Development of molecular tools for pathogens detection in Aquaculture

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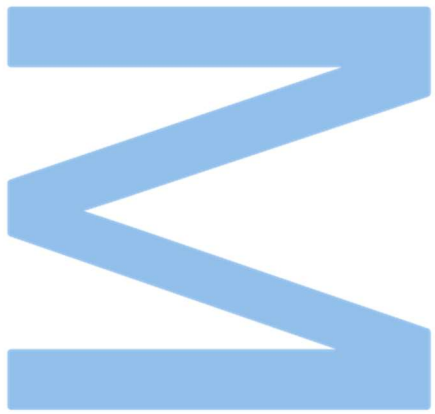

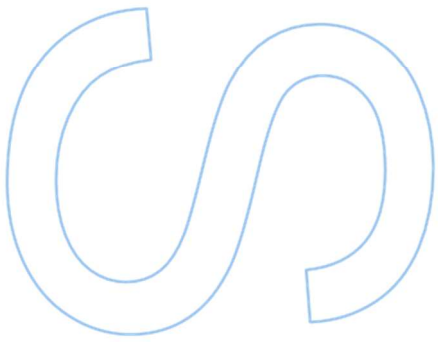

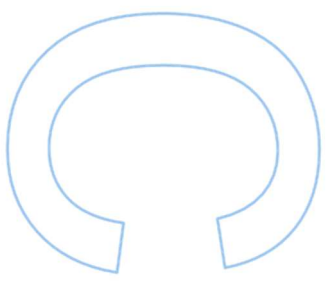

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Resumo

O setor da aquacultura é o setor alimentar com o maior crescimento nas últimas décadas, prevendo-se o aumento da sua produção de forma a satisfazer as necessidades de uma população também em crescimento. No entanto, esta intensificação enfrenta vários desafios, nomeadamente um aumento da ocorrência de doenças infeciosas de etiologia bacteriana e parasitária, uma das principais razões que levam a elevadas perdas económicas em aquacultura. Assim, garantir um rápido diagnóstico das mesmas desempenha um papel crucial no que diz respeito à contenção das suas consequências, minimizando as perdas económicas. As técnicas de identificação molecular revelam-se um método robusto, preciso e altamente sensível de deteção de agentes patogénicos com potencial de minimizar este impacto. No entanto, ferramentas moleculares de diagnóstico estão ainda por desenvolver para algumas das doenças mais prevalentes em aquacultura. Nesse sentido, o primeiro objetivo deste trabalho foi otimizar ferramentas moleculares para a deteção de parasitas que frequentemente afetam o trato gastrointestinal de diversas espécies de peixes cultivados em aquacultura. Para tal, seriam conciliados um diagnóstico clássico e uma análise molecular de amostras do trato gastrointestinal de peixes com sinais de doença de etiologia parasitária. Devido à impossibilidade de obter DNA dos parasitas para otimização da análise molecular no contexto do presente trabalho, esta ficou sem efeito. O segundo objetivo deste trabalho foi, então, a otimização e desenvolvimento de uma ferramenta molecular para a deteção de espécies bacterianas de elevado impacto no setor da aquacultura. Para tal, a técnica de PCR foi utilizada na identificação das espécies bacterianas selecionadas e as condições de PCR devidamente otimizadas e validadas para o desenvolvimento de uma reação de PCR multiplex, capaz de identificar diferentes espécies patogénicas simultaneamente. Seguiu-se a esta otimização a realização de ensaios de infeção em larvas de peixe-zebra com o objetivo de validar a ferramenta molecular desenvolvida através da identificação de infeções bacterianas em amostras experimentais. Os resultados obtidos confirmaram que, apesar de ter sido possível aplicar a ferramenta molecular com sucesso *in vitro*, esta careceu da desejada validação *in vivo*. Embora se tenha verificado a necessidade de realizar diversos passos para a sua otimização, a ferramenta molecular desenvolvida ao longo do presente trabalho revela-se promissora no que diz respeito à deteção de espécies bacterianas relevantes no contexto da produção em aquacultura.

Palavras-chave: Aquacultura, doenças infeciosas, PCR multiplex

Abstract

The aquaculture sector is the fastest-growing food sector in recent decades and, in order to meet the needs of a growing population, its production is expected to increase. However, the intensification of this sector faces a number of challenges, including an increase in the occurrence of infectious diseases, particularly bacterial and parasitic diseases, that have proven to be one of the main drivers of high economic losses in aquaculture. For this reason, ensuring a rapid diagnosis of these diseases plays a crucial role regarding containing their repercussions, thus avoiding such economic losses. Molecular identification techniques have proven to be a robust, accurate and highly sensitive method for detecting pathogens, revealing a high potential for minimizing the impact of infectious diseases. However, molecular diagnostic tools are yet to be developed for some of the most prevalent diseases in aquaculture. In this sense, the first objective of this work was to optimize molecular tools for the detection of parasites that frequently affect the gastrointestinal tract of various species of fish reared in aquaculture. To this end, classical diagnosis and molecular analysis of samples from the gastrointestinal tract of fish showing signs of parasitic disease would be combined. Due to the impossibility to obtain parasitic DNA for optimization of the molecular analysis in the context of this work, this was not possible. The second objective of this work was to optimize and develop a molecular tool for the detection of bacterial species with a high impact on the aquaculture sector. To this end, the PCR technique was used to identify the selected bacterial species and the PCR conditions were properly optimized and validated to develop a multiplex PCR reaction capable of identifying different pathogenic species simultaneously. This optimization was followed by an infection challenge assay on zebrafish larvae with the aim of validating the molecular tool developed by identifying bacterial infections in experimental samples. The results obtained confirmed that although it was possible to successfully apply the molecular tool *in vitro*, it lacked the desired *in vivo* validation. Although there was a need to carry out several steps to optimize it, the molecular tool developed during this work is promising in terms of detecting bacterial species that are relevant in the context of aquaculture production.

Keywords: Aquaculture, infectious diseases, multiplex PCR

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CFUs	Colony Forming Units
FAO	Food and Agriculture Organization of the United Nations
GI	Gastrointestinal
mPCR	Multiplex Polymerase Chain Reaction
Mt	Million tonnes
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
Pdd	*Photobacterium damselae* subsp. *damselae*
Pdp	*Photobacterium damselae* subsp. *piscicida*
Sa	*Streptococcus agalactiae*
Si	*Streptococcus iniae*

1. Introduction

1.1. Aquaculture

Aquaculture, which can also be referred to as “underwater agriculture”, may be described as the farming of fish, shellfish and aquatic plants. In 2020, global fisheries and aquaculture reached a new record regarding their production, with aquatic animals accounting for 178 million tonnes (Mt) of which 87 Mt came directly from aquaculture. Excluding algae, around the last 60 years, an average annual rate of 3% characterized the increase of per capita consumption of aquatic foods, doubling the world’s population growth rate. Due to the COVID 19 pandemic, in 2020, this consumption suffered a slight decrease. In spite of this decline, nowadays, aquaculture not only provides one-third of the seafood consumed around the world as it remains the fastest growing food sector in all continents since the last decades (Carbone & Faggio, 2016; Mugimba et al., 2021) (FAO, 2022).

By 2050, the human population is expected to grow to nearly 10 billion people. The expected population growth and global economic development will ultimately contribute to an increased global fish consumption. Since wild-capture fisheries are in decline, the aquaculture sector currently is and will continue gaining a crucial importance regarding filling the gap between food supply and the need to meet its growing demand (Huston et al., 2020; Ma et al., 2020). According to the Food and Agriculture Organization of the United Nations (FAO), by 2030, most of the growth in the fisheries and aquaculture sector will come from aquaculture, growing by 22% and contributing to 54% of total fish production and 60% of fish used directly for human consumption, dominating the future worldwide fish supply (Norbury et al., 2022) (FAO, 2022).

To achieve the estimated fish production through aquaculture, there’s an important commitment between intensification and sustainability that needs to be guaranteed. Following the need for this commitment, FAO proposes the concept of “Blue Transformation” that envisions a sustainable expansion and intensification of the aquaculture sector to meet the worldwide demand for aquatic food as well as an equitable distribution of the aquaculture’s production benefits while contributing to reach global food security targets. The sustainable growth of the aquaculture sector faces, however, many challenges. On one hand are the environmental concerns that jeopardize the sustainability of aquaculture’s expansion, for example: competition for the use of resources such as land, water and ingredients with other sectors; this sector’s frequently associated aquatic pollution leading to the deterioration of water supplies or

complications regarding the integration of aquaculture and different farming activities. On the other hand, due to the aquaculture's dependance on the natural environment, its fast expansion and intensification, the sector's vulnerability to stressors such as pollution, harmful algal blooms, climate change and pathogens is consistently increasing (Bayliss et al., 2017; Naylor et al., 2021).

Despite de numerous challenges affecting the sustainable expansion of the aquaculture sector, several studies point to infectious diseases as the most dangerous concern regarding the consequences of this sector's intensification and globalization. Infectious diseases are caused by a wide range of pathogens such as bacteria, viruses and parasites that have the ability to impact fish health causing adverse effects, frequently resulting in the most important cause of economic loss, hindering the aquaculture development (Bayliss et al., 2017; Carbone & Faggio, 2016; Mugimba et al., 2021; Norbury et al., 2022).

1.2. Diseases in aquaculture

Since the occurrence of infectious diseases in aquaculture has revealed itself to be the most important cause of economic loss to the aquaculture sector, it is essential to recognize the causes that actively contribute to this major issue.

The intensification of aquaculture per se is associated with a high stocking density which consequently results in the need for a higher amount of feed supply, water reuse and treatment procedures, factors that contribute to a significant degradation of the water quality. Water quality parameters such as the concentration of dissolved oxygen and temperature have a critical influence regarding the animals' vulnerability to pathogen exposure. For example, water temperature has a crucial role on the replication rate of pathogens and, being poikilothermic animals, a great impact on the fish's immune response. Considering the lack of physical barriers, the aquatic environment is an inherently conducive medium for the transmission of diseases. Besides that, high stocking densities are also associated with high stress levels and a closer proximity between the farmed fish resulting in a significant increase of not only infectious and opportunistic pathogens' activity and virulence, but also uni- and multicellular parasitic infestation. The combination of all the aforementioned factors promotes the initiation of infectious diseases outbreaks and hinder the decisive detection of early signs of an illness affecting the cultured species. By being associated with a higher number of affected individuals, the late detection of aquaculture diseases hampers their treatment, potentially causing the loss of entire cultures of farmed species (Bayliss et al., 2017; Dawood et al., 2021; Kibenge et al., 2012; Miller et al., 2017).

