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# HOST-PATHOGEN INTERACTIONS IN GRAM-POSITIVE BACTERIOSIS IN FARMED TURBOT (*Scophthalmus maximus*)

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# INTERAÇÃO PATOGÉNIO-HOSPEDEIRO NA BACTERIOSE PROVOCADA POR BACTÉRIAS GRAM-POSITIVAS NO RODOVALHO (*Scophthalmus maximus*)

Tese de Candidatura ao grau de Doutor em Ciência Animal, Especialidade em Morfologia e Fisiologia submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Dissertation submitted for the acquisition of Doctoral degree of Animal Science, Expertise in Morphology and Physiology at Institute of Biomedical Sciences Abel Salazar of Porto University.

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“You learn that time isn't something you can turn back,

Therefore you must plant your own garden and decorate your own soul,

Instead of waiting for someone to bring you flowers.

And you learn that you really can endure.

You really are strong.

And you can go so farther than you thought you could go.

And that life really has a value.

And you have value within the life.

And that our gifts are betrayers,

And make us lose

The good we could conquer,

If it wasn't for the fear of trying.”

*In* Minstrel (William Shakespeare)

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# Abbreviations

CFU – Colony forming unit

DMSO – Dimethyl sulfoxide

FCS – Fetal calf serum

FIA – Freund's incomplete adjuvant

FITC – Fluorescein isothiacyanate

i.p. – Intraperitoneal

KOH – Potassium hydroxide

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBT – Nitroblue tetrazolium

$NO_2^-$ - Nitrite

$NO_3^-$ - Nitrate

$O_2^-$ - Superoxide anion radical

OD – Optical density

OIE – World Organization for Animal Health

PBS – Phosphate buffer saline

PMA – Phorbol myristate acetate

PRF-HBSS – Phenol red-free Hank's balanced salt solution

P/S – Penicillin/Streptomycin

RNI – Reactive nitrogen intermediates

ROI – Reactive oxygen intermediates

ROS – Reactive oxygen species

SOD – Superoxide dismutase

TB – Trypan blue

THKM – Turbot head-kidney macrophages

TSA1 – Tryptic soy agar containing sodium chloride

TSB1 – Tryptic soy broth containing sodium chloride

7H9 – Middlebrook 7H9 broth

7H10 – Middlebrook 7H10 agar

µmax – Maximum specific growth rate

λ – Lag time

# SUMÁRIO

A produção de rodovalho (*Scophthalmus maximus*) é afetada, na sua maioria pela streptococcose e pela tuberculose. Após o aparecimento de uma epizootia de tuberculose numa produção de rodovalho em Portugal, o estudo desta patologia, bem como da sua interação com o rodovalho foram o ponto de partida desta longa jornada. Quer a streptococcose, quer a tuberculose são causadas por bactérias Gram-positivas. Os seus agentes etiológicos: o *Streptococcus parauberis* e a *Mycobacterium marinum*, foram descritos. A taxa máxima de crescimento específico e o tempo de crescimento máximo foram estimados por contagem direta e através da leitura das absorvâncias. Os valores da taxa máxima de crescimento variaram entre 0.01 e 0.0336 $h^{-1}$ nas estirpes de *M. marinum* (MM-SP e MM-PT, respetivamente) e 0.2463$h^{-1}$ na amostra de *S. parauberis.* Os resultados relacionados com o tempo de crescimento confirmaram os anteriores, mostrando que as estirpes de *M. marinum* são de crescimento lento, quando comparadas com a do *S. parauberis*. Para conhecermos a reação imune perante um agente patogénico ativo ou inativo, temos sempre que conhecer a população leucocitária em repouso. Por isso, após injeção de um tampão fosfato (PBS) na cavidade peritoneal do rodovalho, observámos que os resultados não foram diferentes daqueles que haviam sido documentados para outras espécies de peixes, mostrando-se que a população de macrófagos era a mais abundante e a de neutrófilos a menos abundante. A nível citoquímico podemos usar a esterase e a peroxidase como marcadores de macrófagos e neutrófilos, respetivamente, uma vez que reagem positivamente a cada uma delas individualmente. A avaliação da resposta inflamatória do rodovalho é uma mais valia no estudo da interação patogénio/hospedeiro. Esta resposta imune foi estudada através da resposta peritoneal leucocitária, tendo-se observado uma resposta significativa ao bacterin da *M. marinum*. Em relação à MM-PT inativada, os neutrófilos mostraram um aumento significativo inicial, no entanto, tal resposta padronizada não foi observada em relação à MM-SP inativada. A resposta do rodovalho ao adjuvante foi característica de uma resposta inflamatória que persiste ao longo do tempo, mostrando que este agente flogístico é de difícil degradação pelo rodovalho. A infeção experimental *in vivo* é de importância crucial para os imunologistas e patologistas. O desenvolvimento da doença, a oscilação nas populações leucocitárias e os efeitos observados são relevantes no estudo do agente patogénico e da sua patogenia. O rodovalho mostrou-se sensível à infeção experimental com as duas estirpes de *M. marinum* (MM-PT e MM-SP), tendo-se observado que os macrófagos foram a população leucocitária predominante. No ensaio da infeção, observou-se que no grupo infectado com a *M. marinum* ocorreu um aumento

inicial no número de macrófagos e de neutrófilos. Os primeiros aumentaram até 17 vezes nos casos mais severos. Até aos 90 dias do ensaio, durante a resposta inflamatória não específica, observou-se um pico leucocitário ao dia 4, com ausência de granulomas, mas sendo os macrófagos as células predominantes. Contudo, não foram encontradas úlceras cutâneas, alterações de pigmentação ou exoftalmia uni- ou bilateral. No rim-anterior foram encontrados aglomerados bacterianos durante um maior período de tempo, quando comparado com o baço ou o exsudado peritoneal. Após a análise dos resultados obtidos a partir do estudo destas duas estirpes bacterianas, realizámos um ensaio mais preciso para estudar os seus efeitos na função e integridade dos macrófagos do rim-anterior do rodovalho. Esses ensaios basearam-se na deteção da produção do anião superóxido. As estirpes MM-PT e MM-SP são capazes de induzir uma "explosão respiratória" nos macrófagos do rim-anterior do rodovalho. Contudo, a resposta sofreu um aumento quando a MM-PT foi previamente inativada, e um decréscimo quando a MM-SP se encontrava inativada. Tais diferenças não fazem parte de um cenário normal, observando-se que a MM-PT inibe a libertação do anião superóxido para o espaço extracelular, quando comparado com a MM-SP. A citometria de fluxo é uma das tecnologias mais usadas, quando se estudam as populações celulares nos mamíferos e em algumas espécies de peixes. A população leucocitária do rodovalho mostrou ser uma forte candidata para ser estudada através da citometria de fluxo e o azul de tripano mostrou ser um bom agente para eliminar a fluorescência extracelular dos nossos estudos. Constatou-se que a espécie bacteriana intracelular (MM-PT) exibe um padrão de fagocitose diferente, quando comparada com a extracelular (*S. parauberis*). Porém, a estirpe Espanhola: MM-SP, comporta-se como o *S. parauberis*, sugerindo que poderá existir uma influência geográfica na infeção e modelo de virulência evidenciados pelas mesmas espécies bacterianas. A origem geográfica é muito importante e até mesmo considerada um aspeto fundamental na distinção e caracterização de diversas espécies. Durante alguns ensaios experimentais, encontrámos certos resultados intrigantes a respeito das duas espécies bacterianas estudadas. Foram realizadas várias abordagens, para analisar em maior profundidade estas diferenças, nomeadamente estudos da explosão respiratória e da libertação de intermediários nitrogenados dos macrófagos do rim-anterior do rodovalho, após estimulação bacteriana. A estirpe Portuguesa da *M. marinum* é semelhante à descrita na literatura para as espécias micobacterianas. Todavia, a estirpe Espanhola comportou-se como se se tratasse de uma bactéria extracelular, tal como o *S. parauberis*. Talvez tenha ocorrido uma separação destas duas estirpes de *M. marinum* no passado e elas tivessem que ter desenvolvido diversas adaptações para sobreviverem. Em detrimento da nossa preocupação, aliada ao facto da imunologia e da vacinologia de peixes, apesar de em progresso, estarem apenas no

começo, estes ensaios preliminares são de uma importância crucial por forma a dar continuidade aos trabalhos vindouros.

# SUMMARY

Turbot's (*Scophthalmus maximus*) production is mainly affected by streptococcosis and fish tuberculosis. The tuberculosis outbreak in a Portuguese turbot's farm helped the study of this pathogen and its interaction with turbot and was responsible for the beginning of these works. Streptococcosis and tuberculosis are caused by Gram-positive bacteria. Their etiological agents are: *Streptococcus parauberis* and *Mycobacterium marinum*, respectively and both were described. The maximum specific growth rates and lag times were estimated by direct viable count and absorbance data. The maximum specific growth rates varied between 0.01 and 0.0336 $h^{-1}$ in both *M. marinum* strains (MM-SP and MM-PT, respectively) and 0.2463 $h^{-1}$ in *S. parauberis*. Results concerning lag times confirmed the previous ones, showing that *M. marinum* strains are slow growers, when compared to *S. parauberis*. To describe turbot's immune response against an active or an inactive pathogen, we have to know turbot's unstimulated leucocyte population. So, after the intraperitoneal injection of phosphate buffer saline (PBS), we have observed that results were not different from those previously described for other fish species, showing that macrophages were the predominant cell type and neutrophils the less abundant one. Citochemically, we can use both esterase and peroxidase to distinguish between macrophages and neutrophils, because they reacted positively to each of them (macrophages to esterase and neutrophils to peroxidase). The evaluation of turbot's inflammatory response is worthwhile in the study of the interaction between the pathogen and its host. This immune response was studied observing the kinetics in the peritoneal leucocyte population of turbot and it was observed a significant response to inactivated *M. marinum*. When inactivated MM-PT was injected, neutrophils showed an increase at the beginning, but this typical response was not observed when inactivated MM-SP was injected. Turbot's immune response to adjuvant was consistent to an inflammatory response persistent during the period studied. This showed that adjuvant was hardly degraded by fish. The *in vivo* experimental infection is very important for immunologists and pathologists. The development of a disease, the oscillation between the leucocyte populations and its observed effects, are relevant in the study of the pathogen and its pathogenicity. Turbot was shown to be sensitive to the experimental infection with both strains of *M. marinum* (MM-PT and MM-SP) and macrophages were the predominant cell type. The group infected with *M. marinum* showed an increase in the numbers of macrophages and neutrophils. The first increased up to 17 times in the most severe cases. Till 90 days post-infection it was observed high levels of leucocytes at day 4, but with no granulomas. Macrophages were the predominant cell type, however we could not

find skin granulomas, pigmentation alteration, uni- or bilateral exophthalmia. We observed bacterial aggregations in the head-kidney, during a higher time period, when compared to the spleen or the peritoneal exudates. After the analysis of the achieved results, a more precise study was conducted to observe their effects in the function and integrity of turbot's head-kidney macrophages. These studies were related to the detection of superoxide anion production. MM-PT and MM-SP strains are capable of inducing a respiratory burst in turbot's head-kidney macrophages. However, the response was higher when MM-PT was previously inactivated and led to a decrease when MM-SP was inactivated. Those differences were not typical. MM-PT was shown to inhibit the release of superoxide anion to the extracellular space, when compared to MM-SP. Flow cytometry is one of the most used technologies when we are studying the cellular population in mammals and some fish species. Turbot's leucocyte population showed to be a strong candidate to be studied using this technique and trypan blue was a good agent to eliminate the extracellular fluorescence of our studies. We have observed that MM-PT showed a different pattern of phagocytosis, when compared to *S. parauberis*. However, MM-SP behaved as the previous bacterial species, suggesting that it may exists a geographic influence in the infection and virulence patterns evidenced by these two *M. marinum* strains. The geographic origin is very important and is even considered a fundamental issue in the distinction and characterization of several species. Among several experimental trials we came across with some intriguing results, concerning these two bacterial species. We have made different approaches to analyze these differences mainly in respiratory burst studies and in the release of nitrogen intermediates from turbot's head-kidney macrophages after bacterial stimulation. The Portuguese strain of *M. marinum* is similar to that encountered and described in literature for mycobacterial species. However, MM-SP behaved as if it was an extracellular bacteria, like *S. parauberis*. It may have occurred a separation between the two *M. marinum* strains in the past, and they have developed different survival strategies. It is well known that fish immunology and vaccinology are in progress and these preliminary assays are very important and a wonderful tool for the forthcomings works.

# CHAPTER 1
# Introduction

World aquaculture has grown dramatically in the last 50 years. From a production of less than one million tonnes in the early 1950s, production in 2009 was reported to have risen to 55.1 million tonnes, being the marine aquaculture responsible for more than 36% of the total value (FAO, 2010). However, the total fisheries captures have not decreased to levels wanted (FAO, 2010). If we look more deeply in values related to flatfish production, we can see that from 2000 to 2008, there was an increase of more than 122 000 tonnes, being Spain and China the leading producers. Among all the flatfish species, turbot (*Scophthalmus maximus*) was considered the most important one (FAO, 2010).

Turbot (*S. maximus*) is a flatfish species that belongs to the **Scophthalmidae** family. It is a demersal fish that preferentially lives in marine or brackish waters of the North Atlantic, Baltic and the Mediterranean Sea. When looking on an anatomically basis, we see that turbot is a marine teleost with an oval body, almost circular in shape. As a flatfish it has got a dorsoventral symmetry: on its dorsal or ocular side, there is some pigmentation and we can find both eyes, because turbot's right eye migrates during metamorphosis. Its ventral side is also known as the blind one. Turbot has not got scales on the skin and its dorsal fin begins near the above eye. Its mouth has an arc form, due to its movement during metamorphosis. The swimbladder only exists during its larval stage, disappearing later on, when turbot becomes a benthonic species. It lives between 20 and 100m below water (Carvalho and Diniz, 1998; Owen, 2011).

Why producing turbot? Well, among several fish species, turbot (*S. maximus*) is an economically important rearing species. Turbot aquaculture began in the 1970s in Scotland (UK) and it was subsequently brought to France and to Spain. At first, the number of facilities in Spain was rather limited due to the scarcity of juveniles. The technological development of juvenile production changed this. At the beginning of the 1990s, there were already 16 producers in Spain. A significant crisis in turbot aquaculture occurred in 1992; there was an increase of 52 percent in production but the industry lacked a consolidated commercial marketing network. Another factor that contributed to this crisis was that farms were small and had very high production costs. This crisis caused the closure of some farms. From that moment onwards a reorganization of the

sector began, which gave rise to a growth both in production and in the number of countries where turbot was farmed. Spain, with its highly suitable oceanographic conditions, is now the major producer worldwide but turbot is also currently farmed in Denmark, Germany, Iceland, Ireland, Italy, Norway, Wales (UK), France and Portugal, and was previously reared in the Netherlands. Turbot has also been introduced to other regions (notably Chile in the late 1980s), and more recently, China (FAO, 2010). Besides commercial investment to improve facilities or built new farms, other decisive factors have assisted in the consolidation and development of this species production. These have included the production of dry feed and the development of vaccines for the most important diseases affecting turbot (FAO, 2010).

During the last decades, we have witnessed the dissemination of several diseases among aquacultures and Portugal was not an exception. It is well known that conditions in fish farms lead to the appearance of severe epizootic outbreaks, not only due to overcrowding, but also due to intensive farming conditions (Jacobs *et al*, 2009). Moreover, intensive rearing of aquatic species in aquaculture facilities provides an opportunity for the amplification of both native and exotic diseases (Jacobs *et al*, 2009). According to the World Organization for Animal Health (OIE), an emerging disease is defined as "...a recently admitted serious illness, whose aetiology can, or not, have already been established, and which is likely to be propagated within a population or between populations" (Renault and Guichard, 2007; Saulnier *et al*, 2007).

Fish diseases have been characterized and it was observed that some of the diseases affecting fresh water fish species productions were also considered as emerging diseases in marine fish species (Toranzo, 2005). Fish are continuously exposed to viral and bacterial pathogens, however, in this thesis, we will only focus on the bacterial pathogens associated to outbreaks in aquacultures.

When we look at bacterial diseases, we need to distinguish between Gram-negative and Gram-positive bacteria. Turbot production is affected by some Gram-negative bacterial species, such as: *Lestionella anguillarum* (formerly known as *Vibrio anguillarum*), the etiological agent of vibriosis; *Aeromonas salmonicida* subsp *salmonicida*, the etiological agent of furunculosis; *Pseudomonas anguiliseptica*, the etiological agent of "winter disease" or pseudomonadiasis, as well as *Tenacibaculum maritimus* (formerly known as *Flexibacter maritimus*), the etiological agent of flexibacteriosis. Besides these bacterial species, turbot production can also be affected by some Gram-positive species. Among these we can point out: *Streptococcus parauberis*, the etiological agent of streptococcosis and *Mycobacterium marinum*, the etiological agent of mycobacteriosis or fish tuberculosis (Toranzo, 2005).

More than 150 species of salt water and freshwater fish are affected by mycobacteriosis or fish turberculosis (Toranzo, 2005) and its main etiological agent is *M. marinum* (as mentioned before). However, *M. fortuitum* and *M. chelonae* are also capable of inducing tuberculosis in fish (Toranzo, 2005). The first report of a mycobacterial infection in fish has been attributed to Bataillon *et al* (1897), who isolated acid-fast bacilli from a tuberculous lesion in a common carp. Mycobacteria are disseminated in the environment, so fish are continuously exposed to these bacterial agents. Why some fish develop disease and others don't? Well, with all the conditions observed in aquacultures, such as: overcrowding, poor nutrition, deteriorated water quality and advanced age, fish become more susceptible to opportunistic agents (Toranzo, 2005). Turbot was not an exception and it showed to be sensible to these agents during its entire production cycle.

Streptococcal diseases are also responsible for important epizootic outbreaks in freshwater fish facilities, as well as, in saltwater farms (Toranzo, 2005). *Streptococcus parauberis*, classically known as the etiological agent of mastitis in cattle, was first described in 1958, affecting a culture of rainbow trout in Japan (Toranzo, 2005). In 1993, was responsible for an important disease outbreak in turbot in Galicia (NW Spain) (Toranzo, 2005). Streptococcosis was considered to be the cause of heavy economic losses, because affected fish became unmarketable due to their poor external appearance (Toranzo, 2005; Toranzo *et al*, 2009).

Turbot is susceptible to streptococcosis at all stages of the production cycle. This fish pathogen forms a biochemically and antigenically homogenous group, being that an advantage when developing a vaccine against it (Toranzo *et al*, 2009). The vaccines developed against streptococcosis only where effective when administered intraperitoneally (Toranzo *et al*, 2009). With the development of these vaccines, turbot production became more protected, but even though sensible to other diseases outbreaks, such as mycobacteriosis. And for this special one, there are no vaccines available to prevent it (Toranzo *et al*, 2009), which makes turbot susceptible to this disease during its production life cycle.

In 2004, in a Portuguese Turbot Aquaculture, located in the north of the country, an outbreak of mycobacteriosis was witnessed and followed. For obvious reasons, this Farm will not be identified in this thesis, but was the motor that led to its development.

After the observation of a mycobacteriosis infection in the turbot farm, and using the peritoneal cavity as a model, we tried to induce an experimental inflammation and infection by two strains of *M. marinum*: a Portuguese and a Spanish one, both isolated from farm outbreaks. For a better comparison, *in vitro* studies were also conducted.

We will now provide an overview of each theme that will be discussed during this thesis. On Chapter 2 we will provide the characterization of the resting peritoneal

population of turbot, essential for the forthcoming works. On Chapter 3 we will characterize *M. marinum*, on Chapter 4 we will discuss the experimental inflammation trial, on Chapter 5 the experimental infection trial and on Chapter 6 we will discuss the *in vitro* studies conducted with *M. marinum.* Finally on Chapter 7 we will correlate the behaviour of *M. marinum* with *S. parauberis* and the results achieved will be discussed on Chapter 8.

The results of this thesis, as well as others related to some of the themes discussed, have been documented in a number of publications, which will be attached at the end.

# FISH TUBERCULOSIS

Fish tuberculosis is caused by a bacteria belonging to the genus *Mycobacterium*. Mycobacteria are Gram-positive, usually acid and alcohol fast at some stages of its growth, non-motile, do not form capsules, endospores or conidia, rarely exhibit grossly visible aerial hyphae, produce oxidatively acid from sugars and with some exception of those that do not grow *in vitro*, can be divided into rapid- and slow-growing strains (Godfellow and Wayne, 1983). The genus was established by Lehmann and Neumann in 1896 (Godfellow and Wayne, 1983), because it included tubercle and leprosy bacilli, being considered atypical or nontuberculous (Chemlal *et al*, 2002), but it now encompasses 41 approved species (Godfellow and Wayne, 1983). The past 40 years have witnessed a marked change in the outlook of bacterial systematic, due to an introduction of new and more exacting biochemical, chemical, genetical and numerical methods, which have led to rapidly changing views of how bacteria ought to be classified and identified (Alkhodair *et al*, 2010).

Tuberculosis in fish is a subacute to chronic wasting disease capable of affecting fresh and salt water fishes (Saulnier *et al*, 2007). It typically includes granulomas in the spleen, kidney and liver. External manifestations are scale loss, hemorrhagic lesion (Alkhodair *et al*, 2010). As a chronic progressive disease, affected fish may lose their appetite, appear debilitated and emaciated, have impaired growth and become more susceptible to infection by opportunistic bacteria (Heckert *et al*, 2001).

