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Shewanella putrefaciens Pdp11 and arginine as feed additives for gilthead seabream (*Sparus aurata* L.): Effect on skin regeneration and intestinal function in wounded fish

Shewanella putrefaciens Pdp11 y arginina como aditivos alimentarios para dorada (*Sparus aurata* L.): Efecto sobre la regeneración de la piel y la función intestinal en peces heridos

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2022

I want to dedicate my thesis to my parents and my love.

任何成就都是经过几次、几十次、甚至成百上千次失败而取得的。

因此不要一碰到困难就苦恼、动摇起来。

——华罗庚

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INDEX

LIST OF ABBREVIATIONS I

LIST OF FIGURES 1

SUMMARY 1

1. INTRODUCTION 5

1.1. Skin mucosal barrier 5

1.1.1. Microscopic features of fish skin 6

1.1.1.1. The mucous layer 6

1.1.1.2. Epidermis 6

1.1.1.3. Dermis 7

1.1.2. The skin mucosal immunity of fish 8

1.1.2.1. Innate immunity 8

1.1.2.2. Adaptive immunity 16

1.2. Skin wound healing in fish 18

1.2.1. Inflammation in skin wound healing 18

1.2.2. Re-epithelialization 19

1.2.3. Granulation tissue formation 20

1.2.4. Remodeling 21

1.3. Intestinal mucosal barrier 21

1.3.1. The mucus layer 22

1.3.2. The physical barrier of the intestine 22

1.3.3. The intestinal immunity 23

1.3.3.1. Macrophages 23

1.3.3.2. Granulocytes 24

1.3.3.3. B lymphocytes 24
1.3.3.4. T lymphocytes 25
1.3.4. Toll-like receptors (TLRs), NF-κB signaling, and the intestinal inflammation 26
1.3.4.1. Toll-like receptors (TLRs) and NF-κB signaling 26
1.3.4.2. The skin damage and the intestinal inflammation 28

1.4. Feed Additives: probiotics and immunostimulants 28
1.4.1. Probiotics: SpPdp11 29
1.4.2. Immunostimulants: Functional amino acids - Arginine 30

2. OBJECTIVES 5

3. EXPERIMENTAL CHAPTERS 31

CHAPTER I 33

Effect of dietary administration of S*hewanella putrefaciens* Pdp11 (SpPdp11) on skin regeneration and intestinal function in experimentally wounded gilthead seabream 33

Chen Z, Ceballos-Francisco, D., Guardiola, F. A., & Esteban, M. (2020). Dietary administration of the probiotic Shewanella putrefaciens to experimentally wounded gilthead seabream (Sparus aurata L.) facilitates the skin wound healing. Scientific Reports, 10(1), 1-13.

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CHAPTER II 63

Effect of dietary administration of arginine on skin regeneration and intestinal function in experimentally wounded gilthead seabream **63**

Chen Z, Ceballos-Francisco, D., Guardiola, F. A., Huang, D., & Esteban, M. Á. (2020). Skin wound healing in gilthead seabream (Sparus aurata L.) fed diets supplemented with arginine. Fish & Shellfish Immunology, 104, 347-358.

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4. GENERAL DISCUSSION **87**

5. CONCLUSIONS **89**

6. RESUMEN EN CASTELLANO **91**

6.1. Introducción **91**

6.2. Objetivos **92**

6.3. Principales resultados **92**

6.4. Discusión **94**

6.5. Conclusiones **96**

7. REFERENCES **99**

1. LIST OF ABBREVIATIONS

ALB, albumin

ARG, arginine

AST, aspartate aminotransferase

BSA, bovine serum albumin

BT, bony tubercle

C, complement

Ca^{2+}, calcium

CAT, catalase

CD, cluster of differentiation

cDNA, complementary deoxyribonucleic acid

cfu, colony forming units

CK, creatine kinase

COLIα, collagen type I, α

COLXα, collagen type X, α

CSF1R, colony-stimulating factor 1 receptor

CTLA4, cytotoxic T-lymphocyte-associated protein 4

CTLs, cytotoxic T lymphocytes

DE, dermis

DNA, deoxyribonucleic acid

EMC, extracellular matrix

EP, epidermis

EP, external perimeter

FasL, Fas ligand

FN1α, fibronectin 1α

GLOB, globulin

GLU, glucose

H&E, hematoxylin and eosin

HBSS, hank's buffer

IFN, interferon

Ig, immunoglobulin

IGF-1, insulin-like growth factor 1

IGHT, immunoglobulin T heavy chain

IKK, inhibitor of nuclear transcription factor-κB kinase

IL, interleukin

IMUC, intestinal mucin

iNOS, inducible nitric oxide synthase

IP, internal perimeter

IκBα, inhibitor of nuclear transcription factor-κB α

JAM, junctional adhesion molecule

K^+, potassium

KRT1, keratin type 1

MALT, mucosa-associated lymphoid tissue

Me, melanophore

MMP, matrix metalloproteinase

MPO, myeloperoxidase

MUC18, mucin 18

MUC2, mucin 2

MyD88, myeloid differentiation factor 88

Na^+, sodium

NF-κB, nuclear transcription factor-κB

NLR, Nucleotide-binding and oligomerization domain (NOD)-like receptor

NO, nitric oxide

OD, optical density

PAMP, pathogen-associated molecular patterns

PBS, phosphate buffered saline

PBS-T, phosphate buffered saline supplemented with tween-20

PCNA, proliferating cell nuclear antigen

PHOS, phosphorus

PR, perimeter ratio

RBLs, rhamnose-binding lectin

RNA, ribonucleic acid

RPS18, ribosomal protein S18

RT, room temperature

RT-PCR, Real-time polymerase chain reaction

SC, stratum compactum

SEM, standard error of the mean

SHH, sonic hedgehog

SOD, Cu Zn-superoxide dismutase

SpPdp11&SP, *Shewanella putrefaciens* Pdp11

SPSS, statistical package for the social science

SS, stratum spongiosum

TCA, trichloroacetic acid

TCR, T-cell receptor

TGF, transforming growth factor

Th, T helper

tLP, thickness of the lamina propria

TLR, Toll-like receptor

TNF, tumor necrosis factor

TNFR, tumor necrosis factor receptor

TSB, tryptic soy broth

UA, uric acid

X, xanthophore

ZO, zona occludens protei

2. LIST OF FIGURES

Figure 1.	Microscopic features of a teleost fish (Oncorhynchus kisutch) skin, quoted from (Elliott, 2000). Me, melanophore; X, xanthophore.5	**5**
Figure 2.	Skin of turbot. Staining: hematoxylin-eosin. Scale bar = 50 μm. EP, epidermis; DE, dermis; SS, stratum spongiosum; SC, stratum compactum; BT, bony tubercle; MU, muscle (Unpublished).	**8**
Figure 3.	Suppositional activation and regulation of complement system in teleost fishes (Liao *et al.* 2019).	**12**
Figure 4.	The histology of the intact skin (A) and inflamed skin (B) of turbot (*Scophthalmus maximus* L.). Black arrows indicate the infiltration of admixed leucocytes in the lamina propria. Staining: hematoxylin-eosin. Scale bar = 50 μm (Unpublished).	**16**
Figure 5.	Representation of a cross-section through the epidermis at the wound edge in an adult zebrafish 5 minutes post-wounding (mpw), one hour post-wounding (hpw), quoted from (Richardson and Hammerschmidt 2018).	**21**
Figure 6.	Diagram of the intestinal mucosa in teleost fish (Martin *et al.* 2016).	**27**
Figure 7.	Representative histological appearances of normal (a) and inflammed intestine (b) (Hu *et al.* 2016).	**29**

3. SUMMARY

The present study focuses on two major organs, the skin and intestine, which are in direct contact with, persistently exposed to, the external milieu, providing a tight barrier against pathogenic infections and coexisting with a myriad of commensal organisms. The aim of the present Doctoral Thesis is to explore the effect of dietary administration of *Shewanella putrefaciens* Pdp11 (SpPdp11) and arginine (ARG) on skin regeneration and intestinal function in experimentally wounded gilthead seabream. The results could make strides in replacing antibiotics and promoting safer aquaculture. The experimental chapters have been divided into two parts.

Firstly (**Paper I&II**), the feeding trial was conducted to investigate the effects of dietary administration of SpPdp11 on skin regeneration and intestinal function in experimentally wounded gilthead seabream as follows. Two replicates (n = 12) of fish were fed a commercial diet (control, CON) and the CON diet enriched with 10^9 cfu g^{-1} SpPdp11 (SP) for 30 days. Afterward, half of the fish from each diet group were sampled while the other half were injured and continued to be fed the same diet for an extra week. **In paper I**, results by image analysis of wound areas showed that SpPdp11 inclusion facilitated wound closure. The skin wounds showed some negative changes in the metabolic parameters in the serum of gilthead seabream. Compared with the CON group, fish in SP group sampled seven days post-wounding had a significantly decreased serum AST and increased ALB/GLOB ratio. Furthermore, protease and peroxidase activities were significantly increased in skin mucus from fish in SP group sampled 7 days post-wounding compared with those from CON diet. Additionally, SP diet up-regulated the gene expression of antioxidant enzymes, anti-inflammatory cytokines, and re-epithelialization related genes in the fish skin. Furthermore, significant decreases in pro-inflammatory cytokines expression were detected in fish from SP group, respect to control ones. Furthermore, the fibronectin and collagen deposition during the granulation tissue and formation, and improvements in vascularity were observed in the SP group. **In paper II**, the intestinal histology and gene expression of different genes relevant for the intestinal barrier function were

studied. The results showed that injured fish had a disordered enterocyte nucleus disposition, a more intense infiltration of mixed leucocytes and a thicker lamina propria in the intestine compared to the control fish. However, the fish in the SP+W group did not present these pathological symptoms in the intestine. No significant variations in the number of goblet cells were detected among the different experimental groups. Pro-inflammatory cytokines (CSF1R, MPO, and IL-1β), mucins (IMUC and MUC2), and IGHT were up-regulated, while tight junction protein Occludin was down-regulated in the intestine from fish of the CON+W group. Similarly, the dietary administration of SpPdp11 markedly depressed the gene expression of pro-inflammatory cytokines, MUC2, and IGHT, but increased the gene expression of anti-inflammatory cytokine TGF-β1 and the tight junction proteins Tricellulin and Occludin after wounding.

The second part includes the effect of dietary administration of arginine on skin regeneration and intestinal function in experimentally wounded gilthead seabream as follows (**Paper III&IV**). And two replicates of fish (n = 8) were fed with either a commercial diet (control, CON) and the CON diet supplemented with 1% arginine (ARG1) or with 2% arginine (ARG2) for 30 days. Afterward, half of the fish from each diet group were sampled while the other half were injured and continued to be fed the same diet for an extra week. **In paper III**, results by image analysis showed that the wound closure rate was significantly improved in fish that were fed the ARG1 diet, compared with those in the CON group. After seven days of wound healing, the AST and CK levels in the serum and the protease and peroxidase activities in the skin mucus were down-regulated, while the IgM level in the skin mucus was up-regulated in the ARG1 group after wounding and in the CON group before wounding. Compared with the CON diet, the ARG1 diet remarkedly depressed the gene expression of MPO, IL-8, and TNF-α, and enhanced the gene expression of TGF-β1, IGF-1, PCNA, KRT2, MMP9, FN1α, and COLIα and the antioxidant enzyme CAT in the skin tissues after wounding. Furthermore, compared with both the ARG1 and the CON groups, negative effects of the ARG2 diet on wound healing were demonstrated. **In the paper IV**, the intestinal histology results showed that a more intense infiltration of mixed leucocytes

was evident in the wounded fish, which was remarkably reduced in fish that were fed the ARG1 diet. Serum IgM levels were significantly higher in the ARG1 group than levels in the CON group at 7 days post-wounding. Compared with the fish in the CON group after wounding, dietary administration of 1% arginine markedly downregulated the gene expression of TLRs (TLR2 and TLR5), MyD88, and proinflammatory cytokines (CSF1R, IL-1β, and TNF-α), but significantly enhanced the gene expression of IκBα, the anti-inflammatory cytokine TGF-β1, and tight junction proteins (Tricellulin and Occludin) in wounded fish. Furthermore, the ARG2 diet demonstrated no additional benefits on intestinal barrier function, compared to both the ARG1 and the CON diets, and it even appeared to induce negative effects.

In conclusion, SpPdp11 and arginine inclusion facilitated the skin wound healing and maintained the intestinal barrier function in the in experimentally wounded gilthead seabream.

1. INTRODUCTION

1.1. Skin mucosal barrier

The prominent mucosal surface constructed with skin, gill, intestine, and the urogenital system is the crucial interface contact and communication between fish and its external milieu (Esteban 2012, Salinas 2015). As shown in **Figure 1**, the skin of fish is composed of the outer epidermis, the inner dermis, unicellular glands (goblet cells, granule cells, serous cells, and club cells), multicellular glands (larval adhesive organs, venom glands, luminous organs) as well as scales. Especially, the fish skin, together with its outermost mucous layer, forms the mucosal barrier on the surface of fish, separating the individual from its environment (Esteban 2012).

