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Sulphated polysaccharides extracted from brine water and its effects on fish immune response

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Gonçalo Pereira de Castro Pinto)

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Resumo

O peixe-zebra (*Danio rerio*) é um ciprinídeo de água doce nativo da Ásia Meridional. Nestas regiões pode ser encontrado em águas calmas, pouco profundas e com vegetação cujas condições conseguem ser facilmente mimetizadas em tanques com baixo volume. Nestes ambientes o peixe-zebra é capaz de ocupar toda a coluna de água alimentando-se de presas como zooplâncton, insetos, ovos de pequenos crustáceos e, muitas das vezes, algum material inorgânico o que o torna bastante responsivo tanto a alimento vivo, como náuplios de Artémia e rotíferos, como também a dietas inertes, normalmente usadas em contexto laboratorial. A sua reprodução é também facilmente atingida, uma vez que o peixe-zebra consegue desovar numa variedade de condições laboratoriais otimizadas, com o desenvolvimento de técnicas de reprodução através do uso de pequenos tanques, desenhados para promover o comportamento reprodutivo e maximizar o número de ovos.

Para além da facilidade de manutenção, existem mais algumas características que tornam o peixe-zebra um recurso valioso para os cientistas como, por exemplo, o seu tamanho reduzido e o seu rápido desenvolvimento, com um curto período entre gerações que os torna ideais para o planeamento de experiências, e o facto de serem translúcidos durante as primeiras fases de vida facilitando a observação de estruturas internas. Todas estas características contribuem para a sua elevada resistência em várias condições ambientais quando mantidos em cativeiro e são a razão pela qual esta é uma espécie tão bem estudada e utilizada como animal-modelo, com diversas aplicações em diferentes ramos de investigação incluindo biologia de desenvolvimento, ecotoxicologia, neurobiologia, biomedicina e aquacultura.

O peixe-zebra surge também, devido à sua praticabilidade, como complemento em estudos de genética aplicada à imunologia com a possibilidade de detetar fenótipos de interesse já estudados em mamíferos, tais como o rato, que contribuíram para a compreensão do desenvolvimento do sistema imune e de doenças a ele associadas. O uso de mamíferos como animais-modelo vem com a desvantagem de estes terem fertilização interna e não serem translúcidos nas fases larvares tornando impossível o estudo do sistema imune na sua génese, em sistemas *in vivo*. O ramo da imunologia tem vindo a utilizar o peixe-zebra precisamente devido ao facto de serem translúcidos durante as fases embrionária e larvar. Diversas linhas transgénicas e de sinalização ("reporter lines") foram desenvolvidas para identificar, mapear, quantificar e compreender a função e interação de diversas células do sistema imune. Exemplos disso são os neutrófilos e macrófagos que, após marcação com um fluorocromo expresso sob controlo de promotores de genes marcadores, tornam possível a análise da sua origem e desenvolvimento em organismos vivos como também uma melhor avaliação dos efeitos de manipulação química e genética relacionadas com a triagem de drogas que possam alterar, ou não, a função destas células.

Outro aspeto importante da imunologia, que tem sido objeto de estudo, passa pela formação inicial de um sistema imune inato e das suas funções durante uma resposta inflamatória induzida. É o sistema imune inato que confere proteção ao peixe-zebra durante as suas primeiras fases de desenvolvimento sem qualquer interferência de uma resposta imune adaptativa que apenas se estabelece entre 4 e 6 semanas após fertilização. Contrastando com os mamíferos e também com os peixes vivíparos, as larvas do peixe-zebra eclodem em contacto direto com o meio ambiente e, como tal, para ultrapassarem a exposição prematura a diversos patógenos, têm de contar com um rápido desenvolvimento do seu sistema imune.

De todas as células que desempenham um papel na resposta alérgica e inflamatória em peixes, são os macrófagos e os neutrófilos os mais importantes. Estes dois tipos de leucócitos apesar de estarem presentes às 15 e 48 horas pós-fertilização (hpf), respetivamente, apenas adquirem função às 72 hpf, com os neutrófilos a atuarem em primeiro lugar, seguidos dos macrófagos, que uma vez no local de inflamação fagocitam patógenos e resíduos existentes.

Os neutrófilos vão iniciar a reposta inflamatória recorrendo à fagocitose e à libertação de produtos antimicrobianos e de moléculas sinalizadoras com o objetivo de recrutar outras células imunes. Esta migração/ação dos neutrófilos tornou-se possível de seguir *in vivo* com a criação de uma linha "reporter" de peixe-zebra através da incorporação da proteína verde fluorescente (Green Fluorescent Protein - GFP) controlada pelo promotor de um gene codificante para uma enzima específica destas células, a mieloperóxidase (*Mpx*).

Os macrófagos são também constituintes essenciais da resposta imune inata capazes de eliminar bactérias inoculadas, não só da corrente sanguínea, como também de cavidades corporais fechadas mostrando mecanismos de sensibilidade e migração ativos. Tal como para os neutrófilos, foi também desenvolvida uma linha "reporter" para os macrófagos através de um marcador genético específico destas células – macrophage-expressed gene (*mpeg*) – através da utilização de proteínas com diferentes fluorescências mCherry (*Tg*(*mpeg1:mCherry*) e GFP (*Tg*(*mpeg1:EGFP*).

A resposta inflamatória começa com o reconhecimento de moléculas e outros patógenos ou de moléculas libertadas por células danificadas. Este reconhecimento é feito por proteínas transmembranares associadas a células do sistema imune inato. Após o reconhecimento, passamos à parte efectora da resposta inflamatória com a ativação de uma cascata molecular que começa com a libertação de citoquinas que recrutam os neutrófilos e os macrófagos já no local, através da sua ação autócrina e parácrina, e que vão também alterar os níveis de citocinas e prostaglandinas na corrente sanguínea, desta vez através da sua ação endócrina. Esta alteração no gradiente das quimiocinas vai, por sua vez, influenciar a migração de leucócitos em direção ao local da inflamação.

A utilização de lipopolissacarídeos bacterianos (LPS) – uma endotoxina existente nas paredes de bactérias Gram-negativas – na indução química da inflamação é um dos modelos mais estudados. É sabido que a estimulação com LPS leva à produção de espécies reativas de oxigénio e de azoto provocadas pelo aumento dos níveis da enzima óxido nítrico-sintase induzida - iNos (Inducible Nitric Oxide Sysnthase). Os níveis da enzima cicloxigenase-2 - Cox-2 (Cyclooxygenase-2) - são também aumentados após estimulação com o LPS. Esta enzima para além de produzir prostaglandinas que promovem a inflamação também dá origem a níveis elevados de RNA mensageiro (mRNA) que codificam pro-citoquinas. O desenvolvimento deste modelo de inflamação criou uma ferramenta para testar e validar diferentes tipos de drogas anti-inflamatórias que podem ir desde drogas bem estudadas na indústria farmacêutica que procuram encontrar novas aplicações, a compostos naturais como extratos de micro- e macroalgas ricos em polifenóis.

Podemos destacar também os polissacarídeos, que tal como outros compostos naturais, têm vindo a ser alvo de estudo com o objetivo de encontrar propriedades bioativas que possam ser aplicadas ao melhoramento da saúde e bem-estar. Os polissacarídeos estão constantemente a serem produzidos por organismos marinhos, fazendo com que a obtenção destes compostos possa ser feita de maneira substancial e sustentável. Estes podem ser classificados de acordo com a sua origem e estrutura, sendo, por exemplo,

identificados como ulvanos, aqueles que são obtidos a partir das algas verdes (*Chlorophyta*) e como fucoidanos, aqueles obtidos a partir de algas castanhas (*Phaeophyta*).

O nosso interesse foca-se, no entanto, numa classe de polissacarídeos, identificados como polissacarídeos sulfatados, que são descritos na literatura como compostos extremamente bioativos a diferentes níveis, contando com propriedades antibacterianas, antivirais e antioxidantes, como também anticoagulantes, osteogénicas e imuno-estimulantes/imuno-modulatórias. São compostos extremamente solúveis em água e como tal constituem a maior parte dos componentes orgânicos encontrados no material polimérico que está associado ao sal marinho. Estudos realizados com estes compostos extraídos da água de salmoura – um subproduto da cristalização do sal – mostraram que são dotados de propriedades imuno-estimulantes.

Os ensaios realizados *in vitro* com células SHK-1 sugerem que essas propriedades imuno-estimulantes realmente existem nos polissacarídeos sulfatados estudados. A exposição a estes compostos foi capaz de estimular a expressão de alguns genes relacionados com a resposta imune (*tnfα, il-1β e il-8*) e com o stress oxidativo (*cox-2*). A actividade mineralogénica destes compostos também foi avaliada através do ensaio do opérculo em larvas de peixe-zebra. No entanto, não existe qualquer indicação de que estes compostos em específico são dotados de propriedades pro-osteogénicas. Após a inclusão destes compostos em ração comercial, verificou-se que podem influenciar a resposta imune do peixe-zebra após infeção provocada pela bactéria *Aeromonas hydrophilla.*

Nesta tese, os polissacarídeos sulfatados, originários da água de salmoura, foram estudados de maneira a avaliar a sua bioatividade ao nível da resposta imunitária e da capacidade de modular a mineralização em peixes.

Palavras-chave: *Danio rerio*; Polissacarídeos Sulfatados; Lipopolissacarídeos; *Aeromonas hydrophila***.**

Abstract

Zebrafish (*Danio rerio*) is a model animal with increasing applications in several research fields. It arose as a particularly interesting model in the immunology field making possible the study of the immune system at early stages of formation in *in vivo* systems due to its optical transparency during embryo and larval stages. Apart from that, zebrafish gives the opportunity to study the innate immune system isolated from any kind of adaptive immunity, that will only be established 4 to 6 weeks after fertilization.

Neutrophils and macrophages are the principal intervenient cells of the innate immune system. Zebrafish reporter lines with fluorescent proteins, were developed in order to easily recognize, trace and quantify these cells during an inflammatory response. Several approaches to induce inflammation in zebrafish were developed and are described in the literature. The lipopolysaccharides (LPS)-induced inflammation model is one of the most studied, which created an opportunity to test and validate different anti-inflammatory drugs. The operculum assay also arose as a screening method to evaluate compounds with potential mineralogenic effects in zebrafish larvae. Sulphated polysaccharides belong to the most abundant natural compounds produced by living organisms being described as compounds with health improvement and wellbeing effects, such as antibacterial and osteogenic activity and immunomodulatory/ anti-inflammatory properties.

In this context, this thesis aims to study the immunostimulant effects of brine derived sulphated polysaccharides on inflammation-induced larvae and juvenile zebrafish and in SHK-1 cells. Also, the mineralogenic effects of these compounds will be accessed *in vivo*. Overall, survival rates and disease signals will be recorded, and innate immune system mediators and effectors will be measured. Based on the literature, we hypothesise that sulphated polysaccharides may have a positive effect on the inflammatory response, increasing survival rates and reducing inflammation induced damages. We also hypothesise that, based on previous studies performed in zebrafish, the expression of several inflammation modulators and promoters can be decreased by treatment with sulphated polysaccharides, promoting a modulatory effect on inflammation response.

The *in vitro* studies, performed with SHK-1 cell line suggest the existence of immunostimulatory properties in the SP extracts analysed. The cell exposure to these compounds revealed an increment in the expression of genes related to the immune response (*tnfα, il-1β* and *il-8*) and oxidative stress (*cox-2*). The mineralogenic activity was also accessed, under the operculum essay performed in zebrafish larvae, but with no indication of the presence of pro-osteogenic properties. The sulphated polysaccharides studied apparently influence zebrafish immune system response when under a bacterial challenge induced by *Aeromonas hydrophilla* infection.

Keywords: *Danio rerio*; Sulphated Polysaccharides; Lipopolysaccharides; *Aeromonas hydrophila*

Index

Abbreviations List

NCC – Neural Crest Cells

hpf – hours post fertilization

GFP/EGFP – Green Fluorescent Protein

mpo/mpx – myeloperoxidase

Tg – Transgenic

PAMPs – Pathogen Associated Molecular Patterns

DMAPs – Damage Associated Molecular Patterns

PRRs – Pattern Recognition Receptors

TLRs – Toll-like Receptors

NLRs – NOD-like Receptors

NOD – Nucleotide Oligomerization Domain

RLRs – RIG-like receptors

RIG – Retinoic acid-Inducible Gene I

CLRs – C-type Lectin Receptors

TNF-α – Tumor Necrosis Factor α

IL-1β – Interleukine 1β

IL-8 – Interleukine 8

IL-6 - Interleukine 6

iNOS – Inducible Nitric Oxide Synthase

NO – Nitric Oxide

ROS – Reactive Oxygen Species

NOS – Nitrogen Oxidative Species

COX-2 – Cyclooxygenase-2

SOD – Superoxide Dismutase

CAT - Catalase

mRNA – messenger Ribonucleic Acid

LPS – Lipopolysaccharide

dpf – days post fertilization

SP – Sulphated Polysaccharides

EPS – Extracellular Polymeric Substances

SHK-1 – Salmon Head Kidney 1

ZFIN – Zebrafish Information Network

ID – Identification in ZFIN database

°C – degrees Celsius

L – Litre

µWs/cm² - microwatt second per square centimetre

µS – microsiemens

mg/mL – miligram per mililitre

CCMAR – Centro de Ciências do Mar

RO – Reverse Osmosis

ppt – parts per trillion

mL – mililitre

mL/L – mililitre per litre

TAN – Total Ammonium Nitrate

UA – University of Aveiro

BW – Brine Water

AS – Artifitial Salt

NE – Not Exposed

%(w/v) – concentration expressed in weight volume percentage

LEOA – Laboratório Experimental de Organismos Aquáticos

h – hours

AR-S – Alizarin Red S

min – minutes

MS-222 – Tricaine Methanosulfonate

λex – Exposure wavelength

ms – milliseconds

nm – nanometer

NaOH – Sodium Hydroxide

mM – millimolar

qPCR – quantitative polymerase chain reaction

FBS – Fetal Bovine Serum

PBS - Phosphate-buffered saline

µL – microlitre

µg/mL – microgrma per mililitre

g – gravitational force

g – grams

V – Volts

UV – Ultraviolet

M-MLV RT - Moloney Murine Leukaemia Virus Reverse Transcriptase

dNTP - Deoxynucleotide triphosphates

DTT – Dithiothreitol

RNase – Ribonuclease

DNA – Deoxyribonucleic Acid

cDNA – complementary deoxyribonucleic acid

MM – master mix

FW – primer forward

RE – primer reverse

Nº - number

n – number of specimens

Op – Operculum

Hd – Head

$\bar{x}$ - mean

CFU – Colony Formation Unit

OD – Optical Density

nm – nanometres

LD50 – Lethal Dose 50

TSA – Trypticase Soy Agar

TSB – Tryptic Soy Broth

G – gauge

(´´) – inches

CA – Control Amputated and Not Exposed

CB – Control Not Amputated and Exposed

+C – Positive Control

-C – Negative Control

S – Sample

CTRL – Control group

BE – Before Exposure

1. **Introduction**

The Zebrafish, *Danio rerio* (Hamilton, 1822) (Figure 1.1), is a freshwater cyprinid that can be found in tropical areas of South Asia, distributed across India, Bangladesh and Nepal with reported observations amongst other places of the Indian subcontinent (Spence et al., 2006).

Figure 1.1: Zebrafish (Danio rerio) (Adapted from smithsonianmag.com, 2022)

Studies conducted in India (McClure et al., 2006) and Bangladesh (Spence et al., 2006) describe zebrafish preferential habitats as slow, quiet water locations such as irrigation ditches, shallow ponds and rice fields, most of them associated with aquatic vegetation, which conditions can be easily reproduced in small volume tanks. At these environments zebrafish is able to occupy the entire water column. Gut content analysis showed a preference for a variety of preys such as zooplankton, insects, mostly of terrestrial origin, small crustacean eggs and, often, inorganic material. Several other studies suggest feeding habits near water surface as well as near the substrate (Mcclure et al., 2006; Spence et al., 2006, 2007). Altogether, makes them positively responsive to live feed diets, such as rotifers and artemia nauplii, as well as inert feeds commonly used in laboratory environment. Being native from monsoon regions, with water temperatures ranging from 6 to 38 ºC, zebrafish reproduction is likely to be triggered by the arrival of the rains and the food availability that comes with it (Spence et al., 2006). Wild zebrafish take part in pair spawning with males actively chasing the females and sexual competition between males as also regarding male mate selection (Hanak et al., 2010). However, zebrafish can spawn under a wide range of laboratory conditions, plus, breeding techniques have been developed with the use of small, dedicated tanks to promote mating behaviour and maximize egg collection (Lawrence, 2007).