*M. marinum* is a pathogen of poikilothermic hosts, such as frogs and fish (Prouty *et al*, 2003), being closely related to *M. tuberculosis*. This has been confirmed using gas chromatography of fatty acids and alcohols, DNA-DNA hybridization and 16S rRNA gene sequence analysis (Tonjum *et al*, 1998). Moreover, it is a photochromogenic organism

that causes disease in fish and self-limiting skin granulomas in man. Its natural reservoir appears to be water however, results published by Le Dantec *et al* (2002), showed that mycobacteria are highly resistant to chloride disinfection. They have shown to be able to replicate in biofilms, and solid-liquid interfaces may be regarded as sites of selective enrichment for these bacteria. On a primary isolation, it grows readily at 30º C but not at 37º C, but after several subcultures it can become adapted to growth at the mentioned temperature, as stated by Godfellow, but also confirmed by us (Godfellow and Wayne, 1983). Its optimal temperature growth may be the primary reason why it only causes lesions on the extremities of humans (EL-ETR *et al*, 2001). *M. marinum* has a generation time of 4 hours compared to 20 hours of *M. tuberculosis*, being a biosafety level two organism (EL-ETR *et al*, 2001). *M. marinum* is an intracellular pathogen, capable of developing several survival strategies in a normally hostile environment. It can escape from its vesicles into the cytoplasm of the host cell, be resistant to the lysosomal compartment that does not become lysosomal in nature (Decostere *et al*, 2004). Pathogenic mycobacterial species are believed to survive by use of the latter strategy, although the mechanisms by which these bacteria avoid phagosome-lysosome fusion appear to be different from those of other organisms studied (Decostere *et al*, 2004). Barker and colleagues (Decostere *et al*, 2004) showed that live *M. marinum*, like other pathogenic mycobacterial species, reside in a compartment which is not acidified and does not contain lysosomal enzymes. These bacteria are capable of replication and persistence in cultured macrophage cell lines (Decostere *et al*, 2004).

Patients with swimming-pool granuloma are usually tuberculin positive, probably by the Koch reaction. Indeed the lesions caused may be a continuous response to a few extremely superficial bacilli (Alkhodair *et al*, 2010).

Aetiological factors related with the mycobacterial granuloma are: chemical constituents, virulence, hypersensivity (Decostere *et al*, 2004). A granuloma may be defined as an inflammatory lesion in which there is a conglomeration of cells of the mononuclear phagocyte series. Granulomas may be classified as: non-toxic, due to a chemically and biologically inert substance such as carbon particles; toxic, due to certain substances such as colloidal silica or asbestos, which act by increasing the permeability of the lysossomal membrane; immunological, mediated by humoral antibody, cell mediated immunity or both. The type of granuloma is consistent to the predominant cell type encountered (Decostere *et al*, 2004) and there are several structures of granulomas, such as: organized or unorganized granulomas. These structures are related to the presence or absence of concentrically arranged or whorled structures (Houben *et al*, 2009). However, this organization is not always observed in mycobacterial granulomas and never dominates the entire granuloma. The unorganized granulomas have several

variants and represent the form and distribution of the antigen, the number, size, density and uniformity of the microorganism (Houben *et al*, 2009).

When inside the mononuclear phagocytes, what happens to mycobacteria is very important, mainly because it is related to the pathogenesis of tuberculosis (Houben *et al*, 2009). Mycobacteria multiply inside mononuclear phagocytes in susceptible animals, as well as other opportunistic pathogens (Houben *et al*, 2009). But, what we cannot forget is that mycobacterial inhibition or death in a granuloma may be regarded as an extracellular process mediated by the accumulation of high concentrations of products from the mononuclear phagocyte's metabolism, as well as from its degeneration or secretion (Houben *et al*, 2009).

There is not a vaccine available against fish tuberculosis. However, Pasnik and Smith (2005) developed a DNA vaccine encoding a potent immunostimulator, which elicited high levels of protective immune responses in hybrid striped bass (*Morone saxatilis* x *M. crysops*). The degree of protection was dose- and route-dependent, so it became impracticable, but was an important step in the development of a protective vaccine against fish tuberculosis.

# HOST-PATHOGEN INTERACTIONS

Charles Chapin once wrote that "as it takes two to make a quarrel, so it takes two to make a disease, the microbe and its host" and he could not be more right. During the first pages of this thesis we mentioned our host: turbot and our pathogen: *M. marinum*, so now we will try to correlate both.

Organisms, such as fish, have an important immune system capable of destroying any microorganism and preventing its entrance. Pathogens, in turn, possess self-defending systems, that will overcome the innate and adaptive host immune responses.

The non specific immune response is very important, and it was considered essential when fighting against pathogens, because the adaptive immune system is limited, not only due to the poikilothermic nature of fish, but also due to their few repertoire of antibodies, deficit in proliferation, maturation and memory existent in their lymphocytes (Ellis, 2001; Uribe *et al*, 2011). Fish have got physical barriers (skin, scales, mucus) and chemicals barriers (lysozyme, lectins) which prevent the entrance and attachment of pathogens in their cells. The protective effect of mucus has been established in turbot (Fouz, 1990) but the exact mechanisms involved in this protection are not known. Lysozyme is an enzyme capable of affecting the peptidoglycan layer of bacterial cell walls,

and was also found in fish mucus, in peritoneal macrophages and blood neutrophils (Ellis, 2001). Phagocytosis, is one of the most important activity of the immune system of fish, as it is not dependent of temperature. The main cells involved in this defence mechanism are neutrophils and macrophages. They remove bacteria by the production of reactive oxygen species, such as superoxide anion, during respiratory burst (Uribe *et al*, 2011).

The complement will have an important function in activating cellular defences. It is a system of serum proteins and can be activated in three ways: the alternative pathway, the classical pathway and the lectin pathway. The first is considered very important in fish, because it is found in high doses in fish serum, when compared to mammals (Ellis, 1999; Nakao *et al*, 2011). By releasing components (such as C3b and C5a), it can activate the recruitment of phagocytes (Ellis, 2001).

However, primary immune defences can be deficient and pathogen may be able to enter in the host cell and there, it needs to survive against the innate and adaptive immune system of the organism invaded. The site of infection and the type of pathogen is clearly, important to decide which immune response will be effective.

Pathogens are extremely diverse, but based on the pathogenesis of infection and on the immune response elicited in the host, they can be classified as intracellular and extracellular.

Bacteria causing extracellular infections, like *S. parauberis*, are preferentially localized in body fluids and other extracellular spaces, being killed if captured by phagocytes. Therefore, the host defence against these bacteria is related with the activation of the non-classical complement, neutralisation by antibodies, release of cytokines, inflammation and efficient phagocytosis (Ellis, 2001). The key mechanism and importance demonstrated by the last in the defence against infection, was first proposed by Metchnikoff, more than 100 years ago.

Intracellular bacteria, as the one used in our studies (*M. marinum*), induce a chronic inflammatory response on the host. These kinds of bacteria survive and replicate inside host cells, making their internal environment, their “home”. Intracellular bacteria are able to survive due to their ability of interfering with bactericidal activities inside the host cell. Professor Kaufmann has enumerated some of these mechanisms, such as inhibition of fusion of lysossomes with the phagossome, a common strategy demonstrated by mycobacteria (Kaufmann, 1989). Moreover, after infecting macrophages, T lymphocytes are responsible for their recognition and development of several protective responses. However, macrophages protect microorganisms from these immune responses, making the eradication of the pathogen far more complicated. During the last years, investigations were conducted and Galluzzi *et al* (2011) correlated the autophagy with bacterial invasion. Autophagy may be associated with the engulfment of intracellular components by double-

membrane organelles, known as phagossomes. Later on, by the action of lysossomes these components will be degraded. In stress conditions, cells may activate this process of autophagy, though contributing to the clearance of bacteria inside host cells (Galluzzi *et al*, 2011).

# CHAPTER 2

# Peritoneal leucocyte population in *Scophthalmus maximus*

The initial investigation in this thesis concerned the study of the resting peritoneal leucocyte population of turbot (*S. maximus*).

The use of the peritoneal model (Silva *et al*, 1989) was advantageous because the inflammatory and infection events that occur inside it could be studied both quantitatively and qualitatively, in contrast to what happens in solid organs. Moreover, the leucocyte population within the peritoneal cavity is dispersed and can be easily processed for cytological analysis.

## MATERIAL AND METHODS

Turbot, weighting 65±5.0 g (from Piscicultura Marinha do Rio Alto – A. Coelho & Castro, Lda, in Póvoa do Varzim, Portugal) were used. Fish were kept at 18-22º C in a recirculating aerated salt water (35‰) system at Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR, Porto, Portugal). Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pelleted feed (SORGAL, S.A.; Ovar, Portugal).

Turbot were divided into 2 groups of 40 fish each. Each group was intraperitoneally injected (i.p.; 100 µl) with Phosphate Buffer Saline (PBS: 1.47 mM $KH_2PO_4$, 7.82 mM $Na_2HPO_4$, 0.169 mM NaCl, 13.21 mM $Na_2CO_3$; pH 7.2), after being anaesthetized with 0,03% (v/v) ethylene glycol monophenyl ether (Merck).

The peritoneal response was observed by studying quantitatively the leucocytes at 12, 24 and 48 hours, as well as, 4, 7, 15, 30, 60 and 90 days after i.p. injection.

Leucocytes were collected from groups of 10 fish (5 per each group). Turbot were killed by an overdose of anesthesia (0.06% (v/v)). Peritoneal exudates were collected using a previously described technique (Afonso *et al*, 1997; Afonso *et al*, 1998). Briefly, PBS was injected into the peritoneal cavity (0.5 mL per fish). Fluid containing the

peritoneal cells was then collected and maintained on ice until processing for cytospins preparations and for cytochemical analysis by electron microscopy.

## CYTOSPINS PREPARATIONS

Cytospins preparations of peritoneal leucocyte suspensions were made using a Cellspin I apparatus. Cytospins were fixed with formol-ethanol (10% of 37% formaldehyde in absolute ethanol) for one minute and stained with Wright's stain (Haemacolor Merck). For peroxidase detection, the Antonow's technique (Afonso *et al*, 1998) was used. Differential counts of macrophages, neutrophils and other small mononuclear cells were made under x100 oil immersion. Preliminary studies developed in other fish species (Afonso *et al*, 1997; do Vale *et al*, 2002), confirmed that the population of small mononuclear cells is almost entirely composed by lymphocytes, but by light microscopy it is difficult to differentiate between lymphocytes and other mononuclear cells such as small monocytes and thrombocytes. Thus, these were classified together as small mono cells. To overcome the weight variation between individuals, leucocyte numbers were converted to leucocytes per gram of body weight.

## CYTOCHEMICAL ANALYSIS

Peritoneal leucocyte suspensions collected, were then fixed in 5% Gluteraldehyde in PBS and processed for electron microscopy. Briefly, after fixation, the peritoneal exudates were washed in 0.1M Cacodylate Buffer (pH 7.3) during the course of one hour. Later on, they were post-fixed in 1% Osmium Tetroxide in Cacodylate Buffer for 3 hours at room temperature, incubated during 30 minutes in 0.5% Uranyl Acetate and then embedded in Epon. Ultrathin sections were obtained using an Ultramicrotome, contrasted with Uranyl Acetate and Lead Citrate (Silva *et al*, 1987) and examined with a JEOL JEM 100CXII Electron Microscope. Detection of peroxidase activity by electron microscopy was carried out by the method of Robbins *et al* (1971). The technique described by Robinson and Karnovsky (1983) was used for the detection of esterase.

# RESULTS AND DISCUSSION

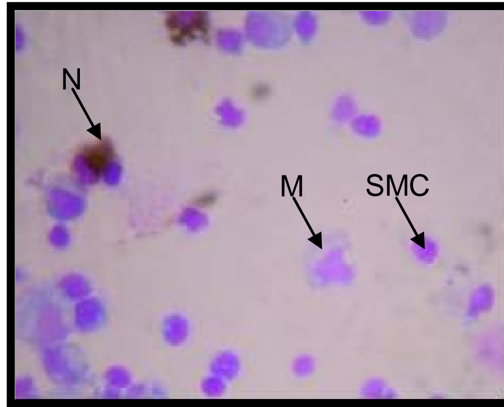



Figure 1. Citological preparation from normal turbot peritoneal washes stained by haematoxylin-eosin (M – macrophage; N – neutrophil; SMC – small mono cell).

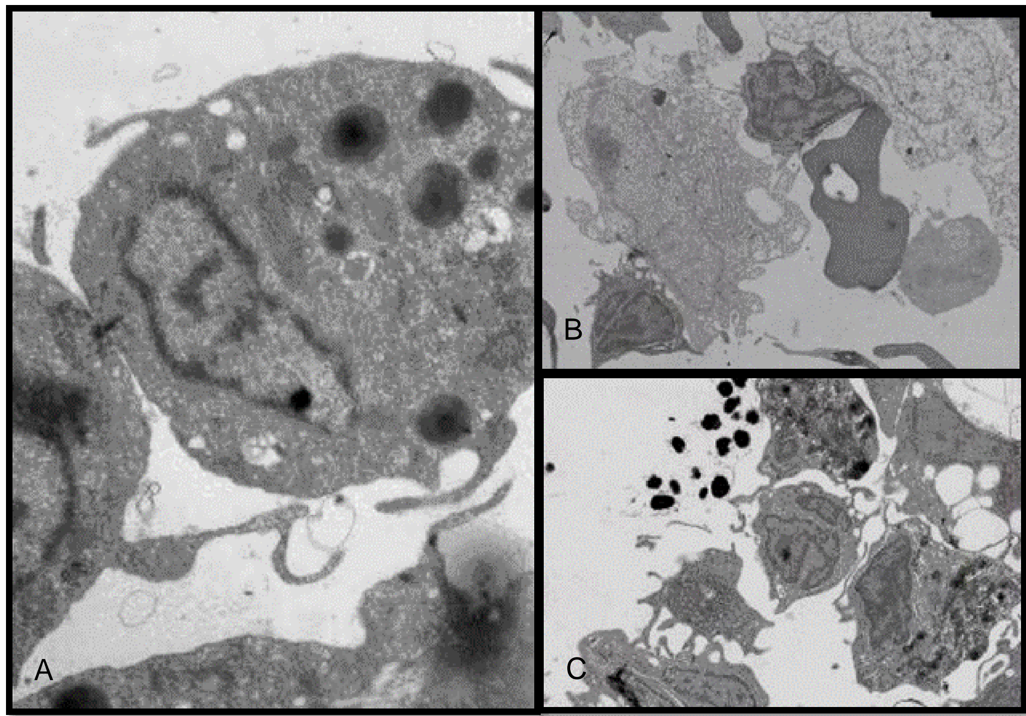



Figure 2. A) A peritoneal macrophage in a sample of a control turbot processed for conventional microscopy (X10000; note the existence of pseudopodia); B) and C) Peritoneal exudate processed for ultrastructural cytochemistry for peroxidase (X5000).

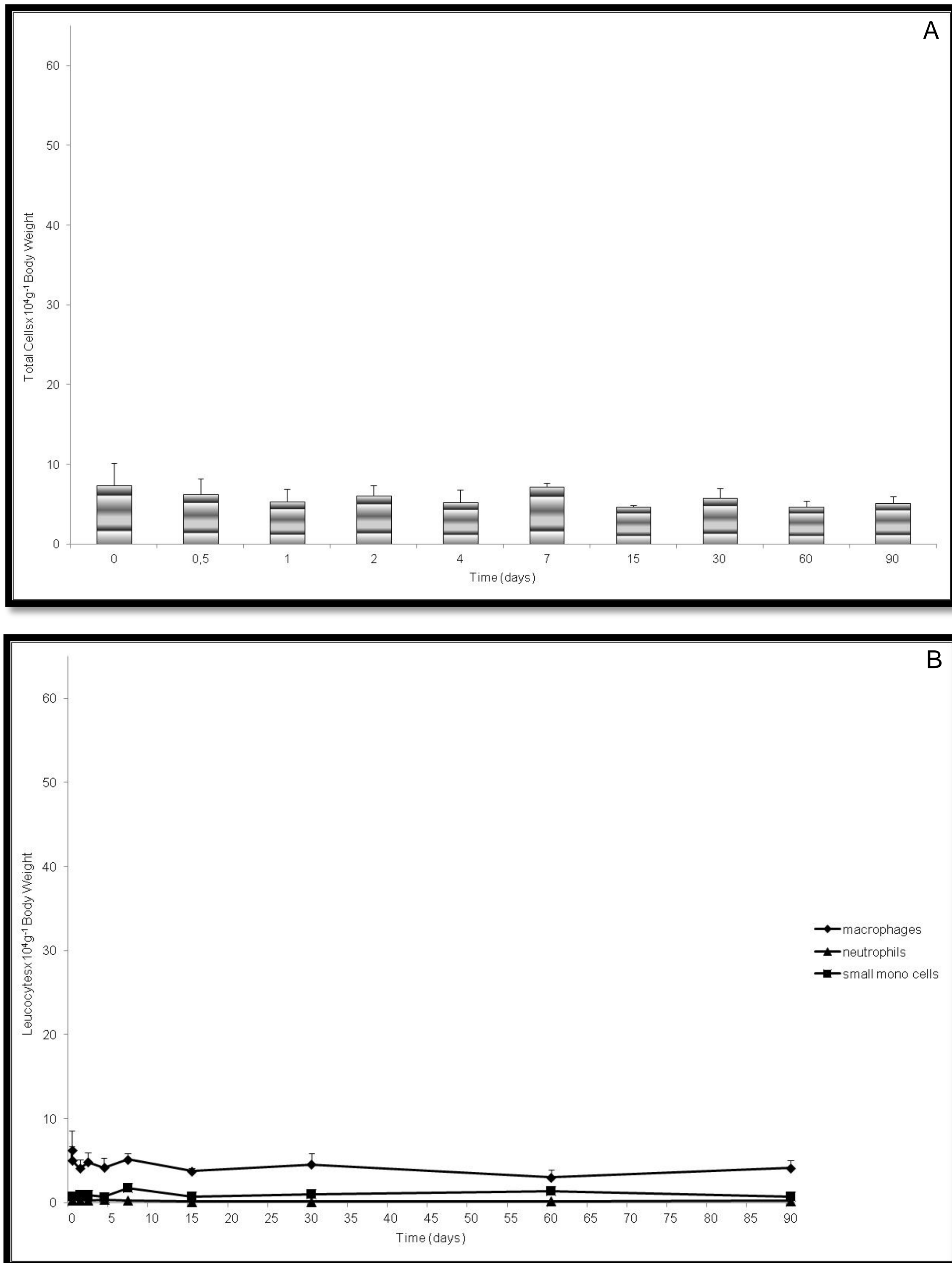



Figure 3. A) Total peritoneal leucocytes from control turbot; B) Turbot peritoneal leucocyte response after intraperitoneal injection of PBS.

The peritoneal leucocyte response to i.p. injection of PBS was observed and both total and differential counts were made. The total peritoneal leucocytes, per peritoneal

cavity, of control fish were $2.14 \pm 1.10 \times 10^6$, which corresponded to $3.81 \pm 1.82 \times 10^4$ per gram of body weight (Figure 3.A). Differential leucocyte counts were: macrophages 53.20%, small mono cells 40.80% and neutrophils 6% (Figure 3.B). The control group showed small insignificant variations over time (Figures 3.A and 3.B).

As observed, the relative percentages of each leucocyte type, in the unstimulated peritoneal leucocyte population was not different from those previously described for other fish species (Afonso *et al*, 1997; Afonso *et al*, 1998), as well as in recent mammals studies (Pires *et al*, 2008). In resting conditions, the concentrations of the two professional phagocytes were very dissimilar. Macrophages were the predominant phagocyte and neutrophils the less abundant one.

Peritoneal leucocytes from turbot are mainly composed by macrophages, which can be observed in our cytological preparation (Figure 1.). They are round in shape, with a basophilic cytoplasm, their nucleus is in general eccentric and occasionally horse-shoe shaped. Besides macrophages, we could also find neutrophils, which reacted positively to peroxidase. These are round and large, with fine neutrophilc granules in the cytoplasm, their nucleus is small, rod-shaped and occasionally segmented (Figure 1.). The other cell type, which we named small mono cells were mainly composed by small round cells, with a large nucleus, which occupies almost all the cytoplasm (Figure 1.). When observing electron microscopy photos we observed that macrophages were negative for peroxidase (Figures 2.B and 2.C) and positive for esterase (electron photography not included) and neutrophils were positive for peroxidase (Figures 2.B and 2.C) and negative for esterase (electron photography not included). Moreover, we could also observe the existence of a vacuolated cytoplasm in macrophages, which is a common feature among this cell type (Figure 2.A). When regarding their morphological features, we observed that, once again, turbot leucocytes were not different from other fish leucocytes (Afonso *et al*, 1997; do Vale *et al*, 2002; Tavares-Dias *et al*, 2005). Data concerning cytochemistry studies was in accordance with previous documented studies (Afonso *et al*, 1998; do Vale *et al*, 2002). Moreover, flatfish neutrophils seem to have similar fibrillar granules to those firstly described for amphibians and mammals (Hine and Wayne, 1988) and this similarity may be explained on a common ancestor basis.

These results strongly show that even if there could be doubts in identifying peritoneal turbot leucocytes, we can do it using cytochemical methods such as the identification of peroxidase or esterase, because they are a distinctive feature between macrophages and neutrophils.

# CHAPTER 3

# *M. marinum*'s growth behavior

When studying a relationship between a host and a pathogen, we need to characterize each of them individually and only then, establish their interaction.

We have obtained the growth curve for *M. marinum* (Portuguese and Spanish strains) and for *S. parauberis* (for comparison).

## MATERIAL AND METHODS

We used, as mentioned, two bacterial species, in a total of three strains.

One strain of *M. marinum* was kindly supplied by Dr. Nuno M.S. dos Santos (Institute for Molecular and Cell Biology, IBMC, Porto, Portugal) (dos Santos *et al*, 2002), and we named it Portuguese *M. marinum* (MM-PT). The other was kindly supplied by Dr. Jesús Romalde (University of Santiago de Compostela, USC, Spain), and we named it Spanish *M. marinum* (MM-SP). The last one had already been biochemically characterised. Both bacteria were thawed from a frozen stock (-70º C) and platted onto Middlebrook 7H10 Agar (7H10, Difco). Cultures were incubated one week at 30º C, and used to inoculate in 100 mL Middlebrook 7H9 Broth (7H9, Difco) in 500 mL Erlenmeyer flasks. Ziehl-Nielsen stain (Merck, Germany) was used for presumptive identification of each culture.

*S. parauberis* strain was kindly supplied by Dr. Jesús Romalde (USC, Spain) (Romalde *et al*, 1999). It was thawed from a frozen stock (-70º C) and plated onto Tryptic Soy Agar (TSA, Difco) supplemented with sodium chloride (NaCl) to a final concentration of 1% (TSA1). Cultures were incubated 48 hours at 22º C and used to inoculate in 100 mL Tryptic Soy Broth (TSB, Difco), supplemented with NaCl to a final concentration of 1% (TSB1) in 500 mL Erlenmeyer flasks, after a preliminary confirmation of the purity of the culture, using the Gram-Hucker Stain (Panreac Química S.A., Barcelona, Spain).