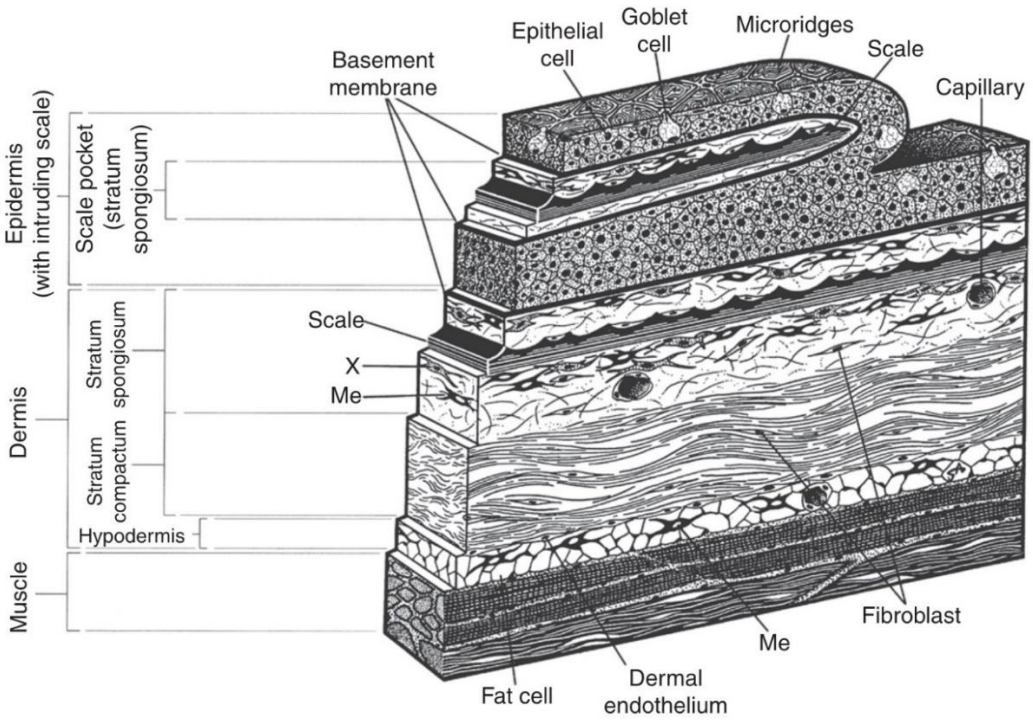

Figure 1. Microscopic features of a teleost fish (*Oncorhynchus kisutch*) skin, quoted from (Elliott 2000). Me, melanophore; X, xanthophore.

3.1.1. Microscopic features of fish skin

3.1.1.1. The mucous layer

Gland cells abundant in the fish skin can secrete mucus to cover the external surface of the fish. The mucus contains mucopolysaccharides and fibers, which swell to form mucus when exposed to water (Harris and Hunt 1975). The mucus could act as coagulants and flocculating agents to precipitate suspended matters in water, prevent the invasion of pathogenic microorganisms (Hattingh and Van Warmelo 1975), maintain the osmotic pressure (Zaccone 1981), moisturize the skin, and minimize the friction (Uribe and Sibbing 1984). The mucus on the surface of the fish body contains a large amount of mucins, which provides the lubrication and increasing the elasticity of the mucus (Wickström *et al.* 1998). Besides, The abundant antimicrobial peptides, interferons, immunoglobulin, lysozyme, complements, proteases and protease inhibitors, lectins and natural antibodies in mucus play an important role in the immune system of fish (Esteban and Cerezuela 2015). The acid and alkaline phosphatases, and superoxide dismutase are also evidenced in the skin mucus to show protective effects of fish (Liu *et al.* 1999, Huang and Ma 2010).

3.1.1.2. Epidermis

The epidermis originates from the ectoderm. The epidermis of fish is composed of several cell layers, and the number of epidermal cell layers is related to species, location, age, condition, sex, sexual maturation, and other factors (Elliott 2011). Epithelial cells are the basic structural units of the fish epidermis as occurs in other vertebrates (Rakers *et al.* 2010). Besides, there are many kinds of gland cells in the outermost layer of the epidermis, which migrate to the skin surface after being produced from the basement membrane. According to the morphological structure of cells and their staining reaction, they can be divided into many different unicellular glands, including goblet cells, granular cells, serous cells, club cells, and thread cells (Quagliata *et al.* 2006). Among them, goblet cells are the most common gland cells in the skin. The goblet cells are generally goblet-shaped, and a few are spherical or tubular, with the nucleus near the

cell base. Newborn goblet cells are in the deep layer and gradually move to the surface layer after maturation. Secretions from goblet cells contain substances such as mucins and fibers, which swell to form mucus when exposed to water (Montagna 2012). As mentioned above, many cells are produced from the basement membrane. The basement membrane is a single cell layer located at the base of the epidermis, these cells are cylindrical, neatly arranged, and uniform in size. The basement membrane is where the epidermal cell proliferation occurs, can produce new cells, and push the cells to the outer layer of the epidermis (Whitear *et al.* 1980).

3.1.1.3. Dermis

The dermis is located below the epidermis, originates from the mesoderm, and is mainly composed of two layers of fibrous connective tissue (Sire *et al.* 2009). These two layers are the *stratum spongiosum* and the *stratum compactum* from the outside to inside (Gupta 2002). In the *stratum spongiosum*, the fibrous connective tissue is loosely arranged. In addition to the fibrous connective tissue, the *stratum spongiosum* also contains fibroblasts, melanophores, and xanthophores. There are abundant blood vessels in this layer (Sayed *et al.* 2021). In the *stratum compactum*, the fibrous connective tissue is dominated by collagen fibers, which are bundled and arranged tightly. This arrangement can increase the toughness of the skin (Sire *et al.* 2009). The dermal endothelium is a single layer of cells bounds the deep face of the dermis. The hypodermis under the dermal endothelium is a layer of loose connective tissue with abundant blood vessels, which separates the dermis from the skeletal muscle (Elliott 2011). Scales are derivatives of fish skin and have protective effects as the mucous layer. Fish scales can be divided into three types: placoid, ganoid, and bony scales. The bony scales are unique to teleost. The body surface of most fish is coated with scales (White 2018). Besides *Cyclostomata*, *Torpediniformes*, and a few of the *Cottoidei*, the *Siluriformes* (*Osteichthyes*) have no scales (Xie 2010). Moreover, some *Scophthalmus*, such as turbot, had neither scales nor scale pockets. On the contrary, skin showed bony tubercles with conical shape located in the dermis, as shown in **Figure 2**. These tubercles were composed by a great amount of collagen fibres and small amounts of

calcium on the top,which may serve as breeding tubercle or contact organ (Willey 1970, Faílde *et al.* 2014).

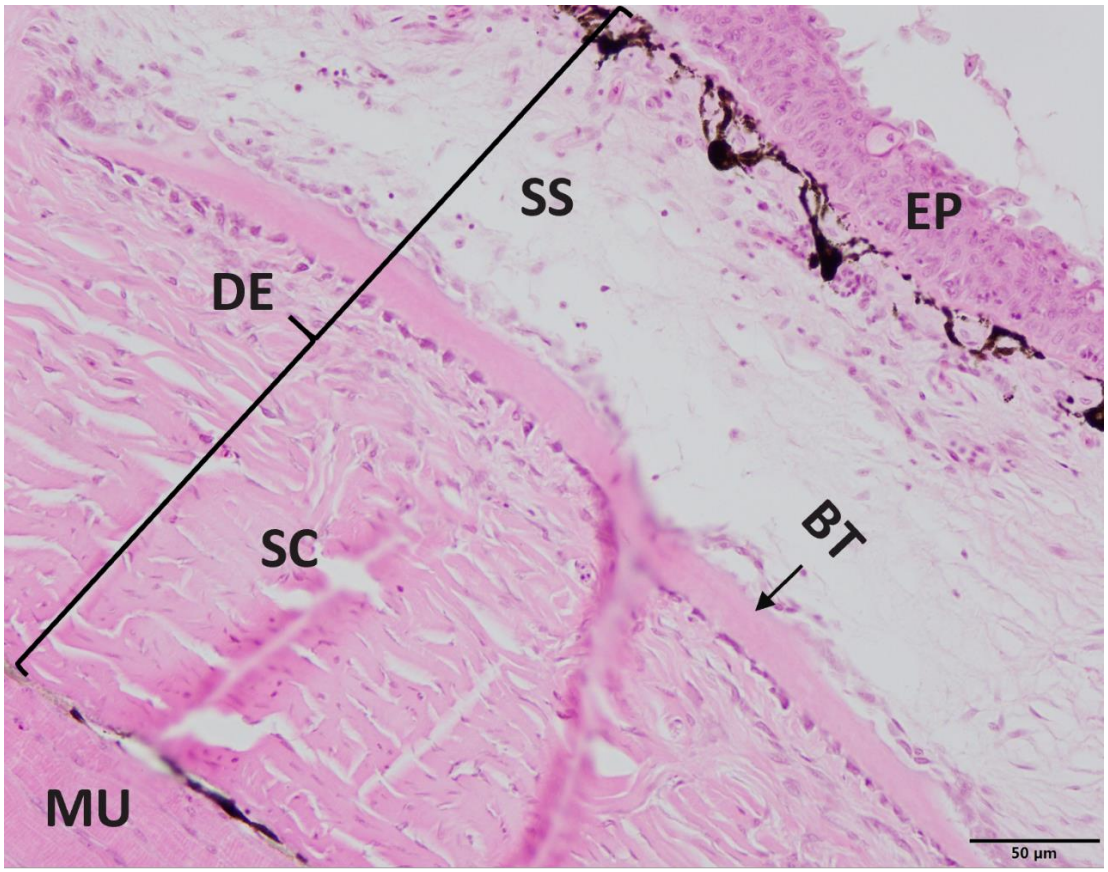

Figure 2. Skin of turbot. Staining: hematoxylin-eosin. Scale bar = 50 µm. EP, epidermis; DE, dermis; SS, stratum spongiosum; SC, stratum compactum; BT, bony tubercle; MU, muscle (Unpublished).

3.1.2. The skin mucosal immunity of fish

3.1.2.1. Innate immunity

The physical barrier

Skin, mucus, and the symbiotic bacteria constitute the primary defense barrier to prevent invasion of various pathogens in the aquatic environment (Shephard 1994, Sepahi *et al.* 2016). As a kind of colloid, the mucus can effectively prevent the bacterial translocation (Benhamed *et al.* 2014). The mucus contains a great amount of immune factors, which play different immune functions (Palaksha *et al.* 2008, Nigam *et al.*

2012). Skin, like intestine and gill, constitute a part of the mucosa-associated lymphoid tissue (MALT), which are in direct contact with the external milieu, providing a tight barrier against pathogenic infections and coexisting with a myriad of commensal organisms (Salinas *et al.* 2011, Salinas 2015, Yu *et al.* 2020). Various immune cell types are distributed in the skin, including lymphocytes, macrophages, and granulocytes, so this organ has the ability to independently complete local immune responses (Cesta 2006, Esteban and Cerezuela 2015). Many bacteria live on the skin surface of fish and develop a symbiotic relationship with them, thereby inhibiting the growth of pathogenic microorganisms (Pickard *et al.* 2017). When the external environment changes, the composition of the skin bacteria is destroyed, and the defense ability of the skin mucosal barrier will be affected (Gallo and Nakatsuji 2011, Byrd *et al.* 2018). The following parts introduce the skin mucosal immunity of fish from the components.

Phagocytes and phagocytosis

The phagocytes of fish skin mainly include **monocytes**, **macrophages**, and various **granulocytes**. Most phagocytes are vital components of the innate immune system (Secombes and Fletcher 1992). Phagocytes in the skin mucosal barrier constitute the first barrier against infection. Monocytes and granulocytes in blood serve as the second barrier to removing pathogenic microorganisms in the internal environment (Dalmo *et al.* 1997). Cells with phagocytic activity in fish skin mucosal barrier are involved in the ingestion and degradation of microorganisms and their products (Esteban 2012). In fish, the monocytes phagocytosis and digest foreign substances in the blood because of the strong adhesion and phagocytic ability. Monocytes are incompletely differentiated cells that enter various tissues through the blood to differentiate into phagocytes (Zhu and Su 2021). The macrophages in fish skin take part in many processes involved in the innate immunity (Buchmann 2014). When pathogenic microorganism and antigens invade the body through skin barrier, macrophages can recognize through the specific receptors and eliminate these microorganisms (Greenberg and Grinstein 2002). Besides, the macrophages are defined

as the antigen present cells that present antigens to B cell by phagocytosis, processing and delivery (Secombes 1994). Moreover, the macrophages secret many bioactive substances (interleukin-1, fibronectin, prostaglandin, lymphocyte activating factor, macrophage activating factor, interferon α&β, and tumor necrosis factor), enhance the immune status. There are three types of granulocytes in fish, neutrophils, eosinophils, and basophils. Neutrophils are the most common in the fish skin and have active phagocytosis and killing functions against pathogenic microorganisms by producing reactive oxygen species (Castro and Tafalla 2015).

