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Fish Skin. Application of Imaging Techniques

La Piel de los Peces. Aplicación de Técnicas de Imagen

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A ti, mi princesa que me has dado tanto sin saberlo,
porque cada día lucho por ser tu mejor espejo.
Siempre repite lo que te dice papá:
"Sí puedo", porque si te esfuerzas se puede.

“Nunca desistas de un sueño. Sólo trata de ver las señales que te lleven a él”.

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Castillo, Y., Ceballos-Francisco, D., De La Rosa, F., Vásquez, W., Esteban, M.Á. (2018). "Inmunoestimulant and bactericidal effect of guava *(Psidium guajava* L.*)* in a hybrid of *Oreochromis niloticus* and *Oreochromis mossambicus*". Oral communication in XIV *Congreso Internacional de Investigación Científica*. Santo Domingo, Dominican Republic.

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LIST OF
ABBREVIATIONS

16s rRNA	16S ribosomal RNA
AFM	Atomic force microscopy
ALL	Above lateral line
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
BLL	Below lateral line
B-mode	Brightness mode
BSA	Bovine serum albumin
BSL-I	*Bandeiraea simplicifolia* agglutinin
cat	Catalase
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
ConA	Concanavalin A
CT	Computed tomography
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ef1a	Elongation factor 1 alpha
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FBP	Filtered back projection
GALT	Gut-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
grhl1	Grainyhead-like transcription factor 1

HBSS	Hank’s buffer
HEWL	Hen egg white lysozyme
HU	Hounsfield units
Ig	Immunoglobulin
ighm	Immunoglobulin M heavy chain
ight	Immunoglobulin T heavy chain
IL-1α	Interleukin 1 alpha
il1b / IL-1β	Interleukin 1 beta
il6 / IL-6	Interleukin 6
IL-8	Interleukin 8
il10	Interleukin 10
krt1	Keratin type 1
kVp	Kilovoltage
LD	Light-dark
LL	Lateral line
MALT	Mucosal-associated lymphoid tissue
Micro-CT	Micro computed tomography
MHz	Megahertz
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
mSv	Milisievert
MTT	3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide
µA	Microamps
NALT	Nasal-associated lymphoid tissues
NaOH	Sodium hydroxide
nt	Nucleotide
NW	No wounded
OD	Optical density
oligo-dT	Oligo deoxy-thymine nucleotides
OTU	Operational taxonomic unit
PBS	Phosphate buffer saline

PBS-T	Phosphate buffered saline supplemented with Tween-20
PGI2	Prostacyclin
PNA	Peanut agglutinin
PUFA	Polyunsaturated fatty acids
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
rpm	Revolutions per minute
rps18	Ribosomal protein S18
s	Second
SALT	Skin-associated lymphoid tissue
SB	Swim bladder
sem	Scanning electron microscopy
SEM	Standard error of the mean
sod	Superoxide dismutase
SONAR	Sound navigation and ranging
SPSS	Statistical Package for the Social Science
TCA	Trichloroacetic acid
tgfb	Transforming growth factor beta
TMB	Tetramethylbenzidine hydrochloride
tnfa/TNFα	Tumor necrosis factor alpha
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UEA-I	*Ulex europeaus* agglutinin I
UPGMA	Unweighted pair groups method with arithmetic averages
WFA	*Wisteria floribunda* agglutinin
WGA	Wheat germ agglutinin

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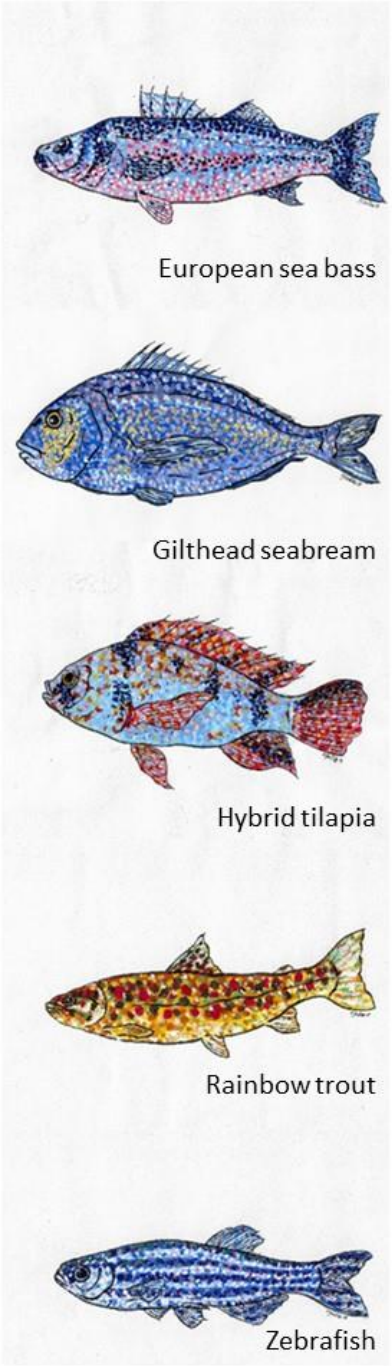
LIST OF FISH
SPECIES USED IN
THIS WORK

Common Name (Scientific name)	Photograph
European sea bass (*Dicentrarchus labrax*)	
Gilthead seabream (*Sparus aurata*)	
Hybrid tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*)	
Rainbow trout (*Oncorhynchus mykiss*)	
Zebrafish (*Danio rerio*)	

GRAPHICAL ABSTRACT

Fish skin study



Stressors

Circadian rhythm

Environment

Bacterial Skin Infection

Pathogens

Experimental wounds healing

Handling

Diet

Prevention / treatment

GRAPHICAL ABSTRACT


SUMMARY

Aquaculture is probably the fastest growing sector in the food industry, producing almost 50 percent of the fish destined for food worldwide. However, to supply this high demand, fish are farmed in intensive systems, where they are exposed to stress conditions and a deteriorating environment. These factors promote the appearance of diseases, which are manifested mainly on the skin.

In teleost fish, the skin fulfils a number of important functions: it maintains the body shape, improves hydrodynamics, is an osmotic barrier and is rich in mucous cells that produce antifungal and antibacterial substances, among others. Therefore, fish welfare depends on skin integrity and its defence mechanisms against external agents, since many opportunistic pathogens found in the aquatic environment can quickly colonize the skin, creating a wound or aggravating an existing one.

This Doctoral Thesis aims to study the skin from fish of interest in aquaculture and to apply medical imaging techniques to fish and it is divided in two parts: Part 1, the response of the skin and its mucus against different external factors in some aquacultural and experimental fish species (gilthead seabream, European sea bass, hybrid tilapia, rainbow trout and zebrafish); and Part 2, the application of modern imaging techniques (ultrasonography and X-ray micro computed tomography) to study gilthead seabream and European sea bass.

Part 1 of this Doctoral Thesis contains three chapters. In Chapter I, we analyse the possible daily changes in different immune parameters in the skin mucus of gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) specimens, exposed to a constant light–dark photoperiod (12 h L:12 h D). The results showed that, during the dark period, the levels of IgM in gilthead seabream increased significantly with a significant daily rhythm. By contrast, no variations were observed in immunoglobulin M

(IgM) levels of European sea bass at the different experimental times, although the daily cycle was the inverse of that observed in the gilthead seabream. This study also demonstrates that different enzymes (protease, antiprotease, peroxidase and lysozyme) related to the immune system and the bactericidal activity of skin mucus varied markedly during the daily light–dark cycle in gilthead seabream and European sea bass.

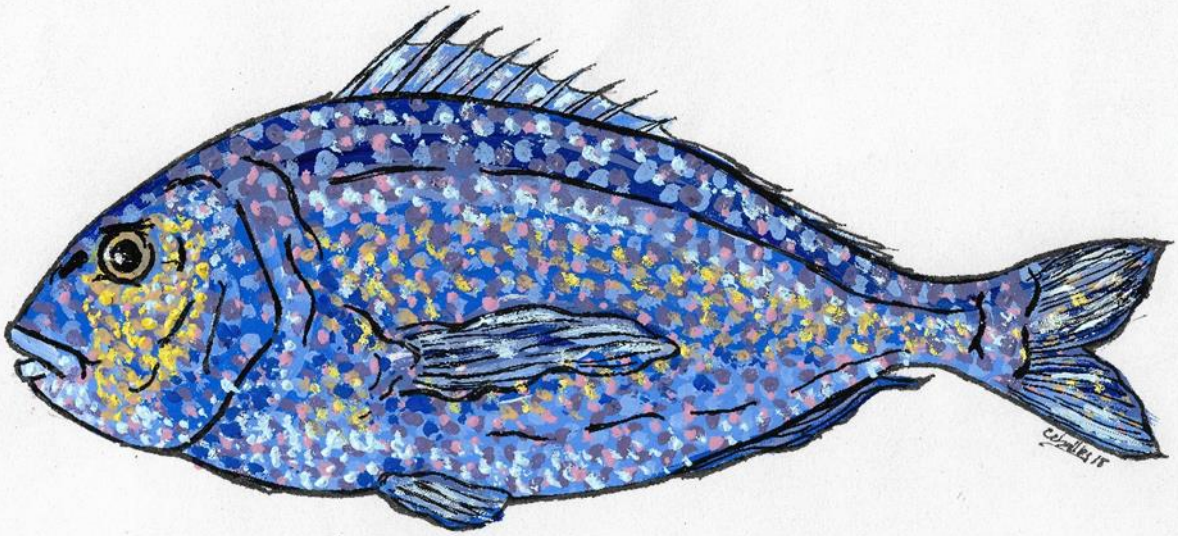
In Chapter II, we manipulated fish diet by supplementation with guava leaf (*Psidium guajava* L.) at different concentrations (1.5% and 3%) to study the dietary effect in the skin mucus of hybrid tilapia *(Oreochromis niloticus* × O. *mossambicus*) and the possible microbicidal effect against *Vibrio harveyi* on the skin of this fish species. Statistically significant increases in some immune parameters (protease, antiprotease and peroxidase activities) were detected in the skin mucus from fish consuming a diet supplemented with 1.5% guava leaf, compared to the values obtained in skin mucus of the control group (non-supplemented). In addition, guava leaf supplementation of hybrid tilapia significantly reduced the bacterial load after *V. harveyi* infection. These results demonstrate that the dietary intake of guava leaf (1.5%) increases the immune activity in skin mucus from hybrid tilapia and protects the skin against *V. harveyi* colonization.

Chapter III of this Doctoral Thesis is sub-divided in three sections. In section III.1, our aim was to study the skin healing process after making experimental wounds in two body locations (above or below the lateral line) in gilthead seabream (*S. aurata*) specimens. Further, skin mucus and serum immunity was studied by analysing some important immune parameters (protease, antiprotease, peroxidase, lysozyme and bactericidal activities), as well as the gene expression profile of genes relevant for immunity and cell regeneration in skin from wounded fish. Macroscopic results showed that the healing process is faster below the lateral line than above it. The immune parameters analysed in skin mucus and serum from experimentally wounded gilthead seabream showed significant variations depending on the site of the wound in the skin. At the same time, the gene expression profile of several immune-relevant genes, including pro-inflammatory *(il1b, il6),* anti-inflammatory *(tgfb),* immunoglobulin T *(ight)*, as well as the gene implicated in skin regeneration *(krt1)* showed significant variations, depending on the wound side. These results throw further light on the complex process of skin wound healing in fish, since substantial changes in skin mucus and serum, in addition to skin gene expression, were seen to result from the presence of wounds.

In section III.2, we performed experimental skin ulcers in gilthead seabream specimens and evaluated the changes in mucus composition and microbial diversity associated with the ulcerated skin. The abundance of terminal carbohydrates and immune parameters (protease, antiprotease, peroxidase and lysozyme activities and total immunoglobulin M levels) were evaluated in skin mucus. A significant decrease in the terminal abundance of α-D-mannose, α-D-glucose and N-acetyl-galactosamine residues was determined in the mucus from ulcerated skin, compared to control fish (non-ulcerated). The levels of IgM and the tested enzymatic activities decreased in the mucus from ulcerated fish (compared to control fish) although the observed decreases were only statistically significant in the case of protease and antiprotease activities. Concomitantly, analysis of the skin microbiota showed clear differences between ulcerated and non-ulcerated areas. The taxonomic analysis showed that *Staphylococcus* and *Lactobacillus* genera were more abundant in non-ulcerated skin, whereas *Streptococcus* and *Granulicatella* were more abundant in ulcerated areas. Substantial decreases in the number of sequences related to *Alteromonas*, *Thalassabius* and *Winogradskyella* were detected in ulcerated skin, whilst sequences related to *Flavobacterium*, *Chryseobacterium* and *Tenacibaculum* genera increased slightly. Overall, these results demonstrated that the presence of skin ulcers provide microenvironments that disturb the mucus composition, immune potential and microbial biodiversity of this important external surface. In section III.3, we aimed to optimize a fluorescein test to detect initial skin damage in fish. Rainbow trout specimens were used to detect experimental skin lesions in the initial stage and zebrafish were used to study wether possible damage due to bacterial contamination in the experimentally sensitized skin could be detected. In both cases, after a fluorescein bath and exposure to ultraviolet light, fluorescence was detected in the damaged areas and also in some injuries that were not perceived by the naked eye. Interestingly, fluorescence was sometimes perceived outside the experimental area which may have been due to previous undetectable lesions or to fish manipulations. These findings confirm that the fluorescein test can be regarded as an easy and cheap tool to detect small or incipient skin lesions.

Finally, Chapter IV is divided in three sections, in which we focus on the study of gilthead seabream and European sea bass through the application of image analysis techniques: real-time ultrasonography (section IV.1) and X-ray micro computed tomography (micro-CT) (sections IV.2 and IV.3). The results showed that the skin thickness

along the fish varies depending on the body region (section IV.1). Furthermore, in section IV.2 a complete segmentation of fish body was carried out by CT and the results showed that density values for the whole fish were in the range -1,000 to +2,500 Hounsfield Units (HU), while, values for skin and subcutaneous fat were between -400 and -50 HU. Furthermore, the skin varied in thickness in both, gilthead seabream and European sea bass. Curiously, the skin values partly coincide with those for fat segmentation (-115 to +50 HU), and so, a separate study was made to identify fat depots in gilthead seabream. The image analysis identified those segmented areas that topographically coincided with the fat depots. Finally, to corroborate the presence of fat in the fish body, the fat content was measured in gilthead seabream specimens maintained under starvation for 60 days



Unpublished paint inspired in gilthead seabream *(Sparus aurata)* (C. Ceballos 2018).


INTRODUCTION

According to the Food and Agriculture Organization (FAO) aquaculture is the term given to the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants in both coastal and inland areas, involving interventions in the rearing process to enhance production, similarly to what on land are livestock raising and agriculture (FAO, 1988; APROMAR, 2017). Fisheries and aquaculture are important sources of food, nutrition, income and livelihoods for hundreds of millions of people around the world. In 2016, FAO reported an all-time high global farmed fish production of 171 million tonnes, of which 88 percent was destined for direct human consumption (FAO, 2018). This rapid growth of aquaculture has led to intensive fish production to cover market demands. Nevertheless, there are still some important challenges to overcome in order to develop productive, feasible, and sustainable aquaculture in present intensive systems. One of these challenges is that in large-scale production facilities aquatic animals are exposed to stressful conditions, problems related to diseases and the environmental deterioration, which often results in serious economic losses (Balcázar *et al.*, 2006). The consequences of these threats to fish health are skin lesions caused by fish-to-fish contact in crowded installations, nutritional defects or pathogen outbreak. All these problems together make it necessary to deepen on the knowledge of fish skin and its mucus, to enable strategies for the prevention and treatment of common diseases in aquaculture to be implemented.

1. An overview of teleost skin

Teleost skin is a living non-keratinized organ that covers the body and protects it not only from the entry of pathogens or allergens, but also from the leakage of water, solutes or nutrients (Vieira *et al.*, 2011; Esteban, 2012). The skin, sometimes referred to as the integumentary system and the largest organ of the body, is continuous with the linings of all body openings and also covers the fins (Esteban, 2012; Esteban and Cerezuela, 2015). Skin thickness varies between individuals as well as species and is influenced by factors such as

sex, life stage, season, reproductive condition, nutrition, water quality, location on the body and general health status (Henrikson and Matoltsy, 1967; Bullock and Roberts, 1974; Iger and Abraham 1990; Groff, 2001; Fontenot and Neiffer, 2004). Its normal structure and function in fish reflects the adaptation to the physical, chemical, and biological properties of the aquatic environment, and the natural history of these organisms (Esteban and Cerezuela, 2015). Hence, teleost skin in particular is unique and histologically diverse (Fast *et al.*, 2002) with a well conserved organization (Fig. 1) (Raj *et al.*, 2011; Vieira *et al.*, 2011; Esteban and Cerezuela, 2015).

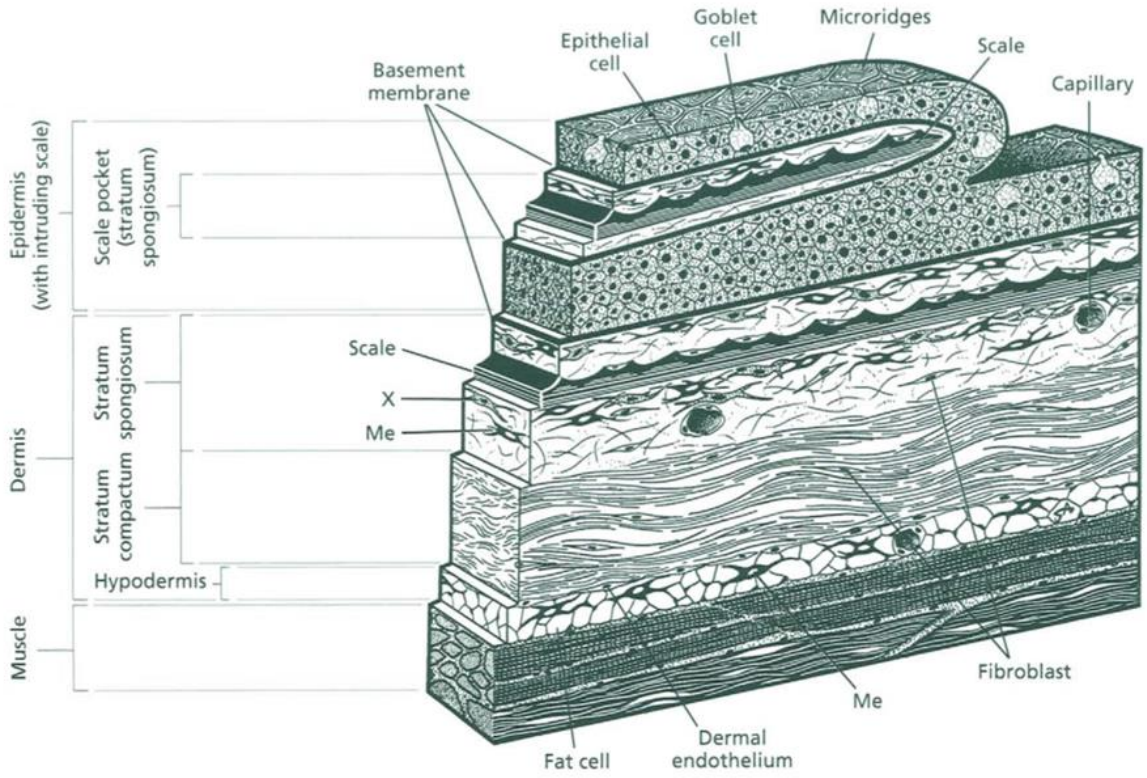


Figure 1. Representation of a three-dimensional section of the skin of a teleost fish (*Oncorhynchus kisutch*), showing the microscopic structures and some specific structures of the dermis and epidermis. Me: melanophore; X: xanthophore, (adapted from Elliott, 2000).

1.1. Mucus layer

The most superficial layer of the skin is the mucus layer or cuticle where the microorganisms forming the microbiota are present (Larsen *et al.*, 2013). This protective layer is composed primarily of sloughed epithelial cells, cell protoplasm, and mucus produced by the epidermal goblet or mucus cells (Henrikson and Matoltsy, 1967; Bullock and Roberts, 1974; Stoskopf, 1993; Esteban, 2012). The skin mucus is continuously secreted

and is mainly composed of water (approximately 95%) with glycoproteins and fatty acids as additional components, antibodies, enzymes and lytic agents (Henrikson and Matoltsy, 1967; Bullock and Roberts, 1974; Stoskopf, 1993; Jais *et al.*, 1998).

1.2. Epidermis

The epidermis, separating the individual from its environment, consists entirely of live cells, of which the majority are squamous cells and the minority mucous cells (Zhao *et al.*, 2008; Rakers *et al.*, 2010). The squamous cells are characterized by numerous desmosomes and associated cytoplasmic filaments with only minimal quantities of keratin in the cells of the superficial layer, whose cells show microridges that contain mucus secreted to the surface from mucous goblet cells (Fig.2), located in the intermediate stratum of the epidermis (Brown and Wellings, 1970; Mittal and Whitear, 1979). The epidermis is avascular and its number of cell layers may vary from two in larval fish to ten or more in adult fish (Whitear, 1986).

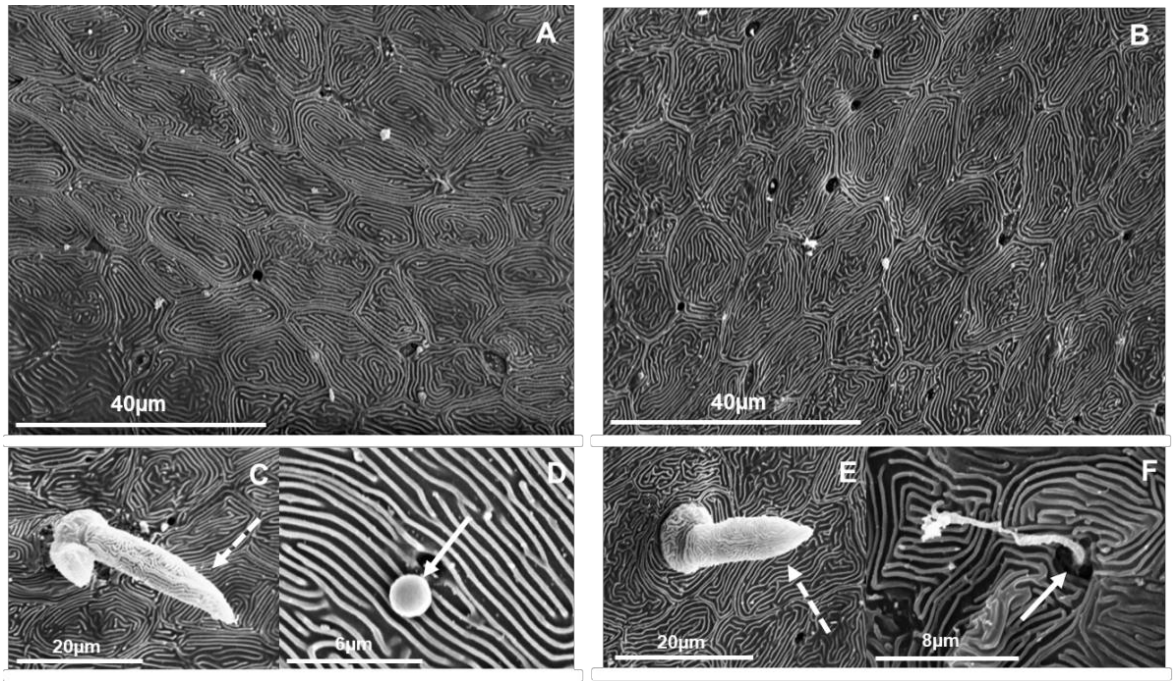


Figure 2. Analysis of fish skin by scanning electron microscopy (SEM). Representative images of dorsal (A, C, D) and ventral (B, E, F) skin from gilthead seabream (*Sparus aurata*) by SEM. Skin structures, including "sensory cells" (discontinuous arrows; C, E) and skin mucus secretions (continuous arrows; D, F), are detailed (Cordero *et al.*, 2017).

1.3. Dermis

The dermis, or corium, gives structural strength to the skin and is composed of two layers of loose and dense connective tissues. These layers provide a matrix for blood and lymphatic vessels, and nerves. The two layers of the dermis are termed the *stratum spongiosum (stratum laxum)* and the *stratum compactum*.

The *stratum spongiosum* contains a loose construction of collagen that is well vascularized, and is adjacent to the basement membrane of the epidermis (Bullock and Roberts, 1974; Groff, 2001). This layer contains, and primarily supports, the scales. Scales, although appearing to be epidermal projections, are actually of dermal origin and are the main component of the dermal skeleton. Scales have been classified according to size, shape, structure or a combination of these criteria (Ubels and Edelhauser, 1982; Stoskopf, 1993; Groff, 2001). The *stratum spongiosum* also contains smooth muscle, sensory papillae of various sensory organs, chromatophores or pigment cells, white blood cells, mast cells, osteoblasts, scleroblasts, vascular and nerve tissues (Bullock and Roberts, 1974; Groff, 2001). Pigment cells are located within the superficial layer of the dermis, and are identified according to the substances they produce (Ubels and Edelhauser, 1982; Stoskopf, 1993).

The second layer of the dermis, the *stratum compactum*, is a dense network of collagen over the hypodermis, which is penetrated by vascular and neural tissues that reach the more superficial layer of the dermis (Fontenot and Neiffer, 2004). This layer is relatively acellular as it only contains fibroblasts (Bullock and Roberts, 1974; Groff, 2001).

1.4. Hypodermis

The upper portion of the hypodermis, immediately below the dermal tissue, is generally occupied by the deep chromatophore layer (Whitear, 1986). It lies beneath the dermis and above the musculoskeletal tissue of the fish, and is composed of loosely organized connective tissue. This layer allows for movement of the integument between the *stratum compactum* of the dermis and underlying musculature. The hypodermis also contains abundant pigment cells, vascular and neural tissues, lipid cells, and fibroblasts. Due to its loose structural composition and blood supply, pathogens can easily infiltrate this layer through a skin lesion (Bullock and Roberts, 1974).

2. Skin functions

As in terrestrial species, the primary function of the teleost integumentary system is to create a protective barrier between the animal's internal organs and the environment (Fontenot *et al.*, 2004). However, the skin is a metabolically active and multipurpose tissue that fulfils many other functions. The unique mucus layer of the skin provides mechanical and chemical protection against pathogenic microorganisms and desiccation (Bullock and Roberts, 1974; Noga, 2000; Groff, 2001; Esteban, 2012).

The other skin layers encase the body maintaining the body shape, protecting the fish from insults such as physical damage and microorganism invasion, and preservation of hydrodynamics, locomotion, sensory perception, osmotic balance, respiration, ion regulation, excretion, and thermal regulation (Elliott, 2000; Marshall and Bellamy, 2010; Esteban and Cerezuela, 2015). Skin structures include the scales of the dermis and venom glands, alarm substance glands, pigment cells, and light organs located in the epidermis to deter predation either directly or through aiding in camouflage (Bullock and Roberts, 1974; Noga, 2000; Groff, 2001). Scales, which have always been assigned a merely protective role, are proving to have more functions than those traditionally ascribed, playing a crucial role in maintaining the structure of the upper layers of skin (Hawkes, 1974; Casado *et al.*, 2018). In addition, the microscopic study of scales is proving useful for species identification and even sex differentiation (Gholami *et al.*, 2013). It may also be possible to assess the fish general status by studying the ultrastructure of the scales under non-invasive conditions, such those used in the atomic force microscopy (AFM) (Casado *et al.*, 2018).

Fish represent the most ancient vertebrates with their mucosal adaptive immune system (Salinas *et al.*, 2011), which can be subdivided according to the anatomical location. The mucosal-associated lymphoid tissue (MALT) in teleost fish is subdivided into gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and the last discovered nasal-associated lymphoid tissues (NALT) (Salinas *et al.*, 2011; Tacchi *et al.*, 2014; Esteban and Cerezuela, 2015). Our interest is focused on SALT, because the skin is the largest MALT acting as the first line of defence against pathogenic agents. As the aquatic environment is rich in these pathogenic organisms, the skin of aquatic vertebrates plays an extremely important role as a protective barrier.

As previously mentioned, mucus is continuously secreted and replaced, immobilising or washing out foreign particles, bacteria and viruses before they can adhere to, or make contact with, epithelial cells since they are removed from the skin mucosa by the surrounding water, which prevents colonization by potential infectious agents. Thus, the most important immune function of the skin occurs in the mucus layer (Esteban, 2012). For this, the 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.*, 2018), proteases (serin-proteases, aspartic-proteases, cysteine-proteases and metalloproteases) as well as protease inhibitors (Bowden *et al.*, 1997; Rao *et al.*, 1998). Other enzymes such as esterases and alkaline phosphatases are less known but also important in mucus defences (Ross *et al.*, 2000; Guardiola *et al.*, 2014a,b). In addition, lysozyme deserves special mention since this enzyme is able to hydrolyse the peptidoglycan present in Gram + and Gram – bacterial cell walls (Grinde, 1989; Saurabh and Sahoo, 2008), a critical process in the skin mucus to protect against foreign pathogens. The composition of the skin mucus also determines its adhesiveness, viscoelasticity, transport and protective capacity (Gómez *et al.*, 2013).

3. Stress affects skin integrity

Stress is the non-specific response of the body to any demand for change (Selye, 1936). Throughout the animal kingdom, many types of stressful *stimuli* (stressors) are universal because the basic needs of most animals are similar. Examples of universal stressors include deviations from optimal ranges of environmental parameters (e.g., ambient temperature, oxygen supply, etc.), insufficient food availability, inadequate refuge from sunlight or predators and the demands of social interactions such as territorial disputes, while other stressors are unique to certain animal groups or habitats (Harper and Wolf, 2009) and very important for poikilotherm animals, including fish. In fish, physiological activities, among them the immune function, are greatly influenced by the environment and in most cases the outbreak of fish disease is dependent on environmental and endogenous factors, and unfavourable conditions result in opportunistic infection (Yada and Nakanishi, 2002).

Fish skin is metabolically very active and quickly responds to stressors (Noga, 2000). Common stressors in both wild and farmed fish are associated with environmental factors, infections or social behaviour causing skin damage (Yada and Nakanishi, 2002). Changes in

the skin (in one or more parts) are the most readily visible clinical features in fish, including haemorrhages, traumatic lesions, ulcers and changes in pigmentation (Esteban and Cerezuela, 2015). However, when fish are under intensive systems most of this damage is not clearly visible by the farmer until fish are quite seriously affected due to many circumstances such as the high number of specimens, the depth of water, decrease in light with increasing depth or organic particles, for example. Furthermore, skin lesions may be due to a primary disease condition of the integument or a manifestation of a systemic disease with concurrent involvement of the integument. Nevertheless, the pathogenesis of primary cutaneous lesions in fish is often complicated by secondary infections of the lesions (Groff, 2001).

In addition, epidermal damage not only provides access for infectious agents, but also produces an osmotic stress that can be life-threatening (Noga, 2000). In summary, the effect of a stressor, single or combined with any other detrimental factor, can produce skin damage or trigger a disease in fish (Fig. 3).

Figure 3. Representation of different stressors causing skin injury in fish.

4. Skin healing

Fish skin is susceptible to damages (skin lesions, abrasions or ulcers) from handling, fighting, physical trauma, predation, environmental irritants and pathogens, and the damage can lead to skin colonization by commensal (typically with low pathogenicity) and opportunist pathogenic microorganisms, which are present in abundance in the aquatic environment (Law, 2001; Harper and Wolf, 2009). As mentioned above, skin and its mucus provide an important barrier against infection and it is very important for this barrier to be reconstructed quickly after injury (Groff, 2001).

In addition to the skin layers described above, the internal layers and tissues, including sub-cuticular muscle, cartilage (more common in young teleost and cartilaginous elasmobranchs) and bone, might be compromised when fish skin is damaged (Stoskopf, 1993).

Although the susceptibility of the skin to damage, whether acute or chronic, can be influenced by the physical properties imposed by the thickness of the epidermis and its mucus secretions, living cells exist in all layers of the skin, providing fish with quicker healing abilities compared to terrestrial vertebrates (Noga, 2011). Therefore, after tissue damage, a sequence of temporarily overlapping events is triggered, to control the injury and restore homeostasis, combat infection, clear cellular debris and regain cellular composition and the extracellular matrix (ECM) architecture (Cordeiro and Jacinto, 2013). After wounding, the skin surface of healthy fish is quickly covered by mucus and re-epithelisation from the margins of the wounds occurs within a few hours (Iger and Abraham, 1990; Quilhac and Sire, 1999; Vieira *et al.*, 2011). This healing occurs in four phases (Fig. 4).

1) Haemostasis and clotting: The fish epidermis is avascular and so haemorrhaging may not be observed until the dermis is disrupted. If the dermis is affected, this process is initiated within the first seconds or minutes after wounding (Bullock and Roberts, 1974; Noga, 2000; Groff, 2001). Blood vessels constrict to prevent excessive blood loss, thrombocytes aggregate and blood coagulates to plug the wound and provide a matrix for infiltrating cells (Lorenz and Longaker, 2003; Artlett, 2013). Then, minutes after wounding, when the clot is formed, factors such as serotonin, 5-hydroxytryptamine, prostacyclin (PGI2) and histamine induce the reversal from vasoconstriction to vasodilation (Basu and Shukla, 2012). Some

microbes that enter the circulation can activate the alternative or lectin pathway of the complement system. The complement's degradation fragments serve as chemoattractants for neutrophils and as opsonins to promote phagocytosis, occurring in the next step (Tsirogianni *et al.*, 2006).

2) Inflammation: After haemostasis has been achieved, immune cells start to migrate into the wound space and an inflammatory response is initiated, becoming evident 1 to 3 hours following injury and being maintained for 3 to 4 days (Fontenot *et al.*, 2004; Tsirogianni *et al.*, 2006). While the immune cells secrete pro-inflammatory cytokines, including interleukin-1α (IL-1α), IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNFα), the inflammatory cells, notably neutrophils, also produce large amounts of reactive oxygen species (ROS), which are essential to protect the body from developing an infection but, when present in excess, can simultaneously damage the surrounding tissues (Kurahashi and Fujii, 2015). Then, phagocytes (neutrophils, monocytes-macrophages) ingest necrotic tissue and cell remnants (Fontenot *et al.*, 2004). It is also believed that the epidermal cells are capable of phagocytosis (Quilhac and Sire, 1999). In the normal process of wound healing, immune cells and inflammatory cytokines are reduced within a few days after an injury, at which time migrating keratinocytes, fibroblasts and endothelial cells start to secrete various growth factors (Kurahashi and Fujii, 2015).

3) Proliferation: The wound closes through a combination of re-epithelization and contraction processes. In many aquatic organisms the epidermis rapidly seals the affected area to regain osmotic homeostasis (Schmidt, 2013). Proliferation, organization and differentiation of the cells in the healing epidermis occurs 9 to 48 hours after re-epithelization and are typically completed in 3 to 4 days. This process has been throughly studied in a cichlid species, *Hemichromis bimaculatus*, using scanning and transmission electron microscopy (Quilhac and Sire, 1999). Then, in the proliferative phase, along with re-epithelization and angiogenesis, matrix deposition and collagen synthesis result in the formation of granulation tissue. Epithelial cells start to move laterally until they meet those migrating from the other side. Accordingly, fibroblasts are attracted from the wound edge, proliferate and then stimulate the migration and proliferation of keratinocytes. Neovascularization occurs and begins to supply nutrients and oxygen to the emerging tissue. Afterwards, proliferated fibroblasts secrete matrix proteins such as collagen to build the ECM and, together, this results in the formation of connective tissue. The resulting initial

dermal tissue thus compensates for the lost dermis and forms granulation tissue (Kurahashi and Fujii, 2015). In *H. bimaculatus*, cell proliferation peaked at 4 days post-injury and then decreased, the restoration of all cell layers being complete by 6 days post-trauma (Quilhac and Sire, 1999). If muscle tissue is injured, its regeneration starts at 7 to 8 days post-trauma and is completed by 10 to 15 days (Ramachandran and Thangavelu, 1969). By this time, differentiation of the cells of the epidermis has occurred accompanied by the formation of new scales (if applicable) (Fontenot *et al.*, 2004). Once the wound has contracted and enough amounts of new ECM have been produced, the granulation tissue develops more mature tissue through the apoptosis of endothelial cells and fibroblasts and by remodelling of the ECM (Schmidt, 2013).

4) Remodelling: In this final stage of healing, wounds are fully re-epithelialized, and the final steps of dermal reorganization take place (Braiman-Wiksman *et al.*, 2007). The wounded skin regains its strength and elasticity and proceeds through reorganization of the collagen and elastic fibers until the final reconstruction of the dermis. Collagen degradation is dependent on specific proteolytic enzymes known as matrix metalloproteinases that are produced by macrophages, keratinocytes and fibroblasts (Angelov *et al.*, 2004). Thus, there must be a delicate balance between proteases, their inhibitors and ECM molecules (Schmidt, 2013). Normal remodelling is also consistent with another healing stage: scar-tissue formation. This stage marks the termination of the healing process since all of the other assessed parameters have returned to pre-wounding levels (Braiman-Wiksman *et al.*, 2007). Remodelling starts from a few weeks after injury but may take several years (Stashak and Theoret, 2009; Schmidt, 2013).

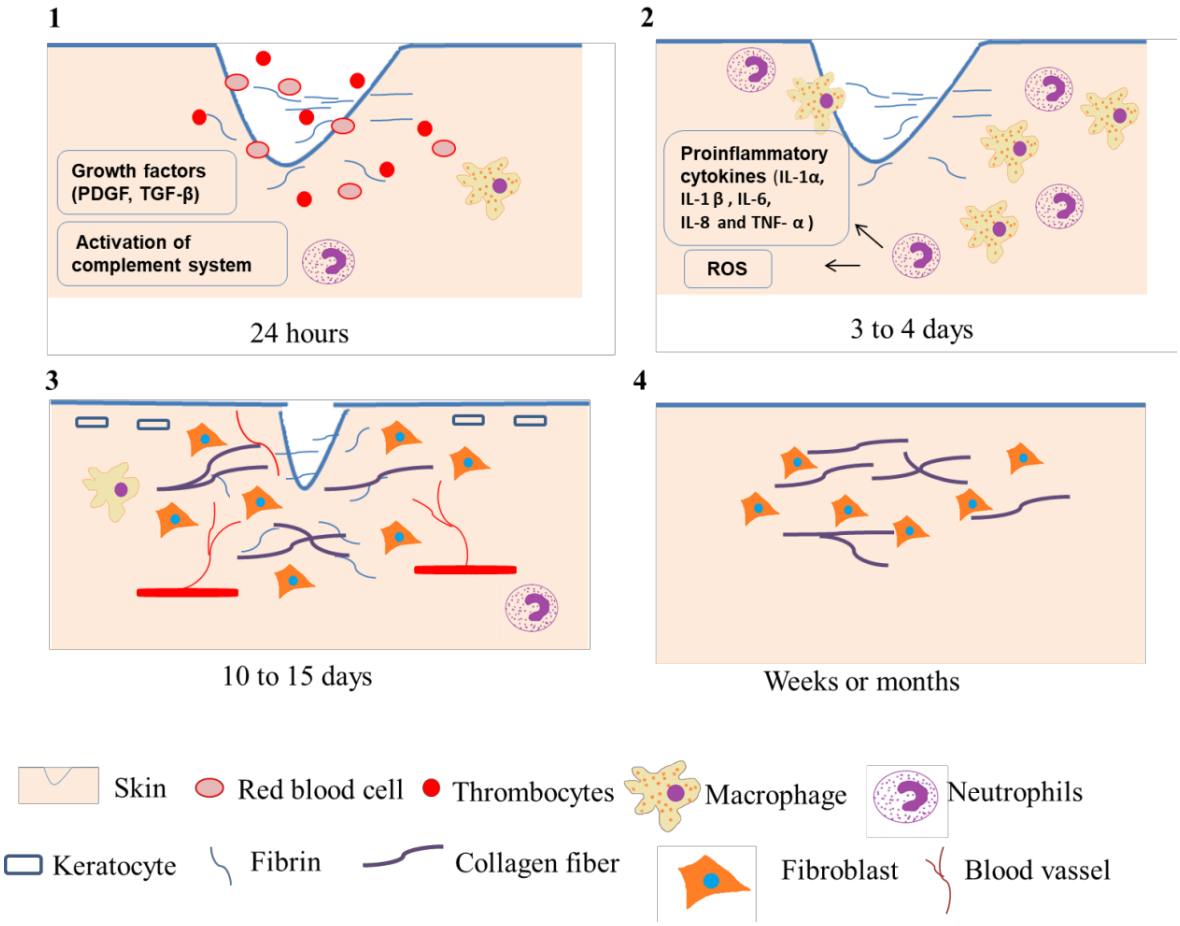


Figure 4. Stages of fish skin healing. 1. Haemostasis and clotting. 2. Inflammation. 3. Proliferation. 4. Remodelling.

It is known that stress factors, which vary in magnitude and duration, affect the healing process in humans and animals, causing a delay in the same (Christian *et al.*, 2006). Thus, although healing is a consistent and regulated process, stress can significantly affect its progression via multiple neuroendocrine and immune pathways. In this sense, glucocorticoid function is a key-related mechanism in the stress-healing association. Glucocorticoids, which are responsive to stress, affect the inflammatory processes by suppressing pro-inflammatory cytokines (Lowry, 1993). At this regard, impaired wounding or delayed healing may result in the increased occurrence of secondary infections (Noga, 2000; Roberts, 2012). Hence, the rapid healing of skin wounds is necessary to prevent the invasion of pathogens and to maintain the integrity of the surrounding tissue (Kumari *et al.*, 2017). Thus, the optimal management of wound infections is essential not only to promote a good healing response but also because of the significant morbidity and mortality associated with wound infections (Jones *et al.*, 2004).

5. Imaging techniques to study fish

The success of modern aquaculture is based on the control of many parameters, among which can be highlighted: reproduction, good knowledge of the biology of the farmed fish, technology innovation and the development of a specific feed (Esteban, 2012). Parameters corresponding to knowledge of fish biology and the innovation are closely associated due to the need to obtain as much information as possible with the lowest number of sacrificed animals in a short period of time (Romvári *et al.*, 2002).

In this sense, knowledge of the skin structure and function is critical to any improvement in the development of aquaculture (Casado *et al.*, 2018) due to the important role played by this organ in fish defence. For this reason, skin research has increased in the last decade, with the development of updated and innovative methodologies. Although many techniques have been used to study the skin, certain structural characteristic of fish skin still remain unrevealed (Casado *et al.*, 2018). Traditional techniques (light and confocal microscopy, transmission or scanning electron microscopy) often require fish dissection and tissue preparation in order to reveal anatomical and morphological details, so they are very expensive and need the fish to be sacrificed (Berquist *et al.*, 2012). Further, international norms for animal handling refer to the use of non-invasive techniques [according to the Guidelines of the European Union Council (2010/63/EU)]. Fortunately, there are techniques that can be used to acquire three-dimensional information of the body with high-spatial resolution. Moreover, recent technological advances have led to digital imaging becoming a familiar and versatile part of modern science, including Biology and Veterinary science (Nelson, 2006). Furthermore, the application of these techniques is necessary to understand fish skin behaviour *in situ*, in the face of different threatening situations and to provide rapid answers to common problems in aquaculture. These techniques provide a lot of information in a short time, involve the sacrifice of few animals and decrease the costs of analysis. In this sense, different non-destructive techniques of image analysis are fast increasing, among them, real-time ultrasonography and X-ray computed tomography (CT), both of which have been extensively used to describe human and animal anatomy (Ellis *et al.*, 1999; Lauridsen *et al.*, 2011; Drake *et al.*, 2017). However, their application in aquaculture is still limited (Romvári, 2002; Brinkman *et al.*, 2006; Babei, 2012; Carvalho *et al.*, 2018).

5.1. Real-time ultrasound (ultrasonography)

Ultrasound is one of the most used *in vivo* imaging techniques for studying internal body structures in a non-invasive way; it is an economical and flexible technique that offers anatomical and dynamic information (Domínguez, 2011). Ultrasound is defined as a series of mechanical waves, usually longitudinal, originated by the vibration of an elastic body (crystal piezoelectric) and propagated by a material medium (body tissues), whose frequency exceeds the sound audible by the human (Villaseñor *et al.*, 2012).

The ultrasound equipment is composed of a transducer or probe, the gain button, the gain and curve buttons according to the depth and a screen to display the image (Segura *et al.*, 2014). The technique is based on the emission of an ultrasound wave and the reception of the reflected wave (Fig. 5). Incident ultrasonic energy is partially transmitted and partially reflected at the boundary between adjacent structures; this generates echoes, whose amplitudes are characteristic of the nature of the medium. Then, an image is formed which can be measured either manually or with computer assistance to determine the thickness of the structure (Waller and Maibach, 2005). The technique is similar to the echolocation used by bats, whales and dolphins, as well as Sound Navigation and Ranging (SONAR) used by submarines (Freudenrich, 2001).