In order to perform growth measurements, two times per day, each bacterial density was measured by spectrophotometry at 550 nm (Jenway 6405 UV/VIS) and serial dilutions in 7H9 (*M. marinum*) or TSB1 (*S. parauberis*) were made to estimate the number

of Colony Forming Units (CFUs). Growth measurements were made until each culture reached the death phase.

Maximum-Specific Growth Rate ($\mu_{max}$) values were estimated from viable counts data (μVC) by the log-transformed four parameter Logistic Model (Dalgaard and Koutsoumanis, 2001) (Table 1).

$$Log(N_t) = Log\,[N_{min} + \frac{N_{max} - N_{min}}{1+e^{(-\mu VC(t-t_i)}}]$$

Lag times (λ) values were estimated from viable count growth data (λVC) (Dalgaard and Koutsoumanis, 2001) (Table 1).

$$Lag\ time\ (\lambda VC) = t_i - \frac{1}{\mu_{max}} x \ln \frac{N_{max} + N_{max}\ x\ e^{(\mu_{\max x}\, t_i)}}{N_{max} + N_{min}\ x\ e^{(\mu_{max}\ x t_{i})}} - 1$$

Table 1. Nomenclature

| Symbol | Description and Units |
|---|---|
| $\mu_{max}$ | Maximum specific growth rate ($h^{-1}$) |
| μVC | $\mu_{max}$ determined from viable count data |
| λVC | λ determined from viable count data |
| $N_{min}$, $N_{max}$ | Parameters corresponding to asymptotic minimum and maximum cell concentrations (CFU/mL) |
| t | Time (h) |
| $t_i$ | Time at inflection point (h) |

# RESULTS AND DISCUSSION

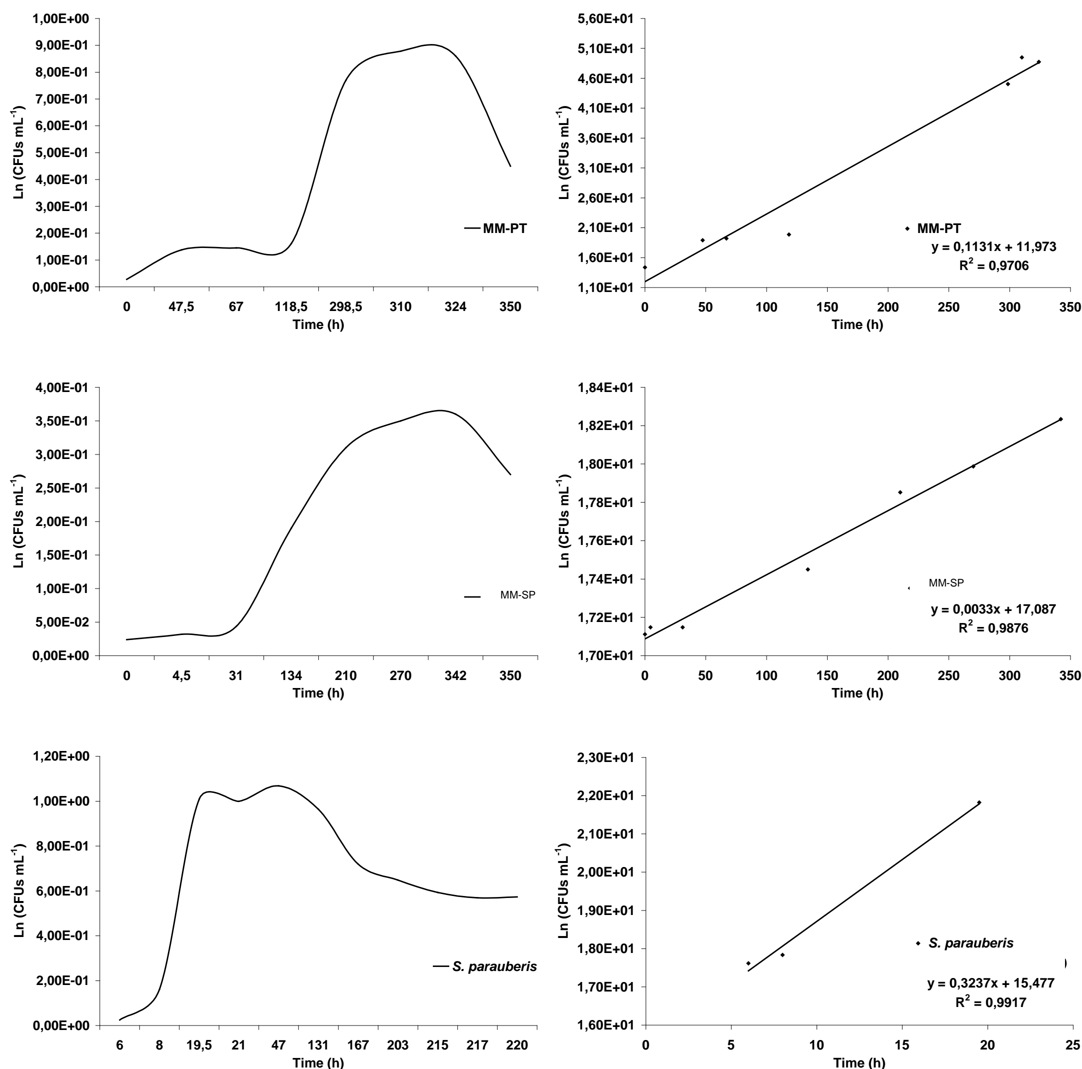



Figure 4. Growth curves.

| | Temporal Length (hours) | |
|---|---|---|
| Bacteria | Exponential Phase | Stationary Phase |
| MM-PT | 192 | 14 |
| MM-SP | 239 | 72 |
| *S. parauberis* | 21 | 26 |

Table 2. Temporal length of each of the growth curves parameters.

| Bacteria | Maximum Specific Growth Rate (µVC) |
|---|---|
| MM-PT | 0.0336 |
| MM-SP | 0.01 |
| *S. parauberis* | 0,2463 |

Table 3. Maximum specific growth rates.

| Bacteria | Lag Times (λVC) |
|---|---|
| MM-PT | 100.92 |
| MM-SP | 278.58 |
| *S. parauberis* | 4.26 |

Table 4. Lag times.

The three bacterial strains were confirmed to be Gram-positive and the two strains of *M. marinum* could be included in the acid-fast group because, when stained by Ziehl-Nielsen, could not be decolorized by acid.

The bacterial species were able to grow, showing that growth media were appropriate. Furthermore, it was possible to observe each of the characteristic growth phases. Lag phase was characterised by an initial slow growth, corresponding to the time required by bacteria to accommodate to food and nutrients of their new habitat. Once its metabolic machinery was running, they started to multiply exponentially (log or exponential phase). As more bacteria were competing for dwindling food and nutrients, booming growth stopped and the number of bacteria tended to stabilize (stationary phase). Finally, toxic waste products built up, food became depleted and bacteria began to die (death phase).

Growth curves (Figure 4.) were used to determine the temporal length of the exponential and stationary stages of development and exponential stage growth rates could be quantified (Table 2.). Values of maximum-specific growth rates (µVC) varied from 0.01 to 0.2463 $h^{-1}$ (Table 3.) and lag times (λVC) estimated from viable count growth curves varied between 4.26 and 278.58 h, in the three growth experiments (Table 4.).

The difference between a fast and a slow grower is mainly in its ability of inducing acute or chronic infection and inflammatory responses on the host, respectively. Results achieved, showed that *S. parauberis*, an extracellular bacteria, is also a fast grower (µVC

= 0.2463 $h^{-1}$; λVC = 4.26 h). This is in accordance with the temporal length of each growth phase, as they take less time to be achieved and less time in duration, as well as with previous detailed works conducted by Dalgaard and Koutsoumanis (2001). Moreover, these results show that, *S. parauberis* mainly induces an acute inflammatory response on the host. In contrast, the other two strains of *M. marinum* are acid-fast bacilli and are known as intracellular pathogens. Comparing the results achieved, with those from *S. parauberis*, we observe that these bacteria are slow growers, not only by the maximum-specific growth rates, but also by the lag time's results. Once again, and based in these results, *M. marinum* is capable of developing a chronic inflammatory on the host.

All these results are in accordance to that described in literature (Kaufmann, 1992; Ellis, 1999, 2001, Galluzzi *et al*, 2011). As a slow grower, *M. marinum* studies its host and the best entrance to survive and replicate. This intracellular pathogen has chosen macrophages as its home and survives because it is difficult for the immune system of the host to recognize where the invaders are and kill them. There is no doubt that is a well developed and clever way of surviving within the host and that is why these pathogens subsist. In comparison, extracellular bacteria, as *S. parauberis*, as they live in the extracellular space have more difficulties in circumventing host's immune system and so they need to be fast growers, to replicate as fast as possible and establish the disease.

What seemed to be a routine experiment, the determination of bacterial growth curves, turned to the beginning of an interesting issue. In fact, results showed that MM-SP appeared to be slower grower than MM-PT, not only by the values obtained for the maximum-specific growth rate and lag times, but also because it took more time to reach the stationary phase. This kind of behavior has not been documented elsewhere and works documented later in this thesis are important in supporting these differences.

# CHAPTER 4
# Turbot inflammatory response

In order to evaluate the phenomena associated with turbot inflammation, we decided to perform an inflammatory trial using *M. marinum* inactivated, a control group and an adjuvant group, so as to characterise turbot peritoneal leucocyte response to phlogistic agents.

## MATERIAL AND METHODS

We have used turbot, weighting 65±5.0 g (from Piscicultura Marinha do Rio Alto – A. Coelho & Castro, Lda; Póvoa de Varzim, Portugal). Fish were kept in the same conditions as mentioned before (Chapter 2 – Material and Methods).

The bacterial cultures (MM-PT and MM-SP) were inactivated. Briefly, *M. marinum* was thawed form a frozen stock (-70º C) previously prepared and inoculated onto 7H10. Cultures were grown during 1 week and ressuspended into 7H9. The bacterial density was measured by spectrophotometry at 520 nm and dilution (in 7H9) were made until the chosen number of CFUs (predicted by the curve obtained previously, corresponding to 200 µg of protein/mL was achieved (Chapter 3). Then, cultures were inactivated by adding 8% formol and left at 4º C during 3 consecutive days. Later on, it was washed in PBS by centrifuging five consecutive times (3900 rpm during 20 minutes). Between each wash the supernatant was discarded and the pellet ressuspended in PBS. Finally, PBS with 15% of glycerol was added and stored at -70º C for posterior use. Freund's Incomplete Adjuvant (FIA, Sigma), was also used as a phlogistic agent.

Turbot were divided into 4 groups of 80 fish each. Briefly, a non-injected, control and 3 other groups. All fish were starved 24 h prior to inflammatory trial. After anaesthetizing turbot (0.03% (v/v)), groups were i.p. injected (100 µL) with the following substances:

Group I – PBS (Control)

Group II – Inactivated MM-PT

Group III – Inactivated MM-SP

Group IV – FIA

As mentioned in Chapter 2, the acute phase of the inflammatory response was evaluated by studying quantitatively the peritoneal leucocytes at 12, 24 and 48 hours after i.p. injection. To evaluate the chronic response, peritoneal leucocytes were collected at 4, 7, 15, 30 and 60 days after i.p. injection.

Leucocytes were collected from groups of 10 fish for each time period, following the method described before (Chapter 2, Material and Methods). Differential counts of macrophages, neutrophils and other small mononuclear cells (small mono cells) were made under a x100 oil immersion. Once again, to overcome weight variation between individuals, leucocyte numbers were converted to leucocytes per gram of body weight.

At each collection time, internal organs were observed. Sixty days after i.p. injection of the several phlogistic agents, internal organs (kidney, liver, spleen, intestine) and gills of one fish per group randomly chosen, were collected and fixed in formol 4% for histological analysis. Tissues samples were embedded in paraffin wax and 5 μm sections were cut with a rotary microtome (American Optical, Buffalo, NY). After dewaxing, the sections were stained with haematoxylin/eosin. Further tissue sections were evaluated for the presence and type of pathological lesions.

# RESULTS AND DISCUSSION

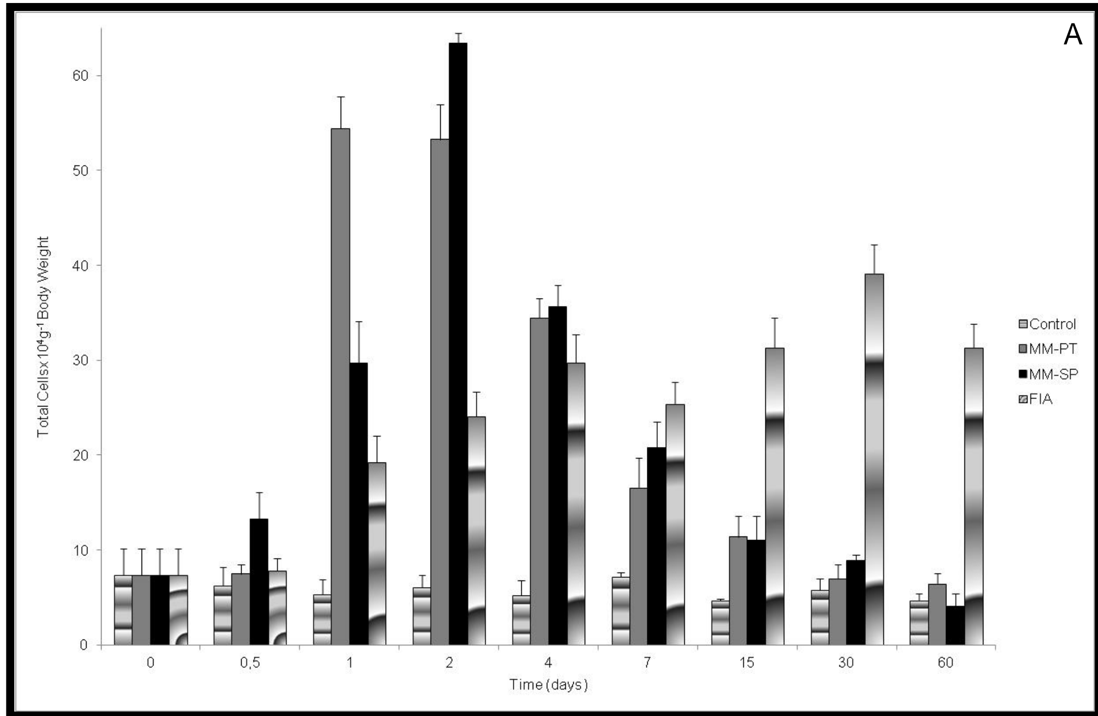
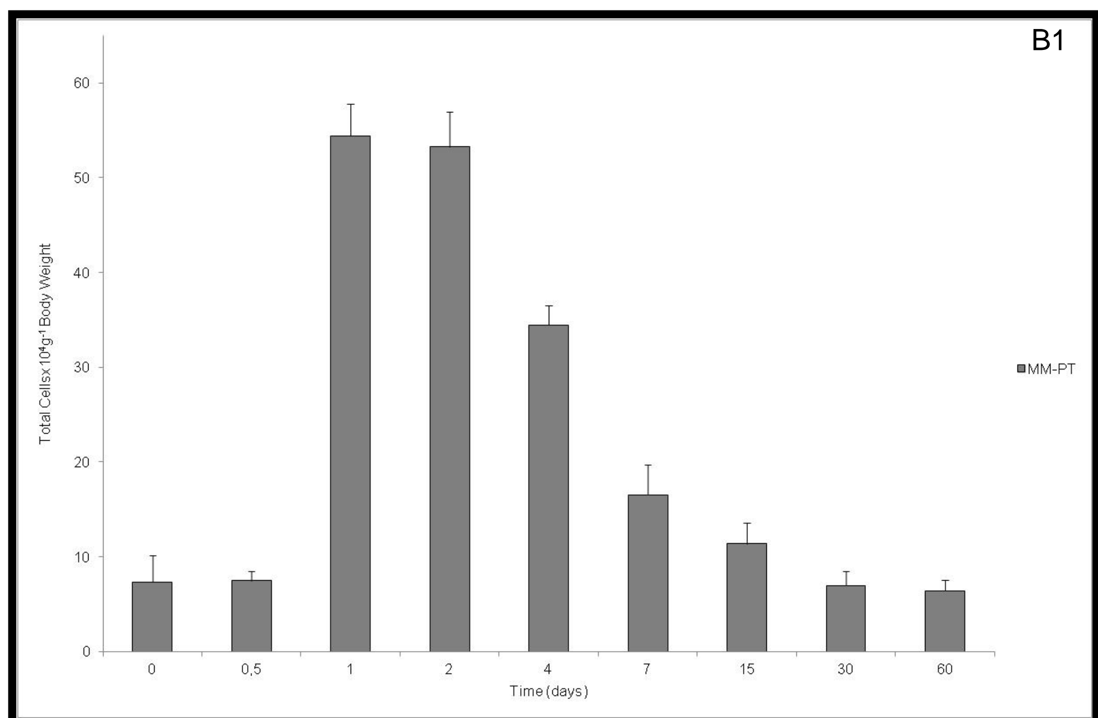



Figure 5. Peritoneal leucocytes after intraperitoneal injection of several phlogistic agents.



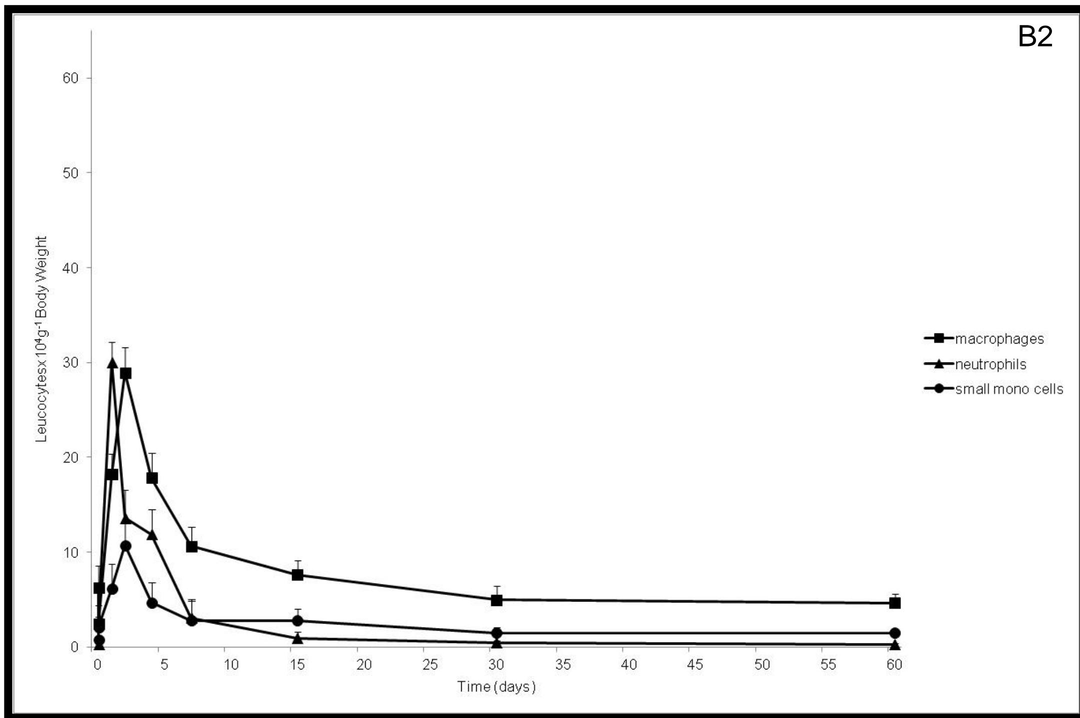
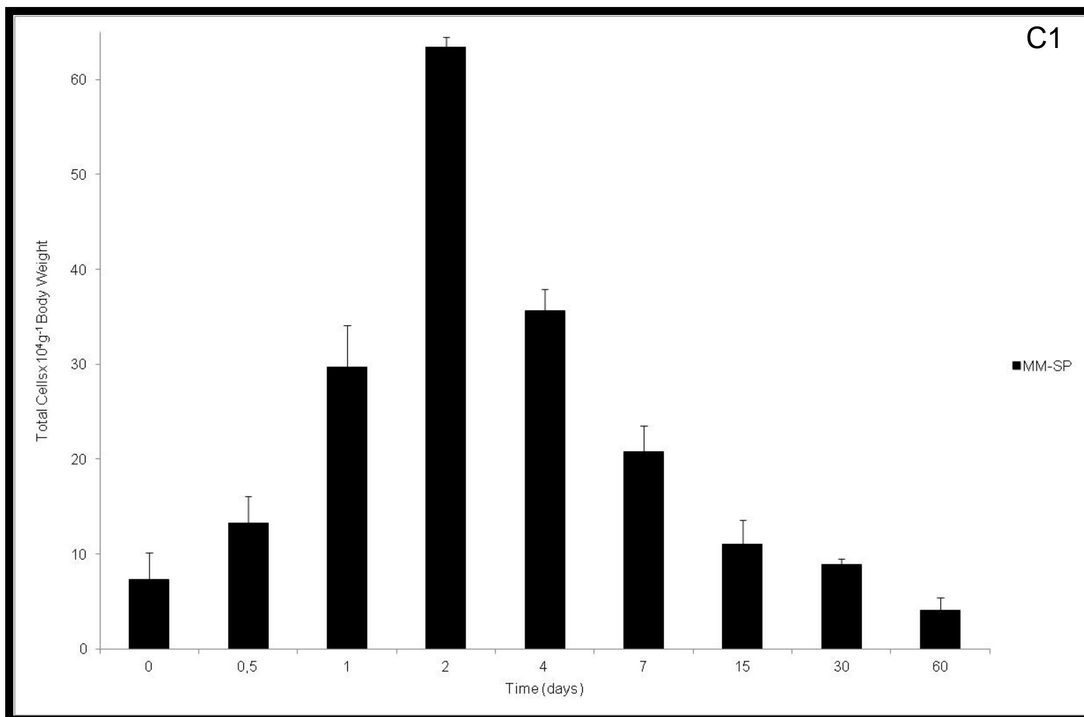



Figure 6. Turbot peritoneal leucocyte response after intraperitoneal injection of inactivated MM-PT.



