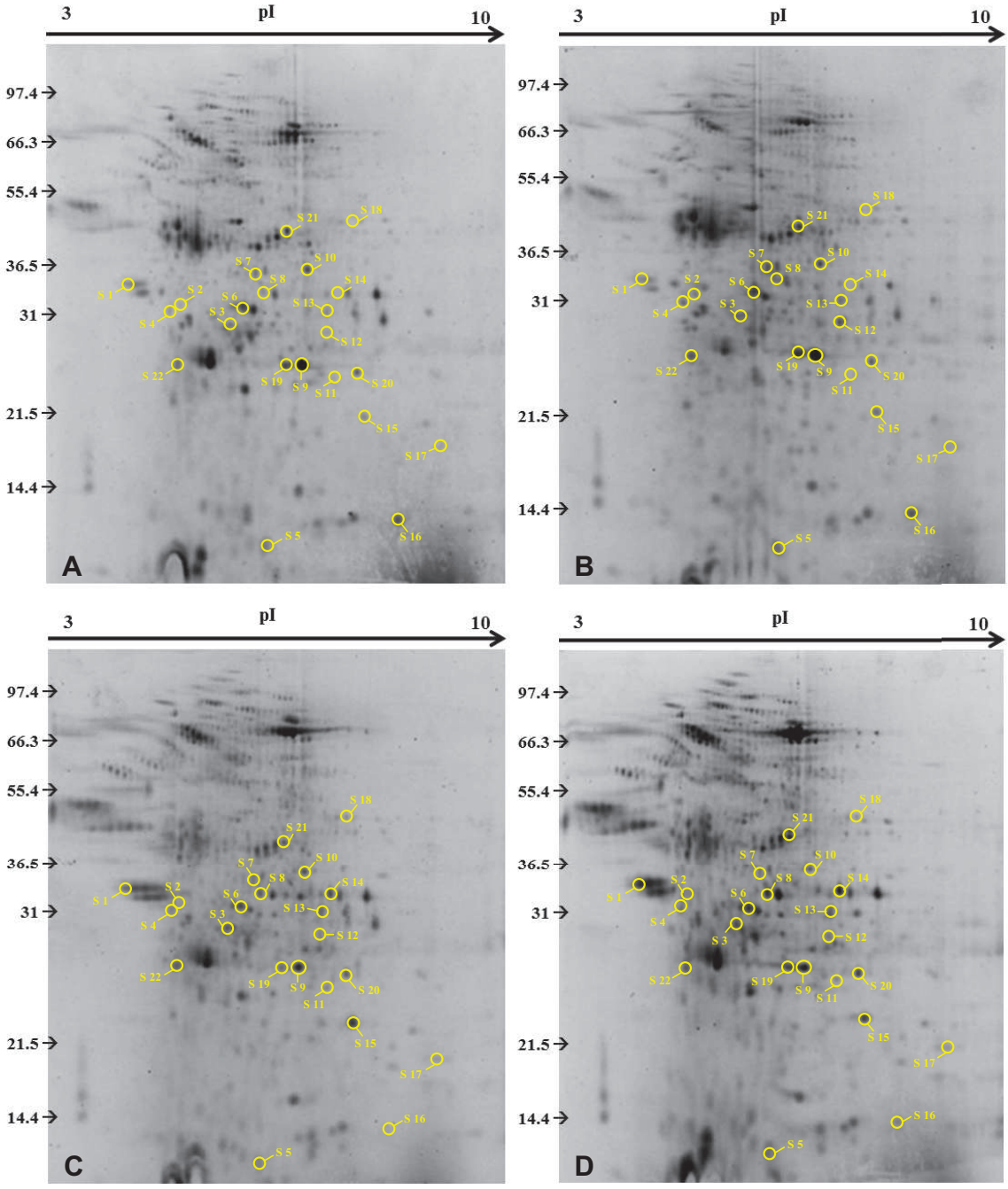


Figure 20. Representative 2-DE gels of skin mucus of *S. aurata* for each experimental group: commercial diet (A), probiotic diet (B), overcrowding stress (C) and probiotic diet and overcrowding stress (D). All the four gels were generated from samples at 30 days of treatment in triplicates. Skin mucus proteins were isoelectrically focused on 17 cm IPG strips (pI 3–10) and subjected to 12.5% SDS-PAGE. The 2-DE gels were stained with SYPRO® Ruby protein

gel stain and the spots identified were annotated using the data from LC-MS/MS. The spot numbers represented in gels correspond to the protein identities mentioned in **Table 8**.

The intra-group variability of differentially expressed spots was in a range between 0.5% and 9.3% (**Table 9**). Further, a literature-based comparison is showed in **Table 10**.

Spot	Protein name	Fold change relative to control group (CV intra-groups)		
		Probiotic	Overcrowding	Probiotic + Overcrowding
Fifteen days				
S12	Complement c3 (C3)	↑ 1.95 (2.8%)		↑ 1.6 (3.9%)
S19	Peroxiredoxin 6-like (PRDX6)		↓ 0.63 (1.8%)	
S20	Glutathione S-transferase (GST)	↑ 1.97 (6.3%)		↑ 4.20 (3.3%)
S21	Beta-actin (ACTB)		↓ 0.56 (2.1%)	
S22	14-3-3 (YWHAB)	↑ 1.58 (5.2%)	↓ 0.55 (3.3%)	↓ 0.38 (1.7%)
Thirty days				
S1	C-type lectin (CLEC)		↑ 1.58 (3.6%)	↑ 2.63 (3.2%)
S2	Leucocyte elastase inhibitor (LEI)	↑ 2.18 (4.1%)	↓ 0.64 (1.4%)	↑ 2.36 (1.9%)
S3	Nonspecific cytotoxic cell receptor protein 1 (NCCRP-1)	↑ 3.27 (0.8%)	↑ 2.56 (5.8%)	↑ 2.05 (2.8%)
S4	Apolipoprotein A-1 (APOA1)	↑ 2.31 (1.6%)		↑ 1.67 (2.5%)
S5	Profilin (PFN)			↑ 1.85 (8.2%)
S6	Inositol monophosphate (IMPA)		↓ 0.39 (3.7%)	↓ 0.51 (4.9%)
S7	Actin beta (ACTB)	↑ 1.95 (7.2%)		
S8	F-type lectin (FBL)	↑ 1.54 (4.3%)	↑ 1.59 (2.1%)	↑ 2.79 (3.6%)
S9	Triose phosphate isomerase A (TPIA)		↓ 0.43 (1.9%)	↓ 0.57 (3.4%)
S10	Aldose reductase-like (AR)			↓ 0.41 (0.5%)
S11	Natural killer cell enhancing factor b (NKEF2)		↑ 9.69 (3.9%)	↑ 4.79 (5.6%)
S13	Complement c3 (C3)	↑ 1.74 (2.3%)	↑ 1.52 (3.5%)	↑ 1.64 (2.5%)
S14	Keratin, type I cytoskeletal 50 kDa (KRT1)		↑ 3.40 (5.0%)	↑ 4.31 (4.8%)
S15	Lysozyme (LYZ)	↑ 1.27 (6.1%)	↑ 4.58 (2.3%)	↑ 2.80 (1.8%)
S16	ADP-ribosylation factor (ARF) GTPase-activating protein (GAP)	↑ 1.39 (5.5%)	↓ 0.01 (2.7%)	↓ 0.14 (9.3%)
S17	Glutathione S-transferase (GST)		↑ 1.54 (3.1%)	↑ 1.58 (4.2%)
S18	NADP-dependent isocitrate dehydrogenase (IDH)		↑ 5.12 (4.4%)	↑ 5.20 (3.9%)

Table 9. List of proteins that are differentially expressed in skin mucus of *S. aurata* after dietary probiotic administration and/or overcrowding stress for 15 and 30 days. ↑ and ↓ indicate over- and under-expression of the proteins at $p<0.01$, respectively. Coefficient of variation (CV) in percentage (%) from different pools (n = 3) is represented in brackets.

Table 10. List of differentially expressed proteins in the present study: literature-based comparison about presence of these proteins in skin mucus of other fish species after 2-DE spot detection.

Protein	Reported in skin mucus?	Fish species	Reference
14-3-3	Yes	*D. labrax* *G. morhua* *S. aurata* *S. salar*	(Cordero et al., 2015a) (Rajan et al., 2011) (Jurado et al., 2015) (Provan et al., 2013)
ACTB	Yes	*D. labrax* *G. morhua* *S. aurata*	(Cordero et al., 2015a) (Rajan et al., 2011) (Jurado et al., 2015)
APOA1	Yes	*D. labrax* *G. morhua* *S. aurata* *G. morhua*	(Cordero et al., 2015a) (Rajan et al., 2011) (Jurado et al., 2015) (Rajan et al., 2013b)
AR	No	*S. aurata*	(Cordero et al., 2016c)
ARFGAP	No	*S. aurata*	(Cordero et al., 2016c)
C3	Yes	*D. labrax*	(Cordero et al., 2015a)
CLEC	Yes	*Anguilla japónica*	(Suzuki et al., 2003)
FBL	Yes	*D. labrax*	(Cordero et al., 2015a)
GST	Yes	*D. labrax* *G. morhua* *S. aurata*	(Cordero et al., 2016c) (Rajan et al., 2013b, 2011) (Jurado et al., 2015)
IDH	No	*S. aurata*	(Cordero et al., 2016c)
IMPA	Yes	*G. morhua* *S. aurata*	(Rajan et al., 2011) (Sanahuja and Ibarz, 2015)
KRT1	Yes	*S. aurata*	(Sanahuja and Ibarz, 2015)
LEI	Yes	*D. labrax* *G. morhua*	(Cordero et al., 2015a) (Rajan et al., 2011)
LYZ	Yes	*D. labrax* *G. morhua* *S. aurata*	(Cordero et al., 2015a) (Rajan et al., 2011) (Jurado et al., 2015)
NCCRP-1	No	*S. aurata*	(Cordero et al., 2016c)
NKEF2	Yes	*S. aurata*	(Jurado et al., 2015)
PFN	Yes	*D. labrax* *S. aurata*	(Cordero et al., 2015a) (Sanahuja and Ibarz, 2015)
PRDX6	Yes	*G. morhua*	(Rajan et al., 2013b)
TPIA	Yes	*S. aurata*	(Cordero et al., 2016c)

3.1 Effects of Pdp11 and/or overcrowding stress on structural proteins

Structural proteins are a major group in seabream mucus proteins and include several isoforms of actins, keratins, tubulins, tropomyosin 4-2, cofilin-2 and filamin-A-like (Jurado et al., 2015), and some of them are modulated in the present study (**Tables 8, 9**). First, actin beta (ACTB; spots 7 and 21) was found differentially expressed in both experimental times (**Table 9**). At 15 days, the levels of ACTB (around 42 kDa) in skin

mucus were decreased in seabream specimens under overcrowding stress compared to the control group. However, a different ACTB (around 35kDa) was found up-regulated almost 2-fold after probiotic dietary administration in non-stressed specimens compared to the control group. ACTB is among the most common structural proteins in the skin mucus and seems to be a normal protein with some functionality in this surface rather a product of the epidermal cell disruption (Easy and Ross, 2009). In fact, the presence of its proteolytic products, as in our study, has been related to some stressful situations and these products could enhance the immune response (Easy and Ross, 2010). Moreover, the decrease in the 42 kDa isoform after overcrowding stress could also indicate that this isoform is fragmented in several different products from the 35 kDa isoform, a hypothesis that should be further evaluated.

