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Differentially expressed immune genes in response to *Streptococcus agalactiae* infection in Nile Tilapia

Faculdade de Ciências e Tecnologia

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Statement of authorship

I hereby declare that I am the author of this work, which is, to the best of my knowledge and belief, original, except as acknowledged in the text. Authors and work consulted are properly cited in the text and listed in the references in the required format. The material has not been previously submitted, in whole or in part, for a degree at this or any other university.

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Signature

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"Aquaculture is a risky business. You don't go into aquaculture
for the money. You do it because you love it."
Michael Matthews

Abstract

As the world population grows it becomes harder to fulfil the demand for protein. Aquaculture, an activity that consists in the farming of aquatic organisms, is one of the most important global sources of fish. Asia, and particularly China, represent almost 70% of this production, although, one of the biggest obstacles to the development of the aquaculture industry is infections and mortality caused by pathogens. All around the world, Nile tilapia (*Oreochromis niloticus*) emerges as an economically important species. Even though it is an alien organism in Asia, the Chinese aquaculture of this fish represents almost 50% of the world total production. Also known as group B streptococcus (GBS), *Streptococcus agalactiae* is a Gram-positive pathogenic bacterium. This pathogen is not only a problem for modern aquaculture, but it affects a broad range of hosts including humans and other mammals, and a wide range of fish species including the Nile tilapia. The fish gills are an important immune barrier, and part of the first line of defence against pathogens as physical contact is the first step for pathogen infection. This study aimed to identify immune related genes and characterize the Nile tilapia immune response to *S.agalactiae* infection in the gill using RNA-Seq. Nile tilapia gills were collected 6, 9, 15 and 18 hours post infection, and their isolated RNA was sequenced. A differential gene expression and functional enrichment analysis was performed. At 6, 9, 15, and 18 hours post infection 2122, 1851, 1791, 2395 differentially expressed genes were identified. These represented a significant enrichment of different immune related pathways in response to the pathogen infection such as the Cytokine-cytokine signalling pathway, the Toll-like receptor pathway, the NOD-like receptor pathway, the phagosome pathway, and others. These results provide an insight into the response to an important bacterial pathogen as a basis for management of infection in aquaculture.

Keywords: Nile Tilapia, Immune response, *Streptococcus agalactiae*, Transcriptomics, Gill

Resumo

Com o aumento da população, torna-se difícil responder às necessidades nutricionais da mesma. A aquacultura, atividade que consiste no cultivo de organismos aquáticos, surge nos dias que correm como uma importante fonte de proteína animal. A Ásia, e particularmente a China, representam quase 70% dessa produção, embora um dos maiores obstáculos para o desenvolvimento da indústria da Aquacultura e sua produção sejam os agentes patogénicos. A produção de tilápia do Nilo (*Oreochromis niloticus*), uma das espécies economicamente mais relevantes, representa quase 50% da produção total mundial. *Streptococcus agalactiae* uma bactéria Gram-positiva também conhecida como Streptococcus do grupo B, é atualmente um problema para a aquacultura moderna, mas não só. Esta bactéria afeta uma ampla gama de hospedeiros, incluindo humanos e outros mamíferos, e uma ampla gama de espécies de peixes, incluindo a tilápia do Nilo. Como parte integrante do sistema imune dos peixes, as branquias são uma das barreiras do sistema imune que constituem parte da primeira linha de defesa contra patógenos. Este estudo teve como objetivo identificar genes relacionados com a resposta imunológica e caracterizar a resposta imune da tilápia do Nilo à infeção por *S.agalactiae*, ao nível do transcriptoma branquial. As brânquias de tilápias do Nilo juvenis foram coletadas 6, 9, 15 e 18 horas após a infeção, e o respetivo RNA isolado foi sequenciado por sequenciação de última geração. Foram realizadas analises de expressão genética diferencial e enriquecimento funcional. Foram identificados 2122, 1851, 1791, 2395 genes diferencialmente expressos, 6, 9, 15 e 18 horas após a infeção, respetivamente. Os resultados apresentaram um enriquecimento significativo de diferentes vias imunológicas relacionadas com a deteção e resposta à infeção por esta bactéria, como a via de sinalização de citocina-citocina, a via do recetor Toll-like, a via do recetor semelhante a NOD, a via do fagossoma, entre outras. Estes resultados fornecem uma visão sobre a resposta a uma importante bactéria patogénica como base para o controlo da sua infecção na aquacultura.

Palavras-chave: Tilapia, Imunidade, *Streptococcus agalactiae*, Transcriptomica, Branquia

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List of Abbreviations and acronyms

FAO – Food and Agriculture Organization

GBS – Group B streptococcus

CFU – Colony forming units

BHI – Brain heart infusion

hpi – Hours post injection

PCR – Polymerase chain reaction

GIFT – Genetically improved farmed tilapia

DEG – Differentially expressed gene

BP – Biological process

MF – Molecular function

CC – Cellular component

CAM – Cell adhesion molecules

TLR – Toll-like receptor

RNA – Ribonucleic acid

DNA – Desoxyribonucleic acid

cDNA – Complementary desoxyribonucleic acid

GO – Gene ontology

bp – Base pairs

PBS – Phosphate buffered solution

mRNA – Messenger ribonucleic acid

ncRNA – Noncoding ribonucleic acid

P – Phosphate buffered solution injection

B – Bacterial injection

RNA-seq – Ribonucleic acid sequencing

KEGG – Kyoto Encyclopedia of Genes and Genomes

PAMP – Pathogen-associated molecular pattern

PRR – Pattern recognition receptors

NOD - Nucleotide-binding Oligomerization Domain

°C – degrees Celsius

µL – microliter

h – hours

g – grams

ml – milliliter

L – liter

cm – centimeters

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CHAPTER I

Introduction

Introduction

As the world population grows it becomes harder to fulfil the demand for protein. A total of 9 billion humans is expected on Earth by 2050 and it will be a problem to feed them (FAO, 2016). In 2017, 17% of the animal protein globally consumed came from fish and this percentage is growing, which makes it a valuable source to fight the future needs for sustenance. In 2018 the world has reached a fish consumption of 20.5 kilograms per capita (FAO, 2020).

Aquaculture, an activity that consists in the farming of aquatic organisms, started thousands of years ago and is the fastest growing food production sector, according to the United Nations Food and Agriculture Organization (FAO, 2020). The first evidence of fish culture goes back eight thousand years in China, with the domestication of the common carp (*Cyprinus carpio*) (Nakajima *et al.,* 2019).

With capture fisheries, aquaculture is one of the most important global sources of fish. It provides today 52% of the fish consumed around the world. Thanks to both sources, in 2018 the world fish supply for human nutrition reached 156 million tonnes (FAO, 2020). The number for wild capture has remained relatively static since the 80's, and the growth in fish supply is from aquaculture production. Over the last decades, aquaculture has improved at an impressive rate and because of that the fish production increased while prices were kept relatively low, making it more available to the consumers (FAO, 2016, 2020). Aquaculture is concentrated in tropical (23ºN to 23ºS) and subtropical (24-40ºN and 24-40ºS) regions and globally, the inland production of freshwater finfish predominates, achieving 30% of the global fish supply in 2006 and approximately 65% of world aquaculture production (De Silva and Soto, 2009; FAO,2020).