Apart from the easy maintenance in laboratory settings, there are a few more characteristics that make zebrafish such an extraordinary resource for scientists: i) its small size and fast development with a short generation time make them ideal when planning for experiments, ii) the optical transparency present throughout early live stages makes it much easier to observe the fish internal organs, iii) established fluorescent reporter lines that allow us to see specific components or cells (Ferrero et al., 2020; Kolb et al., 2018; Lawrence, 2007; Svoboda et al., 2016; Varga et al., 2018). Altogether, zebrafish natural characteristics are the reflection of its high tolerance for a wide range of environmental conditions in captivity and the reason why this species is a well-studied and used experimental animal model, with an increasing range of applications in numerous research fields including developmental biology, ecotoxicology, neurobiology biomedicine and aquaculture (Fernández et al., 2018; Kolb et al., 2018; Lawrence, 2007; Ulloa et al., 2014; Varga et al., 2018).

Due to its practicability, zebrafish also emerged as a model animal to complement studies already made in mammalian models, regarding genetics applied to immunology with the possibility of detecting a phenotype of interest (Trede et al., 2004). Despite the several advantages of mammalian models, such as the mice, that contributed in great manner to the understanding of immune deficiencies and the immune system development (reviewed in Fischer & Malissen, 1998; Fischer, 2001), they come with the disadvantage of internal fertilization and lack of optical transparency making impossible to study the immune system at its earliest stages of formation as *in vivo* systems (Traver et al., 2003). The immunology field has been using zebrafish due to its optical transparency during embryo and larval stages. Several reporter and mutant lines have been generated to track, quantify and understand the function and interaction of a variety of immune cells. One example are neutrophil and macrophage cells labelled with fluorochromes. Not only the analysis of the origin and development of these cells become possible in living organisms, but it also allows a better understanding of what are the effects of genetic and chemical manipulations such as the screening of drugs that may or may not alter cell function(s) (Trede et al., 2004).

Another important aspect of immunology that has been object of study, is the early establishment of an innate immune system and its functions when inducing an inflammatory response. In zebrafish the innate immune system acts by itself during the first stages of development with no interaction with an adaptive immune system that will

only be established several weeks after fertilization (Trede et al., 2004). The zebrafish innate immune system is present at the start of embryogenesis (Herbomel et al., 1999) and immune defence, at this point, is only depending on this system up until the morphological and functional maturation of an adaptive immune system, that occurs between 4 to 6 weeks after fertilization (Davidson & Zon, 2004; Trede et al., 2004; Willett et al., 1999). Innate immunity is defined as the first line of defence against invasive pathogens and is mediated by mechanisms that include the production of cytokines and interferons and enhanced by a system complement activation that will recruit and stimulate phagocytic cells as inflammatory response effectors (Trede et al., 2004). Unlike mammals and viviparous fishes, zebrafish larvae hatch from eggs that are exposed to the natural environment and, as so, exposed from the very beginning to several pathogens. This requires a fast development of their immune system (Du Pasquier, 2000; Poorten & Kuhn, 2009).

When it comes to components and mechanisms of the innate immune system, teleosts including zebrafish, can count with leukocytes, a complement system, an inflammatory process and non-specific, non-phagocytic cytotoxic cells (NCC) culminating on the impact that this quick array of responses end up having on the development of an adaptive, more specific, immune system (Traver et al., 2003). Different types of leukocytes were described in teleosts such as monocytes, granulocytes and tissue macrophages. Fish also have a different type of macrophages, when compared to the ones found in mammals, due to their pigmentation. For this reason, they are called melanomacrophages and can be found in lymphoid tissues (Herráez & Zapata, 1991). Granulocytes classes can vary amongst fish species (Traver et al., 2003). In zebrafish and other cyprinids (Bennett et al., 2001; Jagadeeswaran et al., 2000; Lieschke et al., 2001), neutrophils/heterophils and eosinophils are found in two lymphoid organs, head kidney and spleen, as well as in blood, in which over 90 % of the granulocytes are in fact neutrophils. Despite the observation of eosinophils as well as mast cells, that play important allergic and inflammatory roles, like their mammalian counterparts (Dobson et al., 2008), neutrophils and macrophages are still the major players of the innate immune system of fishes. During zebrafish ontogeny, macrophages are the first to arise, being recognizable at 15 hours after fertilization (hpf) followed by neutrophils that can be identified around 48 hpf (Lieschke et al., 2001). These two types of leukocytes are present but are not active until hatching at 72 hpf. When in response to an injury, neutrophils are

the first to act, followed by macrophages that once at an inflammation site start to phagocyte existing pathogens and debris (Novoa & Figueras, 2012).

Zebrafish neutrophils are characterized by having a pale cytoplasm with a large number of granules and a multilobulated segmented nucleus, as it is typical for other cyprinid species (Bielek, 1981; Imagawa et al., 1989; Lieschke et al., 2001). Kolaczkowska and Kubes (2013) described that once released, neutrophils migrate to the infection *loci* following a series of chemoattractant signals. This chemotaxis process is first stimulated by endogenous chemokines and then, at a later stage, by bacterial peptides or complement signals. Neutrophils will initiate inflammation resolution through phagocytosis, releasing of antimicrobial products and further signalling to recruit other immune cells. In order to facilitate the visualization of this migration/action mechanisms Renshaw et al. (2007) developed a reporter zebrafish line with a green-fluorescent protein (GFP) at the neutrophil-specific myeloperoxidase (*mpo*, also called *mpx*) promoter site. This model not only enables *in vivo* analysis of the innate immune response by labelled fluorescent neutrophils (Figure 1.2 – A), but also allows experimental manipulation of the inflammation with consequent quantification of the response by counting of fluorescent cells.

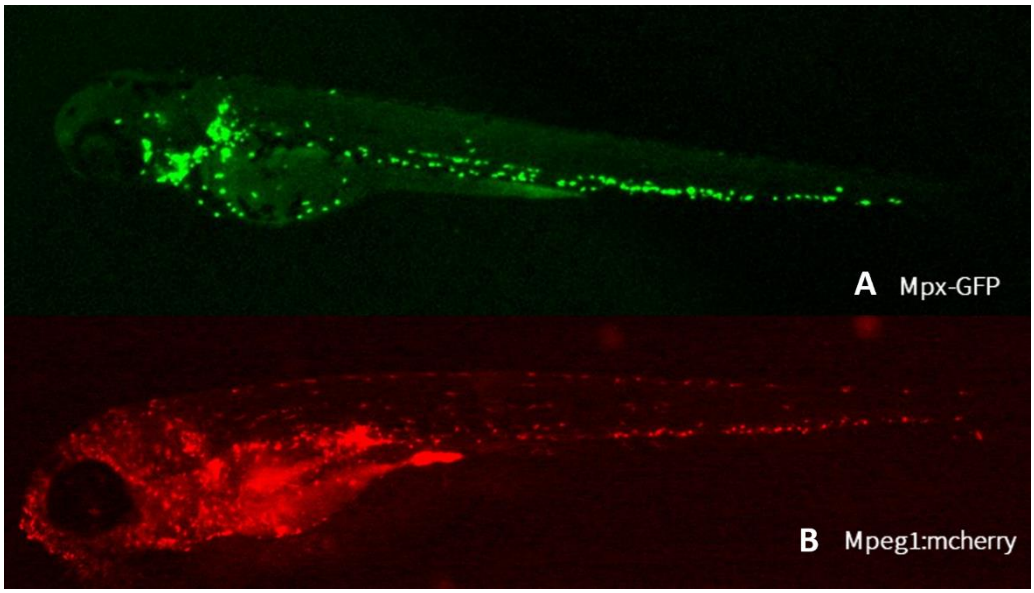

Figure 1.2: A - zebrafish reporter line Tg(mpx:GFP) signalling the presence of neutrophils (green fluorescent dots); B –zebrafish reporter line Tg(mpeg:mCherry) signalling the presence of macrophages (red fluorescent dots). Image adapted from saferworldbydesign.com, 2022.

Macrophages ontogeny and behaviour were described in detail by Herbomel et al. (1999). In their work the authors recognize an early population of "amoeboid cells with bean-shaped eccentric nuclei", some of them with phagosomes that gather around the yolk sac resisting to the blood stream flow before colonizing other tissues. On top of that they also observed these cells engulfing debris and remains of apoptotic erythroblasts. Apart from neutrophils, macrophages are essential effectors of the innate immune response. According to Herbomel et al. (1999), macrophages were shown to be capable of clearing injected bacteria, not only from the blood stream, but also from closed body cavities showing sensing and active migration mechanisms. These capabilities are also involved in tissue regeneration as reported in zebrafish caudal fin regeneration trials (Nguyen-Chi et al., 2017) as well as their performance against several kinds of bacteria and fungus (Linnerz & Hall, 2020; Prajsnar et al., 2008). As for the neutrophils, a reporter zebrafish line was designed for macrophages, that allow its visualization through a macrophage-specific genetic marker. According to Ellett et al. (2010) several transgene constructs were built to observe the macrophage-expressed gene *(mpeg*) in live animals. The transgenic (Tg) reporter lines using the genes for the fluorescent proteins mCherry (*Tg*(*mpeg1: mCherry*) (Figure 1.2 – B) and GFP (*Tg*(*mpeg1: EGFP*) as *tags* were the ones with higher transgene expression. The authors also highlight the usefulness of such lines by demonstrating that these cells can be quantifiable and their behaviour analysed. The action of the above-described cells is activated when the body is under some kind of harmful stimuli, being that a wound, a pathogen or a dysfunctional/dying cell. The innate immune system will be activated, and an inflammatory response initiated (Chen et al., 2018; Netea et al., 2017).

The inflammatory response begins with the recognition of molecules frequently found in microbes and other pathogens, the Pathogen-Associated Molecular Patterns (PAMPs), or, on the other hand, molecules released by damaged cells, the Damage-Associated Molecular Patterns (DAMPs) (Akira et al., 2006; Netea et al., 2017). Either of this "alarm" patterns are recognized by transmembrane proteins associated with innate immune cells known as Pattern Recognition Receptors (PRRs). There are four major sub-families of this germline encoded receptors: Toll-like receptors (TLRs); NOD-like receptors (NLRs); RIG-like receptors (RLRs) and C-type lectin receptors (CLRs) (Walsh et al., 2013). After recognition, a molecular cascade takes course. First, the release, by the immune cells, of pro-inflammatory cytokines such as tumour necrosis factor -α (Tnf-

α) and interleukine-1β (Il-1β) takes place with autocrine and paracrine functions over local neutrophils and macrophages and endocrine functions changing serum concentrations of inflammatory mediators such as chemokines and prostaglandins (Akira & Takeda, 2004; Netea et al., 2017). The gradient changes on chemokines levels will influence the leukocytes migration towards inflammation *loci* as inflammation resolution effectors (Bonecchi et al., 2009; MacLeod & Mansbridge, 2015; Netea et al., 2017). This series of events will result on the appearance of the classical inflammation symptoms such as redness, warmth, pain and swelling (Netea et al., 2017) that can ultimately lead to loss of function. However, in order to study the inflammation response in zebrafish at cellular and molecular level, there is first the need of an induced inflammatory status.

There are several described approaches, with different developed disease models in zebrafish. Those models can be summarized (Xie et al., 2021) in different categories, namely, i) wounding-induced inflammation, in which part of the fin tail is amputated or an incision is made with a scalpel or needle (Lee et al., 2005; Mathias et al., 2006; Renshaw et al., 2007); ii) chemical-induced inflammation that can be performed using bacterial lipopolysaccharides (LPS) that will be recognized as a PAMP; iii) copper that will disturb internal balance of this nutrient, leading to inflammation due to oxidative stress (Pereira et al., 2016) and, iv) Leukotriene B4 (LTB4), a pro-inflammatory mediator that will stimulate leukocyte´s accumulation at inflammation sites (Peters-Golden et al., 2005; Yokomizo et al., 2001).

The LPS-induced inflammation model is one of the most studied models in zebrafish. This molecule is an endotoxin present in Gram-negative cell walls that, as stated above, will be recognized by TLRs as PAMPs. The recognition patterns for LPS are not fully understood in zebrafish as comprehended in mammals, however, a similar response has been observed (Forn-Cuni et al., 2017). It is known that LPS stimulation leads to oxygen and nitrogen reactive species production (ROS and NOS) caused by increased levels of Inducible Nitric Oxide Synthase (iNos) enzyme. Nitric Oxide (NO) production by this enzyme can have beneficial microbicidal, antiviral, antiparasitic and antitumoral actions (Kleinert & Forstermann, 2007). The LPS stimulation also leads to increased levels of Cyclooxygenase-2 (Cox-2) enzyme that, in its turn, produces prostaglandins that promote inflammation, as well as increased levels of mRNA for genes encoding pro-cytokines (Ko et al., 2017; Watzke et al., 2007b). The administration of this endotoxin can be performed by a non-invasive manner, in which larvae of different ages (starting at 3 dpf – days post

fertilization) are immersed in rearing water containing increasing concentrations of LPS solution, and survival rates are measured on a constant basis (Novoa et al., 2009). The same protocol can be applied in embryos with the added task of removing the chorion at 28 hpf (hours post fertilization) (Watzke et al., 2007a). Another exposure method is through injection. To execute this procedure larvae are first anaesthetized and then microinjected with an LPS solution into the yolk sac and monitored for any disease signal or mortality as described by Yang et al. (2014).

The development of LPS-induced inflammation model in zebrafish created an opportunity to test and validate different anti-inflammatory drugs, making zebrafish an excellent model for drug discovery (Kim et al., 2014; Wang S. et al., 2020). In these studies, not only the leukocyte migration upon infection is recorded but also the ROS and NOS formation. The latter are influenced by the compounds that are being studied and therefore used as indicators of those compounds activity/efficacy in controlling the induced inflammatory effects.

Many of these studies were performed, studying the anti-inflammatory capacities of several kinds of products from various origins. Those products can be divided as well-studied drugs that are under repositioning studies in order to find new applications (Hall et al., 2014); pharmacological pathway signalling inhibitors influencing LPS tolerance (Novoa et al., 2009) and several natural compounds, such as: i) frog skin, used in traditional Chinese medicine and tested for the anti-inflammatory capacities of its molecular constituents against LPS-induced inflammation (Zhang et al., 2019); ii) polyphenol-rich extracts from an edible brow algae by-products, *Ecklonia cava*, that were tested for anti-inflammatory activity in LPS-induced macrophage cells (Kim et al., 2014); iii) several powdered material extracts from different medicinal plant species, tested for its anti-inflammatory properties in zebrafish larvae upon incubation with LPS and injured with tail fin transection (Cordero-Maldonado et al., 2013)

Polysaccharides as well as other natural products present in marine environments have been, lately, a research target with the goal to find new bioactive compounds due to its wide range of applications, when it comes to health and well-being improvement. Marine organisms, driven by the challenge of living in everchanging environments, with constant variations in temperature, light and nutrient availability produce a wide variety of bioactive metabolites, as an adaptation response, that cannot be found in other type of

organisms (Lordan et al., 2011). Polysaccharides, in general, are present in a variety of marine organisms. They are constantly being produced by these organisms what makes them eligible for production in substantial amounts (Nunes et al., 2019; Paulsen, 2002). Nunes et al. (2019) summarize these compounds considering its origin and structure: i) red seaweeds (*Rodophyta*) have sulphated galactans, carrageenan and agarans, ii) brown seaweeds (*Phaeophyta*) have fucoidans, iii) green seaweeds (*Chlorophyta*) have ulvans, while iv) sea cucumbers and cartilaginous fish have chondroitin sulphates in its tissues, v) sponges have sulphated glucan and heteropolysaccharides and vi) microalgae have different heteropolysaccharides.

Sulphated polysaccharides (SP) belong to the most abundant natural compounds produced by living organisms. The literature describes them as compounds with antibacterial (Liu et al., 2017) antiviral (Subramaniam et al., 2015) and antioxidant (Wang et al., 2019) activity, but also with anticoagulant (Jin et al., 2013) and immunomodulatory/anti-inflammatory (Geng et al., 2018) properties. This makes these molecules excellent targets for use as potential prebiotics (Gotteland et al., 2020).