1.2.1. Parasitic diseases in aquaculture

Viruses and bacteria are, undoubtedly, the most prevalent infectious diseases' causative agents regarding their occurrence in aquaculture. However, recently, the role of parasites is progressively gaining attention as growing literature shows a significant increase in parasitic epidemics not only in farmed fish but also in wild populations (Shivam et al., 2021). It is very likely for any aquaculture fish species to host at least one parasitic species throughout its production lifetime. Even closed, recirculation systems, with good biosecurity measures, often regarded as less prone to disease outbreaks, can be subject to parasitic infections (Gratzek et al., 1983; Jørgensen et al., 2009; Moestrup et al., 2014). Parasites with direct life cycles, or those with simple life cycles with a water-dependent infectious stage, tend to dominate within the aquaculture scenario and can have a devastating outcome at economic, ecological and welfare levels. Besides the direct heavy impact to the aquaculture production due to the economic loss, some parasites also have the ability to cause toxicity of infected fish to the final consumer (Ohnishi et al., 2018; Shin et al., 2016) or even result in cases of zoonosis (Freitas et al., 2020). Essentially, by offering ideal conditions for the completion of parasite's life cycle, the presence of a wide spectrum of these organisms infecting fish in the aquatic environment is ubiquitous and diverse. Such diversity affecting fish species encompasses, for example, parasitosis caused by unicellular and metazoan parasites, which can be divided into ectoparasites, living in the external surface of their host and endoparasites, living inside the host, in both cases, completing their life cycle in a specific location or distinct sites in the same host. Moreover, such parasites might depend on only one or multiple hosts for the completion of their life cycle, being considered parasites with direct and indirect life cycles, respectively (Abd-Elrahman et al., 2023; Barber et al., 2000; Dykman et al., 2023).

Among the most important endoparasites affecting aquaculture production, parasites that inhabit the gastrointestinal (GI) tract of their host species, have specially adapted to obtain nutrients and cope with the local intestinal host immune response. Intestinal parasitism in fish, poses as a significant stress factor with the ability to induce malnutrition and lowered performance in the affected fish stocks, not only resulting in direct mortalities but also morbidity, poor growth, higher susceptibility to opportunistic pathogens and overall lower resistance to stress. Some of the main intestinal intracellular parasites of fish belong to the Apicomplexa Phyla and are obligate parasites whose life cycle takes place within the host cells after their penetration trough an active process. In consequence of the invasion and destruction of the intestine's epithelial cells, these intracellular parasites not only jeopardize the absorption of nutrients but also these cell's

role as the first line of defense against potential ingested pathogens by disrupting the integrity of the intestinal barrier (Sitjà-Bobadilla et al., 2016).

1.2.1.1. Coccidiosis

Piscine coccidia, including apicomplexan parasite genera such as *Eimeria* spp., *Goussia* spp. and *Cryptosporidium* spp. are commonly found infecting the majority of fish, being responsible for intestinal coccidiosis. Although this disease's pathogenicity greatly depends on the rearing conditions, immune status and individual sensitivity of the potential hosts, coccidiosis represents one of the major causes of illness and mortality in the fish population (Ananda Raja, 2022; Suyapoh et al., 2022). Morbidity caused by coccidia infections is reflected on the cultured species' poor growth rate despite being adequately fed to guarantee the fulfillment of their nutritional demands. Such morbidity and increased mortality intimately related to the occurrence of coccidiosis in the aquaculture sector are responsible for a significant economic impact (Golomazou & Karanis, 2020).

The life cycle of apicomplexan parasites is characterized by alternating asexual and sexual multiplication and can be generically divided in three different phases, namely sporogony, merogony and gamogony, that include four distinct cell types: the haploids sporozoite, merozoite and gametes and the diploid zygote. The invasion of host cells by sporozoites follows their release from the infectious oocyst, which marks the beginning of the infection process. After invading the host cells, sporozoites transform into trophozoites initiating the merogony phase. Merogony is characterized by a sequential number of asexual reproduction steps ultimately resulting in the development of merozoites that continue invading, replicating and leaving other host cells until the final generation of merozoites, thought to be genetically committed to begin sexual multiplication. The next phase, gamogony, is characterized by some merozoites becoming macrogamonts (female macrogametocytes) and, eventually, immobile macrogametes, while the major part of merozoites becomes microgamonts (male microgametocytes) and, later, motile flagellated microgametes. The fusion of a microgamete and a macrogamete leads to fertilization and the formation of a diploid zygote. Completing the life cycle, in the last phase, sporogony, the zygote develops into the haploid sporozoite through various cell divisions. Thus, inside the host cells, apicomplexan parasites undergo various replication steps while invading neighboring epithelial cells and ultimately produce sporozoite containing oocysts that maintain the infection in the host through autoinfection and environmentally resistant infectious oocysts, released in the host's feces, that have the ability to infect the next host following

their ingestion (Cruz-Bustos et al., 2021). Given that a single oocyst can be immediately infectious, promoting the initiation and development of disease, the aquatic habitat of fish greatly promotes the success of apicomplexan parasites infections by facilitating the distribution and transmission of such oocysts to other hosts. The particularities of the different stages of their life cycle vary according to the considered species and, despite the progress that has been made in understanding the life cycle of the Apicomplexa, the current knowledge regarding fish coccidia and respective life cycles in fish is still scarce (Golomazou et al., 2021; Saraiva et al., 2023).

Parasites belonging to the genus *Eimeria* spp. are responsible for important economic losses in various food production sectors, besides aquaculture, by also having the ability to infect, for example, poultry, cattle, swine, rabbits and turkeys (Ahmad et al., 2023; Ashfaq et al., 2023; Ovington et al., 1995; Serbessa et al., 2023). *Eimeria* spp. parasites have a monoxenous life cycle, completing it in the same host, and most species belonging to this genus develop in the epithelial tissue of intestinal and adjacent tissues in the host's GI tract. By replicating inside the enterocytes, infections caused by these parasites severely impair those cells' function. Although information regarding these parasites effect on fish intestinal integrity is scarce, according to studies carried out evaluating the effect of *Eimeria* spp. infections in poultry, for example, such impairment is due to the structure and morphology modifications as well as destruction of epithelial cells, inflammation and alterations in tight junction proteins responsible for maintaining the integrity of the intestinal barrier. Consequently, nutrient malabsorption, jeopardized immune responses and bacterial infections are frequently recorded alongside severe diarrhea, weight loss and potentially sudden death (Cruz-Bustos et al., 2021; Felici et al., 2021; Galli et al., 2019; Graham et al., 2023).

The genus *Goussia* spp. was considered a synonym of *Eimeria* spp. for a long time until differences between sporocysts characteristically belonging to each genus were discovered. In spite of such discovery, both genera remained regarded as synonyms by some authors until recently. However, aided by the continuous development of molecular biology, recent studies were able to find significant differences between molecular sequences of *Eimeria* spp. and *Goussia* spp. affecting fish (Molnár & Ogawa, 2000; Molnár & Székely, 2017; Rosenthal et al., 2016). Opposed to *Eimeria* spp., *Goussia* spp. have only been described parasitizing diverse fish species, being the most commonly reported piscine coccidia infecting fish globally, with some species also infecting amphibians (Jowers et al., 2023). Most *Goussia* spp. species have an homoxenous life cycle and, besides being frequently found in the intestine of parasitized fish, infections

of other organs such as kidney and spleen are common. In the intestine, intense *Goussia* spp. infections can be responsible for microvillar atrophy of epithelial cells and, consequentially, formation of multiple secondary mucosal folds. As a result, some important detrimental effects frequently encountered on the hosts include focal necrosis, mal-absorption and starvation (Dogga et al., 2015; Jowers et al., 2023).

Regarding *Cryptosporidium* spp., this genus includes widespread protozoan parasites with the ability to infect a number of vertebrates, including humans, being considered the fifth most important food-borne pathogen. *Cryptosporidium* spp. have a monoxenous life cycle and an infection with parasites belonging to this genus also typically initiates with the release of sporozoites in the GI tract of their host, followed by the invasion of intestinal cells, upon the ingestion of sporulated oocysts. Despite having the ability to infect other organs, these parasites are commonly found parasitizing the stomach and intestine of their hosts. *Cryptosporidium* spp. infections trigger the occurrence of mild to moderate gastritis. Moreover, necrosis, increased vacuolization, degradation, disaggregation and sloughing of epithelial cells are reported as the main histopathological effects detected in infected tissues (Golomazou et al., 2021; Hassan et al., 2020; Moratal et al., 2020; Moratal et al., 2022).

Besides fish, coccidiosis affects various other animals such as poultry, cattle or swine and, consequently, their production (Ahmad et al., 2023; Ashfaq et al., 2023; Serbessa et al., 2023). Regarding the poultry production, for example, coccidiosis caused by parasites belonging to the genus *Eimeria* spp. is the most significant ubiquitous disease affecting this industry, being responsible for a significant economic impact annually. Contrary to the aquaculture sector, preventive and treatment measures regarding coccidiosis affecting the poultry industry are well studied as increasingly successful strategies are continually developed to combat this infection, including vaccination, anticoccidial drugs and feed supplementation with natural products such as probiotics and phytochemicals (Fatoba & Adeleke, 2020; Madlala et al., 2021). In regards to *Cryptosporidium* spp. infections, therapeutic options are very limited. That being the case, the lack of treatment is reflected in efforts to prevent and control this disease based on prophylaxis (Golomazou et al., 2021). All things considered, the continuous monitoring and screening for this pathogens' presence is extremely relevant in the aquaculture sector.