Innate immune factors

Lysozyme is a thermolabile alkaline protein that acts exclusively on the hydrolysis of microbial cell walls, mainly secreted by neutrophilic granulocytes and monocytes (Castro and Tafalla 2015). Lysozyme can dissolve bacteria by hydrolyzing the peptidoglycan on the gram-positive bacteria cell wall (Saurabh and Sahoo 2008). But lysozyme has a weak ability to remove gram-negative bacteria. Also, lysozyme can activate complement and promote phagocytosis of bacteria (Magnadóttir 2006, Saurabh and Sahoo 2008). Therefore, the detection of lysozyme activity is very important for diagnosing fish diseases (Ellis 1990).

Proteases are proteolytic enzymes present in the skin mucus of fish, which can directly act on invading pathogens by proteolysis. Besides, proteases can also regulate bacteria adhesion by altering mucous consistency, thereby preventing the pathogen invasion or strengthen the immune system in terms of antibacterial peptides, complements, or immunoglobulins (Esteban 2012, Esteban and Cerezuela 2015). There are many **protease inhibitors** in fish mucus, including serine proteinase inhibitor, cysteine proteinase inhibitor, metalloproteinase inhibitor, and α-macro globin (Bowden *et al.* 1997, Xiao 2011). The serine proteinase inhibitor, cysteine proteinase inhibitor, and metalloproteinase inhibitor can specifically bind to the corresponding proteases. However, the α-macro globin can bind with all the proteases. The basic function of protease inhibitors is to maintain the stabilization of homeostasis, and to regulate the

activity of the complement system and agglutination test (Magnadottir 2010). Protease inhibitors can inhibit the activity of extracellular enzymes of pathogenic microorganisms and regulate antigen presentation, thereby participating in specific immunity (Xiao 2011).

The complement system comprises more than 35 distinct soluble blood plasma proteins and cell membrane proteins in higher vertebrates, whose biological function is to non-specifically remove antigen-antibody complexes in the body, and plays a pivotal role in innate immunity (Janeway Jr *et al.* 2001, Liao *et al.* 2019). As shown in **Figure 3**, the activation of the complement system in fish depends on three different routes: classical, alternative, and lectin pathway. The complement 1q (C1q) synthesized by the monocyte-macrophage lineage cells leads to the activation of the classical pathway activated by pentraxins (c-reactive protein, serum amyloid-p, and pentraxin 3). The alternative pathway starts with the spontaneous activation of C3 by hydrolysis, which is further triggered by contact with microorganisms and other foreign surfaces (Harboe and Mollnes 2008, Liao *et al.* 2019).The mannan-binding lectin, ficolin, and collectin bonded to carbohydrate moieties on bacterial surfaces initiate the lectin pathway of complement (Runza *et al.* 2008). Subsequently, these three pathways participate in the formation of C3 convertases. The C3 convertases can lyse the C3 into C3a and C3b. The C3b is involved in forming a membrane attack complex by the assembly of C5b~C9, leading to cell lysis including microbes (Gudding *et al.* 2014, Liao *et al.* 2019). The role of fish complement is multi-faceted, and it participates in the protective immune response of antibody, maintaining homeostasis, and also immunopathological damage (Yu *et al.* 2021). In these processes, it is generally caused by various bioactive substances generated after complement activation. In addition to direct antimicrobial effects such as lysis of bacteria, neutralization of exotoxins, lysis of parasites, etc., the complement of teleost fish also shows a promotion effect on the activity of phagocytes (Magnadóttir 2006).

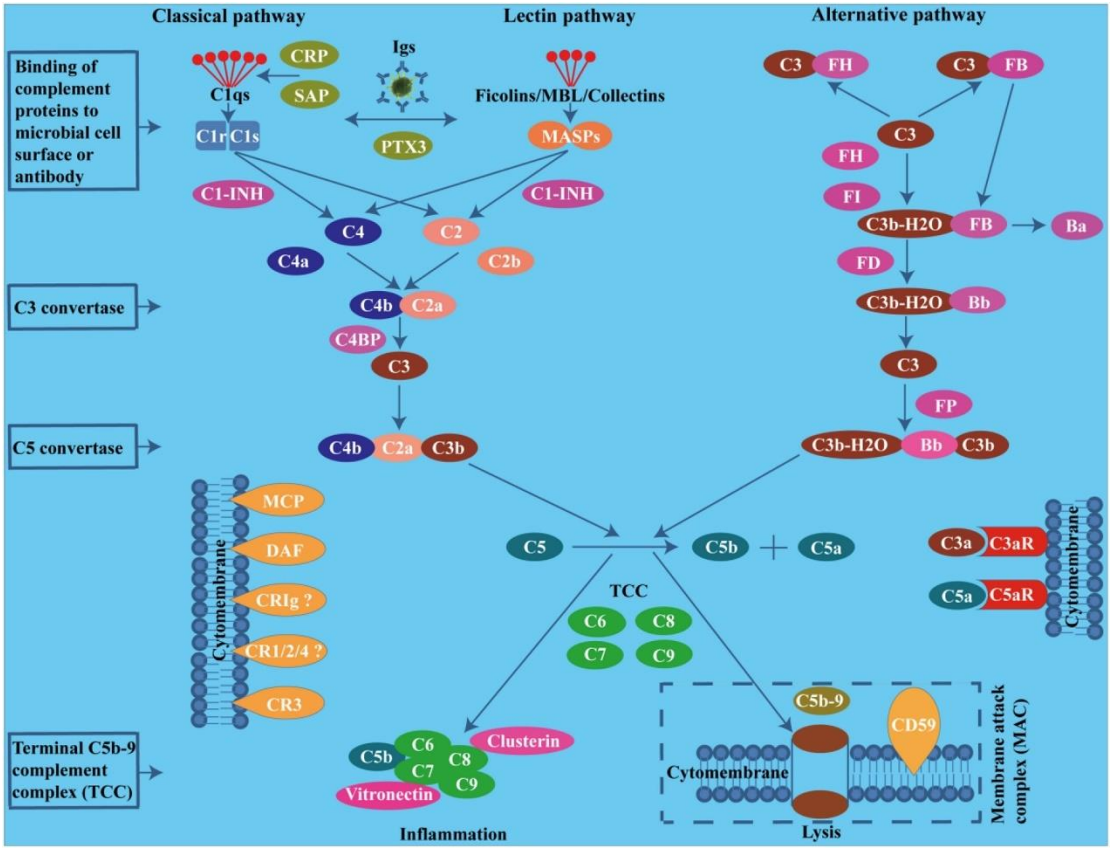

Figure 3. Suppositional activation and regulation of complement system in teleost fishes (Liao *et al.* 2019).

Lectins are a series of proteins or glycoproteins without enzymatic activity, including C-type lectins, galectins, rhamnose-binding lectin (RBLs), F-type lectins, lily-type lectin, and pentraxins in fish (Elumalai *et al.* 2019). Specifically, the galectin, typical C-type lectins, and RBLs were identified in the skin mucus of fish. The galectin AJL-1 from *Anguilla japonica* is similar in structure to congerin from *Conger conger,* which was highly expressed and only identified in the skin mucus and exhibits agglutination against *Streptococcus difficile* (Shirai *et al.* 1999, Tasumi *et al.* 2002). The lectin AJL-2, a Ca^{2+}-independent C-type lectin, was specially isolated from the skin mucus of *Anguilla japonica*. The agglutination experiment showed that AJL-2 specifically agglutinate with pathogenic bacteria *Escherichia coli* K 12, inhibiting the invasion of pathogens from protecting the fish, which is similar to C-type lectin mannose-binding protein (Tasumi *et al.* 2002). PFL-1and PFL-2 were specially

revealed in the skin mucus from pony fish (*Leiognathus nuchalis*). Because PFL-1 and PFL-2 is highly homologous to L-RBLs in the fish eggs, so are classified into the RBLs family (Okamoto *et al.* 2005). These lectins also have the important immune function of agglutinating bacteria, preventing the invasion of pathogens. Suzuki *et al.* (2003) identified pufflectin from surface mucus of *Takifugu rubripes* as 'Lily-type lectin'. Pufflectin showed no effect on the agglutination with bacteria, but against parasites. The finding confirmed that lectins have innate immunity to both bacteria and parasite.

Antimicrobial peptides are a class of small molecule peptides that are ubiquitous in organisms and have broad-spectrum antimicrobial activity. It plays an essential role in the non-specific immune system of fish (Masso-Silva and Diamond 2014). When the fish body is damaged or attacked by pathogens, the antimicrobial peptides could be quickly produced to prevent the invasion of bacteria, viruses, fungi, and protozoa (Chia *et al.* 2010, Mihajlovic and Lazaridis 2010, Valero *et al.* 2020). Antimicrobial peptides could act on the cell membrane of bacteria to induce the membrane depolarization, or the pores on the cell membrane, causing the release of cellular content and quickly kill invading pathogens (Brogden 2005, Mihajlovic and Lazaridis 2010). In addition, antimicrobial peptides can bind to bacterial DNA or RNA to inhibit protein synthesis and cause bacterial death (Zhang and Falla 2006). Antibacterial peptides can also exert their activity by inhibiting the respiration of cells or combining with heat shock proteins of bacteria (Izadpanah and Gallo 2005). Differing from the other biological molecules (such as antibodies) and immune cells, the antibacterial peptides could be synthesized quickly and spread to the whole body (Zasloff 2002).

Cytokines are a class of small molecular polypeptides synthesized and secreted by immune cells, which regulate a variety of cellular physiological functions and play an important part in the innate immunity of fish (Bayne and Gerwick 2001, Whyte 2007). Under normal conditions, the secreted cytokines are in a low level or in an inactive state. Once immune cells or tissues are stimulated, the signal transduction pathway regulating cytokine synthesis will be activated. The increased expression of cytokines recognized specific receptors on the cell membrane and further function on

the immune regulation (Uribe *et al.* 2011). **Interleukin 1β** (**IL-1β**) is generated by the combination of pattern recognition receptor (PRR) and pathogen-associated molecular patterns (PAMP), which is an important inducer of Interferon γ (IFN-γ) and a promoter of T cells (Zou and Secombes 2016). Similar to the IL-1β, **tumor necrosis factor** (**TNF-α**) is a proinflammatory cytokine that is expressed in the early phase of the infection in fish that plays a key role in the regulation of inflammatory response (Reyes-Cerpa *et al.* 2012). **Interleukin 6** (**IL-6**) family includes IL-6, IL-11, ciliary neurotrophic factor (CNTF)-like, and M17 in fish. The recombinant IL-16 protein can promote the growth of macrophages and have pro- and anti-inflammatory properties (Huang *et al.* 2019, Sakai *et al.* 2021). As a chemokine, **interleukin 8** (**IL-8**) can induce intracellular signal transduction by activating G protein-coupled receptors (GPCR), chemotactic neutrophils, and macrophages, and participate in the occurrence of inflammatory responses in the body. Usually, **transforming growth factor** (**TGF-β**) and **interleukin 10** (**IL-10**) are defined as the anti-inflammatory cytokines in fish. TGF-β has three subtypes: TGF-β1, 2, and 3, which show the immunosuppressive effects and immunobiological activity in teleost peripheral blood lymphocytes (Yang and Zhou 2008). IL-10 acts as a suppressor in dampening inflammatory responses (Sakai *et al.* 2021), whose functions has been characterized in goldfish (*Carassius auratus* L.) and European common carp (*Cyprinus carpio* L.) (Grayfer *et al.* 2011, Piazzon *et al.* 2015, Piazzon *et al.* 2015).

Inflammatory response

The inflammatory response is a pathological process caused by the infection, antigen challenge or tissue injury (Kumar *et al.* 2004, Sherwood and Toliver-Kinsky 2004). The leucocyte, red blood cells, platelets, histamine, and serotonin play a key role in the early phase of inflammation (Schmid-Schönbein 2006). Inflammation can attract large numbers of phagocytes at the needed site (Fujiwara and Kobayashi 2005). The rapid expansion and increased permeability of micro-vessels accelerate blood flow, resulting in a localized concentration of antibacterial substances at the site of infection,

and the accumulation of dead host cells also releases some antibacterial substances (Xiao 2011). Decreased oxygen concentrations and increased lactate concentrations at infected spot inhibit the growth of a variety of pathogens (Ohishi 2000). All the above processes are designed to remove pathogens or stimulations and enhance tissue repair. However, excessive inflammation may lead to tissue injury and cause physiological decompensation, organ dysfunction and death. (Sherwood and Toliver-Kinsky 2004). In fish, the inflammatory response is accompanied by the release of cytokines (Secombes *et al.* 2001). No matter in the intestinal inflammation, liver damage, and inflammatory response process in the skin wound healing, the gene expression of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, and IFN-γ) is significantly induced, while the gene expression of anti-inflammatory cytokines (IL-10 and TGF-β) is remarkably decreased (Ceballos-Francisco *et al.* 2017, Tan *et al.* 2017, Chen *et al.* 2018). Moreover, there are also some histological changes in the inflamed skin. As shown in the **Figure 4**, the epidermal cell layers and goblet cells are well-arranged in epidermis of intact skin. Besides this, the infiltration of leucocytes, red blood cells, and platelets was evident in the muscle, dermis, and even epidermis.