Being molecules with a great range of bioactive properties, the sulphated polysaccharides also present themselves as potential osteogenic compounds, improving bone mineralization and preventing osteoclastogenesis, a process that gives origin to the osteoclasts that degrade bone tissue. This is shown in studies that focused on a well-known, highly sulphated polymer, a fucoidan extracted from the sea cucumber *Apostichopus japonicus* and from brown algae species (Kariya et al., 2004; Kim et al., 2014).

An analysis through the literature showed an increasing interest on the discovery of new, bioactive, marine derived, molecules and compounds that can upregulate the osteogenesis such as, fucoidans (Hwang et al., 2016), phenolic compounds from green algae (*Cladophora rupestris* and *Codium fragile*) (Surget et al., 2017) and a powder extract from the red algae *Ceramium pallidum* (Carson et al., 2018). This diversity of compounds calls for a screening method that is simple, robust, and capable of giving high data output. Tarasco et al. (2017), have created a method resorting to the zebrafish operculum, one of the first bones to calcify on the zebrafish larvae head (Gavaia et al., 2006; Kimmel et al., 2010). The opercular bone is located on the side of the head, very near to the surface and its structure is flat making the imaging and consequent morphometric analysis easily

accomplished in a timeframe as little as four days from exposure to data collection making this protocol useful for large-scale analysis and validation of several potential drug compounds.

One characteristic of these compounds (SP) is its high solubility in water. The Extracellular Polymeric Substances (EPS) analysis tells us that they are present in high amounts at intertidal areas (Pierre et al., 2013). Besides that, SP represent most organic components present in sea salt polymeric material (Silva et al., 2015) that originate from marine organisms trapped in the crystalline matrix (Engel & Händel, 2011). Nunes et al. (2019), has studied the salt pan brine water (Figure 1.3), a salt crystallization by-product, as SP source as well as its immunostimulant properties, opening the door for, as stated by the authors, the incorporation of these compounds in functional foods with health therapeutic applications.

Figure 1.3: Salt pan explored by Necton S.A. Olhão, Algarve - Portugal. Image from necton.pt

In this thesis the brine derived polysaccharides were studied in order to understand the immunostimulant and mineralogenic effects of these compounds.

1.1. Objectives of the work

The present work is divided in 3 main objectives: the first objective is to access the mineralisation potential of the SP by measuring the opercular bone growth in zebrafish larvae exposed to different concentrations of these compounds The second objective is to elucidate the molecular mechanisms of defence involved in response to SP in an Atlantic salmon head-kidney cell line (SHK-1) under LPS-induced inflammation. The third objective is to evaluate the immunomodulation capacity of SP enriched microdiets on juvenile zebrafish under a bacterial challenge with *Aeromonas hydrophila*.

2. Material and Methods

2.1. Zebrafish Rearing Conditions

Fish used in this work were obtained from the Centre of Marine Sciences (CCMAR, Portugal) facilities. Two zebrafish reporter lines were maintained in distinct breeding populations for this study, *Tg(mpx:GFP)* (ZFIN ID: ZDB-TGCONSTRCT-070118-1) and *Tg(mpeg1:mCherry)* (ZFIN ID: ZDB-TGCONSTRCT-120117-2). The fish room operates under a 14:10 light: dark photoperiod, with an independent air conditioning and air extraction system keeping air temperature at 26 ± 1 ºC and air humidity around 60%. Fish were kept in 3.5 L tanks in a 980 L recirculating housing system (ZebTEC®, Tecniplast, Italy) with a dedicated water treatment unit. Water quality is achieved by a 10% daily water renewal and through filtration, including mechanical filtration with 50 µm plated cartridge filters, biological filtration with ceramic beads, a carbon filter with granular activated charcoal and an ultraviolet sterilization system operating under 180 000 µWs/cm². Water conditions were set at: 28.0ºC ± 0.5ºC, pH 7.5 ± 0.1 and conductivity 750 ± 30 µS. Nitrogenous compounds, ammonia, nitrites and nitrates, were monitored weekly and kept below toxic limits (0.1 mg/L for ammonia and nitrite and 100 mg/L for nitrate).

2.2. Rotifer Rearing Conditions

Rotifer (*Brachionus plicatilis* – L) stocks were obtained at Centre of Marine Sciences (CCMAR, Portugal). The rotifers were cultured in 27 L fermenters (Fermzilla 27L, KegLand, Australia) with artificial saltwater produced using reverse osmosis (RO) water (RO Water Maker - MROT-4000 Aquaneering, Inc., USA) and Tropic Marine® synthetic salt (Tropic Marine Centre, United Kingdom) to reach a salinity of 16 ppt. Water temperature was kept around 25 ºC with mild aeration.

Rotifer population was controlled by daily counting of the total number of rotifers per 1 mL and the total number of females carrying eggs. All the counts were performed under a stereomicroscope Leica MZ6 (Leica, Germany). After accessing the rotifer´s population, the amount of food needed was calculated. Rotifer´s were fed twice a day with Phytobloom Green Formula® (Necton SA, Portugal) using 2.5 mL per million rotifers in culture. During the last feeding of the day, a high nutritional culture medium for microalgae, Phytobloom NutriBloom (Necton SA, Portugal) was added to the culture

water at a concentration of 0.5 mL/L and an ammonia neutralizer ClorAm-X (AquaScience Research Group, Inc.) at a concentration of 31.9 mg/L, as recommended by the manufacturer, to avoid total ammonia nitrogen (TAN) concentration. Once a week the rotifers were concentrated and washed using a 50 µm sieve in order to clean debris accumulation in the containers and to prevent contamination by ciliates.

2.3. Sulphated Polysaccharides

For this study four different samples of polymeric material extracted from salt pan brine water were used, with different concentrations of SP. The salt pan brine water samples were collected by Necton – Companhia Portuguesa de Culturas Marinhas S.A. and shipped to Universidade de Aveiro (UA). The four samples have gone through different purifying methods applied by UA, freeze dried and kindly provided to our laboratory for testing. The concentrations used were calculated taking into account the amount of SP in each sample. Table 2.1 shows the total amount of each sample that was available to us as well as the SP concentration in each sample.

Table 2.1: Sulphated polysaccharides sample characterization, as supplied by University of Aveiro.

Samples	Sample (original name)	Description	Total weight (g)	Sulphated Polysaccharides (mg/g)
SP1	E8_2021 Conc_8	Hooven dried	0,1153	311
SP2	E8_2021 Conc_8		0,13	293
SP3	2021-C4	Crystalizer water	0,109	313
SP4	E9_2022 Diafiltrado 7	Diafiltered	0,1173	324

2.4. Operculum assay

2.4.1. Tested Conditions

Before the samples described in table 2.1 were purified and made available to us, we had received other samples with very low degree of purification regarding SP from Necton

S.A.. They contained a very low amount of SP and high quantity of salt what made difficult for us to test them in zebrafish larvae, hence mineralization results of this samples will not be considered for this thesis.

Nevertheless the tests conducted served to determine the range of concentrations we would have to use in further tests. Hence the concentrations (weight/volume) used to test these four different samples were as follows: 0.0001%, 0.0033%, 0.01%. Plus the following controls: Control with brine water (BW) – to understand if the brine water by itself (brine water from which the samples were purified) had some type of effect regarding the opercular bone mineralization. This control ended up being removed from the experiments and therefore from the results presented due to the presence of heavy metals whithin the samples of unpurified brine water that we were using; Negative control with artificial salt (AS) – The salt from TropicMarin® was used to normalize the salt concentration for each condition and to compare directly with the brine water to help us understand if there was any kind of effect of the brine water by itself; Negative control with non exposed (NE) larvae. The solutions for all the concentrations were made using distilled water as the vehicle to carry the molecules of interest. The controls were also made with the same water.

The salinity of the four SP samples was not described when sent to us. Since we needed to know this parameter in order to establish the concentrations to be tested and also to make sure that in every condition there was the same amount of salt we made a stock-solution at 0.1% (w/v) so we could measure the salt concentration, using a refractometer. For each sample the salt concentration was zero or very near to zero, so we decided to match the salinity in every condition for all the samples, apart from the NE control, with the salinity of the system where the fish are reared, that is 750 µS or 480 mg/L.

2.4.2. Experimental Design

2.4.2.1. Zebrafish breeding

Wild-type AB zebrafish couples were selected from the broodstock available at LEOA facilities and were placed in breeding tanks for around 16 hours. After this period the fertilized eggs were selected and placed to incubate in 1 L tanks with system water and methylene blue (0.05%) acting as an anti-fungic for 3 days (figure 2.1).

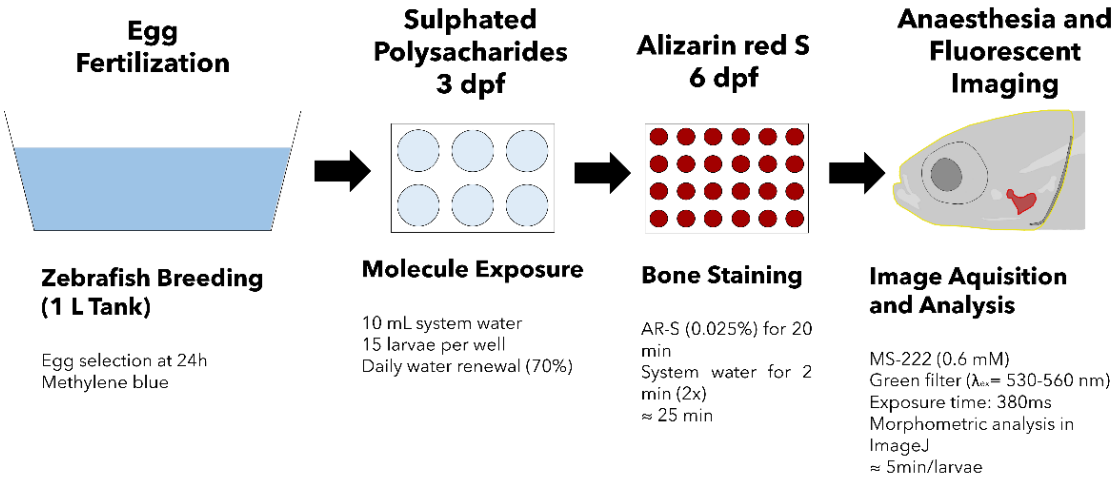

Figure 2.1: Operculum assay protocol flowthrough illustration.

2.4.2.2. Molecule exposure

At 3 dpf larvae were exposed to the SP dilutions of interest (Figure 2.1). For each sample 5 conditions were tested: 0.0001%, 0.0033%, 0.01%, AS, NE. Exposure took place in 6-well plates with 10 mL/well and 15 larvae/well (Figure 2.2). The exposure was done in triplicates, so there were 45 larvae exposed to each condition. During the exposure period the plates with the larvae were mantained in an incubater set to 28° C with a 14:10 light:dark photoperiod. A 70% water renewal was made every day in each well.

Figure 2.2: 6-well plates where zebrafish larvae were exposed to the SP extracts.

2.4.2.3. Bone staining

At 6 dpf, after the exposure period, larvae were transfered to 24 well plates with 2 mL/well where they were exposed to an alizarin red S (AR-S) solution (0.025%) for staining bone (Figure 2.1).

A 0.1% AR-S solution was first made by mixing 50 mg of AR-S powder with 45 mL of system water in an erlenmeyer. After homogenizing the solution the pH was measured while a magnet kept on agitating the solution and sodium hidroxide (NaOH) was added

until pH = 7.4. System water was added until the final volume was 50 mL. The solution was kept in a 50 mL centrifuge tube wrapped in alluminium paper to avoid exposure to light since it decreases the dye intensity.

For preparing the work solution, we used 2.5 mL from the stock solution that were dilluted in 7.5 mL of system water in order to achieve 10 mL of 0.025% AR-S solution. During the staining procedure we transferred the larvae from the 6-well plate to the 24-well plate, removed most of the water they were in and immidiatly fill the well with 2 mL of the AR-S working solution. They were kept in this solution during 20 minutes. After this period the AR-S solution was removed and the larvae were washed two times for 2 minutes in system water.

2.4.2.4. Image Acquisition and Analysis

Zebrafish larvae were anaesthetised with a tricaine (MS-222) solution (0.2 mM) in the 24-well plate with system water. In order to have a clear picture of the opercular area the fish larvae were placed in an agarose plate with a small hole so the fish head could be supported by it and the fish was able to be positioned laterally. With the fish immobilized, pictures were taken, under a green fluorescent filter with the ZEISS® Axio Zoom. V16 stereomicroscope, in order to observe and analyse the operculum bone and head area using ImageJ® software. The quantification of the mineralized operculum was achieved after taking the measurements of the operculum area (Op) and head area (Hd), and organizing and correcting the data using a normalization technique to minimize redundancy and so that data from each condition was relative to the control (AS). The following equation was used: $Corrected\ value = \frac{\frac{Op}{Hd}*100}{\bar{x}(\frac{Op}{Hd}(AS))}$.

2.5. SHK-1 essay – Total RNA isolation and gene expression analysis by qPCR

2.5.1. Experimental Design

2.5.1.1. Cell culture and Plate Seeding

Head kidney salmon cells (SHK-1) (Dannevig et al., 1997) were cultured using Gibco® L-15 (ThermoFisher, Germany) culture media supplemented with 15% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, 1% L-gluthamine and 0.2% Fungizone in an incubator (Binder KB53, Germany) at 20°C. Cells were checked daily and the plate divided reaching a 70-80% confluency.

After checking the cell culture under an inverted microscope with phase contrast we aspirated the culture media with a glass Pasteur pipette connected to a vacuum pump and washed the cells with a PBS solution. After washing we added a trypsin solution. The digestive action of this enzyme will make the cells detach from the bottom of the plate giving them a different phenotype, rounded and shiny compared to elongated and opaque when attached. After a quick check under the microscope to make sure of the cell detachment we added culture medium (L-15). At this point the trypsin will start to digest the protein in the medium and stop digesting the cells. Then we homogenized the cell culture by pipetting up and down while slowly agitating the plate and the cells were transferred to a 50 mL centrifuge tube. We homogenized the culture one more time and used 12 µL for counting purposes in a Neubauer chamber. Cells were added to a 6-well plate at a concentration of $5x10^5$ cells/well. Image 2.3 summarizes the protocol followed.

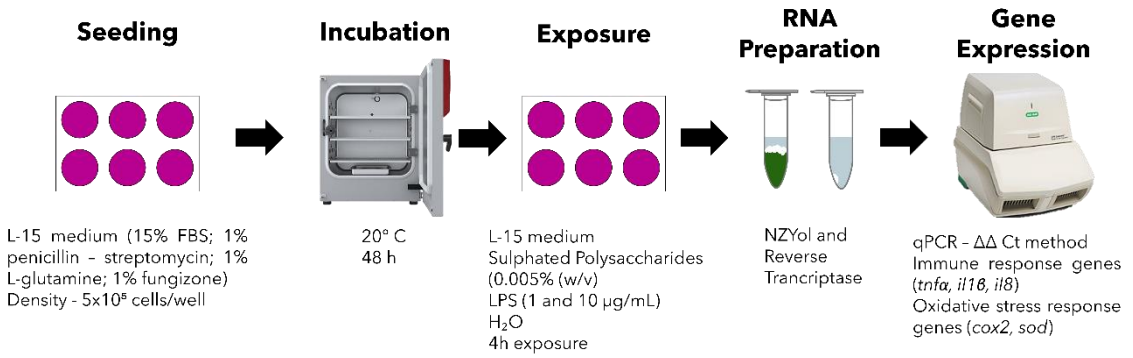

Figure 2.3: Illustration of the SHK-1 assay protocol flowthrough.

2.5.1.2. Exposure

Initially four stock solutions at 0.5 % (w/v) concentration (Figure 2.4) were prepared from the four different samples.