Keratin, type I cytoskeletal 50 kDa (KRT; spot 14) protein was also found over-expressed in seabream specimens under overcrowding stress after 30 days compared to the control group (Table 9). At this point, profilin (PFN; spot 5) was also increased in fish fed the probiotic diet under overcrowding stress compared to the control group. These data suggest a role different than the strictly structural, at least in the epidermal mucus. For example, KRTs of fish mucus have shown some antibacterial activity due to their pore-formation ability (Molle et al., 2008), and this fact points to the increase of this immune activity in seabream specimens. In fact, seabream exposed to heavy metal stressors showed significant increments in the bactericidal activity of the skin mucus and altered protein profiles (Guardiola et al., 2015). These data together suggest that the altered structural proteins in the skin mucus might have some unknown biological role at present, which merits further evaluation.

3.2 Effects of Pdp11 and/or overcrowding stress on metabolism proteins

The presence of some proteins involved in the cellular metabolism in seabream mucus is also regulated by dietary probiotic administration and/or overcrowding stress (Tables 8, 9). Apolipoprotein A-1 (APOA1; spot 4) was over-expressed with 2.31-fold increase

after probiotic diet both in non-stressed group and overcrowding stressed group compared to the control group at 30 days (Table 9). Despite of primary role of APOA1 in the reverse cholesterol transport, its presence in the skin mucus and over-expression after probiotic Pdp11 administration suggest not only an extracellular role of antimicrobial activity against pathogens in gilthead seabream, as occurs in striped bass (Johnston et al., 2008), but also an improving of the antimicrobial properties inferred by the Pdp11 probiotic diet. In fact, this protein has been shown to be increased in infected Atlantic salmon and Atlantic cod and showed bactericidal activity (Concha et al., 2004; Easy and Ross, 2010; Rajan et al., 2013b, 2011).

The predicted 14-3-3 protein (spot 22) was over-expressed in probiotic fed fish 1.58-fold but under-expressed in stressed fish (0.55-fold) and stressed and Pdp11-fed fish (0.38-fold), compared to the control group at 15 days (Table 9). Despite the fact that this protein was previously identified in skin mucus of gilthead seabream (Jurado et al., 2015), to our knowledge this is the first time that over-expression has been found after any probiotic diet, Pdp11 in this study. In addition, this protein has also reported in skin mucus of teleosts such as European sea bass (Cordero et al., 2015a) or Atlantic cod (Rajan et al., 2011) but further studies are needed to clarify its implication in skin mucus.

NADP-dependent isocitrate dehydrogenase (spot 18) was over-expressed more than 5-fold in both overcrowding groups compared to the control group at 30 days. At cytosolic level, isocitrate dehydrogenase catalyses oxidative decarboxylation of isocitrate to 2-oxoglutarate and require NAD+ or NADP+, producing NADH and NADPH, respectively (Koshland et al., 1985), playing a critical role protecting cells against oxidative stress (Lee et al., 2002). At extracellular level, this protein is for the first time reported in fish mucus, however it has been previously described in human cervical mucus (Panicker et al., 2010).

Inositol monophosphate (IMPA; spot 6) and triosephosphate isomerase A (TPIA; spot 9) were under-expressed in overcrowding groups compared to the control group at 30 days (**Figure 20C**). In certain agreement with our data, *impa* gene was up-regulated in European eel and Nile tilapia fish species stressed by seawater change (Kalujnaia et al., 2013). At proteome level, this protein was identified in skin mucus of Atlantic cod after infection (Rajan et al., 2013b), suggesting a role in disease protection on fish.

The predicted aldose reductase-like (spot 10) was under-expressed in fish fed the probiotic diet under overcrowding stress after 30 days, compared to the control group (**Figure 20D; Table 9**). Aldose reductase catalyses the NADPH-dependent conversion of glucose to sorbitol (Petrash, 2004), and it has been associated to oxidative stress (Spycher et al., 1997), which may suggest a putative role against overcrowding stress but more information is needed to confirm this.

Glutathione S-transferase (GST; spots 17 and 20) was over-expressed in both probiotic fed groups at 15 days, as well as over-expressed in overcrowding groups at 30 days, compared to the control group (**Table 9**). GST is a family of proteins which are involved in biotransformation of compounds including toxic substances and oxidative stress products, transport of ligands, and regulation of signalling pathways (Burmeister et al., 2008). It was reported that GST levels increased in the liver of fish after exposure to pyrethroids (Sayeed et al., 2003). In addition, GST omega 1 was over-expressed in skin mucus of Atlantic cod after infection (Rajan et al., 2013b). According with these studies, after 30 days of overcrowding stress the oxidative stress by GST over-expression in skin mucus of gilthead seabream could increase.

ADP-ribosylation factor GTPase-activating protein (ARFGAP; spot 16) was over-expressed in probiotic diet fed group, and under-expressed in both overcrowding stressed groups, compared to the control group, at 30 days (**Table 9**). ARFGAP has been associated with Golgi organization and actin cytoskeletal organization, mediating cell adhesion (Mazaki et al., 2001). In relation with that, probiotic diet promotes over-

expression of beta actin (1.95-fold) as well as ARFGAP over-expression (1.39-fold) in skin mucus of gilthead seabream. Similarly, ACTB and ARFGAP showed under-expression in skin mucus of gilthead seabream under overcrowding stress at 15 and 30 days.

Overall, our data show some important regulation of seabream skin mucus metabolic proteins after feeding a probiotic and/or after overcrowding stress. However, the implications of these proteins in the surface mucus should be further ascertained in order to relate them, as some studies suggest, with other different roles such as immunity.

3.3 Effects of Pdp11 and/or overcrowding stress on immune-related proteins

Another group of proteins with known immune functions have been found to be regulated in our study (**Tables 8, 9**). First, C3 (spots 12 and 13) was over-expressed in both probiotic fed groups at both trial times, and overcrowding stress group at 30 days (**Table 9**). The highest over-expression was found in specimens fed Pdp11 probiotic diet with 1.95-fold and 1.74-fold at 15 and 30 days, respectively (**Figure 20B**). In previous studies, complement activity was undetected in the skin mucus of seabream (Guardiola et al., 2014b; Sanahuja and Ibarz, 2015), but it was reported in sea bass (Cordero et al., 2015a). At gene level, a *c3* up-regulation was found after yeast dietary administration in gilthead specimens (Reyes-Becerril et al., 2008) whilst bathing of turbot with acid lactic probiotics also up-regulated its transcription in several tissues (Muñoz-Atienza et al., 2014). Taking this into account, our results at protein level of C3 over-expression in skin mucus of seabream after Pdp11 probiotic diet may indicate beneficial effects in the key component C3 in skin mucosal immunity, which acts against pathogens. Furthermore, the two identified spots with a little difference in MW could indicate isoforms which are influenced not only by probiotic intake but also by overcrowding stress at 30 days.

Lectins are a group of sugar binding proteins which are involved in both innate and adaptive immunity (Vasta et al., 2004), including pathogen recognition and neutralization (Weis et al., 1998). Furthermore, a diversity of lectins was reported in fish skin mucus (Suzuki et al., 2003). On one hand, in our study, a C-type lectin (spot 1) was identified as over-expressed in both overcrowding stressed fish, reaching 2.61-fold in the group fed with probiotics under stress conditions, compared to the control group (**Table 9, Figure 20D**). On the other hand, F-type lectin (spot 8) was over-expressed after probiotic diet and/or overcrowding stress, with the maximum protein expression (2.79-fold) in the skin mucus of seabream specimens under both treatments (**Table 9**). The fact of finding the maximum up-regulation in Pdp11 probiotic diet groups could suggest the improvement of skin mucosal immunity by Pdp11 probiotic effect and no negative impact of the tested stress. In this regard, skin mucus of seabream specimens under stress caused by exposure to heavy metals showed increased levels of F-lectin as detected by western blotting (Guardiola et al., 2015).

Leucocyte elastase inhibitor (LEI; spot 2) was over-expressed in the skin mucus of specimens fed with probiotic, stressed or not, but and under-expressed in those under overcrowding stress, compared to the control group (**Table 9**). LEIs are inhibitory proteases regulating tissue destruction and inflammation, commonly associated to several skin diseases (Matejusová et al., 2006). Our results suggest that Pdp11 may avoid deleterious effects in skin. In addition, it was found that LEI has antimicrobial properties in mucosal fluids (Ashcroft et al., 2000) and is up-regulated by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Sallenave, 2000). Based on this, LEI under-expression in overcrowding stress fish may suggest an increased susceptibility of skin to infections.

Nonspecific cytotoxic cell receptor protein 1 (NCCRP1) is the receptor of NCC, evolutionary precursors of mammalian NK cells, which are involved in innate cell-mediated cytotoxicity on fish, including gilthead seabream (Cuesta et al., 2005). It was

reported that *nccrp1* is up-regulated by overcrowding stress (Tapia-Paniagua et al., 2014). Besides, other study with probiotics has demonstrated up-regulation of *nccrp1* after *in vitro* incubation in fish leucocytes (Lazado et al., 2010). NCCRP1 (spot 3) was over-expressed in the overcrowding groups fed with probiotic or with control diets after 30 days (**Table 9**).