1.2.2. Bacterial diseases in aquaculture

As mentioned above, the intensification of aquaculture is often associated with deteriorated zootechnical conditions such as low and suboptimal oxygen and pH levels,

respectively, high ammonia concentrations and host stress. These conditions have revealed to be intimately related with a microbial imbalance, or dysbiosis, traduced by an increased abundance of opportunistic and pathogenic bacteria with the ability to colonize the mucosa of fish and subsequently increase their host's susceptibility to various illnesses (Rosado et al., 2023). Notwithstanding, only a few pathogenic bacterial species causing such diseases are accountable for serious economic losses regarding the aquaculture sector worldwide (Diyie et al., 2022). Photobacteriosis and streptococcosis, for example, are included in the most prevalent diseases currently affecting the aquaculture production (Santos et al., 2021; Ziarati et al., 2022). The use of antibiotics as a prophylactic and treatment measure poses as the first line of defense regarding controlling bacterial diseases in the aquaculture sector, namely photobacteriosis and streptococcosis. However, there is a growing concern regarding antibiotic use and associated issues, such as the emergence and spreading of antibiotic resistance among bacterial species and the occurrence of antibiotic residues in aquatic environment, contributing to its pollution, which jeopardize both human and animal health and the sustainability of the aquaculture sector (Gouife et al., 2022; Silva et al., 2021).

1.2.2.1. Photobacteriosis

The bacterial species *Photobacterium damselae* includes two significantly distinct subspecies: *Photobacterium damselae* subsp. *damselae* (Pdd) and *Photobacterium damselae* subsp. *piscicida* (Pdp), the causative agents of photobacteriosis, one of the most threatening bacterial diseases affecting several wild and cultured marine fish species (Baseggio et al., 2022; Eissa et al., 2018). Formerly known as pasteurellosis, this disease generally causes septicemia and internal lesions in the liver, spleen and kidney in its hosts, also having the ability to produce external lesions in the head, gills and skin (Santos et al., 2022). Both *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* are associated with considerable mortality rates among marine species, being responsible for serious economic losses regarding the aquaculture production of important fish species due to photobacteriosis (Su & Chen, 2022).

Photobacterium damselae* subsp*. damselae

P. damselae subsp. *damselae*, formerly known as *Vibrio damsela*, is a Gram-negative, mesophilic ubiquitous marine bacterium which has been increasingly gaining importance in the aquaculture sector as an emerging aquaculture pathogen. Being a virulent generalist and opportunistic pathogen of compromised hosts, *P. damselae* subsp. *damselae* affects an extensive range of not only aquatic organisms such as molluscs, crustaceans, cetaceans and marine fish species, but also humans (Baseggio et al., 2022;

Gouife et al., 2022; Matanza & Osorio, 2018; Matanza & Osorio, 2020; Morick et al., 2023). Although in some species it is supposedly a commensal member of the gut microbiome, in several fish species, hemorrhage of the liver, spleen and kidneys and ulcerative lesions around the mouth and pectoral fins often manifest as clinical symptoms of *P. damselae* subsp. *damselae* infection, which can be fatal in reduced time frames such as 24 hours. Devastating outbreaks in cultured marine fish species including seabass, seabream, turbot and rainbow trout have been responsible for immense economic loss. Moreover, many antibiotic-resistant strains of *P.* damselae subsp. damselae already exist. This scenario is encouraging an increased concern regarding developing novel effective preventive and treatment measures regarding the incidence of outbreaks prompted by *P. damselae* subsp. *damselae* (Su & Chen, 2022; Suzzi et al., 2023).

Photobacterium damselae* subsp*. piscicida

P. damselae subsp. *piscicida*, formerly known as *Pasteurella piscicida* is a Gram-negative facultative intracellular halophilic bacterium highly specialized, highly virulent and strictly pathogenic for fish species. *P. damselae* subsp. *piscicida* was first isolated six decades ago from natural white perch and striped bass populations affected by, at the time, an unknown bacterial species. Since then, it has been linked to devastating consequences in marine aquaculture, being responsible for great financial losses in this sector due to its outbreaks leading to mortalities up to 80% of the infected stock. High water temperatures, generally above 23°C, and detrimental water conditions are characteristically associated with such outbreaks (Abushattal et al., 2022; Baseggio et al., 2022; Santos et al., 2022; Teixeira et al., 2023). The acute form of *P. damselae* subsp. *piscicida* infection, although clinical signs are usually unnoticeable besides the appearance of slight hemorrhages on the host, is characterized by the occurrence of a generalized bacteremia and extensive cytopathology with abundant tissue necrosis. The chronic form, on the other hand, is characterized by typical skin wounds in the pectoral fin and caudal peduncle that manifest as granulomatous ulcerative dermatitis. Internally, whitish tubercle-like deposits consisting of accumulations of bacteria, apoptotic cells, and necrotic debris are frequently present in organs such as the liver, spleen and head-kidney of infected fish. Notwithstanding the development of vaccine strategies against *P. damselae* subsp. *piscicida* during the last 30 years, the prevalence of severe outbreaks in several countries, including vaccinated stocks, continuously proves that there are no reliable control measures against *P. damselae* subsp. *piscicida* as both antibiotics and vaccines remain ineffective or only partially effective and improved methods considering

the prevention of this disease are still required (Lattos et al., 2022; Pečur Kazazić et al., 2019; Rudenko et al., 2023; Teixeira et al., 2023).

1.2.2.2. Streptococcosis

Streptococcal disease or streptococcosis is a widespread bacterial disease caused by Gram-positive streptococci-shaped bacteria, including warm-water pathogens such as *Streptococcus agalactiae* (Sa) and *Streptococcus iniae* (Si). These bacteria affect both freshwater and marine fish species such as tilapia, rainbow trout and European seabass and are associated with extensive morbidity and mortality rates (Pirollo et al., 2023; Vela-Avitúa et al., 2023). Moreover, *S. agalactiae* and *S. iniae*, being zoonotic streptococcal species, are extremely important for human health. Thus, besides their relevance in the aquaculture sector regarding an economic impact, their ability to cause opportunistic invasive infections in humans, also posing as a significant cause of worldwide morbidity and mortality among humans, justify the present concern regarding these pathogens (Sapugahawatte et al., 2022; Saralahti & Rämet, 2015; Varga et al., 2022).

Streptococcus agalactiae

Streptococcus agalactiae infection represents a severe threat to both humans and animals, including fish, by causing neonatal sepsis and meningitis in the first and resulting in high economic loss in aquaculture (Zhu et al., 2023). *Oreochromis* spp. (tilapia) is one of the most severely affected fish species regarding *S. agalactiae* infections with the GI epithelium being this pathogen's main route of entry. In fish, *S. agalactiae* is responsible for the destruction of the immune system and damage of the liver and kidneys, predominantly causing fish septicemia and meningoencephalitis (Mian et al., 2009; Suwanbumrung et al., 2023). Currently, prevention and control of *S. agalactiae* infection in tilapia greatly depends on the use of antibiotics. However, considering the above-mentioned growing concern regarding its use, vaccination has become the preferred method providing protection regarding *S. agalactiae* infection, achieved by the development of inactivated vaccines, attenuated vaccines, DNA vaccines and subunit vaccines (Chen et al., 2023; Zhu et al., 2023).

Streptococcus iniae

Streptococcus iniae is a largely distributed pathogen in aquatic environments, able to infect at least 27 different freshwater and marine teleosts, as well as humans, transmitted by *S. iniae* infected fish handling. Depending on the infected fish species, different clinical signs of the disease can be found such as erratic swimming, exophthalmia, corneal opacity, hemorrhage, ascites and lesions of the liver, kidney, spleen, and intestine. Despite the variety of symptoms, an *S. iniae* infection commonly results in meningitis

and total ophthalmitis that culminate in large-scale mortality among wild and farmed fish with the aquaculture sector being the most impacted regarding infection-related economic losses (Cheng et al., 2010; Maekawa et al., 2023; Suanyuk et al., 2010). Besides the use of antibiotics and chemicals, prevention of this disease's outbreaks through the improvement of fish resistance to *S. iniae* has also been achieved using baits supplemented with micro-ecological agents such as *Bacillus subtilis* and *Saccharomyces cerevisiae* (Sheng et al., 2023). Moreover, the urgency to improve this disease's treatment discarding the reliance on antibiotic use has stimulated a wide study regarding vaccine development in several species, for example, by using inactivated vaccines against *S. iniae* in Nile tilapia (*Oreochromis niloticus*), zebrafish (Danio rerio) and rainbow trout (*Oncorhynchus mykiss*) (Tanpichai et al., 2023).

1.3. Diagnostic and detection methods in aquaculture

Disease control in production sectors such as aquaculture, to be effective, requires rapid and reliable identification methods to not only detect the specific pathogens responsible for the disease, but also monitor changes in their abundance. Only by following these criteria it is possible to prescribe adequate treatment, if existing, and control measures (Diyie et al., 2022; Pečur Kazazić et al., 2019). Currently, there are limited diagnostic and detection methods regularly used in aquaculture management.

1.3.1. Detection of target parasites

Parasites affecting fish in aquaculture, namely coccidia, are usually identified through traditional diagnostic techniques such as histopathological and ultrastructural descriptions of the infectious agents, for instance, through the characteristic morphology of their various life-stages. Different techniques are used in this identification, including light microscopy methods such as phase contrast, bright-field and differential interference contrast microscopy. The use of *in situ* hybridization with monoclonal and polyclonal antibodies and lectins, has been associated with the improvement of light microscopy methods by facilitating the visualization of protistans, aiding their detection, as well as the use of transmission electron microscopy, extremely important to adequately diagnose protistans due to their distinct internal cellular structures, allowing the discrimination of different species and genera. These techniques, however, not only tend to be labor-intensive and time-consuming, as they are frequently unable to specifically confirm the disease's causative agent. As the aquaculture sector faces the growing issue of an increased occurrence in infectious diseases and since providing appropriate treatment continues to be the most effective way of fighting outbreaks, the availability of quicker and reliable diagnostic methods is indispensable. Thus, the

application of molecular tools in the detection of parasites affecting the aquaculture sector is increasingly important (Ador et al., 2021; Gibson-Kueh et al., 2011; Moreira et al., 2021; Paladini et al., 2017).

1.3.2. Detection of target bacteria

Regarding bacterial species affecting the aquaculture sector, conventional microbiology methods including isolation and culturing of bacteria, serology and histology are often used in their detection (Martins et al., 2013). Being considered laborious and time-consuming, such techniques have been progressively substituted by DNA-based technologies, for instance, the use of Polymerase Chain Reaction (PCR) as the development of molecular tools has aided research in this field to overcome the limitations associated with culture-based approaches.