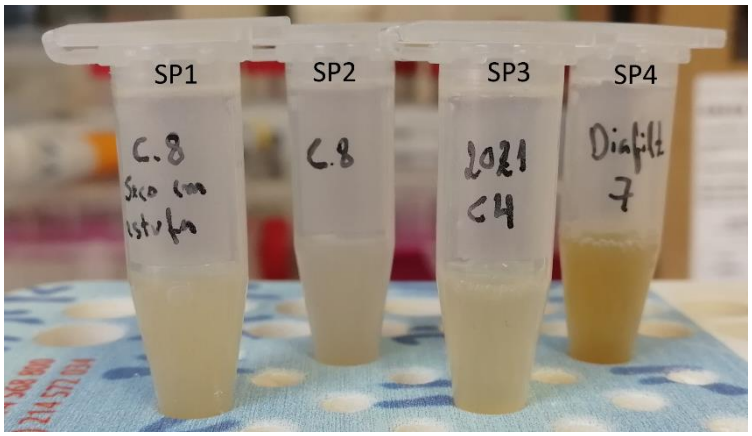

Figure 2.4: Four different SP extracts (SP1, SP2, SP3, SP4). Stock solutions made at 0.5% (w/v) concentration.

As a preliminary trial, exposure was done at a concentration of 0.001% (w/v). Further exposures were done at a 0.005% (w/v). Table 2.2 shows a description of the volumes used to make the exposure solution concerning the two different concentrations.

Table 1.2: Description of the solutions to which the cells were exposed. Volumes used from the culture medium (L-15), from the four different SP samples (SP1, SP2, SP3, SP4), from the positive control (LPS1, LPS10) and from the negative controls (H_2O (4), H_2O (15).

		CONCENTRATIONS %(W/V)			
		0,001		0,005	
		L-15 Vol (mL)	Sample Vol (µL)	L-15 Vol (mL)	Sample Vol (µL)
CONDITIONS	SP1	1.5	3	1.5	15
	SP2	1.5	3	1.5	15
	SP3	1.5	3	1.5	15
	SP4	1.5	3	1.5	15
	LPS1	1.5	4.75	1.5	4.75
	LPS10	1.5	4.75	1.5	4.75
	H_2O (4)	1.5	4.75	1.5	4.75
	H_2O (15)	1.5	3	1.5	15

The positive control used was a lipopolysaccharide (LPS) from the Gram-negative bacteria *Escherichia coli*. It was used at two different concentrations. Hence in table 2, LPS1 refer to 1 µg/mL and LPS10 to 10 µg/mL. The negative control was the water used to make the sample and LPS solutions. So, as a negative control for the LPS solutions (H_2O (4)), we used 4.75 µL of SIGMA water, and as a negative control for the sample

solutions (H_2O (15)) we used 15 µL of distilled water. All the exposures were done for period of 4 hours.

2.5.1.3. Isolation of total RNA

After exposure, 1 mL of NZYol was added to each well (1 mL/10 cm^2 growth) pipetting up and down several times to ensure cell disruption. The lysate was transferred to 1.5 mL cap tubes and stored at -80 °C for posterior RNA extraction.

We, then, proceeded to the phase separation. First, we let the samples for 5 minutes at room temperature. We added 0.2 volumes of chloroform per 1 volume of NZYol used and shook the cap tubes by hand for about 15 seconds. We let the samples rest for 2-3 minutes at room temperature and centrifuged at 12 000 g for 15 minutes at 4 °C. After this we were able to see a 3-phase separation: a colourless upper aqueous phase that contains the RNA, an interphase containing cell debris and the DNA and a lower green phenol phase also called organic phase because it contains organic material such as proteins and lipids. The next step had to be done with extreme care since we had to remove the aqueous phase without making contact with the other two phases to avoid contamination. We removed around 400 µL to a new 1.5 mL Eppendorf tube.

RNA precipitation was done by adding 1 volume of isopropanol to 1 volume of aqueous phase recovered, so in this case 400 µL. We inverted the tubes several times and incubate them at -20 °C for 3 to 4 hours. The longer the incubation time at this step more precipitation will occur. Off course, we should keep in mind that, this is valid for RNA as well as for any other contaminating molecule that may be present.

After incubation the samples were removed from the freezer and centrifuged at 12 000 g for 15 minutes at 4 °C. At this point we should be able to see a pellet formed at the bottom of the tube. After centrifugation, the supernatant was discarded with a micropipette with the tip placed away from the pellet. Then, to wash the RNA we added 1 volume of 75% ethanol (made with RNase-free water) per 1 volume of aqueous phase. In reality, we added 100 µL more to make sure that the samples were properly washed. We shacked the tubes by hand and centrifuged them at 12 000 g for 5 minutes at 4 °C. After this, again with extra care not to remove the pellet, we discarded the supernatant as much as possible and let the tubes open to air dry the pellets for at least 10 minutes.

After making sure that the pellet was well dried, it was resuspended in 30 µL of Rnase-free water (SIGMA® water), allowed to rest for 5 minutes at room temperature, homogenized by giving it a little flick and allowed to rest for 5 more minutes at room temperature. After being homogenized, 1 µL was removed to use at a UV-VIS spectrophotometer – NanoDrop® (Thermo Scientific)– to measure RNA concentration (µg/µL) as well as purity according to two ratios: A260/A280 – with optimum values between 1.8 and 2.2. Values above 2.2 means protein contamination and values below mean DNA contamination; A260/A230 – with optimum values above 1.7. Values below it means phenolic substances contamination such as guanidine-thiocyanate. The remaining 29 µL were stored at -80 °C.

2.5.1.4. RNA Electrophoresis – Integrity Check

In order to check the RNA integrity, we performed an electrophoresis expecting to have sharp, clear 28S and 18S rRNA bands. First, we washed and rinsed all the material needed for the gel preparation with a 0.1M NaOH solution (4g of NaOH in 1 L of MiliQ water). Then we prepared a 1X TAE solution (20 mL of TAE 50X in 1 L of MiliQ water) that we used to make the agarose gel and to run it during electrophoresis. To make the gel we used 0.75g of agarose and diluted it in 50 mL of TAE 1X in an Erlenmeyer flask. We melted the agarose in a microwave. After achieving total dilution of the agarose powder, we let the flask cool down until we were able to handle it and we added the nucleic acid stain Safe-Green (0.65 µL in 50 mL) to be able to visualize the nucleic acids in the agarose gel. The samples were prepared in order to contain 500 ng of RNA. Given the RNA concentration values that we obtained at the NanoDrop® we calculated the volume of the RNA samples that we needed to pipette and the volume of water that we had to add to have a final volume of 5 µL for each sample to load in the gel. Hence, we loaded the 5 µL samples plus 1 µL of the loading buffer Safe-Green (1:5). Finally, we run the gel at 120 V for 20-30 minutes and checked it under UV light to see the RNA bands.

2.5.1.5. DNase Treatment - RNA Samples

After the RNA integrity check we performed the DNase treatment of the RNA samples. This step was made to remove the genomic DNA from RNA samples. Since it is a DNA-

specific enzyme it will digest single and double-stranded DNA without interfere with RNA structure.

Having into account the RNA concentrations, we calculated the volume in microliters to have 1 µg of RNA per sample. The volume of water (SIGMA® water) was also calculated in order to have a 7.55 µL volume of RNA plus water for each sample. To each PCR tube was also added 0.95 µL of RQ1 DNase reaction buffer (10X) and 1 µL of RQ1 DNase completing a final volume of 9.5 µL per PCR tube. The tubes were flicked and spined down to ensure homogenization. In order to reduce the number of tips used the mixture in each tube was performed in the following order: water; buffer; RNA and the enzyme. When taking the enzyme from the freezer we should always put it in a small cooler designed to accommodate it and to avoid frequent temperature changes. The samples were then incubated at 37° C for 30 minutes, to which succeed the adding of 1 µL of RQ1 DNase stop solution and the incubation at 65° C for 10 minutes to inactivate the enzyme.

2.5.1.6. Reverse transcription

Once the DNase treatment was completed, we proceeded to the cDNA synthesis using Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT). We first added 1 µL of Oligo dT (50 µM) complimentary to the poli-A sequences at the mRNA strands. Then, we added 1 µL of dNTP - free nucleotides, that are used by the enzyme to synthesize the complementary chain. The samples were homogenized by flicking, followed by a quick spinning and incubated at 65°C for 5 minutes to denature RNA, followed by a quick chill on ice. With the samples on ice, we added 4 µL of 5X first strand buffer, 2 µL of DTT (0.1M), a reducing agent that will optimize the enzyme function, and 1 µL of Ribolock, an RNase inhibitor that prevents RNA from degrading. After this the samples were incubated at 37° C for 2 minutes. We added 1 µL of the enzyme M-MLV RT and incubated the samples at 37° C for 50 minutes followed by an incubation at 70° C for 15 minutes. Finally, we diluted the cDNA in a 1:10 ratio by adding 180 µL of SIGMA® water.

2.5.1.7. Semi quantitative PCR (qPCR)

qPCR was used to analyse expression levels of the genes of interest related to the immune response: *tumor necrosis factor alpha* (*tnfα*), *interleukine 1β* (*il1β*), *interleukine 8* (*il8*);

and oxidative stress response - *cyclooxygenase 2* (*cox2*), *superoxide dismutase* (*sod*). The expression of this genes was normalized using the ΔΔCt method and having as reference the housekeeping genes - *elongation factor 1alpha* (*ef1α*) and *beta actin* (*βact*) – to obtain the relative expression of the genes of interest (Table 2.3).

Table 2.3: Primers sequences of the genes used in qPCR reactions. The sequences are written in 5´ to 3´ orientation.

Gene	**Primer**	**Sequence (5´- 3´)**
ef1a	Forward	GTGGAGACTGGAACCCTGAA
	Reverse	CTTGACGGACACGTTCTTGA
βact	Forward	CTGGACTTTGAGCAGGAGAT
	Reverse	GGAGTTGTAGGTGGTCTCGT
tnfα	Forward	AGGCTTTTTCCCAGGGC
	Reverse	GAGTCCGAATAGCGCCAA
il1β	Forward	ACAAGTGCTGGGTCCTGATG
	Reverse	TAGGGCTACAGGTCTGGCTT
il8	Forward	AGAATGTCAGCCAGCCTTGT
	Reverse	TCTCAGACTCATCCCCTCAGT
cox2	Forward	TCGCTCACATTTGGTGGACA
	Reverse	GCGGTTCCCATAGGTGTAGG
sod	Forward	CCGTATTCTTTGAGCAGGAG
	Reverse	AGCCGTTGGTGTTGTCTC

We had first to prepare two master mixes (MM), from which we could then pipette to the 96-well plate, considering the number of genes (master mix containing the specific primers – MM-primers) and samples (master mix containing the cDNA samples – MM-RT) that we wanted to analyse. For the MM-primers we added, for each gene, 10 µL of SensiFAST™ SYBR® Green- that intercalates between the nucleotides and emit fluorescence; 0.8 µL of primer forward (FW) and 0.8 µL of primer reverse (RE). Primers were diluted in a 1:10 ratio, diluting 5 µL of the stock solution in 45 µL of SIGMA® water to have a working solution at 10 µM. For the MM-RT we added, for each sample, 2 µL of cDNA and 6.4 µL of SIGMA® water. To make sure that the MM were enough we always made them in excess (one time more for the MM-RT and half time more for the MM-primers).

As an example, if we had 6 samples and 4 genes to test, the calculation would have been performed as it is depicted in table 2.4.

Table 2.4: Example of calculations made concerning the master mixes MM to perform qPCR

MM-primers	Nº of samples	Final Volume	MM-RT	Nº of genes	Final Volume
10 µL SensiFAST	6	10*6,5= 65 µL	**2 µL RT**	4	2*5= 10 µL
0,8 µL FW	6	0,8*6,5= 5,2 µL	**6,4 µL SIGMA® water**	4	6,4*5= 32 µL
0,8 µL RE	6	0,8*6,5= 5,2 µL			

As we can see, the final volumes were achieved considering 6.5 samples instead of 6 and 5 genes instead of 4 to have an excess of the mixes has a backup to any technical problem or accident. This procedure was made with the tubes inserted in a styrofoam box full of ice. Once the mixes were made, it was time to fill the 96-well Bio-Rad® plate to run in the qPCR machine.

In each well of the plate, we pipetted 11.6 µL of MM-primers and 8.4 µL of the MM-RT, so that each well had a final volume of 20 µL. During this procedure the plate was placed in a cooler to avoid drastic changes in temperature. After pipetting, the plate is covered with a plastic sealing and a spatula is used to make sure that the seal is well glued to the plate surface. Finally, we made a fast spin to the plate in a centrifuge and started the qPCR cycle, as shown in Figure 2.5.

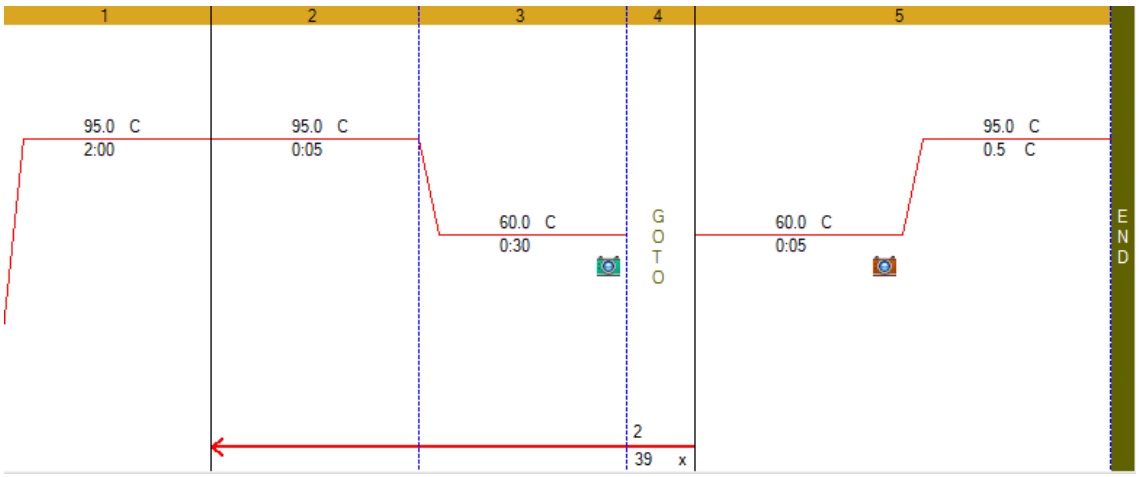

Figure 2.5 Illustration of the cycle used in qPCR. Two minutes at 95° C at step 1, followed by 0.5 seconds at 95° C at step 2 and 30 seconds at 60° C at step 3. Steps 2 and 3 repeated 39 times. After the cycle a melting curve was made in a ramp from 60° C to 95° C with steps of 5 seconds.

2.6. Bacterial Challenge

In order to test the sulphated polysaccharides immunostimulant capabilities in a *in vivo* system we decided to challenge zebrafish juveniles, with 40 dpf, with a bacterial infection by exposing them to the LD50 concentration of a particular strain of the bacteria *Aeromonas hydrophila*. In this trial we pre-conditioned the fish with a dietary treatment with three different SP concentrations and a negative control before the exposure to the bacteria.

All the fish used for this trial were kept at CCMAR facilities and transferred to a temperature-controlled room at the Aquaculture Research Station at the Portuguese Institute for the Ocean and Atmosphere, in Olhão, Faro, to perform the bacterial infection.

2.6.1. Pre-trial

A pre-trial was carried on in order to access the bacterial concentration correspondent to the LD50 to then use it to challenge the fish pre-conditioned with the SP-incorporated diet. The bacteria used – *A. hydrophila* (AH-1) - was obtained from a laboratory in Ghent University - Belgic and shipped to Portugal in 1.5 mL tubes with Trypticase Soy Agar (TSA) solid medium and then stored at – 80 ^{o}C.

2.6.1.1. Experimental Design

Wild-type AB zebrafish juveniles, raised under standard feeding protocols, with ages around 40 dpf, were randomly selected from the existent stock at LEOA facilities, mixed together and exposed to three different concentrations of the bacteria - 10^5, 10^6; 10^7 - plus two controls: CA – amputated and not exposed – a control to the amputation procedure, to confirm that the fish don´t die because of the amputation but because of the exposure to the bacteria; CB – not amputated and exposed – to evaluate the virulence of the bacteria in unharmed fish.

Exposure to the bacteria was done via water bath during 5 hours in 1 L tanks with 15 fish per tank, each tank corresponding to a different bacterial concentration.

After the exposure time, the 15 fish of each condition were placed at a density of 5 fish per litre so we could have three different tanks per condition.