Peroxiredoxin 2 (PRDX2) or natural killer enhancing factor B (NKEF-B; spot 11) was over-expressed in overcrowding stress groups at 30 days, fed with the Pdp11 probiotic or not, compared to the control group. Additionally, a predicted peroxiredoxin 6-like (spot 19) was under-expressed in overcrowding stress group at 15 days (**Table 9**). PRDXs are a family of antioxidant enzymes which act protecting cells from oxidative damage (Rhee et al., 2005) but also show an important role in immunity since extracellular NKEF-A or PRDX1 acts as "endogenous" danger signal by binding to danger signal sensors (Riddell et al., 2010). We suggest a similar mechanism in skin mucus of gilthead seabream under overcrowding stress since NKEF showed almost 9.7 and 4.8-fold increase and also because recently, not only in gilthead seabream but also in European sea bass, it has been demonstrated *nkefa* and *nkefb* up-regulation after infection (Esteban et al., 2013).

Last, LYZ (spot 15) was over-expressed in fish fed probiotic diet and/or under overcrowding stressed groups, compared to the control group, at 30 days (**Table 9**). In the present study, the theoretical and practical MW and pI converge with 20.3 kDa and 6.9, respectively. LYZ, widely known in fish, is an enzyme which hydrolyses N-acetylmuramic acid and N-acetylglucosamine which are constituents of the peptidoglycan layer of bacterial cell walls (Elllis, 1999). For this reason, LYZ is a key molecule in skin mucosal immunity, conferring protection against pathogens. In agreement with our results it has been demonstrated that different stressors, including overcrowding stress, increase lysozyme levels in rainbow trout (Demers and Bayne, 1997). Furthermore, some probiotics such as *Bacillus* ssp. or *Lactobacillus* ssp.

increase LYZ levels in fish (review in (Bidhan et al., 2014)]. Our results demonstrated that dietary Pdp11 increases the LYZ expression in skin mucus of gilthead seabream even under overcrowding stress situation which could be considered very interesting results for fish farmers.

3.4 Gene expression profile

The analysis of four selected genes (*c3*, *nkefb*, *nccrp1* and *lyz*) in the skin of gilthead seabream was performed by qPCR (Figure 21). Strikingly, neither *c3*, *nkefb* nor *nccrp1* gene expression was significantly regulated by probiotic and/or overcrowding stress and these results were not correlated to the regulations observed at protein level. This could indicate differential half-lives for mRNA and proteins, regulations out of the transcriptional control for these proteins or that the mRNA studied and the proteins detected are not the same isoforms. Furthermore, the option that mucus proteins are not produced by epidermal cells can be also considered but needs further confirmation. Our results are in an agreement with a previous study which demonstrated that overcrowding stress did not modify *c3* mRNA levels in liver from gilthead seabream (Mauri et al., 2011). To our knowledge, there is no available information about probiotic or stress regulation of *nkefb* and *nccrp1*. Strikingly, *lyz* gene expression showed up-regulation in probiotic and/or overcrowding stressed groups compared to the control group, at 15 days (Figure 21D), although the highest increase of *lyz* gene expression was found in probiotic fed under non-stressed conditions, around 8-fold, compared to the control group. Present results suggest that Pdp11 probiotic diet may confer protection in skin mucosal immunity through an increase of lysozyme, not only at protein level in mucus but also at gene expression level in skin of gilthead seabream.

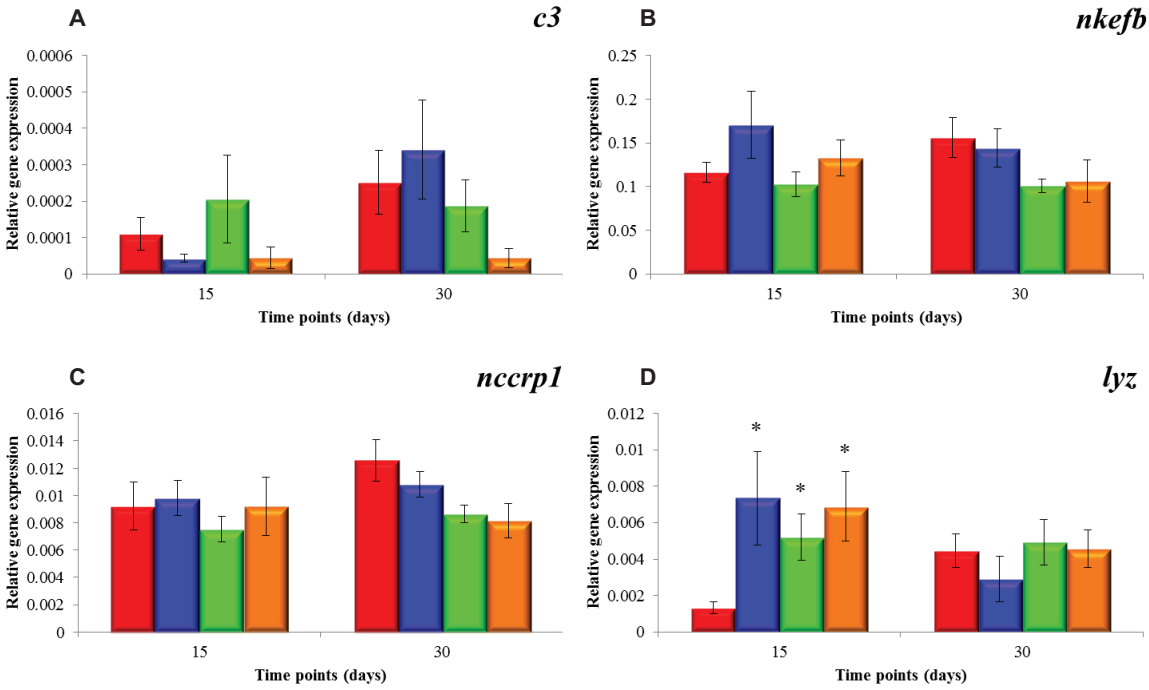


Figure 21. Gene expression mRNA levels of four selected genes *c3* (A), *nkefb* (B), *lyz* (C) and *nccrp1* (D) in skin from *S. aurata* were analysed. Control groups are represented with red bars, probiotic diet groups with blue bars, overcrowding stressed group with green bars, and probiotic diet and overcrowding stressed group with orange bars. Transcripts were quantified by qPCR and normalised using the geometric average of the reference genes elongation factor 1 alpha (*ef1a*) and ribosomal protein S18 (*rps18*). The values are presented as mean ± SEM (n = 5). For the statistical analysis, ANOVA and Tukey's post-hoc test was performed in the case of *nkef* and *nccrp1* (Levene's test values were p = 0.052 and p = 0.232, respectively); for *c3* and *lyz* Kruskal-Wallis H test was performed (Levene's test values are p = 0.000 in both genes). The asterisks indicate significant difference ($p<0.05$) in expression levels of each treatment group compared to the control group at the same time points.

Functional and nutritional feedings such as probiotics are taking importance in aquaculture in order to prevent infections. The great interest of this study was to find out the molecules which are involved in the mechanism of protection at a mucosal level, as well as studying if Pdp11 is useful against overcrowding stress. Molecules such as F-type lectin, C3, LEI, NCCRP1 and LYZ, through over-expression by Pdp11 intake could be directly involved in mucosal protection, and consequently in fish well-

being. Moreover, overcrowding stress had positive (such as in the case of C3 and LEI) or little negative impact on the skin mucus proteomic profile, which was even improved by probiotic feeding, suggesting that the skin mucus is not affected in a negative way by overcrowding. And last but not least, in the case of LYZ there is positive correlation between mRNA levels and protein expression in skin mucus suggesting a local synthesis of one of the most important antimicrobial protein.

Chapter 6

Chronic wounds alter the proteome profile of gilthead seabream skin mucus

Abstract

Wounds are probably the most important and frequent lesions on fish body surface. In the present paper, we have studied the alteration in the proteome map of skin mucus of gilthead seabream through 2-DE followed by LC-MS/MS approach after chronic wounds caused with an electric toothbrush. Our study revealed a general decrease of immune-related, stress-related and metabolic and structural molecules at protein level. This study showed for the first time the important and notable alteration caused by chronic wounds in the skin mucus proteome, whose molecules involved are mostly immune-related (C3, APOA1, H2A, H2B, H4 and IgM) and stress-related (NKEF1, NKEF2, SOD, GST3 and HSC-70) molecules. These results help to understand why fish with skin wounds are more susceptible to infection than fish with a normal skin. In addition, we postulate these molecules as biomarkers of fish health, which can be studied with non-invasive techniques in skin mucus.

1. Introduction

Intensive fish farming increases the occurrence of injuries and diseases, commonly associated with the appearance of ulcers or wounds in the skin causing major economic losses (Balcázar et al., 2006; Esteban, 2012). Specifically in the market of gilthead seabream, these injuries and diseases in the skin such as the white nodules from lymphocystis disease (Borrego et al., 2015; Cordero et al., 2016a; Sarasquete et al., 1998) or the physical wounds that increases the susceptibility of bacterial vibriosis (Balebona et al., 1998) are critical.

Skin mucus is mainly secreted by goblet cells present in the epidermis of fish, protecting as a mechanical, physical, chemical, biological and immunological barrier against any external stressors (Esteban, 2012; Shephard, 1994). In recent years, skin mucus has becoming in a hot topic as a faithful mirror of the immune status of fish (Brinchmann, 2016). Thus, many humoral immune activities such as proteases, antiproteases, peroxidases, esterases, alkaline phosphatases, lysozyme or immunoglobulins have been evaluated in skin mucus (Cordero et al., 2016b; Guardiola et al., 2014a; Ross et al., 2000). Apart from the individual characterization of antimicrobial peptides (Rakers et al., 2013), immunoglobulins (Xu et al., 2013) or lectins (Ng et al., 2015), the recent advances in high-throughput proteomics research methods have been used for identification and quantification of proteins (Gstaiger and Aebersold, 2009). Homology-driven proteomics is a major approach for identification of proteins whose sequences are available (Junqueira et al., 2008); however, identification of unknown proteins often relies on the similarity (rather than identity) comparing with homologous protein sequences from phylogenetically related species (Liska and Shevchenko, 2003), especially, as happens in gilthead seabream, when the specific genome is not publically available and/or the transcriptome data are scarce.