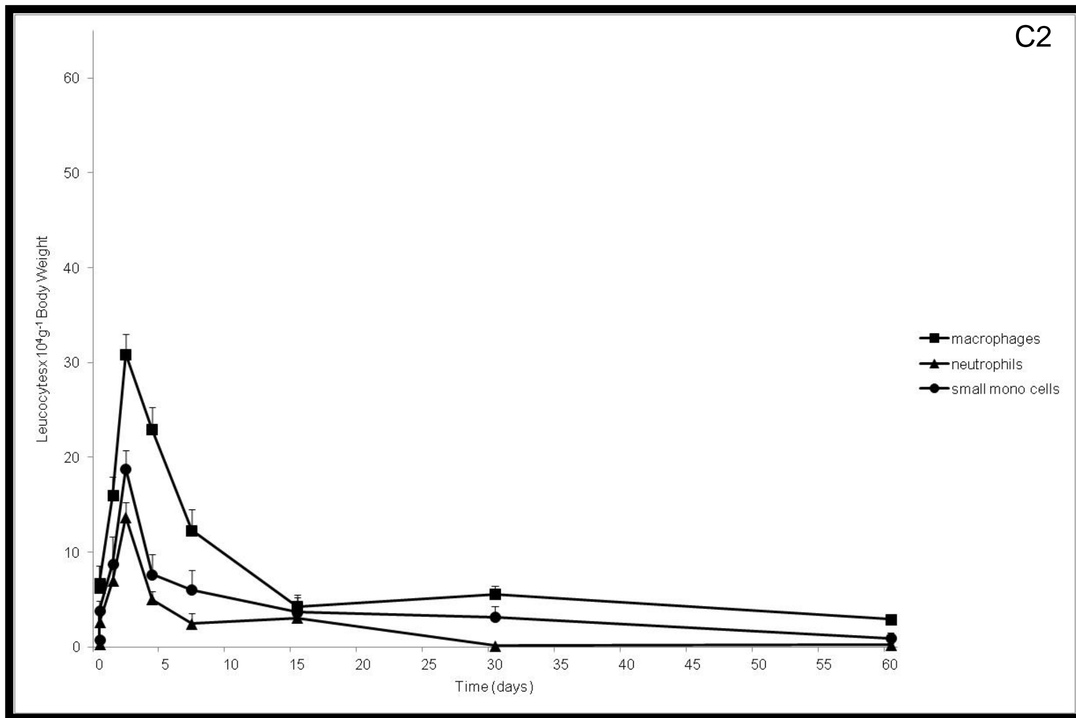
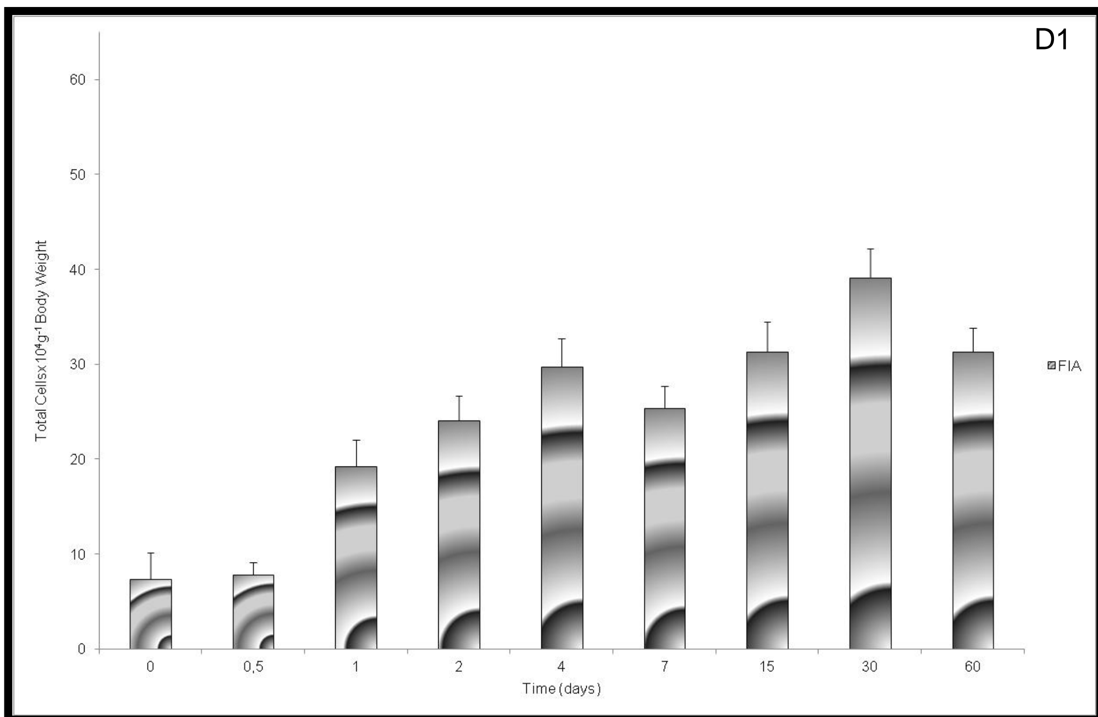



Figure 7. Turbot peritoneal leucocyte response after intraperitoneal injection of inactivated MM-SP.



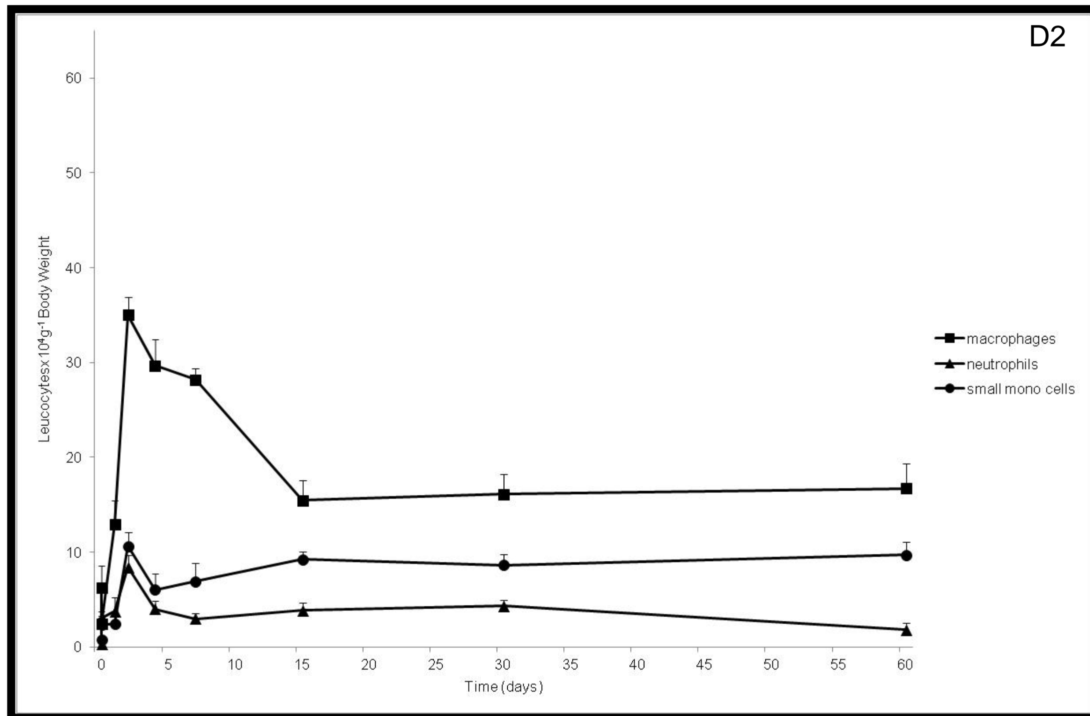



Figure 8. Turbot peritoneal leucocyte response after intraperitoneal injection of Adjuvant (FIA).

Results achieved, showed that leucocyte mobilization to the peritoneal cavity depended on the type of phlogistic agent injected (Figure 5.).

Values obtained for both inactivated *M. marinum* (MM-PT and MM-SP) were not very dissimilar (Figures 6. and 7.).

Turbot i.p. injected with inactivated MM-PT, showed a statistical significant increase in the number of total cells 24 h after injection ($P<0.05$), when compared with the non-injected and the control groups (Figure 6.B1). The most prominent increase, in the inactivated MM-SP group (Figure 7.C1), was 48 h post-injection when compared with the non-injected and the control groups ($P<0.05$). The rise in macrophages was more extensive as compared to that of neutrophils, in the MM-SP group (Figure 7.C2). The number of macrophages increased up to 48 h post-injection and then decreased till the end of the experiment. Thirty days post-injection they have returned to non-injected values. On the other hand, in the MM-PT group (Figure 6.B2), neutrophils numbers showed a significant increase 24 h post-injection when compared with the non-injected and control groups ($P<0.05$), being higher than the macrophage numbers. From 24 h till the end of the experiment, neutrophils have showed a significant decrease when compared with the control group ($P<0.05$). Forty-eight hours post-injection, small mono cells showed a significant increase when compared to the non-injected and the control

groups (P<0.05). But this was followed by a decrease till the end of the experiment (Figures 6.B2 and 7.C2).

The adjuvant group (Figure 8.) could be characterized by a fluctuation in the number of leucocytes over time. Again, there was a significant increase in the number of total cells 4 days post-injection, when compared with the non-injected and the control groups (P<0.05), and has maintained the same pattern till the end of the experiment, without achieving till 60 days post-injection, the resting values. The observed increases were mainly due to the increment in the number of macrophages (Figure 8.D2). The neutrophils and the small mono cells populations were the most constants (Figure 8.D2).

As showed, data observed during the inflammatory trial was also very interesting, because MM-PT had a characteristically neutrophilic response and MM-SP did not. Moreover, turbot, in contrast to that observed in other fish species (Afonso *et al*, 1998; do Vale *et al*, 2002; Afonso *et al*, 2005) and in mammals (Pires *et al*, 2008), did not seem to be capable of developing an early neutrophilic response, during an inflammatory status, in the group i.p. injected with inactivated MM-SP. Moreover, macrophages appeared to be the most sensitive cell type in presence of phlogistic agents, making them good indicators of inflammation, as demonstrated by the increase in macrophage numbers up to 17 times in the most severe cases, usually 48 hours post-injection. There was also maintenance of macrophage values, up to 90 days in turbot injected with adjuvant, showing that a chronic inflammation had been established, as in accordance to what was observed in other fish species (Afonso *et al*, 2005). It seems feasible to suggest that inactivated *M. marinum* acted as transient inflammatory stimulants and the adjuvant, which is hardly degraded by fish, acted as an inflammatory stimulant, as it was capable of inducing and perpetuating the inflammatory reaction. However, differences were observed in the MM-SP group and as we are using the same host (turbot), there should be a strain variation not only related to the incoming of cells to the peritoneal cavity, as well as in their number. Moreover, it is well known the important function developed by macrophages during an inflammation event (Kolaczkowska *et al,* 2007) and data achieved in this study clearly shows that fish behaves in the same manner.

Several specimens were opened and it was not found any adhesions or granulomas inside the peritoneal cavity. However, we observed a slight alteration in the kidney's cellular population where there was a proliferation of leucocytes.

Some of the results achieved were not in accordance to what was described in other fish species, such as sea bass or rainbow trout (Afonso *et al*, 1998; do Vale *et al*, 2002; Afonso *et al*, 2005), where the neutrophilic population was considered a good indicator of inflammation. Our findings suggest that in turbot, macrophages are the

population responsible for this indication, when looking at “our” strain of *M. marinum* (MM-PT).

# CHAPTER 5
# Turbot infection trial

The study of the inflammatory response in turbot peritoneal cavity is the first step among others not less, nor more important. The *in vivo* experimental infection is of crucial interest for immunologists and pathologists. The development of disease, the oscillation of leucocyte populations and the effects observed are of major importance to study the pathogen, the disease and then work on a way to accomplish the task of developing a bacterin/vaccine.

## MATERIAL AND METHODS

For this assay, turbot, weighting 100±5.0 g, were supplied by Aquacria (Torreira, Portugal). Fish were kept in the same conditions, as mentioned before (Chapter 2, Material and Methods). All fish were starved 24 h prior to experimental infection.

The two strains of *M. marinum* (MM-PT and MM-SP) were thawed from a frozen stock (-70º C) and inoculated onto 7H10. Cultures were grown for one week at 30º C and ressuspended into 7H9. The bacterial density was measured by spectrophotometry at 520 nm and dilutions (in 7H9) were made until $10^7$ CFUs/mL were achieved (corresponding to approximately 200 µg of protein/mL). Actual CFUs counts, used as experimental infection dose were confirmed, by viable counts of dilutions plated on 7H9 plates and incubated during one week at 30º C.

Turbot were divided into four groups of 60 fish each. After anaesthetizing turbot (0.03% (v/v)), groups were i.p. injected (100 µL) like the scheme below:

Group I – Non-injected group
Group II - Control; injected with PBS
Group III – injected with MM-PT
Group IV – injected with MM-SP

Groups III and IV were infected and put into two different tanks in an "isolated infection room"[1].

Mortalities were monitored during the entire assay (6 months) and dead fish were examined for the reisolation of the inoculated bacterial strain from the head-kidney, by streaking it directly onto 7H9. Shape of colonies and Ziehl-Nielsen stain (Merck) were used for presumptive identification.

Each turbot collected (8 fish per group) was bled from the caudal vein. The blood was allowed to clot for 15 min, centrifuged (2100xG for 10 min) and the serum collected. Serum was stored at 4º C for one day before use. In some cases, serum was heat-inactivated at 50º C for 30 min.

Then, their peritoneal leucocytes (8 fish per group) were collected at 12, 24, 48 hours and 4, 7, 15, 30, 60 and 90 days. Turbot were killed with an overdose of anesthesia (0.06% (v/v)).

Peritoneal exudates were collected using a previously described technique (Chapter 2, Material and Methods) and three sets of cytospins of peritoneal leucocyte suspensions were made using a Cellspin I apparatus. The first set of dry cytospins were fixed with formol-ethanol for one minute and stained with Wright's stain. For peroxidase detection, the Antonow's technique was used (Chapter 2, Material and Methods). The second set of cytospins was stained with Gram-Hucker stain (Deltalab, Barcelona, Spain) and the third were stained with Ziehl-Nielsen technique, using acid Fucsin (Merck) and contrasted with methylene blue.

Differential counts of macrophages, neutrophils and small mono cells were made under x100 oil immersion. Once agains, to overcome weight variation between individuals, leucocyte numbers were converted to leucocytes per gram of body weight.

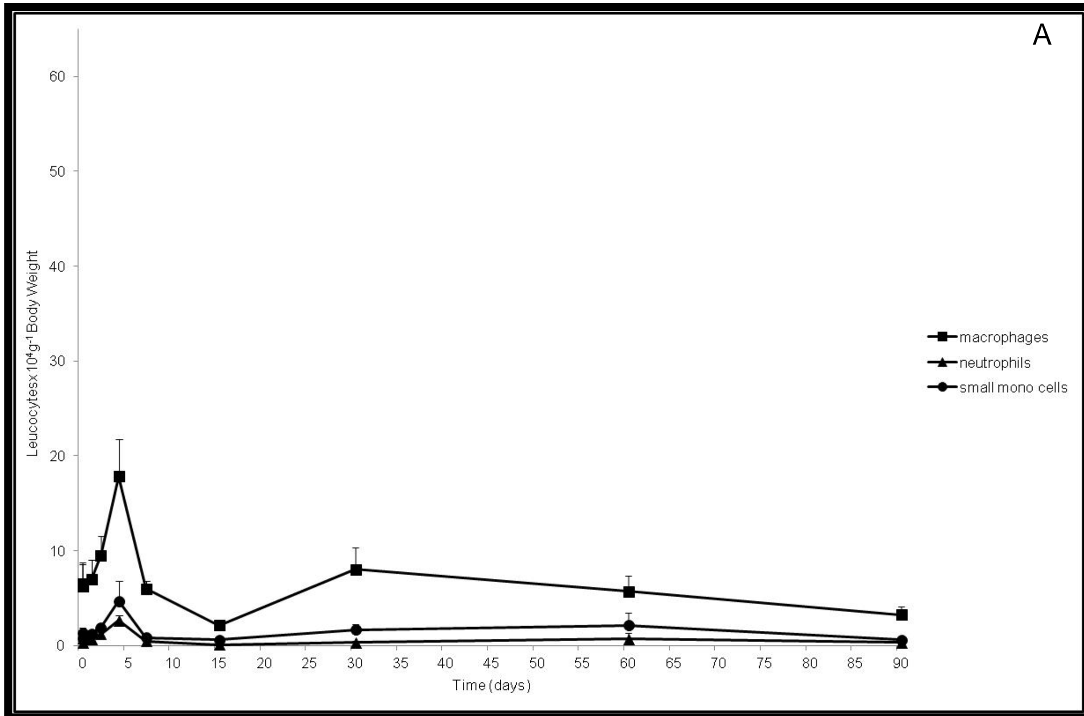
At each collection time, internal organs were observed. Internal organs (kidney, liver, spleen and intestine) and gills, from half of the sample, were collected and fixed in formol 4% for histological analysis. Tissues samples were embedded in paraffin wax and 5 µm sections were cut with a rotary microtome (American Optical, Buffalo, NY). After dewaxing, the sections were stained with haematoxylin/eosin.

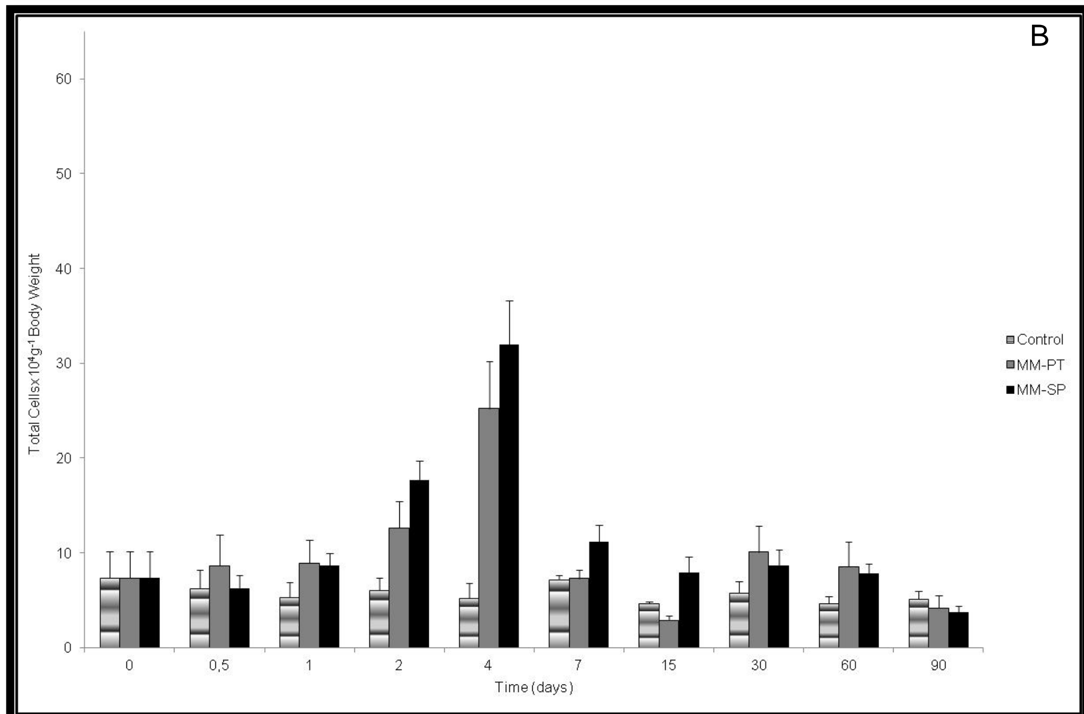
The number of CFU of each strain injected, MM-PT and MM-SP, in spleen and head-kidney of infected turbot, was determined, by serial diluting and plating the tissue homogenates, from 4 fish per sample, onto 7H10 medium. Results are expressed as the growth index, which represents the difference between the $\log_{10}$ mean of CFU at day 90 and the $\log_{10}$ mean of CFU at day 7. The CFU number in the peritoneal cavity was

[1] The "infected room" had a "pediluvium" at the entrance before and after the door, with diluted hypochloride, and before entering, specific boots, white laboratory coat and latex gloves were obligatory.

determined in the same way as with the tissue homogenates, but using the collected lavage fluid.