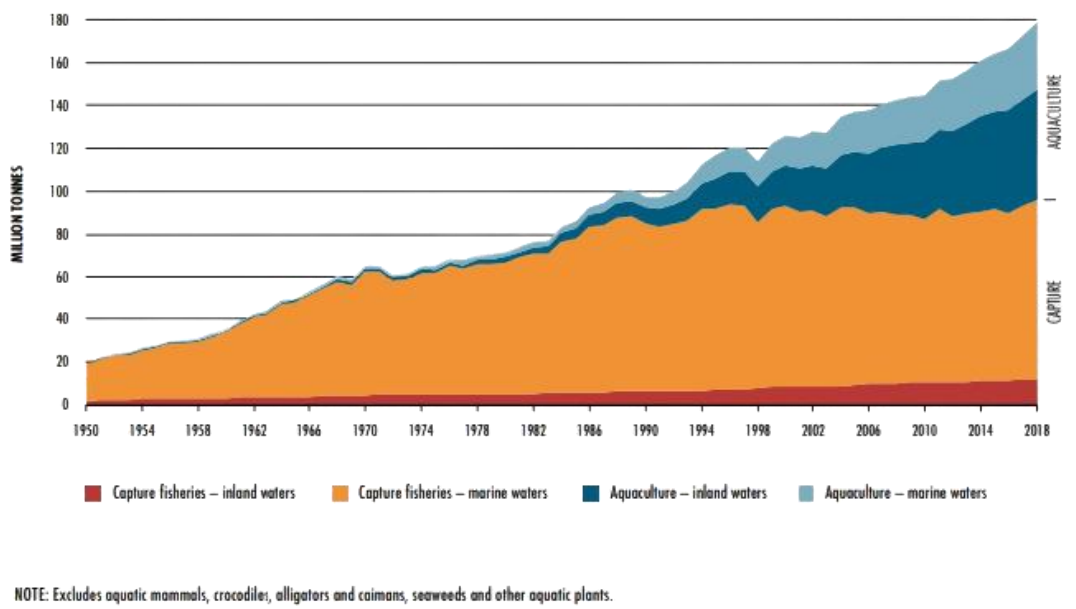

Fig. 1.1 Global fish captured or produced in aquaculture in million tonnes between 1950 and 2018. Source: FAO (2020).

Inland freshwater aquaculture represents an important source of food for populations inhabiting rural areas in Asia, where it can help fight poverty and malnutrition (Bailey and Skladany, 1991). Asia, and particularly China, represent almost 70% of the world production (FAO, 2020). More specifically, in 2014 China produced 45.5 million tonnes (FAO, 2016). In 2018, this country contributed with almost 60% of the aquatic cultured food and 35% of the world fish production.

Among the most produced finfish species in China and in the world are the grass carp (*Ctenopharyngodon idellus*), the silver carp (*Hypophthalmichthys molitrix*), the common carp (*Cyprinus carpio*) and the Nile tilapia (*Oreochromis niloticus*). All of them belong to inland freshwater ecosystems and together they correspond to more than 35% of the global finfish aquaculture (FAO, 2020).

One of the biggest obstacles to the development of Aquaculture industry is mortality caused by infectious pathogens. The losses caused by pathogen outbreaks reach 10% of total cultured organisms and in some cases, there is no cure, therapy, or prophylaxis (Heppell *et al.*, 2000).

1.1 Nile tilapia (*Oreochromis niloticus*)

All around the world, Nile tilapia (*Oreochromis niloticus*) emerges as an economically important species for Aquaculture. Originally from Africa and the Middle east regions, tilapias (*Oreochromis sp.*) belong to the third largest family, the *Cichlidae* (Trewaves, 1983), within the class Osteichthyes. Its first appearance in China goes back to 1956, imported from Thailand, but the industrial production of this species started with the Nile tilapia (*Oreochromis niloticus*) aquaculture in 1978, owing to appropriate climatic and environmental conditions (Yuan *et al.*, 2017). Although an alien to Asia, the Chinese aquaculture of this fish represents almost 50% of the world total production (Zhu *et al.*, 2017), thrice the quantity of the next leading species, being cultivated normally in freshwater ponds (Wang *et al.*, 2015). This tropical species prefers to live in warm shallow waters, at temperatures around 25ºC and can reach sexual maturity at 5-6 months old in ponds (Beveridge and McAndrew, 2000; Lacerda *et al.*, 2006). Different characteristics make this species a suitable candidate for large-scale aquaculture: fast growth and easy reproduction, feeding versatility, and resistance to poor water conditions (Nandlal & Pickering, 2004). However, its resistance to pathogens, which was considered to be high, has been called into question (Klesius *et al.*, 2008; Amal *et al.*, 2011). It is now accepted that Nile tilapia is susceptible to both bacterial and parasitic diseases, such as *Streptococcus* sp., *Flavobacterium columnare*, *Aeromonas hydrophila*, *Edwarsiella tarda*, *Ichthyophitirius multifillis*, *Tricodhina* sp., and *Gyrodactylus niloticus* (Klesius *et al.*, 2008). This species is particularly susceptible to one of the biggest problems affecting the southern Chinese aquaculture production, the bacterium *Streptococcus agalactiae*. This infectious disease causes high mortality and economical loss in fish farms (Zhu *et al.*, 2017).

1.2 *Streptococcus agalactiae*

Also known as group B streptococcus (GBS), *Streptococcus agalactiae* is a Gram-positive pathogenic bacterium. This pathogen is not only a problem for modern aquaculture, but it affects a broad range of hosts including humans and other mammals, and a wide range of fish species including the Nile tilapia (Zhu *et al.*, 2017). The infection by this pathogen causes meningoencephalitis, perceptible by symptoms such as septicaemic infection, exophthalmia, corneal opacity, and different swimming abnormalities. There is a worldwide concern with the outbreaks of this zoonosis causative agent, and it has already reached infection rates of 50% and mortality rates of 95% (Ye *et al.*, 2011). The outbreaks of this infection occur

mainly at high temperatures, above 26ºC, high stocking densities, and intensive production seems to potentiate the occurrence of this infection (Mian *et al.,* 2009). There is still a lack of research about this subject and the immune mechanisms against this infection are not yet understood, preventing the development of effective measures to control and prevent this disease (Zhu *et al.,* 2017).

1.3 The immune response

The immune response is a physiological mechanism of great importance in defence against infections and in the preservation of internal homeostasis (Saurabh & Sahoo, 2008). Physiological changes caused by stress conditions affect metabolism and cellular processes, including immune processes, compromising the innate response, and consequently increasing the likelihood of disease (Ellis, 2001).

The immune system protects organisms through cells and molecules responsible for the immune response. Healthy individuals protect themselves from diseases through this system that identifies and kills a wide variety of pathogens, such as viruses, bacteria, parasites or even tumour cells (Litman *et al.,* 2005). There are two types of immune system, the innate immune system and the specific or adaptive immune system. The innate immune system provides an immediate but not specific response and can be found in all animals and plants (Medzhitov & Janeway, 1997; Litman *et al.,* 2005), being composed by soluble factors and different types of cells (Raven, 2014). The adaptive response occurs through mechanisms involving a complex network of specialized cells, proteins, genes, and biochemical messages that provide the body with the necessary means to specifically react with antigens, antibodies (immunoglobulins) and effector cells with high specificity and affinity (den Haan *et al.,* 2014, Uribe *et al.,* 2011). This information is stored in the form of immune memory, which allows the body to prepare a stronger and faster defence in a future similar situation (Mayer, 2006). Adaptive immunity is characterized by genetic rearrangements that generate a diverse set of molecules capable of recognizing virtually any invading pathogen (Raven, 2014).