Through this approach, the proteomic mapped of skin mucus proteome have been recently studied in several fish species such as Atlantic cod (Rajan et al., 2011),

European sea bass (Cordero et al., 2015a), and gilthead seabream (Jurado et al., 2015; Sanahuja and Ibarz, 2015). These proteomes have allowed the discovery of new molecules involved in protection and immunity located at this mucosal surface. Besides, changes of skin mucus proteome with differentially expressed proteins have been studied after infection (Easy and Ross, 2009; Rajan et al., 2013b; Valdenegro-Vega et al., 2014), both handling or crowding stress (Cordero et al., 2016c; Easy and Ross, 2010) and after parental care (Chong et al., 2006). However, despite of being relevant for fish health, to the best of our knowledge, there are no available studies regarding the changes on the skin mucus proteome after wounds.

The aim of this work was to study the alteration of the skin mucus proteome after chronic wounds in gilthead seabream using 2-DE followed by LC-MS/MS. This study provides a suitable idea about the changes of specific proteins involved in immunity, stress and metabolism as well as structural proteins related to regeneration and cicatrization present skin mucus of gilthead seabream.

2. Materials and methods

2.1 Animal care

Forty specimens of gilthead seabream (4.7 ± 1.3 g and 7.4 ± 0.6 cm), obtained from a local farm (Murcia, Spain), were kept in running seawater aquaria (flow water 900 l h^{-1}) at 28‰ salinity, 22ºC and a photoperiod of 12h light: 12h dark. Fish were fed daily at 2% rate of fish biomass per day with commercial diet (Skretting). All the fish handling procedures were approved by Ethical Committee of the University of Murcia.

2.2 Chronic wounds

Chronic wounds were performed in the skin with an electric toothbrush for 30 sec in both sides of the fish body. The procedure was repeated once each two days for four days, and two days later samples were collected. Control group was handled in similar conditions without triggering wounds.

2.3 Mucus collection and protein purification

Forty specimens (twenty fish per group) were anesthetized with 100 mg l^{-1} MS222 prior to sampling. After causing the wounds, mucus was gently scraped off from the skin surface, avoiding blood, urine and faeces during collection (Palaksha et al., 2008). In order to obtain enough amount of mucus, samples were pooled as described elsewhere (Cordero et al., 2016c), divided into four groups (n = 10), two control and two wounds, respectively. Mucus was transferred into tubes of 15 ml and stored at -80ºC until use as described elsewhere (Cordero et al., 2016b).

The pooled samples were treated as described in section 2.5 of Chapter V (page 115).

2.4 2-DE and LC-MS/MS analysis

The samples for 2-DE were treated as described in section 2.6 of Chapter V (page 116). Spots from SYPRO-stained gilthead seabream skin mucus 2-D gels (n = 3) were picked, excised and subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 ng μl^{-1} trypsin (V511A; Promega) as described elsewhere (Shevchenko et al., 1996). Peptide mixtures containing 0.1% formic acid were loaded onto a nanoACQUITY UltraPerformance LC (Waters), containing a 5 µm Symmetry C18 Trap column (180 µm × 20 mm; Waters) in front of a 1.7 µm BEH130 C18 analytical column (100 µm × 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with a flow of 0.4 µl min^{-1} eluted to a Q-TOF Ultima mass spectrometer (Micromass/Waters). The samples were run in data dependent tandem mass spectrophotometry (MC/MC) mode. Peak lists were generated from MS/MS by Mascot Distiller Workstation and submitted to MASCOT search engine (version 2.5.1) and searched against NCBInr with the following parameters: maximum one missed cleavage by trypsin, peptide mass tolerance 100 ppm, MS/MS ion tolerance set to 0.1 Da, carbamidomethylation of cysteine selected as fixed modification and methionine oxidation as variable modification. Protein hits not satisfying a significance threshold

(p<0.05) or with low sequence coverage were further searched against Swissprot and vertebrate EST (expressed sequence tags) databases, taxonomy *Actinopterygii*.

2.5 Statistical analysis

Protein spots were considered as differentially expressed when expression level was at least 1.5-fold different compared to the control group and when the differences were detected as significant at p<0.01 by two tailed Student's t-test according to PDQuest Advanced software version 8.0.1. (Bio-Rad).

3. Results

A wide range of proteins differentially expressed in skin mucus of gilthead seabream were identified (**Figure 22, Table 11**), and they have been clustered in four groups according to the main functional role: immune-related, stress-related, metabolic and structural.

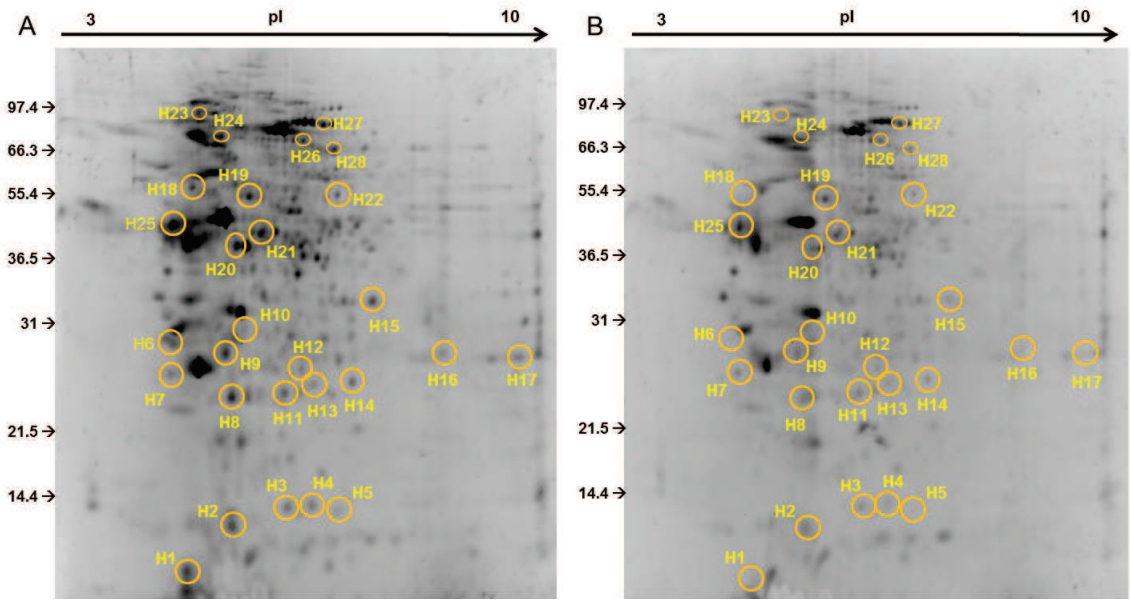


Figure 22. Representative 2-DE gels of skin mucus of *S. aurata* for both control (A) and wounded (B) groups. Skin mucus proteins were isoelectrically focused on 17 cm IPG strips (pI 3–10) and subjected to 12.5% SDS-PAGE. The 2-DE gels were stained with SYPRO® Ruby protein gel stain and the spots identified were annotated using the data from LC-MS/MS. The spot numbers represented in gels correspond to the protein identities mentioned in **Table 11**.

Table 11. Details of the differentially expressed protein spots in skin mucus of *S. aurata* after chronic wounds.

SN[a)]	Protein name	Organism AN[b)]	pI/MW[c)]	S/C[d)]	M_p/U_p[e)]	Peptide sequence and e-value[f)]
H1	Histone H4	*Oncorhynchus mykiss* P62797	11.4/11.4	76/19	2/2	**VFLENVIR** ($2.9*10^{-5}$) **TVTAMDVVYALK** (0.002)
H2	Apolipoprotein A1	*Sparus aurata* AAT45246	5.3/15.9	151/39	3/3	**LLNLLSQAQTASGPMVEQASQDGR** (0.0068) **EYAETLQAKPEFQAFVK** (0.025) **VATALGEEASPLVDK** (0.016)
H3	Histone H2B	*Danio rerio* Q5BJA5	10.4/13.6	28/7	1/1	**LLLPGELAK** (0.0016)
H4	Cu/Zn Superoxide dismutase	*S. aurata* CAI79044	5.4/7.0	66/44	2/2	**HVGDLGNVTAGADNVAK** (4) **MLTLSGPLSIIGR** (0.14)
H5	Histone H2A	*D. rerio*	10.6/13.5	49/7	1/1	**AGLQFPVGR** (0.00014)
H6	14-3-3 protein beta/alpha-1	*O. mykiss* Q6UFZ9	4.6/27.7	143/15	2/5	**YLSEVASGDSK** ($2.6*10^{-8}$) **YLSEVASGDSKK** (0.35) NLLSVAYK ($8.3/10^{-5}$) VISSIEQK (1.3) DSTLIMQLLR ($1.5*10^{-5}$)
H7	Apolipoprotein A1	*S. aurata* O42175	5.2/29.6	232/29	5/5	**AVLDVYLTQVK** (0.02) **AVNQLDDPQYAEFK** (0.0032) **IEEMYTQIK** (0.00025) **SSLAPQNEQLK** (0.00099) **TLLTPIYNDYK** (0.0014) **EVVQPYVQEYK** (0.092) **ITPLVEEIK** (0.0024)
H8	Phosphatidylethanolamine-binding protein 1	*S. aurata* FM145015	9.1/29.7	174/13	3/2	**LYDQLAGK** (28) **LYTLALTDPDAPSR (**0.0019) YGSVEIDELGK (0.00074)
H9	Apolipoprotein A1	*S. aurata* O42175	5.2/29.6	183/19	5/5	**IEEMYTQIK** (1.2) **SSLAPQNEQLK** (3.5) **TLLTPIYNDYK** (0.14) **EVVQPYVQEYK** (0.42) **ITPLVEEIK** (0.87)
H10	Actin cytoplasmic 1	*Ctenopharyngodon idella* P83751	5.3/42.1	53/7	3/3	**AGFAGDDAPR** (0.085) **DLTDYLMK** (0.089) **GYSFTTTAER** ($6*10^{-5}$)
H11	Natural killer enhancing factor 2	*Larimichthys crocea* XP_010732927	5.9/21.8	278/26	6/2	**DYGVLKEDDGIAYR** (0.22) **EDDGIAYR** (21) IPLVADLTK ($1.3*10^{-5}$) GLFVIDDK (0.41) QITINDLPVGR (0.00085) LVQAFQHTDK (0.34)
H12	ADP-ribosylation factor 3	*Takifugu rubripes* P61207	6.8/20.7	106/24	4/4	**ILMVGLDAAGK** ($4*10^{-7}$) **MLAEDELR** ($3*10^{-5}$) **DAVLLVFANK** (0.056) **QDLPNAMNAAEITDK** (0.17)
H13	Natural killer enhancing factor 1	*Osmerus mordax* ACO 09982	5.8/22.3	102/14	3/3	**LAPDFTAK** (26) **AVMPDGQFK** (18) **QITINDLPVGR** (0.0028)
H14	Glutathione S-transferase 3	*S. aurata* AFV39802	6.9/25.5	206/19	5/3	**FTGILGDFR** (0.00069) **MTEIPAVNR** (0.1) **TVMEVFDIK** (2.2) YLPVFEK (11) AILNYIAEK (0.79)
H15	Triosephosphate isomerase A	*S. aurata* FG266106	8.7/28.8	203/18	5/4	**IIYGGSVTGATCK** (0.3) **NVSEAVANSVR** (0.0059) **KNVSEAVANSVR** (1200) **GAFTGEISPAMIK** (4.9) FGVAAQNCYK (11)
H16	Triosephosphate isomerase B	*D. rerio* Q90XG0	6.5/27.1	76/12	3/3	**FFVGGNWK** (0.065) **GAFTGEISPAMIK** ($5.7*10^{-7}$) **WVILGHSER** (0.037)