# RESULTS AND DISCUSSION



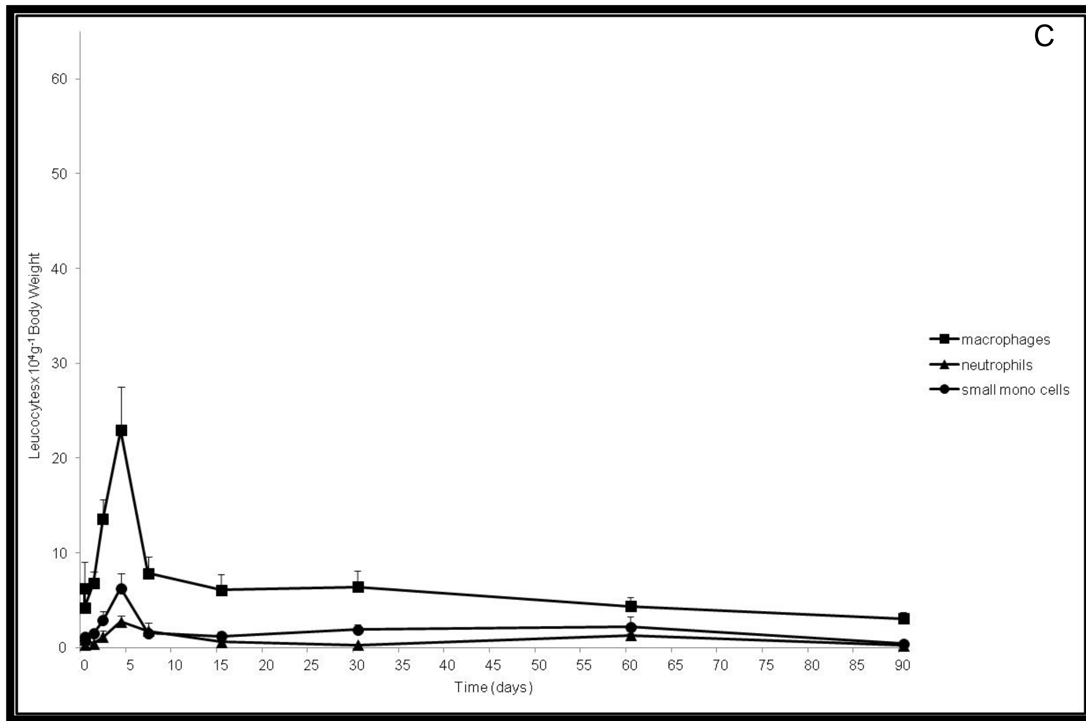



Figure 9. A) Peritoneal leucocytes after intraperitoneal injection of PBS, MM-PT and MM-SP; B) Turbot peritoneal leucocyte response after intraperitoneal infection with MM-PT; C) Turbot peritoneal leucocyte response after intraperitoneal infection with MM-SP.

| | MM-PT Group | | | | | | | | MM-SP Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tissue | 12h | 24h | 48h | 4d | 7d | 15d | 30d | 60d | 12h | 24h | 48h | 4d | 7d | 15d | 30d | 60d |
| Liver | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Head-kidney | - | + | + | + | + | - | - | - | - | + | + | + | + | - | - | - |
| Spleen | + | + | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| Gills | - | - | + | - | - | - | - | - | - | - | + | - | - | - | - | - |
| Heart | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Table 5. Presence (+) or absence (-) of *M. marinum* in several tissues collected during the experiment.

| | MM-PT Group | MM-SP Group |
|---|---|---|
| Exudate | 2.705 | 1.988 |
| Head-kidney | 2.891 | 1.888 |
| Spleen | 1.91 | 1.926 |

Table 6. Growth index in turbot i.p. infected with *M. marinum*.

The two strains of *M. marinum* (MM-PT and MM-SP) were consistent with the results achieved (Figures 9.B and 9.C).

Both groups of *M. marinum*, showed a statistical significant increase in the number of total cells 4 days post-infection ($P<0.05$), when compared with the non-injected and the control groups (Figure 9.A). The rise in the number of macrophages was more extensive as compared to that of neutrophils (Figure 9.B and 9.C). The number of macrophages increased up to 4 days post-infection and then decreased till the end of the experiment. The increase observed 30 days post-infection in the MM-PT group was not statistically significant (Figure 9.B). Sixty days post-infection leucocyte numbers have returned to values observed in the non-injected group (Figures 9.B and 9.C). The number of neutrophils or small mono cells did not show any statistically significant difference, during the 90 trial days (Figures 9.B and 9.C).

Gross examination of turbot infected with *M. marinum* (MM-PT and MM-SP) did not reveal any abnormality, such as bleeding on internal organs, in contrast to the results showed by *in vivo* infection (Decostere *et al*, 2004; Gauthier and Rhodes, 2009). However, histopathological examination showed the existence of bacteria in several tissues.

Briefly, 12 h after i.p. infection, no bacteria were detected in liver, kidney, spleen and gills. However, in MM-PT group we observed bacteria in the spleen that persisted till 24 h post-infection, when it was observed for the first time in turbot infected with MM-SP. Bacteria were observed in the kidney of turbot infected with MM-PT and MM-SP, 24 h post-infection and persisted more 6 days. Moreover, bacteria were only observed in gills 48 h post-infection in both groups: MM-PT and MM-SP (Table 5.).

Till the end of the experimental trial, no bacteria were detected in heart and/or skin.

In the control group, bacteria did not grow, showing that animals were not contaminated and validating our experiment. Till 7 days post-infection there was an increase in the number of CFUs in each tissue and exudates. However, these values began to decrease till 60 days post-infection, indicating that after 7 days post-infection, turbot was able to control the infection, even though without complete elimination of the bacteria during the period studied. These data clearly show that turbot may be capable of releasing the bacteria to the medium, as described by Avendaño-Herrera *et al* (2006), however, attempts to recover them were not successful.

Growth index in turbot i.p. infected with MM-PT was higher than in turbot i.p. infected with MM-SP, showing that MM-PT proliferates more extensively in turbot, than MM-SP (Table 6.).

During the course of infection, the infecting organism (pathogen), uses host's resources in order to multiply, which in turn will interfere with normal functions developed by the host, and may lead to chronic wounds, grangrene, loss of an infected limb or even death (Bassett *et al*, 2003). As observed previously, reaction to phlogistic agents was more severe, because microorganisms were inactivated and could not interfere with host's resistance mechanisms, when compared to an experimental infection.

In this trial, turbot was shown to be sensitive to the experimental infection by *M. marinum*, and recovery of bacteria from organs and peritoneal exudates was described. Moreover, we observed that head-kidney tended to exhibit bacterial aggregations for a longer time period, when compared to spleen and the peritoneal exudate. Even still, typical infection signs were not encountered till 60 days post-infection and that may be related to non-specific immunossuppression in turbot, by mycobacterial infection (Appelberg *et al*, 1989). Avendaño-Herrera *et al* (2006) showed that turbot is capable of releasing bacteria to the water, but we have analysed water and no mycobacteria could be recovered (data not shown). Differences between MM-PT and MM-SP were only encountered when calculating growth index, where we have observed that it was higher in turbot i.p. infected with MM-PT, than in turbot i.p. infected with MM-SP. This showed that MM-PT was capable of proliferating more extensively in turbot, than MM-SP. As described elsewhere (Olsson *et al*, 1998), tests were made to evaluate the persistence of a bacteria inside the host, by infecting its intestines. However, due to etiological and deontological reasons, these trials were done conducted.

During the infection trial, we have calculated the difference between weights and we have obtained a decrease in weight till 4 days post-infection, in groups i.p. infected with *M. marinum*, but not statistically significant (data not shown). However, the decrease was in accordance with Stamm & Brown (2004), who also observed that mycobacterial infection was almost always accompanied by weight loss. We have also noticed that fish tended to separate from each other, becoming solitary individuals in the corner holding facilities. Once again, this behaviour is in accordance with Decostere *et al* (2004).

# CHAPTER 6

# In vitro studies with M. marinum

After the inflammatory and infection trials, we have decided to study the effect of two strains of *M. marinum* on the function and integrity of turbot (*S. maximus*) head-kidney macrophages. During this chapter, we will detect the production of superoxide anion and not only due to the fact that counting the number of engulfed particles under a microscope, to evaluate phagocytosis, is tedious, laborious and time consuming, but also because mycobacteria have a high capacity to aggregate, we decided to develop a flow cytometric assay to quantify phagocytosis.

## MATERIAL AND METHODS

Turbot weighting 200±20 g, were purchased from a commercial fish farm located in the North of Portugal. Fish were kept in the same conditions as mentioned before (Chapter 2 – Material and Methods). All fish were maintained for at least 15 days and starved 24h prior to cell collection. Turbot were bled from the caudal vein. The blood was allowed to clot for 15 min, centrifuged (2100xG for 10 min) and the serum collected. Serum was stored at 4º C for one day before use. In some cases, serum was heat-inactivated at 50º C for 30 min.

Two virulent strains of *M. marinum* (MM-PT and MM-SP) were thawed from a frozen stock (Chapter 4, Material and Methods).

Turbot were killed by an overdose of anesthesia (0.06% (v/v)). Then, the head-kidney was aseptically dissected, as previously described (Chung and Secombes, 1988) and pushed through a 100 µm nylon mesh with L-15 medium containing 2% Fetal Calf Serum (FCS, Gibco), 100 u/mL Penicillin/Streptomycin (P/S, Gibco) and 20 u/mL heparin (Sigma). The resultant suspension was layered onto a 30-45% discontinuous Percoll gradient previously set up. The gradient was then centrifuged at 1000xG for 40 min at 4º C, and the leucocytes were recovered at the 30-45% Percoll interface. The macrophage enriched isolated cells (THKM) were counted in a haemocytometer, adjusted to $10^7$ cells/mL in L-15, 5% FCS, P/S, heparin and plated in 96 well plates (100 µL/well). Viability

of cells monitored by means of trypan blue exclusion assay was above 92%, in all preparations. After overnight at 20º C, non-adherent cells were discarded by carefully washing the cultures twice with phenol red-free Hank's Balanced Salt Solution (PRF-HBSS, Gibco).

## DETECTION OF EXTRACELLULAR SUPEROXIDE ANION

The method based on the reduction of ferrocytochrome C was used for the detection of extracellular superoxide anion (Secombes, 1990). Monolayers of THKM were washed twice with PRF-HBSS and 100 µL of live or inactivated MM-PT and MM-SP in ferrocytochrome C solution (2 mg ferrocytochrome C $mL^{-1}$ PRF-HBSS) were added. Serial dilutions ranged from 3.1 x $10^7$ to 10 x $10^8$ bacteria $mL^{-1}$. Ferrocytochrome C solution containing 1 µg $mL^{-1}$ phorbol myristate acetate (PMA, Sigma) was added to the positive control, as a soluble stimulant of the respiratory burst. Wells with THKM and ferrocytochrome C were used as negative control, for spontaneous and background production of superoxide anion. Ferrocytochrome C with bacteria or PMA and 300 units of superoxide dismutase $mL^{-1}$ (SOD, Sigma) was used to confirm the specificity of the reaction. Bacteria previously opsonised with 1:500 diluted serum for 30 min were used in some samples.

For each sample, 4 wells of macrophages per fish were assayed. Plates were read immediately after addition of the bacteria and/or reagents to the THKM monolayers and reading were then taken every 60 s for 60 min, on a multiscan spectrophotometer (BioTek, Power Wave 440) at 550 nm. Data were expressed at the $V_{max}$ rate of the response in mOD $min^{-1}$ (optical density, OD). Data using opsonised bacteria were expressed as an index (opsonisation index) relative to results using unopsonised bacreria and allowed pooling of data.

## DETECTION OF INTRACELLULAR SUPEROXIDE ANION

For the detection of intracellular superoxide anion, the method of the reduction of the redox dye nitroblue tetrazolium (NBT) was used. Briefly, NBT was dissolved at 1 $mg.mL^{-1}$ in PRF-HBSS, and PMA added at 1 $µg.mL^{-1}$. SOD was also added to the same solution, as a negative control. THKM were washed twice with PRF-HBSS, 100 µL of live or inactivated MM-PT and MM-SP in a NBT solution, containing 2.5 x $10^8$ $bacteria.mL^{-1}$ were added. Microplates were incubated for 60 min. Medium was then removed and cells fixed in 70% methanol and allowed to air dry. The formazan in each well was dissolved in 120 µL 2M potassium hydroxide (KHO) and 140 µL dimethyl sulfoxide (DMSO, Fluka),

with mixing. The turquoise-blue coloured solution was then read in BioTek at 630 nm using KHO/DMSO as a blank. The OD of control macrophages incubated with NBT only was subtracted from the OD of well with macrophages, bacteria and NBT.

FLOW CYTOMETRIC ASSAY

The phagocytic capacity of fish leucocytes has been studied, each phagocytic assay being carried out in triplicate.

Samples of 100 µL of THKM suspensions were placed in 5 mL tubes. Then 10 µL of FITC-labelled bacteria were added to each tube and the samples centrifuged at 400xG for 5 min. Afterwards, the samples were ressuspended in PBS and incubated at 25º C for different times ranging from 0 to 120 min. At the end of each incubation time, the samples were placed on ice to stop phagocytosis and 500 µL of ice-cold PBS was added to each sample. The fluorescence of bacteria that adhere to phagocytes but were not ingested was quenched by adding 125 $\mu g.mL^{-1}$ of trypan blue. To exclude cell debris and non phagocytised bacteria, propidium iodide (PI) was added to a final concentration of 10 $\mu g.mL^{-1}$.

The samples were analysed in a FACScan flow cytometer with an argo-ion laser turned to 488 nm. The instrument setting was adjusted to obtain an optimal discrimination of the different cell populations present in THKM. Data were collected in the form of two parameter dot plots and histograms of side scatter (granularity), forward scatter (size), green fluorescence (FL1; FITC) and red fluorescence (FL2; PI) on a computer system. Each of the analysis was performed on 10000 cells which were acquired at the rate of 300 $cells.s^{-1}$. The quantitative study was made using a statistical option of the Lysis Software Package. FITC-labelled bacteria, bacteria and fish leucocytes were used as standard samples.

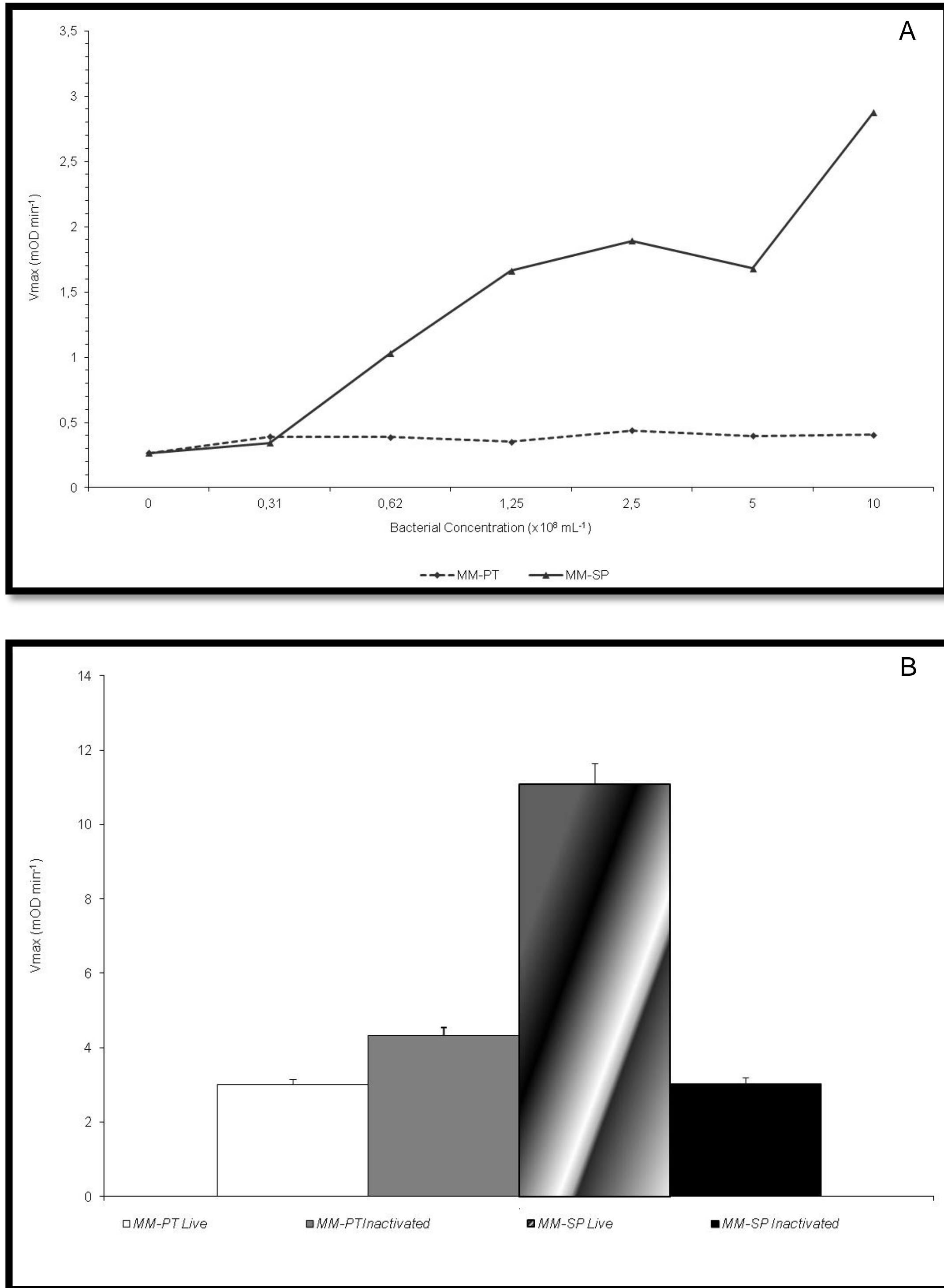



Figure 10. A) Superoxide anion production from unopsonised THKM after stimulation with different concentrations of live *M. marinum* (MM-PT and MM-SP). Data are presented as

$V_{max}$ rates of ferrocytochrome C reduction ($V_{max}$ for PMA was 5.66 $mOD.min^{-1}$); B) Superoxide anion production from THKM, after stimulation with live or inactivated *M. marinum* (MM-PT and MM-SP). Data are presented as $V_{max}$ of ferrocytochrome C reduction, expressed as the means ± S.E.M. of 3 fish.

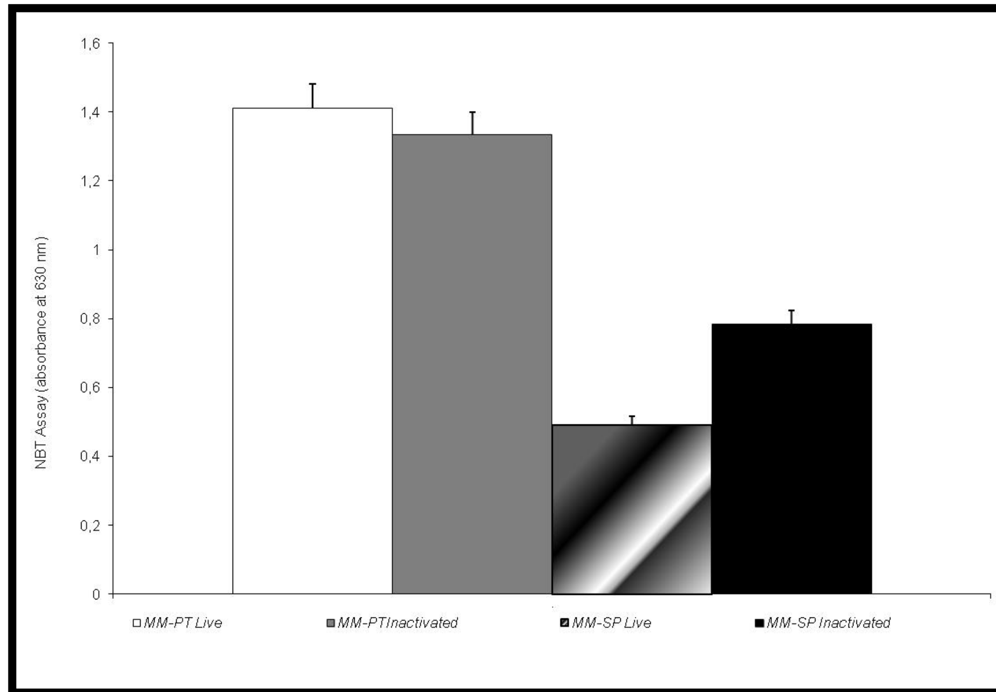



Figure 11. NBT reduction from THKM, after stimulation with live or inactivated *M. marinum* (MM-PT and MM-SP). Data are presented as the means ± S.E.M. of 3 fish (OD for PMA stimulated macrophages was 0.66).

In both assays, SOD and PMA were used as negative and positive controls, respectively, and validated our experiment.

Using a concentration of $10^9$ bacteria, the effect of challenging turbot head-kidney macrophages with MM-PT and MM-SP, elicited a respiratory burst (Figure 10.), being that in accordance with Stave *et al* (1984), Bandín *et al* (1995) and Chaves-Pozo *et al* (2005). MM-SP elicited a higher response, when compared to MM-PT ($P<0.05$).

In the detection of intracellular superoxide anion (Figure 11.), we observed that there was a higher increase in its production in THKM stimulated with MM-PT, when compared to the response elicited by THKM stimulated with MM-SP ($P<0.05$), being that in accordance to Salinas *et al* (2006).

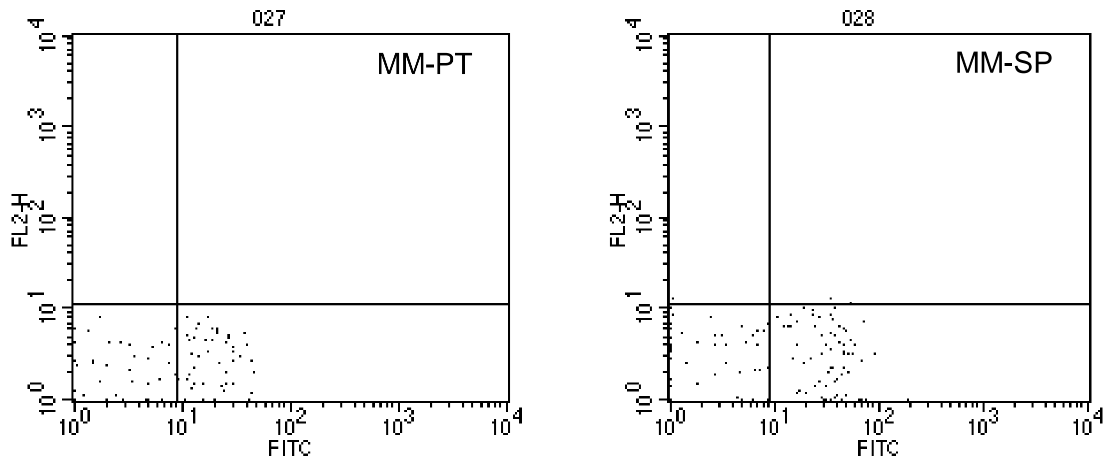



Figure 12. Trypan blue treated THKM phagocytosing FITC-labelled MM-PT and MM-SP analysed by fluorescence flow cytometry. Dot plot of the distribution og green (FL1-FITC) and red fluorescence (FL2 - PI). Two distinct cell subsets are identified, which coincide with head-kidney leucocytes/bacteria interactions: quadrant A, no interaction; quadrant B, ingestion.

| | | | | | Incubation time (minutes) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacteria | FITC | TB | SB+I | I | 0 | 10 | 30 | 60 | 90 | 120 |
| MM-PT | X | | X | | 39.1% | 40% | 54.3% | 53.9% | 49.1% | 56.2% |
| MM-PT | X | X | | X | 23.6% | 37.9% | 51.4% | 39.8% | 47.4% | 54.7% |
| MM-SP | X | | X | | 66.8% | 63.6% | 63.5% | 67.7% | 74.5% | 65.6% |
| MM-SP | X | X | | X | 47.2% | 60.6% | 60.6% | 63.4% | 56.3% | 60.7% |

Table 7. Percentage of attached plus ingested and ingested *M. marinum* (MM-PT and MM-SP) particles per phagocytosing THKM at different incubation times (samples were prepared and analysed as described; the results are the means ± S.E.M. of three experiments (TB- Trypan blue; SB – Surface bound; I – Ingested).