It has long been believed that the response of vertebrates to microbial invasions was divided into specific and non-specific defence (Raven, 2014). It is now thought that both responses are embedded in vertebrates as a single immune system, since the innate response precedes and is required in the adaptive response (Medzhitov & Janeway, 1997; Litman *et al.,* 2005).

1.4 Fish Immune system

Within the fish immune system two types of mechanism of defence against pathogens can be found, humoral and cellular. Within the non-specific humoral response factors, there are substances that inhibit the growth of bacteria, such as anti-proteases, lysins, such as lysozyme, proteases, antimicrobial peptides and complement factors (Ellis, 1999). These can be found in the plasma and mucus of the skin, gut and gills, preventing adhesion and colonization of microorganisms (Alexander & Ingram, 1992), with the last three being the mucosal tissues directly linked to the immune response (Kiron, 2012). Also, these tissues contain immunocompetent cells like leukocytes and intraepithelial plasmatic cells (Moore *et al*., 1998).

The mucus that covers the surface of the epidermis is the first line of defence against invading pathogens, also containing humoral immune response factors. The epidermal layer of the skin secretes mucus, in addition to providing physical protection (Dalmo *et al.,* 1997; Kiron, 2012; Guardiola *et al.,* 2014). The rate of mucus secretion may increase in response to an infection (Nilsen, 1995). Certain organisms can bypass the skin mucus antimicrobial action and permeate host tissues, causing disease to develop. When pathogens penetrate through physical barriers, fish activate biological responses, including specialized cells capable of destroying and digesting the invaders (Aoki *et al*. 2008). Granulocytes, monocytes or macrophages, lymphocytes (including T and B cells), and non-specific cytotoxic cells are the key biological components engaged in immunological defense against microbial invasion (Neumann *et al*. 2001).

If pathogens pass this first line of defence and reach circulation, a set of bloodborne substances originated in the anterior kidney and spleen are activated (Press & Evensen, 1999). These organs have a key function in the functioning and regulation of the immune system, participating in the immunoendocrine and neuroimmunoendocrine interactions (Tort *et al.,* 2003).

Innate responses can block or deter microbial infections until a more effective adaptive immune response is formed. It is significant for a variety of reasons, one of which is the specific acquired immunity system, which is temperature dependent and takes a longer time to respond (Magnadottir 2006).

It is important to notice that being the first line of defence, the innate immune system is responsible for the detection of the pathogen. That detection is possible through the action of a group of receptors called pattern recognition receptors (PRRs) (Medzhitov & Janeway, 1997). These receptors recognize the presence of pathogen-associated molecular patterns (PAMPs), such as peptidoglycans, lipoteichoic acid, lipoproteins, among others (Mogensen, 2009). This information is transmitted to the relevant lymphocytes, activating the intracellular signalling flow, and inducing humoral and cellular immune response. One example of this response is the expression of proinflammatory molecules. This process constitutes the host first response to the infection and promotes the activation of the adaptive immunity (Covello *et al.,* 2009).

Teleost fish have a diverse set of PRRs that play a role in the immunological response to infections. Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and PGRPs (peptidoglycan recognition proteins) are the four primary types of PRRs that have been identified in fish (Boltaña *et al*. 2011). The TLR family, which includes membrane and endolysosomal receptors, is a well-known family (Kaway & Akira 2010). More than 21 TLRs have been discovered so far in teleost fish (Liao *et al*., 2017). It is thought to be the most important group of recognition receptors involved in inflammation. Boltaña *et al*. (2011) reported that all TLRs include an external domain with a series of leucine-rich repeats (LRRs) and an intracellular region with a common signaling module known as a Toll/IL-1 receptor (TIR) domain. Many phagocytic cells, such as macrophages, neutrophils, and dendritic cells, express TLRs, which identify specific components of bacteria, viruses, fungi, and protozoa to activate them (Akira *et al*. 2006). TLRs' primary function in mammals is to trigger inflammatory pathways such as NFkB, IFN-regulatory factor (IRF), and transcription factors (Kawai & Akira 2007). These molecules control the release of cytokines and chemokines such IL-1, IL-6, TNF-, CC-1, and MCP-1, which produce an initial inflammatory response in response to invading pathogens (Taylor *et al*. 2005).

NLRs are cytosolic receptors that play a role in autoimmunity. They may identify a variety of pathogens, including bacteria and viruses, as well as some apoptotic processes (Inohara *et al*. 2005).

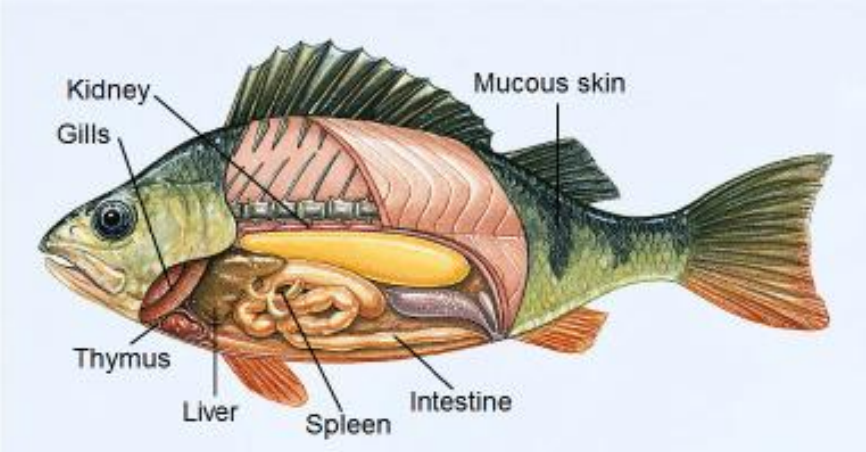

Fig. 1.2 Immune structures in teleost fish. Source: Kum and Sekkin (2011)

1.5 Fish Gills

The fish gill is more than a respiratory tissue. It plays an important role in many different biological functions, such as osmotic regulation, nitrogen compounds excretion, ion exchange, and others (Hughes, 1984). As stated before, gills, are also an imperative immune barrier, and part of the first line of defence against pathogens as physical contact is the first step for pathogen infection (Zhou *et al.,* 2020). This mucous covered tissue is in constant contact with the environment and pathogens like bacteria, fungi, viruses, and others may expose the gill to immune challenges and lead to an immune response (Wang *et al.,* 2015; Koppang *et al.,* 2015). This response aims to resist the infection through a series of cellular and biological processes (Zhou *et al.,* 2020).

A few studies have already investigated the action of *S.agalactiae* at the spleen and kidney level but there is still a lack of information on the role of the gill on the immune response to this pathogen (Abrahams, 2011; Cho *et al.,* 2003; Yamamoto-Furusho *et al.,* 2010). A previous study in young grass carp (*Ctenopharyngodon idella)* demonstrated the imparity in the immune response and even fish growth disturbance associated with gill malfunction, both as immune defence and as a first line of defence (Chen *et al.,* 2015). The innate immunity function of fish gills has already been described by Rebl *et al.,* (2014), referring the important roles of antibacterial compounds such as the acid phosphatase, lysozyme, complement, and antimicrobial peptides.