There are three basic ways of presenting ultrasound images: the A or amplitude mode, was used initially to distinguish between cystic and solid structures and it is used to represent a signal graphically; the M-mode is used for structures in movement such as the heart; in a graphic representation of the signal, the amplitude is the vertical axis, while time and depth are the horizontal axis; finally, the B-mode is the pictorial representation of the sum of the echoes in different directions (axial, lateral). Each echo is displayed at a point in the image, which corresponds to the relative position of its origin within the body cross section, resulting in a scaled map of echo-producing features. The brightness of the image at each point is related to the strength or amplitude of the echo, giving rise to the term B-mode (brightness mode) (Hoskins *et al.*, 2010; Díaz, 2014). The B-mode is the modality used in all ultrasound equipments in real time and it is a static two-dimensional image. The acoustic reflectance is measured by the echogenicity, i.e. the ability of a tissue to reflect an ultrasound wave. Thus, echogenic structures appear bright on ultrasound (the higher the amplitude of the reflected wave, the brighter the pixel) (Villaseñor *et al.*, 2012).

Ultrasound is characterized by its penetration, which is inversely proportional to the emitted frequency; therefore, the more superficial the structure being investigated, the higher the emission frequency. Thus, transducers of 7-13 MHz are needed to study skin (Alfageme *et al.*, 2011). Modern ultrasound systems are able to make detailed measurements of blood movements in blood vessels and tissues, visualize moving structures in 3D and make measurements related to the stiffness of body tissues (Hoskins *et al.*, 2010)

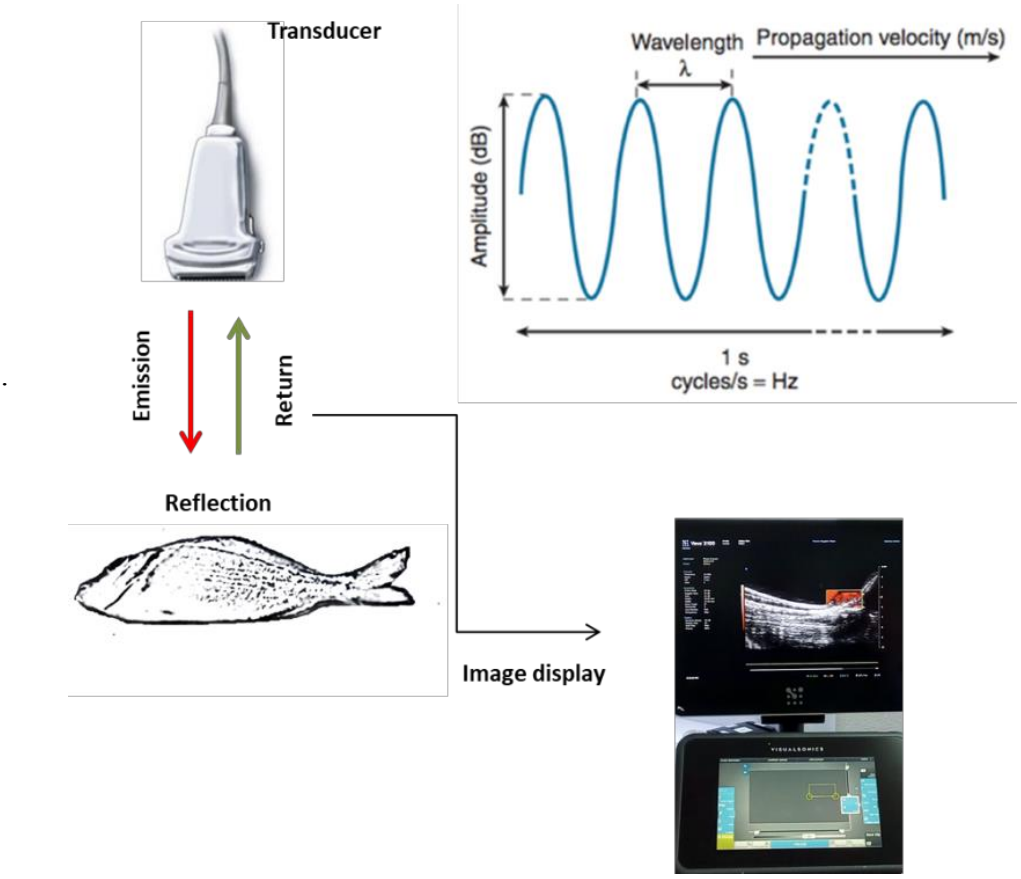


Figure 5. Real-time ultrasound scan scheme. The ultrasound beam from the transducer converts the electrical energy to sound waves, wich propagate through different tissues and return to the transducer as reflected echoes. The generated echoes are converted into electrical pulses by the transducer and an image is displayed in the screen.

5.2. X-ray computed tomography

X-ray computed tomography is a non-destructive technique for visualizing interior features within in biological specimens based on their differences in contrast and obtaining digital information on their 3D geometries and properties (Ketchman and Carlson, 2000; Gremse *et al.*, 2014).

Tomography refers to the cross-sectional imaging of a subject from either transmission or reflection data collected by illuminating the subject from many different directions. The elements of X-ray computed tomography are a X-ray source and a series of detectors that measure X-ray intensity attenuation along multiple beam paths, associated to a gantry that rotates around the subject, which is positioned in the centre of the scanner (Ketchman and Carlson, 2001; Lauridsen *et al.*, 2011). Different configurations of these components can be used to create CT scanners optimized for imaging subjects of various sizes (Ketchman and Carlson, 2001). CT is routinely used to diagnose diseases in humans and animals. Moreover, there are numerous applications for CT in research and smaller versions are now manufactured for small animal research (micro-CT) (Haacke *et al.*, 1999; Lauridsen *et al.*, 2011; Berquist *et al.*, 2012; Sasser *et al.*, 2012). Micro-CT provides sectional images of specimens of interest without disturbing internal structures.

During micro-CT scanning, electromagnetic radiation (X-rays) penetrates the study specimen from 360° (Fig. 6A). Because some radiation is absorbed by the tissues, the initial data processed by the computer system are, in fact, shadow projections of the absorption from various angles. These data are then reconstructed, typically with a filtered back projection algorithm (Lauridsen *et al.*, 2011; Wathen *et al.*, 2013). The pre-defined field of view is divided into volume elements (voxels) and the absorption of the X-ray beam when it passes through the specimen, measured in Hounsfield units (HU). Then, voxels are computed into elements (pixels), creating thin cross-sectional images of the study specimen that are displayed as individual grey shades on a screen, allowing differentiation between tissues and structures according to their radio-density (Fig. 6A), where black represents the negative values (e.g. air) and white the positive values (e.g. bones). Density ranges vary between -1,000 HU for air and +1,000 HU for bones (Fig. 6B). Average values correspond to liquid structures, with approximate values of 1-10 HU. A negative value corresponds to fat. These ranges may vary depending on the specimen being studied (Lauridsen *et al.*, 2011; Wathen *et al.*, 2013).

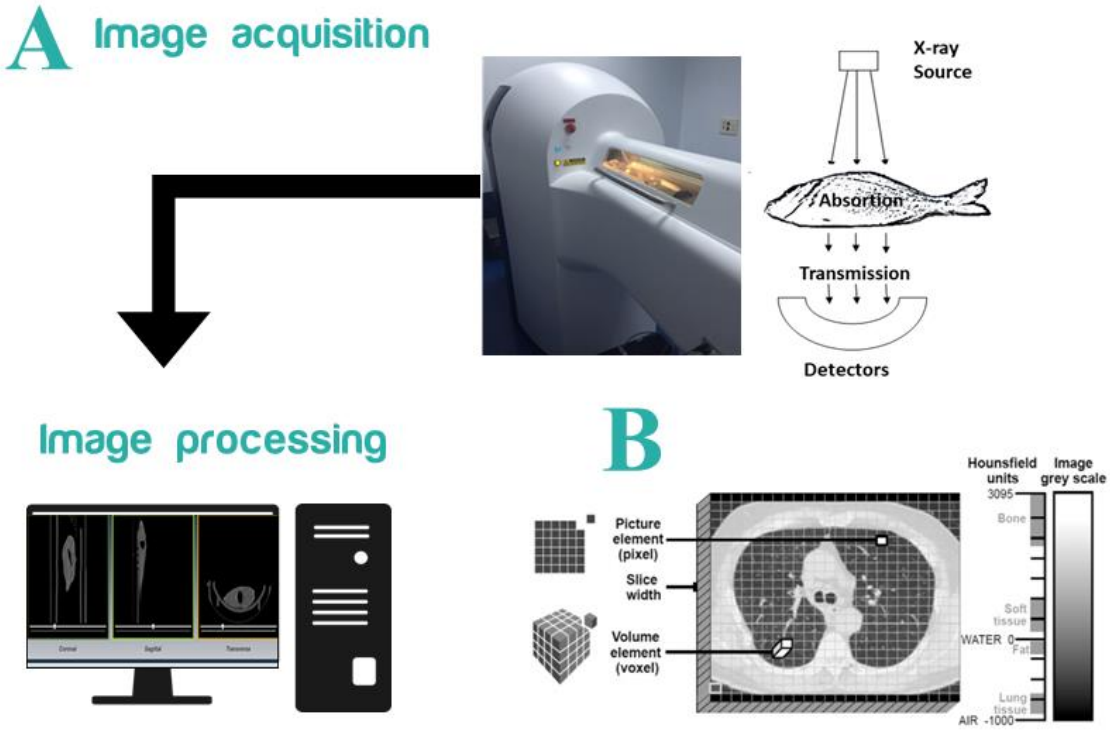


Figure 6. A) Computed tomography (CT) scan scheme. The X-ray source emits a beam that passes through the material under study, suffering an absorption process that impresses some detectors and which after being processed, will allow us to obtain an image (pixel) of the volume junction (voxel) of the material studied. B) Visual depiction of the Hounsfield scale, representing the radio-densities of various tissues/materials (Modified from Campos and Díaz, 2017).

Micro-CT is distinguished from other imaging techniques for its ability to acquire high-resolution images based on the physical density of the tissues (Lauridsen *et al.*, 2011). Among other strengths of this technique are that it represents entirely non-destructive 3D imaging, little or no sample preparation is required and reconstruction is generally attenuation-conservative, allowing sub-voxel level details to be extracted. The only preparation necessary for micro-CT scanning is to ensure that the subject fits inside the field of view and that it does not move during the scan. Micro-CT data generally take the form of a sequence of image files, which can be visualized and analysed using a wide variety of 2D and 3D-based image processing tools (Ketchman and Carlson, 2001).


OBJECTIVES

This Doctoral Thesis aims to improve our knowledge of fish skin due to the importance of this organ, the many functions which it performs and its interest to the immunity, since it forms part of the organs which have associated lymphoid tissue.

The specific objectives are:

1. To ascertain wether some variations in the skin mucus immune activities as consequence of the circadian cycle.
2. To check wether it is possible to strengthen the skin mucosal immunity and to reduce its pathogen colonization by manipulating fish diet.
3. To increase knowledge of the processes of healing, regeneration and skin ulceration in fish, as well as their involvement in the skin mucus immune response and the associated microbiota.
4. To test a technique to visualize cutaneous wounds in their initial stages.
5. To help fish characterization by using imaging techniques.


EXPERIMENTAL
CHAPTERS

PART 1

CHAPTER I

Effects of dark-light cycle on skin mucosal immune activities of gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*)

CHAPTER II

Effect of dietary supplementation of guava leaf (*Psidium guajava* L.) on the skin of hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*)

CHAPTER III

Healing and immune response in gilthead seabream (*S. aurata*) after experimental injury

III.1. Healing and humoral immune parameters in skin mucus and serum of gilthead seabream after induced skin injury

III.2. Mucus glycosylation, immunity and bacterial microbiota associated to the skin of experimentally ulcerated gilthead seabream

III.3. Optimization of fluorescein test to detect initial skin lesions in fish

PART 2

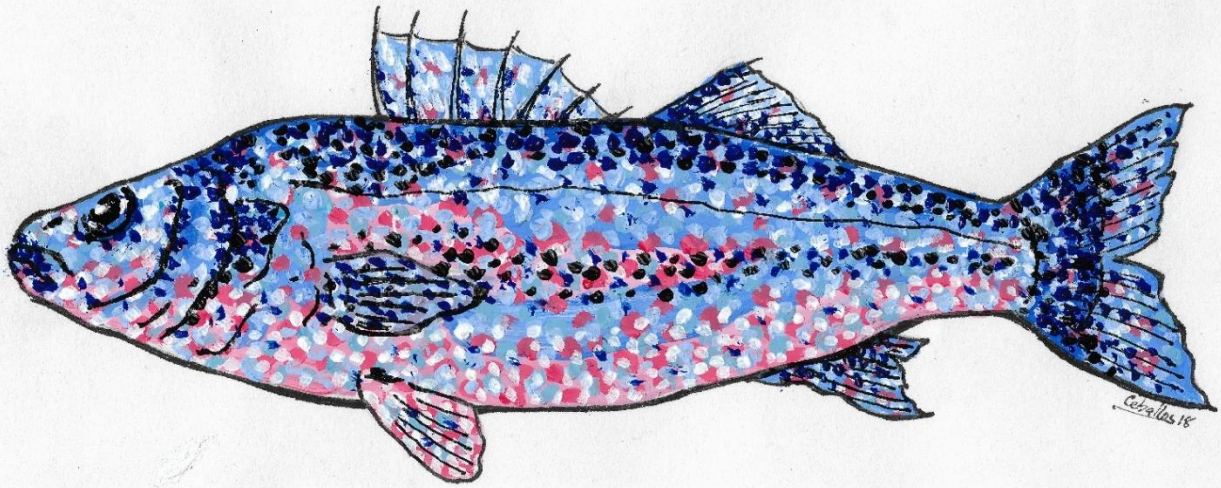
CHAPTER IV

Imaging techniques to study fish

IV.1. Skin imaging by real time ultrasound (ultrasonography)

IV.2. Skin imaging by X-ray computed tomography (micro-CT)

IV.3. Fat imaging by X-ray computed tomography (micro-CT)



Unpublished paint inspired in European sea bass *(Dicentrarchus labrax)* (C. Ceballos 2018).

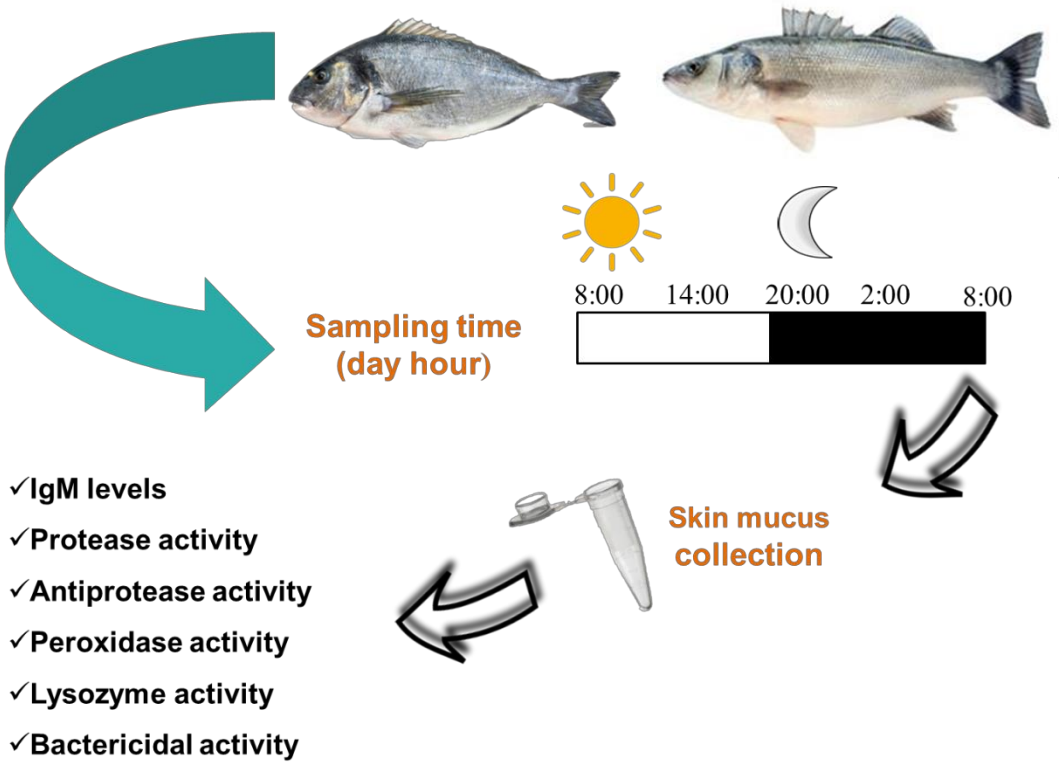
CHAPTER I

Effects of dark-light cycle on skin mucosal immune activities of gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*)

1. Objective

Many environmental factors may influence the life cycles of fish (Valero *et al.*, 2014). Fortunately, most organisms (including fish) are equipped with a biological system that allows them to maintain a circadian rhythm to adapt to daily environmental changes in 24-h cycles generated by the Earth's rotation (Bowden *et al.*, 2007). Circadian rhythms are endogenously generated in vertebrates and play central roles in the maintenance of homeostasis and growth (Boeuf and Le Bail, 1999; Kalra *et al.*, 1999; Huang *et al.*, 2011). In this sense, although artificial photoperiods have been used to improve fish growth and manipulate reproduction (Maitra and Hasan, 2016), there is still very little information about the possibilities of altering photoperiod to improve fish immune status (Bowden *et al.*, 2007). Thus, in the present study we aimed to analyse the changes in different immune activities in the skin mucus of gilthead seabream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) specimens exposed to a constant light–dark photoperiod (12 h L:12 h D). This knowledge about how the daily rhythms regulate the main fish immune activities will help us to try to improve fish welfare and health.

2. Graphical abstract



3. Materials and Methods

3.1. Fish

Thirty specimens of the hermaphroditic protandrous seawater teleost gilthead seabream (*S. aurata*) and 30 specimens of the gonocoric seawater teleost European sea bass *(D. labrax)* obtained from a local farm were kept in re-circulating seawater aquaria (400 L), with a flow rate of 900 L h^{-1} at 22 ± 2°C and 25‰ of salinity in the Marine Fish Facilities at the University of Murcia. Commercial diet (Skretting) was administrated at rate of 2% body weight day^{-1}. An individual light source, consisting of a blue bulb (Sylvania Grolux) was located at the top of each tank. The light–dark (LD) cycle (artificial photoperiod 12 h L: 12 h D) was programmed by an electronic timer and set to switch on at 8:00 h and off at 20:00 h. Specimens were reared under a 12 L: 12 D daily cycles for a month.

All the experimental protocols made in this Doctoral Thesis were approved by the Ethical Committee of the University of Murcia (Permit Number: A13150104), following the guidelines of European Union for animal handling (2010/63/EU).

3.2. Experimental design and sampling

Skin mucus samples were collected at 8:00, 14:00, 20:00, 2:00 and 8:00 h (six fish per time and per fish species). Sample collection during the dark phase was conducted with a red light. Prior to sampling, fish were anesthetized with 20 mg L^{-1} of clove oil (Guinama®). Skin mucus was gently collected with a cell scraper (Sigma-Aldrich) from the whole skin surface, avoiding blood, urine and faeces during collection (Palaksha *et al.*, 2008). Mucus samples were vigorously shaken and centrifuged (1,400 × g, 10 min, 4°C) being the supernatant collected and kept frozen at -20°C until use (Cordero *et al.*, 2016d). Protein concentration in each sample was determined by the Bradford method (Bradford, 1976).

3.3. Total immunoglobulin M (IgM) levels

Total IgM levels were analysed for gilthead seabream and European sea bass using the enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Cuesta *et al.*, 2004). Thus, 10 µg $well^{-1}$ of skin mucus proteins were coated in flat-bottomed 96-well plates (Nunc) in triplicate by overnight incubation at 4°C with 100 µL of carbonate-bicarbonate buffer (35

mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBS-T [phosphate buffer saline (PBS) and 0.05% Tween 20], plates were blocked for 2 h at room temperature with 200 µL per well with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS-T (blocking buffer) and rinsed three times with PBS-T. The plates were then incubated for 1 h with 100 µL per well of mouse anti-gilthead seabream or anti-sea bass IgM monoclonal antibody (1:100 in blocking buffer; Aquatic Diagnostics Ltd.), washed and incubated for 1 h with the secondary antibody anti-mouse IgG-HRP (1:1,000 in blocking buffer; Sigma-Aldrich). After exhaustive rinsing with PBS-T the samples were developed using 100 µL of 0.42 mM 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB; Sigma-Aldrich) containing 0.01% hydrogen peroxide (H_2O_2). The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µL of 2 M sulfuric acid (H_2SO_4). The plates were read at 450 nm in a plate reader (BMG; Labtech). Negative controls consisted of samples without skin mucus samples or without primary antibody, whose optical density (OD) values were subtracted for each sample value.

3.4. 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 125 µL of 0.7% azocasein (Sigma-Aldrich) for 24 h at 30°C. The reaction was stopped by adding 4.6% of trichloroacetic acid (TCA; Sigma-Aldrich) and the mixture was centrifuged (13,000 × g, 5 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µL $well^{-1}$ of 0.5 N sodium hydroxide (NaOH), and the OD was read at 450 nm using a plate reader. Skin mucus was replaced by trypsin solution (5 mg mL^{-1}; Sigma-Aldrich), as positive control (100% of protease activity), or by buffer, as negative control (0% activity).

3.5. Antiprotease activity

Total antiprotease activity was determined by the capacity of the skin mucus to inhibit trypsin activity (Hanif *et al.*, 2004). Briefly, 10 µL of skin mucus samples were incubated (10 min, 22°C) with the same volume of standard trypsin solution (5 mg mL^{-1}). After adding 100 µL of 100 mM ammonium bicarbonate buffer and 125 µL of 0.7% azocasein, samples were incubated (2 h, 30°C) and, following the addition of 250 µL of 4.6% TCA, a new

incubation (30 min, 30°C) was done. The mixture was then centrifuged (13,000 × g, 5 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µL well 0.5 N NaOH, and the OD read at 450 nm using a plate reader. For a positive control, buffer replaced skin mucus (100% protease and 0% antiprotease activity), and for a negative control, buffer replaced the trypsin (0% protease and 100% antiprotease activity). The percentage of inhibition of trypsin activity by each sample was then calculated.

3.6. Peroxidase activity

The peroxidase activity in skin mucus was measured according to Quade and Roth (1997). Briefly, 10 µL of skin mucus were diluted with 40 µL of Hank's buffer (HBSS; Panreac) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. As substrate, 100 µl of 10 mM TMB solution containing 0.015 % H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µL of 2 M H_2SO_4 and the OD 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 expressed as U mg $protein^{-1}$.

3.7. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Parry *et al.* (1965) with some modifications. Briefly, 20 µL of skin mucus were placed in flat-bottomed 96-well plates. To each well, 180 µL of freeze-dried *Micrococcus lysodeikticus* (0.2 mg mL^{-1}; Sigma-Aldrich) in 40 mM sodium phosphate (pH 6.2) was added as lysozyme substrate. As blanks of each sample, 20 µL of skin mucus were added to 180 µL of sodium phosphate buffer. The absorbance at 450 nm was measured after 20 min at 35ºC in a microplate reader. The amounts of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL; Sigma-Aldrich) through serial dilutions in the above buffer. Skin mucus lysozyme activity is expressed as U mg $protein^{-1}$ equivalent of HEWL activity.

3.8. Bactericidal activity

An opportunist marine pathogenic bacterium (*Vibrio harveyi*) was used to determine the bactericidal activity present in skin mucus samples. Bacteria were grown in agar plates at 25°C in tryptic soy agar (TSA; Sigma-Aldrich). Then, fresh single colonies of 1-2 mm were

diluted in 5 mL of tryptic soy broth (TSB; Sigma-Aldrich), cultured for 16 h at 25ºC on an orbital incubator at 200-250 revolutions per minute (rpm) and adjusted to 10^8 bacteria mL^{-1} TSB. The absorbance of bacteria cell cultures was measured at 600 nm and used to know the concentration based on growth curves.

The mucus bactericidal activity was then determined following the method of Graham *et al.* (1988) with some modifications. Briefly, 20 µL of skin mucus was added to triplicate wells of U-shaped 96-well plates (Nunc). HBSS was added to some wells instead of sample and served as positive control (100% bacterial growth is 0% bactericidal activity). Then, 20 µL of *V. harveyi* was added and plates were incubated for 5 h at 25ºC. To each well, 25 µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg mL^{-1}; Sigma-Aldrich) were added and incubated for 10 min to allow the formation of formazan. Plates were then centrifuged (2,000 × g, 10 min) and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich), being transferred to flat-bottomed 96-well plates and the absorbance of the dissolved formazan was measured at 560 nm in a plate reader. Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving in each sample compared to the number of bacteria from positive controls (100%).

3.9. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). All data were analysed by one-way ANOVA followed by Tukey's post-hoc analysis to determine differences among groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. All the statistical analysis was conducted using Statistical Package for Social Science (SPSS 23.0 software). In addition, daily rhythms for each activity were analysed using the Ritme© software package v. 1, 179 by Dr. Diez-Noguera (University of Barcelona). Differences for both analyses were considered statistically significant when $P<0.05$.

4. Results

4.1. Total IgM levels

Immunoglobulin M levels in skin mucus of gilthead seabream showed the highest values at 08:00 h (when lights were turned on) and decreased all through the light cycle reaching the minimum values at 20:00 h (when the lights were switched off), at this time statistically significant variations were recorded for this parameter (Fig. 7 A). During the dark period, the gilthead seabream IgM were increasing (Fig. 7A). By contrast, no significant variations were obtained in European sea bass for IgM levels at the different experimental times, though an inversed pattern was observed (Fig. 7 B). Therefore, a significant daily rhythm was found for skin mucus IgM levels in gilthead seabream but no in European sea bass (Fig. 7 A).

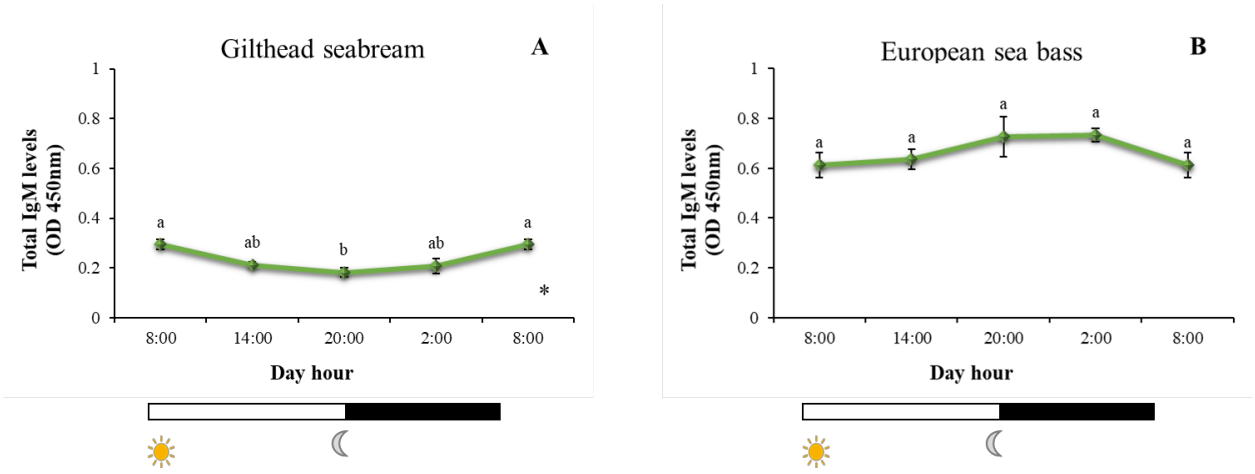


Figure 7. Photoperiod effect on total immunoglobulin M (IgM) levels found in skin mucus of gilthead seabream (*S. aurata*) (A) and European sea bass (*D. labrax*) (B), sampled at 6-h intervals throughout the day. Symbols and indicate the lights on and off, respectively. Results are expressed as mean ± SEM (n=6). Different letters denote significant differences among groups. An asterisk indicates that the activity during a daily cycle followed a significant rhythmic pattern. The levels of significance were set at $P<0.05$.

4.2. Enzymatic activities in skin mucus

Protease, antiprotease, peroxidase and lysozyme activities were studied in skin mucus of both fish species during the light–dark cycle. Regarding protease and antiprotease activities, a similar pattern was observed in both fish species and no significant changes were observed for any studied time and besides this, no significant daily rhythms were found in these activities (Fig. 8 A-D). Contrarily, peroxidase activity reached its maximum and its minimum at 02:00 h in gilthead seabream and European sea bass, respectively (Fig. 8 E, F). The lower and higher levels were recorded at 08:00 h (when lights were turned on) for gilthead seabream and European sea bass, respectively. Significant daily rhythm was obtained for European sea bass peroxidase activity, but no for gilthead seabream (Fig. 8 F). On the other hand, lysozyme activity in gilthead seabream skin mucus was not affected (Fig. 8 G, H). However, in European sea bass, lysozyme activity decreased at 14:00 h respect to the values recorded at 8:00 h (when lights were switched on) but the values recovered to similar levels to the rest of sampling. By contrast, significant daily rhythmicity was found in this activity for both fish species (Fig. 8 G, H).

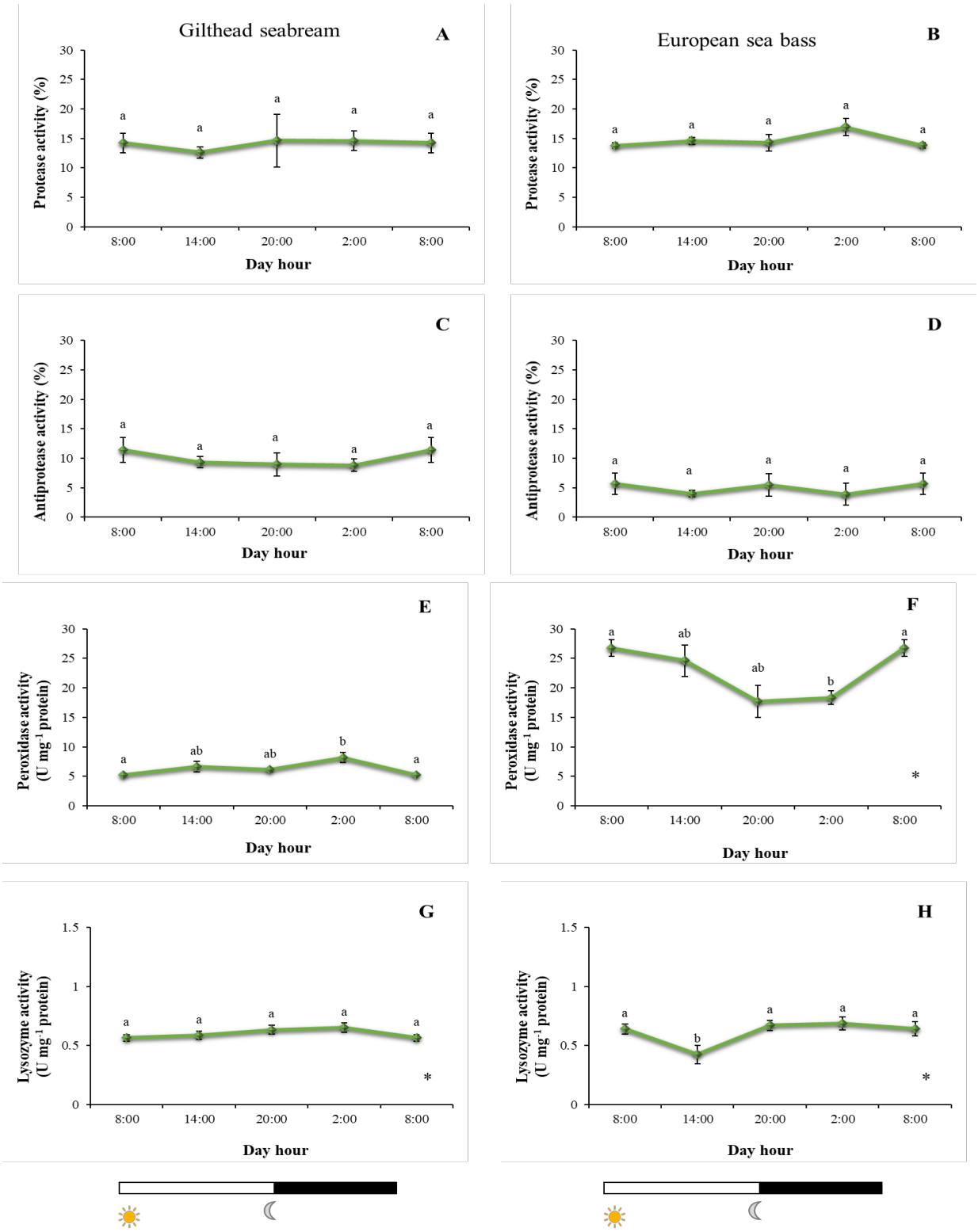


Figure 8. Photoperiod effect on protease (A, B), antiprotease (C, D), peroxidase (E, F) and lysozyme (G, H) activities in skin mucus of gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*), sampled at 6-h intervals throughout the day. Symbols ☀ and ☾ indicate the lights on and off, respectively. Results are expressed as mean ± SEM (n=6). Different letters denote significant differences among groups. An asterisk indicates that the activity during a daily cycle followed a significant rhythmic pattern. The levels of significance were set at $P<0.05$.

4.3. Bactericidal activity

Bactericidal activity against *V. harveyi* was differently affected by the photoperiod in gilthead seabream and European sea bass (Fig. 9). In the case of gilthead seabream, the activity increased throughout the day reaching a significant increase at 2:00 h. From this time, the activity was decreasing again till reaching the same values at 8:00 h (Fig. 9 A), with no significant daily rhythm. On the contrary, European sea bass bactericidal activity was significantly lower from 14:00 to 20:00 h, respect to the values recorded for skin mucus collected at 8:00 h, showing also a significant daily rhythm (Fig. 9 B).

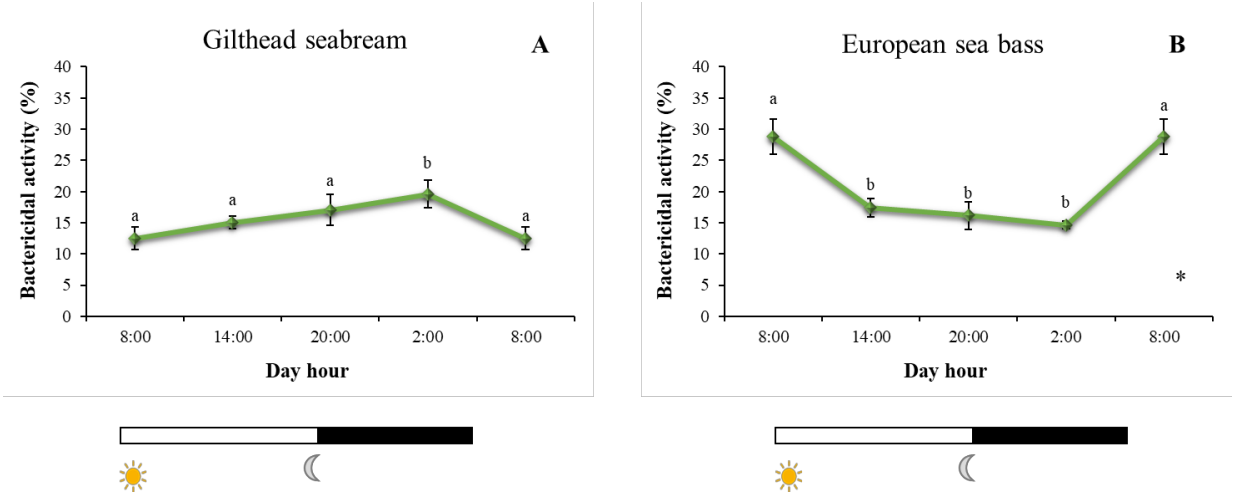
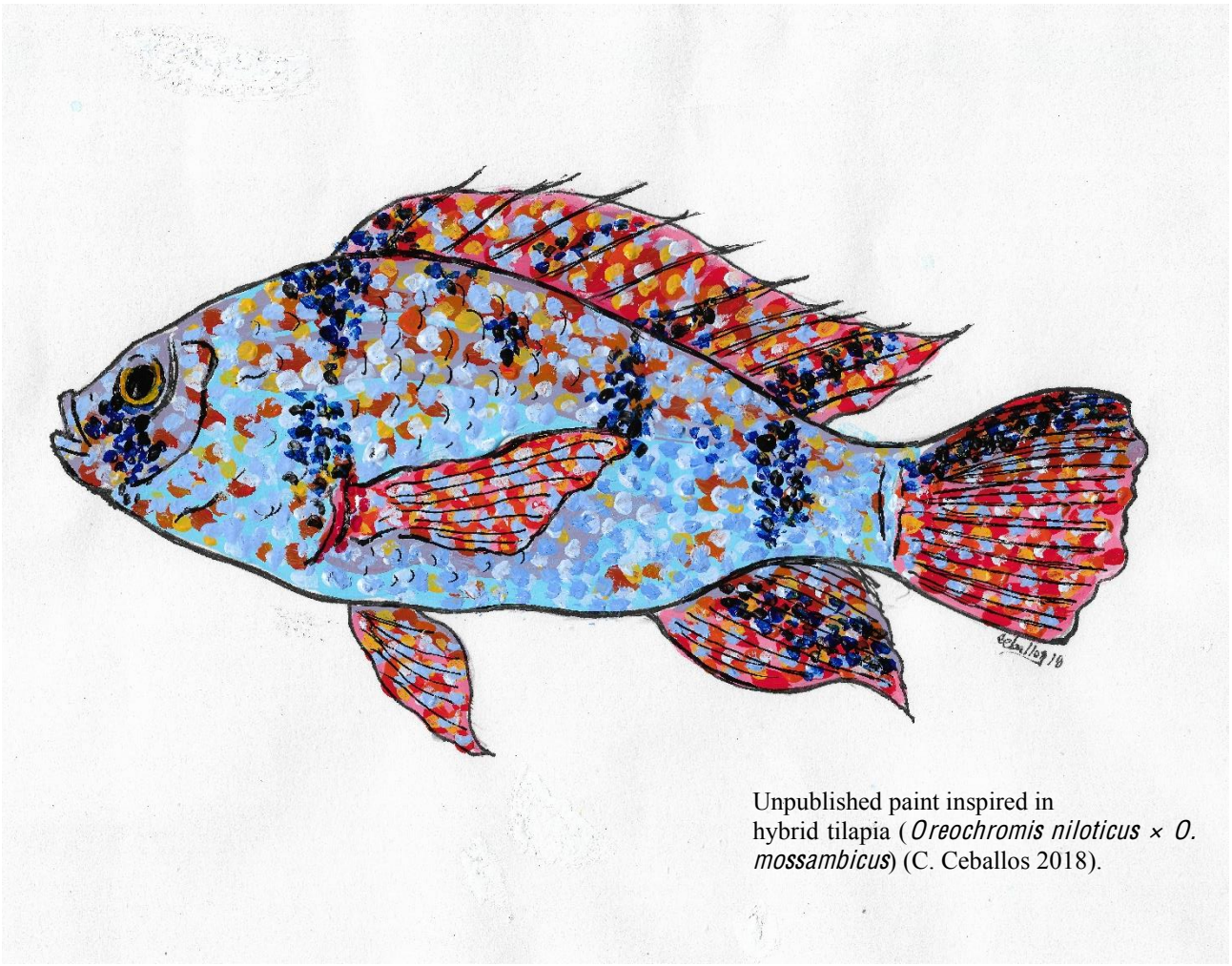


Figure 9. Photoperiod effect on bactericidal activity against *V. harveyi* in skin mucus of gilthead seabream (*S. aurata*) (A) and European sea bass (*D. labrax*) (B), sampled at 6-h intervals throughout the day. Symbols and indicate the lights on and off, respectively. Results are expressed as mean ± SEM (n=6). Different letters denote significant differences among groups. An asterisk indicates that the activity during a daily cycle followed a significant rhythmic pattern. The levels of significance were set at $P<0.05$.



Unpublished paint inspired in hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) (C. Ceballos 2018).

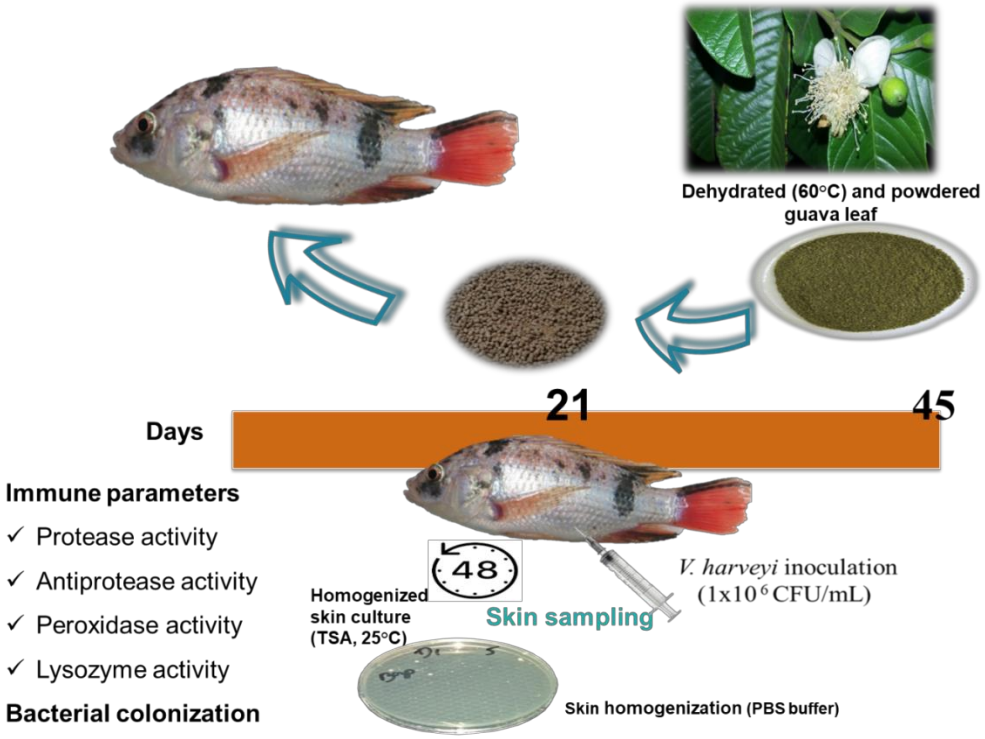
CHAPTER II

Effect of dietary supplementation of guava leaf (*Psidium guajava* L.) on the skin of hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*)

1. Objective

Fish skin is a primary target organ for a number of common infectious agents normally found in the aquatic environment (Noga, 2011) between them *Vibrio* genera, which are among the most important, affecting wild and aquaculture fish, while also affecting humans (Austin, 2010). In the last decade, bacterial resistance has become an important issue because it represents a threat to global health, food security and development (Aidara-Kane *et al.*, 2018). As a consequence, the study of alternatives to antibiotics in aquaculture has increased. Of these alternatives, plants are considered the most reliable source of compounds to prevent and/or treat infections in both animals and humans (Rios *et al.*, 1987; Vieira *et al.*, 2001; Villamor and Fawzi, 2005; Wang *et al.*, 2009; Dawood and Koshio, 2016; Dotta *et al.*, 2018). The most important advantage of using plants as immunostimulants in aquaculture is that they contain natural organic materials that are safe to fish health, to the environment and to human health (Talpur *et al.*, 2013). Thus, in this chapter we aimed to study the dietary effects of guava (*Psidium guajava* L.) leaf on the skin mucus of hybrid tilapia (*Oreochromis niloticus* × O. *mossambicus*) and the possible microbicidal effects in fish skin against *V. harveyi*.

2. Graphical abstract



3. Materials and Methods

3.1. Preparation of diets

Guava leaves were collected from guava trees growing on a private property (Santo Domingo Este, Dominican Republic). The leaves were washed and dried for 24 h and stored at room temperature. Dried guava leaves were ground using a mixer grinder (Moligrano; Becken).

Three diets containing different concentrations of guava leaf were prepared (0%, control, 1.5% and 3%). A commercial pellet diet (GISIS) was crushed and mixed with tap water before the correct amount of guava leaf powder was added and pelleted again to obtain the control and supplemented diets. All the experimental diets were allowed to dry and were then stored at 4°C.