H17	Triosephosphate isomerase B	*D. rerio* Q90XG0	6.5/27.1	131/23	5/5	**FFVGGNWK** (6.1) **GAFTGEISPAMIK** ($1.7*10^{-6}$) **WVILGHSER** (0.001) **HVFGESDELIGQK** ($2.9*10^{-6}$) **VVLAYEPVWAIGTGK** (0.022)
H18	ATP synthase subunit beta	*Cyprinus carpio* Q9PTY0	5.1/55.3	317/28	10/10	**TIAMDGTEGLVR** (0.0043) **VLDTGAPIR** ($1.8*10^{-6}$) **IPVGPETLGR** ($7.4*10^{-8}$) **IMNVIGEPIDER** ($1.1*10^{-6}$) **VVDLLAPYAK** ($3*10^{-5}$) **IGLFGGAGVGK** ($6.8*10^{-6}$) **TVLIMELINNVAK** (0.022) **VALVYGQMNEPPGAR** ($5.4*10^{-5}$) **IPSAVGYQPTLATDMGTMQER** (0.0006) **AIAELGIYPAVDPLDSTSR** (0.0045)
H19	Actin-related protein	*T. rubripes* O73723	5.6/47.9	33/8	3/3	**FSYVCPDLVK** (0.062) **DYEEIGPSICR** (0.0066) **EVGIPPEQSLETAK** (0.14)
H20	Actin cytoplasmic 1	*Oreochromis mossambicus* P68143	5.3/42.1	144/15	5/5	**AGFAGDDAPR** ($3.8*10^{-8}$) **VAPEEHPVLLTEAPLNPK** (0.0038) **DLTDYLMK** (0.024) **GYSFTTTAER** ($1.5*10^{-5}$) **EITALAPSTMK** (0.066)
H21	Macrophage-capping protein	*L. crocea* XP_010735467	5.8/38.7	185/12	5/4	**TQVEILPQGK** (0.022) **MKTQVEILPQGK** (0.45) **MPELAESTPEEDSK** (0.16) **EIASLIR** (10) EGGVESGFR (1.8)
H22	Citrate synthase	*Katsuwonus pelamis* Q6S9V7	8.5/52.4	95/8	4/4	**DVLSDLIPK** (0.25) **ALGFPLERPK** (0.061) **VVPGYGHAVLR** ($3.7*10^{-5}$) **IVPNVLLEQGK** ($1.1*10^{-6}$)
H23	Heat shock cognate 71 kDa	*Oryzias latipes* Q9W6Y1	5.8/76.6	476/20	13/3	**NQVAMNPTNTVFDAK ($1.8*10^{-7}$)** **SFYPEEVSSMVLTK ($1.2*10^{-5}$)** **GQIHDIVLVGGSTR (0.0077)** VEIIANDQGNR ($5.8*10^{-6}$) MKEIAEAYLGK ($7.2*10^{-5}$) EIAEAYLGK (0.02) DAGTISGLNVLR ($3.6*10^{-5}$) IINEPTAAAIAYGLDKK ($1*10^{-6}$) STAGDTHLGGEDFDNR (0.0014) ARFEELNADLFR ($5.5*10^{-5}$) FEELNADLFR ($7.6*10^{-7}$) LLQDFFNGK ($9.2*10^{-6}$) NGLESYAFNMK (0.00053)
H24	Heat shock cognate 71 kDa	*Ictalurus punctatus* P47773	5.2/71.6	306/14	8/2	**TTPSYVAFTDSER ($1.8*10^{-6}$)** **FELTGIPPAPR (0.00019)** VEIIANDQGNR ($2.3*10^{-7}$) MKEIAEAYLGK (0.096) DAGTISGLNVLR ($7.8*10^{-7}$) STAGDTHLGGEDFDNR (0.00012) FEELNADLFR ($7.2*10^{-8}$) LLQDFFNGK (0.0026)
H25	Keratin type I	*O. mykiss* NP_001117848	5.2/51.9	521/14	9/0	KLEAANAELELK ($1.7*10^{-9}$) LEAANAELELK (0.00012) LAADDFR (0.0068) TKYENELAMR (0.041) QSVEADIAGLKR (43) SDLEMQIEGLK ($9.2*10^{-5}$) NHEEELLAMR (1.6) TRLEMEIAEYR (0.18) LEMEIAEYR (0.029)
H26	Complement component 3	*S. aurata* ADM13620	8.1/186.9	152/4	7/6	**TLYTPESTVLYR** (18) **DITYLILSR** (0.87)

						VTGDPEATVGLVAVDK (62) **SVPFIIIPMK** (13) **DSSLNDGIMR** (21) **VVPQGVLIK** (11) EYVLPSFEVK (100)
H27	Gelsolin	*S. aurata* HS984154	6.0/31.6	548/45	9/7	**QPGLQVWR** (0.035) **GGVASGFQHVVTNDMSAK** (13) **GDSFILDLGK** (0.059) **LHMVEEGEEPK** (25) **AFTEALGPK** (2.1) **TAIAPSTPDDEKADISNK** (0.00049) **GALYMISDASGTMK** (0.0044) **VSSVAPSSPFK** (0.0033) QAMLSPEECYILDNGVDK (1600) IENLDLKPVPK (54)
H28	Immunoglobulin M heavy chain	*S. aurata* AFN20639	6.1/51.2	50/2	1/1	**GFSPNSFQFK** (0.039)

a) Spot number.
b) Accession number according with NCBI and SwissProt databases.
c) Theoretical isoelectric point and molecular weight (kDa).
d) Total score and coverage (%)
e) Total matched peptides (Mp)/total unique peptides (Up).
f) Unique peptides are in bold. Expect value (e-value) is noted for each peptide sequence.

3.1 Immune-related molecules

The proteome profiling of skin of gilthead seabream showed a general decrease of some proteins involved in several immune routes (Table 12). One of the most important components of both innate and adaptive immunity, C3 (spot H26), was identified and down-regulated after chronic wounds. APOA1 was identified in different part of the gels (spots H2, H7 and H9) and also showed a down-regulation of all the analysed protein levels.

It is relatively well-known that some histones act as antimicrobial peptides (Valero et al., 2013). We have identified H2A (spot H5), H2B (spot H3) and H4 (spot H1) differentially down-regulated in the skin mucus from wounds of gilthead seabream. Finally, the main component of the adaptive immunity, IgM (spot 28), who was identified for first time in fish skin mucus after 2-DE methodology, showed an important down-regulation after chronic wounds in skin mucus from wounded fish, respect to the expression level recorded for mucus samples.

3.2 Stress-related molecules

Chronic wounds in the skin also altered some stress-related proteins in the mucus of gilthead seabream. Peroxiredoxins are a family of antioxidant enzymes that protect cells from oxidative damage (Valero et al., 2015b). The most studied peroxiredoxins, identified here such as NKEF1 (spot 13) and NKEF2 (spot 11), were down-regulated after chronic wounds in skin mucus of gilthead seabream. Furthermore, we have identified SOD (spot H4), GST3 (spot 14) and HSC70 (it was identified in two parts of the proteome map, spots H23 and H24), which were down-regulated after chronic wounds (Table 12).

3.3 Metabolism molecules

Important proteins involved in several metabolic routes were identified in the present study. Thus, we have found differentially expressed YWHAZ (spot H6), PEBP1 (spot H8), ARF3 (spot H12), TPIA (spot H15), TPIB (spot H16 and H17), ATPB5B (H18) and CS (H22), being all of them down-regulated after chronic wounds in skin mucus of gilthead seabream (Table 12).

3.4 Structural molecules

Structural proteins are playing a major role in chronic wounds of skin. We have identified ACTB (spots H10 and H20), ARP (spot 19), CAPG (spot H21), KRT1 (spot H25) and GSN (spot H27), showing down-regulation in all cases with the lowest levels in KRT1 (Table 12).

Table 12. List of differentially expressed proteins in skin mucus of *S. aurata* after chronic wounds. ↓ indicates under-expression of the proteins at $p<0.01$ with two-tailed T-test.