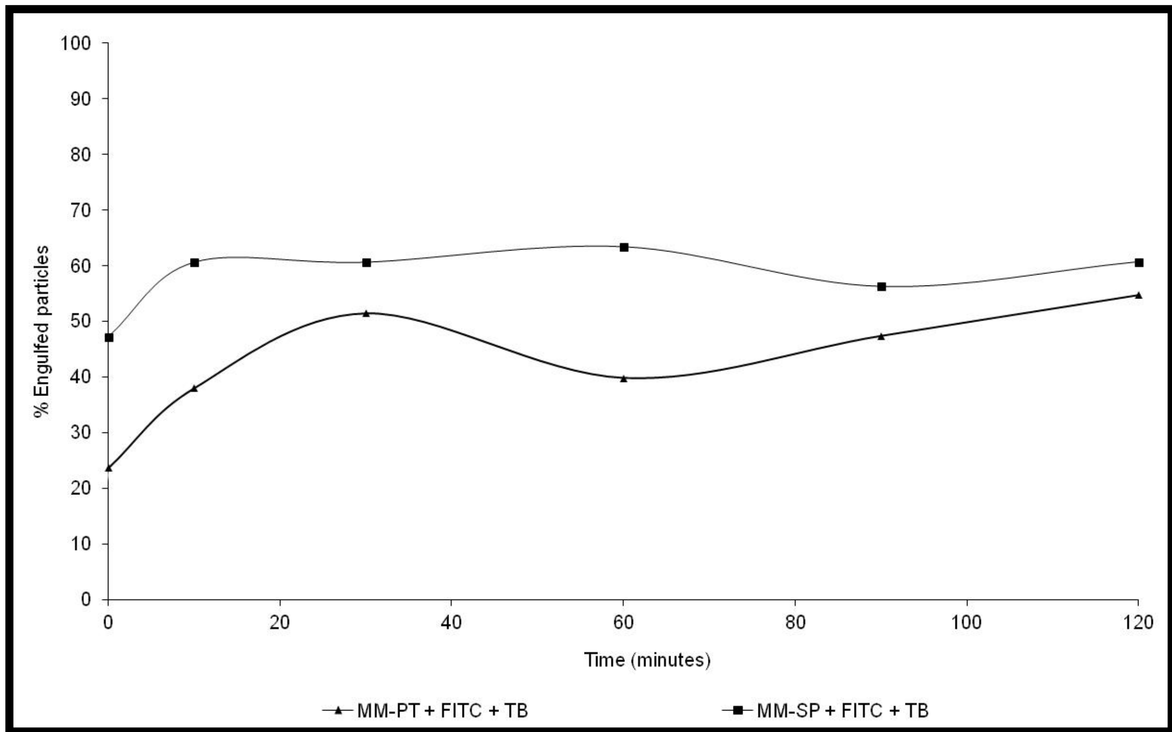



Figure 13. Percentage of phagocytosis of FITC-labelled *M. marinum* (MM-PT and MM-SP), by THKM, using trypan blue exclusion in a flow cytometric assay.

We have "observed" THKM phagocytosing the two strains of *M. marinum* (MM-PT and MM-SP), as detected by FL1 (green fluorescence) and FL3 (red fluorescence) photomultipliers. Two different cell populations were identified: quadrant A, encloses non-fluorescent events, corresponding to head-kidney macrophages without any associated particles and quadrant B, corresponds to green fluorescent events, which are head-kidney macrophages bearing only ingested bacteria (Figure 12.).

For the distinction between internalised and surface bound FITC-labelled particles, trypan blue was used for quenching surface-bound fluorescence. Table 7 clearly shows that the percentage of phagocytosed bacteria decreases when using trypan blue, meaning that we are counting the ingested ones and not the attached plus the ingested microorganisms. Our results are in accordance to Nuutilla & Lilius (2005). Moreover MM-PT exhibits a different pattern, once again, of phagocytosis, when compared to MM-SP (Figure 13.).

The response of turbot to a mycobacterial infection was observed and we have also conducted studies to observe the influence of the two strains of *M. marinum* (MM-PT and MM-SP) in the homeostasis of turbot head-kidney macrophages. Results presented show that early contact of macrophages with MM-PT or MM-SP induced respiratory burst release of reactive oxygen intermediates, including superoxide anion. Moreover, there was also a more intense response elicited by live microorganisms, than inactivated ones, observed when turbot head-kidney macrophages were stimulated with MM-SP. This is in accordance to studies developed in other fish species (Stave *et al*, 1984; Bandín *et al*, 1995; Chaves-Pozo *et al*, 2005). However, when stimulating with MM-PT, inactivated bacteria elicited higher superoxide anion production by macrophages when compared to the response of the live pathogen, in accordance to Salinas *et al* (2006). These results were further confirmed when intracellular superoxide anion was performed to both MM-PT and MM-SP. It is evident, by results, that MM-PT is capable of inhibiting and MM-SP of stimulating the release of superoxide anion to the extracellular space.

It seems feasible to suggest that the Portuguese strain of *M. marinum* does not inhibit the respiratory burst, and is capable to stimulate bacterial enzymes, such as superoxide dismutase, thus augmenting the production of hydrogen peroxide.

Intracellular pathogens, as *M. marinum*, are considered virulent due to their ability of resist host microbicidal mechanisms (Gupta, 2012), however, during the course of my thesis, some inconsistencies were found. These continued even in the cytometric analysis. Once again, MM-PT exhibited a different pattern of phagocytosis, when compared to MM-SP.

# CHAPTER 7

# In vitro studies with S. parauberis

Results achieved during the course of this Thesis, showed that the two strains of *M. marinum*, behave in different ways. As intracellular bacteria, but isolated from different fish farms, we have decided to compare them with an extracellular bacteria, *S. parauberis*. This Gram-positive bacterium is also the etiological agent of severe disease in turbot, as mentioned before.

Studies of the respiratory burst and the release of nitrogen intermediates from THKM after bacterial stimulation were conducted.

## MATERIAL AND METHODS

Turbot weighting 200±20 g, were purchased from a commercial fish farm located in the North of Portugal. Fish were kept in the same conditions as mentioned before (Chapter 2 – Material and Methods). All fish were maintained for at least 15 days and starved 24h prior to cell collection.

*M. marinum* strains (MM-PT and MM-SP) were thawed from frozen stocks (Chapter 4 and 5, Material and Methods), inactivated and live, respectively.

A *S. parauberis* strain, was thawed from a frozen stock (Chapter 3, Material and Methods) and inoculated onto TSA1. Cultures were grown during 48 h at 22º C and ressuspended into TSB1. The bacterial density was measured by spectrophotometry at 550 nm and dilutions, in TSB1, were made until $10^7$ $CFUs.mL^{-1}$ were achieved. Then, one part of the culture was inactivated, as previously described (Chapter 4, Material and Methods).

Turbot were killed with an overdose of anesthesia (0.06% (v/v)) and head kidney was aseptically removed and processed as previously described (Chapter 6.1, Material and Methods).

Detection of extracellular and intracellular superoxide anion was conducted as described in Chapter 6.1 (Material and Methods).

Nitrite concentration in the medium was measured by a microtitre assay method described elsewhere (Ding *et al*, 1988). Briefly, 50 μL of each bacterial sample live and inactivated ($10^9$ bacteria.mL$^{-1}$) were added to monolayers with THKM and incubated with equal volume of Griess reagent (1% sulphanilamide; 0.1% naphthylethylene diamine dihydrochloride; 2.5% phosphoric acid; Sigma) at room temperature for 30 min. The absorbance at 550 nm was determined using BioTek. Nitrite concentration was determined by using sodium nitrite as a standard.

# RESULTS AND DISCUSSION

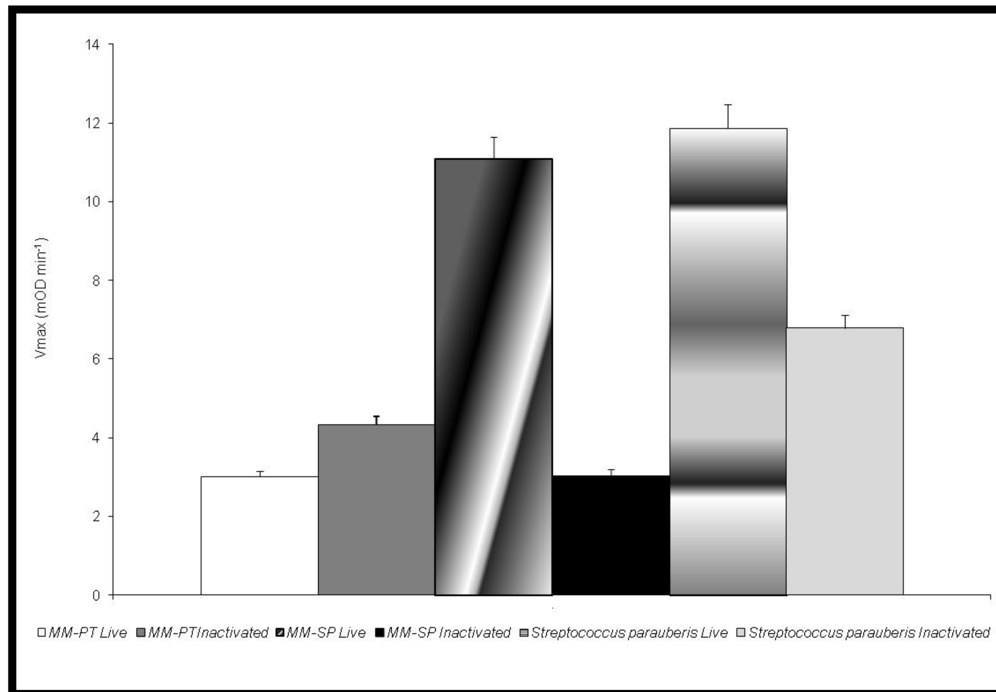



Figure 14. Superoxide anion production from unopsonised THKM after stimulation with different concentrations of live and inactivated *M. marinum* (MM-PT and MM-SP) and *S. parauberis*. Data are presented as $V_{max}$ rates of ferrocytochrome C reduction, expressed as the means ± S.E.M. of 3 fish ($V_{max}$ for PMA was 5,66 mOD.min$^{-1}$).

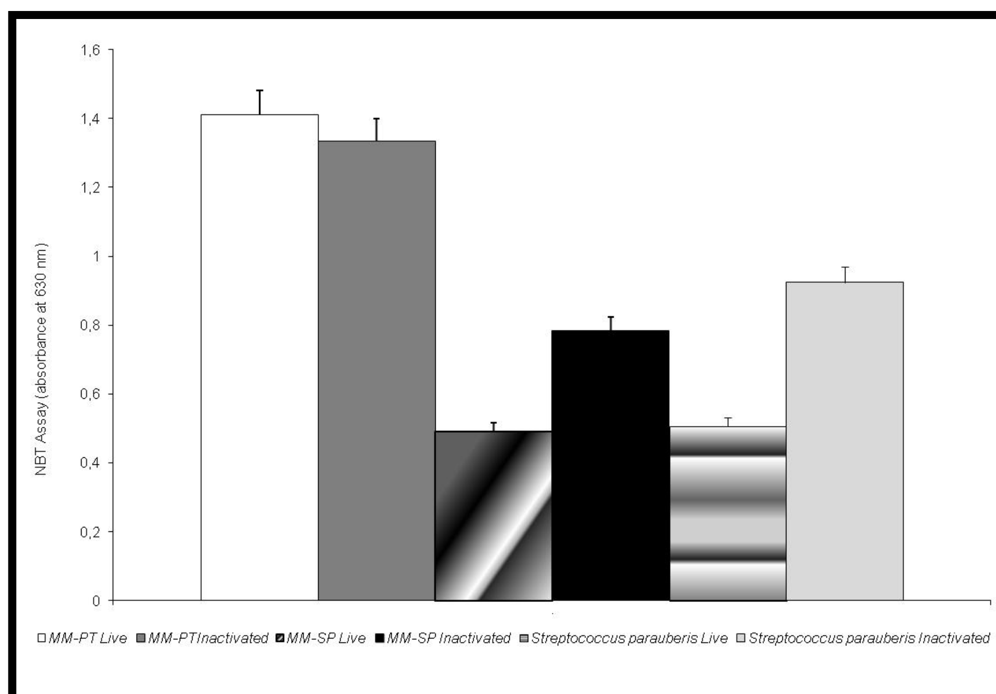


Figure 15. NBT reduction from THKM, after stimulation with live or inactivated *M. marinum* (MM-PT and MM-SP) and *S. parauberis*. Data are presented as the means ± S.E.M. of 3 fish (OD for PMA stimulated macrophages was 0.66).

| | Bacteria | Nitrite concentration (moldm$^{-3}$) |
|---|---|---|
| *M. marinum* | Live MM-PT | $1.72 \times 10^{-4}$ |
| | Inactivated MM-PT | $3.76 \times 10^{-2}$ |
| | Live MM-SP | $3.55 \times 10^{-4}$ |
| | Inactivated MM-SP | $3.77 \times 10^{-4}$ |
| *S. parauberis* | Live | $5.76 \times 10^{-4}$ |
| | Inactivated | $5.81 \times 10^{-4}$ |

Table 8. Results concerning nitrite concentration in MM-PT, MM-SP and *S. parauberis.*

After observing the date presented, we can say that MM-PT, when inactivated elicited a higher, although not statistically significant response ($P<0.05$) than live bacteria. In contrast, live MM-SP exhibit a similar behaviour of that observed for *S. parauberis*, eliciting a statistically significant higher response ($P<0.05$), when compared to its inactivated form (Figure 14.).

The results concerning the detection of intracellular superoxide anion, showed that, there was an increase in its production in THKM stimulated with live MM-PT, in comparison with the inactivated one, although not being statistically significant ($P>0.05$). In contrast, MM-SP behaved like *S. parauberis* (Figure 15.).

Once again, when looking at the results concerning nitrite concentration, MM-SP behaved in a different pattern, when compared to MM-PT (Table 8.).

Release of reactive oxygen intermediates from phagocytes in response to bacterial pathogens is well known in fish, and it is generally accepted that live microorganisms may elicit a more intense response than inactivated ones (Stave *et al*, 1984). In our study, this was observed when turbot macrophages were stimulated with *S. parauberis*. However, when stimulating with *M. marinum* (MM-PT strain), it was observed that inactivated bacteria elicited higher superoxide anion production by macrophages when compared to the response of the live pathogen. These results were further confirmed when detection of intracellular superoxide anion was performed to both pathogenic bacteria. The phenomenon evidenced by *M. marinum* has also been noticed in mammalian studies using the intracellular pathogen *M. lepraemurium* (Brett and Butler, 1988; Mor *et al*, 1988;

Ellis, 1999). It is evident that *M. marinum* is capable of inhibiting and *S. parauberis* of stimulating the release of superoxide anion to the extracellular space. Results achieved in the detection of intracellular superoxide anion, suggest that *M. marinum* does not inhibit the respiratory burst. The response was greater to *M. marinum* than to *S. parauberis*. Once could say that it is capable of stimulating bacterial enzymes such as superoxide dismutase, thus augmenting the production of hydrogen peroxide. Bacteria which survive within macrophages may be capable of stimulating the burst by binding to the macrophage through its hydrophobic surface and enter the cell at a later stage when the burst is exhausted, as shown for *Renibacterium salmoninarum* (Bandín *et al*, 1993; Bandín *et al*, 1995, Ellis, 1999), but this does not seem to be the case for *M. marinum*.

# CHAPTER 8
# Conclusions

Studies concerning host-pathogen interactions are vast and the best way to accomplish them is subjective. During the development of this thesis, we have tried to maintain a connection line, and sometimes that was not very easy. The epizootic outbreak of fish tuberculosis, observed and witnessed in a Portuguese farm, was the motor for the development of these studies. Fish, as other animals, are difficult to maintain in sterile environments, and external influences are always interfering with experimental results. Hence we did lots of experiments and some of them were only important for validation of previous results.

During the last decades, we have observed great improvements in fish immunopathology field. However, we have considered that, fish mycobacteriosis/tuberculosis studies lacked some of the initial information regarding turbot, the host, and *M. marinum*, the pathogen. Moreover, the initial processes of infection are very important in studies of host-pathogen interactions. Not only they are capable of determining the establishment of a disease, but also its eradication, because successful pathogenic bacterium need to multiply at a degree, so as to subsist in the host or be transmitted effectively.

Results achieved during this thesis led to the following conclusions:

- Turbot peritoneal cavity, in resting conditions is composed by two professional phagocyte populations: macrophages, the predominant leucocyte (53.20%) and neutrophils, the less abundant one (6%).
- Turbot leucocytes are similar to those from other fish species and mammals. Macrophages are round in shape, their cytoplasm is basophilic and nucleus is eccentric and sometimes horse-shoe shaped. Ultrastructurally, there have pseudopodia and are peroxidase negative and esterase positive. Neutrophil is large, round and contains granules in its cytoplasm. Neutrophil’s nucleus is small, rod-shaped and sometimes even segmented. Cytochemically, neutrophils are peroxidase positive and esterase negative. The small mono cell is mainly composed by small round cells, with a large nucleus, occupying almost all the cytoplasm.

- We can identify the different types of peritoneal leucocytes using cytochemical methods such as for the presence of peroxidase or esterase, because they are a distinctive feature between macrophages and neutrophils.
- The two strains of *M. marinum* which were isolated from two mycobacteriosis outbreaks: one in Portugal (MM-PT; dos Santos *et al*, 2002) and the other in Spain (MM-SP, not in publication) were studied. An extracellular bacterium, also isolated from an epizootic outbreak in Galicia (Spain), was used as control: *S. parauberis* (Toranzo *et al*, 1994). *S. parauberis* was confirmed to be Gram-positive bacteria and a fast-grower and *M. marinum* strains, confirmed to be slow growers.
- In the determination of bacterial growth curves, MM-SP appeared to be slower grower than MM-PT.
- Turbot is not capable of developing an early neutrophilic response, during an inflammatory status, in the group i.p. injected with inactivated MM-SP.
- Turbot's macrophages appeared to be the most sensitive cell type in presence of phlogistic agents, making them good indicators of inflammation. Inactivated *M. marinum* acted as transient inflammatory stimulants and the adjuvant, which is hardly degraded by fish, acted as an inflammatory stimulant, as it was capable of inducing and perpetuating the inflammatory reaction.
- In turbot, macrophages are the population responsible for the indication of the presence of an inflammatory status.
- When turbot is experimentally infected with *M. marinum*, they tend to separate from each other, becoming solitary individuals in the corner holding facilities.
- Turbot is sensitive to the experimental infection by *M. marinum*, and recovery of bacteria from organs and peritoneal exudates was accomplished. Turbot head-kidney tends to exhibit bacterial aggregations for a longer time period, when compared to spleen and the peritoneal exudate. However, typical infection signs were not encountered till 60 days post-infection and that may be related to non-specific immunossuppression in turbot, by mycobacterial infection.
- MM-PT is capable of proliferating more extensively in turbot, than MM-SP.
- Early contact of macrophages with MM-PT or MM-SP induces respiratory burst release of reactive oxygen intermediates, including superoxide anion. There is a more intense response elicited by live microorganisms, than inactivated ones. However, when stimulating with MM-PT, inactivated bacteria elicited higher superoxide anion production by macrophages when compared to the response of the live pathogen. MM-PT is capable of inhibiting and MM-SP of stimulating the release of superoxide anion to the extracellular space.

- In the cytometric analysis MM-PT exhibits a different pattern of phagocytosis, when compared to MM-SP.
- MM-PT has a similar behaviour to any mycobacterial species, however, MM-SP seems to behave as an extracellular species, like *S. parauberis.*

We can suggest that geographic coherence, coupled with environmental influences, is able to generate patterns in *M. marinum* capable of inducing differences in their mode of action. Moreover, the Portuguese virulent *M. marinum* strain may be indicated as a possible vaccine component, because is capable of inducing a significant leucocytic response in turbot peritoneal population. Without being too speculative, and due to the existence of a close relationship between *M. marinum* and *M. tuberculosis*, works should be conducted so as to determine a possible role of *M. marinum* as a candidate in the fight against Human Tuberculosis...

# CHAPTER 9

# Future Perspectives

Works conducted in this thesis have opened a new window for future investigations. The two different behaviours witnessed between the spanish and the Portuguese strains of *M. marinum*, have led to the possibility of an environmental influence among this bacterial species. Such a difference may be considered in the area of immunopathology and also in the wide area of the vaccines industries.

Is the environment or the geographic origin, important to take into account when studying a specific bacterial species?

May that be considered a possible reason why some vaccines do not act as they should?

Lots of experimental works can be developed and future is and cannot be a barrier to conquer any innovation in this area...tuberculosis is a bacterial disease and needs to be controlled...fish, in particular, turbot, may be a good tool and the beginning of that battle...

# CHAPTER 10

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# CHAPTER 11

# Attachments

Turbot is affected by streptoccosis during all the production cycle. However, as it has been said, there is already an available vaccine to prevent this disease. At the beginning of this Thesis, we thought it would be important to make a review concerning some important cases etiological diseases, capable of affecting turbot, like lactococcosis. Moreover, and knowing that weight and survival are extremely important when we are dealing with an enterprise, like an aquaculture, the effect of L-carnitine supplemented in turbot's diet was also evaluated.