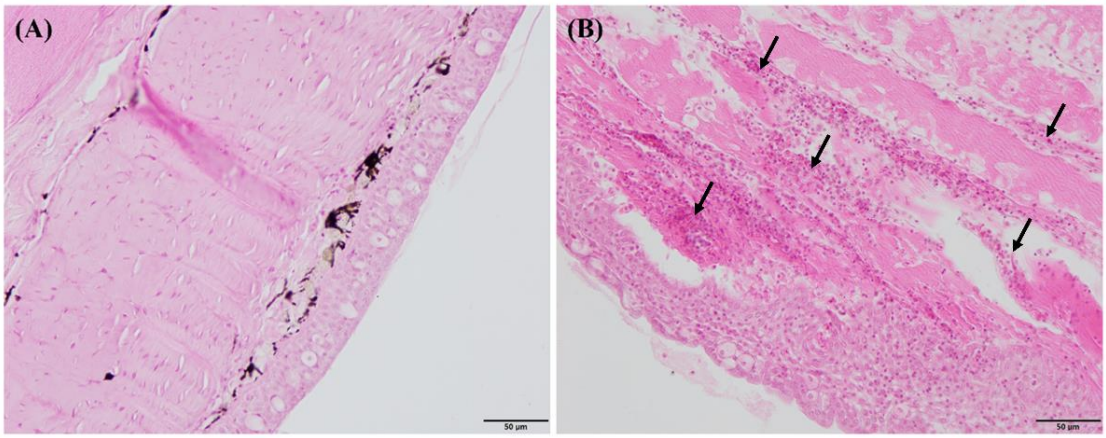

Figure 4. The histology of the intact skin (A) and inflamed skin (B) of turbot (*Scophthalmus maximus* L.). Black arrows indicate the infiltration of admixed leucocytes in the lamina propria. Staining: hematoxylin-eosin. Scale bar = 50 μm (Unpublished).

3.1.2.2. Adaptive immunity

The immune response

As opposed to innate immunity, adaptive immunity, also known as acquired immunity or specific immunity, generates a specific immune response after recognizing different antigens to ensure the maintain homeostasis (Xiao 2011). Specifically, the adaptive immune responses are specific to the corresponding antigens, including the humoral immune system and the cellular immune system. In addition, adaptive immunity differs between different individuals of the same organism or the same individual under different conditions (Mutoloki *et al.* 2014). Antigen stimulations activate, proliferate, and differentiate lymphocytes through antigen-specific lymphocytes' recognition of the antigens. Finally, humoral immunity and cellular immunity are exhibited (Parkin and Cohen 2001, Bonilla and Oettgen 2010).

Cellular immunity

The immune response generated by the immune cells to clear the invading pathogens is called cellular immunity in the broad sense. In the narrow sense, cellular immunity only means T lymphocyte immunity (Xiao 2011). After being stimulated, the T lymphocytes proliferate and differentiate into effector T lymphocytes (Inaba and Steinman 1984). The effector T lymphocytes secrete the cytokines and take part in the immune response. When the antigens invade again, the effector T lymphocytes could clear the antigens directly at the beginning (Broere and van Eden 2019). Once the effector T lymphocytes specifically bind to the target cells with the antigen again, the effector T lymphocytes increase the permeability of the target cell membrane, leading to the swelling and death of target cells (Parkin and Cohen 2001). As a member of effector T lymphocytes, cytotoxic T lymphocytes (CTLs) kill target cells through the granule-dependent pathway and FasL-FAS-mediated apoptotic pathway (Abougergi *et al.* 2005, Pardo *et al.* 2009). Perforins and granzymes are two important granule proteins for CTL to kill target cells in the granule-dependent pathway (Trapani and Smyth 2002). In the Fas-mediated apoptosis pathway, FasL and TNF-α secreted by CTLs bind to Fas and TNFR of target cells to initiate a caspase signal pathway and kill

the target cells (Russell and Ley 2002). Furthermore, CTLs cooperate with lymphokines to kill target cells. T lymphocyte immunity has not been studied clearly in fish skin. Therefore, the roles of T lymphocyte immunity need to be clarified in warrant further research (Gomez *et al.* 2013).

Humoral immunity and immunoglobulin

The humoral immune response of fish includes the following processes: antigen processing and presentation, B cell activation, regulation of suppressor T lymphocyte, B lymphocyte proliferation and differentiation, and antibody production (Parra *et al.* 2015, Wlasow and Jankun 2019). Generally, humoral immunity takes part in both innate immunity and adaptive immunity, only the role of humoral immunity in adaptive immunity is introduced in this part. For a long time, the immunoglobulin M (IgM) had been regarded as the only immunoglobulin in the fish immune system (Sunyer 2013). But, in the subsequent studies on the zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar*), turbot (*Scophthalmus maximus*), Nile tilapia (*Oreochromis niloticus*), the immunoglobulin D (IgD) and T/Z (IgT/Z) were also detected in the systematic immunity of fish (Danilova *et al.* 2005, Hansen *et al.* 2005, Edholm *et al.* 2010, Tadiso *et al.* 2011, Ramirez-Gomez *et al.* 2012, Tang *et al.* 2018, Velázquez *et al.* 2018). The IgM is the major immunoglobulin in the serum of fish, and recent studies also showed that IgM takes part in the mucosal immunity with only very small amounts (Parra *et al.* 2015). The IgD works as a specialized mucosal immunoglobulin in fish, similar to the immunoglobulin A (IgA) in mammals, while the function of IgD has not been figured out and seems not to be associated with the mucosal immunity (Salinas *et al.* 2011, Parra *et al.* 2015). In mammals, it is clear that mucosa organs (skin, intestine, and gut) could produce specific antibodies (IgA or IgM) when the pathogens invaded the epithelial cells (Brandtzaeg and Pabst 2004, Brandtzaeg *et al.* 2008), but it is still unclear in fish.

3.2. Skin wound healing in fish

The skin wound could lead to a metabolic disorder, tissue infection and death, and even endanger lives. Thus, the rapid repair mechanism of skin makes a case for the defensive mechanism against the external environment. In mammals, skin wound healing is a complex process involving blood clot formation, inflammation, re-epithelialization, granulation tissue formation, and remodeling (Singer and Clark 1999, Shaw and Martin 2009). However, the previous studies on the zebrafish showed that the blood-clot formation phase is lacking in the wound healing process of fish, the other stages, including inflammation, re-epithelialization, new tissue formation, and remodeling, are remained (Richardson *et al.* 2013, Costa and Power 2018).

3.2.1. Inflammation in skin wound healing

The studies on adult zebrafish indicated that re-epithelialization is independent of inflammation (Richardson *et al.* 2013), which had also confirmed that the re-epithelialization also happened before the activation of leucocytes and induction of macrophages in scale removed seabream (Costa and Power 2018). In mammals, the inflammatory phase starts with the recruitment of neutrophils and macrophages (Midwood *et al.* 2004, Olczyk *et al.* 2014). During the early phase of the inflammatory response, the neutrophils stay behind the leading edge and play the dominant role in removing cellular debris and pathogens from host tissue (Kim *et al.* 2008, Richardson *et al.* 2013). And then, the macrophages enter the wound area and conduct the phagocytosis as well as the release of cytokines and growth factors for initiating the next phase of the healing process (Leibovich and Ross 1975, Martin and Leibovich 2005, Mori *et al.* 2008).

The inflammatory response ascribes the release of pro-inflammatory cytokines in the wound sites, which is necessary for granulation tissue formation and vascularization (Richardson *et al.* 2013). The inflammatory cytokines and growth factors trigger the fibroblast migration and have the main effect in the synthesis of extracellular matrix (ECM) components (Brown *et al.* 2007, Barrientos *et al.* 2008), which contributes to

the granulation tissue formation (Singer and Clark 1999, Shaw and Martin 2009). At the same time, the process of neovascularization in a wound, driven by the release of cytokines and growth factors, restores blood circulation and prevents ischemia-induced tissue damage, which consequently benefits the tissue repair process (Olczyk *et al.* 2014).

3.2.2. Re-epithelialization

Re-epithelialization is the term used to describe the resurfacing of a skin wound with new epithelium, which relies on the migration and proliferation of keratinocytes (Telgenhoff and Shroot 2005, Rousselle *et al.* 2019). In mammals, the blood-clot is formed by thrombin generation and fibrin formation with the activation and aggregation of platelet in the wound area. The blood clot work as a provisional matrix in which growth factors are in effect and cells migrate, which is the immediate response in the skin wound healing of mammals (Nurden *et al.* 2008, Shaw and Martin 2009). Usually, the formation of the blood clot is mainly for sealing the external environment and preventing blood loss and the entry of pathogens (Metzger 2018). However, the blood-clot formation phase is lacking in the skin wound healing process of fish, as mentioned above, for a rapid re-epithelialization take its place to close the skin wound initially (Richardson *et al.* 2013).

The study on the zebrafish showed the initial re-epithelialization of fish does not require keratinocyte proliferation (Richardson *et al.* 2016). As indicated in the previous study on zebrafish (Figure 5), the migration of the keratinocytes initiates from the basal layers of the epidermis. The basal cells make lamellipodial protrusions that extend to the wound area. Subsequently, the intermediate cells fuse into the basal layer. The epidermic cells gradually covers the wound by forming protrusions and cell elongation (Richardson and Hammerschmidt 2018). The covering of the wound area by the migrating cells will stop when they front meet each other, and then the keratocyte proliferation starts thickening the neo-epidermis when the epidermal thickness recover to normal in adjacent regions (Banerjee and Mittal 1999, Quilhac and Sire 1999, Jensen

et al. 2015, Richardson *et al.* 2016, Sveen *et al.* 2020). As for the goblet cells on the surface of the neo-epidermis, the studies on Atlantic salmon showed that the secreting cells display as beads on a string rather than randomly dispersed, forming a mucosal layer to protect the neo-epidermis and wound bed (Jensen *et al.* 2015, Sveen *et al.* 2019, Sveen *et al.* 2020).

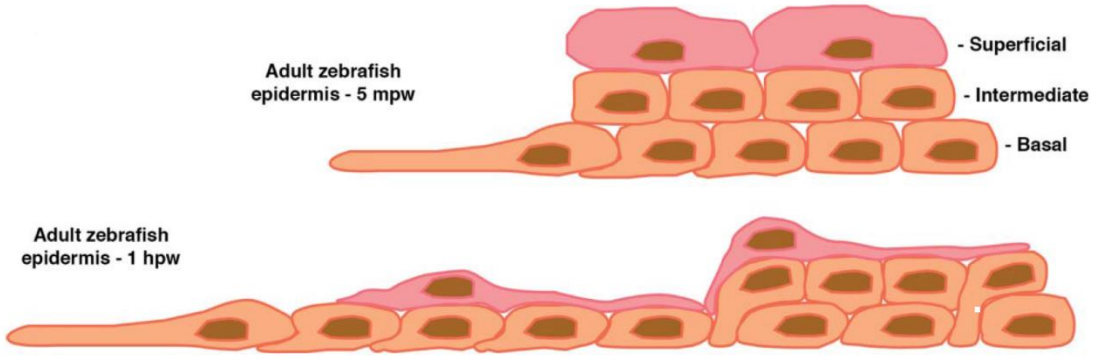

Figure 5. Representation of a cross-section through the epidermis at the wound edge in an adult zebrafish 5 minutes post-wounding (mpw), one hour post-wounding (hpw), quoted from (Richardson and Hammerschmidt 2018).

3.2.3. Granulation tissue formation

In mammals, the granulation tissue formation is mainly driven by the migration of fibroblasts recruited by the release of cytokines and growth factors (Brown *et al.* 2007, Barrientos *et al.* 2008, Olczyk *et al.* 2014). The early matrix of granulation tissue is mainly formed from fibrin and fibronectin networks and abound with a high amount of hyaluronic acid (Midwood *et al.* 2004). The hyaluronic acid molecules create enough space to ensure the leucocytes and fibroblasts can penetrate the granulation tissue by swelling (Martin 1997, Singer and Clark 1999). As the granulation tissue matures, the collagen matrix replaces the temporary matrix and further leads to restoring the structure and function of the proper tissue (Midwood *et al.* 2004). Similar to mammals, the granulation tissue formation of fish also plays the dominant role in skin wound healing. The granulation tissue gradually replaces the damaged tissues and drives the

contraction of the wound border (Roubal and Bullock 1988, Richardson *et al.* 2013, Sveen *et al.* 2019). However, the formation and clearance of granulation tissue are both rapid processes. In the studies on the zebrafish, the formation of granulation tissue formation begins at 2 days post-wounding and is eventually cleared over a six-day period (Richardson *et al.* 2013).