The PCR is a method used to exponentially amplify certain DNA regions through the repetition of three cyclic processes. Firstly, during DNA denaturation, the DNA is denatured into single-stranded DNAs and used as template sequences for the design of synthetic oligonucleotide primers. Following this step, in a process known as primer annealing, such primers will hybridize to specific and complementary opposite locations of the single DNA strands, determining the DNA region to be amplified. Lastly, polymerase extension is characterized by the addition of nucleotides to the end of each primer, a process that is catalyzed by an enzyme called Taq polymerase, resulting in new copies of the target DNA region in each round of the thermocycling reaction (Chuang et al., 2013; Cunningham, 2002).

Besides the conventional PCR, multiplex Polymerase Chain Reaction (mPCR) has also proved to be an important molecular tool regarding the detection of bacterial species affecting the aquaculture sector. The mPCR acts as an extension of the conventional PCR by simultaneously allowing the amplification of numerous targeted DNA sequences in a single reaction. That being the case, using mPCR as a bacterial detection method not only saves time and effort as it also allows the control of false negatives and the diagnostic of multiple infections, preserves DNA templates and is cost-effective as its use requires less reagents (Ador et al., 2021; Diyie et al., 2022; Panangala et al., 2007). Several studies have had encouraging results regarding the development of a mPCR, for example: the detection and quantification of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio anguillarum* in fish and seawater (Kim & Lee, 2014), the detection of *Tenacibaculum maritimum* and *Edwardsiella tarda* in both experimentally and naturally infected fish (Castro et al., 2014), the detection of *Yersinia ruckeri* and *Flavobacterium psychrophilum* to be used in water analyses from Recirculatory Aquaculture System

production sites (Lewin et al., 2020) or the detection of *Vibrio vulnificus* strains hazardous for human and animal health in seafood or feed samples (Roig et al., 2022).

1.4. Zebrafish as a model of infection

The zebrafish have been used as a model in scientific research since the 1980s, playing a key role in the study of diseases that affect humans. Its relevance as a model for scientific research is based on important characteristics inherent to the species *Danio rerio*, such as their transparency in the early stages of life, a growing availability of a wide spectrum of mutant and transgenic lines due to the ease of genetic manipulation, a short generation time combined with continuous egg production and rapid development, promoting a continuous and high production of offspring, as well as a crucial ease of maintenance (Jørgensen, 2020). During the last 20 years, considering the above-mentioned advantages and the development of the aquaculture sector, the use of zebrafish as a study model for fish diseases affecting important production species has gained increasing significance, namely regarding bacterial diseases caused by common fish pathogens such as *Streptococcus* spp. (Neely et al., 2002; Saralahti & Rämet, 2015), *Photobacterium damselae*, *Vibrio* spp., *Aeromonas hydrophila* (Santos et al., 2023; Saraceni et al., 2016) or *Edwardsiella tarda* (Pressley et al., 2005).

1.5. Objectives

Given the increased occurrence of disease outbreaks in the aquaculture sector that commonly result in significant economic losses, rapid and reliable identification methods to effectively prescribe and implement adequate treatment and control measures are urgently needed.

The initial goal of this work was to optimize molecular tools for the detection of endoparasites commonly affecting important fish species produced in the aquaculture industry, and to whom no quick and reliable detection methods are available. Due to the importance of traditional diagnostic methods regarding confirming a parasitosis diagnosis, training in the identification of coccidiosis in field GI samples recurring to classical methods (histology) was carried out. The main objective was to develop a multiplex PCR assay using primers retrieved from the literature targeting the aforementioned coccidia genera. It was expected that this molecular tool could simultaneously detect different parasites from field samples and eventually substitute the laborious and time-consuming histology approach. However, the impossibility to gather parasitic DNA relevant to the development of a mPCR and field parasitized fish samples for PCR analysis, prevented the fulfillment of the initial goal.

Thus, a second goal was established for this work: to develop a multiplex PCR for the identification of relevant bacterial species currently affecting the aquaculture sector. A similar approach was followed including an extensive literature review of specific oligonucleotide primers, testing of their efficiency and specificity and development of multiplex PCR assay to simultaneously detect different bacterial species. To validate the mPCR developed, a zebrafish larvae challenge assay was performed, followed by total DNA extraction at the end of the assay for subsequent use as DNA samples of organisms infected with the bacterial species under study.

2. Materials and Methods

Goussia clupearum DNA samples were obtained from liver, stomach and intestine of *Sardina pilchardus* and *Trisopterus luscus* (**Table 1**). These samples were kindly provided by Doctor Raquel Xavier from CIBIO-InBIO.

Table 1. Provenance of coccidian DNA samples

Sample	Sample ID	Species	Tissue	Host
1	SP37L	*Goussia clupearum*	Liver	*Sardina pilchardus*
2	SP19E	*Goussia clupearum*	Stomach	*Sardina pilchardus*
3	SP61L	*Goussia clupearum*	Liver	*Sardina pilchardus*
4	TL6L	*Goussia clupearum*	Liver	*Trisopterus luscus*
5	TL1I	*Goussia clupearum*	Intestine	*Trisopterus luscus*

2.1. Histology

Histological samples were collected in fish cultivated in earth ponds belonging to a production unit located in Algarve, and exhibited poor growth, which lead to the suspicion of coccidiosis.

2.1.1. Histological processing

Stomach and intestine samples were processed using standard histological techniques. Briefly, samples were dehydrated in an ethanol gradient from 70%, to 100%, cleared in xylene and infiltrated with paraffin. Samples were then transversely oriented and permanently embedded in paraffin and 5 µm sections were cut using a microtome and placed in glass microscope slides. The tissues in each microscope slide were stained with haematoxylin and eosin and mounted with Thermo Fisher's mounting medium and a coverslip.

2.1.2. Quantitative evaluation of parasitized tissue samples

The histological evaluation of parasitized stomach and intestine samples consisted in selecting 5 different optical fields (400x magnification) and count the number of coccidia found in each optical field, calculate the mean count value and attribute a score according to **Table 2**.

Table 2. Guideline table for histopathological evaluation of coccidia infection.

Score	Parasites/Optical field	Intensity
0	0	Null
1	1 - 2	Very low
2	3 - 5	Low
3	6 - 20	Moderate
4	> 21	High

2.2. Bacterial Species and Growth Conditions

The bacterial species used in this work are listed in **Table 3**. The bacterial stocks were stored at -80°C in 30% glycerol, preventing the bacteria's membrane rupture by ice crystals formation.

Table 3. Bacterial strains used in the present study.

Bacterial species	Strain	Origin / Source
Aeromonas hydrophila subsp. *hydrophila*	LMG 2844	BCCM/LMG
Vibrio anguillarum	DSM 21597	DSMZ
Vibrio harveyi	Fish isolate	NUTRIMU collection
Vibrio parahaemolyticus	LMG 2850	BCCM/LMG
Vibrio vulnificus	LMG 13545	BCCM/LMG
Photobacterium damselae subsp. *damselae*	LMG 7892	BCCM/LMG
Photobacterium damselae subsp. *piscicida*	$Lg_{h41/01}$	M.A. Morinigo
Edwarsiella tarda	LMG 2793	BCCM/LMG
Streptococcus agalactiae	LMG15977	F. Tavares
Streptococcus iniae	LMG14520	F. Tavares

Bacterial strains were obtained from bacterial collections (BCCM/LMG, Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University, Ghent, Belgium; DSMZ, DSM Collection, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, from our laboratory stocks (NUTRIMU collection) or kindly supplied by F. Tavares (Centro de Investigação em Biodiversidade e Recursos Genéticos) and M.A. Morinigo (Universidad Málaga).

The selected bacterial species were firstly inoculated through the streak plate method (to obtain single isolated colonies of each bacterial species) using solid Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company) for the growth of *Streptococcus agalactiae* and *Streptococcus iniae* at 37°C for 24 hours and the growth of *Photobacterium damselae* subsp. *damselae* and *Photobacterium damselae* subsp. *piscicida* at 28°C for 48 hours.

2.3. Genomic DNA Extraction

To obtain the bacterial genomic DNA, a single isolated colony of each bacterial species was inoculated in 5 mL of BHI and grown with agitation (120 rpm) at 37 or 28°C, for 24 or 48 hours, depending on the species requirements (described in the previous section).

Subsequently, 2 mL of each bacterial culture were centrifuged at 13 000 rpm for 10 min. The supernatant was discarded and the resulting cell sediments (pellets) were resuspended in 0.8 mL of CD1 solution (QIAGEN), and their DNA extracted using the DNeasy Powersoil Pro Kit (QIAGEN) according to the manufacturer's instructions. Pellet homogenization was done in a Precellys 24 homogenizer (Bertin Instruments, France) three times at 4500 rpm for 1 minute, alternating with 1 minute on ice.

Extraction of DNA from zebrafish larvae was carried out at the end of the zebrafish larvae challenge assay. Following the infected larvae's euthanasia and collection, the DNA extraction was held in a similar way as described above. However, to guarantee the best results possible, an optimization step was previously taken consisting in testing two different homogenization procedures at the beginning of the extraction protocol. While the first procedure (A) maintained the above-mentioned homogenization conditions, the second (B) consisted in using the Precellys 24 homogenizer (Bertin Instruments, France) three times at 6500 rpm for 30 seconds, alternating with 1 minute on ice.

2.4. Polymerase Chain Reaction

2.4.1. Uniplex PCR

The uniplex PCR amplifications were performed in 25 µL reaction volumes, containing 2,5 µL of template DNA, 5µL of 5x MyTaq Reaction Buffer (Bioline), 0,5 µL MyTaq DNA Polymerase (Bioline), 1 µL each primer (**Table 4**) and water in order to complete the final volume of each reaction.

The amplification was conducted using a T100 Thermal Cycler (Bio-Rad), according to the following cycling conditions: initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation for 15 seconds, annealing at 50, 52, 54 or 56°C, depending on the primer's melting temperature (**Table 4**), for 15 seconds and extension at 72°C for 30 seconds.

2.4.2. Multiplex PCR

The multiplex PCR amplifications were performed in 25 µL reaction volumes, containing 2,5 µL of each template DNA, 5µL of 5x MyTaq Reaction Buffer (Bioline), 0,5 µL MyTaq DNA Polymerase (Bioline), 0,2 µL each primer (**Table 4**) and water to complete the final volume of each reaction.