3.2. Fish and experimental design

Fifty-four hybrid fish of *Oreochromis niloticus* × *Oreochromis mossambicus* (160 ± 20 g and 20 ± 4 cm) were obtained from a local farm (Bayaguana, Dominican Republic). Fish were allowed to acclimatise and gradually adapted to brackish water (12‰, 26 ± 1°C) in the fish facilities at the National Aquarium of Dominican Republic for 30 days before starting the trial. The commercial diet was administered at a rate of 2% body weight twice a day. The photoperiod was 12 h L: 12 h D.

Fish were randomly assigned and divided into six tanks (9 fish per tank) of 358 L, in which three experimental groups were established in duplicate: control (non-supplemented diet, 0% guava leaf), 1.5 % and 3% guava leaf supplemented diets. Fish were fed at a rate of 2% body weight twice a day for 45 days.

3.3. Skin mucus immune parameters

After 21 or 45 days of feeding, 6 fish from each experimental group were sedated with 20 mg L^{-1} of clove oil and skin mucus samples were taken as described in Chapter I section 3. Skin mucus samples were adjusted to 500 µg protein mL^{-1} and protease, antiprotease, peroxidase and lysozyme activities were determined following the protocols previously described in Chapter I section 3.

3.4. Experimental infection with V. harveyi

The bacterium (*V. harveyi*) was cultured as in Chapter I section 3. Bacteria were washed twice with PBS and the concentration was adjusted to 10^4 bacteria mL^{-1}.

A preliminary study to evaluate the *V. harveyi* colonization in naïve specimens was carried out. Thus, hybrid tilapia specimens were sedated and intraperitoneally injected with 100 µL of *V. harveyi* at 10^4 bacteria mL^{-1} or PBS (control) using 1 mL syringes. Two days post-injection, fish were sacrificed with an overdose of clove oil (40 mg L^{-1}) and ventral skin, brain, head kidney, spleen, liver and middle gut were removed, weighed and placed in 1.5 mL centrifuge tubes. Samples were homogenized in PBS with a pellet pestle (Fisherbrand™) and centrifuged at 300 × g for 30 s. Supernatants were serially diluted and spread (50 µL) on TSA plates and incubated at 25°C. The number of colony-forming units (CFU) per g^{-1} organ was counted in agar plates after 24 h (Castillo *et al.*, 2016).

After 21 days of feeding with guava leaf supplemented diets, fish were infected with *V. harveyi* and 2 days later the ventral skin was sampled to determine the bacterial load as above.

3.5. Statistical analysis

The results were expressed as mean ± SEM (n=6). All data were analysed by one-way ANOVA followed by Tukey's post-hoc analysis to determine differences among groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. All the statistical analyses were conducted using SPSS 23.0 software and differences were considered statistically significant when $P<0.05$.

4. Results

4.1. Skin mucus immune parameters

The parameters studied in the skin mucus of hybrid tilapia were protease, antiprotease, peroxidase and lysozyme activities (Fig. 10). Only the dietary supplementation with 1.5% guava leaf produced significant effects in the hybrid tilapia skin mucus immune parameters, protease activity (Fig. 10 A) being significantly higher after 21 days of dietary intake, while

antiprotease (Fig. 10 B) and peroxidase (Fig. 10 C) activities increased after 45 days. By contrast, lysozyme activity was not regulated by either of the guava leaf diets (Fig. 10 D).

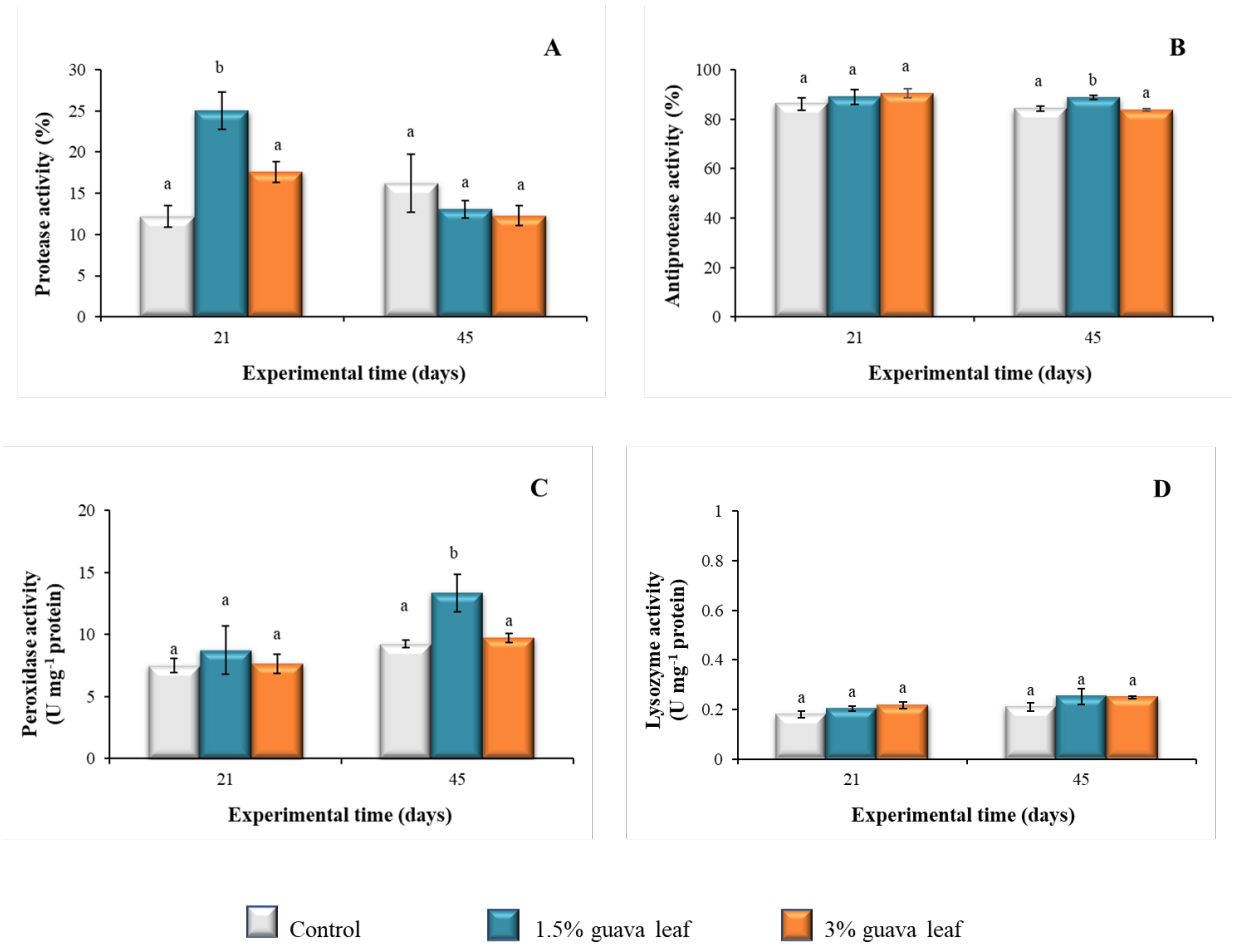


Figure 10. Immune parameters determined in skin mucus of hybrid tilapia (*O. niloticus* × *O. mossambicus*) fed experimental diets for 21 or 45 days. Protease (A), antiprotease (B) peroxidase (C) and lysozyme (D) activities. Bars represent mean ± SEM (n=6). Different letters denote significant differences between groups when $P<0.05$.

4.2. Bacterial infection

In naïve fish, *V. harveyi* infection for 2 days failed to provoke any alteration in their behaviour and no external or internal abnormalities were observed macroscopically (Fig.11).

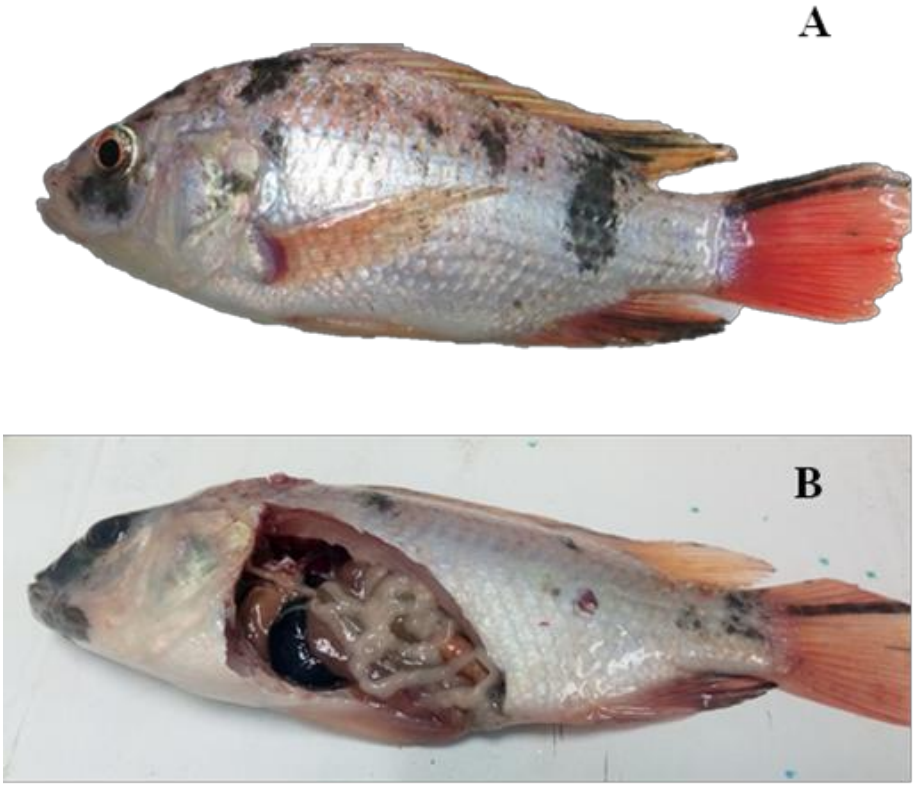


Figure 11. Macroscopic image of hybrid tilapia (*O. niloticus* × *O. mossambicus*) 48 h post-injection with 100 µL *V. harveyi*. Images corroborate the absence of external (A) and internal (B) macroscopic abnormalities in sampled fish.

Furthermore, we determined the bacterial colonization and proliferation in the ventral skin, brain, head kidney, liver, spleen and middle gut after 24 h of incubation. Different rates of bacterial colonization were observed in cultures of skin, spleen and liver, the highest number of CFU being found in the skin (Fig. 12). Curiously, no bacterial growth was observed in agar plates containing homogenates of brain, head kidney and middle gut after 24 h of incubation.

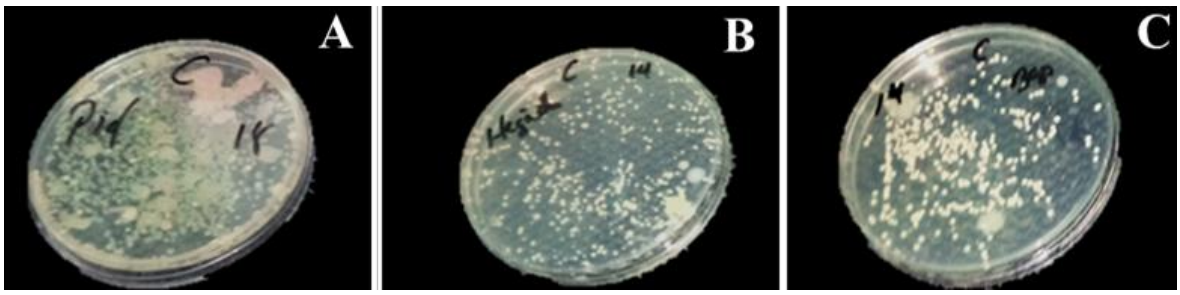


Figure 12. Representative agar plates of bacterial growth in homogenates from skin (A), liver (B) and spleen (C) of hybrid tilapia *(O. niloticus × O. mossambicus)* infected with *V. harveyi*.

4.3. Guava leaf supplemented diet reduce infection

Skin homogenates from fish infected with *V. harveyi* and previously fed different experimental diets (0%, control, 1.5% and 3% guava leaf supplementation) were analysed. The CFU counts showed that the bacterial colonies growing on agar plates of skin homogenates from fish supplemented with both diets (1.5% and 3% guava leaf) were significantly lower compared with the control (non-supplemented) fish 48 h post-injection with *V. harveyi* (Fig.12 A, B).

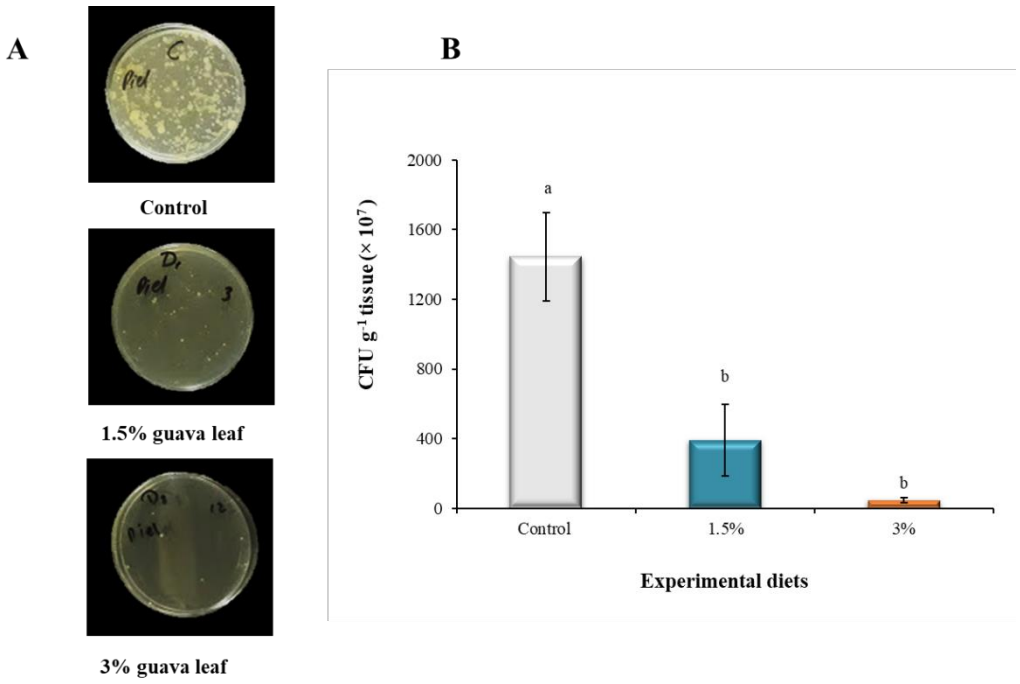
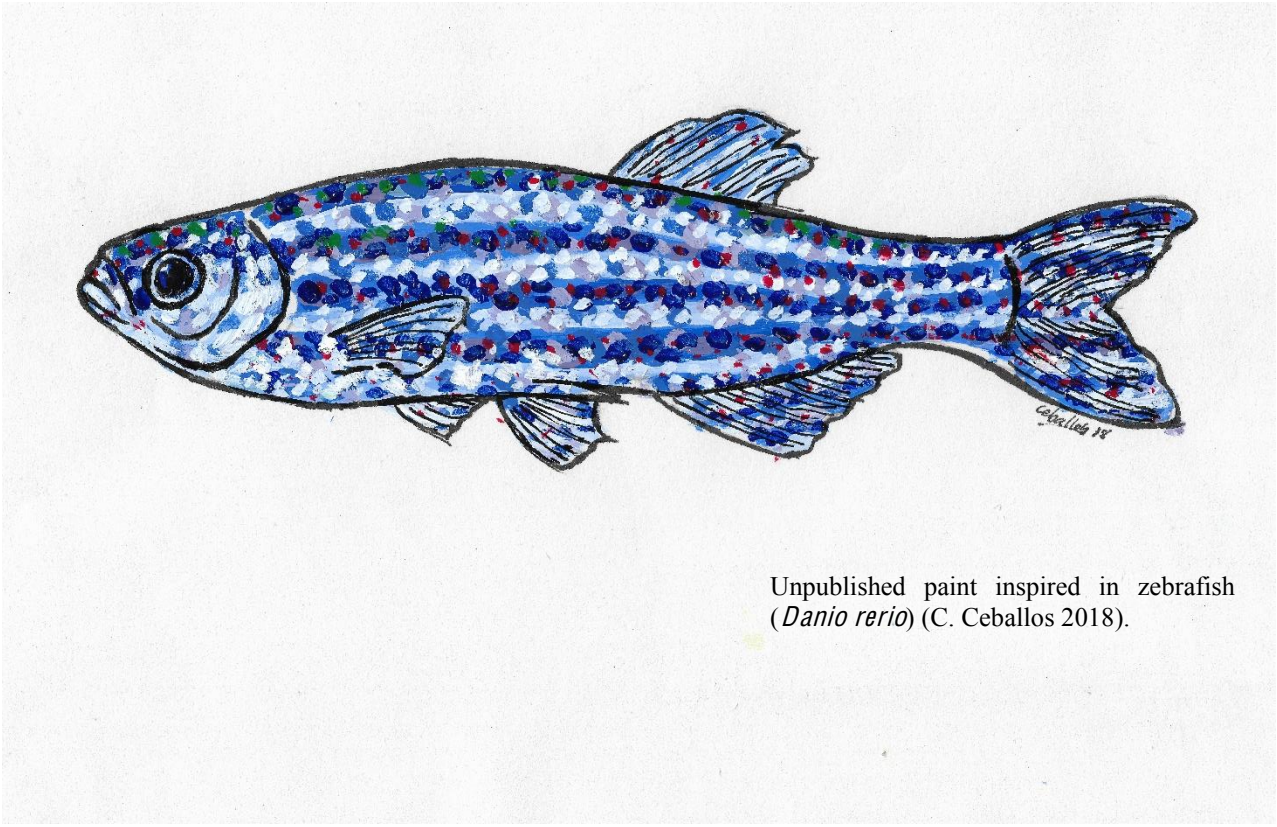


Figure 13. A) Representative agar plates of bacterial growth from skin homogenates obtained from different experimental groups of hybrid tilapia *(O. niloticus × O. mossambicus)* infected with *V. harveyi.* B) Bacterial colonies (CFU) in skin homogenates from hybrid tilapia (*O. niloticus* × *O. mossambicus*) 48 h post-injection with *V. harveyi* and fed with guava leaf experimental diets: control, non-supplemented diet (gray bar), 1.5% guava leaf (blue bar) and 3% guava leaf (orange bar) supplemented diets. Results are expressed as mean ± SEM (n=6). Different letters denote significant differences among groups when $P<0.05$.



Unpublished paint inspired in zebrafish (*Danio rerio*) (C. Ceballos 2018).

CHAPTER III

Healing and immune response in gilthead seabream (*S. aurata*) after experimental injury

III.1. Healing and humoral immune parameters in skin mucus and serum of gilthead seabream after induced skin injury

III.2. Mucus glycosylation, immunity and bacterial microbiota associated to the skin of experimentally ulcerated gilthead seabream

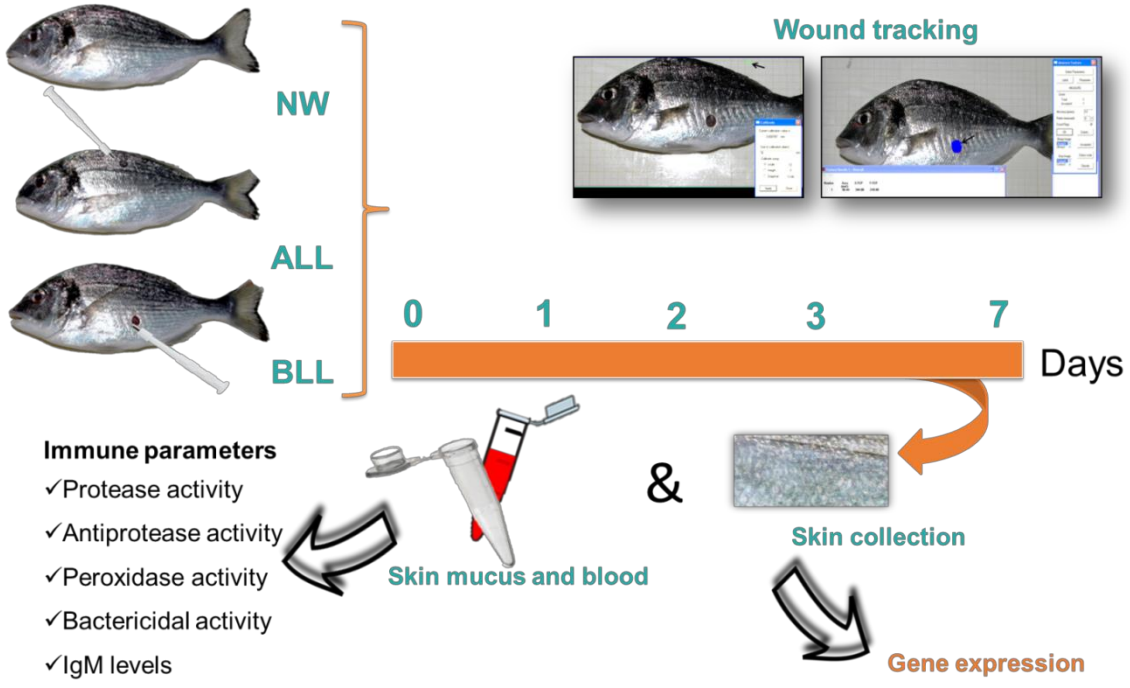
III.3. Optimization of fluorescein test to detect initial skin damages in fish

III.1. Healing and humoral immune parameters in skin mucus and serum of gilthead seabream after induced skin injury

1. Objective

Fish skin is a complex limiting structure that provides an interface between the hostile aquatic environment and the organism. In aquaculture, skin lesions are very common increasing the risk of pathogens entering through the wounded skin of the fish (Fontenot and Neiffer, 2004; Esteban, 2012). Because of this, skin integrity is important to avoid significant morbidity and mortality rates in aquaculture. The aim of the present work was to study the skin healing progress during 7 days after experimental wounds on gilthead seabream (*Sparus aurata*) specimens. Two locations were selected (above and below the lateral line) because it is known that fish integument anatomy can vary with many factors, including species, sex, life stage, season, reproductive condition, nutrition, water quality, location on the body and general health status (Bullock and Roberts, 1974; Iger and Abraham, 1990; Groff, 2001; Fontenot and Neiffer, 2004). Concomitantly, the skin mucosal and serum immunity was studied by analysing some important immune parameters, as well as the gene expression profile of some immune relevant genes and others involved in cell regeneration in skin wounded samples.

2. Graphical abstract



3. Material and methods

3.1. Fish

Fifty-four gilthead seabream (*S. aurata*) specimens (138 ± 10 g and 19 ± 1 cm) obtained from a local farm (San Pedro del Pinatar, Murcia) were kept in re-circulating seawater aquaria (400 L), with a flow rate of 900 L h^{-1} at 22 ± 2°C and 28‰ salinity in the Marine Fish Facilities at the University of Murcia. A commercial diet (Skretting) was administered at a rate of 2% body weight day^{-1}. The photoperiod was 12 h L: 12 h D.

3.2. Experimental trial and wounds

Before making the experimental wounds, fish were sedated with 20 mg L^{-1} of clove oil and divided into three experimental groups of 18 specimens each: the first group (control group or non-wounded group, NW) did not receive any wound although fish were manipulated in the same way as the other two groups; all the fish from the second and the third groups were wounded by using a metallic circular biopsy punch (Fig. 14) with a diameter of 8 mm and 2 mm depth (Stiekel), either above (ALL) or below (BLL) the lateral line (LL). All wounds were made by the same researcher and in the same part of the fish (always in the middle of the left side).

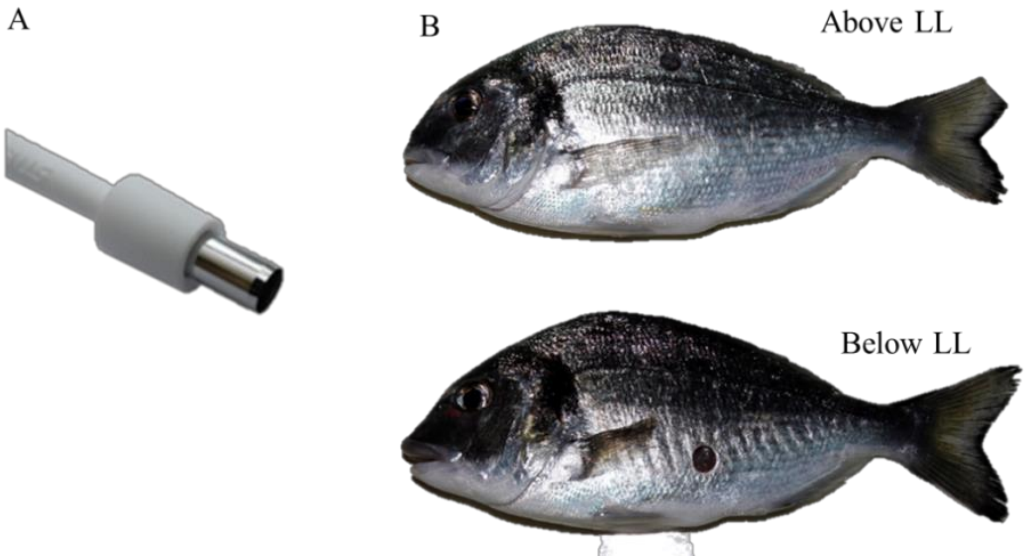


Figure 14. A) Biopsy punch (8 mm) used in this study to perform the experimental wounds. B) Representative images of experimental wounds made above and below the lateral line (LL) in gilthead seabream *(S. aurata)* specimens.

The trial was performed in accordance with the wound healing process, taking the day the wound was made as day 0. The fish were sampled every 24 h during 7 days (Braiman *et al.*, 2007; Deka *et al.*, 2013) as described below. No mortalities were recorded during the experimental trial.

3.3. Image analysis

For macroscopic observation of the healing process, daily images of the wounds were taken with a Canon 7D camera with a wide-angle lens of 22 mm 4.5 (Canon EF) coupled to a ring flash with a tripod. The images were analysed using the Leica QWin image analysis software (Leica Microsystems Ltd.) to determine the wound area in mm^2 (Van *et al.*, 2007).

3.4. Immune parameters in skin mucus and serum

Skin mucus of the wounds and surrounding area and blood samples were taken at 0, 1, 2, 3 and 7 days post-wounding. Skin mucus was collected, centrifuged and stored following the protocol previously described in Chapter I section 3. Blood samples of 1 mL from three fish from each group were taken from the caudal vein. Serum samples were obtained by centrifugation (10,000 × g, 10 min, 4°C) and immediately frozen at -80°C until use. The protein concentration in each sample was determined by the Bradford method (Bradford, 1976) and adjusted to 500 µg protein mL^{-1}.

Protease, antiprotease, peroxidase, and bactericidal activities, and IgM levels, were determined in skin mucus and serum following the protocols previously described in Chapter I section 3.

3.5. Skin gene expression analysis

Skin samples taken from around the wounds were placed in TRIzol® reagent (Life Technologies) and stored at -80°C for later RNA extraction. RNA from the samples was extracted as indicated by the manufacturer's instructions and quantified with Nanodrop®. The 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 SuperScriptIV reverse transcriptase (Life Technologies) with an oligo-dT primer (Life Technologies). The expression of the selected genes was analysed by real-time PCR (qPCR),

which was performed with an ABI PRISM 7500 Instrument (Applied Biosystems) as described elsewhere (Cordero *et al.*, 2015) and using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). For each mRNA, gene expression was corrected by both the elongation factor 1 alpha (*ef1a*) and the ribosomal protein S18 (*rps18*) RNA content in each sample. Details of primers are listed in Table 1.

Table 1. Primers used in this study.

Gene name	Abbreviation	Accession no.	Primer sequence
elongation factor 1 alpha	*ef1a*	AF184170	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA
ribosomal protein S18	*rps18*	AM490061	F: CGAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC
interleukin 1beta	*il1b*	AJ277166	F: GGGCTGAACAACAGCACTCTC R: TTAACACTCTCCACCCTCCA
interleukin 6	*il6*	AM749958	F: AGGCAGGAGTTTGAAGCTGA R: ATGCTGAAGTTGGTGGAAGG
tumor necrosis factor alpha	*tnfa*	AJ413189	F: TCGTTCAGAGTCTCCTGCAG R:TCGCGCTACTCAGAGTCCATG
transforming growth factor beta	*tgfb*	AF424703	F: GCATGTGGCAGAGATGAAGA R: TTCAGCATGATACGGCAGAG
interleukin 10	*il10*	FG261948	F: AGGCAGGAGTTTGAAGCTGA R: ATGCTGAAGTTGGTGGAAGG
grainyhead-like transcription factor	*grhl1*	AM976768	F: GGTGCACCTCCAAACAAGAT R: ATAGCTTCCACCAGGCCTTT
keratin type 1	*krt1*	FJ744592	F: AGAGATCAATGACCTGCGGC R: CCCTCTGTGTCTGCCAATGT
immunoglobulin M heavy chain	*ighm*	AM493677	F: CAGCCTCGAGAAGTGGAAAC R: GAGGTTGACCAGGTTGGTGT
immunoglobulin T heavy chain	*ight*	FM145138	F: TGGCAAATTGATGGACAAAA R: CCATCTCCCTTGTGGACAGT
Cu Zn-superoxide dismutase	*sod*	AJ937872	F: CCATGGTAAGAATCATGGCG R: CGTGGATCACCATGGTTCTG
catalase	*cat*	FG264808	F: TTCCCGTCCTTCATTCACTC R: CTCCAGAAGTCCCACACCAT

3.6. Statistical analysis

The results were expressed as mean ± SEM (n=3). All data were analysed by one- or two-way ANOVA followed by Tukey's post-hoc analysis to determine differences among groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. For gene expression, data were expressed as fold change obtained by dividing each sample value by the mean control value at the same sampling time. Values higher than 1 express an increase, while values lower than 1 express a decrease in the indicated gene (Pfaffl, 2001).

All the statistical analyses were conducted using SPSS 23.0 software and differences were considered statistically significant when $P<0.05$.

4. Results

4.1. Image analysis of wound healing

Experimental wounds were made above (ALL) or below (BLL) the lateral line (LL) of the gilthead seabream specimens and the wounds were photographed daily over a period of 7 days to study the healing progress in both groups of fish (Fig. 15).

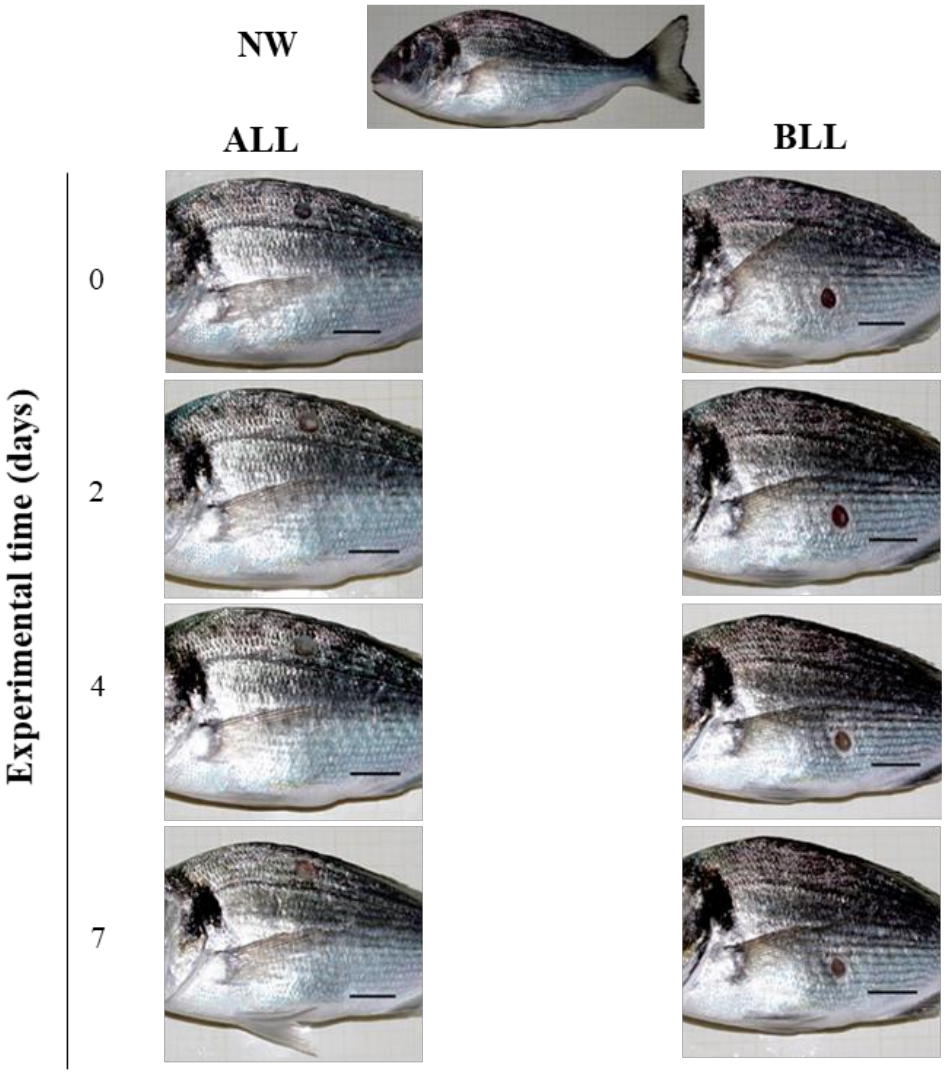


Figure 15. Representative photographs showing wound healing progression from day 0 to day 7 in skin of gilthead seabream (*S. aurata*). Control or non-wounded group (NW); wounded above the lateral line (ALL) and wounded below the lateral line (BLL) groups. Scale bar: 20 mm.

The initial and final stage of wound healing in groups ALL and BLL are shown in more detail in Fig. 16.

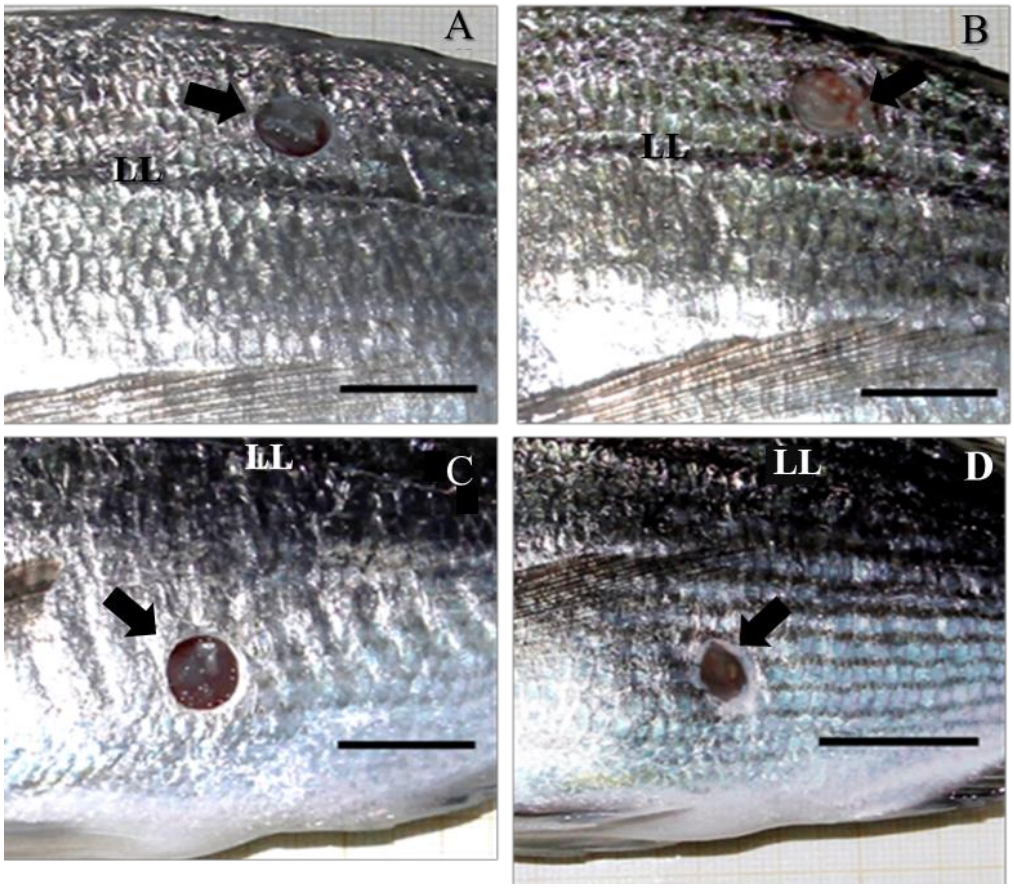


Figure 16. Experimental wounds (8 mm diameter) made in gilthead seabream (*S. aurata*) specimens above (A, B) and below (C, D) the lateral line at 0 (A, C) and 7 (B, D) days. Arrows indicate the wounds; LL = lateral line. Scale bar: 20 mm.

Wound areas were measured from the macroscopic photographs by image analysis (Fig. 17). The results indicate that the area of the wounds made in both groups increased from 1 day post-wounding and then started to decrease until day 7 post-wounding (Fig. 17). However, the area of the wounds in group BLL remained almost stable from 2 to 3 days post-wounding to decrease thereafter. Although no significant differences were found between the areas of the wounds in ALL and BLL fish at any experimental day, the wounds areas in group BLL were smaller from 5 days than those of group ALL at the same time (Fig. 17). Coinciding with the macroscopic images showed in Figs.15 and 16.

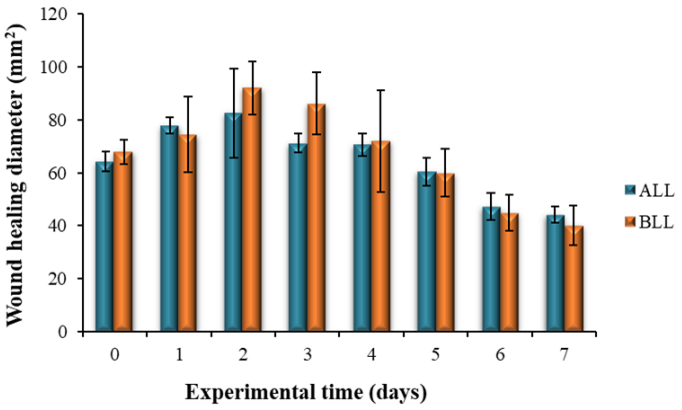


Figure 17. Wound healing area (mm^2) in gilthead seabream (*S. aurata*) specimens after wounding above (ALL) or below (BLL) the lateral line. Results are expressed as the mean ± SEM (n=3).

4.2. Skin mucus and serum immune parameters

Different enzymatic activities related to innate immunity were determined in the skin mucus and serum of wounded fish and the values were compared to those obtained in mucus or serum of the controls, non-wounded fish (NW). No significant differences were observed between the protease activity in the skin mucus of gilthead seabream from ALL and BLL groups compared to the values obtained for the control fish (NW) (Fig. 18 A). However, in serum, a statistically significant increment was observed 7 days post-wounding from wounded fish (both, ALL or BLL; Fig. 18 B). In the case of antiprotease activity, skin mucus values in BLL were significantly lower than that observed in NW group after 7 days (Fig. 18 C); same trend was observed in serum samples, but with no significant results (Fig. 18 D). As regards peroxidase activity, statistically significant decreases and increases, respectively, were detected in skin mucus from BLL and ALL groups 1 or 2 days post-wounding compared with the values recorded in skin mucus from the control group (Fig. 18 E). Similar results were found in serum samples, where higher peroxidase activity was found BLL 2 days post-wounding, compared with the control group (Fig. 18 F).

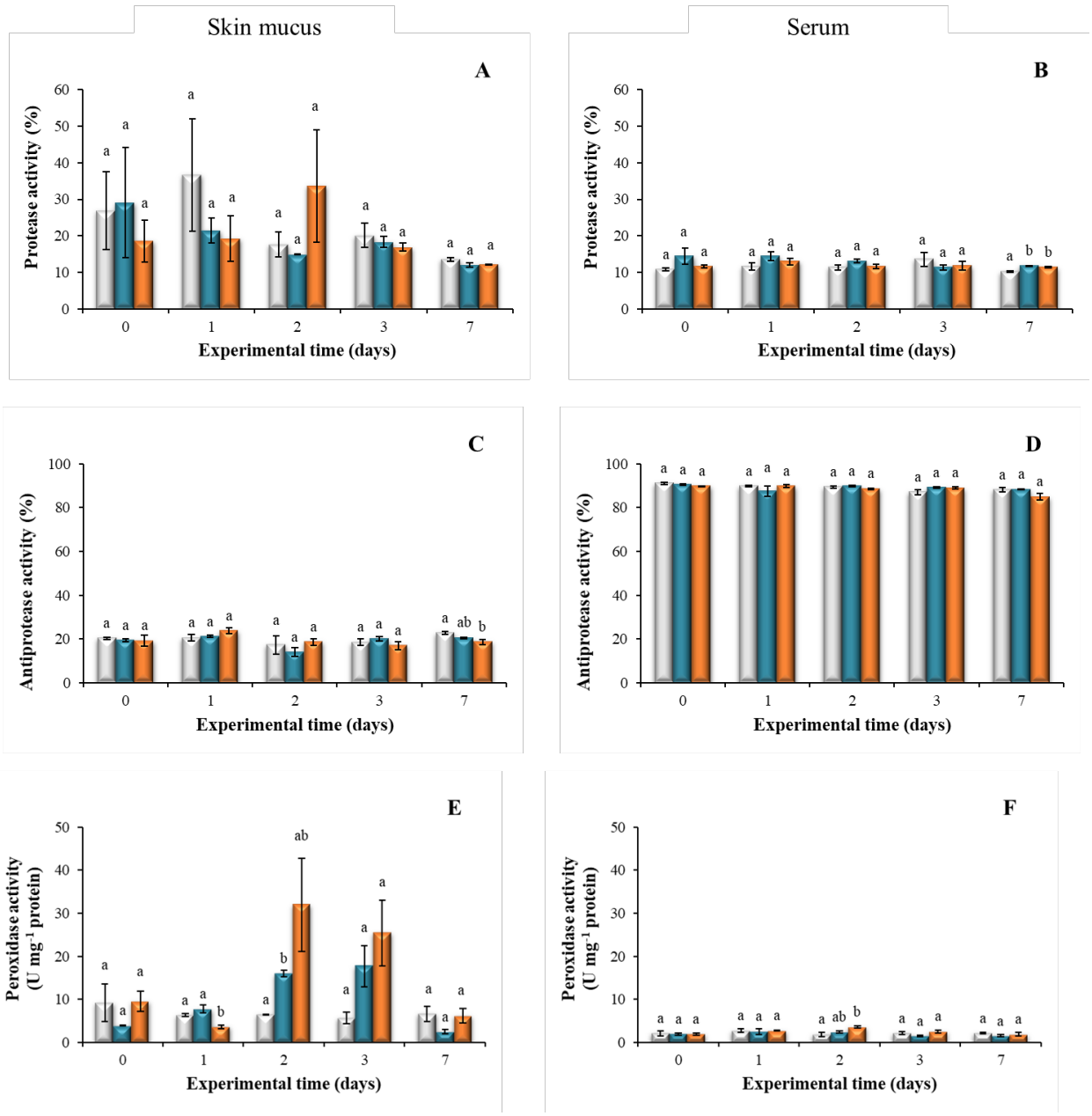


Figure 18. Protease (A, B), antiprotease (C, D) and peroxidase (E, F) activities found in skin mucus and serum samples of gilthead seabream (*S. aurata*) specimens from control (NW; grey) or wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3). Different letters denote significant differences among groups when $P<0.05$

In the present work, the bactericidal activity against two opportunistic pathogenic bacteria (*V. harveyi* and *Photobacterium damselae* subs. *piscicida*) for gilthead seabream was measured in serum. Our results showed that the bactericidal activity against *V. harveyi* decreased after incubation with serum from fish wounded below the lateral line 0 days post-wounding, while it increased 3 days post-wounding in fish wounded both above or below lateral line (Fig. 19 A). However, in the case of bactericidal activity against *P. damselae*, this was significantly higher from 1 to 3 days post-wounding in all wounded fish (Fig. 19 B).