3.2.4. Remodeling

The wound starts with the fibroblasts differentiating into myofibroblasts. In mammals, the granulation tissue gradually matures and remolds to the form of a scar (Olczyk *et al.* 2014). The critical effect cell in the granulation tissue formation retreat, the capillary start to aggregate for establishing the normal blood vessel network gradually (Hoffman *et al.* 2006, Olczyk *et al.* 2014). The content of early granulation tissue such as glycosaminoglycans, proteoglycans, and bound water is replaced by mature, cross-linked collagen fibers of the proper diameter, construct a framework of the newly formed tissue (Kuwaba *et al.* 2001, Reinke and Sorg 2012, Olczyk *et al.* 2014). The studies on the zebrafish showed that the remodeling phase completes quickly, all the components and functions turn into normal conditions within one month, including scales, pigmented cells, and subcutaneous adipocytes (Richardson *et al.* 2013, Olczyk *et al.* 2014, Metzger 2018).

3.3. Intestinal mucosal barrier

To a large extent, the rapid growth, high feed utilization, stable output, and the quality and safety of aquatic products are based on the integrity of the organizational structure and function of the intestine. As the most important digestive organ for absorbing nutrients, water, and electrolytes from food, the intestine is also the largest immune organ (Gosain and Gamelli 2005). It is not only responsible for separating the external environment from host tissues and coexisting with a myriad of commensal organisms, but also actively participating in the fight against the physical, chemical, and microbial challenges (McGuckin *et al.* 2009). Diagram of the intestinal mucosa in

teleost fish is shown in **Figure 6**.

3.3.1. The mucus layer

As the first line of defense against pathogen invasion and external damage, the intestinal mucus barrier consists of mucins and antibacterial substances (Buisine *et al.* 1998, Kim and Ho 2010). It mainly covers the surface of intestinal epithelial cells and is a non-water-soluble structure, which can hinder the direct contact of pathogenic bacteria with the intestinal tissues (Linden *et al.* 2008, Dharmani *et al.* 2009). At the same time, it is responsible for the material transport between the intestinal cavity and the striatum, the digestion and absorption of nutrients, and the lubrication (Kim and Ho 2010).

The balance and dynamic interaction between the mucosal layer, intestinal epithelial cells, microbiota, and host immune defense are essential for maintaining the homeostasis of the intestinal mucosa (Liévin-Le Moal and Servin 2006). The destruction of the intestinal homeostasis will lead to the destruction of the mucous barrier, aggravation of inflammation and damage of the intestinal mucosa (McGuckin *et al.* 2009). As an important component of intestinal mucus, mucins is a type of glycoprotein secreted by goblet cells, participating in the metabolism of epithelial cells, adhesion of bacteria, and cell signal transmission (Carraway III *et al.* 2007, Kufe 2009).

3.3.2. The physical barrier of the intestine

The intestinal physical barrier mainly plays a role in barrier protection (Swidsinski *et al.* 2007). The selective permeability of intestinal mucosa is mainly caused by the difference of cortical components on both sides (Arrieta *et al.* 2006). The transport of substances in intestinal epithelial cells is inseparable from the corresponding receptors on the cell membrane. Besides, the transport of substances can also be carried out through some cell channels or connection structures between cells (Pacha 2000).

The intestinal epithelial cells and the cell junctions between the cells constitute the physical barrier function (Baumgart and Dignass 2002). This structure can effectively

prevent the invasion of bacteria and the harmful bacterium's multiplication, and it is crucial for maintaining the intact form of the intestine (Groschwitz and Hogan 2009). There are many ways of cell junctions between adjacent epithelial cells, such as tight junctions, adhesion junctions, gap junctions, and desmosomes. The tight junction is a complex multifunctional structure between adjacent epithelial cells that occupy the transport channel between cells, functionating the intestinal barrier function by regulating the entry of nutrients, ions, and water and preventing pathogens (Arrieta *et al.* 2006). The tight junction proteins (occludin, claudins, zonula occludens proteins, junctional adhesion molecules, and tricellulin) combine with adipose tissue to form a series of complexes, creating a tight junction structure between intestinal epithelial cells. In addition, the tight junction proteins maintain the integrity of the intestinal mucosal mechanical barrier and prevent bacterial translocation (Schneeberger and Lynch 2004, König *et al.* 2016).

3.3.3. The intestinal immunity

The intestinal mucosa is divided into the intestinal epithelium and the lamina propria. In contrast with mammals, the intestine of fish lacks Peyer's patches and mesenteric lymph nodes present in endotherms (Salinas and Parra 2015). However, as a part of MALT of fish, the intestine contains a large number of immune cells. The granulocytes, lymphocytes, and macrophages in the intestine are mainly located in the intestinal lamina propria, with intraepithelial lymphocytes (T lymphocytes and a few B lymphocytes located among epithelial cells) distributed in the intestinal epithelium (Parra *et al.* 2015, Salinas and Parra 2015). The cellular immunity and humoral immunity in teleost have been introduced in detail in the above paragraphs. The roles of the main immune cells in intestinal immunity are mainly introduced in this part.

3.3.3.1. Macrophages

Macrophages play a critical role in maintaining intestinal homeostasis, which can eliminate pathogens that invade the intestine by identifying pathogenic microorganisms

and the immunoglobulin and complement components on the surface of parasites. Besides, macrophages can also secrete a variety of immune factors and participate in the innate immune response of the intestinal tract (Secombes 1994).

3.3.3.2. Granulocytes

Previous studies have shown that neutrophils, eosinophils, basophils, and mast cells are detected in the lamina propria of the fish intestine (Salinas and Parra 2015). Eosinophils are abundant in the intestinal mucosal barrier and can react strongly to inflammation by migrating and releasing the contents of the particles (Dobson *et al.* 2008, Urán *et al.* 2009). In fish and mammals, neutrophils are abundant in the circulatory system and can quickly accumulate from the blood to the intestinal inflammation site (Do Vale *et al.* 2002). In a healthy state, the number of neutrophils is less than that of eosinophils and basophils. However, neutrophils are easier to penetrate epithelial cells, and their number will increase significantly under infection or irritation (Bernard *et al.* 2006).

3.3.3.3. B lymphocytes

In the context of the innate immune system, the B cells of teleost play a crucial role in phagocytosis and microbicidal abilities (Li *et al.* 2006). Teleost gut-associated lymphoid tissue consists of diffuse elements (Salinas and Parra 2015), and B cells are one of the leading responders in the adaptive immune system. When mucosal pathogen infection occurred in the fish intestine, the B cells are accumulated in the lymphoid tissues (Parra *et al.* 2016). B lymphocytes can recognize the exposed antigenic determinants after the antigen-presenting cells decompose the antigen, and then the body-specific immune response begins (Parra *et al.* 2015). The immunoglobulins (IgM, IgT/Z, and IgD) produced by the B cells are the key to defense against pathogens and prevent the symbiotic flora from entering the intestinal epithelial cells (Parra *et al.* 2016).

3.3.3.4. T lymphocytes

T lymphocytes are abundantly present in the lamina propria and epithelium (Rombout *et al.* 2011). Previous studies showed that the intestinal epithelial T lymphocytes of teleost express multiple key T lymphocyte markers, including the cluster of differentiation 4, 8, 3, 33, and 28 (CD4, CD8, CD3, CD33, and CD28), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), T-cell receptor (TCR), as well as many essential cytokines (Bernard *et al.* 2006, Castro *et al.* 2011, Picchietti *et al.* 2011, Rhee *et al.* 2014, Fink *et al.* 2015). These findings suggest that different T helper (Th) cells subtypes are distributed in the intestine of fish, similar to mammals. They participate in the local immune response and inflammatory response in the intestine by regulating the expression of IL-2, 4, 5, 6, and 10, IFN-γ, and TNF-β (Fujihashi *et al.* 1993, Niessner and Volk 1995, Andrew *et al.* 2001).

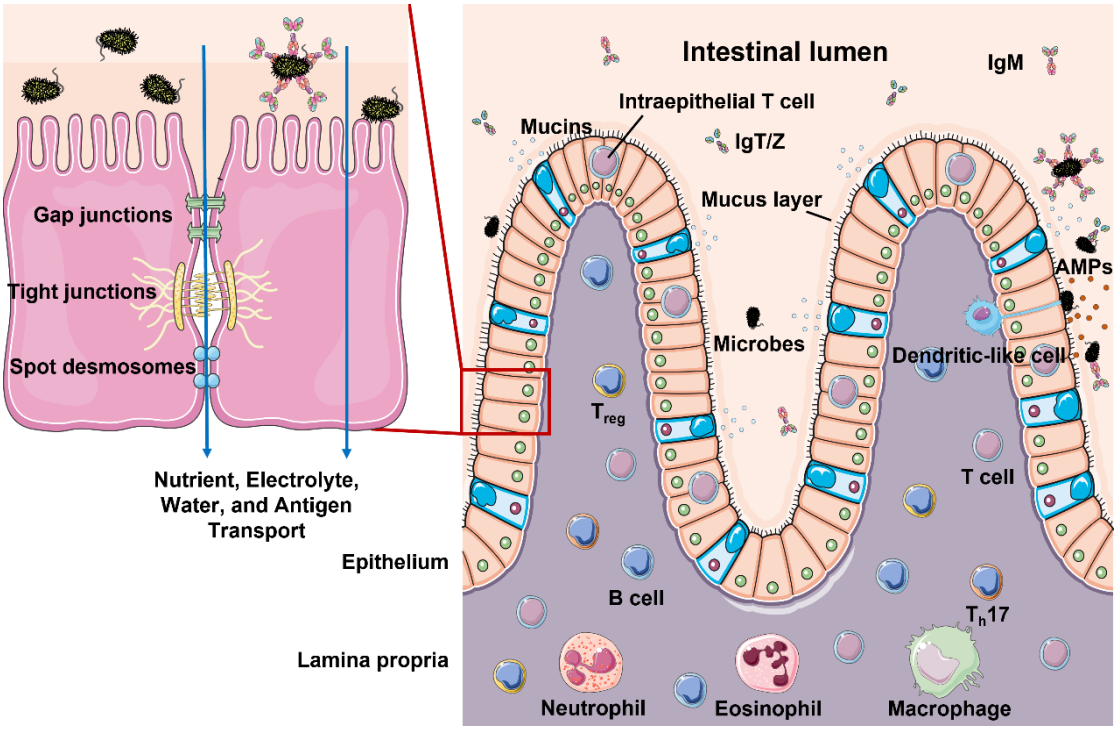

Figure 6. Diagram of the intestinal mucosa in teleost fish (Martin *et al.* 2016).

3.3.4. Toll-like receptors (TLRs), NF-κB signaling, and the intestinal inflammation

3.3.4.1. Toll-like receptors (TLRs) and NF-κB signaling

It is widely confirmed that intestinal epithelial cells participate in the innate immunity (Salinas and Parra 2015). The initial epithelial cells could participate in the phagocytosis of bacteria and the isolation and neutralization of toxins (Neal *et al.* 2006). The innate immune response starts with distinguishing between self and pathogen by recognizing germline gene-encoded PRRs [C3, lectins, nucleotide-binding and oligomerization domain (NOD)-like receptors, REG-1-like receptors, TLRs, etc.] with the PAMPs of microbes (lipopolysaccharide, peptidoglycan, lipoteichoic acid, and polysaccharide of bacteria; β-1,3-glucan and mannose of fungal; bacterial DNA; and viral nucleic acid) (Fujimoto and Uematsu 2020). The TLRs are the best-studied PRRs in fish. TLRs are a series of transmembrane proteins widely found and confirmed in these animals, including mammalian homologous (TLR1, TLR2, TLR3, TLR4 in a few species, TLR5M, TLR9) and fish specific TLRs (TLR5S, TLR18, TLR19, TLR20, TLR21, TLR22, TLR23, TLR25, TLR26, TLR27, and TLR28) (Zhao *et al.* 2019, Wang *et al.* 2021).

Activation of TLRs leads to the recruitment of myeloid differentiation factor (MyD88), resulting in activating NF-κB signaling pathway (Kawai and Akira 2010). The NF-κB regulates the gene expression of the inflammatory cytokines (IL-1, IL-6, and TNF-α) in response to various extracellular signal stimuli (viral infection, bacterial and fungal infection, tumor necrosis factor, and interleukin), inducing the inflammatory response (Zhao *et al.* 2021). When cells are in a quiescent state, NF-κB binds to its inhibitor IκB in the cytoplasm. Once upstream signals are activated, IκB is phosphorylated in the cytoplasm and degraded by the ubiquitin-dependent proteasome. Degradation of IκB results in the release of NF-κB into the nucleus to activate target genes transcription. NF-κB can promote the expression of neutrophil cell surface receptors to facilitate cell migration and stimulate the expression of inducible nitric oxide synthase (iNOS) in response to bacterial infection (Jobin *et al.* 1998, Rogler *et al.*

1998, Yeh *et al.* 2000).