1.6 Transcriptomics

With the development of Next-Generation Sequencing (NGS), new doors were open within the evolutionary biology research (da Fonseca *et al.,* 2016). This kind of technology allows not only genome studies but also large-scale transcriptome analysis, or transcriptomics. This kind of procedure is called RNA sequencing (RNA-seq) (Qian *et al.,* 2014). Transcriptomics allows the identification and characterization of the immune related genes and the pathways involved in the immune response by comparison of transcriptomes with and without immune challenges. This technology can help to understand the origin and evolution of immune system enabling the creation of immune-based therapy for fish diseases and to select disease-resistant fish broodstocks (Qian *et al.,* 2014).

The transcriptome is basically the total amount of transcripts in a cell, tissue or organism, including messenger (mRNAs) and non-coding RNAs (ncRNAs), whose function is to regulate gene expression maintaining cellular homeostasis (Lindberg and Lundeberg, 2010). It changes with external environment, physiological conditions and developmental stage, and it contains splicing isoforms, gene-fusion transcripts, post-translational modifications and epigenetic controls (Mastoridis *et al.,* 2015).

Transcriptomics is a strong tool in the unrevealing of the complex interaction linking genotype and phenotype allowing a better understanding of the molecular mechanisms that control the development and function of the cell, healthy or diseased (Wang *et al.,* 2015). In terms of teleost fishes' transcriptomics, not only the model species benefit from RNA-seq, but the non-model ones too, with both having achieved tremendous advances in the past years. This technology allows to map and annotate fish transcriptome, and also to understand many biological processes, such as development, adaptive evolution, host immune response, and stress response (Qian *et al.,* 2014).

1.7 Objective

There is a lack of information on the gene expression profile of Nile tilapia gills infected with *Streptococcus agalactiae*. This study aims to identify immune related genes and characterize the Nile tilapia (*Oreochromis niloticus*) immune response to *Streptococcus agalactiae* infection, at the gill whole transcriptome level, creating new insights on the molecular mechanisms involved in the host pathogen interaction.

CHAPTER II

Materials and Methods

2. Material and methods

2.1 Experimental design

The Genetically Improved Farmed Tilapia (GIFT) strain, Nile Tilapia (*O. niloticus*), used in this study was provided by the Guangxi Academy of Fishery Sciences with a weight and total length of 5.85±0.63g and 3.34±1.05 cm, respectively. The fish were acclimated for 14 days after the transport and kept at 32 ± 0.5ºC with constant aeration. The Shanghai Ocean University disease lab supplied the bacteria *S. agalactiae* isolate to posteriorly inject the fish. The pathogen concentration was estimated through the plate count method to determine the number of colony-forming units (CFU) per mL. Briefly, 10 ml of 10-fold serial dilutions were inoculated to Brain Heart Infusion (BHI) 1.5% agar plates in triplicates. The plates were incubated for 20 hours at 37ºC, the CFU in each plate were then counted. The whole procedure was performed under an aseptic environment.

The fish were randomly separated into two 30 L tanks, each with 50 juveniles. Before the bacterial challenge, 5 control fish from each tank were euthanized in an ice bath. The second and third gill arch were dissected and immediately frozen in liquid nitrogen before being stored at -80ºC. The remaining fish from one of the tanks were intraperitoneally injected with 1×10^5CFU of *S. agalactiae* in BHI broth, while the fish from the other tank were injected with 10 µl of a PBS solution. The second and third gill arches from 5 organisms in both treatments were collected 6, 9, 15 and 18 hpi (hours post injection) after euthanasia through ice bath and immediately frozen in liquid nitrogen before being stored at -80ºC.

2.2 RNA extraction and isolation

Before the RNA isolation, the tissues were homogenized under liquid nitrogen with a mortar and pestle and kept in a tube with lysis buffer. The total RNA extraction of each tissue was performed using a commercial kit from Tiangen, the RNAsimple Total RNA Kit, following the manufacturer's instructions. All the samples were kept in ice during the procedure and stored at -80ºC until used. A quality analysis was carried for each RNA sample resorting to an Agilent Bioanalyzer Chip RNA 7500 series II and their concentration and quality was assessed with a NanoDropTM 2000 Spectrophotometer.

2.3 RNA sequencing

The mRNA-seq libraries were prepared with three micrograms of RNA from a pool of five samples, using a commercial kit from Illumina, the TruSeq RNA Sample Prep Kit, following the manufacturer's instructions. The libraries were then indexed. Briefly, poly-A-containing RNAs were purified and fragmented at high temperatures using divalent cations. First-strand cDNA was synthetized using the resulting fragments of RNA as templates, using random primers, before second-strand cDNA production and end-repair. The cDNA fragments were flanked by Illumina PE adaptors. The fragments were measured in a 2% low-range ultra-agarose sizing gel, only those with approximately 300 bp were collected. The cDNA fragments already tagged with the adaptor were enriched using the producer's mix and 10 cycles of PCR. To assess the quality and size of the library, a commercial kit was used, the DNA High Sensitivity DNA Kit (Bioanalyzer 2100, Agilent), guarantying a size selection of 300 to 500 bp. Nine sequencing libraries were created from the pools of 5 samples each of gill RNA (C_0h, P6h, P9h, P15h, P18h, B6h, B9h, B15h, and B18h). The C0h refers to the initial time point, previous to any injection. The P and B letters define the PBS and bacterial injection, respectively. The numbers that follow those letters represent the time post injection in hours. Sequencing was carried out in a Illumina HiSeq2000 sequencer.

2.4 RNA reads processing and mapping

Unwanted reads, such as adapter containing, poly-N containing and low quality reads, were removed from the raw data through in-house Perl scripts, to obtain clean reads. The Q20, Q30, GC-content, and sequence duplication level of the clean data were evaluated. The following analysis considered only the high quality clean data. The reference genome from *Oreochromis niloticus* was used to map the reads, each sample separately, with the HISAT2 (Kim *et al*., 2019) program using the default settings.

2.5 Gene Annotation

The gene and protein sequences used for annotation were retrieved from the ensemble database (http://.asia.ensembl.org/info/data/ftp/index.html). The following protein databases were used for gene function annotation according to an e-value threshold of 10-5 by BLASTP algorithm: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot

(A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology). The KASS-KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas/) was used for KEGG (Kyoto encyclopaedia of genes and genomes) annotation.

2.6 Differential expression analysis and enrichment

The EBseq R package was used to analyze the differential gene expression (DEG) between two samples, according to the following threshold: FDR < 0.05 and Fold Change ≥ 2. The GOseq R packages were used for Gene Ontology enrichment analysis of the DEG according to the Wallenius non-central hypergeometric distribution (Young *et al*., 2010). The Kyoto Encyclopedia of Genes and Genomes pathways was analyzed through KOBAS web server (Mao *et al.*, 2005).

CHAPTER III

Results

3. Results

3.1 RNA sequencing results

From the nine libraries, a total of 445,203,168 clean reads were produced and deposited to the National Centre for Biotechnology Information (NCBI) with the accession number of PRJNA751364. The percentage of mapped reads by sample varied between 80.04 and 86.34%. All the information above, as well as the GC, Q20 and Q30 ratio are represented in the Table 3.1. All samples had a Q20 ratio ≥ 97.41 and a Q30 ratio ≥ 93.65.

Table 3.1 Mapping ratio of the sequenced libraries to the Nile tilapia (*Oreochromis niloticus*) genome.