The amplification was conducted using a T100 Thermal Cycler (Bio-Rad), according to the following cycling conditions: initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation for 15 seconds, annealing at 52, 54 or 56°C, according to the used primer pairs (**Table 4**) for 15 seconds and extension at 72°C for 30 seconds

Table 4. List of oligonucleotide primers used in this study.

Primer name	Primer Sequence (5'-3')	Target Species / DNA	Target Gene	Size (bp)	Reference
PhPisA - F	TGCTGGTGGTGTATTCTGGG	*Photobacterium damselae* subsp. *piscicida*	SNP at position 121	130	(Carraro et al., 2018)
PhPisA - R	GTCAACTAGACGATCAATTTCAGTT				
PhPisB - F	TGCTGGTGGTGTATTCTGGG			148	
PhPisB - R	AACAGGTGTCGCATCAACGT				
Car - F	GCTTGAAGAGATTCGAGT	*Photobacterium damselae* subsp. *damselae*	16SrRNA	unknown	(Trevisani et al., 2017)
Car - R	CACCTCGCGGTCTTGCTG				
hdcPdd - F	GGATTAGCGCATGGATTGGT		hdcPdd	unknown	
hdcPdd - R	AACGCCTAAGAAACCCCACA				
dlts - F	AAGTACATGCTGATCAAGT	*Streptococcus agalactiae*	Dlts	952	(Alazab et al., 2022)
dlts - R	TCTTGATCAACTTGTTGTAC				
SAGA - F	GAGTTTGATCATGGCTCA G		16S rRNA	200 - 220	(Diyie et al., 2022)
SAGA - R	ACCAACATGTGTTAATTACTC				
sim - F	TAAAGCATTAGAAGCGGCTAAGAAAGAAG	*Streptococcus iniae*	simA	239	(Wang et al., 2023)
sim - R	CAATAGTTGCTTCAAGTTCTGCTTTTTCA				
SINIAE - F	AAGGGGAAATCGCAAGTGCC		16S rRNA	1100	(Diyie et al., 2022)
SINIAE - R	ATATCTGATTGGGCCGTCTAA				
OrpoB - F	GCAAAGACAGACATGACG	*Mycobacterium marinum*	rpoB	unknown	(Feng et al., 2023)
OrpoB - R	ATGTTGTCCTTCCAGGGT				
IrpoB - F	CCGAGTTCATCAACAACACG			unknown	
IrpoB - R	GTGTTGTCCTTCCAGCGT				
AgryB - F	GCCGAGCCCGACCATCTTCAG	*Aeromonas* spp.	gyrB	875	(Zhang et al., 2014)
AgryB - R	AGATCATCTTGTCGAAACGGGC				
VrpoA - F	AAATCAGGCTCGGGCCCT	*Vibrio* spp.	rpoA	524	
VrpoA - R	ATGTAGTGAATCGCTTCTGCTTT				
etfD - F	GGTAACCTGATTTGGCGTTC	*Edwardsiella tarda*	etfD	445	(Castro et al., 2014)
etfD - R	GGATCACCTGGATCTTATCC				

Table 4. List of oligonucleotide primers used in this study (continued).

Primer name	Primer Sequence (5'-3')	Target Species / DNA	Target Gene	Size (bp)	Reference
16S - 27F	AGAGTTTGATCMTGGCTCAG	Procaryotic organisms	16S	490	(Lane, 1991)
16S - 517R	ATTACCGCGGCTGCTGG				(Muyzer, 1993)
CG - 16S - 358F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG	Procaryotic organisms	16S	159	
16S - 517R	ATTACCGCGGCTGCTGG				
KLJ1 - F	CCACATCTAAGGAAGGCAGC	*Cryptosporidium* spp.	18S rRNA	1056	(Shaapan et al., 2022)
KLJ2 - R	ATGGATGCATCAGTGTAGCG				
CRY18sS1 - F	TAAACGGTAGGGTATTGGCCT		SSU rRNA	240	(Bairami et al., 2018)
CRY18sAs1 - R	CAGACTTGCCCTCCAATTGATA				
EIF1 - F	GCTTGTCTCAAAGATTAAGCC	*Eimeria* spp.	18S rRNA	unknown	(Gibson-Kueh et al., 2011)
EIR3 - R	ATGCATACTCAAAAGATTACC				
EIF3 - F	CTATGGCTAATACATGCGCAATC			unknown	
EIR3 - R	ATGCATACTCAAAAGATTACC				
18E - F	CTACGGAAACCTTGTTACG	*Goussia clupearum*	18S rDNA	unknown	(Friend et al., 2016)
Coc2r - R	CTTTCGCAGTAGTTCGTC				
Coc1f - F	GATTAATAGGGACAGTTG			1000	
18R - R	CTACGGAAACCTTGTTACG				
18E - F	CTGGTTGATCCTGCCAGT			1000	
Gclup2r - R	AGGAGAAGTCGGAGAGACG				
ERIB1 - F	ACCTGGTTGATCCTGCCAG	*Goussia janae*	SSU rDNA	1756	(Jirků et al., 2002)
ERIB10 - R	CTTCCGCAGGTTCACCTACGG				
GJ574 - F	GCAAGTCTGGTGCCAGC			unknown	
ERIB10 - R	CTTCCGCAGGTTCACCTACGG				
GMSSU - F	GAAACTGCGAATGGCTCATT	*Goussia metchnikovi*	SSU rDNA	1524	(Jirků et al., 2009)
GMSSU - R	CTTGCGCCTACTAGGCATTC				

2.5. Agarose Gel Electrophoresis

The DNA products obtained by PCR (5 µL) were resolved by agarose gel electrophoresis (at 1% or 1.2% concentration) for 30 minutes at 100V in 1xTAE (40mM Tris, 20mM Acetic Acid, 1mM EDTA, ph 8.3), and visualized under UV light on GelDoc (BioRad) after staining with 4 µL of GreenSafe Premium (nzytech) per 100 mL of agarose. The Termo Scientific GeneRuler DNA Ladder Mix, with a 10 - 10000bp range, was used as a molecular marker. The gel electrophoresis results were photographed.

2.6. Zebrafish eggs and larvae maintenance

Zebrafish eggs were obtained by pairwise mating of zebrafish adults, collected and raised at 28°C with natural photoperiod, i.e., 14 hours of light and 10 hours of darkness in egg water containing 0.06 g of Instant Ocean®Sea Salts per liter of H_2O. Larvae from 5 days-post-fertilization or above were distributed in 6-well plates containing 10 larvae, per well, in 5 mL of egg water and fed twice a day. Egg water was daily replaced following the second feeding. Larvae with visible abnormalities were retrieved from the wells and replaced by apparently healthy larvae kept under the same egg water and feeding conditions. At the end of the challenge assay, larvae were euthanized via an overdose of tricaine.

2.7. Zebrafish larvae challenge assay

2.7.1. Determination of bacterial concentration

The first step taken to prepare the zebrafish larvae challenge assay consisted in determining the concentration of bacterial cultures by measuring their optical density at 600 nm (OD600) and determining the corresponding colony forming units (CFUs). Bacterial cultures were previously grown under the conditions described in 2.2., followed by their inoculation in 25 mL of BHI medium and growth at 28°C for 48 hours with agitation.

To determine the number of CFUs of each bacterial culture, serial dilutions were prepared as follows: the first Eppendorf tube was filled with 500 µL of bacterial culture, while the remaining 7 were filled with 450 µL of Bott and Wilson (BW) salts: 1.24% K2HPO4, 0.76% H2PO4, 0.1% trisodium citrate, 0.6% [NH4]2SO4, pH 6.7. The serial dilutions consisted in retrieving 50 µL from the first tube and add them to the second, repeating this process from each previous tube to the next. Finally, 8 solutions, each 10 times more diluted than the previous one, were obtained. Following this step, a petri dish containing BHI medium was divided in 8 sections, each destined to the addition of three 10 µL droplets retrieved from the dilution tubes, allowing the growth of bacterial colonies.

After an incubation at 28°C for 24 hours, the petri dish section revealing the best results regarding the growth of single colonies was selected, and the number of single colonies resulting from each pipetted droplet was counted. The mean of single colonies obtained was calculated taking into consideration the dilution of the tube from which such colonies came from and the volume in µL that was plated.

2.7.2. Infection of zebrafish larvae

Bacterial cultures were prepared as previously described in 25 mL of BHI medium, grown at 28°C for 48 hours with agitation, concentrated by centrifugation during 10 min at 10000rpm and finally resuspended in 2.5 mL of 1X Phosphate-buffered saline (PBS) buffer. Ten zebrafish larvae were distributed into 6-well plates containing 5 mL of egg water and 2.0 g/L salts. Different concentrations of each bacterium were added to each well. A negative control group was infected with 1X PBS. Zebrafish larvae were fed immediately after infection and plates were kept at 28 ºC. The cumulative mortalities were registered, and the infection was washed off after larvae reached 50-70% mortalities. Biological replicates were performed with the same bacterial concentrations and infection time.

2.8. Sequencing

The PCR products of interest for the aim of this work were sequenced by the Sanger method, in two directions (direct and reverse), using the respective primers, by the company STABVIDA (Caparica, Portugal). The Bioinformatics resource BLAST of the GenBank nonredundant (nr) nucleotide database (http://www.ncbi.nlm.nih.gov) was used to analyse the sequencing data.

3. Results and discussion

This project started by an extensive literature review to select oligonucleotide primers described as detecting efficiently and specifically the parasitic and bacterial taxa under study. The data collection from PubMed® was accomplished by searching the target species and following terms: “molecular identification”, “detection by molecular methods” and “molecular tools”. The resulting primer pairs retrieved from the literature for detection of both parasite and bacterial species used in this study, are presented in **Table 4**.

3.1. Parasite detection

3.1.1. Histological evaluation

The attempt to develop a multiplex PCR able to identify important parasite species to the aquaculture sector was preceded by a histological assessment of field GI samples from fish parasitized by known coccidia genera. This step served as training for a future histological evaluation of stomach and intestine samples from fish showing signs of parasitic infection, whose infectious agent was unknown. The identification of relevant histological findings characteristically related to a coccidiosis are presented below.