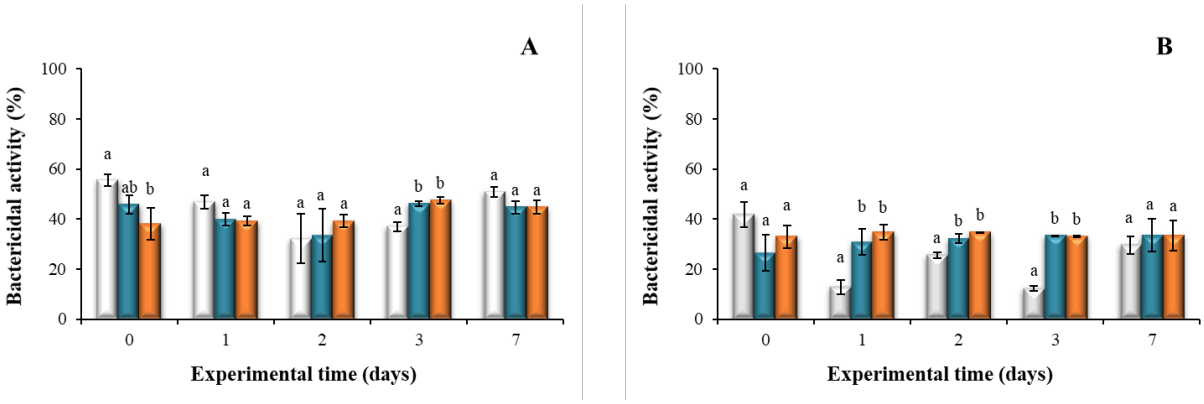


Figure 19. Bactericidal activity against *V. harveyi* (A) and *P. damselae* (B) found in serum samples of gilthead seabream (*S. aurata*) specimens from control (NW; grey or wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3). Different letters denote significant differences among groups when $P<0.05$.

Levels of total IgM in the skin mucus of gilthead seabream were significantly lower in BLL and ALL groups 1 and 7 days post-wounding, respectively, with respect to the values recorded in skin mucus of control fish (Fig. 20 A). In serum samples, IgM levels increased only 2 days post-wounding, in ALL group, compared with the control group (Fig. 20 B).

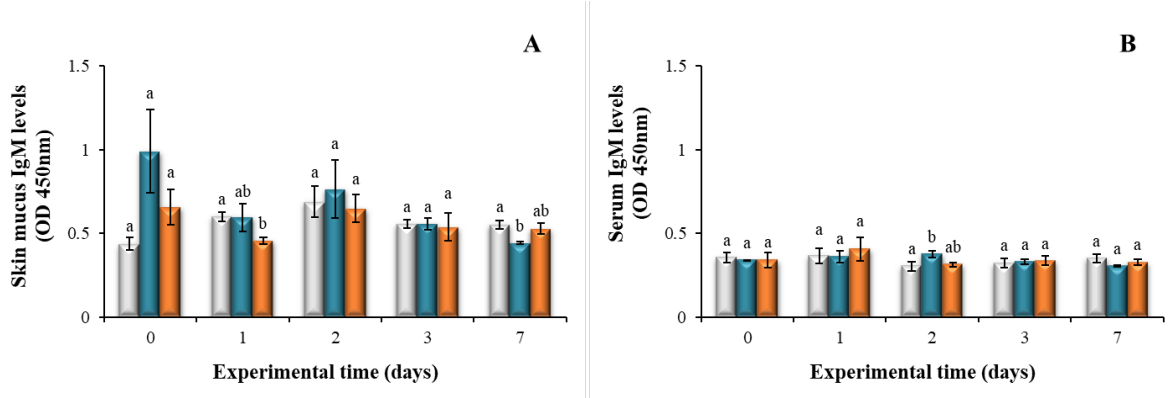


Figure 20. Total immunoglobulin M (IgM) levels found in skin mucus (A) and serum (B) samples of gilthead seabream (*S. aurata*) specimens from control (NW; grey bars) or wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3). Different letters denote significant differences among groups when $P<0.05$.

4.3. Gene expression profile in skin

The expression profile of eleven genes was studied by real-time PCR in the skin of ALL, BLL and NW groups: pro-inflammatory cytokines (interleukin 1beta, *il1b*, interleukin 6, *il6*, tumor necrosis factor alpha, *tnfa*), anti-inflammatory cytokines (transforming growth factor beta, *tgfb*, interleukin 10, *il10*), involved in wound healing (grainyhead-like transcription factor 1, *grhl1* and keratin type 1, *krt1*), immunoglobulins (immunoglobulin M heavy chain, *ighm*, and immunoglobulin T heavy chain, *ight*) and involved in oxidative stress (Cu-Zn superoxide dismutase, *sod*, and catalase, *cat*). As regards expression of the genes involved in immunity, the transcription of the pro-inflammatory genes was very similar. The expression of *il1b* was significantly up-regulated at day 0, 1 and 2 post-wounds in fish from ALL group. However, in BLL group, the expression increased on day 0 and decreased on the other days studied although none of the variations was statistically significant with respect to the values recorded in fish from NW group (Fig. 21 A). Similarly, the expression of *il6* gene increased in fish from ALL group sampled on days 0, 1, 2, and 3 post-wounding. By contrast, in BLL group, significant increases were only recorded in fish sampled on day 0 post-wounding (Fig. 21 B). In the case of *tnfa,* non-significant increases or decreases were detected in skin from fish of ALL and BLL groups, respectively, at all tested times (Fig. 21 C).

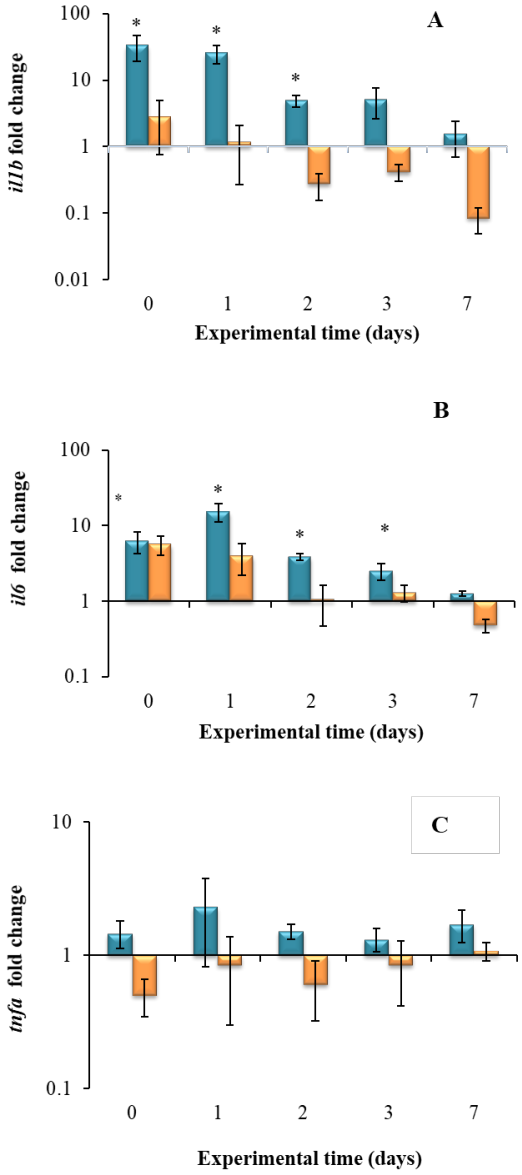


Figure 21. Expression profile of pro-inflammatory *il1b* (A), *il6* (B) and *tnfa* (C) genes determined by qPCR in gilthead seabream (*S. aurata*) specimens wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3) fold increase relative to control. Asterisks denote significant differences respect to control (NW group) when $P<0.05$.

The expression of two anti-inflammatory genes was also studied. The expression of *tgfb* was significantly increased in fish from ALL group sampled at 1 and 7 days post-wound. However, the expression in skin samples from fish of BLL group never reached significance (Fig. 22A). Finally, regarding *il10,* no significant differences were detected (Fig. 22B).

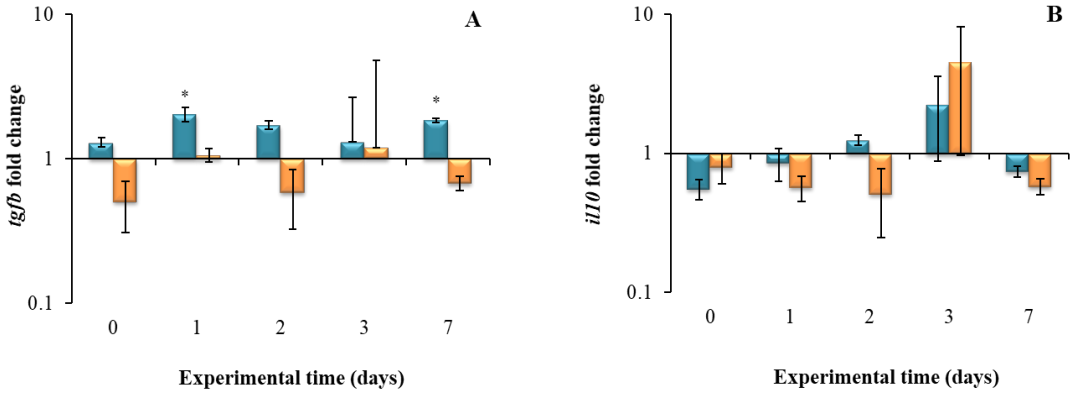


Figure 22. Expression profile of anti-inflammatory *tgfb* (A) and *il10* (B) genes determined by qPCR in gilthead seabream (*S. aurata*) specimens from wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3) fold increase relative to control. Asterisks denote significant differences with respect to control (NW group) when P<0.05.

Regarding immunoglobulins, *ighm* gene expression was lower in the skin of ALL and BLL groups than in group NW, with a slight increase in fish from BLL group, 2 and 3 days after wound, although the variations observed were not statistically significant (Fig. 23 A). The gene expression of *ight* was higher at 1, 3 and 7 days after wound in fish from ALL group although the variation was only statistically significant 1 day post-wounding, however, no significant differences were found in fish from BLL group (Fig. 23 B).

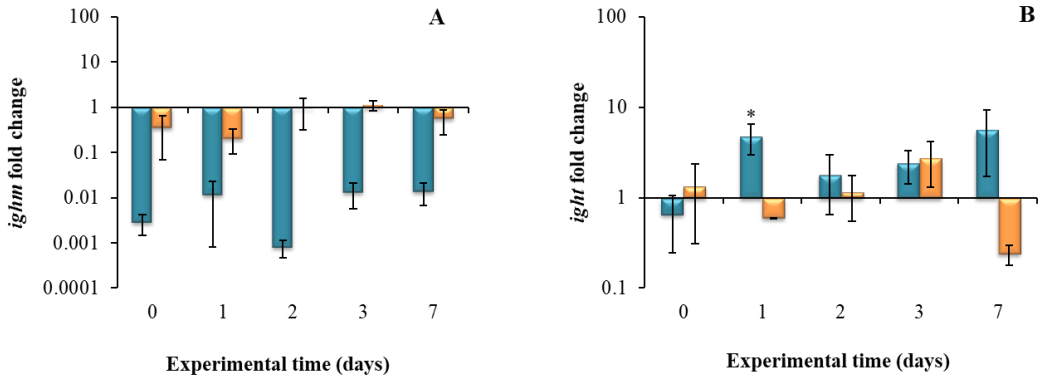


Figure 23. Expression profile of immunoglobulins *ighm* (A) and *ight* (B)genes determined by qPCR in gilthead seabream (*S. aurata*) specimens from wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n = 3) fold increase relative to control. Asterisks denote significant differences with respect to control (NW group) when P <0.05.

The expression of *grhl1* gene was up-regulated (in samples taken from ALL group) and down-regulated or up-regulated in fish from BLL group compared with NW group, but, with no significant differences (Fig. 24 A). In the case of *krt1* gene the expression was down-regulated in both, ALL and BLL groups, during all the experimental time. However,

significant differences were found only in fish of ALL group when compared to NW group at 0, 1 and 2 days after wound (Fig. 24 B).

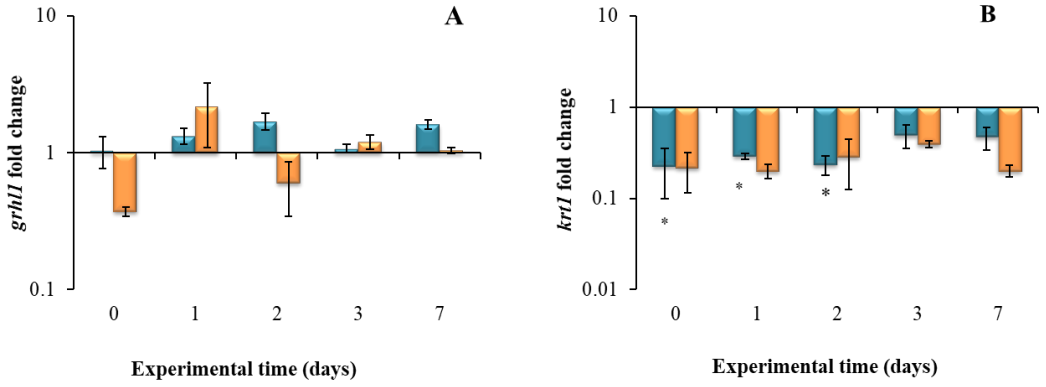


Figure 24. Expression profile of wound healing *grhl1* (A) and *krt1* (B) genes determined by qPCR in gilthead seabream (*S. aurata*) specimens from wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3) fold increase relative to control. Asterisks denote significant differences with respect to control (NW group) when $P<0.05$.

Finally, as regards the expression of genes involved in oxidative stress, the expression of *sod* was higher in fish from ALL group (at all the experimental times) while lower in samples from BLL group although in any case the detected differences were statistically significant (Fig. 25 A). Similarly, the expression of *cat* was higher in samples from ALL group at all the tested times and in BLL group in samples taken at 0 and 1 days post-wound. Afterwards (from day 2 till 7 post-wound), the expression of *cat* decreased although never reached significant extends (Fig. 25 B).

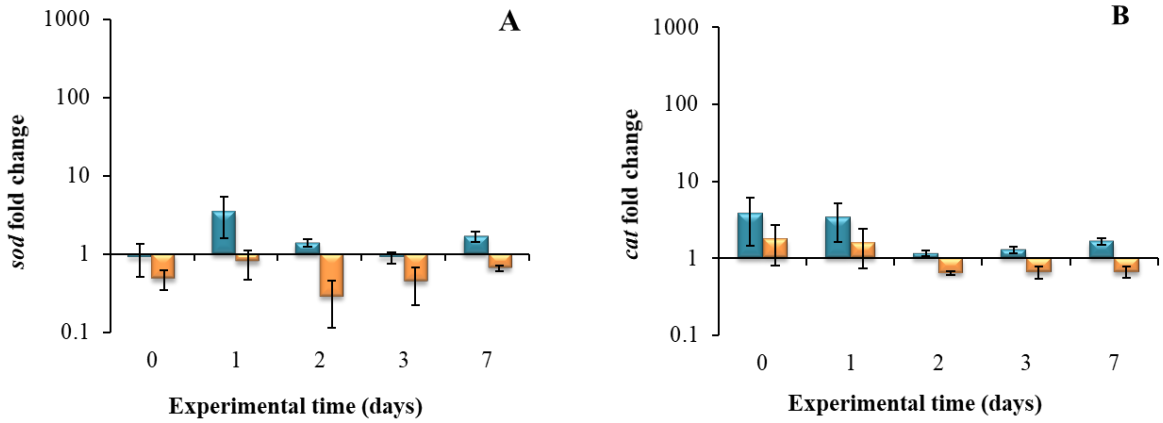


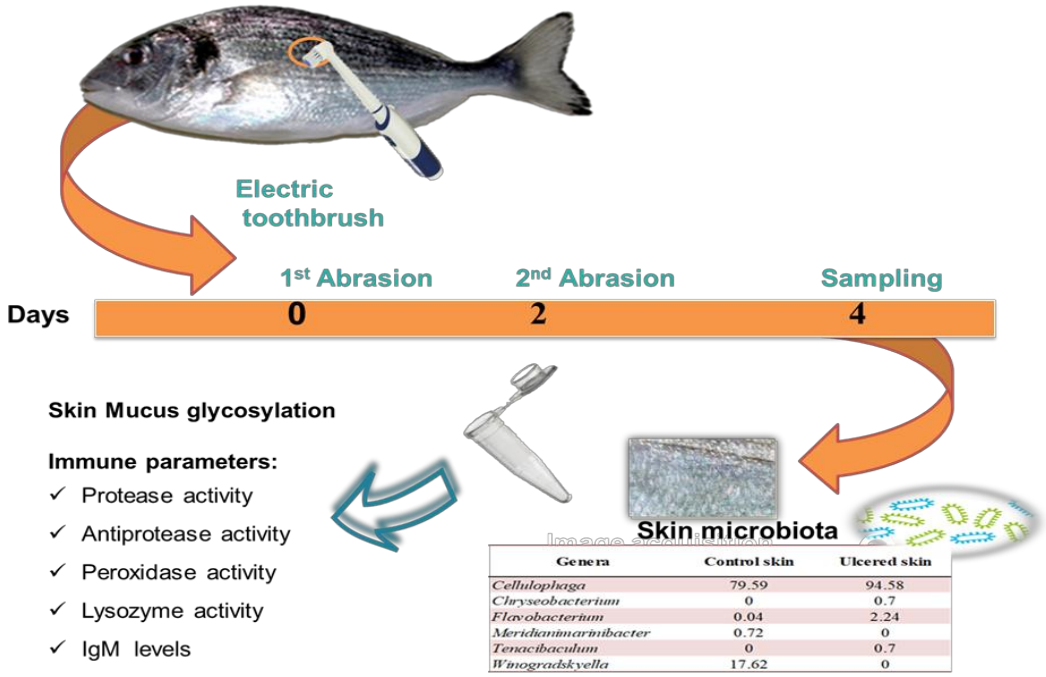
Figure 25. Expression profile of oxidative stress *sod* (A) and *cat* (B) genes determined by qPCR in gilthead seabream (*S. aurata*) specimens from wounded above (ALL; blue) or below (BLL; orange) the lateral line. Results are expressed as mean ± SEM (n=3) fold increase relative to control. Asterisks denote significant differences with respect to control (NW group) when $P<0.05$.

III.2. Mucus glycosylation, immunity and bacterial microbiota associated to the skin of experimentally ulcerated gilthead seabream

1. Objective

Skin mucus layer is a medium in which antibacterial mechanisms may act and depend on many factors including different environmental conditions and intra- or inter-specific variations (Hikima *et al.*, 2000; Fast *et al.*, 2002; Subramanian *et al.*, 2007; Bockelmann *et al.*, 2010; Esteban, 2012; Nigam *et al.*, 2012). This mucus layer is inhabited by a multitude of microorganisms and it is constantly exposed to many others present in the aquatic environment. In fact, all animals live in intimate association with communities of microbes, collectively referred to as their microbiota (Tapia-Paniagua *et al.*, 2018). But, unbalance in the skin-associated microbiota may lead to increases of opportunistic bacteria and result in disease. One of the most frequent pathogenic symptoms associated to skin disease is the production of ulcerative processes (Rizgalla *et al.*, 2016; Småge *et al.*, 2016). Thus, the interest of the present study was to know if skin ulcers may provide microenvironments that perturb both the mucus composition and microbial biodiversity of external surfaces. In this sense we decided to study the skin mucus and microbiota from experimentally ulcerated fish.

2. Graphical abstract



3. Materials and Methods

3.1. Fish

Twenty specimens of gilthead seabream (*S. aurata*) (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}) as described previously (Chapter I section 3). Fish were fed daily at 2% rate of fish biomass per day with commercial diet (Skretting).

3.2. Chronic wounds (ulcers)

Fish were sedated with 20 mg L^{-1} of clove oil and experimental chronic wounds (ulcers) with a diameter of 8 mm and around 50 µm of depth were induced in the skin with an electric toothbrush (PRIMO) for 30 s. All the wounds were performed by the same person and in the same place of the fish (in the middle of the left side below the lateral line, Fig. 26). The procedure was repeated two times with 2 days of resting between the two abrasions. Fish were sampled 2 days after the second abrasion. Control group was handled in similar conditions without triggering wounds.

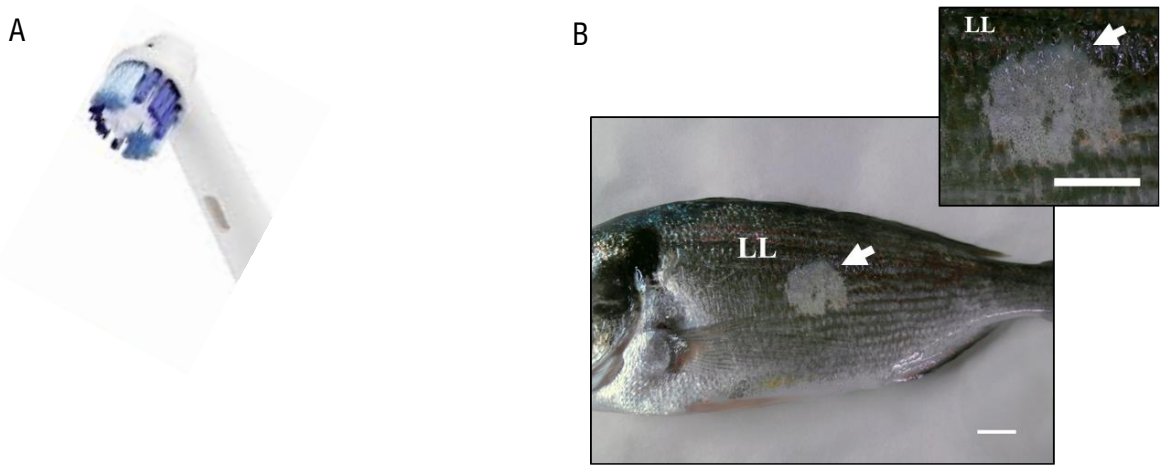


Figure 26. A) Photograph of the electric toothbrush used to perform the ulcers. B) Representative photograph of experimentally ulcerated skin of gilthead seabream (*S. aurata*). Inset zoom image of ulcerated area. Arrow represented the ulcerated area; LL: lateral line. Scale bar: 10 mm.

3.3. Mucus and skin sampling

Fish were sedated with 20 mg L^{-1} of clove oil prior to sampling. Mucus samples (1 mL of media per fish) of the ulcers from 5 ulcerated fish and another 5 skin mucus samples from healthy (control) fish were collected, centrifuged and stored following the protocol previously described in Chapter I section 3.

Protein concentration in each sample was determined by the Bradford method (Bradford, 1976). Skin samples of the ulcers from another 5 ulcerated fish and another 5 skin samples from non-ulcerated (control) fish were aseptically collected. All the samples were immediately frozen at -20°C and sent to the laboratory of the University of Málaga (Spain) for subsequent study of the microbiota.

3.4. Determination of the terminal glycosylation pattern of skin mucus

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 overnight at 4°C. Samples were rinsed three times with PBS containing 0.05% Tween 20 (PBS-T, 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 well^{-1} of biotinylated lectin (Table 2), 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 100 µL of a 0.42 mM solution of TMB, 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. Data are presented as the OD at 450 nm for the skin mucus and lectin used.

Table 2. Lectins used in this study, their acronym and sugar-binding specificities.

Acronym	Lectin source	Sugar binding specificity
BS-I	*Bandeiraea simplicifolia*	α-D-galactose, N-acetyl-α-D-galactosamine
PNA	*Arachis hypogaea*	β-D-galactose
UEA-I	*Ulex europeaus*	α-L-Fucose
ConA	*Canavalia ensiformis*	α-D-mannose, α-D-glucose
WFA	*Wisteria floribunda*	N-acetyl-D-galactosamine
WGA	*Triticum vulgaris*	N-acetyl-β-D-glucosamine, N-acetylneuraminic acid

3.5. Enzymatic activities in skin mucus

Protease, antiprotease, peroxidase and lysozyme activities in skin mucus were determined following the protocols previously described in Chapter I section 3.

3.6. Total immunoglobulin M levels in skin mucus

Total IgM levels were analysed using the ELISA method (Cuesta *et al.*, 2004), previously described in Chapter I section 3 and values expressed in optical density (OD).

3.7. Analysis of the skin microbiota

One mL of PBS, pH 7.2, was added to each one of the individual skin samples before being centrifuged (1,000 × g, 5 min). Total DNA was extracted from each sample as described by Martinez *et al.* (1998).

DNA was amplified using the 16S rDNA bacterial domain-specific primers 677-GC-R (5' CGGGGCGGGGGCACGGGGGGATMTCTACGCATTTCACCGCTAC-3') and 309-F (5' ATCCCTACGGGAGGCWGCAG-3') (Steinum *et al.*, 2009). These primers were used to amplify the V6-V8 regions of 16S rDNA and yield amplicons of 470-bp length. The PCR mixtures and conditions used to perform PCR were adjusted as previously described Tapia-Paniagua *et al.* (2014). The amplicons obtained were separated by Denaturing Gradient Gel Electrophoresis (DGGE) according to previous specifications using a Dcode™ system (Bio-Rad Laboratories, Hercules; Muyzer *et al.*, 1993). The gels were subsequently stained with silver nitrate ($AgNO_3$) (Sanguinetti *et al.*, 1994). DGGE banding patterns were analysed using the FPQuest Software version 4.5 (Applied Maths BVBA). A matrix of similarities for the densitometric curves of the band patterns was calculated using the Dice index. Clustering of DGGE patterns was achieved by constructing dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA).

Based on the high similarity percentage observed in the dendrograms derived from DGGE patterns, DNA from skin samples of fish receiving the same treatment and sampled at the same time were pooled to carry out the subsequent microbiota analysis by Illumina Miseq technology. DNA samples were sent for sequencing to ChunLab (Seoul, Korea) to obtain the microbial DNA sequences of the 16S rRNA gene. Sequences were analysed using CL community™ software (ChunLab). Sequences of less than 200 nt were excluded from the

analysis. The data were filtered for noisy sequences, checked for the presence of chimeras, and binned into operational taxonomic units (OTUs) at the 97% sequence similarity level using the Ezbiocloud database (Peiffer *et al.*, 2013). To determine the level of sequencing depth rarefaction curves were obtained by plotting the number of observed OTUs against the number of sequences. A representative sequence of each OTU was taxonomically classified. In addition, alpha-diversity was determined to describe diversity at local scale and it was analysed using Shannon-Wiener and Chao1 indexes to assay taxonomic and phylogenetic structure diversity, respectively.

3.8. Statistical analysis

The results are expressed as mean ± SEM (n=5). Data were statistically analysed by one-way analysis of variance (ANOVA) followed by Tukey`s post-hoc test to determine differences among groups. All the statistical analyses were conducted using SPSS 23.0 software and differences were considered statistically significant when $P<0.05$.

4. Results

4.1. Skin mucus

All the tested terminal sugar residues were present in the skin mucus samples from ulcers, the levels depending on the lectin studied (Fig. 27). Decreases in the binding of the lectins to the terminal carbohydrates were detected in mucus samples of ulcerated fish, compared to values recorded for skin mucus of control fish (non-ulcerated). However, the decreases were only statistically significant for ConA and WFA lectins (Fig. 27). On the contrary, some increases in the binding patter of PNA were recorded in samples from ulcerated fish although the detected variations were not statistically significant (Fig. 27).

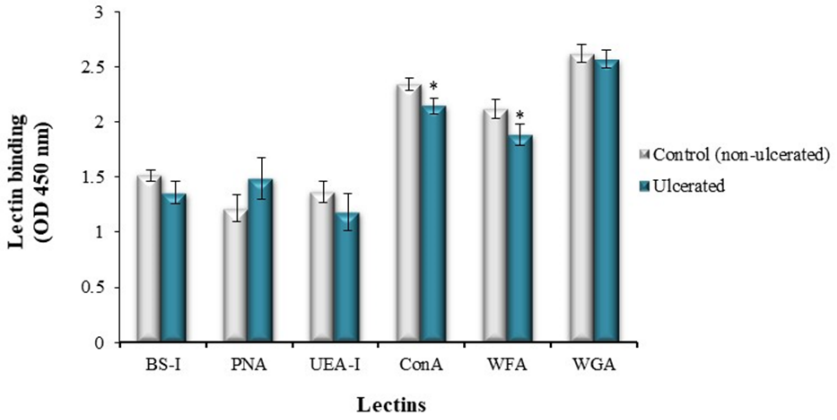


Figure 27. Levels (OD 450 nm) of specific lectin binding measured by ELISA in skin mucus of gilthead seabream (*S. aurata*) specimens. Bars represent mean ± SEM (n=5). Asterisks denote significant differences among groups for each lectin when P<0.05. See Table 2 for lectin specificity.

Regarding the enzymes studied in skin mucus samples, significant decreases were recorded for protease and antiprotease activities in samples from ulcerated fish compared to control fish (non-ulcerated; Fig. 28 A, B), however no variations were recorded to the other activities.

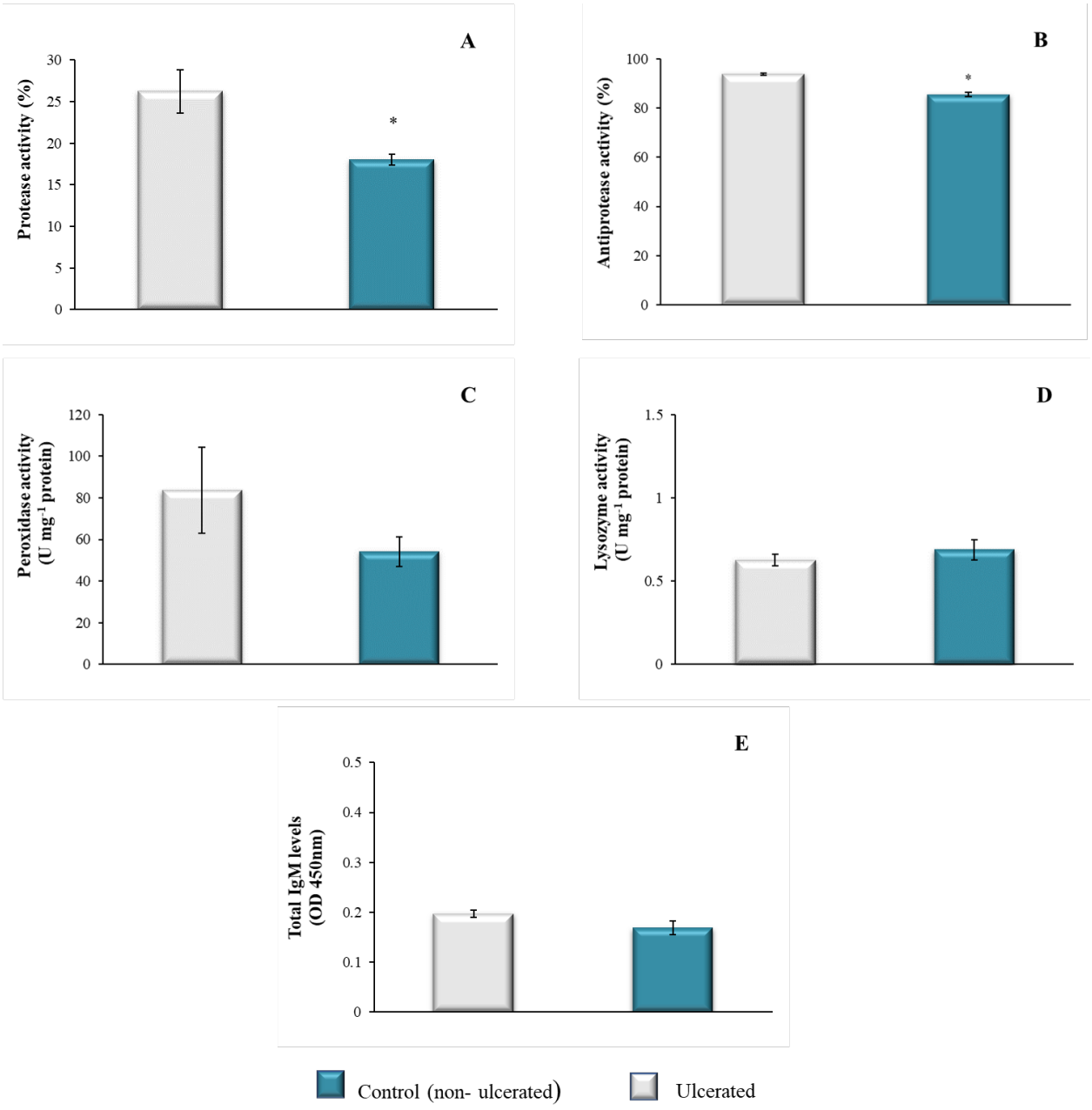


Figure 28. Protease (A), antiprotease (B), peroxidase (C) and lysozyme (D) activities and total IgM levels (E) found in skin mucus samples of gilthead seabream (*S. aurata*) specimens. Bars represent mean ± SEM (n=5). Asterisks denote significant differences with the control group (P<0.05).

4.2. Skin microbiota

Cluster analysis based on the Dice similarity index of DGGE band patterns obtained in the two groups of samples showed intragroup similarity percentages > 85% (Fig. 29). This similarity of the DGGE patterns was considered high enough to justify the pooling of DNA from the same skin samples for the subsequent Miseq study. The DGGE patterns from non-ulcerated or ulcerated specimens were clearly different.

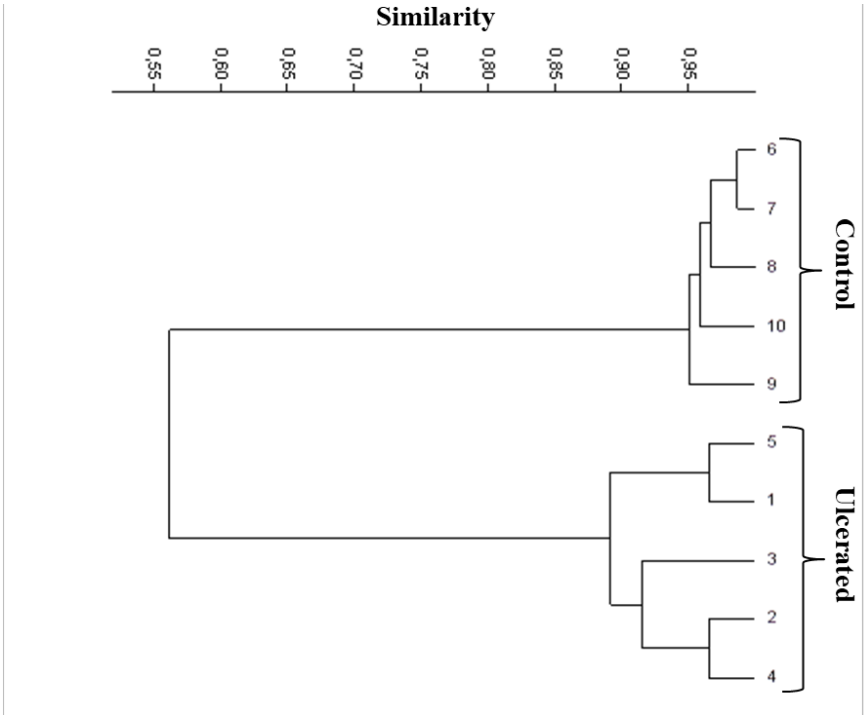


Figure 29. Cluster analysis of DGGE patterns of the analysis of the composition of the microbiota of control (samples 6–10) and ulcerated (samples 1–5) skin of gilthead seabream (*S. aurata*) specimens.

The rarefaction analysis carried out showed slight differences between the microbiota of non-ulcerated and ulcerated skin (Fig. 30).

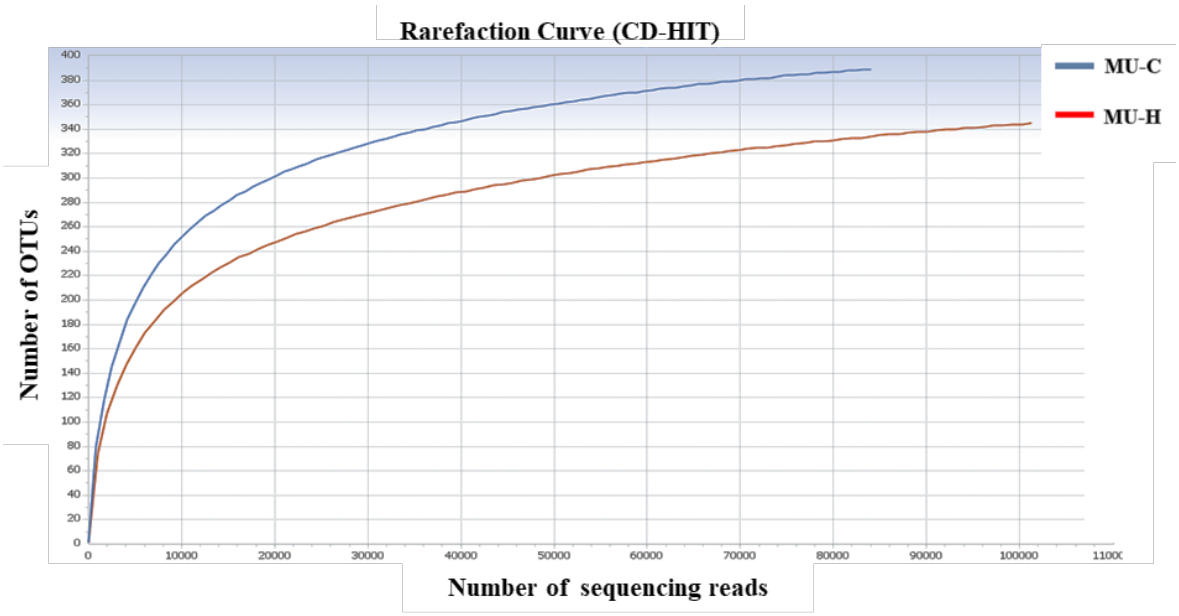


Figure 30. Rarefaction curves obtained from the analysis of the composition of the microbiota of non-ulcerated (MU-C, blue) and ulcerated (MU-H, red) skin of gilthead seabream (*S. aurata*) specimens.

Thus, values of Chao1 and Shannon-Wiener indexes were slightly lower in ulcerated skin samples compared to those healthy (Table 4). The number of families (175) and genera (303) detected in healthy skin was slightly higher than those observed in ulcerated skin (166 and 295, respectively) (Table 3).

Table 3. Number of filtered reads, diversity indexes and assigned taxa present in control (non-ulcerated) and ulcerated skin samples of gilthead seabream (*S. aurata*) specimens.

	Non-ulcerated skin	Ulcerated skin
Reads	88574	106693
Chao 1 index	391.25	372.54
Shannon-Wiener index	2.76	2.58
Number of families	175	166
Number of genera	303	295

The most abundant *phylum* detected in all samples was *Proteobacteria* representing more than 90% of all sequences analysed (Fig. 31 A) followed by *Bacteroidetes* (3.65% and 3.28% for non-ulcerated and ulcerated skin, respectively). On the other hand, *Firmicutes phylum* was higher than 1% only in ulcerated skin whereas in non-ulcerated skin this percentage was lower than 1% of the sequences (Fig. 31 A).

However, the taxonomic analysis at class level showed clear similarity between non-ulcerated and ulcerated skin groups. *γ-Proteobacteria* was the most abundant class in all samples, representing more than 75% of reads, followed by *β–Proteobacteriae* (about 10%) (Fig. 31 B).

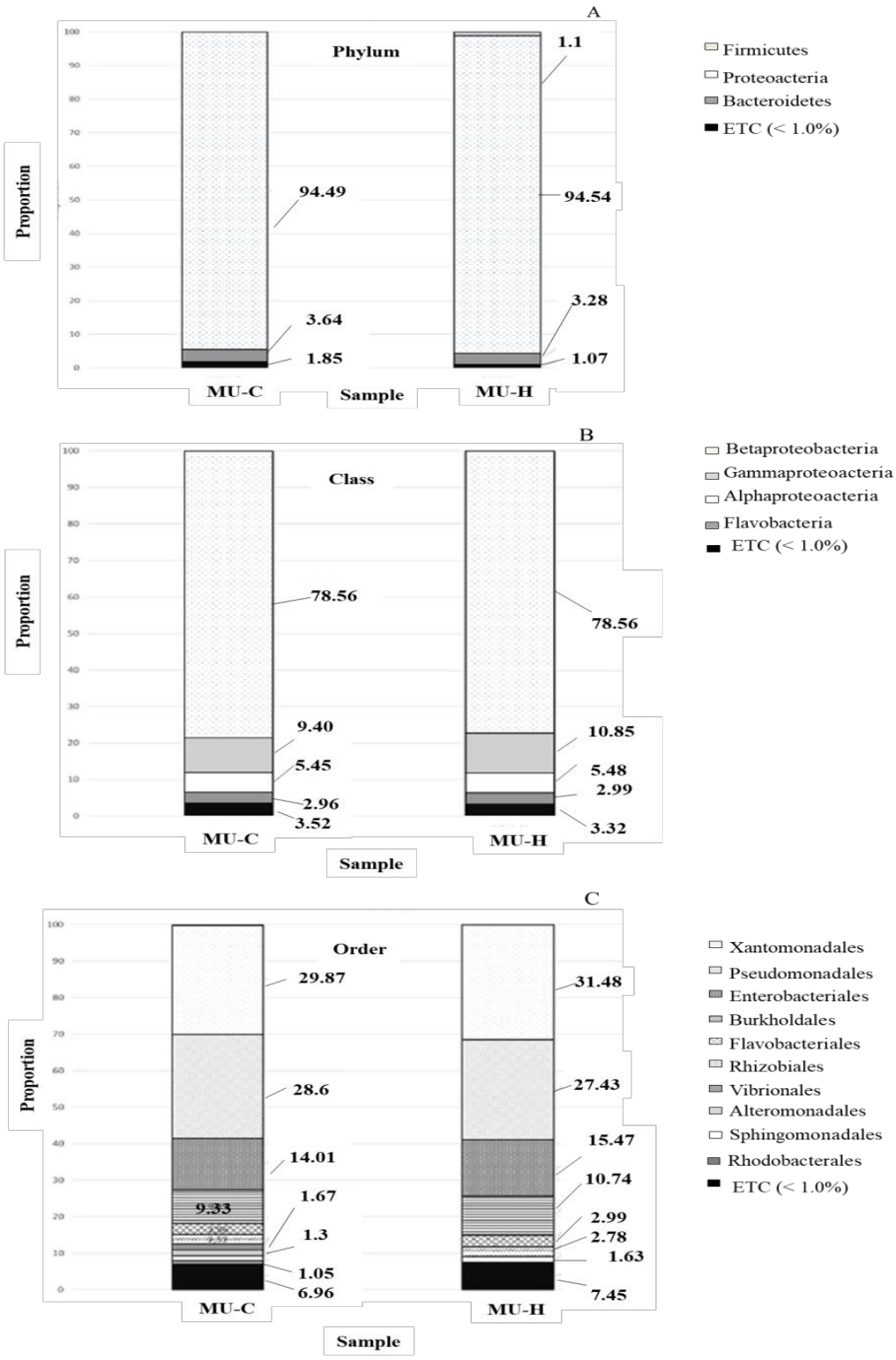


Figure 31. Comparison at level of *phylum* (A), class (B) and order (C) of the composition of the microbiota of non-ulcerated (MU-C) and ulcerated (MU-H) skin of gilthead seabream (*S. aurata*) specimens.

Although *Firmicutes phylum* only represented >1% in ulcerated skin, important differences regarding the composition of this *phylum* were detected between non-ulcerated and ulcerated skin. Thus, *Staphylococcaceae* and *Lactobacillaceae* families were the dominant families in non-ulcerated skin whereas in ulcerated skin were *Streptococcacea*e and *Aerococcaceae* (Fig. 32). *Staphylococcus* and *Lactobacillus* were the dominant genera in non-ulcerated skin and *Streptococcus* and *Granulicatella* in the case of ulcerated skin.

Similarly, the analysis at taxonomic order level showed differences between non-ulcerated and ulcerated skin samples. Orders such as *Rhodobacterales*, *Alteromonadales* and *Vibrionales* showed levels higher than 1% only in non-ulcerated skin (Fig. 31).

In the case of *Alteromonadales* the taxonomic analysis at genus level showed increases of reads related to genera such as *Idiomarina*, *Marinobacter*, *Shewanella* and *Litorilituus* in ulcerated skin, but an important decrease of sequences related to *Alteromonas genus* (Fig. 33A). On the other hand, more than 95% of all reads of *Rhodobacterales* order detected in non-ulcerated skin corresponded to sequences related to *Rhodobacteraceae* family. The taxonomic analysis at genus level showed that the majority of genera of this family corresponded to *Roseobacter* clade in both non-ulcerated and ulcerated skin, and *Thalassobius* being the most abundant genus in both types of samples (Fig.33 B). However, a reduction of reads corresponding to this genus was detected in ulcerated skin. In this study, *Flavobacteriaceae* family represented more than 80% of all reads corresponding to *Flavobacteriales* order in non-ulcerated and ulcerated skin.