Sample	**Total clean reads**	**Total mapped reads**	**GC (%)**	**Q20 (%)**	**Q30 (%)**
Gill-0h	54,708,466	43,788,477 (80.04%)	48.76	97.41	93.67
Gill-B6h	52,297,476	43,565,354 (83.30%)	48.80	97.55	93.92
Gill-B9h	36,636,616	31,095,881 (84.88%)	49.68	97.62	94.00
Gill-B15h	46,424,536	38,965,528 (83.93%)	49.19	97.58	93.91
Gill-B18h	53,185,078	45,678,704 (85.89%)	48.26	97.77	94.23
Gill-P6h	47,948,880	41,022,046 (85.55%)	49.37	97.85	94.40
Gill-P9h	47,849,040	41,310,833 (86.34%)	48.68	97.85	94.44
Gill-P15h	51,421,080	43,273,464 (84.16%)	49.78	97.57	94.03
Gill-P18h	54,731,996	46,234,883 (84.48%)	49.50	97.43	93.65

3.2 Differential gene expression analysis

DEG's were obtained for each comparison between bacterial challenge the bacterial injection (B) and PBS injection (P) that served as a control at each time point (6hpi, 9hpi, 15hpi and 18hpi). Between the B6 and P6 samples, 2122 DEGs were identified, 1074 of them upregulated and 1048 downregulated. Between the B9 and P9 samples, 1851 DEGs were identified, of which 816 were upregulated and 1035 downregulated. Between samples B15 and C15

samples1791 DEGs were identified of which 822 were upregulated and 969 were downregulated. Finally, between samples B18 and C18 2395 DEGs were identified, of which 1134 upregulated and 1261 downregulated. The Venn diagram representing the intersection of all the DEGs identified in these four comparisons, and the volcano-plots according to their fold change can be seen in Fig. 3.1, in which a great number of uniquely expressed unigenes, in each time point, was identified. A hierarchical clustering of the top 20 DEGs was represented by a heatmap based on the gene expression patterns of all the samples (Fig. 3.2).

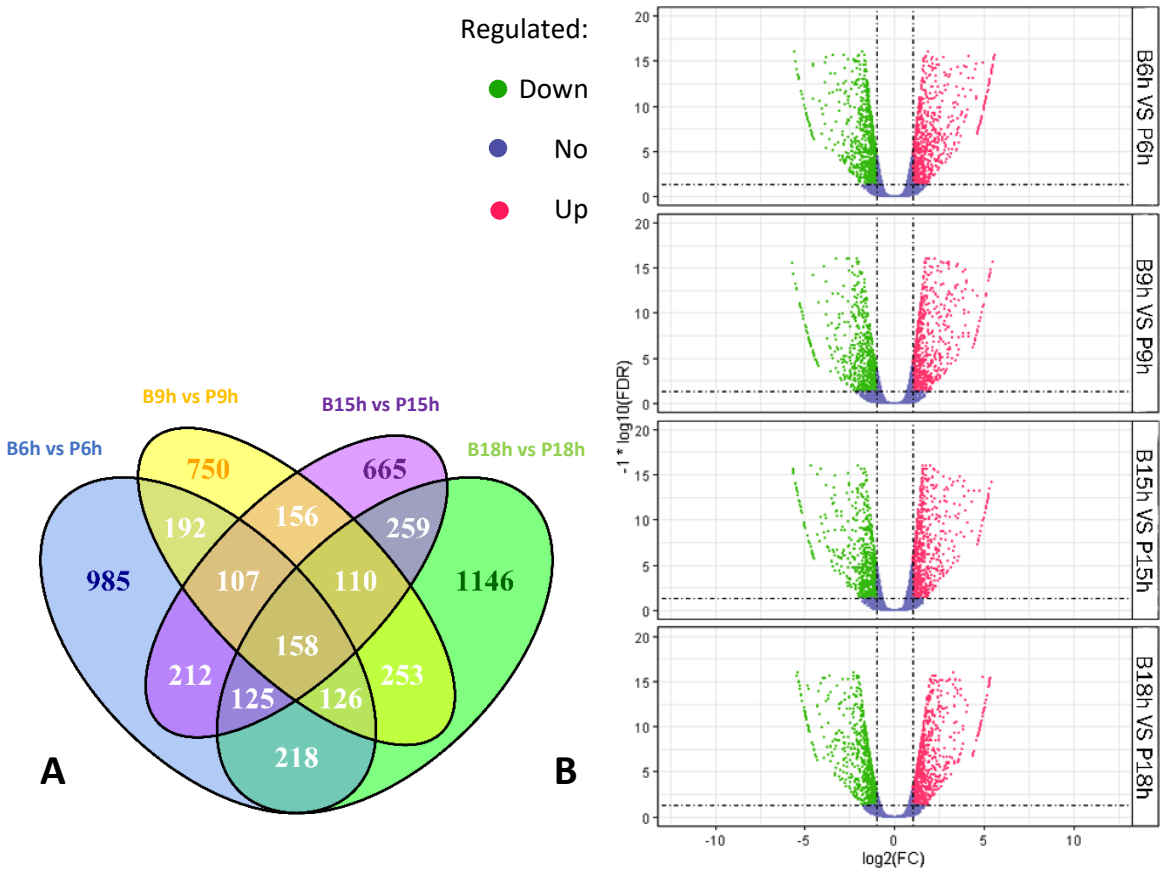

Fig. 3.1 (A) Venn diagram representing the intersection of all the DEGs present in the four group comparisons (B6h vs P6h, B9h vs P9h, B15h vs P15h, B18h vs P18h). (B) Volcano Plot of the distribution of differently expressed genes between the two treatments at four time points. The $\log_2$ fold change indicates the mean expression level for each gene. Each dot represents one gene. Green (downregulated) and red (upregulated) dots represent differentially expressed genes (DEGs). Black dots represent non-differentially expressed genes.

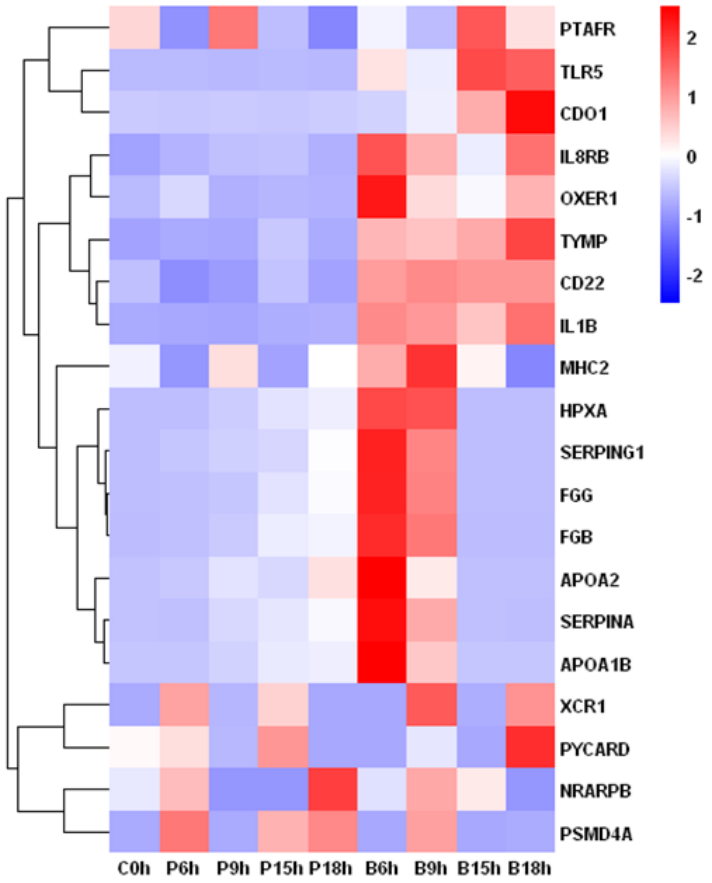

Fig. 3.2 Hierarchical clustering of the 20 most differentially expressed genes in the 9 samples. Heatmap of the differentially expressed genes between C0h, P6h, P9h, P15h, P18h, B6h, B9h, B15h, and B18h groups. Red represents upregulated and purple represent downregulated genes.