Figure 1 shows a *Eimeria* spp. and/or *Goussia* spp. infection affecting the intestine of sea bream and sea bass. Using only classical methods, namely histology, it is very difficult to differentiate these two genera since their development stages are very similar. Such distinction can only be achieved recurring to molecular methods. Although indistinguishable, some histological findings are characteristically related to an *Eimeria* spp. and/or *Goussia* spp. infection. As mentioned before, in the gamogony phase of these parasites’ life cycle, merozoites become female macrogametocytes (macrogamonts) or male microgametocytes (microgamonts) (Cruz-Bustos et al., 2021). A characteristic histological finding during an *Eimeria* spp. and/or *Goussia* spp. infection is, for example, the presence of easily recognizable round bodies corresponding to different forms of the same parasite species, namely micro- and macrogamonts, in the luminal and epicellular spaces of the intestine (indicated in **Figure 1: A**, **B** and **C** through arrows and arrowheads, respectively). Another characteristic is the presence of sporulated oocysts, each containing four spores, in the intracellular space of the enterocytes (indicated in **Figure 1: D** surrounded by a circle). Regarding the histological evaluation of the presented samples and considering **Table 2** as guideline, the intensity of coccidia infection in the four optical fields presented in **Figure 1** was classified between moderate (**A**, **B** and **C**) and low (**D**).

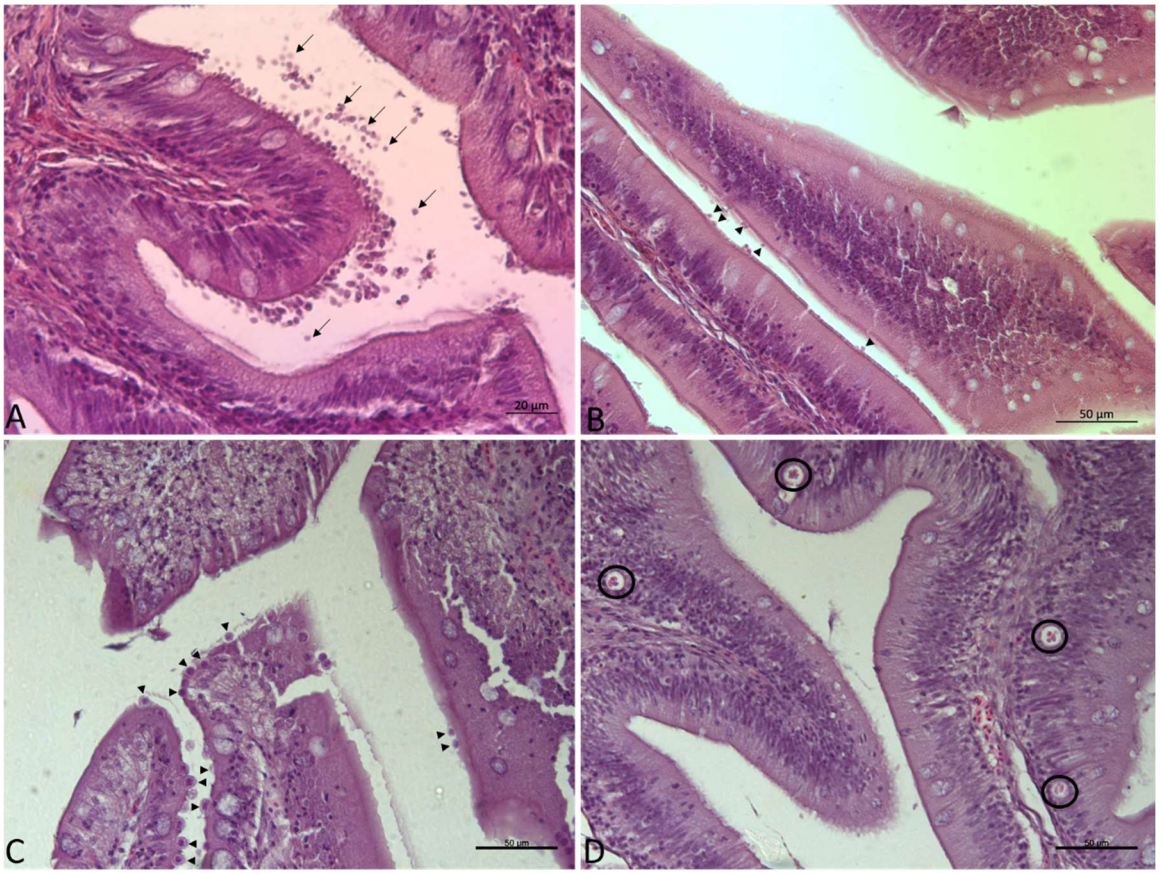

Figure 1. *Eimeria* spp. and/or *Goussia* spp. in the intestine of sea bream (A, B) and sea bass (C, D); parasitic forms occupy different spaces of the intestine: luminal (arrows), epicellular (arrowhead) or intracellular (circles). H-E.

Figure 2 shows a *Cryptosporidium* spp. infection affecting the stomach of sea bass. Infection with this parasite is usually detected by the presence of small structures in the luminal and epicellular spaces of the stomach (indicated in **Figure 2: A** and **B**, through arrows and arrowheads, respectively), namely micro- and macrogamonts. Besides that, the presence of clusters of oocysts is characteristically found in the intracellular spaces of the stomach during *Cryptosporidium* spp. infections (indicated in **Figure 2: A and B**, surrounded by a circle). Regarding the histological evaluation of the presented samples and considering **Table 2** as guideline, the intensity of coccidia infection in the two optical fields presented in **Figure 2** was classified as moderate.

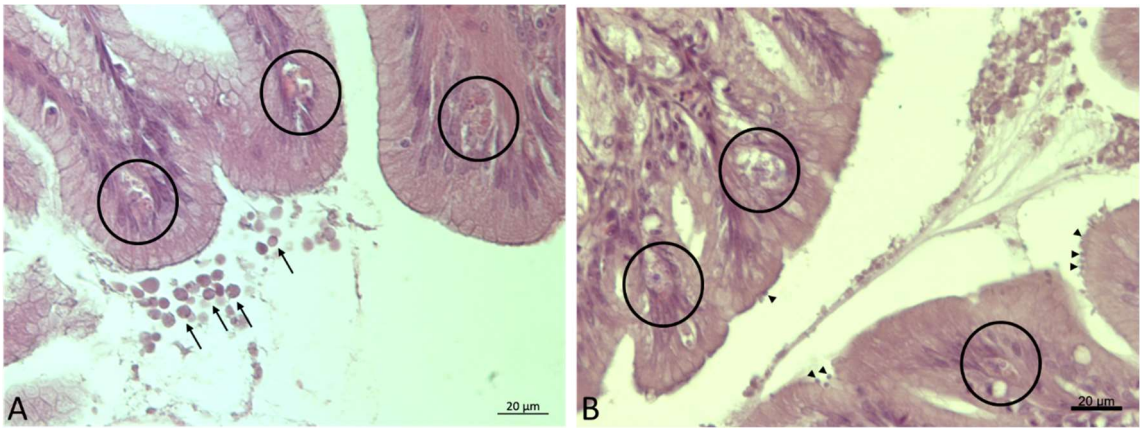

Figure 2. *Cryptosporidium* spp. in the stomach of sea bass; parasitic forms occupy different spaces of the stomach: luminal (arrows), epicellular (arrowhead) or intracellular (circles). H-E.

3.1.2. Primers' specificity and efficiency testing

A given primer pair is considered to be specific for the detection of their target species if the absence of amplicons when performing PCR reactions using DNA from non-target species is verified. On the other hand, such primer pair is considered to be efficient if it reveals the ability to identify their target species using their previously extracted DNA. Although the expected molecular weight of the target amplicons was described for the majority of primer pairs, there were some cases, namely primer pairs EIF1-F/EIR3-R, EIF3-F/EIR3-R, 18E-F/Coc2r-R and GJ574-F/ERIB10-R in which this information was not provided in the original manuscript. *In silico* analysis of each primer sequence could have answered that question, but instead we opted to perform PCR amplification in all cases, with the aim of analyzing the resulting PCR bands by Sanger sequencing.

The first step towards the development of a multiplex PCR targeting different aquaculture parasites consisted in simultaneously test the specificity of all primer pairs retrieved from literature (**Table 4**) and the efficiency of primers targeting *Goussia clupearum*, since these were the first available parasitic DNA samples, obtained from liver, stomach and intestine of *Sardina pilchardus* and *Trisopterus luscus* (**Table 1**). The results of these tests are shown in **Figures 3 to 6**.

Primer pairs KLJ1/KLJ2 and CRY18sS1/CRY18sAs1, targeting *Cryptosporidium* spp., were able to generate amplicons of the expected molecular weight, respectively ~1056 and ~240bp, corresponding to the detection of the target genus in DNA samples 4 and 5 (**Figure 3 - A and B**).

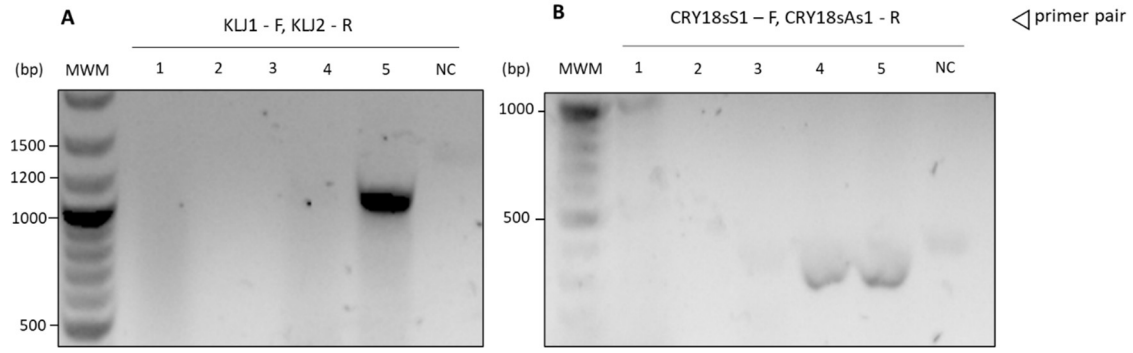

Figure 3. Agarose gel (1%) electrophoresis of the PCR amplification of *Goussia clupearum* DNA samples (1-5) evaluating primer pairs **(A)** KLJ1 - F, KLJ2 – R and **(B)** CRY18sS1 – F, CRY18sAs1 – R specificity, supposedly targeting *Cryptosporidium* spp. Water was used as a negative control (NC). MWM, molecular weight marker.