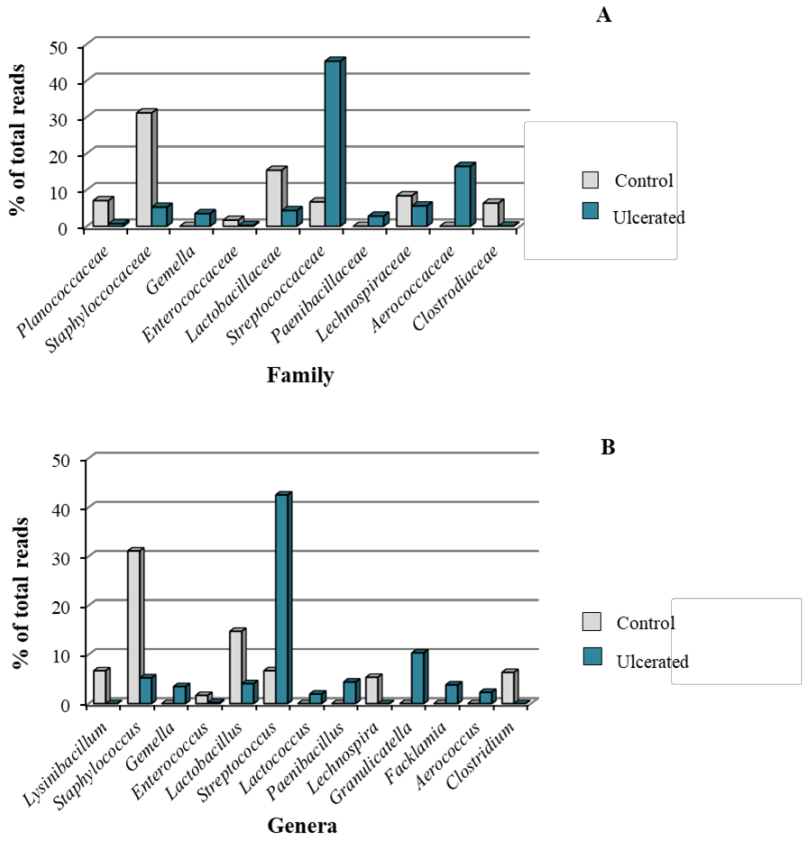


Figure 32. Taxonomic analysis at family (A) and genus (B) level of OTU included in *Firmicutes phylum* from the control (non-ulcerated) and ulcerated skin of specimens of gilthead seabream (*S. aurata*). Percentages are referred to the total number of sequences corresponding to *Firmicutes phylum* and only for families and genera with presence >1% of total *Firmicutes*.

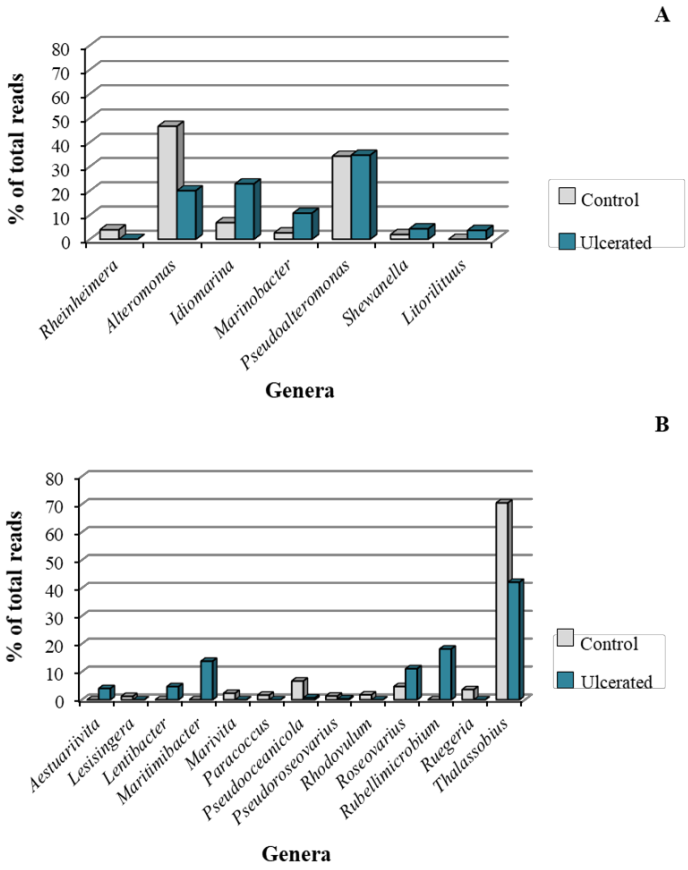


Figure 33. Taxonomic analysis at genus level included in *Alteromonadaceae* (A) and *Rhodobacteraceae* (B) families of the control (non-ulcerated) and ulcerated skin of specimens of gilthead seabream (*S. aurata*). Percentages are referred to the total number of sequences corresponding to *Rhodobacteraceae* family and only for families and genera with presence >1% of total *Rhodobacteraceae* reads.

The taxonomic analysis at genus level showed that the most abundant reads were related to *Cellulophaga* genus (79.59% and 94.58% for non-ulcerated and ulcerated skin, respectively) (Table 5). On the other hand, decreases of the number of reads related to *Winogradskyella* (0 versus 17.62% of all reads corresponding to *Flavobacteriaceae*) and slight increases in reads corresponding to *Flavobacterium*, *Chryseobacterium* and *Tenacibaculum* genera were observed in ulcerated skin (Table 4). The analysis of *Vibrionales* order showed that *Vibrionaceae* family represented >95% of all reads of this order in both types of samples (Table 5). However, clear differences were observed between non-ulcerated and ulcerated skin, because the highest numbers of reads in non-ulcerated skin corresponded to *Photobacterium* genus (about 70%), while *Vibrio* genus was in ulcerated skin (about 85%).

Table 4. Taxonomic analysis at genus level included in *Flavobacteriaceae* and *Vibrionaceae* families of the non-ulcerated and ulcerated skin of specimens of gilthead seabream *(S. aurata)*. Values of percentages are referred only for genera with presence about 1% of total *Flavobacteriaceae* and *Vibrionaceae* and they are referred to the total number of sequences corresponding to these families.

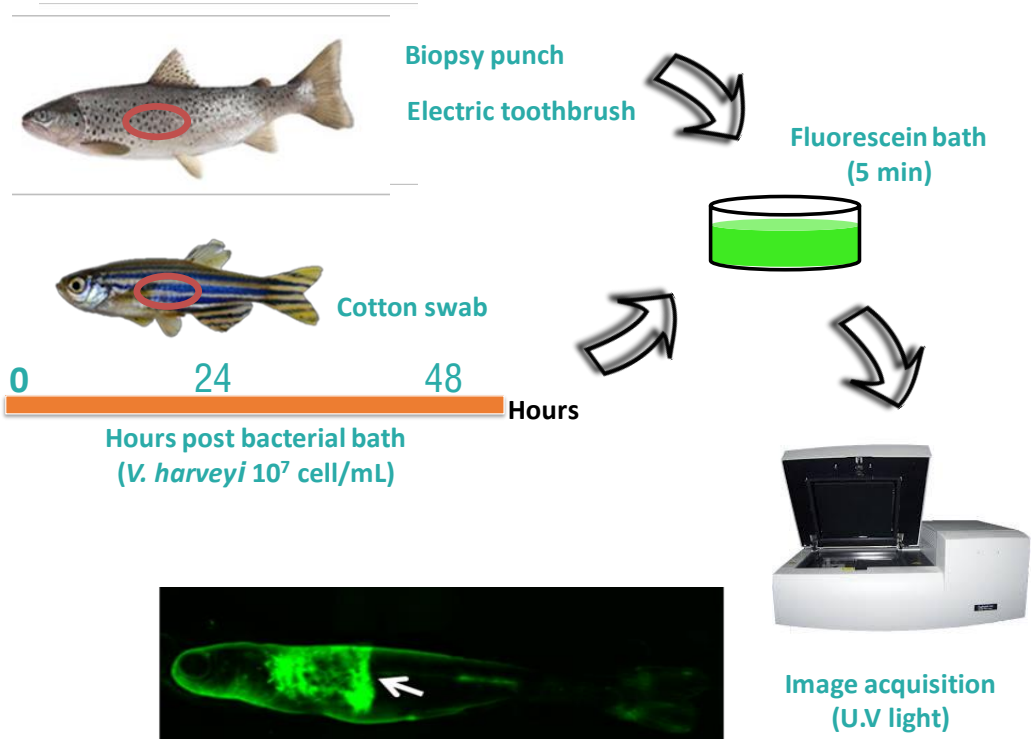
	Non-ulcerated skin	Ulcerated skin
Flavobacteriaceae		
Cellulophaga	79.59	94.58
Chryseobacterium	0	0.7
Flavobacterium	0.04	2.24
Meridianimarinibacter	0.72	0
Tenacibaculum	0	0.7
Winogradskyella	17.62	0
Vibrionaceae		
Photobacterium	30.25	13.17
Vibrio	69.75	86.83

III.3. Optimization of fluorescein test to detect initial skin lesions in fish

1. Objective

The skin of farmed fish is vulnerable to damage by different causes but, whereas advanced or large areas of skin damage are often visible to the naked eye and thus easily identified, small or initial lesions do not usually present any obvious signs of injury. However, these small injuries can act as an entry point for pathogens present in the water, and perhaps even be present on healthy skin, albeit in small numbers (Noga, 2000). In farms, small lesions may be difficult to notice by the naked eye due to the intensive culture conditions, so that the early detection of any lesion to the skin can be considered vital to prevent further damage (Noga and Udomkusonsri, 2002). Therefore, in this work we attempted to imitate a test that is used in amphibians and birds to detect primary lesions in fish skin using a protocol based on fluorescein labeling. This test is based on the use of fluorescein, an inexpensive and non-toxic fluorescent dye that specifically stains ulcerated areas of any size, allowing the detection of even pinpoint ulcers (Noga and Udomkusonsri, 2002). Therefore, if this test can be used in aquacultural practice it would be a useful tool to prevent subsequently diseases.

2. Graphical abstract



3. Materials and Methods

3.1. Fish

Specimens of rainbow trout (*Oncorhynchus mykiss*) (average weight of 4 ± 1 g) and zebrafish (*Danio rerio)* (average weight of 1 ± 0.5 g), obtained from the *Centro Integrado de Formación Profesional Marítimo Zaporito* (Cádiz, Spain) and a local pet store, respectively and kept in the Fish Facilities at the University of Murcia. Fish fish were acclimatized in re-circulating freshwater plastic aquaria (50 L), at 15 ± 15°C (rainbow trout) or 26 ± 2°C (zebrafish). Commercial diet (Skretting or JBL) was administrated at rate of 1% body weight day^{-1}. The photoperiod was 12 h L: 12 h D.

3.2. Physical treatments in rainbow trout

To determine the ability of fluorescein to detect skin lesions, specimens of rainbow trout were sedated with 20 mg L^{-1} of clove oil. Afterwards two physical alterations were made on a defined skin area: 1) marking with a 5 mm biopsy punch or 2) brushing with a rotary electric tooth brush for 2 s. Other fish were kept as control (with no physical treatment). Then, the fluorescein test was carried out as described below.

3.3. Bacterial bath with V. harveyi in zebrafish

In other experiment, specimens of zebrafish were divided in two groups: 1) one group was rubbed with a cotton swab in a delimited skin area for 2 s, after fish were bathed for 1 hour in the presence of *V. harveyi* (2.6×10^7 bacteria mL^{-1} of PBS, Ruangsri *et al.*, 2014); 2) second group was kept in freshwater without bacteria or physical damage (control group). To prepare the bacterial suspension, *V. harveyi* was cultured as previously described in Chapter I section 3. This bacterial suspension was added to a tank. Then fish were exposed for 1 h to the bacterial bath. Bacteria-enriched water was replaced by normal freshwater. After 0, 24 and 48 h zebrafish were taken to perform the fluorescein test.

3.4. Fluorescein test

Fish were immediately placed in a solution of 0.20 mg mL^{-1} fluorescein (Sigma-Aldrich) for 5 min. Afterwards, the fish were removed from fluorescein solution and rinsed on freshwater for 3 min by placing them in three changes of clean freshwater following the protocol of Noga *et al.* (2002).

3.5. Image acquisition

Fish images were acquired under normal white light with a Canon 7D camera with a wide-angle lens of 22 mm 4.5 (Canon EF) or under ultraviolet light in the scanner equipment (Typhoon 9410, GE Healthcare) and processed in the analysis software ImageQuant TL (GE Healthcare).

4. Results

Images of control (non-damaged) or damaged rainbow trout were firstly taken under normal white light after a fluorescein bath (Fig. 34 A, C and E). Results showed that the experimental injury was only visible in rainbow trout damaged with a biopsy punch (Fig. 34 C), while in fish damaged with an electric tooth brush no apparent sign of skin damage was observed (Fig. 34 D). Curiously, in the image acquisition under ultraviolet light (Fig. 34 B, D and F) control fish only showed some very small fluorescent areas coincident with the gills and the base of the fins (Fig. 34 B). However, fish damaged with a biopsy punch (Fig. 34 D) or with the electrical toothbrush (Fig. 34 F) clearly presented visible fluorescent area which mostly coincided with the experimentally damaged skin.

In zebrafish trial, images were acquired under ultraviolet light for control (non-damaged) fish and fish rubbed with a cotton swab and subsequently bathed with *V. harveyi*. The results showed no fluorescence in the control fish (Fig. 35 A), whilst, fluorescent areas were observed after 0, 24 and 48 hours of a bacterial bath in damaged fish, mainly in the pectoral fins (Fig. 35 B, C and D). Images acquired from fish after 24 h of bacterial bath showed greater fluorescence comparing to 0 and 24 h (Fig. 35 C).

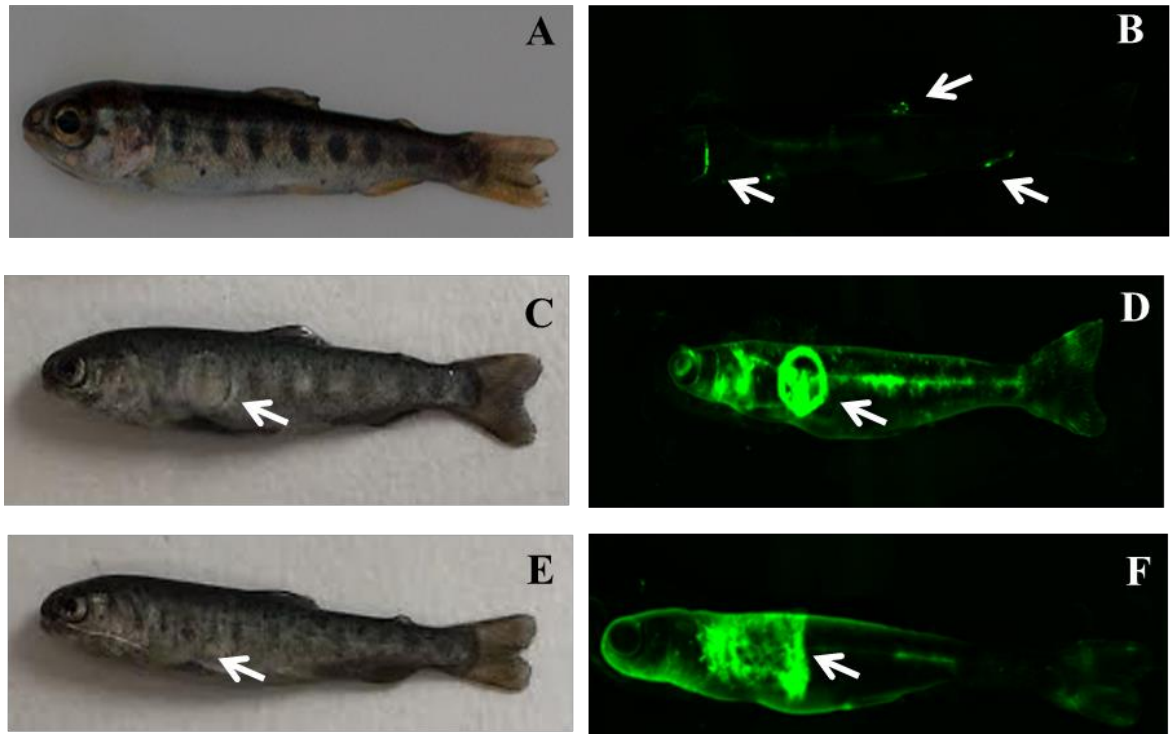


Figure 34. Representative macroscopic images of rainbow trout (*Oncorhynchus mykiss*) specimens bathed in fluorescein solution. Images were taken under normal white light (A, C, E) and under ultraviolet light (B, D, F). White arrows indicate the damaged skin zone under normal (left) or ultraviolet (right) light.

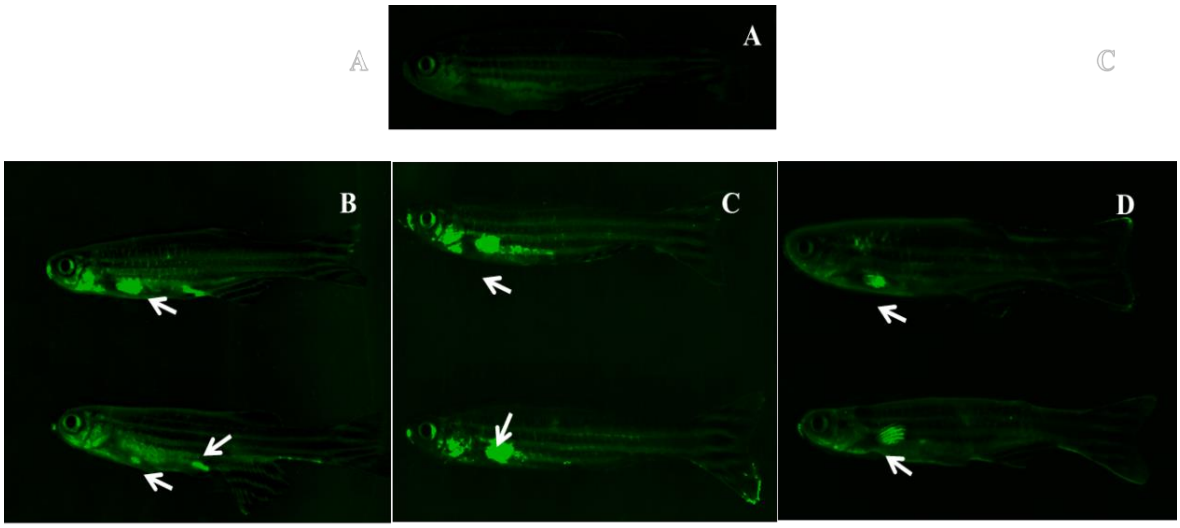


Figure 35. Macroscopic images acquired under ultraviolet light of zebrafish (*Danio rerio*) treated with fluorescein. Control fish (A), fish rubbed with a cotton swab and bathed in presence of *V. harveyi* at 0 (B), 24 (C) and 48 (D) hours post-bath. White arrows indicate the fluorescent areas.

Unpublished paint inspired in rainbow trout (*Oncorhynchus mykiss*) (C. Ceballos 2018).

CHAPTER IV

Imaging techniques to study fish

IV.1. Skin imaging by real time ultrasound (ultrasonography)

IV.2. Skin imaging by X-ray computed tomography (microCT)

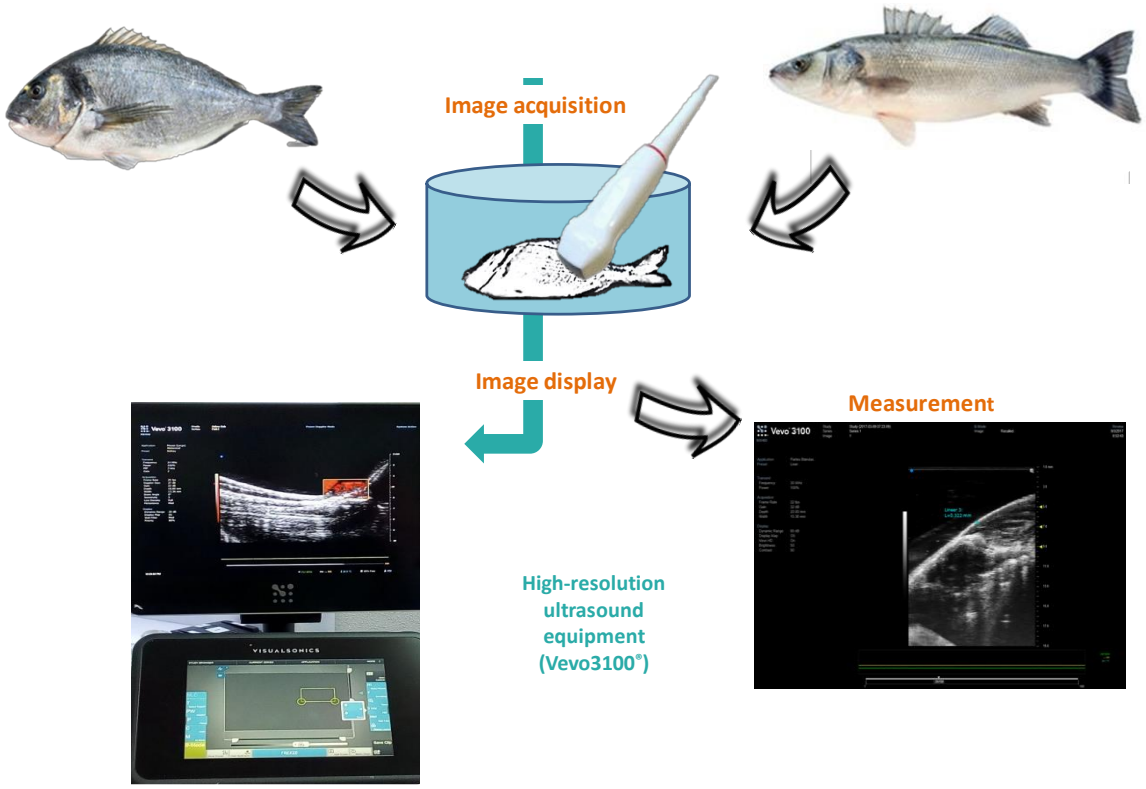
IV.3. Fat imaging by X-ray computed tomography (microCT)

IV.1. Skin imaging by real-time ultrasound (ultrasonography)

1. Objective

Technology and advanced techniques are essential to improve our knowledge of farmed fish biology in order to develop the aquaculture sector. Since fish skin is a live, non-keratinized tissue its study using traditional microscopy techniques is difficult due to the tissue preparation steps needed, in which samples could be affected by the chemical or physical processes involved (Rakers *et al.*, 2010; Alturkistani *et al.*, 2016). In addition, these techniques require animal dissection and do not allow large samples to be studied (Lauridsen *et al.*, 2011). By contrast, ultrasonography allows a detailed study of the animal body without sacrifice and in a short time period. Therefore, the aim of this work was to study for the first time the skin of two marine fish species, gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*), by ultrasonography, an advanced technique of *in vivo* imaging.

2. Graphical abstract



3. Materials and Methods

3.1. Fish

Specimens of gilthead seabream (*S. aurata*) (10 ± 3 g and 8 ± 2 cm) and European sea bass (*D. labrax*) (5 ± 2 g and 7 ± 1 cm) were obtained from a local fish farm and kept in re-circulating seawater aquaria (250 L), with a flow rate of 900 L h^{-1} at 22 ± 2°C and 28‰ of salinity and a 12 h L: 12 h D photoperiod, in the Marine Fish Facilities at the University of Murcia.

3.2. Ultrasonography protocol

Ultrasonographic examination of specimens was performed in a state of sedation, which allowed the fast recovery of the examined specimens. Fish were placed in an anesthetic chamber containing salt water with a low-dose (100 mg L^{-1}) of tricaine methanesulfonate (MS-222; Sigma-Aldrich) to induce sedation without cessation of breathing. Afterwards, the protocol followed two steps:

1. Image acquisition. Sedated fish were transferred into a scanning chamber containing salt water with 100 mg L^{-1} of MS-222 located in the Vevo Imaging Station (VisualSonics) (Fig. 36). The scanning chamber consisted of a plastic container with a smaller plastic contained inside, which served as a support for the fish while under examination. Fish were positioned on the plastic support covered by ~ 30 cm of salt water. The ultrasound transducer (MS700D, band width 30-70 MHz, central operating frequency 40-50 MHz or MS400 band width 18–38 MHz) was then lowered gently into the water over the fish with 3 mm clearance of the body. Fine adjustments of the transducer head position and movement in the longitudinal and transversal axe were achieved by mounting the transducer on a micro-manipulator (Vevo Imaging Station, VisualSonics) (Fig. 36 A). Images were acquired in the Vevo3100® Imaging System (VisualSonics) and typically completed within 15 min. The necessary acoustic coupling was provided by the water itself and assisted ventilation (when necessary) was performed using a portable aerator, while specimens were maintained in lateral, dorsal or ventral position with the transducer in longitudinal or transverse position (Fig. 36 B).

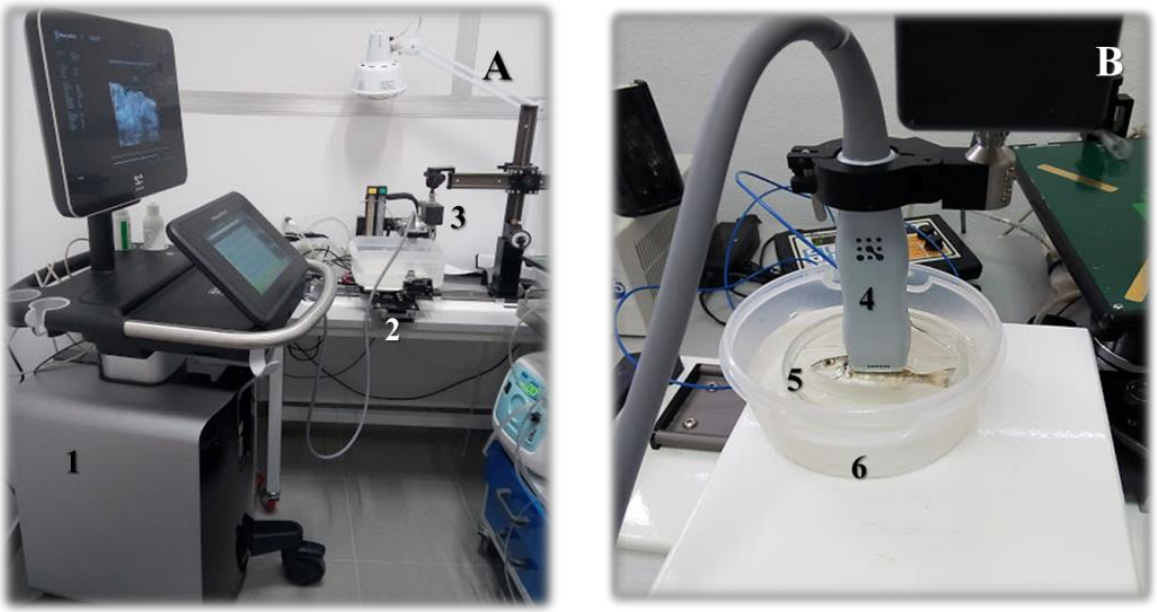


Figure 36. A) High-resolution ultrasound equipment Vevo3100®. 1. Ultrasound equipment Vevo3100®. 2. Vevo imaging station. 3. Micro-manipulator. B) Detailed view of imaging station. 4. High frequency transducer (MS700D, band width 30-70 MHz). 5. Fish in lateral position. 6. Scanning chamber.

2. Image analysis and measurements. Two-dimensional (B-mode) images were recorded in each view. B-mode imaging quality was further optimized by adjusting focal depth, gain, image width and depth. Image analysis was performed using the Vevo Lab™ analysis software package v1.7.1 (VisualSonics). To calculate the skin thickness several straight lines per body section [head (H), anterior section to the dorsal fin (A.S), posterior section to the dorsal fin (P.S) and caudal peduncle (C.P)] were traced manually in a static B-mode image. Images were obtained from a video of the longitudinal section of the fish surface (Fig. 37). Then, straight lines were measured and averaged in each region and the measurements were transferred to an Excel data sheet, and the average value was calculated for the fish as a whole.

3.3. Statistical analysis

Results are presented as means ± SEM (n=5). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis to determine differences. Statistical analyses were conducted using SPSS 23.0 software and differences were considered statistically significant when $P<0.05$.

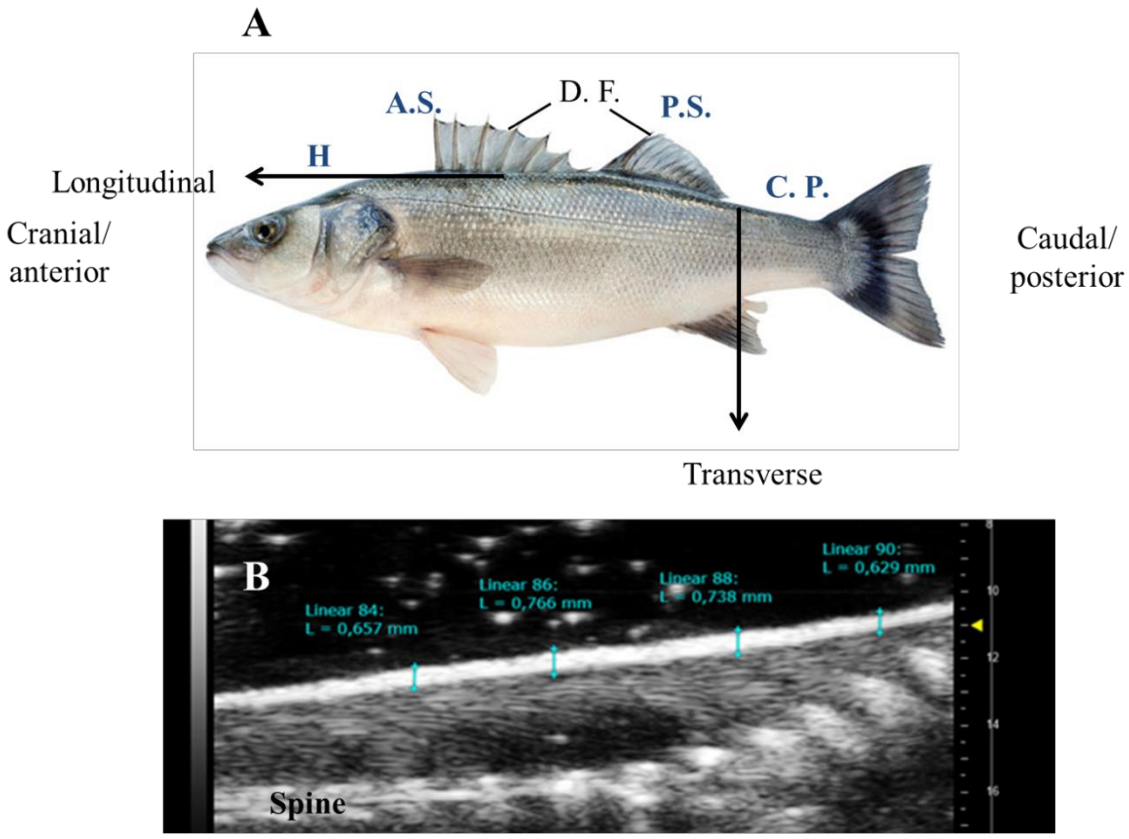


Figure 37. Graphical representation of skin measurement by ultrasound. A) Location of body sections to measure the skin and fish spatial planes in European sea bass (*D. labrax*). H: head; A.S.: anterior section; D.F: dorsal fin; P.S: posterior section; C.P: caudal fin. B) Representative ultrasound image of the skin measurement with the lines indicated in blue in European sea bass (*D. labrax*).

4. Results

The real-time ultrasonography produced high quality images of gilthead seabream (Fig. 38) and European sea bass (Fig. 39). A hyperechogenic (bright) band is showed, which is clearly delimiting the fish body and thus is identifiying the skin (Sk).

When scanning fish specimens with the transducer in transverse (Fig. 38 A, B) or longitudinal (Fig. 38 C) position, we could easily identify the bright band (Sk) and measure it, just bellow the skin the bright hypoechoic area of linear structure that corresponds to the muscle fascia (Msc).

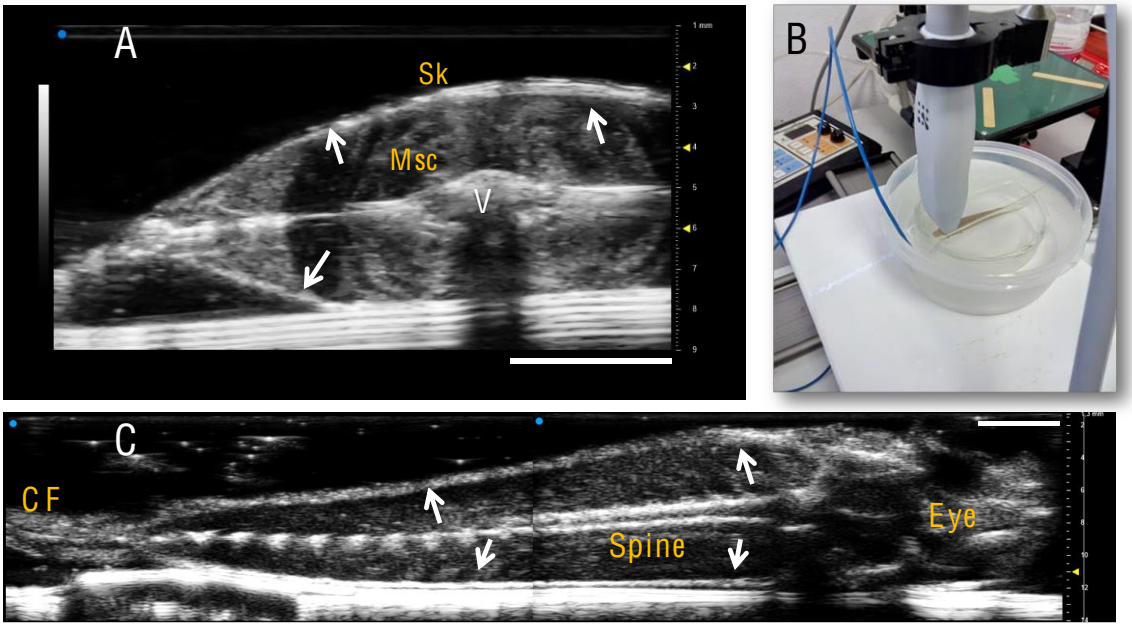


Figure 38. Ultrasonography image to assess skin in gilthead seabream (*S. aurata*). A) Caudal-cranial view in fish transverse axis, the vertebrae (V) are indicated as reference structures; skin (Sk) and muscle fascia (Msc) are indicated. B) Representation of transducer in transverse position to acquire the image in A, with the fish in dorsal. C) Caudal-cranial view in longitudinal plane for whole fish body; caudal fin (CF), spine and eye are indicated. The skin is indicated by white arrows. Scale bars: 5 mm.

Macroscopic analysis of the ultrasound images of European sea bass showed differences among the dorsal (Fig. 39 A, B) and ventral (Fig. 39 C, D) sides of the skin. The skin on the dorsal side is always thinner than on the ventral side. In addition, skin thickness varies along the body, being thinner at the head (Fig. 39 A, C) and thicker in the region between the anterior and posterior section of the dorsal fin (Fig. 39 B, D). In our images, other body structures, such as eyes or spine, can be well visualized.

Four sections were taken as reference points (H, A.S, P.S and C.P) to measure the skin in gilthead seabream and European sea bass (Fig. 40). Our results showed that skin thickness is higher in ventral region compared to dorsal region in both fish species. Greater thickness was observed in the area covered by A.S and P.S regions. Nevertheless, no significant results were found between sections in gilthead seabream or European sea bass.

In both fish species it seems that the epidermal thickness is similar from H to A.S and lower values are in C.P. (Fig. 40).

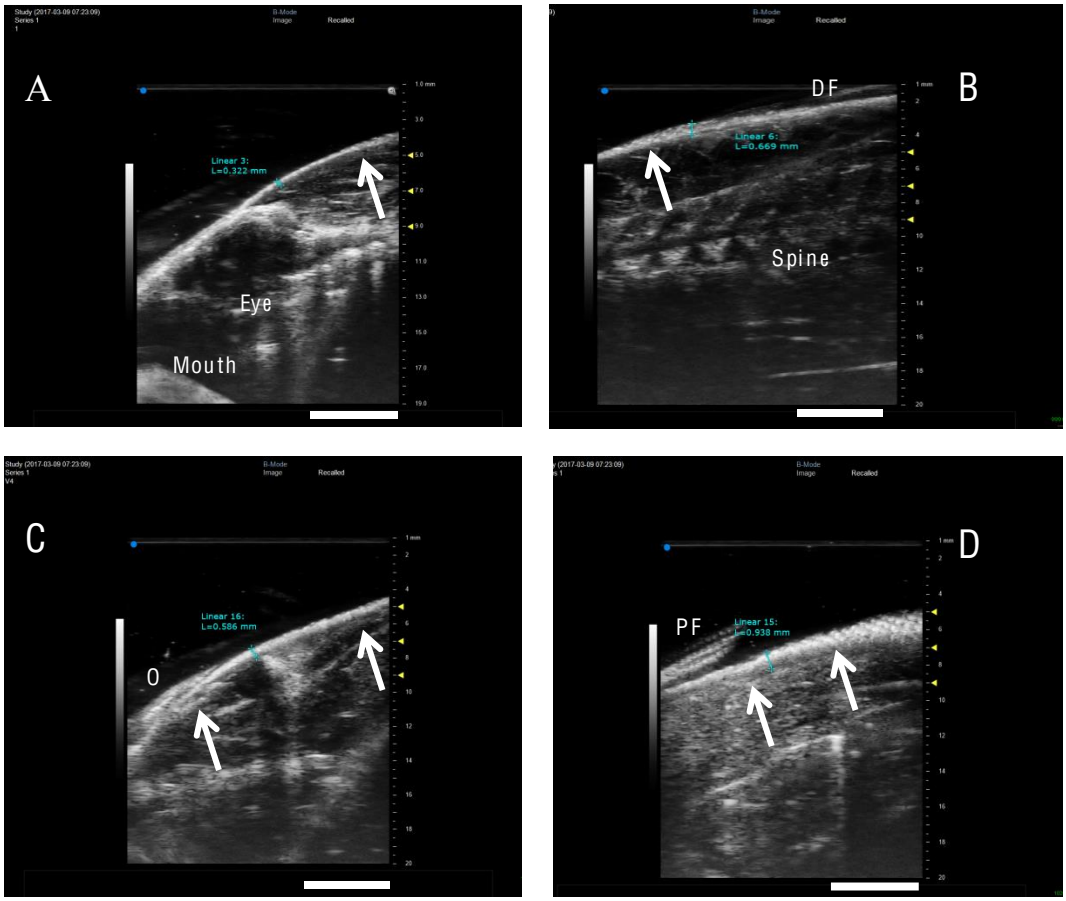


Figure 39. Representative ultrasound images of European sea bass (*D. labrax*) specimens positioned with dorsal (A, B) or ventral (C, D) side touching the transducer. The skin is indicated by arrows. The eye, mouth, dorsal fin (DF), spine, operculum (O) and pectoral fin (PF) are indicated as reference structures. Scale bars: 5 mm.

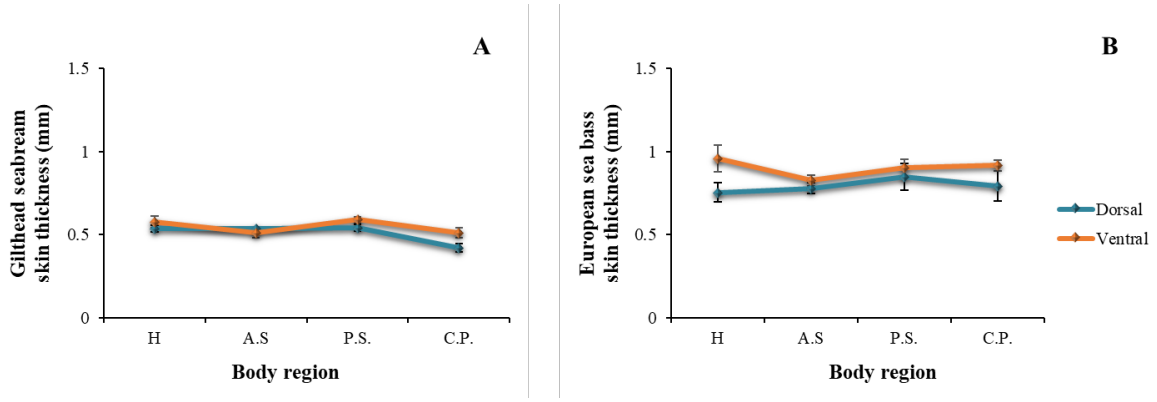


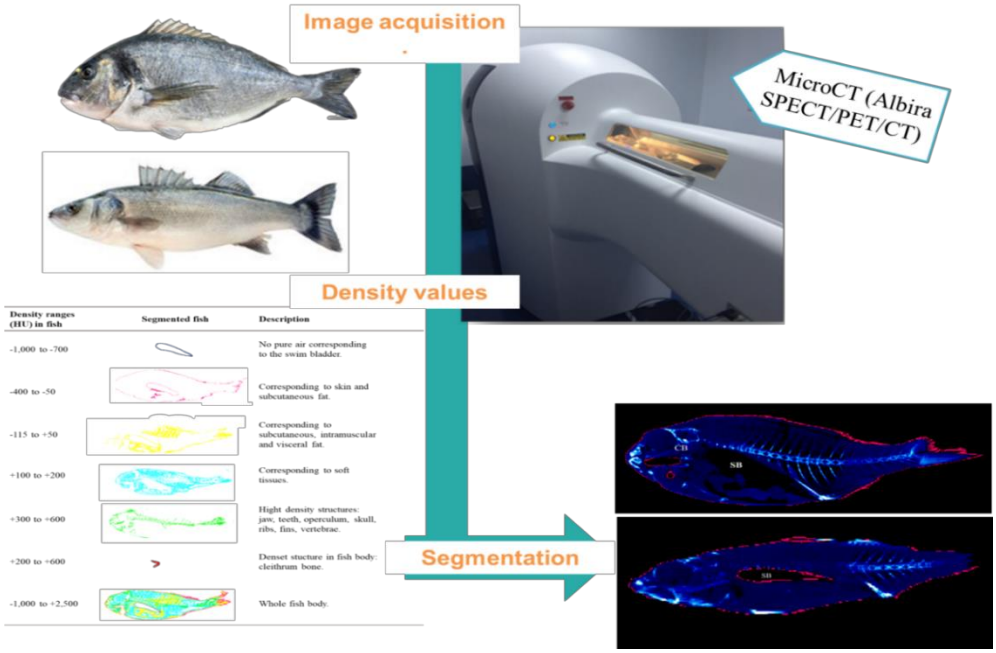
Figure 40. Ultrasound skin measureament (mm) in different regions: head (H), anterior section to the dorsal fin (A.S), posterior section to the dorsal fin (P.S.) and caudal peduncle (C.P.) in gilthhead seabream (*S. aurata*) and European sea bass (*D. labrax*). Results are expressed as mean ± SEM (n=5).

IV.2. Skin imaging by X-ray computed tomography (micro-CT)

1. Objective

Skin structure and function reflects the natural history of fish as well as their most advantageous adaptation to the aquatic environment (Esteban, 2012). Only a few years ago, the economic use of fish skin was important solely for the production of fish oil and fish meal, which explains why biotechnical applications in this topic have long been scarce. However, this is changing by the emerging industrial and biomedical applications of fish skin (Rakers *et al.*, 2010), which go from cosmetology to dermatology or more recently, to the treatment of burns and wounds in humans and animals (Rakers *et al.*, 2013; Silva *et al.*, 2014; Hu *et al.*, 2017; Júnior, 2017; Lima-Junior *et al.*, 2017). Therefore, it is important to study fish skin to fully understand its role on fish survival as well as its application in other fields. In this sense, three-dimensional computer modelling can provide morphological and anatomical information in a non-destructive and much faster way in small animals (Lauridsen, 2011). Thus, this study aimed to analyse the skin of two marine fish species (gilthead seabream and European sea bass) by X-ray computed tomography, one of the most advanced imaging techniques.

2. Graphical abstract



3. Materials and Methods

3.1. Micro-CT protocol

After the ultrasonography study (Chapter IV.1), fish were euthanatized with an overdose of MS-222 (250 mg L^{-1}). Then, to acquire and process micro-CT images several steps were followed (Fig. 42):

1. Image acquisition. Images were acquired using the Albira SPECT/PET/CT tri-modal preclinical-scanner (Bruker® for small animal imaging) (Fig. 41), belonging to the University of Murcia. The X-ray source was set to a current of 200 microamps (μA) and voltage of 45 peak kilovoltage (kVp), using a 0.5 mm filter to harden the beam. A digital flat panel X-ray detector (Bruker®) with 2,400 × 2,400 pixels and a field of view of 70 × 70 mm^2 was used to capture 600 voxel projections of 0.125 mm^3. The total scan exposition per fish was 20-25 min. The approximate radiation deep dose equivalent for micro-CT settings was 220 milisievert (mSv) and the shallow dose equivalent was 357.4 mS.