During the following days the fish were monitored and mortality events were recorded until a 50% cumulative mortality rate was achieved at least in one of the three bacterial concentrations tested. The cumulative mortality rate was used to determine the LD50 with the data collected from three time-points each day (9 a.m., 1 and 5 p.m.).

During the trial all the fish were fed with Zebrafeed® (100 – 200 µm) commercial diet produced by Sparos Lda, and 90% of the water was renewed daily in all the tanks.

2.6.1.2. Infectious Bacteria Production

An aliquot from the stock sample was taken to inoculate TSA solid medium. After 18 to 24 hours a small quantity of the bacteria was collected to inoculate a 3 mL tube with Tryptic Soy Broth (TSB) liquid medium.

The 3 mL tube was left to agitate at 130 rpm and 28 °C in a heater incubator with shaker (Optic Ivymen System COMECTA S100D) and after 18 to 24 hours 1 mL of the culture was transferred to a 50 mL tube with TSB liquid medium and left to grow at the same agitation and temperature conditions.

The bacterial growth was tracked by measuring the culture optical density (OD) in a VWR – UV1600PC spectrophotometer, at 620 nm, right after inoculation and every hour after it, until the culture reached the exponential phase. This phase is determined when the OD value at 620 nm is above 1.0, ideally being 1.3. This value is estimated to be the equivalent to 10^{10} CFU/mL concentration according to the linear regression made with the data collected during the bacterial growth tracking. When this OD value was achieved three serial dilutions were made according to the ratios 1:100, 1:1 000 and 1:10 000 to have, respectively, the concentration of 10^8, 10^7 and 10^6 CFU/mL and 100 µL of each dilution was inoculated in a different plate with TSA solid medium and incubated at 28 °C.

Finally, after 24 to 48 hours the colonies of each plate were counted in order to confirm the 10^{10} CFU/mL concentration estimated by the OD values and the linear regression calculation.

2.6.1.3. Infection Procedure

After making sure that the bacteria were actively growing, we could then induce the infection on the fish. To do that we first had to take a 1 mL sample from the 50 mL tube with TSB liquid medium to measure the OD at 620 nm. According to the value obtained we proceeded to the dilutions to obtain the bacterial concentrations to which the fish were exposed to via water bath - 10^7, 10^6 and 10^5 CFU/mL. The inoculum was then centrifuged at room temperature during 10 minutes at 3 000 rpm. After centrifugation the supernatant was discarded and the pellet was kept and re-dissolved, by agitation, in 50 mL of PBS (1X).

The zebrafish caudal fin was amputated with a sterile scalpel immediately before the start of the fin bifurcation to ensure the existence of an entrance door for the bacteria during exposure. All the fish were anaesthetized with tricaine (MS-222) at a 0.2 mM concentration to endure this procedure. After the caudal fin amputation, the fish were immediately placed in different water baths with the different bacterial concentrations where they recovered from anaesthesia and stayed in exposure for a period of 5 hours.

A volume of 100 µL of each water bath concentration was inoculated in TSA plates and incubated for 24 to 48 hours at 28 ^{o}C and then used to count the existing colonies to compare to the estimated concentration.

2.6.1.4. PCR – Bacterial DNA identification

A tissue sample from one of the dead fish collected during the bacterial exposure was removed and the DNA was isolated and amplified in order to identify the pathogen in study using electrophoresis.

The DNA was isolated using the NZY Tissue gDNA isolation kit from Nzytech® and the protocol followed was the one recommended by the manufacturer. The amplification was done by the enzyme Supreme Taq DNA polymerase and followed the cycle described in the table 2.5. The PCR reaction components are described in table 2.6.

Table 2.5: PCR reaction cycle

Temperature	**Time**	**Cycles**
95ºC	3’	
95ºC	30’’	30x
60ºC	30’’	
72ºC	1’	
72ºC	10’	
4ºC	∞	

Table 2.6: Volumes of the reagents used in the PCR reaction

Reagents	**Volume per sample (µL)**	**Volume Mix (µL) x3,5**
H20	17,79	62,3
Buffer 10x	2,5	8,75
MgCl2 50mM	1,25	4,4
dNTPs 10mM	1	3,5
Primer Reverse (10µM)	0,63	2,2
Primer Forward (10µM)	0,63	2,2
DNA	1 (30ng)	-
Supreme NZYTaq II DNA Pol	0,2	0,7
Final Volume	25	24 (per tube)

The gel was made with 1.5% agarose in TAE (1X) solution and electrophoresis run at 120 V in TAE 1x. The nucleic acid stain used was Safe-Green (1.3 µL in 100 mL).

A DNA sample from an isolated strain of *A. hydrophila* was used as positive control and nuclease-free water was used as negative control.

2.6.1.5. Biochemical Characterization of the Bacteria

After removing the tissue sample from the dead specimen above mentioned a swab was made on that tissue and inoculated in a TSA plate for isolation and purification. A small

sample from the fresh culture was removed and dissolved in 1 mL of PBS (1X); 10 µL of this solution were used to determine the bacteria morphology and motility under a light binocular microscope (Leica® DM750) with an incorporated imaging system. The remaining solution was used to perform additional characterization tests.

Gram test – Levi method

After placing a drop of potassium hydroxide (KOH) (3%) in a Petri dish, we used a sterile toothpick to remove a visible amount of one of the fresh colonies growing at the TSA plate. After homogenization of the sample in the reagent drop, we carefully raised the toothpick about 1 cm above the drop and observed if there was any formation of a string of mucus connecting the tip of the toothpick and the surface of the drop.

For a gram-positive bacteria there is no mucus string formation. For a gram-negative there is. We should wait 5 to 60 seconds to make sure the sample is homogenized in the reagent.

Oxidase test

In this test the presence of the enzyme oxidase was evaluated using oxidase reaction strips, commercialized in a kit – MB0266 Oxidase Reaction Strips - by OXOID®.

With a sterile toothpick we removed a small amount of a fresh colony and spread it on one end of the strip. In about 5 seconds, the presence of the enzyme is revealed by the appearance of a violet/purple colour.

Catalase test

In this test the presence of the enzyme catalase was determined. This enzyme catalyses the decomposition of hydrogen peroxide (H_2O_2) in oxygen and water.

After placing a drop of H_2O_2 (30%) in a Petri dish, a small amount of fresh colony was taken and homogenized in the drop of the reagent.

A positive result is obtained by the formation of gas bubbles (“foam”) due to the O_2 releasing.

Oxidation/ Fermentation test

Difco® OF basal medium containing tryptone, source of amino acids and carbon for the bacteria, and bromothymol blue used as a pH indicator, supplemented with D-glucose (Merk®) as source of carbohydrates, was used for determination of oxidative and

fermentative metabolism of carbohydrates based on an acid reaction. Two tubes with the medium were inoculated with fresh culture and one of them was sealed with melted paraffin to provide an anaerobic environment. Any changes in colour are due to changes in the pH of the medium. The pH variation on the sealed tube is due to fermentative activity while in the open tube is due to the oxidation of the carbohydrates present in the medium.

The ability of the bacteria to oxidise carbohydrates in acidic products can be noticed by a green to yellow change in the medium, only in the open tube.

A fermentative result is obtained when the acid production is visible in both open and closed tubes indicated by a colour change - green to yellow - in both tubes.

A negative carbohydrate utilization - non-oxidizer and non-fermenter bacteria – is indicated by the absence of a yellow colour in both tubes resulting on the remaining of the green colour or in a change to blue.

Incubation time can go up to four days, in our case, at 28 ºC.

Arginine, Lysine and Ornithine test

In this test the decarboxylase broth medium (Fluka®) was used to access if the bacteria could use a certain amino acid as source of carbon and energy resulting in an alkaline shift in the pH of the medium. That shift can be visualized due to the presence of pH indicators such as bromcresol purple and cresol red.

In this case the medium was supplemented with the amino acids arginine, lysine and ornithine. The inoculation was done in 1.5 mL Eppendorf tubes with 20% of the PBS (1X) bacterial suspension in 1 mL of the medium for each amino acid and then covered with melted paraffin to create an anaerobic environment. Incubation time can last between 24 and 48 hours.

A positive result – the amino acid metabolization – is indicated by the appearance of a violet colour in the tube. A negative result – the non-metabolization of the amino acid – is indicated by a translucid yellow colour in the tube.

Triple Sugar Iron (TSI) Agar – Sugar Fermentation test

TSI (Merk®) is used as culture medium in a test that evaluates the microorganism ability to use simple carbohydrates as substrate and produce hydrogen sulphide through a fermentative process. The medium is constituted by the carbohydrate glucose, lactose and sucrose, sodium thiosulphate and the pH indicator phenol red. The medium is solidified in test tubes at an angle allowing the formation of a slant that provides a bigger surface exposed to oxygen for aerobic metabolism. The inoculation was made by perforation of the medium with a sample of fresh culture and then a swab on the slant surface. Incubation period can go up to four days.

Carbohydrate fermentation is indicated by the presence of the yellow colour in the medium. If the medium on the body of the tube turns yellow – acidic pH – but it remains red on the slant – alkaline pH – the microorganism only uses glucose as a fermentative substrate.

The appearance of the yellow colour both in the medium on the body of the tube and in the slant indicates that the microorganism ferments glucose, lactose and/or sucrose.

A red colour – alkaline pH – on the slant and on the medium on the body of the tube can be understood as a negative result – the microorganism is non-fermenter.

The production of hydrogen sulphide is detected by the formation of a black precipitate at the bottom of the tube.

2.6.2. Trial – Challenging Zebrafish Juveniles with *A. hydrophila* Infection.

2.6.2.1. Tested Conditions

The fraction of sulphated polysaccharides used for this trial was the one identified as SP3 and described in section 2.3, table 2.1. As for the SHK-1 essay, a 0.5% (w/v) stock solution of this fraction was made to facilitate the process of inclusion of these molecules in the commercial diet in order to obtain three different concentrations of the experimental diet (Table 2.7).

Table 2.7: Brief description of the different conditions of feed preparation.

Conditions Tested	Description
Negative Control	Re-processed commercial diet
0.005% (w/v)	Re-processed commercial diet + SP3 Fraction
0.01 % (w/v)	Re-processed commercial diet + SP3 Fraction
0.05 % (w/v)	Re-processed commercial diet + SP3 Fraction

2.6.2.2. Experimental Feed Preparation

The amount of feed needed for this trial was estimated considering an adaptation of the methodology described in the work of Martins et al. (2019) with a constant increase of 1 mg in the daily amount of feed. For the 140 larvae per condition that we had we needed around 4 g of feed for each condition in a 50-day treatment. As safety we did an overestimation, regarding the number of days of the treatment, and decided to increase the total amount of feed to 5 g per condition. This would safely ensure that we had enough feed for the entire trial duration. The molecule incorporation was done in Zebrafeed® commercial diet.

In order to obtain a 0.05% (w/v) SP inclusion in 5 g of the commercial feed were needed 2.5 mg of the SP3 extract. From the 0.5% (w/v) stock solution of the fraction of interest - SP3 - were used 500 µL - the volume equivalent to 2.5 mg of the extract that we needed to have 5 g of experimental feed at 0.05% (w/v) concentration. The feeds for the two other experimental conditions – 0.01% and 0.005% (w/v) – were made by mixing the 0.05% diet with the negative control feed, that was re-processed in the same way as the experimental ones, only without the inclusion of the molecules of interest.

Having in mind the fast development of the fish at this stage of life, the experimental and control feeds were made in two different size ranges - 125-180 µm and 180-250 µm.

2.6.2.3. Zebrafish Larviculture

The fish used for this trial were obtained by crossing wild-type AB males and females kept as broodstock at LEOA facilities under the conditions described in section 2.1.

The fish were crossed in large breeding tanks to optimize egg collection.

After collection and rinsing, the eggs were mixed and placed in 1 L tanks with 0.05% methylene blue at a density of 200 eggs/L where they were kept until hatching and larvae reaching 5 dpf.

From 5 to 10 dpf larvae were kept in the same tanks at the same density. At the beginning of this stage the fish requires exogenous food, so we adopted a co-feeding protocol with rotifers (50 rotifers/mL) and 6 mg of Zebrafeed® (<100 µm) commercial diet. The commercial diet feeding was divided into two meals intercalated by one meal of rotifers. A 50% water renewal was made every day.

From 10 to 15 dpf the fish density was adjusted to 100 larvae/L in 1 L tanks. The larvae were fed only with Zebrafeed® commercial diet – 8 mg divided into three meals throughout the day. A 50% water renewal was made every day.

From 15 to 40 dpf the fish were transferred to 3,5 L tanks at a density of 20 larvae/L. The larvae were separated into four different groups, with two tanks per group, corresponding to the three concentrations tested – 0.05%, 0.01% and 0.005% (w/v) - plus the negative control. At his point we adapted a methodology used in the study conducted by Martins et al. (2019), where the total amount of food given to each tank increases 1 mg per day until the end of the treatment. In this case, the 30-day treatment initiated with the administration of 8 mg/tank at 15 dpf and ended with 33 mg/tank at 40 dpf. The total amount of feed given per day was always divided in three meals. A 90% water renewal was made daily to each tank.

2.6.2.4. Infection procedure

The infectious bacteria production was made using the same stock of the cryopreserved bacteria and was performed as described in section 2.6.1.2. for the pre-trial.

The fish were infected with the LD50 concentration previously determined in the pre-trial following the same protocol as described in the section 2.6.1.3. However, unlike for the pre-trial we just included one control group – amputated and exposed. All the fish were submitted to caudal fin amputation and immediately exposed to the bacteria.

After the bacterial exposure period of 5 hours, we assigned 45 fish per condition. Thirty fish were distributed into three 3 L tanks with 10 fish per tank that were monitored for

mortality events. An additional tank with 15 fish was introduced in the experimental setting for making a sampling for total RNA isolation and gene expression analysis.

2.6.2.5. Total RNA isolation and gene expression analysis by qPCR

All the fish sampled were euthanized by overdose of tricaine (MS-222) solution (0.6mM). After euthanized, a pool of two fish from each condition was made for every sampling procedure. The fish were placed in 1.5 mL cap tubes and 1 mL of NZYol was added in each tube after making a few cuts on the fish tissues, with a sterile scissors, to promote the NZYol penetration. After this, the samples were stored at - 80^{o} C for posterior homogenization and RNA extraction.

The homogenization of the fish tissues was performed using 3 mL syringes attached to two different needles by doing up and down movements until the solution could pass through the needle without any resistance. First, an 18-gauge (G) x 2 inches (´´) needle was used followed by an 21G x 2 ´´ needle.

After the tissue homogenization, the remaining protocol of RNA isolation and gene expression analysis by qPCR was followed as described for the SHK-1 essay from section 2.5.1.3. to section 2.5.1.7. qPCR was used to analyse the expression levels of the genes of interest related to the immune response: *tumor necrosis factor alpha* (*tnfα*), *interleukine 1β* (*il1β*), *interleukine 6* (*il6*); and oxidative stress response - *catalase* (*cat*), *superoxide dismutase* (*sod*). The expression of this genes was normalized using the ΔΔCt method and having as reference the housekeeping genes - *elongation factor 1alpha* (*ef1α*) and *beta actin* (*βact*) – to obtain the relative expression of the genes of interest (Table 2.8).

Table 2.8: Primers sequences of the genes used in qPCR reactions. The sequences are written in 5´ to 3´ orientation.

Gene	**Primer**	**Sequence (5´- 3´)**
ef1a	Forward	AGCCCCTCCTGGCTTTCACCC
	Reverse	TGGGACGAAGGCAACACTGGC
βact	Forward	TGATGCCCCTCGTGCTGTTTTC
	Reverse	CTCATTGTAGAAGGTGTGATG
tnfα	Forward	AACAAGATGGAAGTGTGCTGAGAC
	Reverse	TGGTCATCTCTCCAGTCTAAGGTC

il1β	Forward	ATGGCGAACGTCATCCAAGAGCG
	Reverse	CATGCTGAAGCGCACTTTCAAGTCG
il6	Forward	TGAAGACACTCAGAGACGAGCAGTT
	Reverse	AGGTTTGAGGAGAGGAGTGCTGAT
cat	Forward	TATCAGGGATACGCTTCTGTTTCCG
	Reverse	ACTGAACAGGAAAGACACCTGGTG
sod	Forward	TCCTTCTCATGAATCACCATGGTCC
	Reverse	GCCAACCGATAGTGTGAGACACG

3. Results

3.1. Operculum assay

The sulphated polysaccharides studied in this assay were obtain through 4 different purifying methods giving origin to 4 different samples as described in table 2.1 (section 2.3). Zebrafish larvae were exposed to this different samples and their mineralogenic effect was assessed by evaluating the opercular bone mineralization, as described in section 2.4.2. Figure 3.1 shows the imaging results under the stereomicroscope (A) and after the morphometric analysis with ImageJ software (B).