3.3 GO enrichment analysis

To improve the detail and further analyse the relevant function of the DEGs, gene ontology enrichment and KEGG pathway analysis were carried out. GO enrichment analysis was performed for the 4 time point comparisons at the level of Cellular component (CC), Biological process (BP) and molecular function (MF). Comparing the two treatments at 6hpi and 9hpi, it was possible to assign by GO classification, 833 and 747 DEG's, respectively. At 15hpi, a total of 677 DEGs were corresponded. At 18hpi, 983 DEGs were assigned. Figure 3.3 presents the most significantly annotated GO terms in the four time points. In terms of CC, the most represented classifications were Membrane and its respective integral components (GO:0016020 and GO:0016021, respectively) in all comparisons. The most significantly enriched BP, at all time points were Immune response (GO:0006955), followed by the negative regulation of endopeptidase activity (GO:0010951) at 6hpi and cell chemotaxis (GO:0060326) at all the other time points. Other immune related biological processes not present in the figure

were also significantly enriched. For example, the innate immune response (GO:0045087), the antigen processing and presentation (GO:0019882) and the complement activation (GO:0006956). As for MF, Catalytic activity (GO:0003824) was the most significantly enriched term.

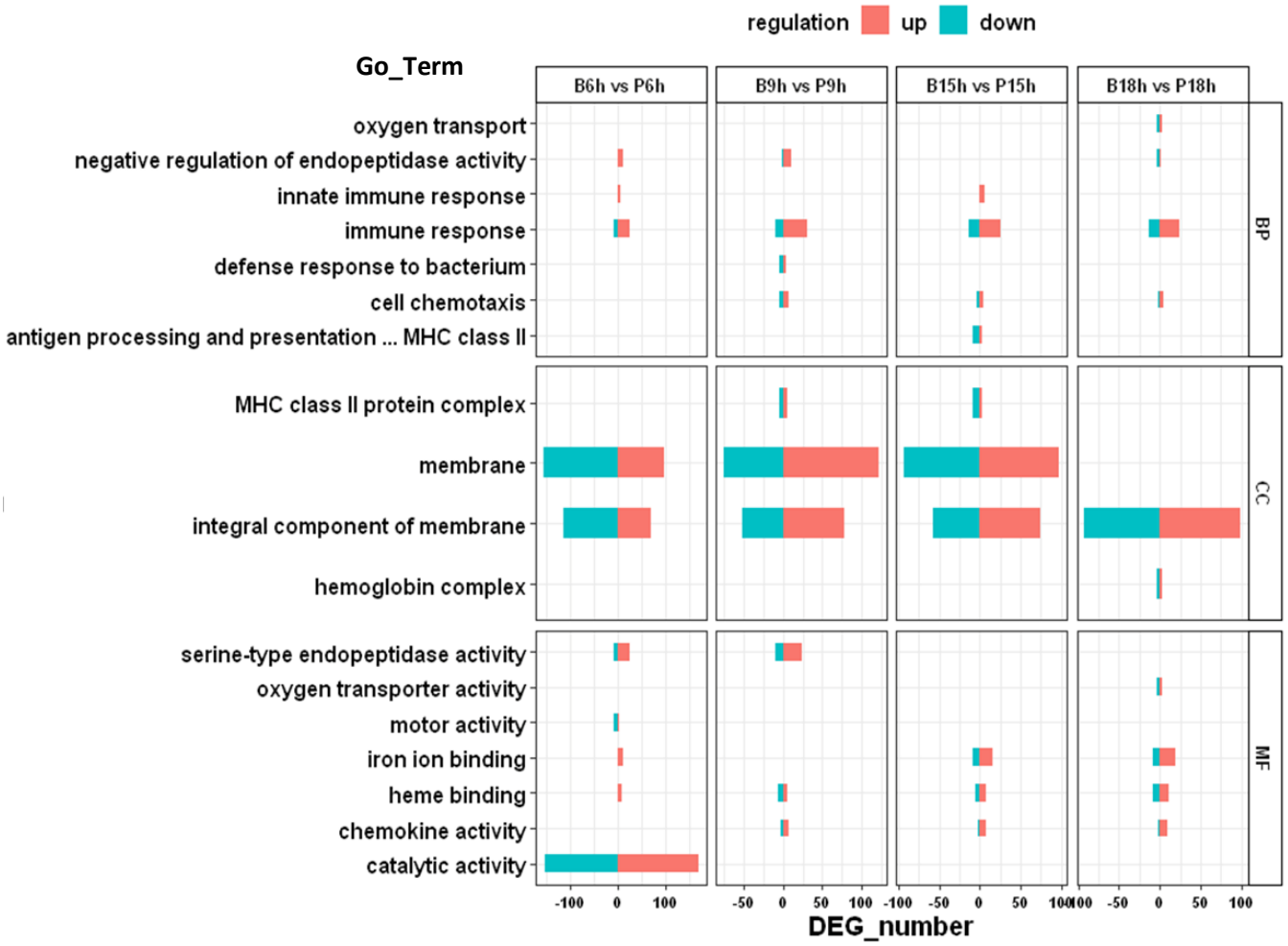

Fig. 3.3 Top 10 significantly enriched GO terms in four comparisons (B6h vs. P6h, B9h vs P9h, B15h vs. P15h, and B18h vs. P18h). BP: biological process; CC: cellular component; MF: molecular function.

3.4 KEGG analysis

The KEGG pathways enrichment analysis assigned DEGs to 337 signalling pathways. From 2122 DEG's in the PBS injection compared to the *S. agalactiae* injection at 6hpi, 1000 were allocated to 143 KEGG pathways. When comparing the same treatments at 9hpi, 802 of 1851 DEGs were annotated to 144 pathways. At 15hpi, 788 of 1791 DEGs were assigned to 133

pathways. At 18hpi 1036 from 2395 DEGs were assigned to 139 pathways. Among the first three time points, the most enriched pathways were the Phagosome (ko04145) and the Cell adhesion molecules (CAM) (ko04514). At the last time point, Phagosome (ko04145) was also the most enriched pathway, followed by the Cytokine-cytokine receptor interaction (ko04060). All these three pathways are immune related. The results from the KEGG enrichment analysis are represented in the Figure 3.4.