Previous studies showed that the inflamed intestine is characterized by: over expression of pro-inflammatory cytokines but suppressed expression of anti-inflammatory cytokines (Zhao *et al.* 2019); the reduced brush border enzyme activities (Chen *et al.* 2018); increased amounts of connective tissue and infiltration of mixed leucocytes in the lamina propria and submucosa; widened lamina propria within mucosal folds; widening and shortening of the intestinal folds; loss of the supranuclear vacuolization in epithelial cells (Baeverfjord and Krogdahl 1996). The representative histological appearances of the normal and inflamed intestine are shown in **Figure 7** (Hu *et al.* 2016).

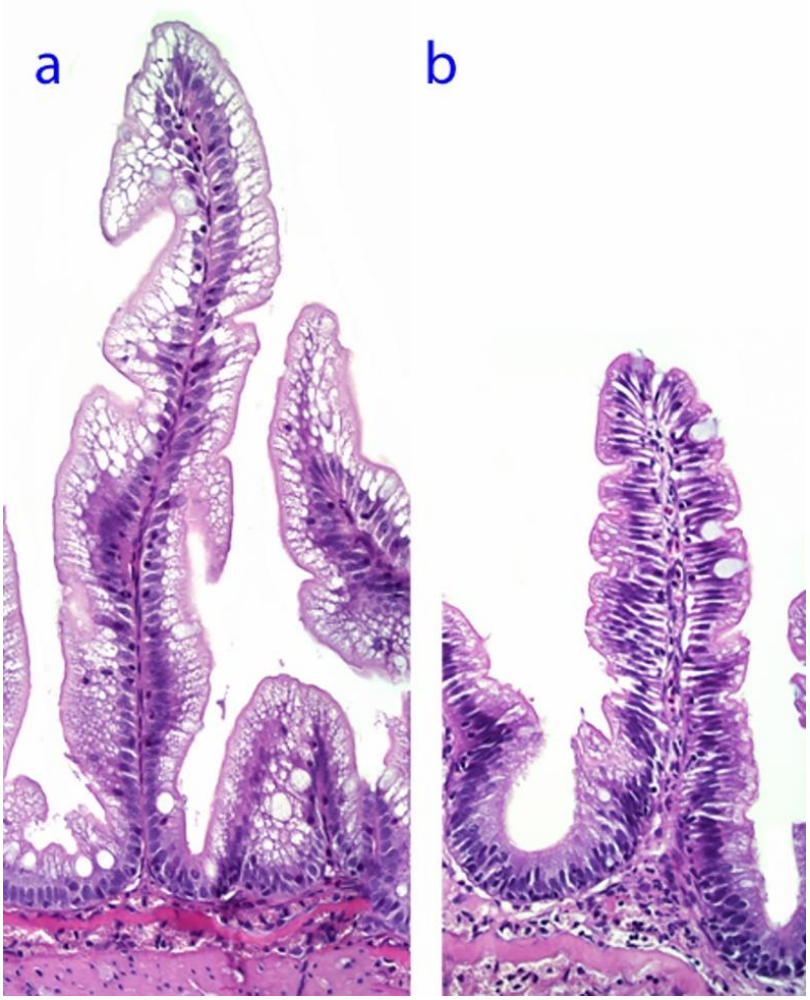

Figure 7, Representative histological appearances of normal (a) and inflammed intestine (b) (Hu *et al.* 2016).

3.3.4.2. The skin damage and the intestinal inflammation

Both skin and intestine are the major interfaces and defenders to prevent the invasion of the pathogens from the external environment (Esteban and Cerezuela 2015, Salinas and Parra 2015). Numerous pieces of evidence have figured the bidirectional connection between the skin and the intestine, such as the skin homeostasis and allostasis related to intestinal barrier functions (Levkovich *et al.* 2013, O'Neill *et al.* 2016). In previous studies carried out in humans, the interaction between skin damage and intestinal inflammation was mainly figured in patients with trauma and burn (Xu *et al.* 1997, Hotchkiss *et al.* 2000, Magnotti and Deitch 2005, Costantini *et al.* 2009, Li *et al.* 2015). The burn and traumatic injuries could immediately induce the disrupted immune system homeostasis of the body. The vulnerable antibacterial function of the organisms to opportunistic infections leads to the systemic inflammatory response, which induces severe septic shock and multiple organ failures, especially the interruption of the intestinal mucosal barrier (decreased small bowel weight and mucosal DNA synthesis, and altered nutrient transport) (Gosain and Gamelli 2005, Magnotti and Deitch 2005, Costantini *et al.* 2009, Stoecklein *et al.* 2012, Li *et al.* 2015). Besides, the increased number of bacteria and the activation of multifunctional immune cells and molecules, frequently detected in the intestine area, result in bacterial translocation and negatively influences the intestinal barrier function (Choudhry *et al.* 2004, Gosain and Gamelli 2005).

3.4. Feed Additives: probiotics and immunostimulants

Theoretically, feed additives refer to non-nutritive components in feed or non-nutritive components in raw materials, including anti-bacterial, antioxidants, binders, colorants, enzymes, organic acids, feed attractants, palatability enhancers, immunostimulants, probiotics and prebiotics, and hormones (National Research Council 2011). However, the recent studies have also expanded to include basic nutrients such as vitamins and amino acids (Lock *et al.* 2010, Andersen *et al.* 2016, Hernandez and Hardy 2020, El-Sayed and Izquierdo 2021, Li *et al.* 2021).

3.4.1. Probiotics: SpPdp11

Probiotics, also known as bio-biotics, refer to "a live microbial feed supplement which beneficially affects the host animal by improving microbial balance" (Zorriehzahra *et al.* 2016). Considering the different modes of addition, the probiotics in aquaculture can be used in two ways. One mixes the probiotics with feed as additives, and the other puts probiotics directly in the aquaculture water (Chauhan and Singh 2019). This part mainly introduces the use of probiotics as additives.

Firstly, the aquatic probiotics struggle for adhesion sites to induce "competitive inhibition", inhibit the adhesion of pathogens, and balance the intestinal bacteria (Martínez Cruz *et al.* 2012). Secondly, probiotics can kill pathogenic microorganisms by producing inhibitory substances (hydrogen peroxide, bacteriocins, lysozymes, siderophores, proteases, and many others) with antibacterial, antiviral, and antifungal activities (Servin 2004, Panigrahi and Azad 2007, Tinh *et al.* 2008, Chauhan and Singh 2019). Thirdly, there are also some probiotics that can produce short-chain fatty acids, promoting the development of the gastrointestinal tract (Dalile *et al.* 2019). Moreover, the reduced pH of the gastrointestinal tract can inhibit the proliferation of opportunistic pathogens (Tinh *et al.* 2008). There are some probiotics that can digest and utilize nutrients that cannot be digested and absorbed in the feed (Dai *et al.* 2021), or even compete with pathogenic bacteria for nutrients to affect their proliferation (Tinh *et al.* 2008).

SpPdp11 is a gram-negative pleomorphic bacterium isolated from the skin of healthy specimens of gilthead seabream (*Sparus aurata* L.). The relative studies showed that SpPdp11 could improve adhesion capacity and antagonistic activity against pathogenic bacteria in the skin and intestinal mucosal barrier (Chabrillón *et al.* 2005, Chabrillón *et al.* 2006). Recently, the genomic characteristics of SpPdp11 analyzed by an automatized workflow called TarSynFlow (Targeted Synteny Workflow) showed that SpPdp11 presents specific proteins for gut colonization, bile salt-resistance, and gut pathogen adhesion inhibition (Seoane *et al.* 2019). These studies reveal the potential

ability of SpPdp11 as a probiotic (Cámara-Ruiz *et al.* 2020). The beneficial effects of SpPdp11 were mainly indicated in the studies on gilthead seabream (*Sparus aurata* L.) and Senegalese sole (*Solea senegalensis*). SpPdp11 has great immunostimulatory effects and antioxidative stress regulation, leading to higher protection against stressors or diseases (Cordero *et al.* 2015, Cerezuela *et al.* 2016, Vidal *et al.* 2016, Jurado *et al.* 2018). Furthermore, dietary administration of SpPdp11 could enhance the innate immune and molecular mechanisms, improving stress tolerance to high stocking densities (Tapia-Paniagua *et al.* 2014, Cordero *et al.* 2016). As well, SpPdp11 increases the transcription of genes related to antiapoptotic effects and oxidative stress regulation of .*S. senegalensis* treated with oxytetracycline (Tapia-Paniagua *et al.* 2015). Furthermore, SpPdp11 was able to ameliorate the effects of the pathogen in the expression of cytokines of the ventral zone of gilthead seabream in response to *Photobacterium damselae* subsp. *Piscicida*.

3.4.2. Immunostimulants: Functional amino acids - Arginine

Immunostimulants, also known as immune enhancers, mainly refer to a class of additives that can promote the body's innate immunity and improve specific immunity (Vallejos-Vidal *et al.* 2016). At present, the most used immunostimulants are mainly synthetic chemicals, microorganisms and their derivatives, animal and plant extracts, vitamins, amino acids, hormones and lactoferrin (Farooqi and Qureshi 2018). In fish, immunostimulants can activate the phagocytosis of leucocytes, stimulate the production of lymphocytes or secrete lymphokines, coordinate cellular and humoral immunity; induce antibody and complement production (Kum and Sekkin 2011). The effects of immunostimulants on fish are preventive, and most immunostimulants have their optimal doses. The higher or lower doses than optimal ones will reduce the immune response, or even show negative effects (Wang *et al.* 2017). In addition, the immune function of fish will affect the effect of immunostimulants, the effect of immune stimulants on fish with low immune levels will be more obvious (Raa 2000). As a member of immunostimulants, functional amino acids refer to amino acids that have

other special functions in addition to synthesizing body proteins. They are not only necessary for the normal growth of animals and maintaining body functions, but also necessary for synthesizing a variety of biologically active substances. Functional amino acids include glutamine, arginine, tryptophan, proline, glycine, histidine, aspartic acid, and asparagine (Wu 2010, Hou *et al.* 2015).

Arginine can be converted into ornithine, which regulates collagen protein synthesis and cell proliferation and differentiation, it is the precursor of several bioactive substances, such as NO, polyamines, proline, and glutamate (Stechmiller *et al.* 2005). NO plays a key role in killing pathogens, increasing blood flow, regulating immune responses, and regulating collagen protein synthesis (Nieves Jr and Langkamp-Henken 2002). Arginine can also be converted to glutamine, thus protecting the shape and function of the intestine. Glutamine can further promote nucleotide synthesis and affect intestinal development (Evoy *et al.* 1998, Stechmiller *et al.* 2005). Besides, arginine is a potential endocrine secretagogue that promotes the secretion of growth hormone and insulin-like growth factor (Gianotti *et al.* 2000). The studies on wounded rodents showed that the treatment of arginine could suppress the inflammatory response. Simultaneously, arginine supplement enhanced collagen deposition by stimulating the TGF-β and restricting NO production, it could also improve the wound breaking strength (Barbul *et al.* 1985, Shi *et al.* 2003, Jerônimo *et al.* 2016). One study on weaned pigs and Jian carp (*Cyprinus carpio* var. Jian) showed that arginine can significantly improve the morphological structure of the intestine, maintain the basic function of the intestine, and improve the digestion and absorption of nutrients in the intestine (Wu *et al.* 2010, Chen *et al.* 2012). Much more studies are related to promoting effect of arginine on the intestinal immunity. Arginine supplementation in the feed of weaned piglets increased the number of immunoglobulin A (IgA) secreting cells, CD^{8+}, and CD^{4+} T cells, while decreased the number of mast cells. The inflammatory response induced by lipopolysaccharide (LPS) was inhibited by arginine (Zhu *et al.* 2013). In addition, the study on mice with mesenteric ischemia-reperfusion injury indicated that arginine could significantly reduce the intestinal inflammation (Taha *et al.* 2016), which

was similar with the study on Jian carp that dietary arginine supplementation could significantly inhibit LPS-induced intestinal inflammation (Jiang *et al.* 2015)

OBJECTIVES

The present Doctoral Thesis aims to improve our knowledge on the regeneration of the mucosal surfaces (both skin and gut) on wounded gilthead seabream (*Sparus aurata*).

1. Deepen the understanding of the progress of teleost skin regeneration.
2. Verify the additives effectiveness of SpPdp11 and arginine on skin regeneration and intestinal barrier function in wounded specimens.
3. Explore the effects of SpPdp11 and arginine on the signaling molecules which are involved in the skin wound healing.
4. Clarify the impacts of skin wounds on the intestinal barrier function.
5. Explore the mechanisms involved in the alleviation of enteropathy by dietary inclusion of SpPdp11 and arginine.

4. EXPERIMENTAL CHAPTERS

CHAPTER I

Effect of dietary administration of S*hewanella putrefaciens* Pdp11 (SpPdp11) on skin regeneration and intestinal function in experimentally wounded gilthead seabream

***Chen Z**, Ceballos-Francisco, D., Guardiola, F. A., & Esteban, M. (2020). Dietary administration of the probiotic Shewanella putrefaciens to experimentally wounded gilthead seabream (Sparus aurata L.) facilitates the skin wound healing. Scientific Reports, 10(1), 1-13.*

***Chen Z**, Ceballos-Francisco, D., Guardiola, F. A., & Esteban, M. Á. (2020). Influence of skin wounds on the intestinal inflammatory response and barrier function: Protective role of dietary Shewanella putrefaciens SpPdp11 administration to gilthead seabream (Sparus aurata L.). Fish & Shellfish Immunology, 99, 414-423.*

CHAPTER I —— Paper I

Dietary administration of the probiotic *Shewanella putrefaciens* to experimentally wounded gilthead seabream (*Sparus aurata* L.) facilitates the skin wound healing.