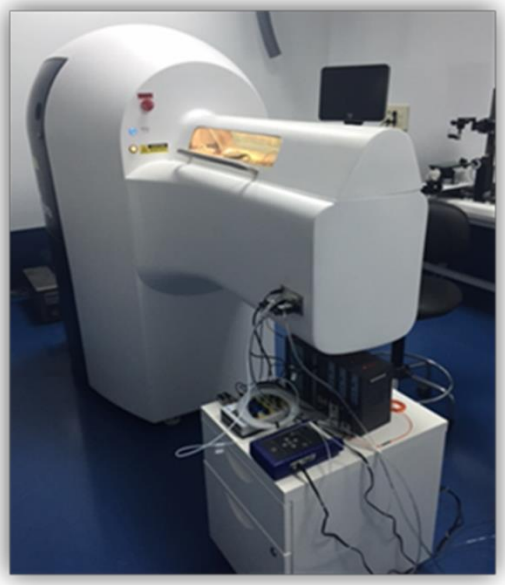


Figure 41. Micro-CT (Albira SPECT/PET/CT) equipment.

2. Image reconstruction. After being scanned with the micro-CT, images were reconstructed in the three spatial planes (transversal, coronal and sagittal) by means the filtered back projection (FBP) algorithm via the Albira Suite 5.0 Reconstructor (Bruker®). These combined acquisition and reconstruction settings produce a final image with 125 μm isotropic voxels, which is considered sufficient for whole animal analysis (Sasser *et al.*, 2012).

3. Image reduction. Images were reduced to minimize computational demands using Pmod (Pmod Technologies LTD) and following the steps described by Sasser *et al.* (2012).

4. Image segmentation. A medical image data examiner (AMIDE: A Free Software Tool for Multimodality Medical Image Analysis) was used to perform both a quantitative and a descriptive analysis of the images.

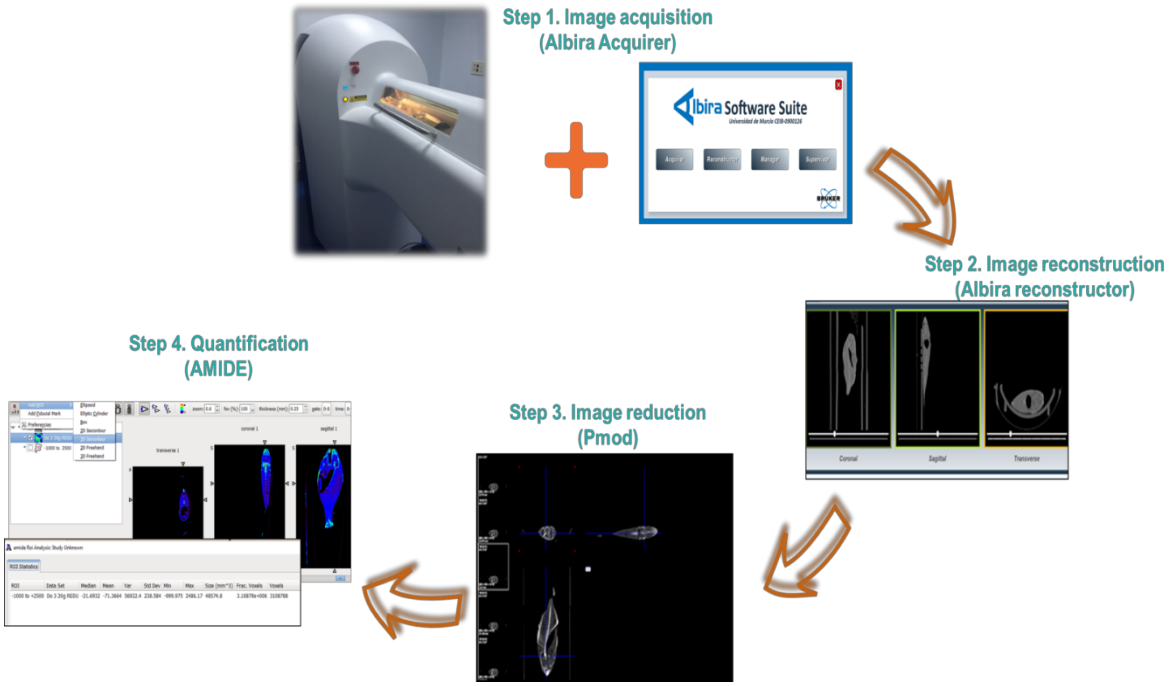


Figure 42. Image acquisition of gilthead seabream (*S. aurata*) and imaging processing steps for skin measurement and visualization. Fish are positioned on the micro-CT scanning bed followed by: 1) Image acquisition in micro-CT + Albira Acquirer. 2) Image reconstruction in Albira reconstructor. 3) Image reduction in Pmod program. 5) Image analisis and quantification in AMIDE software.

3.2. Study of skin density range

To know the density range of fish skin, a biopsy sample of the skin was obtained and immediately analysed. Measurement were carried out by drawing several ellipsoidal regions of interest (ROIs) each of 0.25 mm size over the micro-CT image (Fig. 43).

The pre-defined field of view was divided into volume elements (voxels) and the absorption coefficients were measured in Hounsfield units (HU) (Haacke *et al.*, 1999; Lauridsen *et al.*, 2011). More than 400 voxels were analysed in each ROI to obtain the mean value of the skin in HU.

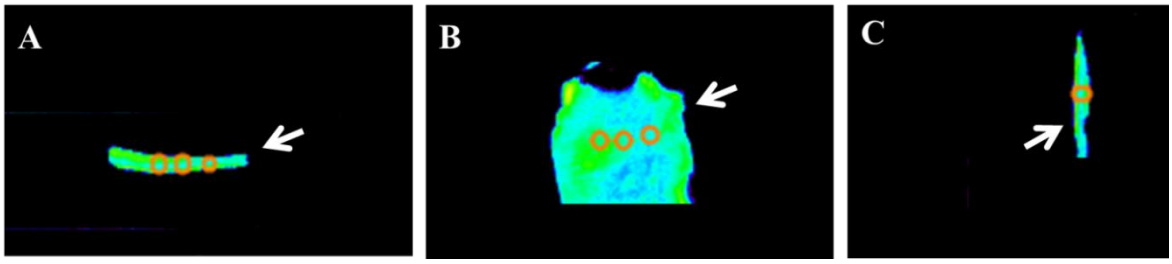


Figure 43. Skin sample (in green) from gilthead seabream (*S. aurata*) (indicated by white arrows), colored according to the AMIDE software and positioned according the three different axes: transverse (A), coronal (B) and sagittal (C); 0.25 mm ellipsoidal ROIs (orange circles) were drawn on these sections.

3.3. Micro-CT skin acquisition, reconstruction and image analysis

Fish were studied individually. Specimens were transferred to a scanning bed in lateral position (right side of the fish lying on the bed) on a tissue paper to provide separation between the fish and bed (Fig. 44). To establish a sensitive protocol with high image resolution and without interference from water and the anesthetic chamber, image acquisitions were performed to scan a bed of 115 mm length, using 600 projections (20-25 min of exposition per fish), always from the mouth to the caudal fin.

Figure 44. Representative photograph of gilthead seabream (*S. aurata*) positioned in the scanning bed of micro-CT equipment, (right side lying to the bed) lying on a tissue paper to provide separation between the fish and bed.

3.4. Fish skin image analysis

Firstly, to study the skin *in situ*, density values for the entire fish had to be determined: with an open image in the AMIDE software, 3D isocontour ROIs in a range of 100 HU or more intervals were drawn [in the HU scale, ranges run from extremely negative values

corresponding to air (-1,000) to extremely positive values corresponding to bone tissue (+1,000), Romvári *et al.*, 2002]. In this work, extreme density values were introduced to include the swim bladder and the densest structure of the fish body (cleithrum bone). Then, the whole fish body was segmented according to tissue density in HU from the mouth to the caudal fin. Afterwards, the means of the analysed voxels were obtained and the volume (cm^3) was transferred to an Excel data sheet.

Secondly, to study the whole skin in the fish body, the same steps described above for the entire fish were carried out. To segment and quantify the skin in the AMIDE software, density ranges obtained in the skin sample analysis were introduced. Then, 3D ROIs from -400 to -50 HU were drawn along the body and statistical data were acquired. The values from 10 fish (per studied species) were averaged.

In cases where the density of the micro-CT bed overlapped the density of some fish portions, the value of the empty micro-CT bed was acquired to be subtracted from the fish values.

3.5. Statistical analysis

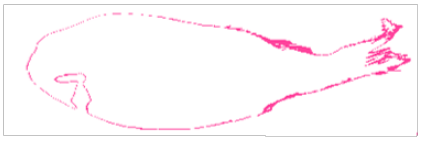
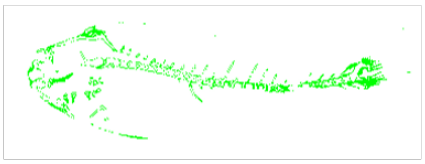
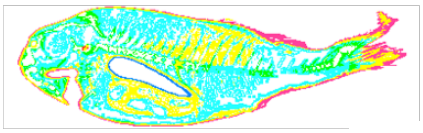
Raw data for each fish were averaged and analysed in the AMIDE software. Then, the results from ten fish per species were averaged and analysed by ANOVA and Tukey's post-hoc test, using SPSS 23.0 software. Data are presented as means ± SEM (n=5) and differences were considered statistically significant when $P< 0.05$.

4. Results

To carry out a radiological study of the skin of gilthead seabream and European sea bass, images were acquired with the micro-CT equipment and different densities of fish body were determined with AMIDE software, as previously explained. The images from fish (n=10 per fish species) were interpreted, first independently and then all together (with the intervention of expert observers in anatomy and radiology). Then, a qualitative and a descriptive analysis was performed and several ROIs (± 50.19655 voxels each) from each fish were analysed.

According to the Hounsfield scale and introducing extreme density structures (swim bladder and cleithrum bone), the density ranges for the whole fish body were established from -1,000 to +2,500 HU. The density values obtained for each fish structure are detailed and represented in Table 5.

Table 5. Representative images created with AMIDE software to display body density ranges (HU) in gilthead seabream (*S. aurata*). Each structure has been segmented with a different color.

Density ranges (HU)	Segmented fish	Description
-1,000 to -700		Non-pure air corresponding to the swim bladder
-400 to -50		Corresponding to skin
-115 to +50		Correspondign to subcutaneous, intramuscular and visceral fat
+100 to +200		Corresponding to soft tissues: tongue, eyeball, muscle, heart, viscera.
+300 to +600		Hight density structures: jaw, teeth, operculum, skull, backbone, vertebrae, ribs, fins,.
+200 to +600		Densest stucture in fish body: cleithrum bone
+1,000 to +2,500		Whole fish body

4.1. Micro-CT analysis in gilthead seabream and European sea bass

After the image acquisition, density ranges were determined in gilthead seabream (Table 5). These values were extrapolated to the European sea bass and the volumes occupied by the density ranges (HU) in each fish species were represented graphically (Figs. 45, 46). According to our results, the highest density range (+600 to +2,500) in the gilthead seabream and European sea bass body corresponds to the cleithrum bone, which represents 0.08 % related of the fish total volume. In addition, density values of +200 to +600 HU corresponded to the rest of the skeletal structures (6% and 8.90% in gilthead seabream and European sea bass, respectively). The density ranges corresponding to soft tissue (+100 to +200) in gilthead seabream represents 25% and in European sea bass represents 31% of the total volume. Curiously, the segmentation with the density range corresponding to the fat content (-115 to 50) were distributed in greater proportion in gilthead seabream (37%), than in European sea bass (2.8%). By contrast, the opposite segmentation was found with the range corresponding to the skin (-400 to -50): in gilthead seabream, skin segmentation occupied 31% and in European sea bass it represented 54.89% of the total volume. Finally, extremely negative values (-1,000 to -700) corresponded to the swim bladder respresenting 2% of the total volume in both fish species.

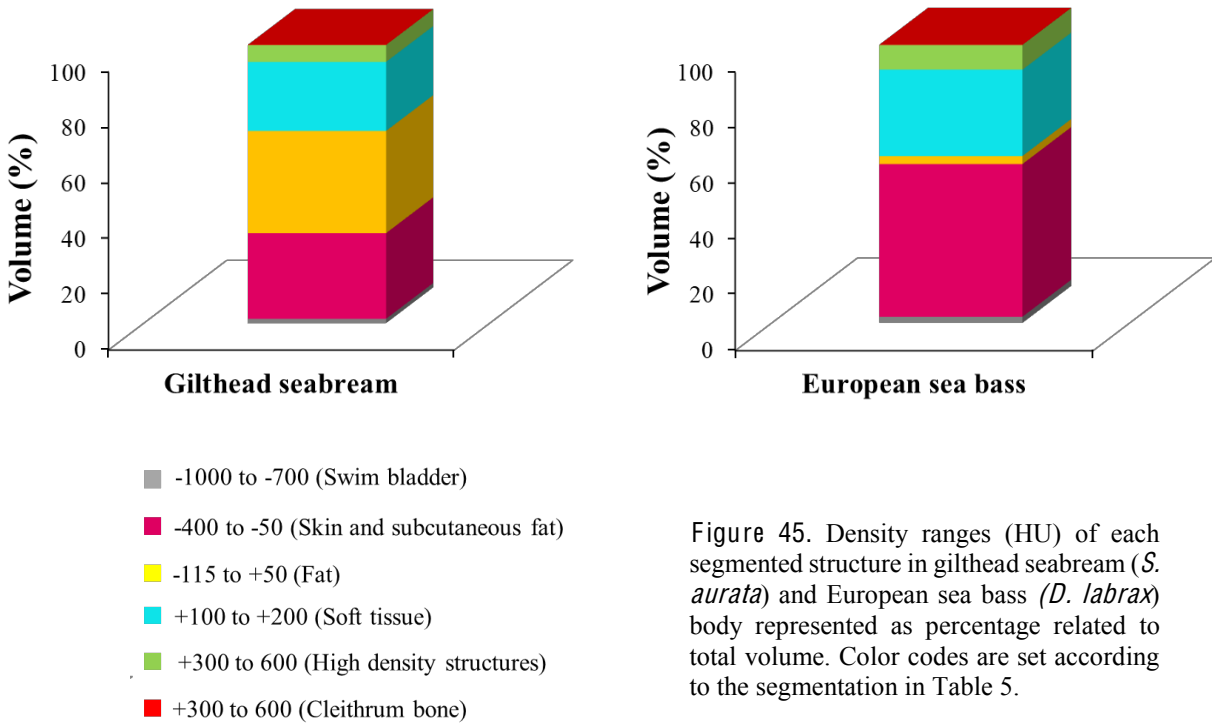


Figure 45. Density ranges (HU) of each segmented structure in gilthead seabream (*S. aurata*) and European sea bass *(D. labrax*) body represented as percentage related to total volume. Color codes are set according to the segmentation in Table 5.

Figure 46 represents the body segmentation according to the density ranges obtained previously (Table 5), in gilthead seabream and European sea bass specimens, supporting the results obtained in the graph shown in Fig. 45.

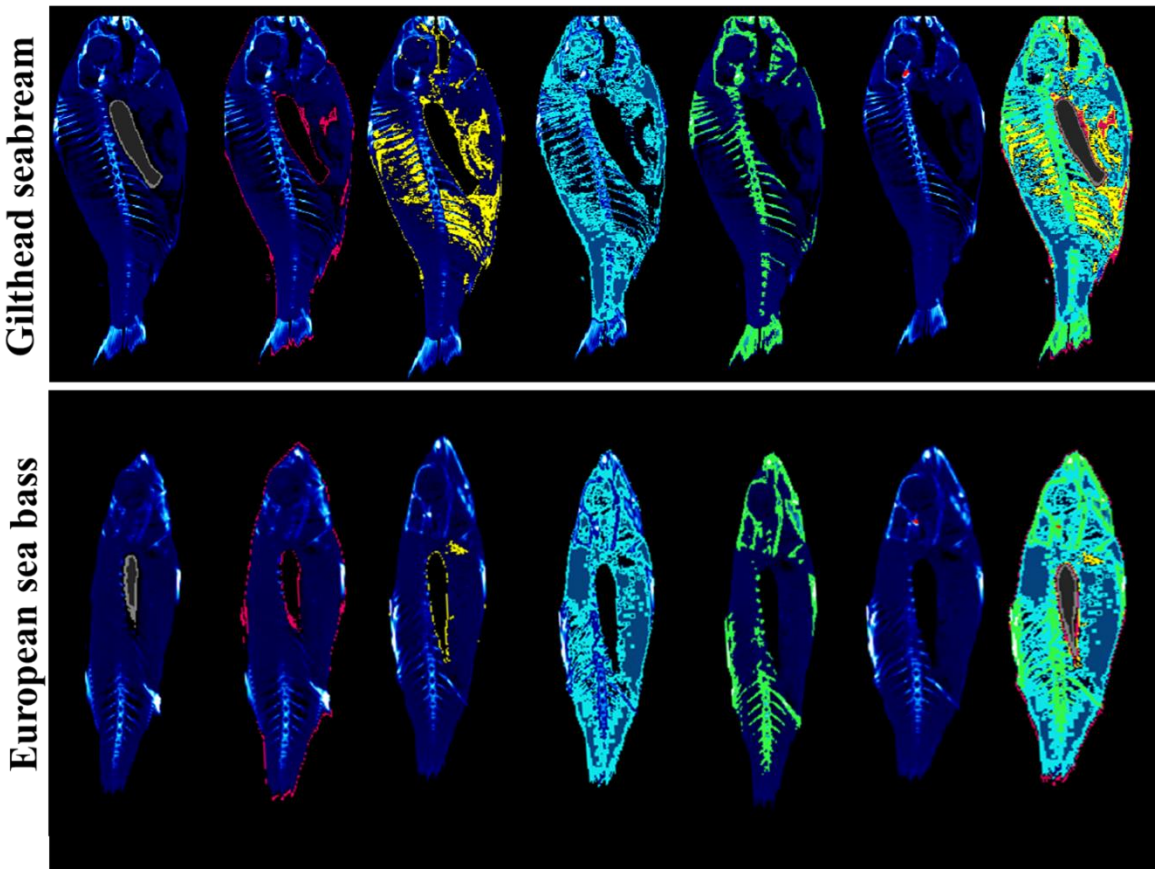


Figure 46. Representative images created with AMIDE software for the visualization of body densities (HU) in gilthead seabream (*S. aurata*) and European sea bass *(D. labrax*). Color codes are set according to the segmentation in Table 5.

4.2. Micro-CT analysis for the skin of gilthead seabream and European sea bass

After determining density values corresponding to the skin in gilthead seabream and European sea bass, fish images were segmented and analysed individually in each fish species. A descriptive study of the micro-CT images for each fish species (gilthead seabream and European sea bass) was made, considering the different anatomical reference points (eye, swim bladder, caudal peduncle and caudal fin). Our results showed that the segmented area (dark pink), with the density range obtained for the skin (-400 to -50 HU), delimits the

whole fish body in both fish species (Figs. 47, 48). In this way, we could know and see the thickness of the fish skin in each part of the fish body.

According to the images obtained for gilthead seabream there were susbtantial variations in the thickness of the segmented area (dark pink) throughout the fish body; this segmentation, which coincides with the topographic location of the skin, is thinner in the head and thicker in the tail. This variability could be better appreciated in transversal view, where more highly segmented areas were observed from the mouth of the fish till the caudal fin (Fig. 47 B).

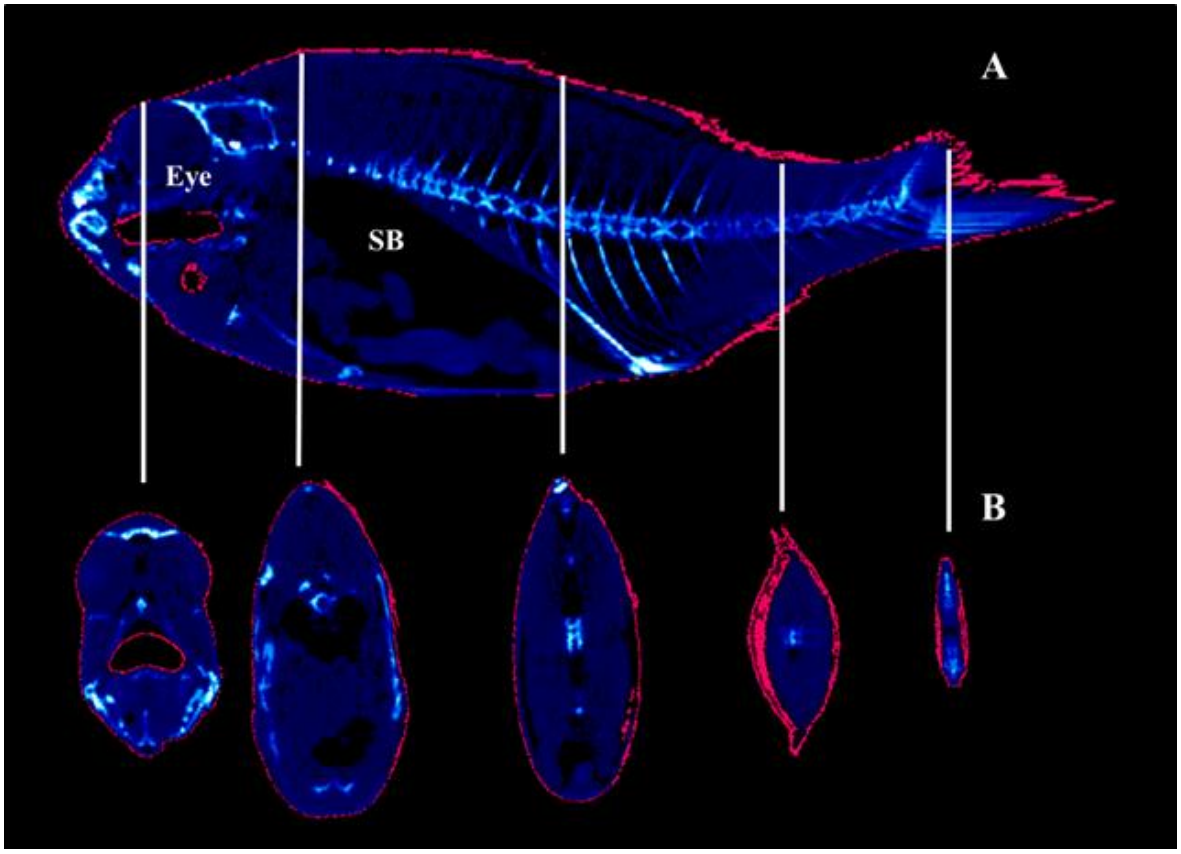


Figure 47. Micro-CT representative images of gilthead seabream (*S. aurata*) coloured according to AMIDE software to display skin segmentation (dark pink) in sagittal (A) and transversal (B) axes from mouth to the caudal fin. White lines are indicating the reference structures where the transversal axis is located. SB: Swim bladder

According to the images obtained for European sea bass, again important variations on the thickness of the segmented area (dark pink) were shown throughout the fish body; segmentation is thinner in the head and thicker in the tail. This variability is better appreciated in transversal view (Fig. 48 B).

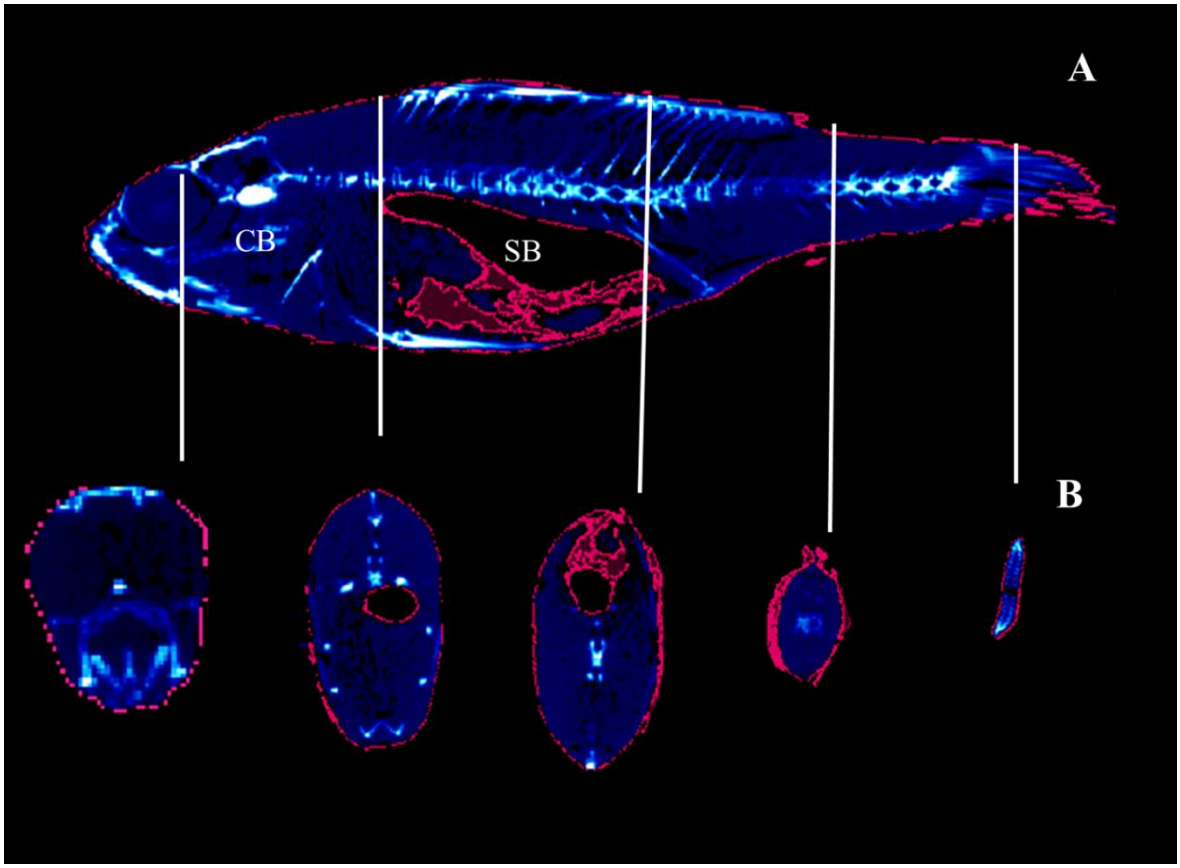


Figure 48. Micro-CT representative images of European sea bass coloured according to AMIDE program to display skin segmentation (dark pink) in sagittal (A) and transversal (B) axes from mouth to the caudal fin. White lines are indicating the reference structures where are located the transversal axis. SB: swim bladder.

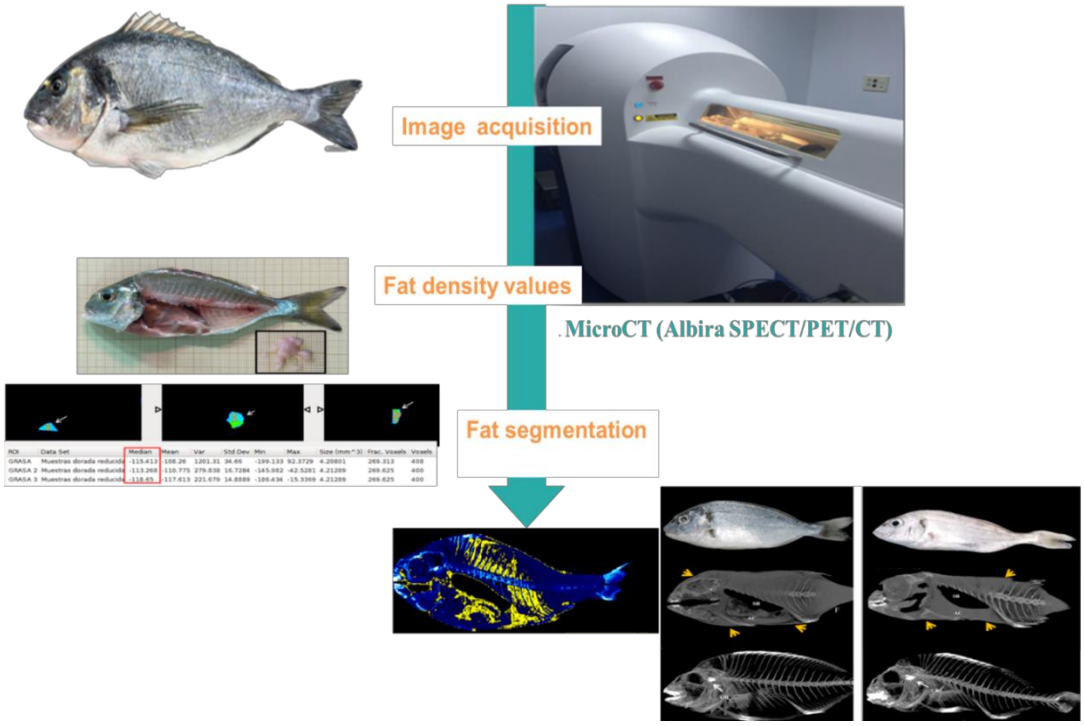
Curiously, in European sea bass there is a segmented area in the abdominal cavity, around the swim bladder and other abdominal organs, which coincides with the density ranges established for the skin (Fig. 48).

IV.3. Fat imaging by X-ray computed tomography (Micro-CT)

1. Objective

After obtaining whole gilthead seabream density ranges, we found that values corresponding to the skin (-400 to -50 HU) overlapped those corresponding to the subcutaneous, intramuscular or visceral fat (-115 to +50 HU). It is known that in fish, the fat content contributes to promoting the nutritional and organoleptic characteristics of flesh (Izquierdo *et al.*, 2005; Cardinal *et al.*, 2011; Silva *et al.*, 2016), which is crucial for consumer acceptance (Probert and Shannon, 2002). Besides, methods to predict the fat composition in fish are important in nutritional and physiological investigations, where body content is traditionally determined by comparative slaughtering followed by chemical analysis (Schwarz and Kirchgessner 1993; Oberle *et al.* 1997). The present study analysed the fat distribution, *in situ,* in whole gilthead seabream body using micro-CT equipment. Moreover, to validate the method changes in fat content in two fish groups (fed and starved for 60 days) were assessed.

2. Graphical abstract



3. Materials and methods

3.1 Fish

Eighteen specimens of the hermaphroditic protandrous teleost gilthead seabream (*S. aurata*) (26 ± 3 g and 12 ± 2 cm) were obtained from a local fish farm and 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 4 weeks. A commercial diet (Skretting, Spain) was administered during acclimatization. The temperature and salinity were 22 ± 2°C and 28‰, respectively. The photoperiod was of 12 h L: 12 h D.

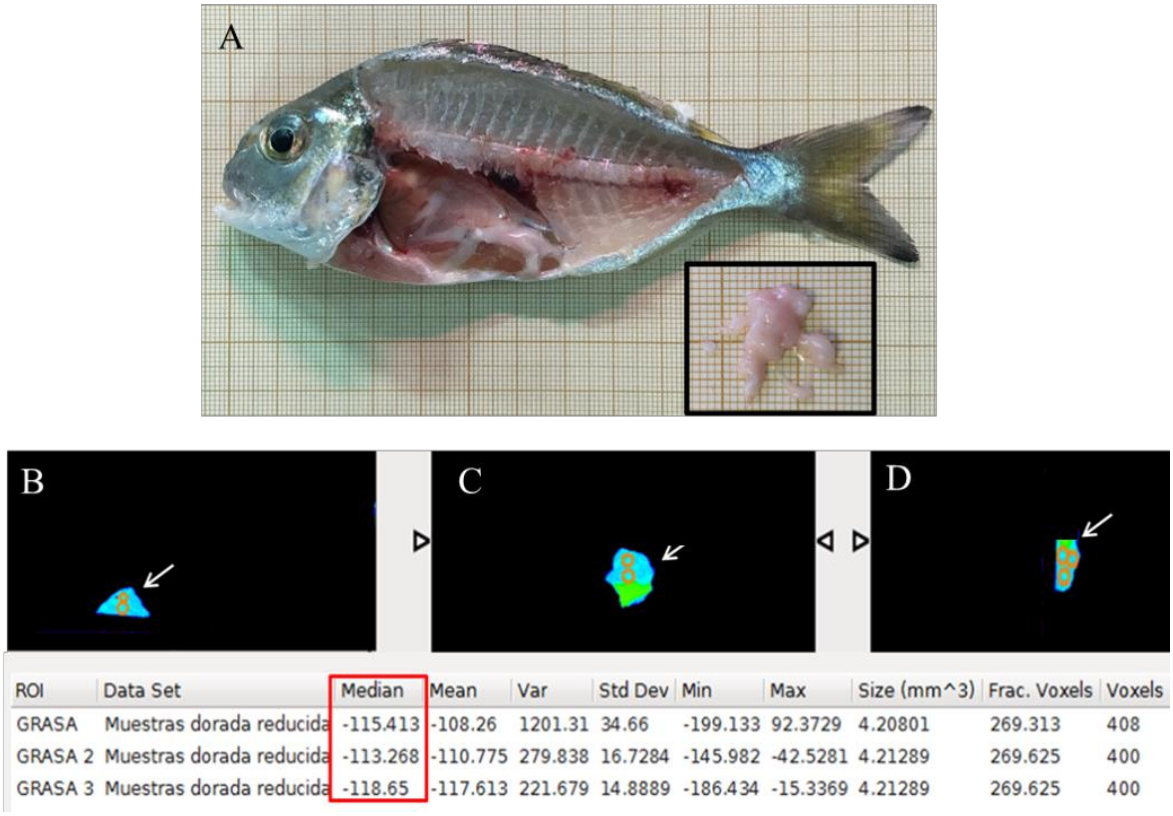
3.2. Experimental trial

Fish specimens were randomly assigned into three groups (n = 6 each), one group was sampled to determine fat distribution and biometric parameters under normal physiological conditions. Then, the rest of the fish were studied in two different conditions (fed and starved) to validate the method and ensure that the image segmentation corresponded to the fat content: 1) The control group (fed fish) was fed with commercial pellets (Skretting, Spain) at a rate of 1.5% body weight day^{-1}; and 2) the last group was starved. Both groups were kept in these conditions for 60 days.

3.3. Micro-CT acquisition, reconstruccion and segmentation

To acquire and process the micro-CT image for fat the protocol previously described in Chapter IV.2 section 3.1 was followed. First, the density value for fat was determined from the isolated visceral fat alone (Fig. 49).

Then, to segment the fat *in situ* 3D isocontour ROIs were manually drawn along the micro-CT image of fish body, HU range based on the value determined for the calibration from visceral fat substracted was introduced in the AMIDE software and an automatic segmentation was carried out, to which we established a yellow color (Fig. 50). In cases where the density of the micro-CT bed overlapped the density of some fish portions, the value of the empty micro-CT bed was acquired to be subtracted from the fish values. To evaluate the fidelity of the automatic segmentation, we have compared the fat segmentation in fed fish and fish under starvation



ROI	Data Set	Median	Mean	Var	Std Dev	Min	Max	Size (mm^3)	Frac. Voxels	Voxels
GRASA	Muestras dorada reducida	-115.413	-108.26	1201.31	34.66	-199.133	92.3729	4.20801	269.313	408
GRASA 2	Muestras dorada reducida	-113.268	-110.775	279.838	16.7284	-145.982	-42.5281	4.21289	269.625	400
GRASA 3	Muestras dorada reducida	-118.65	-117.613	221.679	14.8889	-186.434	-15.3369	4.21289	269.625	400

Figure 49. A) Macroscopic image of a dissected gilthead seabream (*S. aurata*) specimen with part of the muscle removed to show perivisceral fat localization. Inset: Portion of visceral fat obtained from the animal dissected in figure, to be analysed in the micro-CT. Pervisceral fat from gilthead seabream (indicated by the arrows) colored according to AMIDE program and positioned on the different axes: transverse (B), coronal (C) and sagittal (D); 25 mm ROIs (orange circles) are drawn on these sections. In the red box: mean density values for fat in the analysed voxels expressed in Hounsfield units (HU).

3.4. Micro-CT image analysis.

1. Descriptive analysis. Segmented images were visually observed by two experts in anatomy and radiology. A descriptive analysis in the AMIDE software was made on fish images from each experimental group.

2. Quantitative analysis. The quantitative analysis was determined by AMIDE software through a morphometric or volumetric analysis via selecting the option “calculate ROI statistics” after the automatic segmentation; this allowed us to determine parameters as total volume and fat volume in mm^3 (Table 6). All morphometric parameters were normalized to the total volume of the specimen, and thus are independent of the absolute size and its variation between the specimens.

3.5. Statistical analysis

Raw data contained in each isocontour ROI (not less than 33,564) were analysed and averaged in the AMIDE analysis software for each fish. Then, the statistical analysis between groups was performed using SPSS 23.0 software by the t-Student test. Data are presented as means ± SEM (n=6) and differences were considered statistically significant when $P<0.05$.

4. Results

4.1. Fat density values

More than 400 voxels contained in several ROIs were analysed for the visceral fat subtracted and averaged to obtain a final density value of -115 HU (Fig. 49). Then, density ranges for fat in the animal were established from -115 to +50 HU (Table 5).

4.2. Fat analysis in gilthead seabream

The fat content in gilthead seabream was analysed separately (Fig. 50). Our results showed segmented areas in the head (including nostril, mouth, eye and operculum); the fish edge (which coincides topographically with the subcutaneous region); flank (above and below the spine) and abdominal cavity.

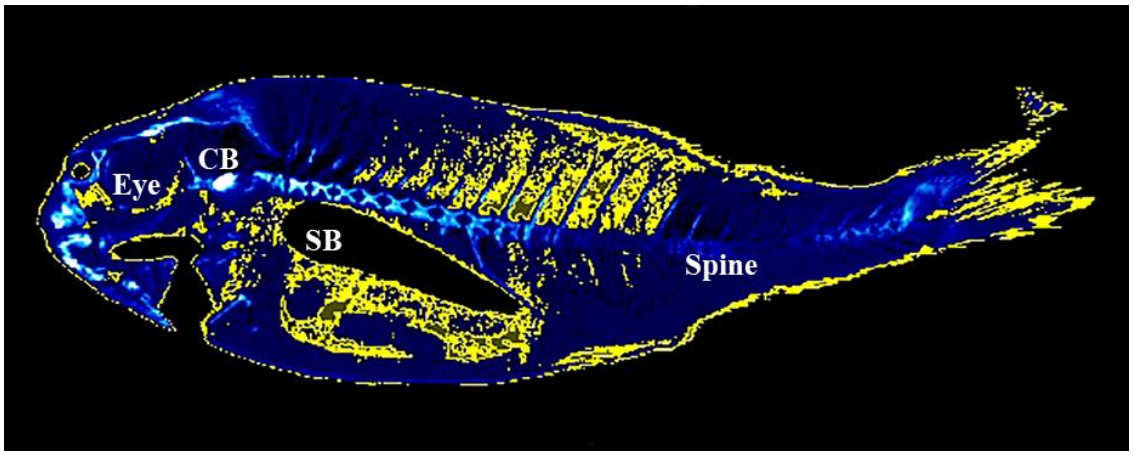


Figure 50. Micro-CT representative image of gilthead seabream (*S. aurata*) coloured according to AMIDE software to display the segmentation of subcutaneous perivisceral and intramuscular fat (yellow). Image is represented in sagittal axe. Eye, cleithrum bone (CB), swim bladder (SB) and spine are indicated as reference structures.

4.3. Fat analysis in fed and starved gilthead seabream

After 60 days of starvation gilthead seabream specimens showed visible differences in body dimensions (Fig. 51), which were also evident in the micro-CT study, where greater body mass was observed in fed fish when compared to those under starvation. Interestingly, the edge delimiting the fish body, which coincides topographically with the skin, was better delineated in control or fed gilthead seabream, being imperceptible in starved fish (Fig. 51 C, D). In addition, when the skeletal structure under Pmod was acquired, visible differences were also detected, mainly in the abdominal region, which seemed compressed (Fig. 51 E, F).

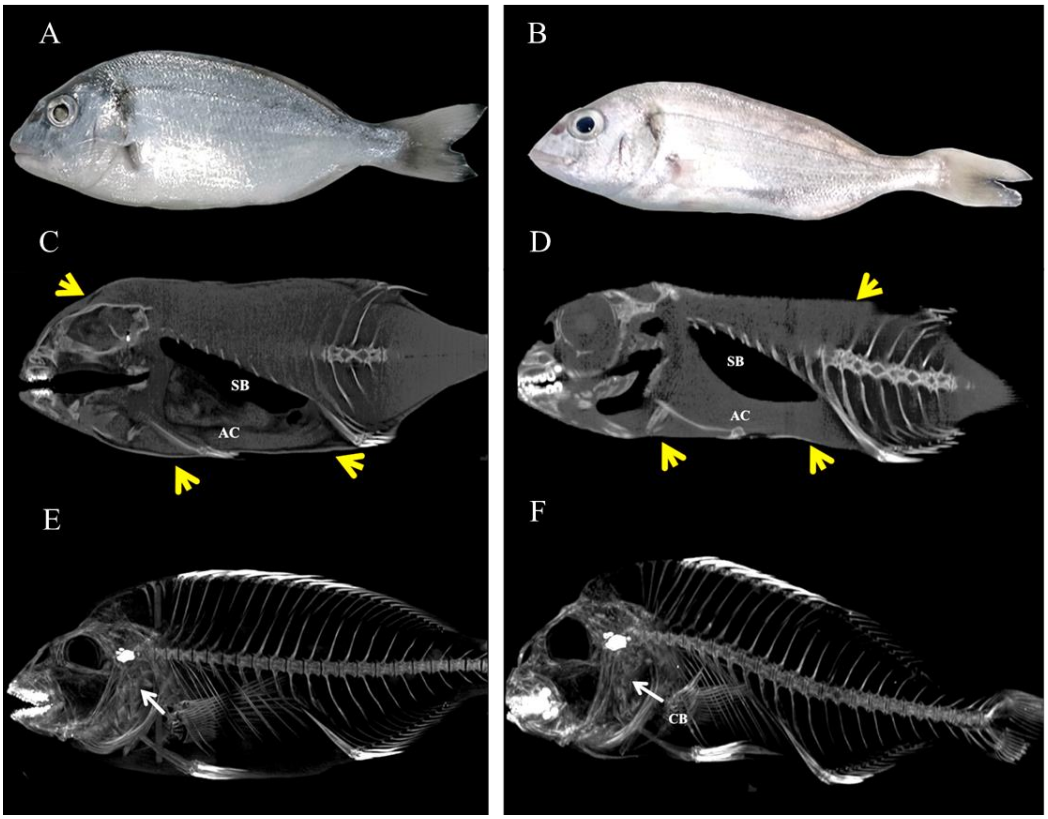


Figure 51. Representative images of gilthead seabream (*S. aurata*) after 60 days of being fed (A, C, E) or left starved (B, D, F). Macroscopic images of fed (A) and starved (B) fish. Micro-CT images of fed (C) and starved (D) fish, images of skeletal structure in fed (E) and starved (F) fish created in Pmod program. Yellow arrows indicated fish edge; swim bladder (SB) and abdominal cavity (AC) as reference structures; white arrows indicated the cleithrum bone (CB) as the densest structure in the fish body.

Image analysis from fed and starved fish showed macroscopic differences between groups, where more segmented areas (yellow) were found in the head (mainly in the orbital region), flanks (intramuscular) and abdominal cavity of fed fish compared to starved fish

(Fig. 52). Body dimension in starved fish was smaller than in fed fish, as well as the eyeball was seen more prominent in starved fish.

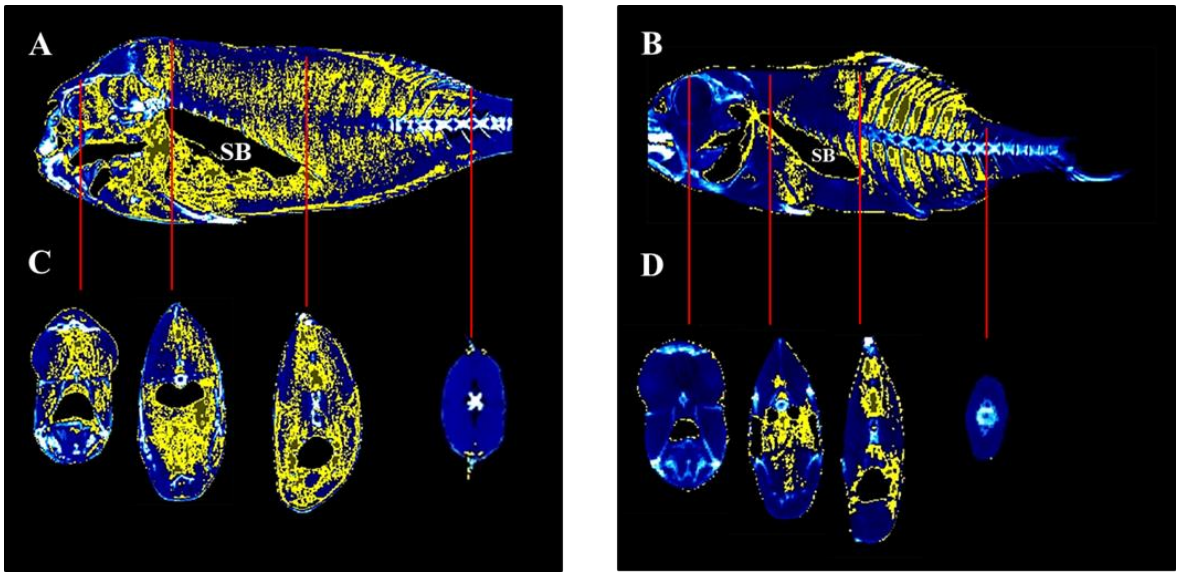


Figure 52. Micro-CT representative images created with AMIDE software to display fat segmentation (yellow) in gilthead seabream (*S. aurata*) fed (A, C) and starved (B, D) after 60 days in sagittal and transverse axes. Red lines are connecting the location of sagittal and transverse axes. Swim bladder (SB) is indicated as reference structure.