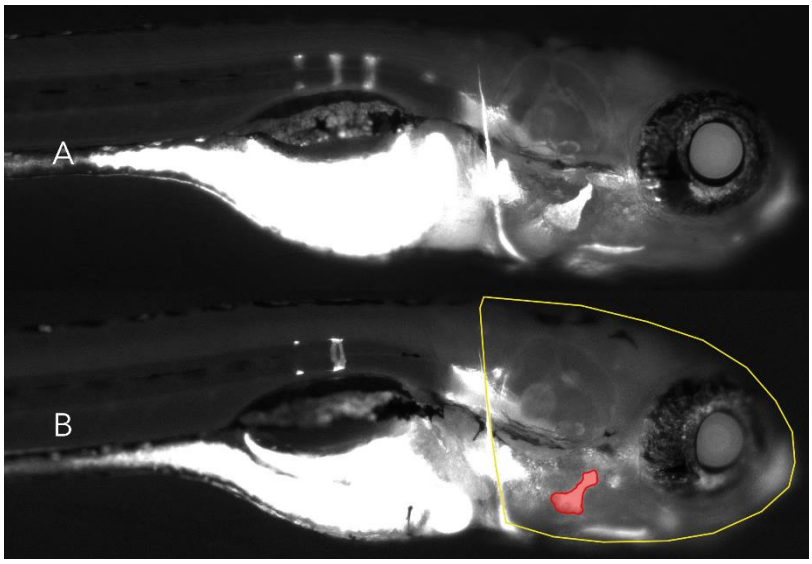

Figure 3.1: A - Zebrafish larvae (6 dpf) visualized under a stereomicroscope equiped with a fluorescence green filter after bone staining with AR-S and anaesthesia. B – Morphometric analysis performed in ImageJ software; Yellow line – Head area; Red line – Operculum bone area.

Table 3.1 summarizes the data by presenting the mean values for each condition tested (0.0001%, 0.0033%, 0.01%, Artificial Salt (AS), Not Exposed (NE)) for each sample (SP1, SP2, SP3, SP4).

Table 3.1: Ratio between the area of the operculum bone area (Op) and head area (Hd). Values corrected against the control values (AS). Only mean values are presented for each condition tested. Number of larvae (N) analysed per each condition is also presented.

SAMPLES	CONDITION	N	CORRECTED OP/HD AREA (MEAN VALUES)
SP1	0.0001	45	95.99
	0.0033	45	99.15
	0.01	45	93.30
	AS	45	100.00
	NE	45	100.92
SP2	0.0001	44	95.99
	0.0033	43	98.39
	0.01	45	101.56
	AS	45	100.00
	NE	44	103.26
SP3	0.0001	43	109.81
	0.0033	30	82.39
	0.01	45	108.62
	AS	45	100.00
	NE	45	108.62
SP4	0.0001	42	104.72
	0.0033	45	102.38
	0.01	45	104.99
	AS	43	100.00
	NE	44	109.10

We should mention a technical problem that occurred concerning the condition 0.00033% tested for the SP3 sample (Figure 3.2). When we were transferring the larvae from the 6-well plate to the 24-well plate to do the bone staining two larvae from the 0.0001% condition were wrongly placed at the same well where the larvae from the 0.0033% were. Not being able to tell them apart inside the same well we did not consider them in the analysis. Hence the loss of 15 larvae from 0.0033% condition and 2 larvae from 0.0001% condition. Apart from that, mortality events that occurred throughout the experiment were minimal.

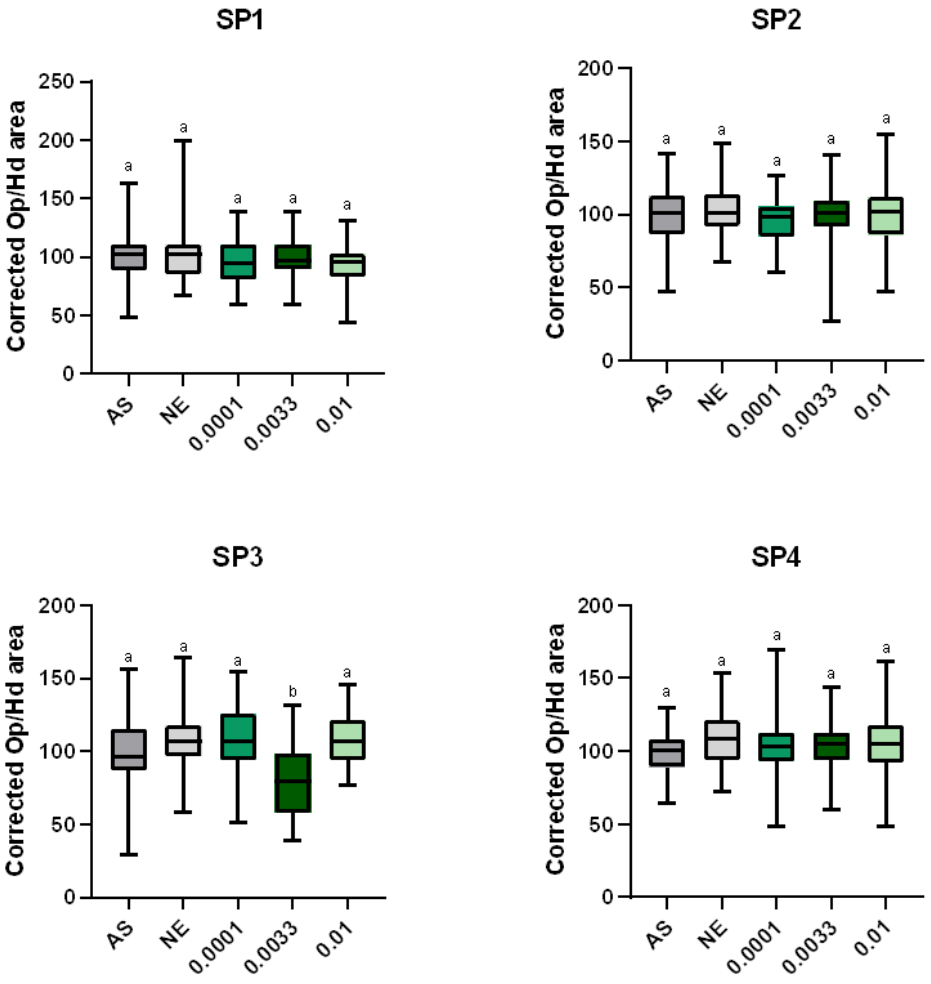

Figure 3.2: Graphical illustration of the opercular mineralization effect of the four different sulphated polysaccharides samples (SP1, SP2, SP3, SP4) on zebrafish larvae for different conditions. AS – Artificial Salt; NE – Not Exposed; 0.0001%, 0.0033%, 0.01% - weight/volume concentrations. Different letters indicate statistical difference (Kruskal-Wallis followed by Dunn´s multiple comparison test, p > 0.05).

Considering the four different samples tested, operculum mineralization was not significantly altered when compared to the negative control (AS). However, SP3 extract at 0.0033% resulted in a significant decrease in mineralization possibly due to a zootechnical problem.

These results suggest that the SP extracts tested in this work do not affect bone mineralization.

3.2. SHK-1 cells assay

The same SP samples tested for mineralogenic activity were then used in an immune response analysis through an *in vitro* exposure of the Atlantic Salmon (*Salmo salar*) head-kidney cell line (SHK-1) as described in section 2.5.1. The effects of the sulphated polysaccharides exposure was measured by considering the expression of genes involved in the immune response: *tumor necrosis factor alpha* (*tnf*α), *interleukine 1β* (*il1β*), *interleukine 8* (*il8*); and oxidative stress response: *cyclooxygenase 2* (*cox2*), *superoxide dismutase* (*sod*). After performing the qPCR, data was organized and treated with the ΔΔCt method. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the gene expression levels, relative to the negative control (H_20). The gene expression values of the different conditions are relative to the respective vehicle, hence the values of the LPS conditions were compared with the values of the negative control – H20(4) – while the values of the SP conditions were compared with the values of the respective negative control – H20(15) (Figure 3.3). This provides a more straightforward comparison of the treatments against the respective negative controls that were normalized with a value of 1. Graphical representation of the data was performed after applying this method.

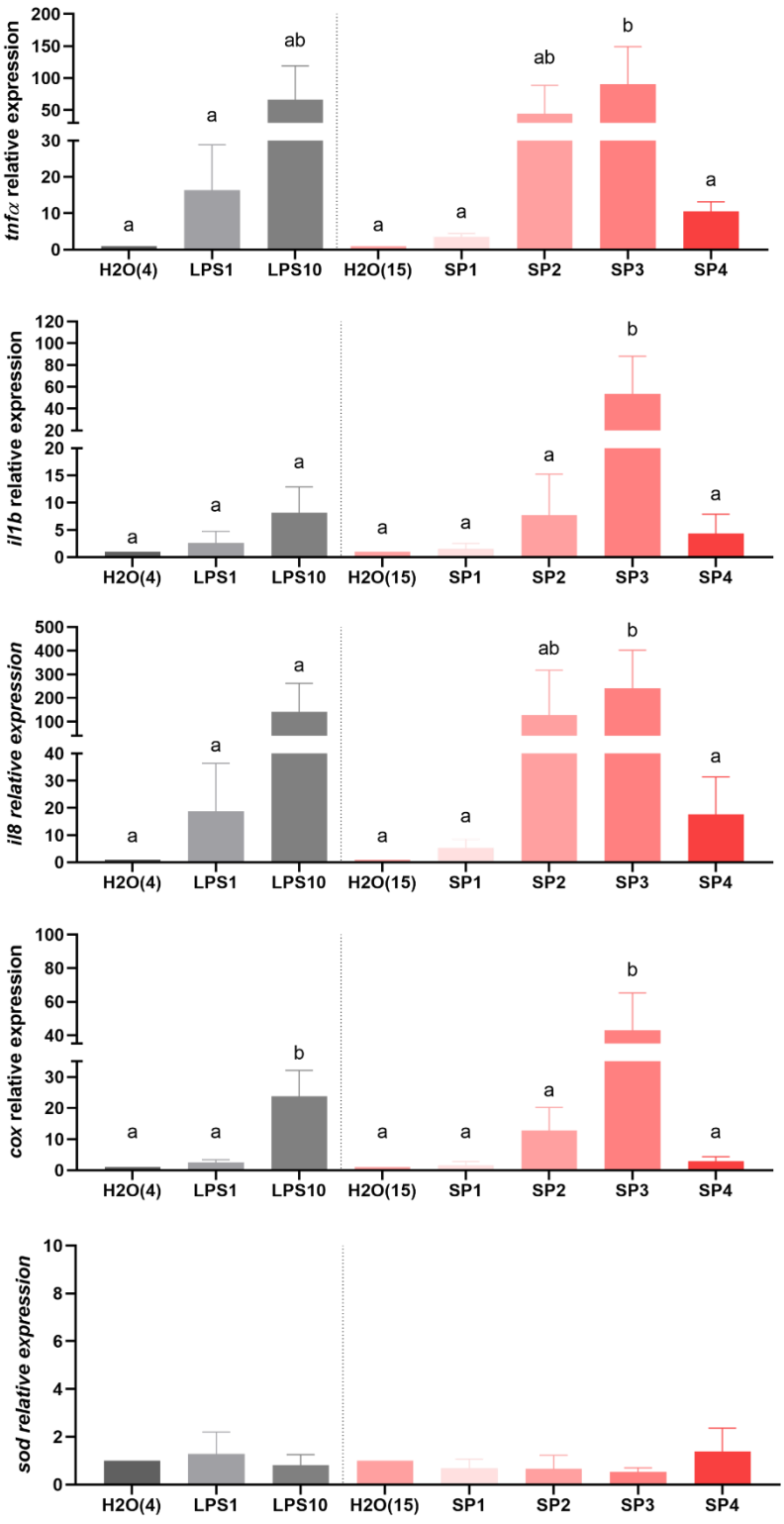

Figure 3.3: Graphical illustration of the relative gene expression after exposure to all sulphated polysaccharides samples (SP1, SP2, SP3, SP4), to LPS as a positive control and to water (H20(4)) as a negative control for the LPS, and water (H20(15)) as a negative control for the SP fractions, concerning genes involved in the immune response (tnfα, il1β and il8) and in the oxidative stress response (cox2 and sod). (One-Way ANOVA followed by Dunnett´s multiple comparison test, $p < 0.05$), ($n = 4$ for all conditions). Different letters mean statistical differences.

Considering the immune response genes, we can point out a significant increase in the expression of *tnfα* with an increment of roughly 90-fold, *il1β,* with an increment of approximately 54-fold, and *il8,* with an increment of around 24-fold when compared to the negative control (H2O(15)) when exposed to the SP3 fraction. Considering the oxidative stress response, we can also see a significant difference on the expression of *cox2* with an increase of around 43-fold for the SP3 fraction over its negative control (H20(15)) and an increase of about 23-fold over the negative control (H20(4)) for the LPS10 condition. However, when analysing the relative *sod* expression, no significant differences were noticed regarding both the SP and LPS treatments.

From the graphical illustration of this data, we could also observe that there were other increments regarding different genes and SP fractions than the aforementioned. For instance, SP2 and LPS10 treatments showed an increase in the relative expression of the *tnfα* and *il8* gene. However, this data lacks the statistical power to have significant differences when compared to the respective negative control. As an overview we can say that SP3 was the fraction that induced the higher response across the set of genes tested.

3.3. Bacterial Challenge

3.3.1. Pre-trial

The OD value at 620 nm of the 50 mL inoculum that we used to make the water baths to expose the fish to the bacteria was 0.945. As explained in the section 2.6.1.2. this value was less than ideal and we had to make adjustments in the dilutions to make sure that there was enough bacteria to achieve the exposure concentrations that we wanted to test. Since this was the case, we decided to use the totality of the 50 mL inoculum and make smaller dilutions of the bacteria to compensate for a smaller OD value. The estimated and real values of the bacterial concentrations for each condition are described in table 3.2. The real concentrations are the result of three serial dilutions of the inoculum concentration – 0.92×10^9 CFU/mL in a final volume of 1 L.

Table 3.1: Estimative versus real concentration of the bacteria included in the water baths during the exposure for all the conditions tested.

CONDITION	BACTERIA (CFU/mL)	
	Estimative	Real
Control Amputated and Not Exposed (CA)	0	0
Control Not Amputated and Exposed (CB)	$1x10^6$	$0,92x10^6 \pm 0,42x10^6$
Low	$1x10^5$	$0,92x10^5 \pm 0,42x10^5$
Medium	$1x10^6$	$0,92x10^6 \pm 0,42x10^6$
High	$1x10^7$	$0,92x10^7 \pm 0,42x10^7$

The mortality events recorded during the pre-trial allowed us to estimate the LD50 to use in the trial with the SP pre-conditioned fish. Figure 3.4 shows the cumulative mortality recorded for each tank.

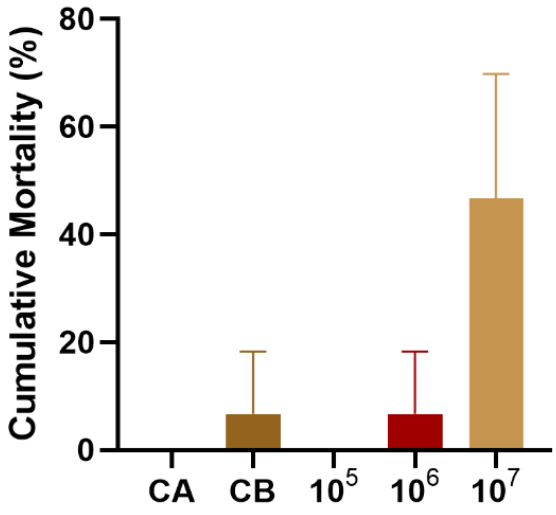

Figure 3.4: Cumulative mortality values obtained for each of the conditions tested during the pre-trial. CA - Control Amputated and Not Exposed; CB - Control Not Amputated and Exposed; 10^5 - Low Dose; 10^6 - Medium Dose; 10^7 - High Dose.