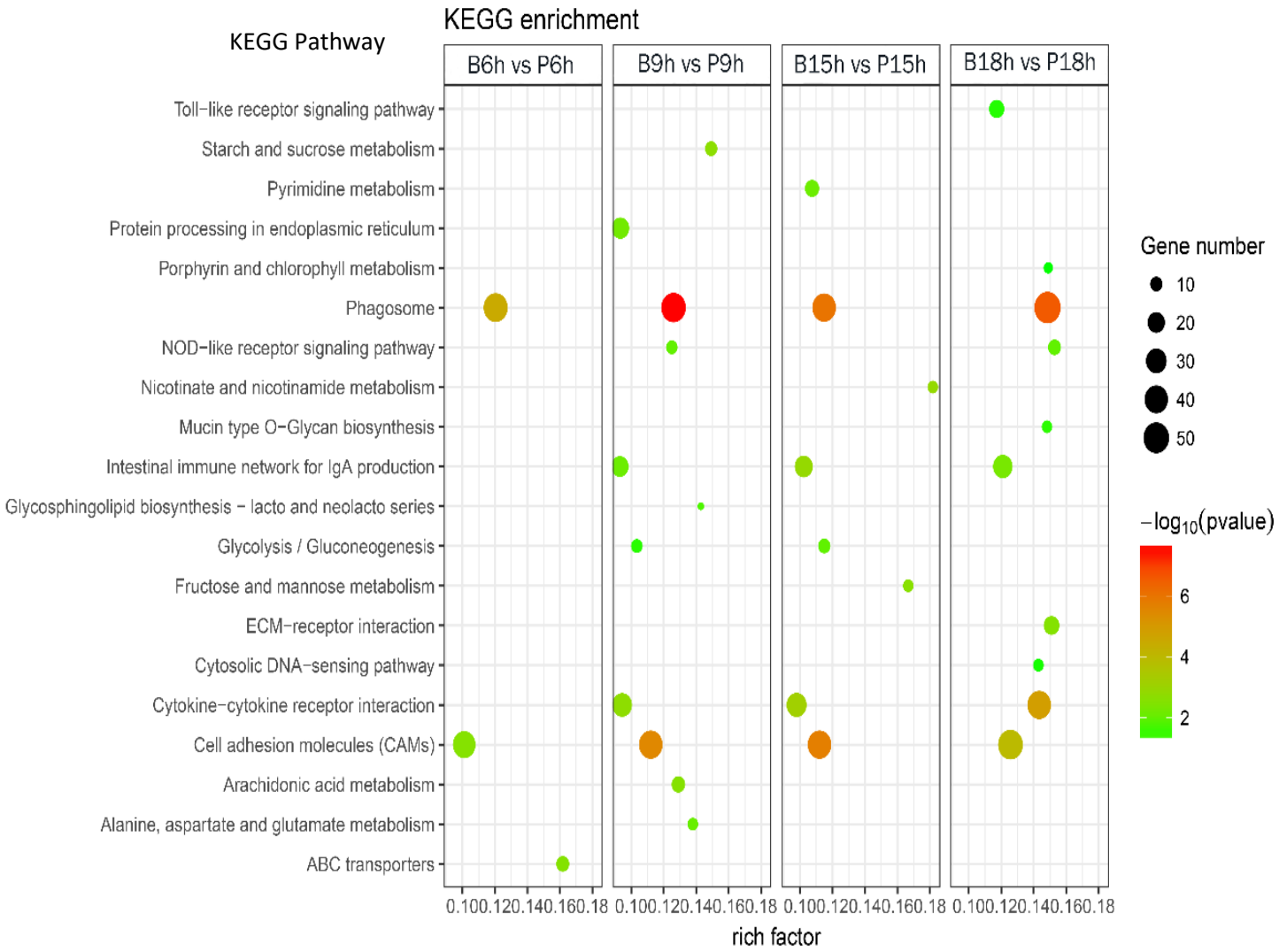

Fig. 3.4 KEGG enrichment analysis scatter plot representing pathways of significant DEGs between GBS and PBS groups in four comparisons. Red color indicates highly significant enrichment according to the P-value range.

3.5 Immune related pathways after infection

Among the different time points and treatment comparisons, different immune related genes were differentially expressed. As a result, some enriched immune related pathways were identified through the KEGG enrichment analysis. When comparing the two treatments at 6hpi, the phagosome and CAMs pathways, two immune related pathways, were identified among all the 7 significantly enriched pathways resulting from this comparison. Two other immune pathways (Intestinal immune network for IgA production; NOD-like receptor signalling pathway) and three immune related pathways (Phagosome; CAMs; Cytokine-cytokine receptor interaction) were identified at the next time point (9hpi) from a total of 17 significantly enriched pathways. At 15hpi, from a total of 13 significantly enriched pathways, the Intestinal immune network for IgA production was also detected, alongside the same 3 immune related pathways as in the previous time point. Finally, at 18hpi, among 12 significantly enriched pathways, 4 immune pathways (Intestinal immune network for IgA production, NOD-like receptor signalling pathway; Toll-like receptor signalling pathway; Cytosolic DNA-sensing pathway) and 3 immune related pathways (Phagosome; CAMs; Cytokine-cytokine receptor interaction) were recognized.

CHAPTER IV

4. Discussion

This research focused on the Nile tilapia gill, 6, 9, 15 and 18 hours post injection to identify immune-related genes and signalling pathways that are active in tilapia during the early stages of infection. As expected, numerous immune-related genes in tilapia were considerably up-regulated after infection with S. agalactiae. Furthermore, there was an enrichment of different immune related pathways at the four selected time points evidencing an activation of the immune system upon infection.

The primary mediation of the fish gill immune response is performed by cytokines, among them, it is possible to reference the tumor necrosis factor-a (TNF-a), interleukin 8 (IL-8), interleukin1b (IL-1b), interleukin10 (IL-10), and transforming growth factor-b (TGF-b) (Chen *et al.,* 2015). It is also important to notice that the gills are mucosal tissues, the mucous secreted by them transport Igs, enzymes, antimicrobial peptides, and other compounds (Gomez *et al.,* 2013; Wilson & Laurent, 2002).

The enrichment analysis of KEGG pathways reported in this study, showed the significant enrichment of the CAM pathway, with an upregulation of the *cd22* and *selp* genes at all time points. CAMs are glycoproteins involved in the inflammatory response, expressed in the surface of leukocytes. It is possible to divide them into the five following groups: the immunoglobulin superfamily, the integrin receptor family, selectins, mucins and cadherins (Samanta & Almo, 2015). Belonging to the immunoglobulin superfamily, the CD22 receptor is enrolled in the activation and management of B lymphocytes in response to alien antigens (Tedder *et al.,* 1997). This type of cells is involved in both phagocytosis and immunoglobulins secretion (Ochando *et al.,* 2006). Immunoglobulins have different tasks, such as the induction of the complement activity, opsonization, attachment prevention and neutralization of toxins (Burton, 1990).