Biometric parameters were compared between the two groups (fed and starved), all of them showed significant differences between groups (summarized in Table 6). First, body weight (g), size (cm) and total volume (cm^3) of starved fish were half the values compared to those fed fish (control). Curiously, the fat volume in relation to total volume was higher in starved fish (49%) compared to the fed group (44%).

Table 6. Biometric parameters of gilthead seabream (*S. aurata*) fed and starved (60 days) groups. Data are represented as mean ± SEM (n=5). Statistical differences between groups are represented by an asterisk when $P<0.05$.

Parameters	Experimental groups	
	Fed	Starved
Body weight (g)	53 ± 4.00	19.66 ± 0.898*
Body size (cm)	16 ± 0.577	12.416 ± 0.663*
Total volume (cm^3)	71.289 ± 0.464	37.101 ± 1.038*
Fat volume (cm^3)	31.804 ± 0.463	18.207 ± 0.387*
Fat related total volume (%)	44.618 ± 0.941	49.172 ± 1.540*


DISCUSSION

The present Doctoral Thesis focuses on the characterization of fish skin in species of aquaculture interest. Aquaculture has grown rapidly, but the farming systems involved cause different types of lesions, abrasions or ulcers in fish, which may appear not only as a result of physical contact but also of poor husbandry practices or traumatic processes due to the confined environment (Law, 2001). These lesions act as entry sites for pathogens, and are a major problem in aquaculture (Tørud and Håstein, 2008), so that fish welfare strongly depends on skin integrity (Noga *et al.*, 2000; Fontenot *et al.*, 2004). For this reason, deepening our knowledge of all aspects related to the skin is a priority in order to improve the health conditions of farmed fish and to prevent excessive economic losses. More specifically, this study aims to look at fish skin immunity, healing processes and new techniques for fish characterization.

Chapter I

All living organisms are influenced by external physical factors, which have a great impact on their physiology (Bowden *et al.*, 2007). In this sense, of the environmental factors that may influence the immune response, photoperiod and temperature are the most important for poikilotherm animals (Esteban *et al.*, 2013; Valero *et al.*, 2014). In mammals, there are many studies on how photoperiod influences neuronal function and melatonin secretion and also about how the hormone melatonin can directly and indirectly govern seasonal changes in the immunity of a variety of vertebrate taxa (Weil *et al.*, 2015) including fish (Nakanishi, 1986; Bly *et al.*, 1997; Esteban *et al.*, 2006, 2013; Valero *et al.*, 2014). Therefore, the behaviour and physiology of fish are strongly influenced by light (both natural or seasonal and manipulated or artificial) conditions (Bowden *et al.*, 2007). The teleost pineal is photoreceptive and considered to be essential to the generation, synchronisation and maintenance of biological rhythms, primarily via melatonin release. In fish, the role of

internal (circadian clock) and external (light) signals control melatonin production in the fish pineal gland (McStay *et al.*, 2014). Melatonin synthesis and secretion is suppressed by light and enhanced by darkness (Liebmann *et al.*, 1997; Esteban *et al.*, 2006). Its peak levels in the dark are associated with age as well as various illnesses (Emet *et al.*, 2016). Thus, the management and control of the physical conditions, in this case the photoperiod, in aquaculture can be considered of great importance for fish welfare and health. In fact, photoperiod manipulation has been successfully used to accelerate growth, development and the survival of young stages in several fish species (Tandler *et al.*, 1985; Kissil *et al.*, 2001) and also to reduce unwanted sexual maturation (Bromage *et al.*, 2001; Chi *et al.*, 2017). However, there are no data about how photoperiod could be altered or modified in order to contribute to making the fish immune status more robust. Regarding fish immunity, several studies have demonstrated the relation between photoperiod and the immune system (Liebmann *et al.*, 1997; Esteban *et al.*, 2006, 2013; Lazado *et al.*, 2015, 2016). More specificaly, a previous study conducted in our group (also in gilthead seabream and European sea bass) reported an effect of photoperiod on several seric immune parameters (natural haemolytic complement, lysozyme and peroxidase activities) (Esteban *et al.*, 2006).

We have studied how the photoperiod affects the levels of some immune parameters (IgM level, protease, antiprotease, peroxidase, lysozyme and bactericidal activities) present in skin mucus from gilthead seabrean and European sea bass. The immune response of fish skin is highly important because this organ is in direct contact with the environment and so the first line of defense. All the parameters analysed were chosen due to their demonstrated presence in skin mucus and implications in the immunity of several fish species (Itami, 1993; Palaksha *et al.* 2008; Guardiola *et al.*, 2014a). Transduction of seasonal information from the environment (*i.e.*, photoperiod and water temperature) and daily rhythms have previously been studied in both fish species and the results clearly suggest that the pineal gland is the major source of plasma melatonin (Molina-Borja *et al.*, 1996; Garcia-Allegue *et al.*, 2001). Present results indicate that different immune parameters followed different patterns dependent of the LD cycle. Furthermore, in this study, notable inter-specific variations were observed between gilthead seabream and European sea bass samples. Skin mucus IgM levels and peroxidase activities showed rhythmicity in gilthead seabream, bactericidal activity showed rhythmicity in skin mucus of European sea bass and lysozyme activity showed rhythmicity in both fish species. Immunoglobulins or antibodies play a vital role in the

immune responses and IgM is considered the most ancient antibody molecule, which shares similar functions in all gnathostomes (Flajnik, 2002). This immunoglobulin has a key role in both innate and adaptive immunity in fish and its effector functions includes complement activation (which both lyses and opsonizes microorganisms) (Boshra, 2004). Furthermore, IgM also mediates agglutination for phagocytosis and removal of pathogens (Ye *et al.*, 2013). Interestingly, present results demonstrated that total IgM levels present in skin mucus were lower during the night with a significant decrease when lights were switched off (8:00 h) in gilthead seabream, while European sea bass IgM levels were lower during the day but without significant differences. As it has been corroborated that specific antibodies can be generated in the mucosal of fish including the skin (Cain *et al*, 2000), new studies are needed to correlate the obtained results on IgM daily changes on skin mucus with the IgM levels in other mucosal surfaces (intestine and gills) and also with the systemic IgM levels present in serum, where is the most prevalent Ig (Flajnik, 2002).

Intriguingly, in the present work, no significant variations were recorded in the levels of protease and antiprotease activities in mucus from both fish species studied. Our results are in agreement with those previously obtained in Nile tilapia (*Oreochromis niloticus*) for both protease and antiprotease activities under LD cycle (Lazado *et al.*, 2016). However, higher protease and antiprotease activities were recorded during light cycle in *Trachinotus falcatus* (Lazado *et al.*, 2015). Future studies aiming to understand why the levels of these enzymes seem to be independent of the LD cycle in some teleost fish species will be welcomed. On the other hand, peroxidase activity in skin mucus reached its maximum and its minimum at 02:00 h in gilthead seabream and European sea bass, respectively. Curiously, when this enzyme was studied in serum from these species maintained at the same LD cycle (12 h L: 12 h D) the peroxidase activity of seabream was significantly higher at 08:00 h than during the rest of the cycle, while in sea bass, it showed little variation (Esteban *et al.*, 2006). These results indicated that the rhythms of this enzyme in the two fish species are different in serum than those registered in skin mucus. Taking into account that peroxidases are important microbicidal agents because they are able of efficiently eliminate H_2O_2 and maintain the redox balance of immune system, its importance for mucosal immunity and skin defense is evident. The scarce available results on the influence of the photoperiod on serum peroxidase activity suggest that peroxidase secretion depends on the fish species (Esteban *et al.*, 2006), as also point out the results obtained in the present study. Lysozymes are ubiquitous

antibacterial enzymes widely distributed within the animals. Lysozymes display hydrolytic activity to specifically cleave the β-1, 4-glycosidic bonds between the *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan (an essential and single cross-linked bacterial cell wall heteropolymer that provides structural strength and protects the osmotically sensitive bacteria). Thus, the disruption of the peptidoglycan present in the bacterial cell walls results in cell lysis and in the destruction of the microorganisms (Watts *et al.*, 2001). Lysozyme activity in fish serum also follows a clear daily rhythm, even though again different interspecific patterns have been observed in gilthead seabream and European sea bass (Esteban *et al.*, 2006). Similarly, different pattern was observed in the activity of lysozyme in skin mucus of these two fish species. Although lysozyme activity increased in both species in dark hours, a significant decrease was found at 14:00 h in European sea bass. Same results were found in gilthead seabream serum where higher values were recorded in dark hours (Esteban *et al.*, 2006). However, in European sea bass, maximum lysozyme activity in serum was observed at 8:00 h (light hour) with no significant differences (Esteban *et al.*, 2006).

Numerous studies have demonstrated the high antimicrobial activity present on skin mucus in fish (Aranichi *et al.*, 1999; Subramanian *et al.*, 2008b; Lazado *et al.*, 2011; Raj *et al.*, 2011; Benhamed *et al.*, 2014). Present results indicated that mucus bactericidal activity against *V. harveyi* was also significantly affected by the LD cycle in gilthead seabream and European sea bass. Our results agree whith those derived from a bacterial endotoxin challenge in *O. niloticus* (Lazado *et al.*, 2016). Furthermore, our results are also in agreement with those obtained in *Oncorhynchus mykiss* focused on the dynamics and interplay of serum-mediated bacterial killing activity against *Flavobacterium psychrophilum* and *Yersinia ruckeri* and on several immune defence factors throughout a daily LD cycle (Lazado *et al.*, 2018). These previous works suggested that responsiveness of humoral factors in serum to a biological insult depend on the time of the day. A deeper knowledge of the daily rhythm of bactericidal activity (not only in fish skin mucus but also in serum) would help us to anticipate to the normal response of the farmed fish in order to improve their welfare, to make more robust their immune system or to prevent and/or treat possible diseases. Similarly, knowing better the periodic changes occurring in fish immunocompetence could aid to know the annual variance in disease incidence and severity in nature, and provide a useful framework to help understand brain-immune interactions.

The immune cells have membrane receptors and nuclear orphan receptors in order to detect the melatonin level (Skwarlo-Sonta, 2002). Comparative studies have demonstrated that the effects exerted by melatonin on immune parameters are different, and depend on several factors (apart from melatonin level); such as species, sex, age of animal, immune system maturation, the parameter examined, experimental procedure, etc. In order to respond to melatonin, the immune cells become activated and secrete lymphoid organ-derived hormones as well as some cytokines. These lymphoid messages can be understood by the pineal gland, closing the bi-directional regulatory loop between both systems (Skwarlo-Sonta, 2002). Many more data are needed to know the different steps that can be involved in these extremely complex networks of communication between neuro-endocrine and immune systems in fish. Therefore, the present findings demonstrate that different enzymes related to the immune system and bactericidal activity of skin mucus remarkably varied during the daily light–dark cycle in gilthead seabream and European sea bass. A better knowledge of the interactions between fish melatonin and immune system will facilitate the development of novel practical suggestions (such as photoperiod manipulation) for enhancing the immune system and welfare of fish in hatcheries and growing farms. The complex interactions of environment, host and pathogens in farmed fish present numerous points of manipulation for research and production improvements.

Chapter II

Fish skin is a primary target organ for a number of common infectious agents which are normally found in the aquatic environment (Noga, 2011). In this respect, to cause an infection, bacterial density and virulence are clearly important factors. However, equally important to the host immune status (Edwards and Harding, 2004) might be skin integrity (Quilhac and Sire, 1999). In aquaculture, vibriosis is one of the most serious diseases that fish farmers have to face. It is caused by a variety of members of the genus *Vibrio (V. anguillarum, V. harveyi, V. parahemolyticus, V. salmonicida, V. viscosus, V. vulnificus,* among others*)*: Gram-negative, rod-shaped, marine bacteria with no geographical limits and also capable of contaminating freshwater fish (Egidius, 1987; Farmer *et al.*, 2005; Haenen *et al.*, 2014). Some species of *Vibrio* are serious pathogens of aquatic vertebrates or invertebrates. Others, including *Grimontia hollisae, Photobacterium damselae* and *V. harveyi,* are thought to cause disease in both aquatic animals and humans (Austin, 2010). *V.*

harveyi, also called *V. carchariae,* is an opportunistic pathogen of marine animals commonly found free-swimming in tropical marine waters. It causes high mortality in marine and freshwater fish and shellfish, leading to great economic losses (Austin and Zhang, 2006). Due to the importance that *V. harveyi* has acquired in aquaculture, its diagnosis has improved in recent years (Pang *et al.*, 2006). However, preventing the disease have proved more difficult, and it can only be controlled with good husbandry practices and antibiotics, the latter contributing to the emergence of bacterial resistance, a natural process that is accelerated by the missuse of antibiotics in food production (Aidara-Kane *et al.*, 2018). In this context, study of natural substances that can be used as alternatives to chemicals has increased. Of the alternatives, which include micronutrients and probiotics, plants are considered the most reliable source of compounds to prevent/treat infections in both animals and humans (Vieira *et al.*, 2001; Wang *et al.*, 2009; Biswas *et al.*, 2013; Van Hai, 2015; Carbone and Faggio, 2016; Dotta *et al.*, 2018).

In aquaculture, the use of natural and innocuous compounds from plants has a great potential due to their immunostimulant effects (Yin *et al.*, 2014; Adel *et al.*, 2015; Awad *et al.*, 2015; Van Hai, 2015; Adel *et al.*, 2016; Guardiola *et al.*, 2016; García-Beltrán *et al.*, 2017, 2018; Hoseinifar *et al.*, 2017; Baba *et al.*, 2018; Mansour *et al.*, 2018). In addition, they can be used as microbicidal agents (Rios *et al.*, 1987; Vieira *et al.*, 2001; Pandey *et al.*, 2012; Biswas *et al.*, 2013; Gobi *et al.*, 2016; Dotta *et al.*, 2018) and promoters of the healing process (Raina *et al.*, 2008; Kumari *et al.*, 2017). Interest in plant compounds has increased world-wide because they are easy to prepare, cheap, and have few side effects on animals and the environment (Van Hai, 2015). Further, increasing evidence of the health-protective benefits of phytochemicals, components derived from plants, has led to great attention in them, warranting further scientific evaluation and mechanistic studies (Thangapazham *et al.*, 2016). Therefore, in Chapter II we evaluated the immunostimulant effect of dietary supplementation with guava (*P. guajava*) leaf on the skin mucus of hybrid fish of *O. niloticus* and *O. mossambicus*. To the best of our knowledge, this is the first time that the guava leaf effect is analysed in fish skin mucus. In addition, the microbicidal effect of guava leaf against an experimental infection with *V. harveyi* was measured in skin from hybrid tilapia. Guava is a plant from tropical and subtropical areas of the world and is adapted to different climatic conditions but prefers dry climates (Stone, 1970). Both the leaves and the fruit have been used in traditional medicine for the treatment of enteritis and dysentery (Jianlin *et al.*, 2005;

Gonçalves *et al.*, 2008). In human medicine, guava leaf has been reported to have clinically relevant functions, including antioxidant, antimicrobial, antihypertensive, anti-inflammatory and antineoplastic effects (Jianlin *et al.*, 2005; Gutierrez *et al.*, 2008; Deguchi and Miyazaki, 2010). As regards our interest (aquaculture), guava leaf extracts have shown immunostimulant, antioxidant, antiviral and antibacterial activities (Vieira *et al.*, 2001; Biswas *et al.*, 2013; Yin *et al.*, 2014; Gobi *et al.*, 2016), protection against white spot syndrome virus (WSSV) and *V. harveyi* infection (Wang *et al.*, 2009; Yin *et al.*, 2014; Gobi *et al.*, 2016) and to act as growth promoter (Gobi *et al.*, 2016; Yin *et al.*, 2014). In this study, the fish diet was supplemented with two concentrations of guava leaf (1.5% and 3%) for 45 days and the skin mucus of the fish showed significant increases in the immune parameters evaluated. Similarly, dietary supplementation with guava leaf aquous and ethanolic extracts (1, 5 and 10 mg g^{-1}) in *O. mossambicus,* resulted in enhanced serum humoral immune parameters (Gobi *et al.*, 2016).

Immune substances such as proteases are essential for the activation of innate and adaptive immune system and they exert a protective role against pathogens (Subramanian *et al.*, 2007). In addition, antiproteases may help combat the proteases produced by many microorganisms, which are crucial proteins contributing to their growth and development. For these important reasons these enzymes play critical roles in microbial infection and disease manifestation. Our results showed that protease activity in skin mucus increased after 21 days in fish feed a diet supplemented with 1.5% guava leaf. By contrast, in the case of antiprotease activity a significant increase was seen after 45 days of dietary supplementation (1.5%). Previous studies in different aquatic species reported significant increases in serum protease activity after dietary supplementation with guava leaf (Yin *et al.*, 2014; Gobi *et al.*, 2016). As regards skin mucus peroxidase activity, our results pointed to a significant increase after 45 days of feeding with 1.5% guava leaf supplemented diet. Similarly, previous findings in Nile tilapia (*O. niloticus*) demonstrated that diets supplemented with aqueous and ethanol extracts of guava leaf (1, 5 and 10 mg g^{-1}) increased the serum myeloperoxidase activity (Gobi *et al.*, 2016).

Lysozyme, is another important humoral component of the immune system, where it acts as a defensive factor against invasive microorganisms (Esteban, 2012). However, in this study no variations were detected in skin mucus lysozyme activity from hybrid tilapia *(O. niloticus* × *O. mossambicus)* in either of the experimental diet groups. These results contrast

with those found by Giri *et al.* (2015) and Gobi *et al.* (2016) who reported a significant increase of serum lysozyme activity in Mozambique tilapia (*O. mossambicus*) and rohu (*Labeo rohita*), respectively, after dietary supplementation with guava leaves. Thus, despite the significant increments previously reported for serum lysozyme, our results suggest that lysozyme levels in the skin mucus of hybrid tilapia (*O. niloticus* × *O. mossambicus*) might be not affected by dietary supplementation with guava leaf.

Regarding the bactericidal activity of *P. guajava,* several authors have demonstrated its effect on different members of *Enterobacteriaceae*, *Vibrionaceae*, *Micrococcaceae* and *Propionibacteriaceae* families (Berdy *et al.* 1981; Vieira *et al.*, 2001; Chomnawang *et al.*, 2005; Qa'Dan *et al.*, 2005). This biocidal activity is attributed to the presence of flavonoids, guajaverine and psydiolic acids, present in guava leaf. In our study, guava leaf supplementation (1.5% and 3%) significantly reduced the bacterial load in hybrid tilapia after *V. harveyi* infection. Similarly, guava supplementation significantly increased the resistence of *O. niloticus* (Pachanawan *et al.*, 2008) and *O. mossambicus* (Gobi *et al.*, 2016) to *Aeromonas hydrophila* infection. Further, Giri *et al.* (2015) reported a significantly higher survival rate (66.66%) post-challenge with *A. hydrophila* in *L. rohita* supplemented with 0.5% guava leaf. In addition, Vieira *et al.*, 2001 demonstrated the inhibitory effect of *P. guajava* sprout extract (ethanol, aqueous and acetone) against *Escherichia coli* and *Staphylococcus aureus* isolated from fish muscle. In another study, with the shrimp giant tiger prawn *(Peaneus monodon),* Yin *et al.* (2014) reported an enhanced non-specific immune response and proposed guava leaf as a safe water disinfectant for use in shrimp culture. Thus, all these findings underline the role of *P. guajava* not only as a good immunostimulant but also as an important source of new antimicrobial compounds to prevent or treat bacterial infections in aquaculture.

Enhancement of the immune system seems to be the most promising method for preventing diseases in fish (Gobi *et al.*, 2016). Therefore, the manipulation of certain environmental factors, as well as the improvement of the diet to strengthen the immune system, could be an effective solution to prevent the appearance of problems related to pathogens in aquaculture.

Chapter III

Failure or disruption of skin anatomical and physiological characteristics often results in apparition and development of cutaneous diseases (Fontenot and Neiffer, 2004) and downgrading of the whole fish or fillets, due to mostly poor visual appeal to the consumer, leading to economic losses. Thus, in Chapter III, we aimed to study the healing proccess and immune response in gilthead seabream (*S. aurata*) after experimental injury. As we previously mentioned, a good response from fish to environmental changes and pathogen entry will greatly depends of skin integrity. Hence, before carrying out a protocol of prevention or treatment of acute or chronic injuries, it is important to know how skin behaves and responds during the healing process (Fontenot *et al.*, 2004). To the best of our knowledge, the process of skin response and recovery after wounding has not been fully understood; even few methods to perform experimental lesions in fish have been published (Davidson *et al.*, 1998; Fontenot *et al.*, 2004; Schmidt, 2013). In this chapter, we made experimental wounds and ulcers in gilthead seabream skin.

Depending on the severity, wounds could be classified in acute or chronic. The term "acute" refers to rapid introduction of the injury and a relatively rapid course of repair that may be compromised. Acute wound models are often parallels of authentic surgical procedures or traumatic injuries (Davidson, 1998). On the other hand, "chronic" wounds do not adhere to the standard time course of cellular and molecular events that lead towards healing of a healthy acute wound (Martin and Nunan, 2015). The treatment and analysis of chronic wounds remains one of the greatest challenges to the wound healing community, because these wounds are highly diverse in their etiology, including infectious agents, toxins, physical, stress and immunologic causes, and nutritional and metabolic perturbations (Davidson, 1998; Law, 2001).

Experimental open wounds were made in two different body locations using a biopsy punch (8 mm). Some authors have described that traditional wound-infliction techniques, which mimic chronic wound-healing models include punch biopsies and excisions, which tend to form non-uniform wounds (Braiman-Wiksman *et al.*, 2007). However, based on the fact that most experimental wounds in animals are made using excisional methods (Gonzalez, 2002; Liu *et al.*, 2010; Deka *et al.*, 2013), we use the biopsy punch, since it is the

most suitable and reproducible method that we have found for fish. According to the figures presented in this study, it can be seen that all the wounds were very similar.

Different body locations (above and below) the lateral line, named ALL and BLL respectively, were selected to perform the wounds, in order to establish possible differences between these two skin zones regarding the healing process (now studied by image analysis) as well as the possible influence of the wound on the skin mucus immunity of the surrounding skin, and in serum. Taking into account that the morphology of the skin can vary depending on several factors such as location in the body, species, sex, life stage, season, reproductive condition, nutrition and water quality (Bullock and Roberts, 1974; Iger and Abraham, 1990; Groff, 2001; Fontenot and Neiffer, 2004), the study was developed using fish of the same origin and size and subjected to the same handling procedures. These two body locations were selected because two recent papers have reported differences in the gene expression profile between these two zones after *in vitro* exposure of skin explants from Atlantic cod (*Gadus morhua*) (Lazado and Caipang, 2014) and gilthead seabream (*S. aurata*) (Cordero *et al.*, 2016c) to both probiotics and pathogens. Results also showed a regional size difference in cells in the epidermis; cells from the dorsal region being significantly larger than those from the ventral region (Lazado and Caipang, 2014). However, it is important to underline that both studies were carried out *in vitro*. Our team previously studied the morphological characteristics of both skin zones selected in the present work (above and below the lateral line, named dorsal and ventral in the manuscript), in gilthead seabream. This previous study, established that the cells isolated from both the dorsal and ventral zones showed a similar cell cycle and gene expression. Nevertheless, the epidermis of the ventral skin was thicker than that of the dorsal zone. These finding also coincides with our results reported below. Moreover, the cell size and area of microridges in the apical part of the dorsal epidermal cells were greater than that recorded in epidermal skin from ventral zone (Cordero *et al.*, 2017). Additionally, experimental wounds were made to compare the wound healing rate between the skin in the dorsal and ventral zones. The results showed a higher ratio of wound healing in the ventral region, whose wounds were closed after 15 days, compared to the dorsal region of the skin (Cordero *et al.*, 2017). These previous results led us to develop the present study in which our attention focused on the first 7 days post-wounding.

After injury, the skin begins to heal in a highly complex biological process, comprising a series of sequential events temporarily overlapped aiming at repairing the injured tissue (Tsirogianni *et al.*, 2006). So, in the present study, the fish were sampled following the healing progression time line described in previous works (Fontenot and Neiffer, 2004; Braiman-Wiksman *et al.*, 2007; Deka *et al.*, 2013). In pigs and rats, it has been described that the healing progression of wounds can be evaluated by means of computerized software and measuring the wound through macroscopic images (Mekkes *et al.*, 1995; Gonzalez, 2002). This method is a simple and fast way to determine the wound area (Mekkes *et al.*, 1995), and in the present study, we have adapted it to skin wounds in fish.

The initial stage of skin wound healing comprises the haemostasis/coagulation and the inflammatory phase. These phases are closely related since inflammation is activated during haemostasis/coagulation (Elliot, 2000). The inflammatory phase plays a central role in wound healing, not only by encountering the invading microbes or new tissue constituents, but also by participating in the tissue repair processes (Abbas *et al.*, 2003; Braiman-Wiksman *et al.*, 2007). Inflammation prepares the wound for the subsequent phases of healing, and could be divided into an early phase and a late phase (Elliot, 2000). In our study, the size of the wounded areas increased immediately after wounding, probably due to an early inflammatory response, which was also evident macroscopically. The area of the wounds started to fall by 2-4 days post-wounding, in accordance with the course of wound healing generally described for fish (Fontenot and Neiffer, 2004; Martin and Nunan, 2015) and from 5 days the healing rate was higher in wounds below the lateral line (BLL) compared to those above the lateral line (ALL) althought no significant diferences were found between them. It has been described that 5 or 6 days post-wounding wounds appear as vascular (Fontenot and Neiffer, 2004). However, in the present work, the wounds made in gilthead seabream skin were not vascularized at any time up to the 7 days. This may have been related to the fact that in our experimental wounds all the skin and probably some layers of muscle were removed, which perhaps caused a delay in the healing process, which is faster if the lamina basal of the epithelium is maintained (Fontenot and Neiffer, 2004; Martin and Nunan, 2015).

In fish, dermal closure is initiated around 6 days post-wounding, concomitant with granulation-tissue formation (Braiman-Wiksman *et al.*, 2007; Esteban *et al.*, 2012). This stage is accompanied by attenuation of the inflammatory response and the start of the proliferative stage. The objective of this stage is to achieve protection of the wound surface

and is characterized by the appearance of red, fleshy granulation tissue, which ultimately fills the wound (Elliot, 2000). Our macroscopic results observed 7 days post-wounding agree with all these descriptions, where the red colour of some areas in wounds was evident. Furthermore, after 7 days of healing, no changes in skin pigmentation were observed in the wounded area, since perhaps more days are needed to see hyper-pigmentation as a consequence of melanocyte recruitment at the wound site. Besides this, melanocytes persist even after a chronic wound has successfully healed (Martin and Nunan, 2015). As mentioned above, no statistical differences were found between the healing processes in ALL and BLL wounds up to 7 days post-wounding, probably due to the high variability of the healing process among fish, as occurs in mammals (Ansell *et al.*, 2014). In fish, an inflammatory response can be observed for 3-4 days and is evident 1-3 h post injury (Fontenot and Neiffer, 2004). In this phase, neutrophils act as a first line of defence in contaminated wounds by destroying cellular debris and bacteria through phagocytosis and subsequent enzymatic and oxygen-radical mechanisms (Elliot, 2000).

It is known that in the complex process of wound healing a wide variety of substances involved in immunity are released, mediating both a local and a systemic immune response (Quilhac and Sire, 1999), including complement system, lysozyme, proteases, lectins, esterases, immunoglobulins and antimicrobial peptides (AMPs), among others (Esteban and Cerezuela, 2015). Thus, the immune response has a fundamental impact on the quality of the tissue response to an injury (Braiman-Wiksman *et al.*, 2007; Esteban *et al.*, 2012). The inflammatory response in the skin is initiated after injury through the active recruitment of granulocytes (neutrophils) from nearby vessels, which is coordinated by growth factor signals from the resident cells and serum (Eming *et al.*, 2009). Neutrophils have an important cleansing role in killing invading microorganisms through several strategies, including the release of proteolityc enzymes and overproduction of reactive oxygen species (ROS) (Dovi *et al.*, 2004; Shaw and Martin, 2009). Some of these important enzymes were selected in this study. One of them, proteases and their inhibitors, which are key elements of tissue repair and play an important role in wound healing mechanisms through the degradation and remodelling of the extracellular matrix (McCarty and Percival, 2013) were studied. A rapid initial increase of protease activity after skin wounds has been reported in some species including humans, although maximum levels are reached after about 3 days, before starting to fall from day 5 (Shaw and Martin, 2009). In our results, not significant increase was

detected in protease activity for skin mucus; however, in serum, protease activity increased significantly in wounded fish (both, ALL or BLL) 7 days post-wounding. Next, antiprotease activity was significantly decreased 7 days post-wounding in skin mucus of wounded gilthead seabream, similar to serum but, with no significant results. Moreover, peroxidase was also analysed because it acts as an important microbicidal agent, which is produced by neutrophils and macrophages (Fontenot and Neiffer, 2004; Cordero *et al.*, 2016c). Its importance is maximum because its uncontrolled release might cause severe damage to normal (non-wounded) tissues of the host near the wounded zones (Eming *et al.*, 2009). Present results demonstrated an increase in peroxidase activity in skin mucus and serum taken from wounded fish, suggesting a greater presence/activity of phagocytes.

The wound healing processes may be strongly affected by bacteria (from inflammation to remodelling stages) and these bacteria are present in the surface and deep tissues of all wounds (Laato *et al.*, 1988). To cause an infection, bacterial density and virulence are clearly important factors. However, similarly important is the host immune status (Harding and Edwards, 2004). In the present work, the bactericidal activity against two opportunistic pathogenic bacteria (*V. harveyi* and *P. damsealae* subs. *piscicida*) increased in the serum of wounded gilthead seabream at early time points. These results underlain the importance of the first few days during the course of the possible infection and agree with those obtained in other animals and humans, where high levels of some immune activities in the initial stage of the wound (described as the inflammatory phase) has been demonstrated (Ubels and Edelhauser, 1982; Elliot, 2000; Tsirogianni *et al.*, 2006; Braiman-Wiksman *et al.*, 2007; Deka *et al.*, 2013).

Regarding immunoglobulins, we detected some increases in the levels of IgM but no clear relations to the wound state or area were observed. Interestingly, we found a reduction in the skin mucus IgM in wounded fish at 1 day after injury, concomitant with an increase in *ight* transcription. This Ig (IgT) represents the most ancient specialized Ig in mucosal immunity. Besides, this IgT plays a key role in the neutralization of bacterial microbiota and pathogens in the skin (Xu *et al.*, 2013), which could explain the up-regulation of this gene in skin of BLL wounds, 1 day post-wounding, when the wound is more susceptible to infection by pathogens and in the skin near the vital organs. In the normal process of wound healing, immune cells and cytokines fall within a few days after an injury (Kurahashi and Fujii, 2015) as found for all the immune parameters analysed in the present study.

During the early inflammatory phase, a production of inflammatory cytokines and growth factors that facilitate wound healing mechanisms is evident (Eming *et al.* 2009; Theoret, 2017). In addition, it is speculated that piscine nucleated thrombocytes persist for some time in the wound, and continue secreting cytokines and growth factors, as well as actively helping to clear the wound of pathogens and cell debris by phagocytosis (Schmidt, 2013). In this same context, our results showed a significant up-regulation of the expression of *il1b, il6* and *tgfb* genes from 0 to 3 days in ALL wounded skin. These data suggest the activation of a mechanism to counteract the inflammation, mechanism that according to the literature is activated few hours post injury (Eming *et al.*, 2009). Several studies have shown that TGF-β promote matrix formation and deposition of collagen fibers at very early stages of wound healing (Finch *et al.*, 1989; Yang *et al.*, 2001), but a persistent inflammatory response does not allow the proper healing of the wound. Some authors propose to learn how to manipulate or reprogram the inflammatory response, in order to prevent infections and resolve the wound in a proper time, to avoid the chronic inflammation that is common in chronic wounds (Martin and Nunan, 2015). Curiously, the expression of the gene which intervenes in epidermis development (McCarty and Percival, 2013) *krt1* gene, was down-regulated at all the experimental times, although only statistically significant in the skin from fish wounded ALL at 0, 1 and 3 days post-wounding. Our results agree with those found for some keratins in gilthead seabream proteome (McCarty and Percival, 2013).

Several mechanisms are involved in the wound healing process; this study provides new insights into the wound healing and immunological properties of different skin zones in gilthead seabream. Our results showed that the healing process is faster for BLL wounds than those ALL. In addition, results suggest that skin cells in gilthead seabream are more sensitive to physical aggression in the BLL area. However, the gene expression of some immune-related genes increased to a greater extent in the ALL skin. Studying the fish skin in both body areas would throw light on how and why the skin of different body locations respond in different manners to the same injury.

The delicate nature of fish skin combined with its intimate contact with the environment explain, at least partially, the fact that epidermal damage, especially ulceration, is considered to be one of the best biomarkers of polluted or stressful environments (Sinderman, 1990). In addition, previous studies reported the presence of pathogenic organisms as the primary

cause of skin ulceration (Noga, 2000; Law, 2001). Further, in a previous study, dermal ulceration was the chief finding in a series of studies in which striped bass (*Morone saxatilis*) and striped bass hybrids (*M. saxatilis* female × *M. chrysops* male) were exposed to acute confinement stress (Noga *et al.* 1998; Udomkusonsri *et al.* 2004). There is evidence that ulceration of as little as 10% of body surface area can result in very high (nearly 50%) acute mortality, probably because of osmotic stress, while the degree of mortality is directly related to the amount of skin loss (Noga, 2000). In Chapter III we also studied mucus glycosylation, immunity and the bacterial microbiota associated to the skin of experimentally ulcerated gilthead seabream compared with non-ulcerated skin, aimed at detecting possible changes in the skin and its mucus provoked by the presence of a skin ulcer.

Skin mucus is composed of hundreds of proteins, but the major structural components are mucins, which are glycoproteins conjugated with a large content of high molecular weight oligosaccharides. Mucins form networks with viscoelastic properties that entrap particles and pathogens while also acting as a framework for interaction with other proteins (Subramanian *et al.*, 2008a; Thornton *et al.*, 2008; Raj. *et al.*, 2011; Dubaissi *et al.*, 2018). Carbohydrate side chains comprise around 80% of the total mucin mass (Spitzer, and Koch., 1998). Our results detected decreases in the binding, in most analysed lectins, to the terminal carbohydrates in mucus from ulcerated fish, compared to the values of control (non-ulcerated) fish skin mucus, although only significant decreases were recorded for ConA and WFA. In contrast, important changes have been demonstrated after bacterial or parasitic infections in the glycosylation pattern of skin and gut mucus in gilthead seabream (Estensoro *et al.*, 2013) and common carp (*Cyprinus carpio)* (Marel *et al.*, 2010), where the release of these mucins from mucosal epithelia was found to be triggered by bacterial products or live bacteria (Marel *et al.*, 2010). Moreover, the analysis of some enzymatic activities (protease, antiprotease, peroxidase and lysozyme) was evaluated in skin mucus from ulcerated fish. Present results showed significant decreases for protease and antiprotease activities in skin mucus from ulcerated fish. Furthermore, a general tendency of decreasing was detected in peroxidase and lysozyme activities, as well as in the IgM levels, in ulcerated fish, but with no significant results. These results are corroborated with those found in a previous study by Cordero *et al.*, (2017b), who analysed the skin mucus proteome in gilthead seabream after chronic wounds and reported decreased expression at both protein and mRNA levels. The

decreases detected in the analysed immune parameters let us to think that the skin mucus from ulcerated fish is less equipped to defend the fish than the mucus of control fish, perhaps due to the intermittent removal of the mucosal layer made during the experimental trial, since these immune substances are mainly present in the mucosal surface (Esteban, 2012). In addition, these results confirm the susceptibility of damaged skin to be contaminated by pathogen organisms.

With the growth of microbiome 16S ribosomal RNA sequencing opportunities, it is now possible to survey the full microbial flora of wounds (Martin and Nunan, 2015). Microbial ecosystem function and stability are influenced by species and functional group richness (Bell *et al.*, 2005; Witebolle *et al.* 2009). In addition, almost certainly some of the pathogens, and even excessive numbers of some otherwise commensal species, might be key in modulating the efficiency of healing, either directly by their actions on keratinocytes or wound fibroblasts, or indirectly by modulating the inflammatory response (Martin and Nunan, 2015). In this context, the analysis of α-diversity and the number of taxonomic groups showed that ulceration slightly reduced the richness of the skin microbiota of gilthead seabream. In the present study, the skin microbiota of gilthead seabream was dominated by *Proteobacteria phylum* followed by *Bacteroidetes*, in contrast with the results obtained by Chiarello *et al.* (2015), who reported that *Actinobacteria* and *Firmicutes* were the most abundant *phyla* in the skin microbiota of gilthead seabream. These differences could be due to the origin of the water of tanks used, because in both studies fish were kept in tanks with a water filtration system in a closed circulating water system without sterilization, but the origin of the water was different because they used subsurface water of the Thay lagoon whereas in our study artificial seawater was employed. However, *Proteobacteria* and *Bacteroidetes* have also been reported as the predominant *phyla* in the skin microbiota of different fish (Larsen *et al.*, 2013; Boutin *et al.*, 2014; Leonard *et al.*, 2014; Lokesh and Kiron, 2016). In our study, *γ-Proteobacteria* was the predominant (more than 75%) class detected. This class has also been reported as the dominant group in the intestinal microbiota of marine farmed fish including gilthead seabream (Estruch *et al.*, 2015) and Senegalese sole *(Solea senegalensis)* (Tapia-Paniagua *et al.*, 2014). *Firmicutes* has been described as a predominant *phylum* in the skin microbiota of gilthead seabream (Chiarello *et al.*, 2015), and a proteomic analysis of the skin mucus of this fish species reported *Lactobacillales*, order included in *Firmicutes*, as the predominant bacterial group in the skin mucus (Jurado *et al.*,

2015). However, these researchers did not carry out the characterization of the skin microbiota. In the present study, *Firmicutes* represented only >1% of total reads in ulcerated skin, but important differences between non-ulcerated and ulcerated skin have been observed. *Staphylococcaceae* and *Lactobacillacea*e families and *Staphylococcus* and *Lactobacillus* genera, well-known probiotics developed for human use and currently extended to other animal species (Salinas *et al.*, 2005; Lazado *et al.*, 2010), were the predominant *Firmicutes* in non-ulcerated skin, whereas *Streptococcaceae* family and *Streptococcus* genera were in ulcerated skin. In agreement with these results, *Streptococcus* has been reported as a predominant group in skin lesions in humans (Chularojanamontri *et al.*, 2016; Tanaka *et al.*, 2016). On the other hand, it has been demonstrated that some *Lactobacillus* strains protected epidermal keratinocytes from the action of pathogens by competitive exclusion (Prince *et al.*, 2015). It would be suggestive to think that the reduction of the level of *Lactobacillu*s in the ulcerated skin of gilthead seabream could be associated with a lower protective effect against the increase of *Streptococcus* strains observed in the ulcerated skin. In this context Hoseinifar *et al.* (2015) demonstrated that the dietary administration of a probiotic (*L. acidophilus)* significantly increased mucosal immune parameters in *Xiphophorus helleri*. It would be reasonable to think that the levels of *Lactobacillus* in the skin could exert a similar effect on the skin mucosal immunity. In this study, *Alteromonadales, Rhodobacterales* and *Vibrionales* families showed higher number of reads in non-ulcerated skin samples than in ulcerated skin. In the first case, the analysis at genus taxonomic level showed increases of reads related to *Shewanella* associated with an important decrease of sequences related to *Alteromonas* genus in ulcerated skin samples, in comparison with non-ulcerated skin. *Alteromonas* genus has not been described as pathogenic for aquatic organisms and on the contrary, there are some studies demonstrating the properties as probiotics in some strains (Kesarcodi-Watson *et al.*, 2010) and even describing their use in the biocontrol of several aquatic pathogens (Isnansetyo, *et al.*, 2009; Jin *et al.*, 2010). It could be suggestive to think that the reduction of the sequences related to *Alteromonas* genus in ulcerated skin could facilitate the raise of genera such as *Shewanella,* which have a pathogenic potential (Chen *et al.*, 2016; PaŽor *et al.*, 2016), suggesting a role for *Shewanella* in the production of the ulcer. In addition, the antagonistic activity against fish pathogens such as *P. damselae* subsp *piscicida* has been demonstrated for strains of *Shewanella* genus isolated from the skin of healthy specimens of gilthead seabream (Chabrillon *et al.*, 2005; Tapia-Paniagua *et al.*, 2014). The high reduction of the level of

reads related to *Photobacterium* genus detected in ulcerated skin could be related to the increase of sequences corresponding to *Shewanella* genus. This reduction of the abundance of *Photobacterium* was associated with the increase of reads related to *Vibrio* genus detected in ulcerated skin and interestingly, different *Vibrio* species have been involved in the production of skin ulcers in fish (Oliver, 2005; Zorrila *et a*l., 2005). More than 95% of all reads of *Rhodobacterales* detected in non-ulcerated skin corresponded to sequences related to *Rhodobacteraceae* family. This family is metabolically and ecologically diverse and it includes aquatic bacteria frequently thrive in marine environments. In this study, the highest number of reads related to genera included in this family corresponded to *Roseobacter* clade. The relative abundance of R. clade sequences is quite different depending on the aquatic environments, representing values higher than 30% of the *α-Proteobacteria* (Ghai *et al.*, 2011; 2012). However, in our study, the abundance of this clade was lower, 18.73% and 5.07% of all α-*Proteobacteria* reads in non-ulcerated and ulcerated skin, respectively. In all cases, the most abundant genus of this family was *Thalassobius* that it has been associated with epizootic shell diseased lesions in lobster. However, this pathology was caused by *Bacteroidetes* members such as *Aquamarina* (Quinn *et al.*, 2012), and reads related to this last genus have not been detected in the present study. Slight increases in reads corresponding to *Cellulophaga, Flavobacterium, Chryseobacterium* and *Tenacibaculum* genera were detected in ulcerated skin samples of gilthead seabream. Species of *Cellulophaga* genus have shown ability to produce α-N-acetyl-galactosaminidase (Bakunina *et al.*, 2014) and it has been reported the presence of N-acetyl-galactosamine in the terminal glycosylation patterns of gilthead seabream (Cerezuela *et al.*, 2016). It is suggestive to think that the ulceration process could produce variations in the levels of N-acetyl-galactosamine and that the increase of reads related to *Cellulophaga* genus was related to its ability to use this sugar. However, more research is necessary to confirm this hypothesis. On the other hand, *Chryseobacterium spp.* was recovered from the mucus of apparently healthy fish (Bernardet and Bowman, 2006). *Chryseobacterium spp* has been reported associated to disease outbreak in farmed turbot (*Scophthalmus maximus*) (Mudarri *et al.*, 1994) and Bernardet and Bowman (2006) recovered strains identified as *C. joostei* from external lesions of farmed Atlantic salmon (*Salmo salar*). Other species such as *C. chaponens* were recovered from the fins, gills, and external lesions of diseased Atlantic salmon, mixed with *Flavobacterium psychrophilum* (Kampfer *et al.*, 2011), and *C. aahli* was isolated from the necrotic fins of brown trout (*Salmo trutta)* (Loch and Faisal, 2014). Finally, *Flavobacterium*

and *Tenacibaculum* are genera known to include species reported as causing skin lesions in farmed fish (Avendaño *et al.*, 2004; Roberts, 2012; Rahman *et al.*, 2015).

Previous changes reported in this study for immune parameters were associated in the case of ulcerated skin with increases of the level of genera including species involved in skin lesions and reductions of the levels of microorganisms considered beneficial such as those included in *Lactobacillus* genus. The results obtained in this study could be applied to design future prophylactic strategies such as use of probiotics to improve the skin mucosal system (Cordero *et al.*, 2016b) and/or to exercise antagonistic effect on pathogenic microorganisms causing skin diseases. However, more research is necessary to unravel the implications of the interactions of skin mucosal immunity on fish welfare and the skin-associated microbiota.