Journal: Scientific Reports

ABSTRACT

The effect of the probiotic *Shewanella putrefaciens* Pdp11 (SpPdp11) was studied on the skin healing of experimentally wounded gilthead seabream (*Sparus aurata* L.). Two replicates (n=12) of fish were fed CON diet or SP diet for 30 days. Half of the fish were sampled while the others were injured and sampled 7 days post-wounding. Results by image analysis of wound areas showed that SpPdp11 inclusion facilitated wound closure. Compared with the CON group, fish in SP group sampled seven days post-wounding had a significantly decreased serum AST and increased ALB/GLOB ratio. Furthermore, protease and peroxidase activities were significantly increased in skin mucus from fish in SP group sampled 7 days post-wounding compared with those from CON diet. Additionally, SP diet up-regulated the gene expression of antioxidant enzymes, anti-inflammatory cytokines, and re-epithelialization related genes in the fish skin. Furthermore, significant decreases in pro-inflammatory cytokines expression were detected in fish from SP group, respect to control ones. Overall, SpPdp11 inclusion facilitated the wound healing and the re-epithelialization of the damaged skin, alleviated the inflammatory response in the wound area through intensifying the antioxidant system, and enhancing the neo-vascularization and the synthesis of matrix proteins in the skin wound sites of fish.

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CHAPTER I —— Paper II

Influence of skin wounds on the intestinal inflammatory response and barrier function: Protective role of dietary *Shewanella putrefaciens* SpPdp11 administration to gilthead seabream (*Sparus aurata* L.)

Journal: Fish and Shellfish Immunology

ABSTRACT

The effects of skin wounds on the intestinal barrier function and the beneficial effects of the dietary administration of *Shewanella putrefaciens* (known as SpPdp11) in gilthead seabream (*Sparus aurata* L.) were studied. Two replicates of fish were fed a commercial diet (control, CON) or CON diet enriched with 10^9 cfu g^{-1} SpPdp11 (SP diet) for 30 days. After this time, half of the fish were sampled, while the others were injured below the lateral line (wounded fish, W) and fed the same diets for an extra week before sampling (CON+W and SP+W groups). The intestinal histology and gene expression of different genes relevant for the intestinal barrier function were studied. The results showed that injured fish had a disordered enterocyte nucleus disposition, a more intense infiltration of mixed leucocytes and a thicker lamina propria in the intestine compared to the control fish. However, the fish in the SP+W group did not present these pathological symptoms in the intestine. No significant variations in the number of goblet cells were detected among the different experimental groups. Pro-inflammatory cytokines (colony-stimulating factor receptor 1, *CSF1R*, myeloperoxidase, *MPO* and interleukin-1β, *IL-1β*), mucins (intestinal mucin, *IMUC* and mucin 2, *MUC2*), and immunoglobulin T heavy chain (*IGHT*) were up-regulated, while tight junction protein *occludin* was down-regulated in the intestine from fish of the CON+W group. Similarly, the dietary administration of SpPdp11 markedly depressed the gene expression of pro-inflammatory cytokines, *MUC2,* and *IGHT*, but increased the gene expression of anti-inflammatory cytokine transforming growth factor-β1 (*TGF-β1*) and the tight junction proteins *tricellulin* and *occludin* after wounding. In brief, the skin wounds provoked an intestinal inflammatory response that

included changes in the mucus layer and tight junction disruptions. Besides this, the preventive administration of SpPdp11 alleviated the intestinal dysfunctions caused by skin wounds in gilthead seabream.

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CHAPTER II

Effect of dietary administration of arginine on skin regeneration and intestinal function in experimentally wounded gilthead seabream

***Chen Z**, Ceballos-Francisco, D., Guardiola, F. A., Huang, D., & Esteban, M. Á. (2020). Skin wound healing in gilthead seabream (Sparus aurata L.) fed diets supplemented with arginine. Fish & Shellfish Immunology, 104, 347-358.*

***Chen, Z.**, Ceballos-Francisco, D., Guardiola, F. A., Huang, D., & Esteban, M. Á. (2020). The alleviation of skin wound-induced intestinal barrier dysfunction via modulation of TLR signalling using arginine in gilthead seabream (Sparus aurata L). Fish & Shellfish Immunology, 107, 519-528.*

CHAPTER II —— Paper III

Skin wound healing in gilthead seabream (*Sparus aurata* L.) fed diets supplemented with arginine

Journal: Fish and Shellfish Immunology

ABSTRACT

Dietary administration of arginine on the wound healing process of gilthead seabream was studied. Two replicates of fish (n = 8) were fed with either a commercial diet [control diet (CON), no arginine added] and the CON diet supplemented with 1% arginine (ARG1) or with 2% arginine (ARG2) for 30 days. Afterward, half of the fish were sampled while the other half were injured and continued to be fed the same diet for an extra week. Results by image analysis showed that the wound closure rate was significantly improved in fish that were fed the ARG1 diet, compared with those in the CON group. After seven days of wound healing, the aminotransferase and creatine kinase levels in the serum and the protease and peroxidase activities in the skin mucus were down-regulated, while the immunoglobulin M level in the skin mucus was up-regulated in the ARG1 group after wounding and in the CON group before wounding. Compared with the CON diet, the ARG1 diet remarkedly depressed the gene expression of *mpo*, *il-8*, and *tnf-α*, and enhanced the gene expression of *tgf-β1*, *igf-1*, *pcna*, *krt2*, *mmp9*, *fn1α*, and *collα* and the antioxidant enzyme *cat* in the skin tissues after wounding. Furthermore, compared with both the ARG1 and the CON groups, negative effects of the ARG2 diet on wound healing were demonstrated. In conclusion, a 1% arginine supplementation facilitates skin wound healing and prevents a systemic inflammation reaction by alleviating the inflammatory response and enhancing the re-epithelialization and ECM biosynthesis in skin wound sites.

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CHAPTER II —— Paper IV

The alleviation of skin wound-induced intestinal barrier dysfunction via modulation of TLR signalling using arginine in gilthead seabream (*Sparus aurata* L)

Journal: Fish and Shellfish Immunology

ABSTRACT

The present study sought to investigate the effect of arginine on the involvement of toll-like receptors (TLRs) in skin wound-induced intestinal barrier dysfunction in gilthead seabream (*Sparus aurata* L.). Two replicates of fish (n = 8) were fed a commercial diet (CON, total 2.75% arginine), CON diet enriched with 1% arginine (ARG1, total 3.65% arginine) and 2% arginine (ARG2, total 4.53% arginine) for 30 days. Half of the fish were sampled, whereas the others were injured and sampled 7 days post-wounding. The intestinal histology results showed that a more intense infiltration of mixed leucocytes was evident in the wounded fish, which was remarkably reduced in fish that were fed the ARG1 diet. Serum IgM levels were significantly higher in the ARG1 group than levels in the CON group at 7 days post-wounding. Compared with the fish in the CON group after wounding, dietary administration of 1% arginine markedly downregulated the gene expression of TLRs (TLR2 and TLR5), *MyD88*, and proinflammatory cytokines (*CSF1R*, *IL-1β*, and *TNFα*), but significantly enhanced the gene expression of *IκBα*, the anti-inflammatory cytokine *TGF-β1,* and tight junction proteins (*tricellulin* and *occludin*) in wounded fish. Furthermore, the ARG2 diet demonstrated no additional benefits on intestinal barrier function, compared to both the ARG1 and the CON diets, and it even appeared to induce negative effects. In summary, dietary administration of 1% arginine significantly inhibited intestinal inflammatory response and tight junction disruption in skin-wounded gilthead seabream by modulating TLR signalling in the intestine.

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5. GENERAL DISCUSSION

In the past decades, the global aquaculture industry has developed rapidly. Although high-density aquaculture and the use of antibiotics have brought huge economic benefits in the initial development period, the negative effects have gradually shown over time, such as the declined animal welfare, emergences of drug-resistant strain, and drug residues. With the advancement of global anti-antibiotic abuse, non-antibiotic breeding has become an inevitable trend in the new era. The search for healthy, harmless, and eco-conscious antibiotic alternatives has become a hot spot in the aquaculture industry. Immunopotentiators used as feed additives have attracted the interest of researchers in the prevention and treatment of diseases. Probiotics and functional amino acids, which have been profoundly verified to be used as immunopotentiators in mammals, are particularly important for building a sustainable or eco-friendly aquaculture industry.

The image analysis results of the macroscopic external wounds showed that dietary administration of SP and ARG1 could significantly facilitate the wound closure. The skin wounds showed some negative changes in the metabolic parameters in the serum of gilthead seabream. Still, the administration of SP and ARG1 could significantly alleviate these impacts and maintain the homeostasis. Besides, the activities of the immune enzymes in the skin mucus were enhanced by the supplementation of SpPdp11 and 1% ARG. Unlike mammals, the blood-clot formation phase is lacking in the wound healing process of fish, although the other stages, including inflammation, re-epithelialization, new tissue formation, and remodeling, are present. The SP and ARG1 diets alleviated the inflammatory response in the skin, which might attribute to the beneficial effect of SpPdp11 and ARG1 on the antioxidant capacity in the skin. In the process of re-epithelialization, dietary administration of SpPdp11 and 1% ARG accelerate the removal of necrotic tissues, releasing growth factors from the wound matrix and helping the migration and proliferation of keratinocytes, and thereby facilitate the re-epithelialization in the skin of wounded gilthead seabream.

The impacts of skin wounds on intestinal function are multifaceted. The intestinal histology showed that more profound infiltrations of mixed leucocytes in the lamina propria were observed in the distal intestine of gilthead seabream 7 days post-wounding. The enzyme linked immunosorbent assay (ELISA) and the real-time polymerase chain reaction (RT-PCR) analysis indicated that skin wounds increased the expression of immunoglobulin level in the intestine. The present study also analyzed the gene expression of inflammatory cytokines, immune-related parameters, mucins, and tight junction proteins, as a crucial points to evaluate the health status of the intestinal barrier function of gilthead seabream. The skin wounds significantly enhanced the intestinal inflammatory response mediated by toll-like receptor (TLR)/nuclear transcription factor-κB (NF-κB) signalling pathway and the secretion of mucins but prominently suppressed the intestinal immune-related parameters and tight junction assembly. However, SP and ARG1 diet administration alleviated the profound infiltrations of mixed leucocytes in the lamina propria and the reduction of the intestinal immune function. Besides, dietary addition of SpPdp11 and 1% ARG could remarkably reduce the intestinal inflammation and over secretion of mucins in the intestinal lumen induced by the skin wound. Moreover, the expression of the tight junction proteins was also elevated.

Overall, SpPdp11 and 1% ARG dietary inclusion facilitated the wound healing and the re-epithelialization of the damaged skin, alleviated the inflammatory response in the wound area, and enhanced the neo-vascularization and the synthesis of matrix proteins in the skin wound sites of fish. Furthermore, the skin wounds provoked an intestinal inflammatory response that included changes in the mucus layer and tight junction disruptions. Besides this, the preventive administration of SpPdp11and 1% ARG alleviated the intestinal dysfunctions caused by skin wounds in gilthead seabream.

6. CONCLUSIONS

Considering the findings presented in the current Doctoral Thesis and based on the interpretation of the results obtained from the *in vivo* experiments carried out, we were able to reach the following conclusions:

1. The skin wound healing of gilthead seabream includes the processes of inflammation, re-epithelialization, and new tissue formation, and remodeling. These processes overlap in time.

2. Dietary administration of SpPdp11 or arginine accelerate the skin regeneration of the gilthead seabream. The beneficial effects are observed on the inflammation, re-epithelialization, ECM biosynthesis, and neovascularization.

3. The addition of SpPdp11 or arginine suppress the inflammatory response through the enhancement of the antioxidant capacity. The re-epithelialization is induced favouring the migration of the keratinocytes and the release of growth factors from the wound matrix cleavage by the MMPs. Moreover, dietary SpPdp11 and ARG contribute to the ECM biosynthesis and neovascularization through shh and cry61.

4. The realization of a experimental skin wound in healthy gilthead seabream impact on the intestinal barrier. Among the observed changes are those affecting the intestinal histology, an excessive secretion of mucins, the depression of the immune response, an intestinal inflammatory response, and the disruption of the intestinal tight junctions.

5. Dietary administration of SpPdp11 or arginine alleviates the intestinal barrier disfunction induced by the skin wounds in terms of the improved intestinal structure, suppressed intestinal inflammation, and recovering the normal assembly of the tight junction proteins.