ARTIGO DE REVISÃO

REVISTA PORTUGUESA DE CIENCIAS VETERINÁRIAS

# Fish vaccination against infections by Streptococcal species and the particular case of Lactococcosis

# Vacinação em peixes contra infecções por espécies de Streptococcus e o caso particular da Lactococcose

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**Summary:** Lactococcus garvieae, a species of the Streptococose-ceae family, is the etiological agent of lactococose. Lactococose has become a worldwide disseminated infection reported in a large number of fish specimens, including salmonids. The increased seriousness of the outbreaks after a first contact is well recognized, being the pathology accompanied by intraocular haemorrhage, anorexia, acute meningitis as well as "oculo-splanchnic dissociation". Treatment is in most cases unsatisfactory and the overall recommendation is to destroy the diseased stock. Immunoprophylaxis seems to be a promising field in the prevention of lactococcosis, especially in freshwater fish, which are the ones experiencing the highest water temperatures, during summer months. Moreover, vaccination has been proved to be a highly effective method controlling diseases in salmonid industry, mainly located in Europe, America and Japan. Although there has been a development of studies on vaccination against lactococcosis, its future still seems to be uncertain, because major efforts need to be done to achieve an effective and long prophylactic method against this disease.

**Keywords:** Streptococcosis, Lactococcosis, Lactococcus garvieae

**Resumo:** O Lactococcus garvieae, pertencente à família Estreptococose, é o agente etiológico da lactococose. A lactococose é uma infecção que se tem vindo a disseminar por inúmeras regiões do Globo, afectando várias espécies de peixes, incluindo salmonídeos. Sendo reconhecido o aumento da gravidade dos surtos após um primeiro contacto, a patologia é normalmente evidenciada pelo aparecimento de hemorragias intra-oculares, anorexia, meningite aguda, bem como por "dissociação óculo-esplâncnica". Os tratamentos terapêuticos não são na sua maioria satisfactórios, e a recomendação aquando de um surto baseia-se na destruição do stock que evidencia a doença. Assim, a imunoprofilaxia parece ser o campo de escolha na prevenção da lactococcose, especialmente nos peixes de água-doce que vivem em ambientes onde se observam temperaturas mais elevadas, durante os meses de Verão. A vacinação tem provado ser um método eficaz na protecção contra doenças em salmoniculturas, na sua maioria localizadas na Europa, América e Japão. Apesar de já terem sido realizados inúmeros estudos na área da vacinação contra a lactococcose, o seu futuro é ainda incerto, havendo ainda um longo caminho a percorrer até que se encontre um método profiláctico eficaz e duradoiro.

**Palavras-chave:** Estreptococose, Lactococose, Lactococcus garvieae

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## Introduction

Fish are a heterogeneous group of animals comprising more than 21 700 species (Fänge, 1994). The presence of a circulatory system filled with blood containing red and white cells (erythrocytes and leukocytes), is a feature shared by all vertebrates and probably inherited from extinct Pre-Cambrian proto-vertebrates (Fänge, 1994).

In fish as in mammals, immune cells and lympho-haematopoietic tissues and organs are responsible for the defence mechanisms in the host against infection. One example is the appearance of an inflammatory status. This results in specific morphological and chemical changes in tissues and cells (Suzuki and Iida, 1992).

An efficient clearance and degradation system may be worthwhile during pathogen invasion, which otherwise would lead to severe inflammation and eventually death (Dalmo et al., 1997). This can be termed "innate immunity" or "non-specific defence mechanisms", which are activated immediately after infection and rapidly control the replication of the infecting pathogen (Medzhitov and Janeway, 2000). On the other hand, there is a more complex system involved in the immune response: "adaptive immunity" or "specific defence mechanism". In the adaptive immune system, the T-cell receptor and the B-cell receptor are generated somatically, during the development of T and B cells, in a way that endows each lymphocyte with a structurally unique receptor (Medzhitov and Janeway, 2000). Moreover, the random generation of a

highly diverse repertoire of antigen receptors allows the adaptive immune system to recognize virtually any antigen (Medzhitov and Janeway, 2000).

**Prophylactic methods against fish diseases**

During the last decades, there has been a continuous growth of aquaculture industry all over the world (Hastein, 1995). The most significant growth has been seen in carp production as well as in the production of other cyprinids and even more so for carnivorous fish species such as salmonids, which are farmed in intensive conditions (Gudding et al., 1999). However, any intensive bioproduction – whether on land or at sea – will experience disease problems (Hastein, 1995). Infectious diseases that occur as sporadic events in wild fish populations may cause high mortalitities when appearing in intensive fish farming (Gudding et al., 1999). For the aquaculture industry to prosper in the future it is a prerequisite that the losses caused by diseases and the use of antibiotics are kept to a minimum. Immunoprophylaxis recognized by stimulation of non-specific and specific immunity is consequently the method adopted for developing aquaculture into a sustainable bioproduction in the aquatic ecosystems.

Fish can be immunized in three ways: by injection preferably intraperitoneally, by immersion, usually by dipping the fish in a diluted vaccine solution, or by oral administration along with food. These methods have different advantages and disadvantages with respect to the level of protection, side effects, practicability and cost-efficiency. Injection and immersion are the two major methods that have been developed for use at an industrial scale and in commercial production of salmonids and both vaccination procedures are established practices and integrated as part of the production routines.

There is still a limited understanding of the mechanisms involved in antigen uptake and presentation after immersion and oral vaccination (Nakanishi and Ototake, 1997; Quentel and Vigneulle, 1997; Moore et al., 1998). Even so, the first encounter with pathogens occurs through the mucosal surfaces and the indisputable presence of a mucosal associated immune system makes antigen delivery through the mucosal surfaces a viable approach. The mucosal immunity of fish is still a poorly explored field of research, although indications are available for the existence of this type of immunity (Rombout and Joosten, 1992). Especially for fish living in a pathogen--rich aquatic environment, the existence of a mucosal immune system may be very important. In fact, compared with higher vertebrates fish have their skin as an extra mucosal barrier. However, an important criterion for the existence of a mucosal immune system is the secretion of antigen-specific antibodies at mucosal surfaces (Rombout and Joosten, 1992).

Oral vaccination research has so far focused on protecting the antigen from digestion and decomposition during passage through the stomach and anterior part of the gut. The ultimate goal has been to stimulate the immunocompetent tissues in the posterior part of the intestine. Different approaches have been tried, one of the latest using antigens encapsulation in alginate and liposome microcapsules (Joosten et al., 1997).

The optimal way of developing an effective vaccine entails the identification of the pathogens' key virulence factors. Further, the induction of the immune response must be optimized so that the vaccinated animal develops a protective immunity against the pathogen (Gudding et al., 1999).

## Vaccines and adjuvants

**Inactivated vaccines (bacterins)**

A common approach in vaccine production is to inactivate the pathogen and toxins by physical (e.g. UV and heat) or by chemical (e.g. kill by formol) means so that it is no longer capable of replication in or damaging the host. It is critically important to maintain the structure of epitopes on antigens during inactivation. Administration of these bacterins is done by injection or immersion. Provided that the serotypes used for vaccine preparation cover the field of virulent strains and that the vaccines are used correctly, these vaccines are usually effective and give negligible side effects (Stevenson, 1997; Toranzo et al., 1997). Most vaccines used in aquaculture to date have been inactivated, bacterial vaccines (Gudding et al., 1999).

**Live vaccines**

Live, attenuated vaccines should potentially have many advantages in aquaculture (Benmansour and de Kinkelin, 1997). Vaccination with a live vaccine is in reality an infection (with an attenuated strain), and if vaccinated fish sheds the vaccine strain, an effective dissemination of the antigen in the production would take place over an extended time period. Live vaccines also have the advantage of stimulating the cellular branch of the immune system (Marsden et al., 1996). Finally, attenuated vaccines have some economical advantages in terms of simple delivery and low dose requirements due to multiplication in the fish (Gudding et al., 1999). However, the risk of reversion to virulence is still poorly understood and there seems to be a general view that the risks associated with live vaccines in aquaculture, whether produced by conventional methods or by recombinant techniques should be heavily scrutinized.

**Vaccines based on DNA-technology**

In recent years, several vectors have been used for cost-effective production of sufficient quantities of protective antigens by recombinant DNA technology.

In aquaculture, research on recombinant vaccines has focused on viral vaccines. Genetic immunization using naked DNA is the most recent approach in vaccine design (Babuik et al., 1996). This technology is based on the observations that skeletal muscle cells injected with purified plasmid DNA express plasmidencoded proteins. DNA vaccines have advantages over conventional vaccines. In mammals, the specific immune response after DNA vaccination encompasses antibodies, T-helper cells, as well as cytotoxic cells. However, before DNA vaccines are applied in commercial enterprises in aquaculture, safety for fish, environment and the consumer have to be addressed. As the DNA-sequence encodes only a single viral gene, there should be no possibility of reversion to virulence, which is a critical factor in relation to environmental safety in aquaculture (Gudding et al., 1999).

**Adjuvants in the vaccines formulation**

In most vaccines for animals, adjuvants are a crucial ingredient for efficacy (Gudding et al., 1999). Briefly, these substances will enhance the immune response in order to make antigens more effective by magnifying the immune pathways and also will be helpful increasing the action of small doses of antigens to enhance the immune response to levels that can prevent diseases in fish culture (Anderson, 1997). In salmonids, a single injection of vaccines with aluminium-based adjuvants or glucans induced an acceptable protection against furunculosis, however, the duration was relatively short (Gudding et al., 1999). In this fish group, various oil-adjuvants induced better and more long-lasting protection than aluminium and glucan against furunculosis in challenge and field trials (Midtlyng, 1996; Midtlyng et al., 1996a; Midtlyng et al., 1996b). Oil-adjuvants are now used extensively in commercial injectable bacterins. A double-adjuvant principle combines glucans and oil-adjuvants and thus induces both short- and long-term protection (Gudding et al., 1999). Adjuvants in injectable vaccines generally create local reactions with granulomas at the injection site. The reactions may vary from mild to severe. Oil-adjuvants generally create the most severe reactions (peritoneal granulomas and pigmentation) and on a few occasions they have affected growth rate of the fish as well as the quality of the final product (Poppe and Breck, 1997; Midtlyng and Lillehaug, 1998; Afonso et al., 2004). There is little doubt that immunoprophylaxis may largely reduce the risks for large scale animal suffering, caused by disease epizootics in fish farming. Side effects due to handling and anaesthesia, which are required to vaccinate fish by injection, can be minimized. While the use of adjuvanted vaccines may cause some undesirable side effects, they are nevertheless acceptable from an animal welfare point of view, especially when taking into account the consequences of leaving the fish unprotected against disease. At present, the reduction of vaccine side effects by maintenance of durable immunity constitutes the presently dominant challenge for fish vaccinology (Midtlyng, 1997).

The ability of fish vaccines to induce a protective immunity is for the most part based on experimental challenge studies and/or field experiments. However, the virulence mechanisms for some fish pathogens have been elucidated, and methods for studying the antibody response and even the cellular response to some protective antigens have been described (Gudding et al., 1999). Consequently data have been generated to describe the relationship between specific parameters of the immune response and protection (Reitan and Secombes, 1997). The environmental temperature has an important influence on the development of immunity in fish (Gudding et al., 1999). The production of antibodies in Atlantic salmon vaccinated and reared at temperatures of 2 $^{0}$C were generally lower than in fish vaccinated at 10 $^{0}$C (Eggset et al., 1997).

Once an effective vaccine has been developed on a laboratory scale, it must be scaled up for production (Ward, 1982). Regulations set up by the licensing authorities ensure that only vaccines, which are safe and effective, are launched on to the market (Ward, 1982). Briefly, after an initial field-testing, developed in populations of fish, there will be a submission for a Product License (Ward, 1982). However, when analyzing this subject on a fish farmer point of view, other factors are also taken into account. For the fish farmer, vaccination is a matter of economy, the use of vaccines at relatively low cost should increase profitability by preventing loss of fish (Horne and Robertson, 1987).

## Bacteriosis caused by streptococcal species

Bacterial infections in aquaculture were mainly originated by Gram-negative microorganisms. However, in the last years, due to unknown reasons, infections provoked by Gram-positive bacteria have developed, being nowadays one of the main problems in the aquaculture industries (Padrós and Furones, 2002). The Gram-positive cocci are grouped together based on their Gram-stain reaction, thick cell wall composition, and spherical shape. In this review we will focus on the Streptococcaceae family. Two genera of cocci are included in this family: Streptococcus and Diplococcus, being primarily differentiated on the basis of arrangement. The primary division of the Streptococcaceae into two physiological groups is based on the difference in fermentation products namely homofermentative and heterofermentative. The first is further divided on the basis of solubility in surface tension reducents into the genera Diplococcus (soluble) and Streptococcus (not soluble) (Frobisher, 1954).

Streptococcosis is a bacterial disease mainly caused by streptococci, lactococci and vagococci bacteria. It occurs in both fresh and seawater and has been a source of major economic losses since the early seventies for the rainbow trout (Oncorhynchus mykiss) and marine fish industry in Asia (especially yellowtail Seriola quinqueradiata) as well as in the United States of America and South Africa. With the development of intensive fish culture practices in Japan, bacterial and viral diseases have spread and caused serious problems for culturists (Kusuda and Salati, 1993). The disease, which is associated with high water temperatures, is now becoming a serious problem for the European trout industry (Ghittino, 1999). Although streptococcosis outbreaks have occurred for four decades in Japanese farms, as mentioned before (Ringo and Gatesoupe, 1998), this disease has been described in other cultured fish species throughout the world, such as hybrid tilapia (Oreochromis aureus x O. niloticus) and striped bass (Morone saxatilis) in North America, or rainbow trout in South Africa and Australia (Ghittino, 1999).

There has been an important controversy about the number and the nature of the bacterial species involved with streptococcosis (Austin and Austin, 1999). In early reports on streptococcosis in fish it was not always possible to assign isolates to a particular species, however some attempt was made to group fish pathogenic strains on the basis of phenotypic traits such as haemolysis and correlate this characte-ristic with a range of pathologies (Miyazaki, 1982). Thus, α-haemolytic isolates responsible for granulomatous inflammation, β-haemolytic isolates, causing systemic infection with septicaemia and suppurative eye inflammation, and non-haemolytic isolates were recognized (Robinson and Meyer, 1966; Plumb et al., 1974; Kusuda et al., 1976; Minami et al., 1979; Iida et al., 1986; Al-Harbi, 1994; Toranzo et al., 1994). Nowadays there is a general acceptance for the division of streptococcosis into two forms according to the virulence of the agents involved at high or low temperatures (Ghittino, 1999). "Warm-water" streptococcosis, causing mortalities at temperatures higher than 15 ºC, typically involves species such as Lactococcus garvieae (formerly Enterococcus seriolicida), Streptococcus iniae, S. agalactiae or S. parauberis. On the other hand, "cold--water" streptococcosis caused by Vagococcus salmoninarum and Lactococcus piscium, occurs at temperatures below 15 ºC.

Toranzo and colleagues described the first epizootic outbreak of streptococcosis in turbot (Scophthalmus maximus) cultured in the northwest of Spain, which occurred in 1993 (Toranzo et al., 1994). Since 1996, preventive measures, based on the employment of a bacterin developed in their laboratory (Toranzo et al., 1995; Romalde et al., 1996), allowed the control of the disease. However, despite this control, streptococcosis seems to be endemic in some turbot farms, posing a potential danger of new outbreaks of the disease (Romalde et al., 1999b).

Streptococcosis can also infect mammals. During December 1995 to February 1996, four cases of a human bacteraemia were identified among patients at a hospital in Ontario (Weinstein et al., 1996). S. iniae, a fish pathogen not previously reported as a cause of illness in humans (Eldar et al., 1994; Eldar et al., 1995; Perera et al., 1995) was isolated from all four patients. All patients were of Chinese descent and had a history of preparing fresh, whole fish. Three of the fish were known to be tilapia that had been purchased from different stores. Patients reported a skin puncture on their hands from a tilapia bone, dorsal fin or from the knife being used to clean the fish (Weinstein et al., 1996).

Streptococcosis is also an important septicaemic disease in pigeons. The most important clinical signs of this disease include sudden death, inability to fly, lameness, emaciation, polyuria and production of slimy green droppings. Most typical lesions consist of extensive, well circumscribed areas of necrosis in the pectoral muscle and arthritis of the knee, the hock and the shoulder joints (Vanrobaeys et al., 1997).

The typical gross pathology observed in salmonid streptococcosis is not significantly different from that described for streptococcosis in other fish species. Apart from elevated rates of mortality (more than 50%), gross external signs include, anorexia, loss of orientation, lethargy, reduced appetite and erratic swimming. Uni or bilateral exophthalmia is frequent with intraocular haemorrhage and clouding of the eye. In many cases abdominal distension, darkening of the skin and haemorrhage around the opercula and anus are also observed (Kusuda et al., 1991; Eldar et al., 1994; Nieto et al., 1995; Stoffregen et al., 1996; Michel et al., 1997; Eldar and Ghittino, 1999). Internally the principal organs affected are the spleen, liver and brain, and to a lesser extent the kidney, gut and heart (Austin and Austin, 1999). The spleen may be enlarged and necrotic and the liver is generally pale with areas of focal necrosis. The intestine usually contains fluid and focal areas of haemorrhage. The abdominal cavity may contain varying amounts of exudates which may be purulent or contain blood. Acute meningitis is often observed, consisting of a yellowish exudate covering the brain surface and often containing large numbers of bacterial cells (Kitao, 1993; Austin and Austin, 1999; Múzquiz et al., 1999; Romalde and Toranzo, 1999).

Experiments carried out by Múzquiz et al. (1999), showed that older fish could eliminate the etiological agent of streptococcosis from internal organs, but it would remain in places from where it could be shed, such as gills or intestines. Thus the organism could establish an asymptomatic carrier state (Múzquiz et al., 1999). Many Gram-positive bacteria are able to survive for a long time under conditions of starvation in the aquatic environment. During this time, they may enter a

"dormant" or "viable but nonculturable" (VBNC) state, in which neither plating onto solid media nor inoculation into liquid media reveals the presence of viable cells (Currás et al., 2002). Although VBNC bacterial cells do not form colonies when plated on culture medium, their direct microscopic counts are constant and they maintain viability (Currás et al., 2002).

**Factors contributing to disease**

Most infectious diseases of fish are opportunistic. This means that the simple presence of the pathogen in the environment of the fish is insufficient to cause a disease outbreak. Stress often plays a significant trigger in outbreaks of opportunistic infectious disease in fish populations. Some stressors which have been associated with streptococcosis outbreaks include high water temperatures, high stocking densities, harvesting or handling and poor water quality such as high ammonia or nitrite concentrations (Yanong and Francis-Floyd, 2002). Streptococcosis affects fish of any size or age, and therefore cautions must be taken during all stages of the production cycle. The main reservoirs of the bacteria are the water and sediments around the farms, where the Streptococcus species can be isolated throughout the year. In addition, a source of infection can be the frozen fish used as a diet in the farms. It has been reported that Streptococcus can survive for more than 6 months in the frozen state. Horizontal transmission has been demonstrated and can occur by two different means: through the water, especially if fish body injuries exist, or by the faecal-oral route. Moreover, carrier status has been described in both susceptible and non-susceptible fish species which can also be important in the spreading of the disease (Romalde and Toranzo, 1999).

**Isolation and identification of fish pathogenic streptococci**

The presence of typical clinical symptoms and the demonstration of Gram-positive cocci from the internal organs, such as kidney or brain, constitute a presumptive diagnosis. Gram-positive cocci can be isolated on standard general purpose media but growth is enhanced by the addition of blood to a final concentration of 5 % (v/v) (Frerichs, 1993). A selective procedure for Streptococcus sp. was described by Bragg et al. (1989), consisting of an enrichment step in nutrient broth supplemented with naladixic acid, oxolinic acid or sodium azide followed by plating the enriched samples onto tetrazolium agar. Preliminary biochemical identification preceded the use of serological analysis, which can be useful to determine the streptococcal species involved in a particular outbreak. Serological confirmation may be performed by a variety of methods such as slide agglutination (Kitao, 1982), or fluorescent antibody staining (Kawahara and Kusuda, 1987). The latter method has been used to differentiate α- and β-haemolytic isolates. An indirect fluorescent antibody procedure has also been used for identification of Streptococcus sp. from pure cultures and smears from experimentally and naturally diseased salmonid fish (Bragg, 1988). More recently, Japanese authors have developed a rapid flow cytometry-based method that proved to be useful to detect the pathogen in mixed cultures (Endo et al., 1998). There is growing evidence that molecular techniques are useful for diagnosis of bacterial diseases in a variety of fish species. However, there are few reports of such work on the bacteria responsible for streptococcosis in fish, perhaps due to the confusion which surrounds the number of Gram-positive species associated with this disease syndrome (Berridge et al., 1998; Zlotkin et al., 1998a; Zlotkin et al., 1998b; Aoki et al., 2000). Among several molecular methods to identify Streptococcus sp. there is Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNA (RAPD), and Ribotyping (RT). Moreover, in experiments developed by Romalde et al. (1999b), RT has proved to be of limited value for epidemiological studies or intraspecific classification of these Gram-positive cocci, and RAPD is considered a better method for discriminating between isolates of Streptococcus. Another identification method developed by a Canadian Research Group and based on the ubiquitous and highly conserved single-copy chaperonin 60 (Cpn 60) gene, was reported to be effective for differentiating various species of Gram-positive cocci including some fish pathogens (Goh et al., 1996; Goh et al., 1997). The procedure relies on the use of both PCR and hybridization, where a set of universal degenerate primers is used to obtain a 600 bp product which is then used as a probe in a reverse checkerboard hybridization experiment (Romalde and Toranzo, 2002).

However, the identification of streptococci currently relies heavily on the serological grouping by the use of Lancefield antisera (Lancefield, 1993) and by analysis of biochemical phenotypes (Bentley and Leigh, 1995).