Spot	Protein name	Fold change in wounds group relative to control group	Previously detected in skin mucus?
H1	Histone H4 (H4)	↓ 0.01	Yes
H2	Apolipoprotein A1 (APOA1)	↓ 0.04	Yes
H3	Histone H2B (H2B)	↓ 0.12	Yes
H4	Cu/Zn Superoxide dismutase (SOD)	↓ 0.09	Yes
H5	Histone H2A (H2A)	↓ 0.06	Yes
H6	14-3-3 protein beta/alpha 1 (YWHAZ)	↓ 0.02	Yes
H7	Apolipoprotein A1 (APOA1)	↓ 0.44	Yes
H8	Phosphatidylethanolamine-binding protein 1 (PEBP1)	↓ 0.09	Yes
H9	Apolipoprotein A1 (APOA1)	↓ 0.06	Yes
H10	Actin cytoplasmic 1 (ACTB)	↓ 0.32	Yes
H11	Natural killer enhancing factor 2 (NKEF2)	↓ 0.13	Yes
H12	ADP-ribosylation factor 3 (ARF3)	↓ 0.06	Yes
H13	Natural killer enhancing factor 1 (NKEF1)	↓ 0.17	Yes
H14	Glutathione S-transferase 3 (GST3)	↓ 0.11	Yes
H15	Triosephosphate isomerase A (TPIA)	↓ 0.01	Yes
H16	Triosephosphate isomerase B (TPIB)	↓ 0.02	Yes
H17	Triosephosphate isomerase B (TPIB)	↓ 0.01	Yes
H18	ATP synthase subunit beta (ATP5B)	↓ 0.07	Yes
H19	Actin-related protein (ARP)	↓ 0.46	Yes
H20	Actin cytoplasmic 1 (ACTB)	↓ 0.25	Yes
H21	Macrophage-capping protein (CAPG)	↓ 0.18	Yes
H22	Citrate synthase (CS)	↓ 0.09	Yes
H23	Heat shock cognate 71 kDa (HSC70)	↓ 0.12	Yes
H24	Heat shock cognate 71 kDa (HSC70)	↓ 0.27	Yes
H25	Keratin type I (KRT1)	↓ 0.11	Yes
H26	Complement component 3 (C3)	↓ 0.36	Yes
H27	Gelsolin (GSN)	↓ 0.41	Yes
H28	Immunoglobulin M heavy chain (IgM)	↓ 0.07	No

4. Discussion

From our own experience working on fish skin mucus (Cordero et al., 2016b; Guardiola et al., 2014a, 2014b) and with proteomic tools (Cordero et al., 2016c, 2015a; Rajan et al., 2013b, 2011), 2-DE followed by LC-MS/MS provides good resolution and high performance for protein detection. Some limitations of this approach could be the limited range of molecular weights, thus mucins and others high molecular weight proteins have been undetected in these works.

Skin mucus is the first barrier of defense in fish, which contains immune components involved in both innate and adaptive immunity. In the present study we have demonstrated the presence of C3, APOA1, H2A, H2B, H4 and IgM. C3 can upon cleavage act as a chemoattractant (recruit immune cells), as opsonin (coat pathogens) to increase phagocytosis, as an agglutinin (coagulate pathogens) (Cordero et al., 2015a). C3 was previously found in skin mucus of European sea bass (Cordero et al., 2015a). While in the present study C3 was under-expressed in chronic wounds, in other study C3 was over-expressed after crowding stress in skin mucus of gilthead seabream (Cordero et al., 2016c).

APOA1 is the major component of high density lipoprotein in serum (Breslow et al., 1982), acting also as a negative acute phase protein (Villarroel et al., 2007), and possessing bactericidal activity *in vitro* (Concha et al., 2004; Johnston et al., 2008); however, despite of being previously found in skin mucus of European sea bass (Cordero et al., 2015a), Atlantic salmon (Easy and Ross, 2009), Atlantic cod (Rajan et al., 2013b, 2011) and gilthead seabream (Cordero et al., 2016c), its role in mucus is still unknown. Our study suggests that this role as a negative acute phase protein may also occur in skin mucus since in our study APOA1 was under-expressed after chronic wounds.

Apart from the classical role of histones folding DNA into chromatin, H2A, H2B and H4 are also known as antimicrobial peptides (Valero et al., 2016, 2013), a role especially notable for H2A and H2B in skin mucus of fish (Bergsson et al., 2005; Fernandes et al.,

2002). The histone H4 deserves more attention since previous studies have found this histone in the skin mucus (Cordero et al., 2015a), but little is known about its role as antimicrobial peptide in this layer. The under-expression of these three histones in skin mucus after chronic wounds may facilitate the entry of potential pathogens resulting in a loss of immune defense.

The main effector of the humoral systemic adaptive immunity, IgM, has been widely studied in skin mucus by ELISA under many conditions and in different fish species (Cordero et al., 2016b; Guardiola et al., 2014a, 2014b). Importantly this is the first time that IgM is identified in a fish skin mucus proteome. The fact of using IgM as biomarker in skin mucus for proteomic studies would be useful to understand the mucosal adaptive immunity in depth.

There is a close relation between stress and immunity, especially in lower vertebrates such as fish, in which, for instance, cytokines and neuropeptides are performing roles in both neuroendocrine and immune system (Tort, 2011). Another example of this relationship between stress and immunity are peroxiredoxins, which may act as modulators of inflammation in pathogen infection and in protection against cell death, tissue repair after damage, and tumour progression (Ishii et al., 2012). According to our results, in which NKEF1 and NKEF2 are under-expressed in skin mucus after chronic wounds, fish NKEFs (mainly NKEF1 and NKEF2) expression, at either gene or protein level, is regulated by LPS treatment, pathogens including bacteria, virus and parasites (reviewed by Valero et al., 2013). Concretely, NKEFs have been previously found in skin mucus of gilthead seabream (Jurado et al., 2015), and over-expressed there after crowding stress (Cordero et al., 2016c). Our results corroborate an opposite expression regulation when fish were stressed by crowding or damaged by chronic wounds.

Also in close relation with the immunity, SOD is an enzyme that protects the tissue against oxidative stress by regulating various ROS and reactive nitrogen species molecules (Break et al., 2012). In addition, T cell activation induces the secretion of

SOD (Terrazzano et al., 2014). SOD was also identified previously in skin mucus of gilthead seabream (Jurado et al., 2015), however, this is the first time that this protein is differentially expressed, and whose role in chronic wounds deserves further considerations.

GSTs are the superfamily of phase II detoxification enzymes that play crucial roles in cellular defense (Cordero et al., 2015a). Some members of this superfamily have been previously identified in skin mucus of fish (Brinchmann, 2016), reducing the amount of proteins in Atlantic cod after *V. anguillarum* infection (Rajan et al., 2013b) or increasing the amount of protein in gilthead seabream after probiotic intake (Cordero et al., 2016c). In the present study GST3 was identified for first time in skin mucus, under-expressed after chronic wounds.

HSPs are part of a superfamily of stress proteins, highly conserved across species, often classified based on their molecular weight (Cordero et al., 2015a). Both HSP70 and HSC70 may have similar cellular roles and have been previously found in skin mucus (Cordero et al., 2015a; Jurado et al., 2015; Sanahuja and Ibarz, 2015). HSC70 can be mildly modulated by stressors such as heat (Jesus et al., 2013), pathogens (S. Das et al., 2015), and heavy metals (Patricia Morcillo et al., 2015). According to these previous studies, at protein level, in the present study demonstrated the under-expression of HSC70 in skin mucus after chronic wounds.

In the present study, some metabolic proteins have been found under-expressed in skin mucus after chronic wounds. PEBP1 was found in the mapping of gilthead seabream skin mucus (Sanahuja and Ibarz, 2015), similar than YWHAZ (Jurado et al., 2015; Sanahuja and Ibarz, 2015). Moreover, YWHAZ was found in skin mucus of other fish species such as Atlantic cod (Rajan et al., 2011) and Atlantic salmon (Provan et al., 2013). In agreement with the present study, it was reported that YWHAZ, ARF and TPIA were under-expressed after crowding stress in skin mucus of gilthead seabream (Cordero et al., 2016c). CS and ATP5B were previously found in the skin mucus of

Atlantic cod (Rajan et al., 2011) and gilthead seabream (Jurado et al., 2015), but this is the first time that these proteins are differentially expressed in skin mucus of fish.

Beta actin is a multifunctional protein involved in cell motility and phagocytosis. It has been reported that ACTB can be fragmented after stress (Easy and Ross, 2009). This fact could explain the under-expression of ACTB found in our study. In agreement with this result, ATCB was also under-expressed after crowding stress (Cordero et al., 2016c). In close relation with ACTB, ARP, CAPG and GSN were previously found in skin mucus of gilthead seabream (Jurado et al., 2015; Sanahuja and Ibarz, 2015), however little is known about the interaction of all these proteins in stress processes since this is the first time that ARP and CAPG are differentially under-expressed after stress by chronic wounds.

KRTs are intermediate filaments that form heteropolymeric filaments containing type I (KRT1) and type II (KRT2) keratins. These molecules have been reported in skin mucus of many fish species (reviewed by Brinchmann, 2016). In the present study KRT1 was under-expressed after chronic wound in a similar fashion than KRT2 was under-expressed in skin mucus after infection (Rajan et al., 2013b). By contrast, KRT1 was over-expressed in skin mucus after crowding stress (Cordero et al., 2016c). It has been reported that KRTs play a role in the regulation stress-resistance in epithelial cells (Marceau et al., 2001). In addition, KRTs have been associated with pore-formation activities in skin mucus of fish (Molle et al., 2008). Overall, it seems that KRTs are essential to maintain the proper function of skin mucus.

Most of the proteins found differentially expressed in the present study have been found previously in skin mucus of gilthead seabream and/or another fish species such as European sea bass and Atlantic cod (Cordero et al., 2016c, 2015a; Jurado et al., 2015; Rajan et al., 2013b, 2011; Sanahuja and Ibarz, 2015), but importantly IgM was identified for first time in a skin mucus proteome.

This study showed for first time the alteration caused by chronic wounds in the skin mucus proteome, whose molecules involved are mostly immune-related (C3, APOA1, H2A, H2B, H4 and IgM) and stress-related (NKEF1, NKEF2, SOD, GST3 and HSC-70), but also molecules involved in metabolism (PEBP1, YWHAZ, TPIA, TPIB, ARF, CS and ATP5B) and structural proteins (ATCB, ARP, CAPG, GSN and KRT1). These early alterations after chronic wounds could increase the possibility of suffering a pathogen infection due to the lower levels of immune-related proteins as immune barrier and because of the lower levels of structural proteins as physical barrier, allowing the penetration of pathogens and, therefore, increasing the vulnerability of the fish.

Conclusions

1. The analysis of the skin cells from gilthead seabream revealed higher cell surface and microridge areas in the dorsal region, but greater thickness and apoptosis cell death in the ventral region.

2. Exposure of seabream skin explants to pathogen and/or probiotics unaltered the cytokine gene expression profile in cells from the dorsal region but greatly altered those from the ventral region.

3. A natural outbreak of lymphocystis in seabream provoked a decrease in adaptive immunity in both skin and head-kidney organs, while the innate immunity was increased. These data point to the activation of innate cytotoxic activity at the site of infection.

4. Fresh mucus samples are preferable for the evaluation of the skin mucus immunity. However, if mucus needs to be stored freezing is preferred to lyophilization.

5. Seventy five proteins from the proteomic map of European sea bass skin mucus were identified. These proteins were related to structure, metabolism, signal transduction and immunity.