Although all DNA samples used in these particular specificity tests belonged to *Goussia clupearum*, similar to the above-mentioned results were obtained when testing other primer pairs, that is, their ability to amplify *Goussia clupearum* DNA though targeting other coccidia species. Primers EIF1/EIR3, targeting *Eimeria* spp., produced an amplicon of ~450bp in DNA sample 4 (**Figure 4**). As well as primers ERIB1/ERIB10, in DNA sample 5, originating an amplicon of ~1750bp, corresponding to the expected molecular weight when targeting *Goussia janae*. Primer pair GJ574/ERIB10, also having been retrieved as able to target the DNA of *Goussia janae*, produced amplicons of ~1200 - 1500 bp in DNA samples 1, 4 and 5 (**Figure 5 – A and B**).

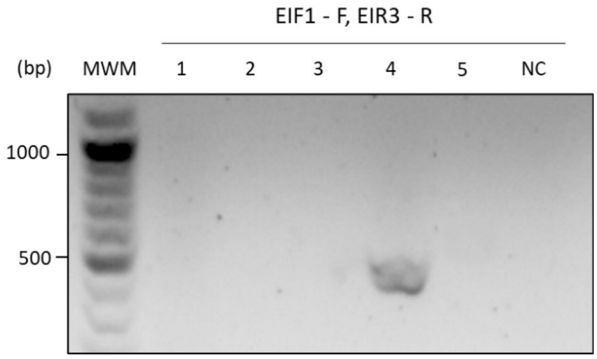

Figure 4. Agarose gel (1%) electrophoresis of the PCR amplification of *Goussia clupearum* DNA samples (1-5) evaluating primer pair EIF1 – F, EIR3 – R specificity, supposedly targeting *Eimeria* spp. Water was used as a negative control (NC). MWM, molecular weight marker.

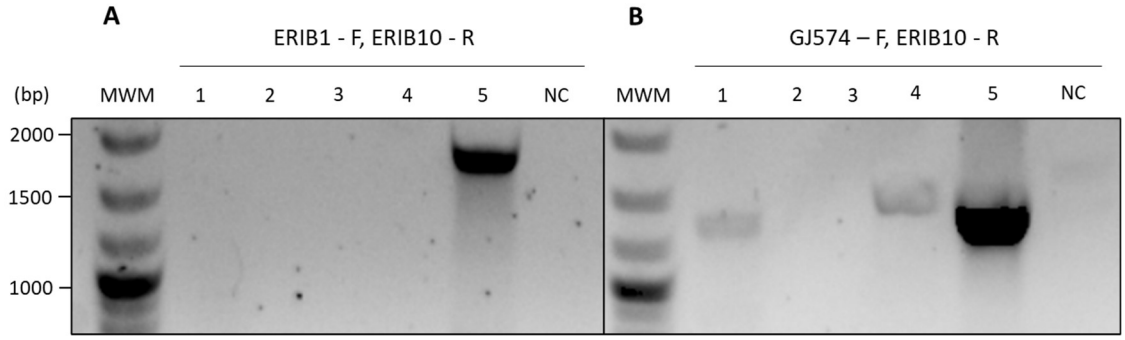

Figure 5. Agarose gel (1%) electrophoresis of the PCR amplification of *Goussia clupearum* DNA samples (1-5) evaluating primer pairs **(A)** ERIB1 - F, ERIB10 – R and **(B)** GJ574 – F, ERIB10 – R specificity, supposedly targeting *Goussia janae.* Water was used as a negative control (NC). MWM, molecular weight marker.

According to **Table 1**, DNA sample 1 corresponded to *Goussia clupearum* DNA extracted from parasitized liver of *Sardina pilchardus* and DNA samples 4 and 5 corresponded to *Goussia clupearum* DNA extracted from parasitized liver and intestine of *Trisopterus luscus*, respectively. There are two possible explanations considering the above-mentioned eletrophoresis results. The first hypothesis is that the primers in question are not specific regarding the detection of their target species, being able to identify other coccidia genera and species, such as *Goussia clupearum*. The second one is that, even though the DNA samples supposedly correspond to *Goussia clueparum* DNA, the fact that these were extracted from parasitized organs from the GI tract of infected fish does not exclude the hypothesis that such infection was not strictly caused by *Goussia clupearum*, but is rather resultant of a synergistic action of different coccidia, including *Cryptosporidium* spp., *Eimeria* spp. and other species belonging to the genus *Goussia* spp.. Moreover, during the organization of the results, an error was detected regarding the selection of primer pair GJ574-F/ERIB10-R from literature, since these primers targeted the SSU rDNA gene of genus *Choleoeimeria* spp., not the species *Goussia janae*. Notwithstanding, the amplification of *Goussia clupearum* DNA samples with such primers originated amplicons, further corroborating the above-mentioned hypotheses.

To effectively validate these hypotheses, further efficiency tests including DNA samples from the primers' target species as well as sequencing of the resultant PCR products would have had to be performed to confirm the primers' ability to detect other species besides their target.

Regarding the efficiency tests performed using three primer pairs retrieved from literature as primers used in the molecular analysis of *Goussia clupearum* DNA samples, the results are shown in **Figure 6**. Primer pair 18E-F/Coc2r-R (**Figure 6 - A**) originated two molecular bands in DNA samples 4 and 5, although in the study performed by (Friend et al., 2016), the same primers were not capable of amplifying the target DNA. Primer pair

Coc1f-F/18R-R (**Figure 6 - B**) generated molecular bands with molecular weights between 1100 and 1350 bp in DNA samples 3, 4 and 5. Since being able to detect molecular bands with a molecular size close to what was expected, this primer pair would have been a good candidate to move forward in the development of the multiplex PCR. Following the confirmation of their efficiency, specificity tests would have been performed to validate their suitability as a good candidate to be included in the multiplex assay. Primer pair 18E-F/Glup2r-R, in turn, was unable to detect their target species, reveling its inefficiency in the detection of *Goussia clupearum*.

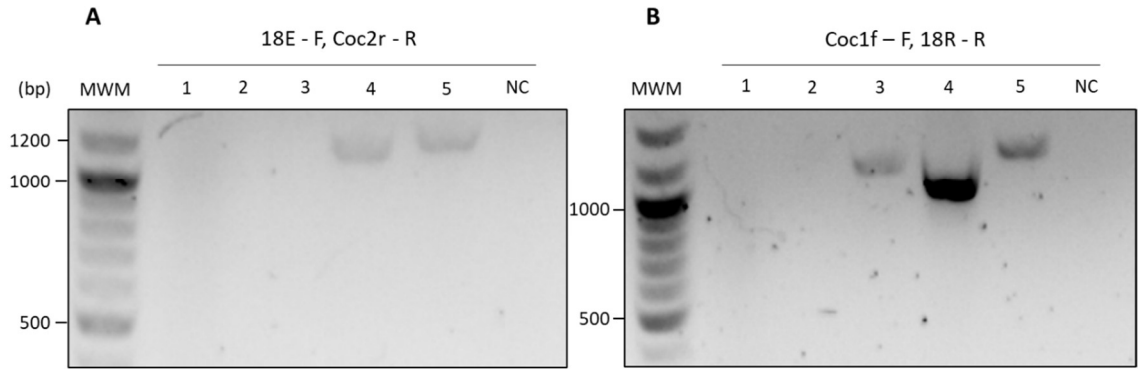

Figure 6. Agarose gel (1%) electrophoresis of the PCR amplification evaluating primer pairs **(A)** 18E – F, Coc2r – R and **(B)** Coc1f – F, 18R - R efficiency targeting *Goussia clupearum* (1-5). Water was used as a negative control (NC). MWM, molecular weight marker.

Lastly the absence of molecular bands in the specificity results regarding primer pairs EIF3/EIR3, targeting *Eimeria* spp. and GMSSU-F/GMSSU-R, targeting *Goussia metschnikovi* confirmed their suitability for the following steps regarding the development of a mPCR, namely testing their efficiency regarding detecting their target species, had it been possible to obtain the corresponding control DNA samples to validate their specificity.

In spite of several efforts, the inability to obtain DNA samples from different parasite species with relevance to the development of the present work at an appropriate time for its completion forced the adjustment of this study's initial purpose.

3.2. Bacterial detection

3.2.1. Primers' efficiency testing

The first step towards the second goal of this work consisted of testing the efficiency of each pair of primers presented in **Table 4**. Once again, the expected molecular weight of the target amplicons was described for the majority of primer pairs, with the exception of primers Car, hdcPdd, OrpoB and IrpoB. As above-mentioned, although *in silico* analysis of each primer sequence could have answered that question, we opted to

perform PCR amplification in all cases, and analyze the resulting PCR bands (**Figure 7**) by Sanger sequencing. All but OrpoB and lrpoB primer pairs originated a single amplicon when used for amplification of DNA isolated from the target pathogens (**Figure 7**).

Amplicons of ~100bp were obtained for *Photobacterium damselae* subsp*. piscicida* with primer pairs PhPisA and PhPisB (**Figure 7**) and further sequencing of the obtained molecular bands confirmed the identification of *Photobacterium damselae* subsp*. piscicida* **(Supplementary Table S1)**.

Photobacterium damselae subsp*. damselae* originated amplicons of ~250bp when amplified with primer pair Car and of ~350bp when amplified with primer pair hdcPdd (**Figure 7**). Sequencing of the obtained molecular bands confirmed the identification of *Photobacterium damselae* subsp*. damselae* when using primer pair hdcPdd. Sequencing results regarding primer Car, however, revealed its inability to distinguish the subspecies *Photobacterium damselae* subsp*. damselae*, identifying only at the species level *Photobacterium damselae* **(Supplementary Table S1)**. Primer Car was retrieved from the literature as able to identify *Photobacterium damselae* subsp*. damselae,* but the fact that the primer's target is the gene 16S rRNA (present in all prokaryotes) and that the PCR conditions were not the ideal ones for this specific primer pair, but rather adapted to all the primers used in the study, allowing the further development of a multiplex PCR, might explain the obtained results upon sequencing. Regardless, this step being at a very early stage of the developed work, it was decided to proceed with the use of primer Car.