In this study, two PRRs pathways were significantly enriched. These are the two most studied families in this group of receptors, the Toll-like receptors (TLRs) and the NOD-like receptors (NLRs). TLR's are able to identify PAMPs such as peptidoglycan (PGN), lipopolysaccharide (LPS), lipoprotein (LP), flagellin, and viral dsRNA and subsequently initiate the innate immune system response (de Nardo, 2015; Poynter & DeWitte-Orr, 2018). Because of the fish innate immunity dependence, the TLR family has been massively expanded in this organism in contrast to mammals (Zhang *et al.,* 2014). It is now possible to identify at least 20 types of TLR's with different recognition capacities, in different fish species (Zhang

et al., 2014). The identification of pathogens by these receptors is followed by the production of proinflammatory cytokines and upregulation of costimulatory molecules (Irizarry-Caro *et al.,* 2020). If we divide this big family of receptors in two subfamilies, in terms of PAMP recognition, TLR1, 2 and 6 are able to identify lipids, TLR7, 8 and 9, are able to detect nucleic acids and TLR4 were reported as able to recognize LPS, mainly present in gram negative bacteria. TLR5 was associated with the identification of the Flagellin protein, present in flagellated bacteria from the gram positive and negative groups (Akira *et al.,* 2006; Chen *et al.,* 2015). Also, previous studies have reported that TLR5 might be responsible for the identification of the pathogen antigen composition (Bai *et al.,* 2017). *S. agalactiae* nucleic acids, for example, are specifically recognized by TLR7 (Mancuso *et al.,* 2009). Upon identification, different TLRs produce a different immune response. That response may be the release of inflammatory cytokines, chemokines, antimicrobial peptides, or interferons (Kawai and Akira, 2010).

The results of the present investigation identified two upregulated DEGs common to all time points, *il-1b* and *tlr5*. This outcome suggests an important role played by these two genes in the response of this species to bacterial infections. Also, and corroborating this data, the TLR signalling pathway suffered a general upregulation at 18hpi, leading to a strong hypothesis that this pathway was only activated upon infection. In general, it is possible to assume that the detection of the pathogen occurred at an early stage, but the activation of the innate immune system was somehow delayed. This fact may be explained by the evolution of the pathogen itself. Nowadays it is possible to assume that these organisms employ different methods and strategies to avoid the host detection (Reddick & Alto, 2014). The important role of cytokines like the IL-1b in the innate immune response of fish have already been referred in different studies (Sullivan & Ulett, 2020; Sakai *et al.,* 2021). This type of interleukins is responsible for the activation of lymphocytes and phagocytic cells and were already related to the resistance to GBS and other bacterial infections, such as the *Aeromonas hydrophila* (Kono *et al.,* 2002; Zhang *et al.,* 2013).

The NOD-like receptors signalling pathway was also significantly enriched upon *S. agalactiae* infection. This type of receptors are also PRRs, involved in the detection of pathogens and in the activation of the innate immune response (Sahoo, 2020). One of the main differences between this family of receptors and TLRs, is their area of action. TLRs are extracellular transmembrane PRRs unlike the intracellular cytoplasmic receptors NLRs (Kawai & Akira, 2009). Both families of PRRs are constituted by three domains (Benko *et al.,* 2008). In fish, the effector domain is formed by one or two caspase-activation and recruitment

domains (CARD) (Wilmanski *et al.*, 2008). The mostly identified NOD receptors among fish species are the NOD1 to 5 (Sahoo, 2020). The NLRs, just like the TLRs, detect the presence of PAMPs. The expression of NLRs like NOD1 and NOD2 in the presence of some of those bacterial components has already been reported in mrigal (*Cirrhinus mrigala*) (Swain *et al.*, 2013). Upon detection, these receptors promote the activation of pro-inflammatory pathways through transcription factors such as NF-$_k$B, and caspase-1 (Zhang *et al.*, 2014). Caspase-1 is an inflammatory protease in control of pro-inflammatory cytokines, such as IL-1b, release (Mariathasan *et al.*, 2004).

The results of this study present an upregulation of many DEGs related to the NLR signalling pathway, such as *il-1b*, *hsp90*, *casp1*, *card9*, *jnk*, *nf-$_k$b*, *p38*, and *tab1*, of which *il-1b* was upregulated at all time points. Between the bacterial injection treatment and the PBS injection treatment, the NLR signalling pathway was enriched at 9hpi and 18hpi. There is a clear indication of the activation of these receptors due to the upregulation of all the DEGs present in this pathway at 18hpi. Other studies regarding the infection of *S.agalactiae* in tilapia have reported the same regulation patterns for some of the DEGs present in this study. For example, Zhang *et al.*, 2013 reported the up regulation of the Heat Shock protein gene *hsp90* in this species liver and spleen following bacterial infection. Moreover, the expression of the proinflammatory cytokine IL-1b gene presented the same behaviour. In that same study was also possible to detect the presence of effector proteins such as CARD9, JNK, p38, NF-$_k$B and TAB1.

The *S.agalactiae* infection caused an enrichment of the Cytokine-cytokine receptor interaction pathway at 9, 15 and 18hpi, as it is possible to see in the results of the KEGG enrichment analysis. It was possible to identify an overall upregulation of two DEGs, the *cxcr3* and the *il-1b*. Cytokines are proteins responsible for cellular interaction and communication, they are not specific to a certain cell type and may have an autocrine or paracrine activity. Normally, a certain cell produces cytokines to stimulate the production of others by the target cells, creating a chain reaction. This type of proteins may induce an anti-inflammatory or pro-inflammatory reaction (Zhang & An, 2007). In contrast to IL-1b, a pro-inflammatory cytokine, CXCR3 is a chemokine. These kinds of proteins are related to the leukocyte migration to a determined target location (Cyster *et al.*, 1999) and may even intervene in the immune response regulation. Chemokines are also linked to the differentiation of the drafted cells, a fundamental step in the early coordination of both innate and acquired immune responses (Esche *et al.*, 2005). The development of the adaptive immunity of organisms like the fish is poor when compared with higher vertebrates, and because of that, they strongly depend on their innate

immune response (Buchmann, 2014; Zhu *et al.*, 2016). In the end, it is considered that both systems, innate and adaptive are correlated, with the first one modulating and orientating the other one (Iwasaki & Medzhitov, 2015).

Fc receptor FCGR1A, a member of the Phagosome pathway, was upregulated at all time points. At 9hpi, all the DEGs related to the phagocytosis-promoting receptors were upregulated, allowing to assume their connection to the immune response to *S.agalactiae* infection. Furthermore, most of the DEGs associated to this type of receptors have presented upregulation at 18hpi. Some examples are the expression of *fcgr1a*, *itgb2*, *mbl*, *tlr2*, *mr* and *marco*.

Fc receptors have been described as phagocytosis enhancers and, cell and antibody dependent cellular cytotoxicity activators, playing an important role in the immune response (Akula *et al.*, 2014). The process of phagocytosis is part of the innate immune response, yet it connects both innate and adaptive immune systems since the phagocytic action over pathogens is imperative in the process of antigen production (Biller-Takahashi *et al.*, 2015). The primary function of phagocytes is the detection and eradication of pathogens or injured tissue, maintaining cellular homeostasis. In fish, the phagocytic cells are mainly neutrophils, a type of leukocyte within the granulocytes group, and macrophages or monocytes, both characterized as mononuclear phagocytes (Secombes & Fletcher, 1992). These cells digest bacterial particles through plasma membrane surrounding (Pauwells *et al.*, 2017). Zhang *et al.*, (2019), reported the major importance of the phagocytic process and interleukins in the immune response of tilapia against bacterial infections. The expression pattern of *mr*, *tlr2* and *il-1b* obtained by authors in this study was similar to the present one.

CHAPTER V

Conclusion

5. Conclusions

This study provides new insight on the Nile tilapia gill transcriptomic changes in response to the bacteria *S. agalactiae*. After analysing and discussing the results it is possible to conclude that this fish immune system was modulated by the bacterial infection. The results of this study may help future research on the defence mechanisms of this species against this bacterium.

CHAPTER VI

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6. References

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