There is a need to develop simple, rapid, and accurate methods for assessing health in fish populations (Noga and Udomkusonsri, 2002). Since epidermal integrity is vital for animal defense, an early detection of any lesion on the skin is vital for maintaining animal health. Nevertheless, in daily practice these lesions are difficult to detect with the naked eye (Law, 2001; Noga and Udomkusonsri, 2002). Thus, we applied a protocol studied by Noga and Udomkusonsri (2002) in which they detected skin ulcers by means of a fluorescein test. Fluorescein is a yellow, relatively nontoxic, vital, hydroxyxanthene dye that produces an intense fluorescence in slightly acid to alkaline (pH>5) solutions (Davis *et al.*, 2008). It exhibits a high degree of ionization at physiological pH and thus neither penetrates intact epithelium nor forms a firm bond with (i.e., stain) vital tissue (Noga and Udomkusonsri; 2002). It is widely used in ophthalmology (to detect corneal ulceration, corneal abrasions and other ocular pathologies) and in angiographic techniques to evaluate blood perfusion of various organs, applied to both human and animal medicine. Fluorescence can be detected in low concentrations if excited with blue or ultraviolet light (Suzuki *et al.*, 2007; Baraboglia, 2009; Rodrígues *et al.*, 2009). In our results, after exposing rainbow trout (*O. mykiss*) to the fluorescein solution and visualizing them under ultraviolet light, fluorescent areas were detected, mostly coinciding with the experimental lesions. In addition, small fluorescent areas were observed in control (not damaged) fish, which could be interpreted as lesions prior to the experiment or provoked by manipulation. Same findings were previously described in rainbow trout (*O. mykiss*), channel catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*) and hybrid striped bass (*Morone saxatilis* male × *Morone chrysops*

female) experimentally ulcerated and exposed to fluorescein solution (0.1 mg mL^{-1}; 3 minutes), where a rapid and easy detection of skin ulcers that were not visible to the naked eye was described, but also in skin areas without presence of experimental ulcers (Noga and Udomkusonsri, 2002). In this sense, another study using rainbow trout, provided evidence that clinically normal fish may have a significant amount of non-apparent skin damage even under presumably non-stressful, routine culture conditions (Kiryu and Wakabayashi, 1999). In the same context, Davis *et al.* (2008) reported that the anesthetic method, specifically non-buffered tricaine, can alter the integrity of fish skin, causing epidermal and corneal damage, which could give false positives in the fluorescein test, interfering with the correct interpretation of the results. The effects of exposure to buffered versus non-buffered tricaine on epidermal and corneal integrity were studied in *O. niloticus* and *Ictalurus punctatus* subjected to the fluorescein test and histological examination. Thus, our findings suggest that fluorescein may be a useful tool for rapid and economic detection of skin ulcers, initial or small lesions, and even partial loss of epithelium (erosion). However, the anesthesia protocol and adequate fish manipulation prior to the test must to be taken into account to avoid misleading interpretations.

Furthermore, the idea of using the fluorescein test to aid in bacterial diagnosis based on the importance of detecting early skin damage in bacterial infections was also published (Noga and Udomkusonsri, 2002). In this sense, a previous study found that *A. salmonicida* which causes furunculosis in salmonids and other fish species, was the primary bacterial pathogen found in ulcers of goldfish (*Carassius auratus)*, present only in the earliest stages of the disease (*i.e.*, small lesions) (Elliott and Shotts, 1980). Thus, the fluorescein test could make possible to identify the earliest lesions, which may not even be visible to the naked eye, to improve the ability to identify important pathogens. In this same context, our results showed that in zebrafish bathed with *V. harveyi* (2.6×10^7 bacteria mL^{-1}) and previously rubbed with a cotton swab on the left flank, fluorescence areas were detected in the rubbed flank after 0, 24 and 48 h of bacterial bath, compared to fish not exposed to experimental damage or bacterial bath. The detected fluorescence was located mainly in the pectoral fin, and we hypothesize that bacteria principally affect the skin of fins due its thickness and subsequent bacterial colonization produces skin damage. Similarly, an *in vivo* bioluminescent study of *Cyprinus carpio* reported that the sites of primary infection are mainly located at the periphery of the fins, when fish were exposed to four physical

treatments in a defined area (rubbing with soft tissue paper, rubbing with a cotton swab, brushing with a rotary electric tooth brush for 2 s or rubbing with sandpaper) to remove skin mucus up to complete erosion of the epidermis and afterwards immersed in water containing Cyprinid herpesvirus 3 (CyHV-3) (Raj *et al.*, 2011). Previously, another study demonstrated, using a CyHV-3 recombinant strain expressing luciferase (LUC) and *in vivo* bioluminescence imaging, that the major portal of entry for CyHV-3 exposed by inmersion is the skin covering the fins (Costes *et al.*, 2009). In another study, striped bass hybrids were exposed to acute stress confinement (2 h) and authors reported skin ulceration on the fins but not on the body (Noga *et al.*, 2011). In the above mentioned study, the presence of ulcers in *O. niloticus,* in areas that were not previously experimentally ulcerated, especially the tips of the fins were reported (Davis *et al.*, 2008). Thus, skin damage, though small, must be detected early to ensure fish protection and welfare.

Chapter IV

As mentioned above, the success of modern aquaculture is based on the control of parameters in which good knowledge of the biology of the farmed fish and technology innovation are closely related (Esteban, 2012). Thus, in the Part 2 of this Thesis, we studied the skin of gilthead seabream *(S. aurata)* and European sea bass (*D. labrax*) through the application of innovative *in vivo* imaging techniques. In the research area, advanced and innovative techniques are implemented to obtain information in a short period of time while sacrificing as few animals as possible. Currently, advanced image analysis techniques such as ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) are used as non-invasive methods to study animal anatomy (Wyatt *et al.*, 2015), but have been scarcely used in studies related to fish of commercial interest (Romvári *et al.*, 2002; Silva *et al.*, 2016) and mostly used in zebrafish (Seo *et al.*, 2015; Babaei *et al.*, 2016) because of their use as animal model. In this study we used real-time ultrasound (ultrasonography*)* and X-ray micro computed tomography (Micro-CT) to study fish.

Skin thickness, structure and the type of cells present in it, can be influenced by several factors (Bullock and Roberts 1974; Stoskopf, 1993; Groff, 2001; Fontenot and Neiffer, 2004). Thus, we focused on skin differences related to body location and the fish species. In this sense, morphometric analysis of images taken from light microscopy, scanning electron microscopy, and transmission electron microscopy, among other methods, has been used to

determine the thickness of various skin layers. However, some histological methods are questioned because of possible artifacts caused by tissue preparation (Waller and Maibach, 2005). So, advanced techniques are necessary to solve the limitations of traditional techniques. In this sense, ultrasonography and CT are useful tecniques. Ultrasound has been used in aquaculture to determine sex and gonad development in several fish species, including European sea bass (Macri *et al.*, 2013). In human dermatology, ultrasonography is the most suitable technique for determining skin thickness, since it enables exact determination without any restriction concerning site and without disadvantages for the subject (Dykes and Marks, 1977). This technique also allows us to evaluate the size and extension of skin lesions, the layers involved and the presence of vascularization (Whittle and Baldassare, 2004). The main limitations of this study are those typically associated with all ultrasound examinations such as error of the operator and the efficiency of the machine (Macri *et al.*, 2013). Two forms of ultrasonography, A and B modes, are available. B-mode ultrasonography requires more time and attention than A mode, but is more reproducible because it enables cross-sectional imaging, allowing then a more reliable determination of skin thickness, even where the dermis–hypodermis interface is unclear (Seidenari *et al.*, 1994; Waller *et al.*, 2005). A limitation in the dermatology field has been the probe emission frequency, until the appearance of the high frequency probes (>10 MHz) (Alfageme *et al.*, 2011).

In this study high frequency ultrasound equipment adapted for small animals was used and great images of the skin in gilthead seabream and European sea bass were acquired allowing its measurement. In humans, literature describes that in the ultrasonographic image the skin (Sk) corresponds to the interface with the gel that is expressed as a hyperechoic (bright) band. In addition, a hyperechoic band bounded below by a hypoechoic area of a linear structure corresponds to the muscle fascia (Msc) (Whittle and Baldassare, 2004; Alfageme *et al.*, 2011). In the image analysis we clearly identified the skin in gilthead seabream and European sea bass and could recognize other layers, such as the muscle (Fig. 38 A). In addition, variations in the skin thickness depending on the body region were detected, showing that the thickness in the ventral region is greater than in the dorsal region. In fish, previous studies by traditional techniques confirmed variations in skin thickness depending on the body region. These differences were previously described by our group, where it was reported that the epidermis thickness of the skin in the ventral region was higher

than that of the dorsal region (Cordero *et al.*, 2017). This study served as the basis to characterize fish skin by *in vivo* and innovative techniques. Futhermore, in gilthead seabream and European sea bass, when measuring the skin, the greatest thickness was found in the region of the dorsal fin compared to the head or caudal peduncle skin, but with no significant differences. In this sense, in human dermatology, it has been reported that comparing measures of thickness in skin layer is challenging because of significant variations between individuals and body sites (Waller *et al.*, 2005). In addition, in a study of the skin of the freshwater fish *capitán* (*Eremophilus mutisii)* in six body regions (jaws, dorsal head, dorsal trunk, caudal trunk, medial and abdominal trunk) variations were reported among regions, the skin on the jaw being significantly thinner than that registered in the abdominal, dorsal and dorsal head regions (Bonilla *et al.*, 2008). These previous finding support our detected differences and confirm that the skin thickness varies not only by the body region but also among fish species. It would be interesting to study possible differences in the skin due to other factors, such as age, habitat, nutrition or temperature.

We also studied the fish by micro-CT. Micro-CT imaging has reached numerous areas of science to facilitate non-destructive, rapid, 3D quantification of morphology and density, both of which are important parameters related to tissue and organ-level homeostasis and for systematically assessing the response to genetic and/or environmental perturbations (Khoury *et al.*, 2015). Micro-CT allows the distribution of different tissues along the body to be visualised and quantified according to its density and thus determines the volume fractions separately. In fish, the distribution of tissue densities along the body is directly related to the centre of buoyancy; for example, heavy tissues such as bone may be concentrated in the head region, whereas special low-density organs such as the swim bladder are located further back. Moreover, the relative amount of tissues of different densities depends on the feeding status of the fish (Varpe *et al.* 2005). Hence, in this work we obtained for the first time, whole fish density ranges (-1,000 to +2,500 HU) and most importantly, we determined density ranges (HU) for several anatomical structures separately. The importance of our results is related to the fact that gilthead seabream and European sea bass are widely appreciated and consumed in Mediterranean countries and the production of both fish species is increasing (Testi *et al.*, 2006).

In terms of consumption, skeletal muscle, which corresponds to the edible part, is the largest organ in fish. In gilthead seabream of commercial size the skeletal muscle represents

34.3-48% of the total body weight (Grigorakis and Alexis, 2005; Testi *et al.*, 2006) while in European sea bass it ranges from 44.2% to 57.5% (Nicolosi Asmundo *et al.*, 1993; Boujard *et al.*, 2004). These finding corroborate our results where the distribution of density ranges corresponding to soft tissue (+100 to +200), including skeletal muscle, is less widely distributed in gilthead seabream than in European sea bass. In terms of consumption, muscle composition is a major quality aspect in fresh fish. On the other hand, the concentration of fat in and around the peritoneal cavity (perivisceral and peritoneal fat, respectively) is another important quality aspect, because the perivisceral fat negatively affects the consumer impressions about the fish, playing an important visual role (Grigorakis, 2007). In this sense, our results showed that fat segmentation in gilthead seabream is widely distributed in relation with total volume and the proportion is greater than in European sea bass. These findings suggest that the nutritional and commercial value of European sea bass is higher than that of gilthead seabream.

Another novelty in this work was to study the skin *in situ* in gilthead seabream and European sea bass. There was a wide distribution of density ranges corresponding to the skin (-400 to -50) compared with the total volume in both fish species, but the volume occupied was greatest in European sea bass. As we mentioned above, density values can vary between species (Romvári *et al.*, 1998; Sasser *et al.*, 2012) thus, although the density ranges found in gilthead seabream were extrapolable to European sea bass, a profound analysis is needed to confirm that these ranges cover all the densities of the European sea bass body, because the skin density in European sea bass could be different than the ranges found for gilthead seabream. In addition, as we previously observed in the ultrasonography study, the micro-CT images showed variations in the segmentation that coincides topographically with the skin, where the segmentation was thinner in the head and thicker from the dorsal fin in both fish species. In contrast to the ultrasonography, the skin segmentation seems to be thinner in ventral region compared to dorsal region in both fish species. A combination of both techniques, ultrasonography and micro-CT, allows different parameters to be analysed but always *in situ* and rapidly, once a protocol has been established (Whittle and Baldassare, 2004).

The hypodermis or subcutaneous layer specializes in the storage of lipids, whose main cells are the adipocytes (Le Guellec *et al.*, 2004). Our results showed that density values for

fish skin (-400 to -50 HU) and fat (-115 to +50 HU) overlapped, thus in the density range stablished for the skin we also have to include part of the subcutaneous fat. Therefore, we analyse the fat content separately by micro-CT. In humans, the use of MRI and CT to assess body composition and determine the fat content is well established (Chiba *et al.*, 2007; Maurovich-Horvat *et al.*, 2007). In rats and mice, micro-CT has been widely used to measure adipose tissue, allowing accurate volumetric quantification with high spatial discrimination (Luu *et al.*, 2009; Sasser *et al.*, 2012). A previous CT study looked at the fillet composition of four freshwater fish species common carp (*C. carpio*), grass carp (*Ctenopharyngodon idella Val.*), silver carp (*Hypophthalmichtis molitrix Val.)* and zander (*Stizostedion lucioperca L.*) (Romvári *et al.*, 2002), but in our study the whole fish was analysed. The fraction of fat is highly variable in fish and may influence the position of the centre of gravity (Brix *et al.*, 2009). Although fat distribution in different anatomical locations in most vertebrate species has been studied the spatial distribution and measurement of fat deposits in fish has not been studied in depth (Saint-Marc *et al.*, 2000; Luu *et al.*, 2009; Sasser *et al.*, 2012). Hence, since the presence of adipose tissue in different fish compartments also plays an important role in product quality, the measurement of the fat content is a valuable information (Weil *et al.*, 2013). As the resolution of micro-CT can be selected to fall into an isometric voxel range of approximately 10 to 200 microns, it is possible to measure not only the total volume of adipose tissue within an animal, but also to identify and quantify very small volumes of fat cells residing in discrete deposits (Luu *et al.*, 2009).

In relation to fish consumption, fat depots that interfere with carcass quality can be roughly divided into those discarded and those consumed. Discarded fat depots include the visceral fat, located in the abdominal cavity around the digestive tract (representing 2–25 % of the body weight depending on fish species and the status of the fish) and the subcutaneous fat, which is located all around the body of the fish. In salmonid species it is more prominent in dorsal or ventral zones (Weil *et al.*, 2013) and, according to our results, the same applies to gilthead seabream. Dorsal subcutaneous fat is highly developed in a region situated between the head and the dorsal fin (Weil *et al.*, 2013), as we could see in the segmented area of our micro-CT images. Moreover, ventral fat is one of the components of the belly flaps localized in the abdomen of fish and represents the part of the flesh that hangs under the spines (Weil *et al.*, 2013), and our findings also showed a large segmented area in the abdominal cavity. In humans, the largest sites of adipose tissue deposition are either

subcutaneous or intra-abdominal (within the abdominal cavity) (Gesta *et al.*, 2006; Karpe and Pinnick, 2015). In addition, analysis of CT and MRI images of Atlantic herring (*Clupea harengus*), Atlantic salmon (*S. salar*) and Atlantic mackerel (*Scomber scombrus*) revealed high concentrations of subcutaneous fat and also around the whole length of the backbone, suggesting that high priority is given to maintaining and safeguarding the levels of fat that are associated with the sensory and motor neurons in the nervous system, and particularly, to ensure swimming activity (Brix *et al.*, 2009). On the other hand, consumed fat depots are located in white and red muscle, with red muscles being richer in lipids than white muscle (Weil *et al.*, 2013). Around 90% of fish skeletal muscle is composed of white muscle (Johnston, 1981). Subcutaneous and peri-visceral adipose tissues influence carcass and fillet yields, while muscle depots modulate flesh organoleptic quality (Weil *et al.*, 2013). In this sense, health benefits of fish consumption, compared to red meat and poultry, are based on its levels of polyunsaturated fatty acids (PUFA) and high levels of eicosapentaenoicacid (EPA)/docosahexaenoic acid (DHA) (Sargent, 1997; Kris-Etherton *et al.*, 2000). In addition, the protective role of fish consumption against coronary heart diseases has been widely demonstrated and has been mainly attributed to the effects of Omega-3 fatty acids and their cardioprotective action (Kris-Etherton *et al.*, 2003). Thus, recently aquaculture feed has been re-formulated to increase the ratio of the fatty acid profile (Izquierdo *et al.*, 2003, 2005; Lenas *et al.*, 2011).

Although there are good reasons to confirm that micro-CT scan precisely quantify fat volume based on voxel densities, the method described above was validated by comparing the results in the segmentation within the range established for the fat in gilthead seabream under two different conditions (fed at a rate of 1.5% body weight or starved). For the present study some fish were food-deprived for 60 days. This period of time was chosen because it could be considered a very long starving period. The reason is that during starvation, the whole body or some organ sizes and weight can change or being significantly altered (Hosoya *et al.*, 2007; Woo *et al.*, 2011). Furthermore, our interest was to provoke the mobilization of lipids, which occurs later than glycogen mobilization (Dutta *et al.*, 2005) and to check whether the segmentation used to analyse fat by micro-CT is adequated or not.

After prolonged food deprivation in sea bass (150 days) global reserve structures were mobilized accompanied by a reduction in muscle protein (Echevarria *et al.*, 1997). In

addition, fasting has been mentioned to cause alterations in muscle quality and fat deposition in gilthead seabream (Grigorakis and Alexis, 2005; Ibarz *et al.*, 2005). After long term fasting, the resulting reduction in muscle connective tissue gives an insubstantial texture to the cooked flesh (Love, 1992). Micro-CT images showed clear differences in body dimensions between fed and fish under starvation, where the fat range in fed and starved fish after 60 days pointed to a lower segmentation of density values corresponding with the fat in the cranial, subcutaneous, muscular and abdominal regions of the starved fish, when compared to fed fish The accuracy of prediction of fat distribution in our study is in accordance with previous studies that reported the influence of starvation in the mobilization of fat tissues in different compartments of fish (Navarro and Gutierrez, 1995; Hosoya *et al.*, 2007; Woo *et al.*, 2011). Food deprivation is also a common practice in fish farming in order to regulate fish stock before marketing or before slaughtering to improve preservation (Bugeon, 2001). Different studies with long periods of food deprivation have been published to evaluate the influence of this condition on different physiological aspects (Yang and Somero, 1996; Einen *et al.* 1998; 1996; Rios *et al.*, 2002). Our results showed that biometric parameters such as body weight and size differ after 60 days of food deprivation. In this sense, a significant decrease of both weight and size was recorded in starved fish compared with fed fish after 60 days. Food deprivation clearly affects fish metabolism, and is reflected in different responses mainly related to the growth rates observed and the functionality of the digestive system (Jobling, 2001). As regards to fat volume, our results pointed to a significant reduction (50%) in starved fish compared to fed fish after 60 days. It is known that quantitative and qualitative modifications of nutriments modulate the global growth of different fish species as well as overall adiposity (Weil *et al.*, 2013).

According to our results, fat represents around 44 and 49% of total volume in fed and starved gilthead seabream, respectively. Previous studies in farmed gilthead seabream in starvation conditions reported an increase in the storage of visceral fat, indicating that a significant proportion of the fed lipids was used to produce fat tissue and lipid reserves rather than being metabolised to support growth and energy demand (Carpene *et al.*, 1998; Mnari *et al.*, 2007), which may explain the high percentage of fat related to total body in starved fish. In a previous study, when comparing wild gilthead seabream with farmed gilthead seabream there was nutritional superiority in the wild one, although with innovative techniques such differences could be solved by evaluating *in situ* and *in vivo* the

improvements in fish feeding and management. Additionally, it was observed that there were no significant differences between using fresh or frozen fish to evaluate fat content, which makes the micro-CT a good tool for analysing the flesh, even when it has been processed for consumption. It is important to highlight that the values in the Hounsfield scale can vary depending on the species, so it is necessary to carry out studies in other fish species.

The application of image analys techniques in aquaculture provides a robust and reliable non-destructive method that can be used to determine and quantify body composition in fish. Ultrasonography allows the fish study *in vivo* and micro-CT offers the possibility to analyse the whole fish body with the intention of deepening their study to analyze live animals. Thus, this work establishes the basis for a depth study in aquaculture fish. In addition, the proposed methodology has great precision and could contribute to the reduction of the number of sacrificed fish. Furthermore, no dissection or additional tissues processing is necessary saving time and reducing costs.


CONCLUSIONS

1. The light–dark cycle has a profound effect on the immunity of the skin mucus of gilthead seabream and European sea bass, with noticeable inter-species differences. A significant daily rhythm was found for IgM and lysozyme in gilthead seabream and for peroxidase, lysozyme and bactericidal activity in European sea bass.

2. The dietary administration of 1.5% dried guava leaves improved the immune status of hybrid tilapia skin mucus with increased protease, antiprotease and peroxidase activities.

3. Intraperitoneal injection of *V. harveyi* in hybrid tilapia provoked bacterial colonization and proliferation mainly in the skin but also in spleen and liver. However, when fish were fed the guava supplemented diets this bacterial colonization in the skin was dramatically reduced. The complex interactions of the environment, host and pathogens in farmed fish involve numerous points of manipulation for research and production improvements.

4. Changes in IgM levels, protease, peroxidase and bactericidal activities, depending on both the place of the experimental wound in the skin (above or below the lateral line) and the time post-wounding (from 0 to 7 days post-wound), were detected in gilthead seabream serum. The highest activities recorded in serum coincided with the inflammatory healing phase, indicating that these substances may be released in order to avoid possible contamination of the wounds.

5. Skin ulcers provide microenvironments that perturb both the mucus composition and microbial diversity of external surfaces in fish. Significant decreases in the terminal abundance of α-D-mannose, α-D-glucose and N-acetyl-galactosamine, and IgM levels, protease and antiprotease activities were detected in skin mucus. These increments were related to an increase in the level of bacterial genera involved in skin lesions, such as *Vibrio,* and a reduction in the levels of microorganisms considered beneficial, such as *Lactobacillus*.

6. The fluorescein test is an easy method to detect initial stages of skin lesions caused by any physical, chemical or biological agent even in small fish specimens.

7. The imaging techniques of ultrasonography and micro-CT enabled us to study the whole fish body obtaining qualitative and quantitative data *in situ* and in a short period of time.

8. The skin thickness in gilthead seabream and European sea bass varied depending not only on the body region but also on the species. The thickness in ventral region was greater than in the dorsal region.

9. Using micro-CT, the density ranges for the whole fish and for several anatomical structures were stablished and determined in fish.

10. The fat density range was determined by micro-CT what allows us to identify and quantify its abundance and distribution in whole fish. The percentage of fat is lower in European sea bass than in gilthead seabream.

11. The analysis of the micro-CT images allows us a quantitative and qualitative evaluation of the fish body. But, it is necessary to determine the density range for each species studied in order to compare distribution and volumes.

RESUMEN EN CASTELLANO

1. INTRODUCCIÓN

La acuicultura es la cría de organismos acuáticos, incluidos peces, moluscos, crustáceos y plantas acuáticas, tanto en la costa como en el interior, que implica intervenciones en el proceso de cría para mejorar la producción (FAO, 1988). Esta actividad es similar a lo que en la parte continental son la ganadería y la agricultura (APROMAR, 2017). La pesca y la acuicultura son fuentes importantes de alimentos, nutrición, ingresos económicos y medios de vida para cientos de millones de personas en todo el mundo. En 2016, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) informó de una producción mundial de pescado de 171 millones de toneladas, de la cual el 88 por ciento se destinó al consumo humano directo (FAO, 2018). El rápido crecimiento de la acuicultura ha llevado a sistemas intensivos de producción para cubrir las demandas del mercado. Sin embargo, existen algunos desafíos importantes para desarrollar una acuicultura productiva, factible y sostenible. Uno de estos desafíos es que en las instalaciones de producción a gran escala los animales acuáticos están expuestos a condiciones estresantes, problemas relacionados con enfermedades y deterioro de las condiciones ambientales, que a menudo resultan en pérdidas económicas (Balcázar y col., 2006). Entre las consecuencias negativas se encuentran las lesiones cutáneas causadas por el contacto entre peces, las condiciones ambientales, defectos de nutrición o patógenos; una situación que también favorece la entrada de muchos patógenos que conducen a pérdidas en la producción de pescado. Todos estos problemas en conjunto hacen necesario profundizar en el conocimiento de la piel de los peces y su mucosa, para implementar estrategias de prevención y tratamiento de enfermedades comunes en acuicultura.

En peces teleósteos, la piel es única e histológicamente diversa (Fast y col., 2002) con una organización bien conservada. En general, la piel de los peces tiene diferentes capas: la cutícula o capa de moco, la epidermis (epitelio escamoso estratificado con células

caliciformes), la dermis (estructura que da firmeza a la piel y se compone de dos capas de tejido conectivo) y la hipodermis o subcutánea (la capa más interna) (Hawkes, 1974). La piel cumple diversas funciones que permiten a los peces sobrevivir en el medio acuático: mantiene la forma del cuerpo, mejora la hidrodinámica, funciona como barrera osmótica y es rica en glándulas mucosas que producen sustancias antifúngicas y antibacterianas (Subramanian y col. 2008a). Por lo tanto, de su integridad va a depender que el animal tenga una defensa contra agentes externos, ya que muchos patógenos oportunistas que se encuentran en el medio ambiente pueden colonizar rápidamente la piel, creando así una herida en ella o agravando una herida ya existente. Estos agentes pueden estar presentes de manera natural en la piel sana, aunque en menor número. Esta estrecha relación entre el daño de la piel y la colonización microbiana es la que, a menudo, dificulta la identificación de la causa inicial del daño en la piel (Noga, 2000). Es por esto que la industria de la acuicultura moderna exige prácticas de prevención alternativas que puedan ayudar a detectar lesiones tempranas a fin de actuar de forma rápida ante la aparición de un patógeno (Subramanian y col. 2008a), además de evitar el uso indiscriminado de agentes antimicrobianos (especialmente los antibióticos), utilizando productos alternativos con el objetivo de reducir la resistencia microbiana. En este sentido, el estudio de productos naturales como sustituto de los fármacos antimicrobianos ha aumentado considerablemente en los últimos años (Van Hai, 2015). Por otro lado, la aplicación de técnicas novedosas y no invasivas ha desempeñado un papel importante no solo en el campo de la investigación, sino también en el desarrollo de la acuicultura moderna (Romvári, 2002; Nelson, 2006; Babei, 2012). Estas técnicas permiten realizar estudios *in vivo* en un tiempo rápido y reducir al mínimo el uso de animales de experimentación (Lauridsen y col., 2011).

2. OBJETIVOS

Esta Tesis Doctoral tiene como finalidad mejorar nuestro conocimiento sobre la piel de los peces debido a la importancia de este órgano, las numerosas funciones que realiza y su relevancia en la inmunidad como parte de los órganos asociados al tejido linfoide.

Los objetivos específicos son:

1. Determinar si existen variaciones en la actividad inmunitaria del moco de la piel como consecuencia del ciclo circadiano.

2. Comprobar si es posible fortalecer la inmunidad de la mucosa de la piel y reducir su colonización por patógenos mediante la manipulación de la dieta.

3. Caracterizar los procesos de curación, regeneración y ulceración de la piel en los peces, así como su implicación en la respuesta inmunitaria de la mucosa y la microbiota asociada.

4. Probar una técnica para visualizar heridas cutáneas en etapas iniciales.

5. Aplicar técnicas de imagen para la caracterización de los peces.

3. PRINCIPALES RESULTADOS Y DISCUSIÓN

Esta Tesis Doctoral se centra en el estudio de la piel de los peces en especies con interés acuícola. La acuicultura ha crecido rápidamente en los ultimos años, pero los métodos intensivos de producción causan diferentes tipos de lesiones, abrasiones o úlceras en la piel de los peces, que pueden aparecer no solo por el contacto físico sino también como resultado de malas prácticas durante el manejo, nutrición inadecuada o por procesos infecciosos de diversos patógenos (Law, 2001). Teniendo en cuenta estos antecedentes, hemos dividido esta Tesis Doctoral en dos partes donde buscamos profundizar en el estudio de la piel de los peces de interés acuícola: en la Parte 1 (Capítulos I, II y III) evaluamos aspectos relacionados con la regulación de la piel y su moco por factores externos y la dieta, así como su curación, regeneración de heridas y la respuesta immune en algunas especies de acuicultura y experimentación (dorada, lubina, tilapia híbrida, trucha arcoiris y pez cebra), mientras que en la Parte 2 (Capítulo IV), usamos técnicas de imagen *in vivo* (ultrasonografía y tomografía computarizada) para estudiar la dorada y la lubina.

La Parte 1 de esta Tesis Doctoral contiene tres capítulos. En el Capítulo I, intentamos analizar los posibles cambios diarios en diferentes parámetros inmunitarios en el moco de la piel de la dorada *(Sparus aurata)* y la lubina (Dicentrarchus labrax), expuestas a un fotoperiodo constante de luz-oscuridad (12 h L: 12 h D). Nuestros resultados demuestran que los niveles de IgM, diferentes enzimas (actividades proteasa, antiproteasa, peroxidasa y lisozima) relacionadas con el sistema inmunitario y la actividad bactericida del moco cutáneo varían notablemente durante el ciclo diario de luz y oscuridad en la dorada y la lubina. Varios estudios han demostrado la relación entre el fotoperiodo y el sistema inmunitario (Liebmann y col., 1997; Esteban y col., 2006, 2013; Lazado y col., 2015, 2016).

Concretamente, un estudio previo realizado en nuestro grupo (también con dorada y lubina) describió un efecto del fotoperíodo en algunos parámetros inmunitarios séricos (actividades del complemento, lisozima y peroxidasa) (Esteban y col., 2006). Nuestros resultados indican que las actividades medidas siguieron diferentes patrones dependiendo del ciclo de luz-oscuridad. Nuevos estudios sobre la expresión de ciertos genes en la piel de los teleósteos podrían ayudar a entender la relación compleja entre el fotoperiodo y la inmunidad de la mucosa de la piel.

En acuicultura, la vibriosis es, presumiblemente, una de las enfermedades más importantes a la que los acuicultores deben enfrentarse. En este sentido, la especie *V. harveyi* ha adquirido gran importancia como patógeno de peces, por lo tanto, el diagnóstico de la enfermedad se ha mejorado en los últimos años (Pang y col., 2006). Sin embargo, la prevención y el tratamiento se ha llevado a cabo exclusivamente mediante mejora en el manejo y el uso de antibióticos, lo que contribuye a la aparición de resistencia a estos fármacos por parte de las bacterias, un proceso natural que se ha acelerado por el uso indebido de antibióticos en la producción de alimentos (OMS, 2018). En este contexto, se ha incrementado el estudio de las plantas como una alternativa a estos fármacos, ya que son una fuente natural, económica y de fácil acceso y su efectividad ha sido probada en el tratamiento de infecciones tanto en animales como en humanos (Vieira y col., 2001; Wang y col., 2009; Biswas y col., 2013; Van Hai, 2015; Carbone y Faggio, 2016; Dotta y col., 2018). En acuicultura, el estudio de estas plantas como compuestos naturales e inocuos tiene un gran potencial debido a su efecto inmunoestimulante (Yin y col., 2014; Adel y col., 2015, 2016; Awad y col., 2015; Van Hai, 2015; Guardiola y col., 2016; García-Beltrán y col., 2017, 2018; Hoseinifar y col., 2017; Baba y col., 2018; 2018; Mansour y col., 2018), además de su uso como microbicida (Rios y col., 1987; Vieira y col., 2001; Pandey y col., 2012; Biswas y col., 2013; Gobi y col., 2016; Dotta y col., 2018) y promotores del proceso de curación (Raina y col., 2008; Kumari y col., 2017). Por lo tanto, en el Capítulo II, manipulamos la dieta mediante la suplementación del pienso commercial con hoja de guayaba (*Psidium guajava*) a diferentes concentraciones (1.5% y 3%) para estudiar el efecto en el moco cutáneo de la tilapia híbrida (*Oreochromis niloticus* × *O. mossambicus*) y el posible efecto frente a infecciones de *Vibrio harveyi*. Se detectaron incrementos estadísticamente significativos en algunos parámetros inmunitarios (actividades proteasa, antiproteasa y peroxidasa) en el moco cutáneo de peces alimentados con pienso suplementado con hoja de

guayaba al 1,5%, en comparación con los valores obtenidos en el grupo control (no suplementado). Además, la alimentación de tilapia híbrida con hoja de guayaba redujo significativamente la carga bacteriana en la piel después de la infección con *V. harveyi*. Estos resultados demuestran que la suplementación con hoja de guayaba (1.5%) aumenta la actividad inmunitaria en el moco de la piel de la tilapia híbrida y la protege contra la colonización por *V. harveyi*. Estos resultados coinciden con estudios previos donde se demostró el efecto inmunoestimulante y microbicida de la hoja de guayaba en *O. mossambicus* (Gobi y col., 2016) y en el camarón (*Penaeus monodon*) (Yin y col., 2014). Además, Yin y col. (2014) propusieron el uso de la hoja de guayaba como un desinfectante seguro del agua para el cultivo de camarones.

Dado que la disrupción o alteración de las características anatómicas y fisiológicas de la piel a menudo conlleva el desarrollo de enfermedades cutáneas, la disminucion de la respuesta inmunitaria (Fontenot y Neiffer, 2004) y la degradación de los filetes debido a una apariencia visual poco atractiva para el consumidor, resultando en pérdidas economicas, en el Capítulo III hemos realizado lesiones cutáneas experimentales con el objetivo de estudiar la respuesta de la piel y su mucosa. Primero, estudiamos el progreso de curación de la piel después de heridas experimentales en dos ubicaciones del cuerpo (por encima y por debajo de la línea lateral) en especímenes de dorada *(S. aurata),* ya que se sabe que la morfología de la piel puede variar dependiendo de varios factores, como la ubicación en el cuerpo, la especie, el sexo, la etapa de la vida o la estación del año, entre otros factores (Bullock and Roberts, 1974; Iger and Abraham, 1990; Groff, 2001; Fontenot and Neiffer, 2004). Además, se estudió la inmunidad sérica y de la mucosa de la piel analizando algunos parámetros inmunitarios importantes (actividades proteasa, antiproteasa, peroxidasa, lisozima y actividad bactericida), así como el perfil de expresión de genes inmunorrelevantes y de regeneración celular en la zona de la piel herida. Los resultados macroscópicos sugieren que el proceso de curación es más rápido por debajo de la línea lateral (LL) que por encima de ella. Por otro lado, los parámetros inmunitarios analizados en el moco cutáneo y en el suero de dorada mostraron variaciones significativas dependiendo del sitio de la herida en la piel en comparación con los peces sin herida. Al mismo tiempo, el perfil de expresión de varios genes inmunorrelevantes (*il1b, il6*, *tgfb* e *ight)*, así como el gen implicado en la regeneración de la piel *(krt1),* mostró variaciones significativas dependiendo del lado de la herida. Las principales variaciones registradas coinciden en el tiempo con la etapa inflamatoria, etapa

que desempeña un papel central en la cicatrización de las heridas, ya que en esta fase se combaten los microorganismos invasores y se forman los nuevos constituyentes tisulares, preparando así la herida para las fases subsiguientes de curación (Raiman-Wiksman 2007; Esteban, 2012).

Uno de los signos clínicos frecuentemente asociados a la enfermedad de la piel es la aparición de procesos ulcerativos (Rizgalla y col., 2016; Karlsean y col., 2017). Estos procesos pueden ser causados por diferentes factores físicos, químicos o incluso ambientales o, más fácilmente, por la simple fricción de un pez con otro que se encuentre en la misma jaula o tanque. Estas úlceras también se han asociado en numerosas ocasiones con el aislamiento de microorganismos oportunistas. Los cambios en la microbiota asociada a la piel pueden llevar a aumentos de bacterias oportunistas y como resultado una enfermedad (Smågeet y col., 2015; Karlsean y col., 2017). Por ello, realizamos úlceras cutáneas experimentales y evaluamos los cambios en la composición del moco, inmunidad y la diversidad microbiana asociadas a la piel ulcerada. Nuestros resultados determinaron una disminución significativa de la abundancia terminal de residuos de α-D-manosa, α-D-glucosa y N-acetil-galactosamina en el moco de la piel ulcerada, en comparación con los peces control. Los niveles de IgM y todas las actividades enzimáticas evaluadas disminuyeron en el moco de los peces ulcerados (en comparación con los peces control), aunque las disminuciones observadas solo fueron estadísticamente significativas para las actividades proteasa y antiproteasa. Concomitantemente, el análisis de la microbiota cutánea mostró claras diferencias entre la piel ulcerada y la no ulcerada. En general, estos resultados demostraron que la presencia de úlceras cutáneas proporciona microambientes que perturban tanto la composición del moco como la biodiversidad microbiana de esta importante superficie externa. Además, este estudio podría aplicarse para diseñar estrategias profilácticas futuras, como el uso de probióticos para mejorar el sistema de la mucosa de la piel y/o para ejercer un efecto antagónico sobre los microorganismos patógenos que causan enfermedades de la piel.

Actualmente, existe la necesidad de desarrollar métodos simples, rápidos y precisos para evaluar el estado de salud y bienestar en las poblaciones de peces (Noga y Udomkusonsri, 2002). Dado que la integridad epidérmica es necesaria para la defensa de los animales, una detección temprana de cualquier lesión en la piel, por pequeña que sea, es vital para su

bienestar, sin embargo, en la práctica diaria estas lesiones son difíciles de detectar a simple vista (Law, 2001; Noga y Udomkusonsri, 2002). Por lo tanto, aplicamos un protocolo estudiado por Noga y Udomkusonsri (2002) para detectar úlceras cutáneas mediante la prueba de la fluoresceína, y el posible daño debido a la contaminación bacteriana en la piel sensibilizada del pez cebra. La fluoresceína es un colorante vital, relativamente no tóxico (Davis y col., 2008) que se ha utilizado ampliamente en oftalmología (Suzuki y col., 2007; Baraboglia, 2009; Rodrigues y col., 2009). Nuestros resultados mostraron que en ambos casos después del baño de fluoresceína y la exposición a la luz ultravioleta, se detectó fluorescencia en las áreas visiblemente dañadas y también se detectaron lesiones que no se percibieron a simple vista. Curiosamente, se detectó fluorescencia fuera del área experimental que podría deberse a lesiones previas indetectables o debido a la manipulación del pez. Nuestros resultados coinciden con los estudios realizados previamente (Noga y Udomkusonsri, 2002, Davis y col., 2008; Raj y col., 2011) y confirman que la prueba de fluoresceína es una herramienta aplicable, fácil y económica para detectar lesiones cutáneas por pequeñas que sean y prevenir enfermedades relacionadas con el daño de la piel en la acuicultura.

Finalmente, en el Capítulo IV nos centramos en estudiar la dorada y la lubina mediante dos técnicas de análisis de imagen *in vivo*: ultrasonografía y tomografía computarizada. Las técnicas tradicionales para estudiar el cuerpo de los animales requieren la disección de los especímenes y el uso de compuestos químicos que pueden ser peligrosos para el investigador y el medio ambiente, además de costosos (Lauridsen y col., 2011). Actualmente, las técnicas avanzadas de análisis de imagen como el ultrasonido, la tomografía computarizada (TC) o la resonancia magnética (RM) se han utilizado como métodos no invasivos para estudiar la anatomía animal (Wyatt y col., 2015), pero esas técnicas se han utilizado poco en estudios relacionados con peces de interés comercial (Romvári y col., 2002; Silva y col., 2016). Nuestros resultados demuestran que el grosor de la piel a lo largo del pez varía según la región del cuerpo. Además, se realizó una segmentación completa del cuerpo del pez mediante micro-TC, mostrando que el rango de valores de densidad en Unidades Hounsfield (UH) para el pez completo fue de -1.000 a +2.500 UH. Por otro lado, en la segmentación de la piel en el rango de -400 a -50 UH, se observó variabilidad en su espesor en ambas especies estudiadas (dorada y lubina). Los rangos de piel se superponen parcialmente con los de la segmentación de la grasa (-115 a +50 HU). Por lo tanto, se realizó un estudio separado para

identificar depósitos de grasa en dorada. El análisis de imagen mostró áreas segmentadas que coinciden topográficamente con depósitos de grasa. Además, se validó esta metodología en condiciones de alimentación e inanición y se encontraron variaciones significativas macroscópicas y cuantitativas entre ambos grupos. Según nuestros resultados cada técnica tiene sus ventajas y desventajas, pero al combinarlas podemos obtener un estudio completo y rápido, con una importante reducción en el número de animales de experimentación, costo y tiempo. Además, estos métodos nos permiten evaluar cambios físicos debidos a una dieta específica o tratamiento *in situ* sin disección del animal, permitiendo tambien analizar animales frescos o congelados sin alterar los resultados.

4. CONCLUSIONES

1. El ciclo luz-oscuridad tiene un profundo efecto sobre la inmunidad del moco de la piel de la dorada y la lubina, con notables diferencias entre especies. Se detectó un ritmo diario significativo para IgM y lisozima en la dorada, y para las actividades peroxidasa, lisozima y bactericida en la lubina.

2. La administración dietética de 1.5% de hojas de guayaba secas mejoró el estado inmunitario del moco de la piel de la tilapia híbrida con un aumento de las actividades proteasa, antiproteasa y peroxidasa.

3. La inyección intraperitoneal de *V. harveyi* en especímenes de tilapia híbrida provocó la colonización y proliferación bacteriana principalmente en la piel, pero también en el bazo y el hígado. Sin embargo, cuando los peces fueron alimentados con las dietas suplementadas con guayaba esta colonización bacteriana se redujo drásticamente en la piel. Las complejas interacciones entre el medio ambiente, el huésped y los patógenos en los peces de piscifactoría implican numerosos puntos de manipulación para la investigación y la mejora de la producción.

4. Se detectaron cambios en los niveles de IgM y las actividades, proteasa, peroxidasa y bactericida del moco y el suero de la dorada, dependiendo tanto del lugar de la herida experimental en la piel (por encima o por debajo de la línea lateral) como del tiempo posterior a la herida (de 0 a 7 días después de la herida). Los mayores niveles de actividades

registradas en el suero coincidieron con la fase inflamatoria de la cicatrización, lo que indica que estas sustancias pueden ser liberadas para evitar la posible contaminación de las heridas.

5. Las úlceras cutáneas proporcionan microambientes que perturban tanto la composición del moco como la diversidad microbiana de las superficies externas de los peces. Se observaron disminuciones significativas en la abundancia terminal de α-D-manosa, α-D-glucosa y N-acetilgalactosamina, y se detectaron niveles de IgM, proteasa y antiproteasa en el moco cutaneo. Estos incrementos se relacionaron con un aumento en el nivel de los géneros bacterianos involucradas en lesiones cutáneas como *Vibrio* y la reducción en los niveles de microorganismos considerados beneficiosos, como *Lactobacillus.*

6. La prueba de fluoresceína es un método fácil para detectar las etapas iniciales de las lesiones cutáneas causadas por cualquier agente físico, químico o biológico, incluso en especímenes de peces pequeños.

7. Las técnicas de imagen, ultrasonografía y micro-TC, nos permitieron estudiar todo el cuerpo del pez obteniendo resultados cualitativos y cuantitativos *in situ* y en un corto período de tiempo.

8. El grosor de la piel de la dorada y la lubina presento variaciones dependiendo no sólo de la región del cuerpo sino también de la especie. El grosor en la región ventral fue mayor que en la región dorsal.

9. Utilizando el microCT, se establecieron y determinaron los rangos de densidad para el pez completo y para varias estructuras anatómicas de este.

10. El rango de densidad para la grasa fue determinado por microCT, lo que nos permite identificar y cuantificar su abundancia y distribución en todo el pez. El porcentaje de grasa es menor en la lubina que en la dorada.

11. El análisis de las imágenes tomográficas nos permite una evaluación cuantitativa y cualitativa del cuerpo del pez. Sin embargo, es necesario determinar el rango de densidad para cada especie estudiada para poder comparar su distribución y volúmenes.

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