6. Immune-related proteins identified in the sea bass skin mucus are mainly involved, directly or indirectly, in the interaction and lysis of pathogens. Moreover, their respective transcription genes were expressed in the skin, suggesting the local production of these molecules.

7. Dietary intake of probiotic Pdp11 provoked an over-expression of lysozyme, fucose-binding lectin, complement component 3, nonspecific cytotoxic cell receptor protein 1 and apolipoprotein, among others, in the skin mucus gilthead seabream pointing to an improvement in mucosal immunity.

8. The proteome of mucus from experimental skin ulcers in gilthead seabream revealed that all identified proteins were under-expressed, suggesting increased vulnerability to any infection in the wounded areas.

Resumen en español

1. INTRODUCCIÓN

La dorada y la lubina son las dos especies de peces marinos más cultivadas (FAO, 2016). El conocimiento de la piel y el moco secretado por ésta, y de la microbiota naturalmente presente allí, todo ello como barrera inmunológica, podría ayudar a prevenir las gravosas pérdidas económicas que causan los brotes de enfermedades, así como prevenir el estrés ocasionado como consecuencia de las condiciones de hacinamiento que generalmente hay en las granjas marinas. La piel y su moco también son la primera barrera de defensa contra cualquier estrés físico, químico o biológico (Esteban, 2012). Los factores de estrés biológico, entre otros, son el estrés por alta densidad o hacinamiento o el estrés producido por las heridas. Dentro también de los factores de estrés biológico, las infecciones causadas por virus, bacterias o parásitos son muy frecuentes. Concretamente en doradas cultivadas, la principal enfermedad vírica es la causada por linfocistis (Hick et al., 2016).

Dentro de la inmunología de la mucosa, la piel tiene su propio tejido linfoide asociado, que se conoce como SALT (sus siglas en inglés) (Salinas et al., 2011). Además, el moco de la piel con sus proteínas y glicoproteínas forma parte de la inmunidad humoral, junto al suero (Yano, 1996). A lo largo de los últimos años, una serie de parámetros humorales de la respuesta inmunitaria tales como los niveles de IgM o las actividades del complemento, proteasa, antiproteasa, peroxidasa, esterasa o fosfatasa alcalina han sido evaluadas bajo distintos retos (por ejemplo, infecciones, variaciones estacionales, variaciones interespecíficas, dietas o tóxicos) en el suero o moco de distintas especies de peces teleósteos (Cordero et al., 2015b; Dawood et al., 2016; Du et al., 2015; Fast et al., 2002; Guardiola et al., 2014b, 2013; Muñoz et al., 2007; Reyes-Becerril et al., 2015; Siwicki et al., 1994).

Más recientemente, con la aparición de la secuenciación masiva y los avances en las técnicas de transcriptómica y proteómica, ha sido posible conocer más en detalle las moléculas involucradas en la inmunidad de la mucosa. Casualmente, durante la

realización de esta Tesis Doctoral han surgido los primeros mapas proteómicos del moco de la piel de dorada (Cordero et al., 2015a; Jurado et al., 2015; Sanahuja and Ibarz, 2015), que junto al realizado en lubina en la presente Tesis Doctoral, han permitido el hallazgo de marcadores de infección o de bienestar en el moco. El siguiente reto, por tanto, será ver como varían esos marcadores bajo distintas condiciones de estrés como las mencionadas anteriormente, que causan grandes pérdidas en el sector acuícola. De esta manera se podrían conocer las alteraciones en estos marcadores a través de técnicas no invasivas en el moco de la piel de estas especies de peces.

2. OBJETIVOS

En la presente Tesis Doctoral tratamos de mejorar el conocimiento de la piel y el moco con especial atención en la inmunidad.

Los objetivos específicos de esta Tesis Doctoral son:

1. La caracterización de la piel de dorada, incluyendo la morfología, el estudio de su ciclo celular, y la expresión de genes comparando entre las regiones dorsal y ventral.

2. La determinación de la respuesta inmunitaria de dorada tras un brote natural de linfocistis.

3. La búsqueda del mejor método de conservación del moco de piel de dorada para evaluar sus actividades inmunes humorales.

4. La caracterización del mapa proteómico del moco de piel de lubina para la búsqueda de nuevas moléculas implicadas en la inmunidad de la mucosa.

5. El análisis de los cambios producidos por la administración del probiótico Pdp11, estrés por hacinamiento o heridas crónicas en el proteoma del moco de piel de dorada.

3. PRINCIPALES RESULTADOS Y DISCUSIÓN

En la presente Tesis Doctoral hemos caracterizado diferentes regiones de la piel de dorada y hemos estudiado su respuesta inmunitaria frente al virus más común de esta especie, el linfocistis (Parte 1). También hemos estudiado qué condiciones de almacenamiento son óptimas para el estudio posterior de diferentes actividades inmunitarias humorales en el moco (Parte 2); y por último, el mapa proteómico del moco de piel y los cambios que se producen en el mismo debido a la ingesta de probiótico, al estrés por hacinamiento y a heridas crónicas, con especial interés en las moléculas inmunitarias implicadas en dichos procesos (Parte 3).

3.1 Caracterización de la piel de dorada

En este primer capítulo experimental se caracterizó la piel a nivel histológico, comparando entre las regiones dorsal y ventral, y midiendo de manera cuantitativa el grosor epidérmico, que resultó ser mayor en la zona ventral que en la zona dorsal, junto con la superficie celular y el área de microcrestas que resultaron ser mayores en las células dorsales que en las ventrales. No hay mucha información sobre la función de estas microcrestas, aunque se cree que están relacionadas con la capacidad para retener el moco (Hawkes, 1974), pudiendo tener también un papel importante en la cicatrización de heridas en la piel de peces (Rai et al., 2012). También se desarrolló un protocolo de aislamiento de células de la piel y se caracterizó por primera vez su ciclo celular en ambas regiones, cuyos ciclos celulares no mostraron diferencias significativas, sin embargo se detectó mayores niveles de apoptosis en la región ventral en comparación con la región dorsal. Finalmente, se comprobó la susceptibilidad de células aisladas de ambas regiones y expuestas *in vitro* al patógeno *Photobacterium damselae* ssp. *piscicida*, solo y en combinación con el probiótico Pdp11, respecto al perfil de transcritos de citoquinas, resultando este perfil prácticamente inalterado en las células de la zona dorsal, y fuertemente inhibido en las de la zona ventral. Estudios previos comprobaron que este probiótico inhibe la

capacidad de adhesión de este patógeno al moco y actúa como exclusión competitiva del mismo (Chabrillón et al., 2005b) lo cual no parece relacionarse con nuestros resultados. Respecto a las diferencias en ambas zonas de la piel, solo hay un estudio previo que describió la capacidad de un probiótico para reducir la apoptosis debido a una infección con *Vibrio anguillarum*, tanto en las células de la parte dorsal como en las de la ventral del bacalao atlántico (Lazado and Caipang, 2014).

En conclusión, en este estudio se ha demostrado que histológicamente existen diferencias entre la zona dorsal y ventral de la piel en cuanto a su grosor epidérmico, superficie celular y área de microcrestas, que no existen diferencias basales en el ciclo celular de las células aisladas de ambas regiones, aunque sí existen diferencias en los niveles de apoptosis, así como mayores cambios en el perfil de expresión de citoquinas frente al patógeno y al probiótico en la zona ventral con respecto a la zona dorsal.

3.2 Caracterización de la respuesta inmunitaria en doradas infectadas por el virus linfocistis

La enfermedad viral del linfocistis o LCD afecta a más de 125 especies de peces, incluyendo peces de agua dulce y agua salada. Esta enfermedad tiene como órgano diana la piel, provocando una hipertrofia de los fibroblastos y actuando a veces como verdaderos tumores epiteliales (Samalecos, 1986). En este segundo capítulo se demostró que el virus replicaba en la piel de los individuos visiblemente afectados y no en aquellos individuos asintomáticos. Respecto a la respuesta inmunitaria, se detectó un descenso de los niveles de IgM en suero, y un aumento de la explosión respiratoria y la actividad peroxidasa en los leucocitos de riñón cefálico. Esta es la primera vez que se analizaron parámetros inmunitarios en una infección con linfocistis y compararon con individuos asintomáticos. Sin embargo, se encontraron patrones de respuesta inmunitaria similares a los obtenidos en otras infecciones virales en la dorada tales como nodavirus o el virus de la septicemia hemorrágica viral (Chaves-Pozo et al.,

2012; Esteban et al., 2008; Valero et al., 2015c) los cuales replican pero son controlados y no producen mortalidad. Por último, a nivel génico, varios genes fueron analizados en la piel como órgano diana y en el riñón cefálico como órgano linfoide. Así, de los tres genes de respuesta antiviral estudiados (*ifn*, *irf3* y *mx*), dos de ellos (*ifn* y *irf3*) mostraban una disminución significativa en la piel, sin embargo el gen *mx* no se encontró diferencialmente regulado ni en la piel ni en el riñón cefálico, en consonancia con otro estudio con la línea celular de dorada SAF-1, en la cual tampoco variaba la expresión del gen *mx* después de una infección *in vitro* con linfocistis (Fernández-Trujillo et al., 2013). Respecto a los genes *csfr1*, *mhc2a*, *tcra* e *ighm*, su expresión se vio fuertemente inhibida tanto en la piel como en el riñón cefálico de los peces afectados, sugiriendo una baja respuesta celular específica a nivel local. En este sentido, no se observó alteración en la expresión de los genes *il1b* y *hamp*, al igual que sucedió después de una infección con nodavirus o con el virus de la septicemia hemorrágica en dorada y trucha arcoíris, respectivamente (Tafalla et al., 2005; Valero et al., 2015a). Respecto al principal marcador de células citotóxicas no específicas, *nccrp1*, se encontró un aumento significativo en su expresión en el riñón cefálico de los peces infectados con linfocistis, tal y como ocurría en doradas infectadas con nodavirus (Chaves-Pozo et al., 2012), sugiriendo una actividad citotóxica innata importante en los ejemplares infectados por linfocistis.

En conclusión, este estudio demostró la replicación del virus linfocistis en la piel de los ejemplares de dorada infectadas, y mostró por primera vez un aumento de las actividades inmunitarias innatas en el riñón cefálico, y un descenso de la inmunidad adaptativa.