**Control measures**

Apart from the usual preventive measures, such as the reduction of overcrowding, overfeeding, unnecessary handling and the prompt removal of diseased or died fish, control of streptococcosis has included vaccination, chemotherapy as well as the use of non-specific immunostimulants (Romalde and Toranzo, 1999). Ideally, after the bacterium has been identified from a sick fish, a sensitivity test should be conducted to select the most effective antibiotic to use. Typically, Gram-positive bacteria are susceptible to erythromycin (Yanong and Francis-Floyd, 2002). Besides this, the majority of streptococci isolated are also sensitive "in vitro" to tetracycline, ampicillin, doxycycline, josamycin. On the other hand, strains resistant to these antibiotics

have been isolated in Japan and other countries in the last years (Romalde and Toranzo, 1999). The production losses produced by the streptococcal septicaemias in cultured fish, the expense involved in using antimicrobial compounds, and the reported increasing drug resistance of the causative Gram-positive cocci (Aoki et al., 1990), point out the need for developing immunoprophylactic measures to prevent these infections (Toranzo et al., 1995). Several attempts have been made to develop appropriate vaccination programs for fish streptococcosis (Iida et al., 1981; Carson and Munday, 1990; Ghittino et al., 1995; Akhlaghi et al., 1996; Romalde et al., 1996; Bercovier et al., 1997; Eldar et al., 1997; Romalde et al., 1999a). The use of bacterins with L. garvieae or S. iniae formalin-inactivated cells rendered good levels of protection in rainbow trout when administered intraperitoneally (Bercovier et al., 1997; Eldar et al., 1997), although differences regarding the duration of protection were observed for each species. Passive immunization of rainbow trout against Streptococcus sp. employing antibodies raised in sheep, rabbit or fish, was also evaluated (Akhlaghi et al., 1996). The results obtained were comparable to those of active immunization in both protective effect and duration (Romalde and Toranzo, 2002). These observations indicate that passive immunization may be important in the prevention of fish streptococcosis. Recently, it has been documented that β-glucans (schizophyllan and scleroglucan) have a protective effect against Streptococcus sp. infection when administered by intraperitoneal injection (Matsuyama et al., 1992). Protection of rainbow trout after intraperitoneal vaccination with a formalin-killed S. iniae vaccine was reported by Eldar et al. (1997). Klesius et al. (1999, 2000) have developed a modified-killed S. iniae vaccine composed of whole cells and concentrated, extracellular products. Turbot were protected against Enterococcus sp. after vaccination with a toxoid-enriched bacterin (Toranzo et al., 1995; Romalde et al., 1996; Romalde et al., 1999a). Rainbow trout immunized with formalin-killed Streptococcus sp. in Freund's Incomplete Adjuvant were protected against homologous Streptococcus sp. whereas trout immunized by bath immersion were not protected (Akhlaghi et al., 1996). Recently, Nakanishi et al. (2002) demonstrated that the protection induced in skin-punctured juvenile rainbow trout immersed in a formalin-killed S. iniae vaccine suspension rivalled that obtained by intraperitoneal injection (Evans et al., 2004).

**Lactococcosis – a particular case of streptococcosis**

The first description of L. garvieae (formerly E. seriolicida) came from an investigation of bovine mastitis in Great Britain (Collins et al., 1984). Later, L. garvieae was isolated from a variety of diseased freshwater and marine fish, and also from humans (Elliot et al., 1991), indicating the increasing importance of this bacterium, as a pathogen of fish and potential zoonotic agent (Romalde and Toranzo, 2002).

The identification criteria for L. garvieae based on biochemical and antigenic characteristics is very similar to L. lactis subsp. lactis, which has also been reported as a human pathogen (Collins et al., 1984; Mannion and Rothburn, 1990; Elliot et al., 1991; Doméneche et al., 1993) and from Enterococcus-like strains isolated from diseased fish (Toranzo et al., 1994; Nieto et al., 1995). Gram-positive cocci which are capable of growth between 10 and 42 ºC, at pH 9.6, in the presence of 6.5% NaCl and on 0.3 % methylene blue-milk agar can be identified as L. garvieae. The recent work of Eldar et al. (1999) and Vela et al. (1999) reveals the phenotypic heterogeneity of L. garvieae. Both workers proposed biotyping schemes which recognized three biotypes of L. garvieae. While based on the same phenotypic traits (acidification of tagatose, ribose and sucrose), there are some inconsistencies between the typing schemes described by the above authors. A possible explanation for this is the use of the Api-20Strep and/or Api-32Strep miniaturized systems for biochemical characterization of the strains. Ravelo et al. (2001) demonstrated that these systems may yield different results depending on the medium used for obtaining the bacterial inocula. In addition, the results achieved for some tests (i.e. acid production from: lactose, maltose, sucrose, tagatose and cyclodextrin) did not always correlate with results obtained with traditional plate and tube procedures. Moreover, although the strains studied by these authors showed variability for some characters, no biotypes with epidemiological value could be established. More recently, Vela et al. (2000) proposed a new intraspecies classification of L. garvieae with 13 biotypes, on the basis of acidification of sucrose, tagatose, mannitol and cyclodextrin and the presence of the enzymes pyroglutamic acid arylamidase and N-acetyl-ß-glucosaminidase, although only 6 of these biotypes were isolated from fish. In 1991, Kusuda et al. (1991) proposed a new species E. seriolicida, in order to bring together a number of Gram-positive isolates recovered from Japanese yellowtail over the preceding 20 years (Kusuda et al., 1976; Kusuda et al., 1991). Subsequent phenotypic and molecular characterization of E. seriolicida demonstrated that this species should be reclassified as a junior synonym of L. garvieae (Doméneche et al., 1993; Eldar et al., 1996; Pot et al., 1996; Teixeira et al., 1996). An interesting feature of these Japanese isolates was the existence of two serotypes, which could not be distinguished from one another biochemically. These two serotypes were associated with the presence (serotype KG-) or absence (serotype KG+) of a capsule. This capsule was reported to confer various properties on isolates, including a hydrophilic character, resistance to phagocytosis, and higher pathogenicity (Kitao, 1982; Yoshida et al., 1996; Yoshida et al., 1997). Freshly isolated cultures of this bacterium consist almost entirely of the KG-serotype. Barnes and Ellis (2004) observed that antisera rose against capsulated isolates regardless of their origin

cross-react with the non-capsulated isolates, showing that protein cell wall components are identical. Moreover, antisera raised against the non-capsulated isolates did not agglutinate any of the capsulated isolates, showing that there is a complete capsular coverage of the protein cell wall components. Whilst there currently appear to be only two distinct capsular serotypes based on geographical source of the isolate, the introduction of vaccination against this pathogen in fish may provide sufficient selective pressure to result in appearance of additional capsular serotypes with consequential failure of the vaccines (Barnes and Ellis, 2004).

For L. garvieae identification, a set of primers designed from the 16S rDNA sequence (EMBL accession number X54262) was selected by Zlotkin et al. (1998a) and used in a PCR protocol. The primers that amplify a fragment of 1.100 bp in size were tested with a collection of rainbow trout and yellowtail isolates from several countries. On the other hand, a Japanese group chose a dihydroperoate synthase gene as a target for a PCR-based diagnosis method for L. garvieae (Aoki et al., 2000). This gene is specially expressed in the KG-strains of this bacterium (Hirono et al., 1999).

## Clinical signs and histopathology of lactococcosis

Macrobranchium rosenbergii (a giant freshwater prawn) is also a host of L. garvieae. When infected, they exhibit anorexia, poor growth and a whitish body colour. Gross pathological changes include whitish muscle and a swollen, yellowish hepatopancreas. Yellowish-white spots were seen on the muscle and varied in size from 0.5 to 2 cm. Smears from fresh hepatopancreas, muscle tissue and tissue from other internal organs of diseased prawns showed the presen-ce of numerous cocci ranging in size from 0.1 to 0.2 μm in diameter. Histopathologically, diseased prawns showed marked oedematous fluid accumulation between the cuticle and underlying muscle tissue and fragmentation of muscle bundles with liquefactive necrosis could also be observed. In the muscle of hepa-topancreas there was also an appearance of necroti-zing foci encapsulated by haemocytes (granulomas). When concerning fish species, there are an overall standard clinical manifestations, like lethargy, anorexia and darkening of the body (Cheng and Wang, 2001). When fish are infected by L. garvieae, they start swimming around the surface, and in the first days a strong and evident bilateral exophthalmia can be observed, accompanied with haemorrhages in the ocular globe. Haemorrhages can also be observed in the base of the fins, around the anus and occasionally in the mouth and tongue. Internal pathological changes consist of haemorrhages in the liver and swim bladder, cerebral oedema, typical pericarditis, splenomegaly, as well as haemorrhages in the intestines, which are the more frequent lesions (AquaTIC, 1998). While the general clinical picture is relatively consistent, some variation in the clinical signs of salmonid lactococcosis have been described depending on which fish species are affected, the stage of infection and the involved etiological agent. Eldar and Ghittino (1999) pointed out that in rainbow trout, L. garvieae produces a hyperacute systemic disease, with the appearance of the so-called "oculo-splanchnic dissociation", which consists of a severe serositis sometimes extended to the myocardium.

## Control of lactococcosis

Effective control measures for Gram-positive infections in fish are important, not only because of the severe economic losses that these diseases can cause in aquaculture, but also because of the potential for some species, such as L. garvieae to infect humans (Elliot et al., 1991; Weinstein et al., 1997; Berridge et al., 1998; Meads et al., 1998). Although various chemotherapeutic agents, i.e. erythromycin, oxytetracycline or enrofloxacin, have proven to be active against L. garvieae "in vitro", therapeutic measures are generally ineffective in the field (Austin and Van Pouce, 1993; Bercovier et al., 1997), probably due to the anorexic condition of diseased fish. Moreover, vaccination seems to be the best alternative to prevent the spread of lactococcosis. Different attempts to develop effective vaccines against lactococcosis have been carried out (Ghittino et al., 1995; Bercovier et al., 1997). As in the case of other Gram-positive fish pathogens (Eldar et al., 1997; Romalde et al., 1999a), good protection levels, in non salmonid species, are only achieved when lactococcosis vaccines are intraperitoneally administered (Bercovier et al., 1997). However, L. garvieae oil adjuvanted bacterin, intraperitoneally injected in trout, provided low levels of protection for only 2-3 months (Romalde and Toranzo, 2002), which is not long enough to cover the warm season when the majority of lactococcosis outbreaks occur (Bercovier et al., 1997). Passive immunisation of rainbow trout against Lactococcus sp. employing antibodies raised in sheep, rabbit or fish, was also evaluated (Barnes et al., 2002). The results obtained were comparable to those of active immunisation in both protective effect and duration. These observations indicate that passive immunisation could have significant potential in the prevention of fish lactococcosis (Romalde and Toranzo, 2002). Moreover, other approaches were considered, including the use of adjuvants in the vaccine formulation, the combination of selection of genetically resistant fish and vaccination, and the use of booster immunisation. For this later approach, oral vaccines have been proposed as good candidates since no handling of fish is required, it is not a stressful method and it does not require extensive labour (Newman, 1993). However, antigens to be used in oral vaccination must be protected in some way to deliver them intact to the lower gut where they could stimulate an immune response

(Horne, 1997). Preliminary results obtained by Romalde et al. (2004) showed that antigen encapsulation in alginate appears to be a promising method for oral vaccination of trout against L. garvieae, at least as a booster immunisation procedure.

Two decades have elapsed since bacteriophages (phages) were reassessed scientifically as a therapeutic and prophylactic agent for bacterial infections (Nakai and Park, 2002). Potential advantages of phage treatment over chemotherapy are: 1) the narrow host range of phages, indicating that the phages do not harm the normal intestinal microflora; and 2) the self-perpetuating nature of phages in the presence of susceptible bacteria, indicating the superfluousness of multiple administrations (Smith and Huggins, 1982; Barrow and Soothill, 1997). However, experiments using phage therapy are so far scarce. "In vitro" studies revealed that the L. garvieae phages are tolerant to physicochemical factors such as water temperature ranging from 5 to 37 ºC and salinity varying from distilled water to double-strength artificial seawater (Nakai et al., 1999). Moreover, it was demonstrated of that intraperitoneal injection of the phage in treating young yellowtail intraperitoneally injected with L. garvieae was effective. The results show that phage administration might be useful as a therapeutic measure at an early stage of systemic infection or with low burdens of bacterial infection in yellowtail (Nakai et al., 1999). If phages are introduced by oral administration into the intestine of fish where virulent L. garvieae cells adhere or commence to multiply, it is expected that the phage will attack the pathogen (Nakai et al., 1999).

The knowledge of a novel vaccine against lactococcosis is worthwhile, not only to prevent the dissemination of such an infection, but also to prevent the destruction of cultured stocks of rainbow trout, among others. The prolonged destruction of the stocks will lead in a forthcoming future to the closure of several aquacultures.

We all hope that from now on, there will be an increase in the importance given to this emerging pathological problem, which seems to be hidden by other neither less nor more important bacterial diseases.

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# Dietary L-Carnitine Supplementation Protected Turbot (*Scophthalmusmaximus*) Against *Streptococcus parauberis* Infection

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*The protective effect of dietary L-carnitine supplementation was accessed in turbot (*Scophthalmusmaximus*) challenged with* Streptococcus parauberis. *Infected fish fed 240mg L-carnitine showed significantly ($P < 0.01$) higher survival (78%) than infected fish fed 40mg L-carnitine (11%). When dietary L-carnitine supplementation was suspended, a significant increase in mortality was observed in infected fish. The present report documents that dietary L-carnitine fed on a continuous basis may act as an immunostimulant in turbot.*

*KEYWORDS* *L-carnitine, turbot, immunostimulant*

## INTRODUCTION

In Portugal, turbot (*Scophthalmusmaximus*) aquaculture accounts for more than 13% of the total marine aquaculture production (Food and Agriculture Organization 2002). Streptococcus spp. are responsible for more deaths in marine aquaculture than any other gram-positive bacterial group (Groberg, Rohovec, & Fryer 1983; Abruzzini, Ingram, & Clem 1982; Toranzo et al.

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1994). *Streptococcus parauberis*, for example, was responsible for heavy economic losses in turbot aquaculture in Galicia, Spain, in 1993.

L-carnitine, a multi-physiological bioactive additive, is not only known to act as a growth-enhancer, but also appears to protect fish against xenobiotic compounds and variations in water quality. It also increases immunocompetence against several bacterial diseases (Harpaz 2005; Ozorio 2009).

Deufel (1990) reported that leucocytes in human subjects, including peripheral blood mononuclear cells, are rich in carnitine, suggesting that L-carnitine and its congeners may modulate immune networks. That is in agreement with Arafa et al. (2003), who observed an increase of immune response in rats following dietary L-carnitine supplementation.

The objective of this study was to evaluate the protective effect of dietary carnitine in turbot experimentally infected with *Streptococcus parauberis* and to determine if dietary supplementation should be on a continuous basis.

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## MATERIALS AND METHODS

Turbot weighing 60 ± 5.0 g (from PisciculturaMarinha do Rio Alto, A. Coelho & Castro, Lda, Póvoa do Varzim, Portugal) were kept at 18–22°C in a recirculating aerated saltwater (35%) system at Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) in Porto, Portugal. Water quality was maintained with mechanical and biological filtration. Turbot were divided into four groups of 60 fish each (30 fish for each of two replicated groups) and were fed daily *ad libitum* for 16 weeks on one of two nutritionally identical diets (crude protein, 39%; crude fat, 13%; gross energy, 19.5 kJ/g), differing only in dietary L-carnitine content (40 or 240 mg/kg diet). The duration of the feeding trial was chosen as the necessary time for the dietary L-carnitine groups (L-C) to develop different body carnitine levels. After 16 weeks, the two dietary treatment groups were sub-divided into two groups each: 40 mg L-C/not infected (G1), 40 mg L-C/infected (G2), 240 mg L-C/not infected (G3), and 240 mg L-C/infected (G4). Fish from G2 and G4 were challenged with *Streptococcus parauberis* (strain RA 83.1). Fish were starved for 24 h, anesthetized with 0.03% of ethylene glycol monophenyl ether (Merck), and intraperitoneally injected with 100 μL of a *S. parauberis* suspension containing $10^8$CFUs/mL.

To determine the importance of continuing to feed L-carnitine, seven days post-infection G3 and G4 were each sub-divided into two sub-groups, one that continued to receive L-carnitine at 240 mg/kg diet (G3, G4) and one for which L-carnitine supplementation was suspended (G5, G^). G5 (240 mg L-C suspended/not infected) was derived from G3, while G6 (240 mg L-C suspended/infected) was derived from G4.

Mortalities were monitored for 18 days post-infection, and dead fish were examined for re-isolation of the inoculated strain from the anterior

kidney by streaking it directly onto TSA1. Gram-Hucker stain (Panreac Química, SA; Barcelona, Spain) was used for presumptive identification.

The L-carnitine content of the diets and muscle samples of the different treatments was determined by a radiochemical method, which is based on the conversion of L-carnitine into [$^3$H] acetylcarnitine by carnitine-O-acetyltransferase (McGarry & Foster 1976).

The potential protective effect of L-carnitine against *Streptococcus parauberis* infection was calculated as relative percent survival (Amend 1981): RPS = [1- (% 240 mg L-C mortality/% 40 mg L-C mortality)] × 100.

Statistical analysis of carnitine intake, muscle carnitine content, and cumulative mortality between treatment groups were performed by one-way ANOVA using a computer software program (STATISTICA; StatSoft). If significant differences were detected, paired comparisons between treatments were analyzed by Tukey's tests. Values were considered significantly different at $P < 0.05$.

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## RESULTS

The dietary L-carnitine intake and its effect on muscle L-carnitine content is presented in Figure 1. Muscle carnitine was positively correlated to dietary L-carnitine level ($P < 0.001$). Groups fed 240 mg L-carnitine/kg diet had significantly higher cumulative L-carnitine intake (81 mg) than the ones fed 40 mg L-carnitine/kg (14 mg). L-carnitine content in the muscle was 92 and 124 mg/kg muscle for groups fed 40 mg and 240 mg L-carnitine/kg diet, respectively ($P < 0.001$).

Cumulative mortalities are shown in Figure 2. Eight days post-infection, mortalities began in fish fed 40 mg L-carnitine (G2), peaking at day 11. In groups G4 and G6, mortalities began day 11 post-infection and stopped

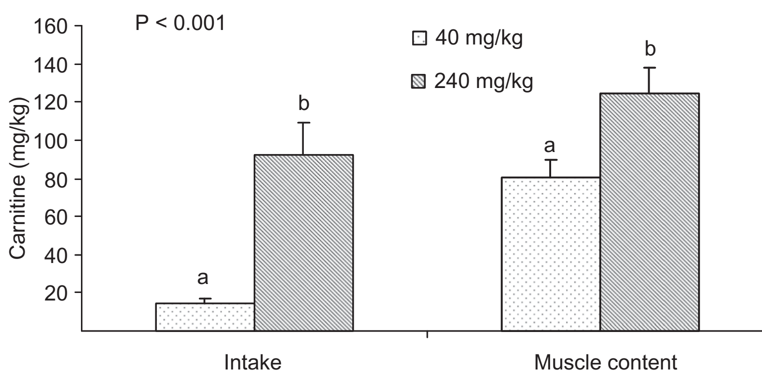


**FIGURE 1** Dietary carnitine intake and muscle carnitine content in turbot juveniles fed 40 or 240 mg L-carnitine/kg diet. Bars not sharing a common superscript letter are significantly different.

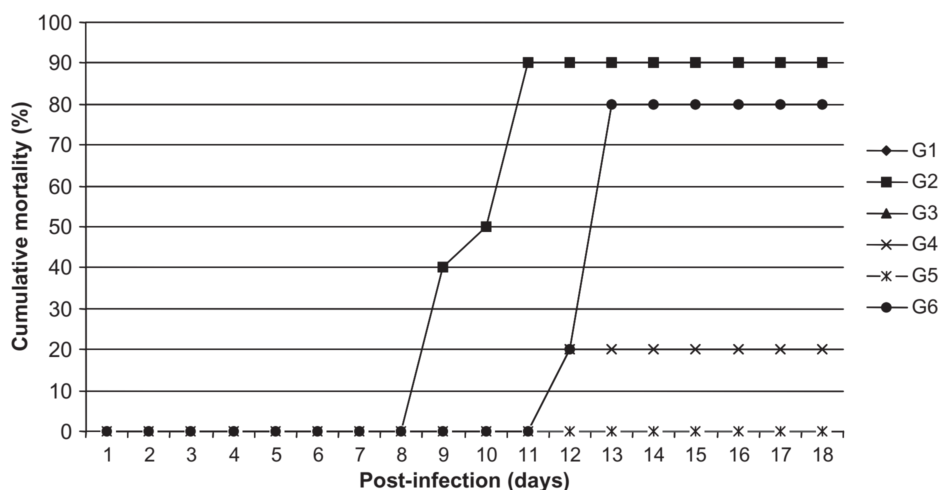


**FIGURE 2** Cumulative mortality (%) of turbot challenged intraperitoneally with *S. parauberis* after treatment with dietary L-carnitine supplements. GI: 40 mg L-C/not infected; G2: 40 mg L-C/infected; G3: 240 mg L-C/not infected; G4: 240 mg L-C/infected; G5: 240 mg L-C suspended/not infected; and G6: 240 mg L-C suspended/infected.

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by day 12 and 13 post-infection, respectively. All deaths were confirmed to be caused by *S. parauberis* by TSA1 plating and by Gram-Hucker's stain.

Significant protection resulting from the diets containing 240 mg/kg L-carnitine was observed when compared with fish fed 40 mg L-carnitine ($P < 0.004$). When dietary L-carnitine supplementation was suspended (G5 and G6), fish showed a reduction in protection. Overall, infected fish fed 240 mg L-carnitine showed significantly ($P < 0.01$) higher relative percent survival (78%) relative to infected fish fed 40 mg L-carnitine (11%). No mortalities were recorded in non-infected groups.

## DISCUSSION

Experiments carried out with L-carnitine have shown contradictory results, probably related to the complexity of the immune system (Athanassakiset al. 2001) interacting with the dosage of L-carnitine, the route of administration, and/or duration of the trial. Under the conditions of our study, dietary L-carnitine supplementation was effective at increasing body L-carnitine concentration, which in turn had a positive effect on the immune system of turbot, resulting in improved resistance to *S. parauberis* infection. That is in agreement with Shug and Gravenstein (1996); Mast, Buyse, and Goddeeris (2000); and Janssens et al. (2000) in work on the effects of oral L-carnitine treatment in pigeons.

Mortality rate associated with *S. parauberis* infection increased significantly when L-carnitine feeding was suspended, indicating a protective effect only as long as L-carnitine is continuously supplemented in fish diets. These immunostimulant properties of L-carnitine in turbot should be evaluated at an industrial scale so as to confirm this protective effect.

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