After the 5 hours bacterial exposure all the fish were found alive. Sixteen hours after the end of the exposure period a 46.7% cumulative mortality was recorded in the tanks with fish infected with the high dose - $0{,}92x10^7 \pm 0{,}42x10^7$ CFU/mL. Forty hours after the end of the exposure period a 6.7% cumulative mortality was recorded in the tanks of the two conditions assigned with the medium dose infection - $0{,}92x10^6 \pm 0{,}42x10^6$ CFU/mL. No mortality events were recorded for the control group with fish injured and not exposed (CA). No further mortality events were recorded during the following 10 days of the trial duration.

The results on the electrophoresis performed after the amplification of the DNA isolated from the tissue sample collected from one of the dead fish after the exposure period came out positive for the bacteria *A. hydrophila* since the 234 bp band was amplified as expected and confirmed by the positive control – an isolated strain from *A. hydrophila*.

Figure 3.5 shows the results from the electrophoresis where, from right to left, we can see the positive control (+C), the result from the tissue sample analysis (S) and the negative control (-C).

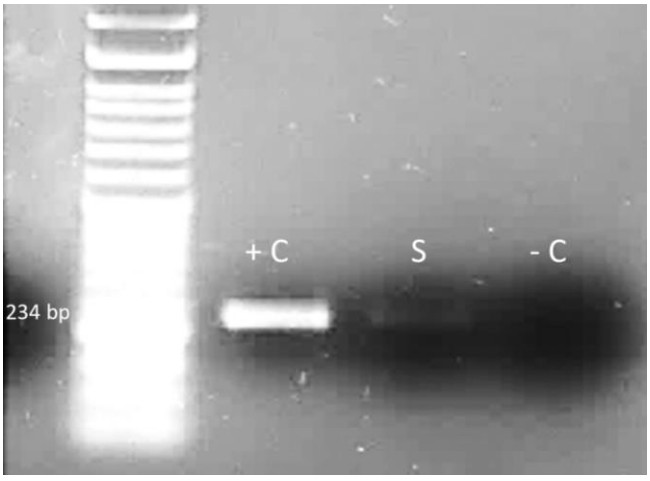

Figure 3.5: Electrophoresis gel performed for bacterial DNA identification. (+C) - Positive Control; (S) - Sample; (-C) - Negative Control.

Regarding the morphology and biochemical characterization of the bacteria the results also suggested that the fish had been infected with *A. hydrophila*. The morphology analysis was made based using an imaging system attached to a light microscope that also allowed us to conclude that we were observing a motile bacteria.

Figure 3.6 shows, highlighted in a red ellipse, two straight rods with rounded ends that can be identified as having the same structure as *A. hydrophila*. However, in the same image there are microorganisms that appear to have a different structure from the one described for the bacteria in study (red dotted circle).

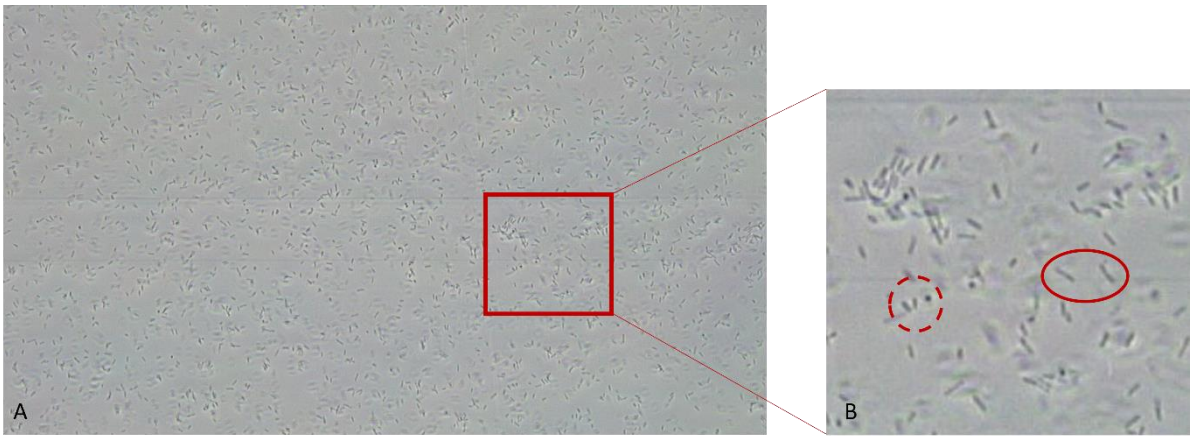

Figure 3.6: Morphology analysis of the bacteria grown from the swab sample removed from one of the dead fish after the infection. A - original image obtained under a Leica® DM750 binocular microscope with the 40x lens; B - Amplification of image A. Red elipse - rods identified as A. hydrophila. Red dotted circle – microorganisms with different morphology.

Finally, the results of the biochemical tests were cross-checked with an identification tree, which also showed that we were analysing a strain of *A. hydrophila*.

In sum, the results from the pre-trial allowed us to determine the LD50 correspondent to the high dose to which the fish were infected that, in this trial, was equivalent to $0{,}92 \times 10^7 \pm 0{,}42 \times 10^7$ CFU/mL. The infectious protocol allowed the bacteria to cause harm to the fish and the identification procedures – PCR, morphology analysis and biochemical tests – indicated the presence of *A. hydrophila* as a possible consequence of the mortality events.

3.3.2. Trial

The OD value at 620 nm of the final inoculum used to infect the fish was 0.930. As it happened in the pre-trial, the value indicated a less than ideal bacterial concentration that led us to use all the 50 mL inoculum available to aim for the estimated LD50 - 10^7 CFU/mL. This was not possible as we came to conclude, after counting the colonies present on a TSA plate with a sample of the water bath, that the real bacterial concentration to which the fish were exposed to was in fact 4×10^6 CFU/mL.

The mortality events were recorded during the 72 hours of the trial duration and the results are depicted in figure 3.7.

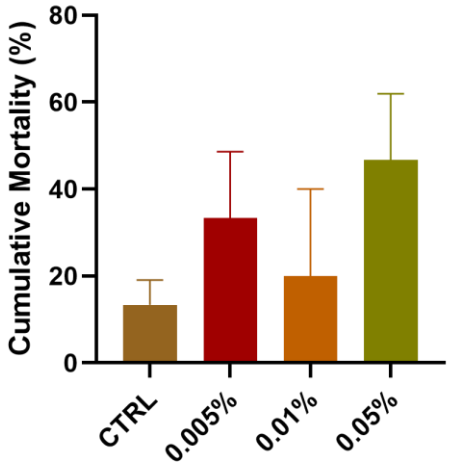

Figure 3.7: Cumulative mortality values obtained for each of the conditions tested during the trial. CTRL (control group) - commercial diet without the inclusion of the SP molecules; 0.005, 0.01, 0.05% (w/v) diets with the different percentages of SP included.

After the 5 hours bacterial exposure 6.67% of the fish were found dead for every condition tested. After 16 hours the cumulative mortality value for the control group (CTRL) was 13.3%, for the 0.005% condition was 33.33%, for the 0.01% condition was 20% and for the 0.05% condition was 46.7%. The highest mortality values were recorded for the condition testing the highest inclusion of the SP3 in commercial diet. No further mortality events were recorded during the following 56 hours of the trial duration.

Sampling procedures were carried on in order to collect fish for gene expression analysis. The fish were collected at four different time points – before exposure to the bacteria (BE) and zero hours (0h), eighteen hours (18h) and seventy-two hours (72h) after the end of the exposure period. The effect of the SP3 included in the diet were measured considering the expression of genes involved in the immune response: *tumor necrosis factor alpha* (*tnfα*), *interleukine 1β* (*il1β*), *interleukine 6* (*il6*); and oxidative stress response: *catalase* (*cat*), *superoxide dismutase* (*sod*).

As for the SHK-1 essay, data was organized and treated with the ΔΔCt method after collecting the raw data from qPCR. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the fold gene expression relative to the negative control – the re-processed commercial diet without the inclusion of the SP molecules before the exposure to the bacteria (BE time point). This

provides a more straightforward comparison of the treatments against the negative control that was normalized with a value of 1.

Figure 3.8 shows the graphical representation of the gene expression levels

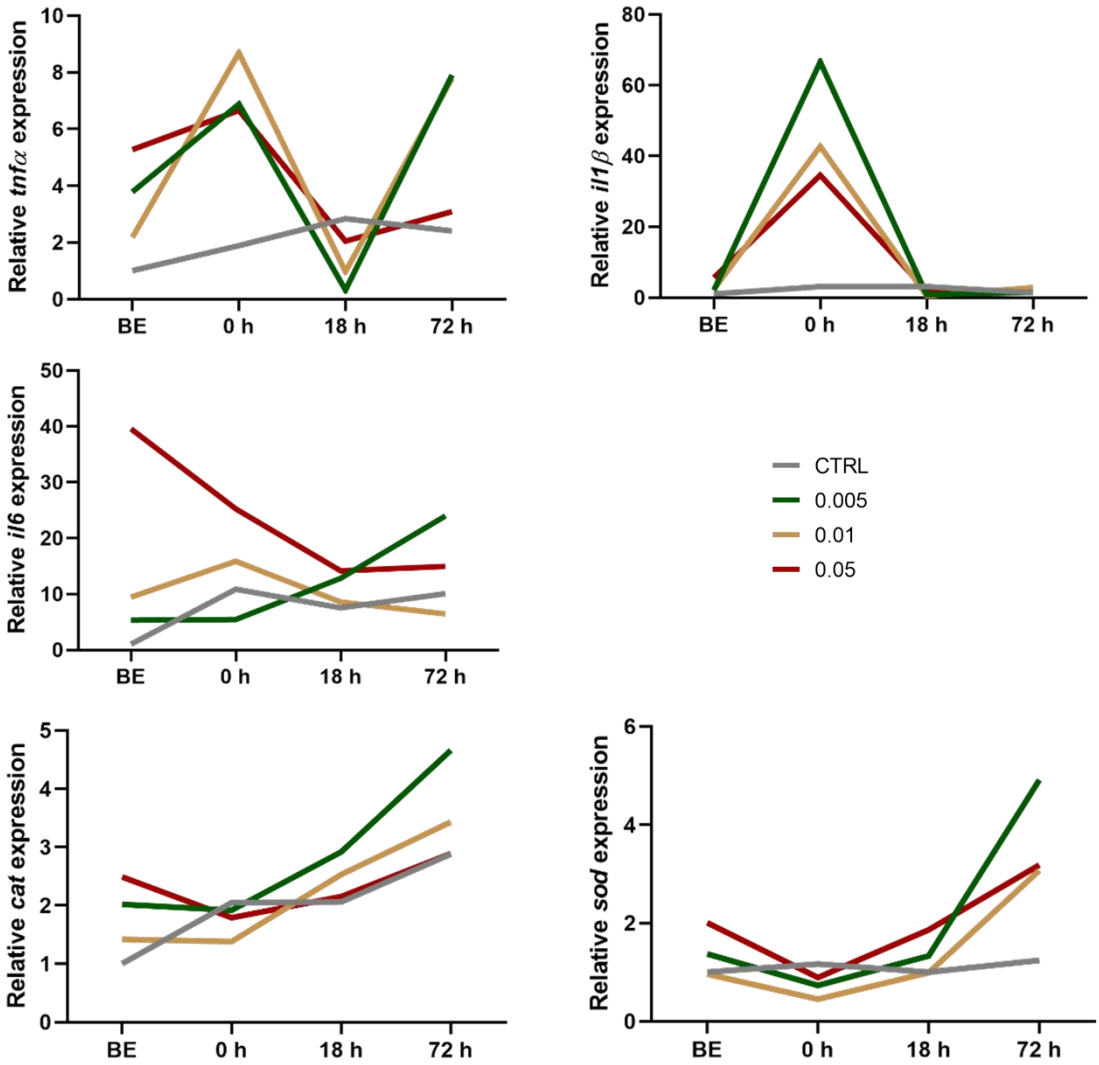

Figure 3.8: Graphical illustration of the relative gene expression in zebrafish juveniles (40 dpf) after a 35 day pre-conditioning with diets with different inclusions of the SP3 fraction (0% (CTRL), 0.005%, 0.01% and 0.05% (w/v)), at different time points before (BE) and after (0, 18 and 72 h) the exposure to the bacteria, concerning the genes involved in the immune response: tnfα, il1β, il6 and in the oxidative stress response: cat and sod. n=1.

It is important to mention that a decision was made in order to compare all the relative gene expression values for each gene, including the ones relative to the control group at the time points recorded after exposure, with the values relative to the control group of the same gene before exposure to the bacteria with the goal to have a representation of how the expression of the genes analysed had evolved in time having as reference a pre-infection status.

Analysing the relative expression levels of the *tnfα* gene, we could notice an overexpression for all the experimental conditions before the exposure to the bacteria (BE), with the higher concentration - 0.05% - showing 5-times overexpression, approximately. Moving forward to the next time point (0h), we noticed an increase in the relative gene expression for all the conditions, with the highest value being reached for the 0.01% concentration with a 9 fold increase, approximately. The opposite was noticed at 18 h after exposure (18h) where all the concentrations caused a reduction of gene expression with the 0.005 and 0.01% concentrations inducing an under-expression of the *tnfα* gene. At 72h the relative expression levels increased again for all the concentrations, with an 8 fold increment for the two lowest concentrations. The values for all the concentrations seem to follow the same pattern with time, peaking at 0h, reaching the lowest value at 18h and increasing again at 72h. On the contrary, the relative gene expression for the negative control (CTRL) followed a different pattern, reaching the highest expression level at 18h and slightly decreasing at 72h.

The relative expression levels of the *il1β* gene evolved in a very similar way to the ones of the *tnfα* gene. All the three concentrations caused an overexpression of the gene at the BE time point, followed by a peak of expression at 0h, immediately after exposure to *A. hydrofila*. At this time point, the 0.005% concentration was responsible for a 67 fold increase in gene expression. After 18h the expression of *il1β* decreased to close to basal levels for all the concentrations.

As for the last two genes, the *il6* relative expression at the BE time point was also stimulated for all the conditions, with a noticeable, near to 40-times overexpression induced by the 0.05% concentration. This high relative expression decreased consistently reaching values of around 14 fold overexpression at 18h. A different pattern was recognized when comparing the response of the other two concentrations, with the expression levels being constant for 0.005% and with an overexpression for 0.01% at 0h and then evolving in different manner, with an increase in expression for the 0.005% concentration and a decrease for the 0.01% concentration.

Focusing on the genes related to the oxidative stress response we could observe an overexpression at the BE time point for both genes with an exception for the *sod* gene, with the 0.01% concentration at the same level as the control. The higher concentration showed a higher expression level before exposure for both genes. An increment in the

expression was registered, with the values reaching their maximum at 72h for all the conditions, for both *cat* and *sod* genes. The lowest concentration was the one responsible for the higher expression values obtained for both genes at this time point. Analysing the control group relative to the *cat* gene, we could also notice a gradual overexpression, with the highest value being recorded at 72h, while the *sod* expression in the control group was kept constant.

The administration of an SP-enriched diet at different concentrations was responsible for a modulation of the expression of genes involved in the immune response and in oxidative stress response before the exposure to the bacteria, with an exception attributed to the 0.01% concentration for the *sod* gene. Although the comparison between genes, regarding their relative expression values is not possible, a similar behavioural pattern seems to be distinguishable for *tnfα* and *il1β* and between both genes analysed for the oxidative stress response.

4. Discussion

The operculum assay is a quick method to screen for compounds with osteogenic properties. As depicted in figure 2.1 (section 2.4.2.1) 3 dpf larvae are exposed to the molecules of interest for 3 days, followed by the opercular bone staining with AR-S prior to imaging and morphometric analysis. It requires a low amount of molecules and a small volume of water to maintain the fish during exposure. The equipment needed, an incubator to maintain the fish at 28° C during exposure in the plates, and the fluorescent stereomicroscope to do the imaging can be found in most research facilities. In addition, the software ImageJ®, used to perform the image analysis, is available to anyone with a computer and can be downloaded free of charge with the advantage of having the already developed macros that can be installed in the software, making the morphometric analysis a relatively fast and easy procedure, considering the experiment settings (number of specimens and number of conditions) (Tarasco et al., 2020).