3.3 Cambios en los niveles de las actividades humorales debido al almacenamiento del moco de dorada

En los últimos años ha habido un aumento enorme del número de trabajos que evalúan los parámetros inmunitarios humorales en el moco en relación con

inmunoestimulantes y resistencia del hospedador a infecciones (Cordero et al., 2015b; Dawood et al., 2016; Du et al., 2015; Fast et al., 2002; Guardiola et al., 2014b; Muñoz et al., 2007; Reyes-Becerril et al., 2015; Siwicki et al., 1994), sin embargo nunca se ha comprobado cuál es el método óptimo de almacenamiento del moco para medir tales parámetros inmunitarios, ya que para algunos estudios lo almacenaban a -20ºC (Chabrillón et al., 2006, 2005a, 2005b; Wold and Selset, 1978), otros a -80ºC (Cordero et al., 2015a; Jung et al., 2012; Jurado et al., 2015; Rajan et al., 2011; Sanahuja and Ibarz, 2015) y otros lo liofilizaban (Ebran et al., 2000; Guardiola et al., 2014b; Nigam et al., 2012). En este tercer capítulo comparamos dichos métodos de almacenamiento para el caso del moco de piel de dorada. Así, se obtuvo un descenso significativo de las proteínas totales evaluadas mediante el método de Bradford (Bradford, 1976) en el moco liofilizado mientras que no se alteraban significativamente en los congelados con respecto al moco fresco. Respecto a los carbohidratos terminales, en general hubo un descenso en dichos niveles al almacenarlos tanto a -20ºC como liofilizado, siendo -80ºC el método de almacenamiento del moco con el que los niveles son más similares a los encontrados en el moco fresco.

En lo relativo a los niveles de IgM, una de las actividades de la inmunidad humoral más evaluadas, no se alteraron por ninguno de los métodos de almacenamiento. Aunque no hay estudios en peces, un estudio en suero humano demostraron que los niveles de IgM no variaron después de la congelación a -20ºC (Tran et al., 2006). También hubiera sido interesante comprobar los niveles de IgT, que tiene una función específica en la inmunidad de la mucosa de teleósteos (Salinas et al., 2011; Zhang et al., 2010). Por último, con respeto a las actividades enzimáticas, antiproteasa, peroxidasa, esterasa y fosfatasa alcalina no se alteraron por la congelación (ni a -20ºC ni a -80ºC), mientras que la actividad proteasa y lisozima si bajaron en el moco congelado con respecto al moco fresco. Además, la liofilización disminuyó significativamente la mayoría de las actividades enzimáticas estudiadas. Esta es la

primera vez que se evalúan cual es el método de almacenamiento de moco más adecuado para estudiar estos parámetros inmunitarios humorales.

En resumen, la congelación resultó ser el método más efectivo de almacenamiento del moco si las actividades no se pueden evaluar en fresco, siendo la liofilización el método de almacenamiento que más cambia los niveles de los parámetros humorales evaluados, y por tanto el menos recomendado, en el moco de dorada.

3.4 Caracterización del mapa proteómico del moco de lubina

Los recientes avances en los métodos de investigación en proteómica han permitido su utilización para identificar y cuantificar proteínas individuales a partir de mezclas complejas (Gstaiger and Aebersold, 2009). En este cuarto capítulo experimental, se analizaron las 100 proteínas más expresadas en el moco de piel de lubina usando la técnica de electroforesis bidimensional seguida por cromatografía líquida acoplada a espectrometría de masas en tándem. Paralelamente a la realización de este estudio, dos estudios similares en el moco de dorada fueron publicados (Jurado et al., 2015; Sanahuja and Ibarz, 2015), poniendo de relieve lo actual de la temática tratada en este capítulo. Entre las proteínas encontradas estaban C3, C1Q, FBL, NKEF, AFP, LYZ, CALM, CALR, APOA1, ERp57, PDI, ASC, CYPA, LEI, GST, HSP70, TF, WAP65, SOD y varias histonas como la H1, recientemente sugerida como péptido antimicrobiano en lubina (Valero et al., 2016), y varias caspasas; todas ellas con una mayor o menor relevancia en la inmunidad de la mucosa de lubina. Además, se evaluaron los niveles de ARNm correspondientes a estas proteínas en la piel, demostrando la expresión constitutiva de todas ellas en la piel de lubina.

3.5 Cambios en el proteoma de moco de piel tras la administración del probiótico Pdp11 en dieta y/o estrés por hacinamiento en dorada

Los cambios en el proteoma han sido estudiados después de la administración de suplementos en dieta y en condiciones de estrés (Cordeiro et al., 2012; Ibarz et al., 2010; Rufino-Palomares et al., 2011). Sin embargo estos estudios han sido realizados

mayoritariamente en el hígado de diferentes peces y muy poco se sabe sobre los cambios a nivel de la mucosa de la piel de peces. En este quinto capítulo se analizaron por primera vez los cambios producidos en el proteoma del moco de piel de dorada debido a la administración de un probiótico en dieta y/o debido al estrés por hacinamiento. Nuestro estudio reveló 19 proteínas identificadas y diferencialmente expresadas. Después de la dieta del probiótico, las proteínas C3, GST y 14-3-3 aumentaron significativamente a los 15 días, mientras que a los 30 días aumentaron las proteínas C3, LEI, NCCRP1, APOA1, ACTB, FBL, LYZ y ARFGAP. Después del estrés por hacinamiento, las proteínas PRDX6, ACTB y 14-3-3 disminuyeron a los 15 días mientras que a los 30 días, aumentaron las proteínas CLEC, NCCRP1, FBL, NKEF2, C3, KRT1, LYZ, GST e IDH; y disminuyeron las proteínas LEI, IMPA, TPIA y ARFGAP.

Cuando se combinó la administración del probiótico y el estrés por hacinamiento, a los 15 días aumentaron las proteínas C3 y GST, y disminuyó la proteína 14-3-3, mientras que a los 30 días, aumentaron las proteínas CLEC, LEI, NCCRP1, APOA1, PFN, FBL, NKEF2, C3, KRT, LYZ, GST e IDH, y disminuyeron las proteínas IMPA, TPIA, AR y ARFGAP. De estas proteínas, la mayoría (79%) ya habían sido previamente identificadas (aunque no diferencialmente expresadas) en moco de dorada (Jurado et al., 2015; Sanahuja and Ibarz, 2015), de lubina (Cordero et al., 2015a) de bacalao atlántico (Rajan et al., 2013b, 2011), de anguila japonesa (Suzuki et al., 2003) o de salmón atlántico (Provan et al., 2013), mientras que el 21% fueron solo detectadas en el moco de dorada en nuestro estudio. Además, de todas las proteínas encontradas y diferencialmente expresadas, seleccionamos, de acuerdo a su importante papel en el sistema inmunitario, 4 de ellas (C3, NKEFB, NCCRP1 y LYZ) para correlacionarlas con los niveles de ARNm de la piel bajo esas condiciones, mostrando en el caso de la LYZ un aumento de expresión en piel correlacionado positivamente con el nivel de proteína en el moco.

En conclusión, la mayoría de las proteínas diferencialmente expresadas en el moco de dorada aumentaron con la dieta del probiótico, tanto cuando el probiótico se administró solo como cuando éste se administró a peces sometidos a estrés por hacinamiento, especialmente aquellas relacionadas directamente con la inmunidad.

3.6 Cambios en el proteoma del moco de piel debido a heridas crónicas en dorada

En los últimos años el mapa proteómico del moco ha sido estudiado en varias especies, incluyendo lubina (Cordero et al., 2015a) y dorada (Jurado et al., 2015; Sanahuja and Ibarz, 2015). Sin embargo, no hay estudios previos sobre el efecto que tienen las heridas en la composición del moco. En el sexto capítulo se estudiaron los cambios producidos en el proteoma del moco debido a heridas crónicas. Como resultado se obtuvo una disminución de los niveles de proteínas relacionadas con el sistema inmunitario (C3, APOA1, H2A, H2B, H4 e IgM), proteínas relacionadas con el estrés (NKEF1, NKEF2, SOD, GST3 y HSC70), proteínas metabólicas (PEBP1, YWHAZ, TPIA, TPIB, ARF, CS y ATP5B) y proteínas estructurales (ATCB, ARP, CAPG, GSN y KRT1). Basados en estudios previos donde se han identificado la mayoría de estas moléculas (Cordero et al., 2015a; Jurado et al., 2015; Rajan et al., 2011; Sanahuja and Ibarz, 2015), podemos concluir que dichas moléculas son marcadores adecuados para el diagnóstico de estrés por heridas en el moco de peces.

4. CONCLUSIONES

1 El análisis de las células de la piel de dorada reveló mayor superficie celular y área de microcrestas en las células de la región dorsal, pero mayor grosor epidérmico y mayor porcentaje de muerte celular por apoptosis en la región ventral.

2 La exposición de explantes de piel de dorada a un patógeno y/o a un probiótico no alteró el perfil de expresión de citoquinas en las células de la región dorsal mientras que sí lo hizo de manera significativa en las células de la región ventral.

3 El brote de linfocistis en dorada provocó un descenso de la inmunidad adaptativa de la piel y el riñón cefálico, mientras que la inmunidad innata fue incrementada. Estos datos apuntan a la activación de la actividad citotóxica innata en el lugar de la infección.

4 El estudio de las muestras de moco fresco es el más adecuado para la evaluación de la inmunidad de la mucosa. Sin embargo, si el moco debe ser almacenado, la congelación es más adecuada que la liofilización.

5 Setenta y cinco proteínas del mapa proteómico del moco de piel de lubina fueron identificadas. Estas proteínas estaban relacionadas con la estructura, el metabolismo, la transducción de señales y la inmunidad.

6 Las proteínas relacionadas con la inmunidad el moco de piel de lubina están principalmente implicadas, directa o indirectamente, en la interacción y lisis de patógenos. Además, sus respectivos transcritos son expresados en la piel, sugiriendo una producción local de estas moléculas.

7 La administración del probiótico Pdp11 en la dieta de doradas provoca un aumento de la expresión de lisozima, de la lectina de unión a fucosa, del componente 3 del complemento, de la proteína del receptor de células citotóxicas no específicas y de la

apolipoproteína A1, entre otras, en el moco de piel, indicando una mejora en la inmunidad de la mucosa.

8 El proteoma del moco de piel de doradas en las que se ha provocado una úlcera reveló una disminución de la expresión de todas las proteínas identificadas, sugiriendo una mayor vulnerabilidad a sufrir una infección en el área dañada.

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