6. The effects caused by the dietary administration of arginine are related to the TLR/NF-κB signalling.

6. RESUMEN EN CASTELLANO

6.1. Introducción

En las últimas décadas, la industria acuícola mundial se ha desarrollado rápidamente. Aunque la acuicultura intensiva y el uso de antibióticos supusieron enormes beneficios económicos en el período de desarrollo inicial, sus efectos negativos han ido aumentando gradualmente con el tiempo. Entre ellos está la disminución del bienestar animal, la aparición de cepas bacterianas resistentes a los medicamentos y a la acumulación de residuos de medicamentos. Debido al abuso global del uso de los antibióticos, su uso ha sido prohibido dando lugar a una tendencia que ya es inevitable. Por ello, la búsqueda de alternativas a los antibióticos, de productos que sean saludables, inofensivos y respetuosos con el medio ambiente se ha convertido en un tema candente de la acuicultura. Entre ellas, los inmunopotenciadores utilizados como aditivos en los piensos han atraído el interés de los investigadores para demostrar tanto la prevención como el tratamiento de enfermedades. En la actualidad, los probióticos y los aminoácidos funcionales, cuyo uso como inmunopotenciadores en mamíferos se ha demostrado, son particularmente importantes para construir una industria acuícola sostenible o respetuosa con el medio ambiente.

El presente estudio se centra en dos órganos cruciales, la piel y el intestino, que están en contacto directo con el medio externo y están permanentemente expuestos a él. Proporcionan una barrera estrecha contra las infecciones patógenas y coexistien con una miríada de organismos comensales. El objetivo de la presente Tesis Doctoral es explorar el efecto de la administración dietética de SpPdp11 y ARG sobre la regeneración de la piel y la función intestinal en doradas heridas. Los resultados indican que ambos suplementos podrían ayudar a reemplazar a los antibióticos y contribuir al Desarrollo de una acuicultura más segura. Los capítulos experimentales se han dividido en dos partes.

6.2. Objetivos

La presente Tesis Doctoral tiene como objetivo mejorar nuestro conocimiento sobre la regeneración de las superficies mucosas (tanto de la piel como del intestino) en la dorada herida (Sparus aurata).

1. Profundizar la comprensión del progreso de la regeneración de la piel de los teleósteos.
2. Verificar la eficacia de los aditivos SpPdp11 y arginina sobre la regeneración de la piel y la función de barrera intestinal en especímenes heridos.
3. Explorar los efectos de SpPdp11 y la arginina en las moléculas de señalización que están involucradas en la cicatrización de heridas en la piel.
4. Aclarar los impactos de las heridas de la piel en la función de barrera intestinal.
5. Explorar los mecanismos implicados en el alivio de la enteropatía mediante la inclusión dietética de SpPdp11 y arginina.

6.3. Principales resultados

En primer lugar (**Trabajos I y II**), el ensayo de alimentación se llevó a cabo para investigar los efectos de la administración dietética de SpPdp11 en la regeneración de la piel y la función intestinal en doradas heridas experimentalmente de la siguiente manera. Se alimentaron dos réplicas (n = 12) de peces con una dieta comercial (control, CON) y la dieta CON enriquecida con 10^9 ufc g^{-1} SpPdp11 (SP) durante 30 días. Posteriormente, se tomaron muestras de la mitad de los peces de cada grupo de dieta, mientras que la otra mitad resultó lesionada y continuó siendo alimentada con la misma dieta durante una semana más. En el **Trabajo I**, los resultados del análisis de imágenes de las áreas de la herida mostraron que la inclusión de SpPdp11 facilitó el cierre de la herida. Las heridas cutáneas mostraron algunos cambios negativos en los parámetros metabólicos del suero de dorada. En comparación con el grupo CON, los peces del grupo SP muestreados siete días después de la herida tenían una AST sérica significativamente menor y una relación ALB/GLOB aumentada. Además, las

actividades de proteasa y peroxidasa aumentaron significativamente en la mucosidad de la piel de los peces del grupo SP muestreados 7 días después de la herida en comparación con los de la dieta CON. Además, la dieta SP regulaba al alza la expresión génica de enzimas antioxidantes, citocinas antiinflamatorias y genes relacionados con la reepitelización en la piel de los peces. Además, se detectaron disminuciones significativas en la expresión de citocinas proinflamatorias en los peces del grupo SP, con respecto a los controles. Además, en el grupo SP se observaron la deposición de fibronectina y colágeno durante la formación y el tejido de granulación, y mejoras en la vascularización al aumentar el reclutamiento de células progenitoras endoteliales en la vasculatura de la herida. En el **Trabajo II**, se estudió la histología intestinal y la expresión génica de diferentes genes relevantes para la función de barrera intestinal. Los resultados mostraron que los peces lesionados tenían una disposición del núcleo de enterocitos desordenada, una infiltración más intensa de leucocitos mixtos y una lámina propia más gruesa en el intestino en comparación con los peces de control. Sin embargo, los peces del grupo SP+W no presentaron estos síntomas patológicos en el intestino. No se detectaron variaciones significativas en el número de células caliciformes entre los diferentes grupos experimentales. Las citocinas proinflamatorias (CSF1R, MPO e IL-1β), las mucinas (IMUC y MUC2) y IGHT se regularon al alza, mientras que la proteína de unión estrecha Occludin se reguló a la baja en el intestino de los peces del grupo CON+W. De manera similar, la administración dietética de SpPdp11 deprimió notablemente la expresión génica de las citoquinas proinflamatorias, MUC2 y IGHT, pero aumentó la expresión génica de la citoquina antiinflamatoria TGF-β1 y las proteínas de unión estrecha Tricellulin y Occludin después de la herida.

La segunda parte incluye el efecto de la administración dietética de arginina sobre la regeneración de la piel y la función intestinal en doradas heridas experimentalmente de la siguiente manera (**Trabajos II y IV**). Y se alimentaron dos réplicas de peces (n = 8) con una dieta comercial (control, CON) y la dieta CON suplementada con arginina al 1 % (ARG1) o con arginina al 2 % (ARG2) durante 30 días. Posteriormente, se tomaron muestras de la mitad de los peces de cada grupo de dieta, mientras que la otra

mitad resultó lesionada y continuó siendo alimentada con la misma dieta durante una semana más. En el **Trabajo III**, los resultados del análisis de imágenes mostraron que la tasa de cierre de heridas mejoró significativamente en los peces alimentados con la dieta ARG1, en comparación con los del grupo CON. Después de siete días de cicatrización de heridas, los niveles de AST y CK en el suero y las actividades de proteasa y peroxidasa en la mucosidad de la piel estaban regulados a la baja, mientras que el nivel de IgM en la mucosidad de la piel estaba regulado al alza en el grupo ARG1 después de la herida y en el grupo CON antes de herir. En comparación con la dieta CON, la dieta ARG1 deprimió notablemente la expresión génica de MPO, IL-8 y TNF-α y mejoró la expresión génica de TGF-β1, IGF-1, PCNA, KRT2, MMP9, FN1α y COLIα. y la enzima antioxidante CAT en los tejidos de la piel después de la herida. Además, en comparación con los grupos ARG1 y CON, se demostraron los efectos negativos de la dieta ARG2 en la cicatrización de heridas. En el **Trabajo IV**, los resultados de la histología intestinal mostraron que una infiltración más intensa de leucocitos mixtos era evidente en los peces heridos, que se redujo notablemente en los peces alimentados con la dieta ARG1. Los niveles de IgM en suero fueron significativamente más altos en el grupo ARG1 que los niveles en el grupo CON a los 7 días posteriores a la herida. En comparación con los peces en el grupo CON después de la herida, la administración dietética de arginina al 1% reguló marcadamente a la baja la expresión génica de TLR (TLR2 y TLR5), MyD88 y citoquinas proinflamatorias (CSF1R, IL-1β y TNF-α), pero significativamente mejoró la expresión génica de IκBα, la citocina antiinflamatoria TGF-β1 y las proteínas de unión estrecha (Tricellulin y Occludin) en peces heridos. Además, la dieta ARG2 no demostró beneficios adicionales sobre la función de barrera intestinal, en comparación con las dietas ARG1 y CON, e incluso pareció inducir efectos negativos.

6.4. Discusión

Los resultados del análisis de imágenes de las heridas externas macroscópicas mostraron que la administración dietética de SP y ARG1 podría facilitar

significativamente el cierre de la herida. Las heridas cutáneas mostraron algunos cambios negativos en los parámetros metabólicos del suero de dorada. Aún así, la administración de SP y ARG1 podría aliviar significativamente estos impactos y mantener la homeostasis. Además, las actividades de las enzimas inmunes en el moco de la piel se vieron reforzadas por la suplementación de SpPdp11 y 1% ARG. A diferencia de los mamíferos, la fase de formación de coágulos de sangre falta en el proceso de curación de heridas de los peces, aunque están presentes las otras etapas, que incluyen inflamación, reepitelización, formación de tejido nuevo y remodelación. Las dietas SP y ARG1 aliviaron la respuesta inflamatoria de la piel, lo que podría atribuirse al efecto beneficioso de SpPdp11 y ARG1 sobre la capacidad antioxidante de la piel. En el proceso de reepitelización, la administración dietética de SpPdp11 y ARG al 1% aceleran la eliminación de tejidos necróticos, liberan factores de crecimiento de la matriz de la herida y ayudan a la migración y proliferación de queratinocitos, y por lo tanto facilitan la reepitelización en la piel de dorada herida.

Los impactos de las heridas de la piel en la función intestinal son multifacéticos. La histología intestinal mostró que se observaron infiltraciones más profundas de leucocitos mixtos en la lámina propia en el intestino distal de doradas 7 días después de la herida. El ensayo inmunoabsorbente ligado a enzimas (ELISA) y el análisis de la reacción en cadena de la polimerasa en tiempo real (RT-PCR) indicaron que las heridas en la piel aumentaron la expresión del nivel de inmunoglobulina en el intestino. El presente estudio también analizó la expresión génica de citocinas inflamatorias, parámetros relacionados con la inmunidad, mucinas y proteínas de unión estrecha, como puntos cruciales para evaluar el estado de salud de la función de barrera intestinal de la dorada. Las heridas en la piel mejoraron significativamente la respuesta inflamatoria intestinal mediada por la vía de señalización del receptor tipo toll (TLR)/factor de transcripción nuclear-κB (NF-κB) y la secreción de mucinas, pero suprimieron de manera prominente los parámetros intestinales relacionados con la inmunidad y el ensamblaje de las uniones estrechas. Sin embargo, la administración de la dieta SP y ARG1 alivió las profundas infiltraciones de leucocitos mixtos en la lámina

propia y la reducción de la función inmune intestinal. Además, la adición dietética de SpPdp11 y ARG al 1% podría reducir notablemente la inflamación intestinal y la sobresecreción de mucinas en la luz intestinal inducida por la herida de la piel. Además, también se elevó la expresión de las proteínas de unión estrecha.

En general, la inclusión en la dieta de SpPdp11 y ARG al 1 % facilitó la cicatrización de heridas y la reepitelización de la piel dañada, alivió la respuesta inflamatoria en el área de la herida y mejoró la neovascularización y la síntesis de proteínas de matriz en los sitios de herida de la piel de pescado. Además, las heridas en la piel provocaron una respuesta inflamatoria intestinal que incluyó cambios en la capa de moco y alteraciones en las uniones estrechas. Además, la administración preventiva de SpPdp11 y ARG al 1% alivió las disfunciones intestinales provocadas por heridas cutáneas en doradas.

6.5. Conclusiones

Considerando los hallazgos presentados en esta presente Tesis Doctoral y en base a la interpretación de los resultados obtenidos de los experimentos in vivo realizados, pudimos llegar a las siguientes conclusiones:

1. La cicatrización de heridas en la piel de la dorada incluye los procesos de inflamación, reepitelización y formación y remodelación de tejido nuevo. Estos procesos se superponen en el tiempo.

2. La administración dietética de SpPdp11 o arginina acelera la regeneración de la piel de la dorada. Los efectos beneficiosos se observan sobre la inflamación, la reepitelización, la biosíntesis de ECM y la neovascularización.

3. La adición de SpPdp11 o arginina suprime la respuesta inflamatoria a través de la potenciación de la capacidad antioxidante. Se induce la reepitelización favoreciendo la migración de los queratinocitos y la liberación de factores de crecimiento de la matriz

de la herida escindida por las MMP. Además, ambos aditivos contribuyen a la biosíntesis y neovascularización de la MEC a través de shh y cry61.

4. La realización de una herida experimental en la piel de una dorada sana es suficiente para afectar a la barrera intestinal. Entre los cambios observados están los que afectan la histología intestinal, una secreción excesiva de mucinas, la depresión de la respuesta inmune, una respuesta inflamatoria intestinal y la disrupción de las uniones estrechas intestinales.

5. La administración dietética de SpPdp11 o arginina alivia la disfunción de la barrera intestinal inducida por las heridas de la piel en términos de estructura intestinal mejorada, inflamación intestinal suprimida y recuperación del ensamblaje normal de las proteínas de unión estrecha.

6. Los efectos causados por la administración dietética de arginina están relacionados con la señalización TLR/NF-κB.

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