An increase or decrease in the corrected operculum area, a measure obtained by the normalization of the operculum area using the area of the head of the fish in order to correct for inter-specimen size variability, mean that the compound has pro or anti-osteogenic properties, respectively (Tarasco et al., 2017). In our trials, we did not obtain

any kind of result that suggest a pro or anti-osteogenic effect in the SP extracts. Apart from the one statistical difference in the treatment with fraction SP3 at the concentration of 0.0033% (Figure 3.2) that was attributed to a zootechnical problem, there were no other significant differences that can be related to any kind of mineralogenic or osteotoxic effect.

It is known, in the literature, that other kinds of sulphated polysaccharides have the capacity to induce mineralogenic effects. The fucoidans, extracted from brow algae and other invertebrates such as the sea cucumber *Stichopus japonicus*, can inhibit osteclastogenesis in *in vitro* systems as shown by Kariya et al. (2004) in cells from the bone marrow of mice. Hwang et al. (2016) also show an increase in 72F cells (a murine pro-osteoblast cell line) viability after treatment with low-molecular weight fucoidan extracted from the brown algae *Sargassum hemiphyllum*. The same study assessed the mineralogenic properties of this fucoidan in *in vivo* systems using aged mice females, although bone density was significant altered at the concentration of 280 mg/Kg (body weight). However, to the best of our knowledge, no studies have focused on the mineralization potential of sulphated polysaccharides on fish bones and, in a general way, there is a lack of *in vivo* experimentation regarding these compounds in a bone formation context (reviewed by Carson & Clarke, 2018). For this reason, and also for the osteogenic activity of these molecules on mammalian models and mammalian derived cell lines we propose that further studies should be carried on, with higher concentrations and/or longer exposure time, to access if there is any kind of significant dose-dependent response.

Concerning the SHK-1 assay, a preliminary test with 0.01% concentration of SP was performed with the objective to access how the cells would react to the exposure to these molecules. The viability was not affected, and we could observe some of the expected results, that is, some stimulation of the expression of the genes related to the immune system and oxidative response. Hereupon, we decided to go for a five times more concentrated solution for exposure (0.05%) to see if an amplification of the signal obtained was verified. Indeed, no toxicity effect was recorded with the increment in the concentration of extract and the results showed a significant difference for one of the treatments (SP3) when compared to the negative control (H2O(15)) in 4 out of 5 genes tested (Figure 3.3).

These results suggest that this fraction induced a similar response to that of LPS, here used as o positive control for the immune response and respiratory burst, that is also stated in other studies across the literature. Wang et al. (2020), has verified that LPS treatment in RAW 264.7 cells, a murine macrophage cell line, significantly promoted the expression *Il-1β* and *Tnf-A*, two of the genes also tested in this work to access the immune response. The same was verified regarding the oxidative response genes. In the same study, they stated that *iNos* and *Cox-2* expression significantly increased when treated with LPS, a result that can be compared to the results here obtained regarding the relative expression of the *cox2* gene for the SP3 treatment (Figure 3.3).

During our trials we also tested the expression of the *inos* gene, however we were having problems with the results obtained after qPCR and we decided not to present them since the primer that we were using was not amplifying correctly. In another study, also with SHK-1 cells, Olavarría et al. (2010) showed that the exposure of these cells to LPS promoted the activation of Nadph oxidase, another respiratory burst pathway that results in the production of reactive oxygen species, much like the iNos enzyme. Fast et al. (2005), also exposed the SHK-1 cells to LPS during 4 hours in which they obtained similar results concerning the *il1β* and *cox2* gene expression that was significantly increased. However, they did not observe any significant differences in the expression of *tnfα-like* gene, probably a polymorphism of the gene that we tested.

Our relative gene expression data suggests that the SP3 treatment have modulated the expression of the majority of the genes tested (*tnfα*, *il1β*, *il8* and *cox2*) resulting in a significant overexpression and that the other treatments, such as SP2, may also increase *tnfα* and *il8* gene expression. However, due to lack of statistical power, due to a low number of replicates, we cannot infer any further. In order to overcome this problem more replicates should be done to try to decrease variability and see if statistical significance is obtained for other conditions. Nonetheless, the results obtained, together with the ones referred in the literature imply the strong immunologic and oxidative response that the sulphated polysaccharides are capable of inducing. Apart from that, our results also highlight the adequacy and practicality of the SHK-1 cell line in the study of immunology aspects related to fish.

The Gram-negative bacteria *A. hydrophila* is a relevant pathogen affecting both fish and humans (Janda & Abbott, 1998). It is a motile, rod-shape, facultative anaerobic bacteria

described as an opportunistic pathogen of fish and terrestrial animals (Igbinosa et al., 2012; Janda & Abbott, 2010). In fish it is responsible for causing a generalized blood infection known as motile aeromonas septicaemia (MAS), leading to high mortalities (Harikrishnan & Balasundaram, 2005). Its virulence has been accounted, across the literature, to several extracellular toxins and enzymes, such as, cytotoxins like the lipopolysaccharide (LPS), haemolysins, enterotoxins and proteases (Allan & Stevenson, 1981; Ljungh et al., 1981). It is also responsible for various diseases in humans such as gastroenteritis, septicaemia, tissue infections amongst others, less frequent complications, such as peritonitis, endocarditis, pancreatic infections, and urinary tract infections (Janda & Abbott, 2010).

Analysing the mortality results of our pre-trial, we could conclude that the $0{,}92x10^7 \pm 0{,}42x10^7$ CFU/mL (10^7 - figure 3.4) concentration was the one closest to achieve the LD50, being responsible for a cumulative mortality of 46.7% 16 hours after exposure. The fish challenged with the medium dose infection - $0{,}92x10^6 \pm 0{,}42x10^6$ CFU/mL (10^6 - figure 3.4) – suffered 6.7% cumulative mortality 40 hours after exposure, while the control group with fish injured – caudal fin amputation – and not exposed to the bacteria (CA – figure 3.4) suffered no mortality throughout the entire trial suggesting that the amputation procedure by itself was not the cause of mortality.

In a similar work, Rodríguez et al. (2008) infected adult zebrafish with $2x10^6$ CFU/mL during a 5-hour immersion. The fish were also injured with a cut in the caudal fin plus a dermis scratch with a sterile scalpel. Furthermore, the control fish were injured but not exposed to the bacteria. They obtained a cumulative mortality of 100% after 24 hours of exposure for the infected fish and 40% cumulative mortality after 4 days for the control group – fish injured but not exposed. The comparison to our results suggests that they might have had some kind of problem related with the caudal fin amputation since in our pre-trial the control group with injured fish and not infected (CA – figure 3.4) did not experience any mortality during the 10 days of the trial duration. Despite this, the bacterial concentration that they used fits in the range of concentrations tested by us during the pre-trial ($0{,}92x10^5 \pm 0{,}42x10^5$ - $0{,}92x10^7 \pm 0{,}42x10^7$ CFU/mL), and while we registered a cumulative mortality of 46.7% after 16 hours of exposure to $0{,}92x10^7 \pm 0{,}42x10^7$ CFU/mL, they have registered 100% mortality 24 hours after exposure to a lower concentration - $2x10^6$ CFU/mL. This differences in the results, apart from any protocol-

related problem, can be associated with differences in the virulence of the bacteria strain used.

In another related work, Saraceni et al. (2016), also performed a water bath infection, this time with AB zebrafish larvae with 4 dpf, healthy and injured by caudal fin amputation. The immersion was done with 10^8 CFU/mL in 6-well plates with 10 larvae/well. Cumulative mortality was recorded until 6 days post infection and significant differences were obtained. A mortality of 33% was recorded for uninjured infected larvae at 96 hours post-infection, while for the injured and infected larvae the mortality values reached 77% by the end of the monitoring. The control group – injured larvae treated with PBS – was accounted for 1% cumulative mortality.

In sum, our results show an acute effect of the bacteria in mortality. Both in our work and in the work of Rodríguez et al. (2008) the main mortality events occurred within the first 24 hours post-infection, while in the work developed by Saraceni et al. (2016), mortality events were recorded until 6 days post infection. This can be related to the fact that the last authors were using 4 dpf larvae while Rodríguez et al. (2008) used adult fish, and we, in our work, used juvenile fish with 40 dpf (around 5 weeks). This differences in age, and in the results can be related to the activation of an adaptive immune system that is mature and functional around 4 to 6 weeks post-fertilization giving the older fish another set of tools to fight against the pathogen (Davidson & Zon, 2004; Trede et al., 2004; Willett et al., 1999). Despite this, innate immunity in teleost fish is considered in the literature of much greater importance than the acquired one when it comes to prevention of infectious diseases, not only because of its later maturation and activation but because it can take several days to be initiated in an already functional adaptive immune system (Nakanishi et al., 2018).

For this reason, we decided to analyse the relative expression of genes related with the immune system, such as the ones encoding for pro-inflammatory cytokines – *tnf-α*, *il-1β* and *il-6* -commonly used immune-regulatory genes in fish (Bird et al., 2002; Roca et al., 2008; Wang & Secombes, 2009; Zou et al., 1999) and genes related with the oxidative stress response (*cat* and *sod*) in zebrafish juveniles after a 35-day pre-conditioning treatment based on a SP-enriched diet.

Cytokines are modulators for both the innate and adaptive immune system and are responsible for small physiological changes and signal transmission between cells (Zou & Secombes, 2016) playing an important role in the innate immune response of fish.

Looking at our results from the bacterial challenge in a holistic way, and trying to correlate the administration of the SP-enriched diet with the bacterial infection and the immune and oxidative response gene expression analysis, it appears that the conditions tested (0.005, 0.01 and 0.05%) did not improve the response to the infectious agent and the overall survival of the fish challenged. We could notice that, despite the lack of statistical significance, the treatment associated with higher mortality was also the one with higher supplementation of the SP molecules with a 0.05% inclusion (figure 3.7). This treatment was also accounted for the higher relative gene expression level for all the genes tested related with the immune and oxidative stress response before the exposure to the bacteria (BE– figure 3.8). The other two experimental conditions also induced modulation of the levels of expression across the panel of genes tested in several time points analysed.

Despite the difference of magnitude of the expression induced by the different conditions they all followed a somewhat similar pattern when considering each gene individually. Hence, we can suggest that, compared to the control group there was, for all the conditions and all the genes tested, an over-stimulation of the immune and respiratory burst activity both before and after exposure to the bacteria. As an alternative way to do the treatment we could perhaps decrease the concentrations to which the fish were exposed, or instead of proceeding to the infection right after the diet-related treatment wait for some time - to be determined - to prevent an overstimulation of the immune system, that when faced with the bacterial infection, cannot react in the same way as it would in a less-stimulated status. Therefore, taking the immune response to the extreme after the already exaggerated immune reaction caused by the pathogen, and leading to the higher mortality recorded for the experimental conditions in comparison to the control group.

Recently, Ehsannia et al. (2022), have shown, that in zebrafish infected with *A. hydrophila* and pre-conditioned with probiotics, the group showing higher *tnfα* and *il1β* expression levels was the untreated group while the one showing the highest survival rate was the one treated with one of the probiotics tested, showing that a higher stimulation of the immune system do not necessarily mean a better performance against an infectious

agent. In another work, conducted by Yang et al. (2021) using another fish species - Korean rock fish (*Sebastes schlegelii*) with an average body weight of 19.20 ± 0.6 g – pre-conditioned them to diets supplemented with 0% (control group), 0.1%, 0.5% and 1% of crude sulphated polysaccharides isolated from a brown algae (*Codium fragile*) for four weeks before challenging them with *Edwardsiella tarda*, a Gram-negative bacteria, administrating 100 μL of $1x10^8$ CFU/mL concentration by intraperitoneal injection. They concluded that the 0.5% group showed a significant overexpression of *il1β* and *il6* genes in the head kidney compared to the control group at day 1 post infection but showed a downregulation of *il1β* in the liver at day 3 post infection, while the 0.1% group showed a downregulation of *il1β* and *il8* genes in the head kidney at day 3 post infection. According to these authors, this might explain the lower mortality in the 0.1% group, suggesting that a lower stimulation of the immune system can be preferable to balance the exaggerated inflammatory response caused in this case by *E. tarda*, leading to a higher survival rate.

Although it would be interesting to make a deeper analysis on the gene response upon infection, we could not find much information to compare our results and to try to find a reason for the different expression patterns that the different genes have across the different time-points analysed. It is also very important to note that this results, despite being a good indication of what might be happening, still require more experimental replicates to reinforce our findings.

As a way to introduce future perspectives that can contribute to a better understanding of the infection mechanisms in place we suggest the use of the already discussed zebrafish reporter lines (Renshaw et al., 2007) in which specific innate immune cells are labelled with fluorescent proteins and imaged in translucid larvae, constituting excellent tolls for visualization of immune cells recruitment and inflammatory resolutions processes in *in vivo* systems (Martin & Renshaw, 2009) such as, the process of phagocytosis (Renshaw & Trede, 2012). As an example, Saraceni et al. (2016), used these reporter lines in conjunction with a strain of *A. hydrophila* also labelled with a red fluorescent protein (*A. hydrophila* DsRed) to better understand the host-pathogen interactions.

Another way to further access the immunogenic potential of SP extracts could be the infection of zebrafish embryos/larvae with different concentrations of LPS and treated with different concentrations of SPs. According to previous studies (Wang et al., 2020) it

is expected that SP treatment decrease cytokine levels (*tnfα; il1β*) and reactive oxygen and nitrogen species, protecting the fish against the LPS inflammation effects. According to the results of Wang et al. (2020) LPS injection showed significant toxic effects, which is in accordance with further published data (Zou et al., 2019). For the *in vivo* study they incubated zebrafish embryos with a 10 µg/mL concentration of LPS as well as with different concentrations of SP isolated from the brown algae *Saccharina japonica* (0, 25, 50, 100 µg/mL).

It was shown that a SP concentration higher than 100 µg/mL was toxic to the fish embryos. This is important information in order to refine the concentrations that could be used in our future trials. The survival rate increased significantly for the fish treated with SP at concentrations of 50 µg/mL and 100 µg/mL when compared with the fish only treated with LPS (positive control). On the other hand, yolk sac oedema size decreased significantly in fish treated with all the different SP concentrations, being more noticeable for the fish treated with the higher SP concentrations (100 µg/mL). This shows that immersion in SP bath can protect zebrafish from toxic damages induced by LPS. This is something that could also be validated by tracking the activity of neutrophils and macrophages using the zebrafish transgenic lines available.

5. Conclusions

The operculum assay, here performed to evaluate the bone mineralization after exposure to the sulphated polysaccharides, showed that these compounds do not have any kind of pro-osteogenic properties at the concentrations tested. The results obtained by treatment of the SHK-1 cell line suggest a strong activity of this sulphated polysaccharides, in particular the SP3 fraction, at the immunologic level. A 4-hour exposure to these molecules resulted, in some cases, in a 240-fold increase in the relative gene expression of some genes related to the immune system and the oxidative response.

Regarding the bacterial challenge we can only say that there are indications that the SP3 molecules influence the immune system of fish when using zebrafish as an *in vivo* model of experimentation. Apart from that, this work showed the suitability of zebrafish as model for research in aquaculture. Beyond the obvious logistical advantages of dealing with a small fish in comparison to one of economic importance for aquaculture, zebrafish

allow us to use tools to make both a holistic and molecular analysis of a problem by analysing it from different perspectives, from nutrition to health management and improvement, utilising very little resources – an advantage by itself when testing experimental molecules that may not be widely available. Testing natural, bioactive molecules can also bring new resources to aquaculture, improving the nutritional conditions in which the fish are reared and opposing to the emergent antibiotic-resistant bacteria that pose an increasing transmission risk to humans through the food chain.

In order to have a more detailed knowledge of how these molecules affect the immune system we propose the realization of some more tests, already discussed in the previous section and here summarized as future perspectives for this study such as utilising zebrafish reporter lines that signal innate immune cells, to perform a bacterial infection with a bacteria labelled with a fluorescent protein to better understand the host-pathogen interactions as well as the exposure of zebrafish embryos/larvae with different concentrations of LPS and co-treatment with different concentrations of SPs.

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