A PCR band of ~200bp was obtained for *Streptococcus iniae* with primer pair sim and a much higher molecular weight band (~1100bp) when amplified with primer pair SINIAE (**Figure 7**). The same was observed for *Streptococcus agalactiae,* originating a ~200bp band when amplified with primer pair SAGA and a ~700bp band when using primer pair dlts (**Figure 7**). Sequencing of the obtained molecular bands corresponding to *Streptococcus iniae* and *Streptococcus agalactiae* confirmed the identification of these species **(Supplementary material Table S1)**. On contrary, neither of the primer pairs targeting *Mycobacterium marinum* (OrpoB and lrpoB) were able to identify its presence in a DNA sample extracted from a *M. marinum* culture (**Figure 7**). That being the case, an attempt to confirm the *M. marinum* DNA's integrity was conducted through a new PCR reaction targeting the 16S rRNA gene, using primers 16S-27F and 16S-517R (**Table 4**), and the results are shown **in Figure 8**. A 1500bp PCR band was obtained corresponding to *M. marinum* 16S rRNA gene. Due to the inability to amplify *M. marinum* specific

amplicons (excluding the 16S rRNA gene) and in the absence of alternative primer pairs, *M. marinum* detection was not further pursued in this work.

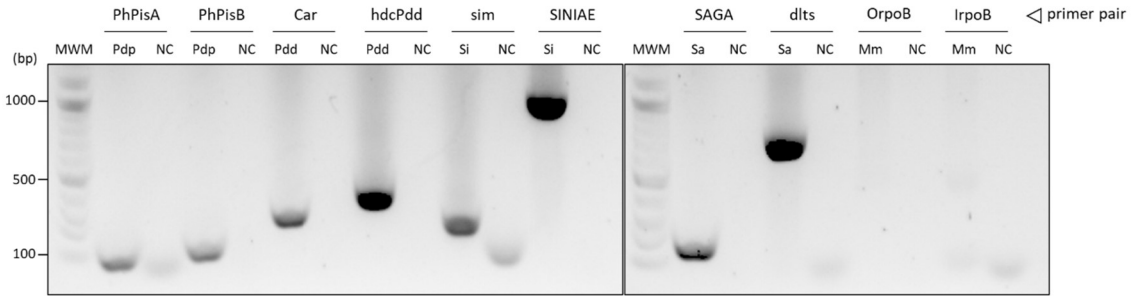

Figure 7. Agarose gel (1%) electrophoresis of the PCR amplification evaluating primer pairs efficiency regarding targeting species *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si), *Streptococcus agalactiae* (Sa) and *Mycobacterium marinum* (Mm). Water was used as a negative control (NC). MWM, molecular weight marker.

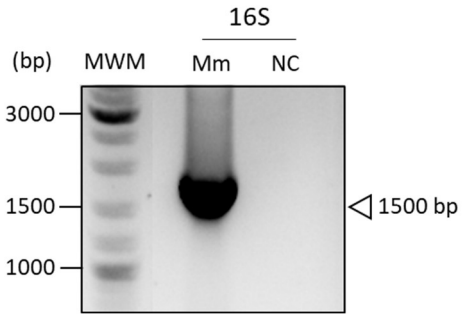

Figure 8. Agarose gel (1%) electrophoresis of the PCR reaction performed to confirm the integrity of *Mycobacterium marinum*'s (Mm) DNA. Water was used as a negative control (NC). MWM, molecular weight marker. The molecular weight of the obtained PCR band is indicated at the right of the figure in bp.

3.2.2. Primers' specificity testing

Considering the overall obtained results regarding the tested primers' efficiency, primers PhPisB, hdcPdd, SINIAE and dlts were selected for the next step of the present work. This step consisted in executing new PCR reactions testing their specificity towards their target species, respectively, *P. damselae* subsp. *piscicida, P. damselae* subsp. *damselae, S. iniae* and *S. agalactiae*.

Regarding the first round of specificity tests, the results are shown in **Figure 9**, only the primer pair dlts proved to be specific for the detection of *S. agalactiae*. Primer pair PhPisB, despite being efficient for the detection of the target species, *P. damselae* subsp. *piscicida,* originated non-specific bands in every other species, including a band of the expected molecular size in the species *P. damselae* subsp. *damselae*, revealing itself unable to distinguish the target species from the latter. Regarding the primer pair SINIAE, although less intense and of different molecular sizes, also originated non-specific bands in every species besides the target *S. iniae* (**Figure 9**).

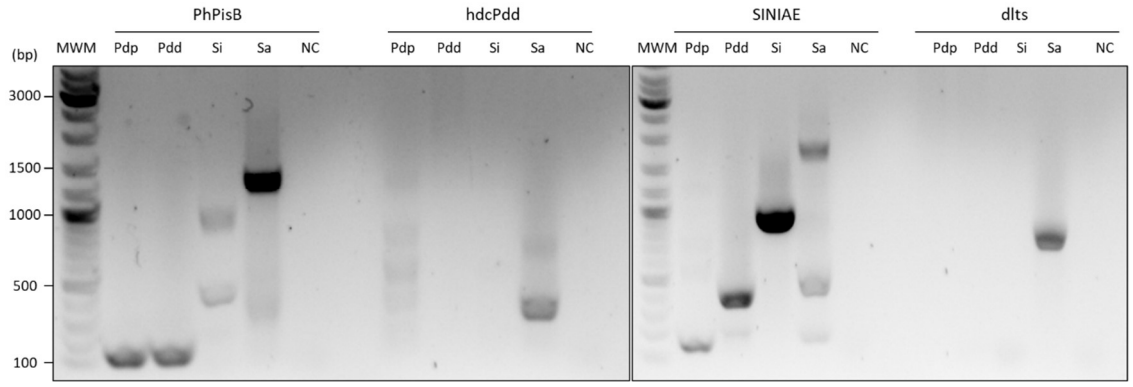

Figure 9. Agarose gel (1%) electrophoresis of the PCR amplification evaluating primer pairs PhPisB, hdcPdd, SINIAE and dlts specificity when targeting *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si) and *Streptococcus agalactiae* (Sa), respectively. Water was used as a negative control (NC). MWM, molecular weight marker.

In spite of the encouraging efficiency results, primer hdcPdd was unable to identify the target species, *P. damselae* subsp. *damselae*, in the specificity tests, only generating one non-specific band identifying *S. agalactiae*. Therefore, it was decided to repeat the PCR reaction to evaluate the specificity of primer hdcPdd (**Figure 10**). The results of the second attempt at testing these primers' specificity corroborated their efficiency in the identification of the target-species, however, also generated, although less intense and distinct in molecular size, non-specific bands targeting *P. damselae* subsp. *piscicida* and *S. agalactiae*.

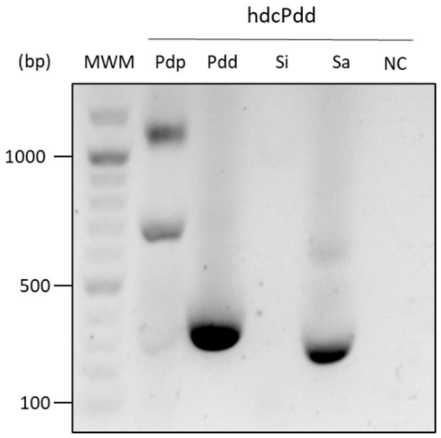

Figure 10. Agarose gel (1%) electrophoresis of a second PCR amplification evaluating primer pair hdcPdd specificity when targeting *Photobacterium damselae* subsp. *damselae* (Pdd). Water was used as a negative control (NC). MWM, molecular weight marker.

Since most of the selected primer pairs revealed to be non-specific to each supposedly target-species, the remaining primer pairs were also tested regarding their specificity (**Figure 11**). Primers PhPisA and Car, targeting *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* respectively, recognized both species originating same-sized bands, preventing their distinction, which was already expected from primer pair

Car following the above-mentioned sequencing results. Primer pair sim recognized every tested species, generating numerous and intense non-specific bands, which led to its retrieval from the present study. Finally, primers SAGA were able to correctly identify *S. agalactiae*, though originating two non-specific almost negligible bands recognizing *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*.

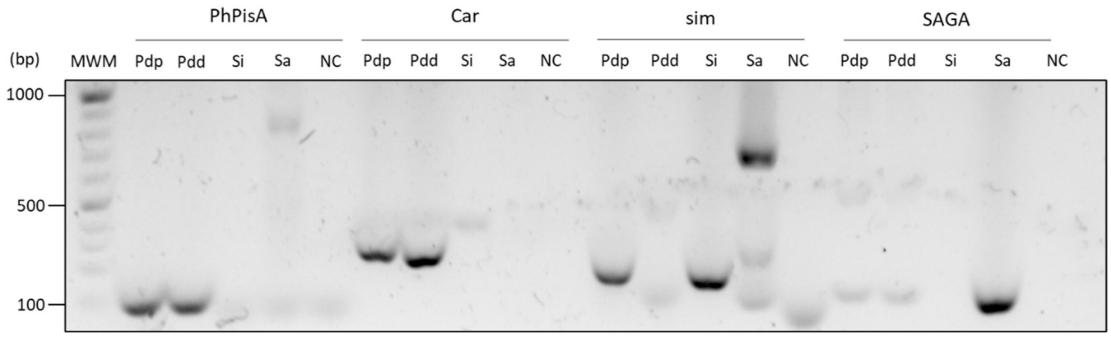

Figure 11. Agarose gel (1%) electrophoresis of the PCR amplification evaluating primers PhPisA, Car, sim and SAGA specificity when targeting *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si) and *Streptococcus agalactiae* (Sa), respectively. Water was used as a negative control (NC). MWM, molecular weight marker.

Since the main purpose of this study consisted on the development of a multiplex PCR able to simultaneously identify different target-species using distinct primers in a single reaction, all primers must be able to maintain their efficiency and specificity in mixed reactions under the exact same conditions, which may require various optimization steps.

Up until this point, all PCR reactions were carried out using a 50°C annealing temperature, either because it followed the PCR conditions used by the authors of the manuscripts from which each primer sequence was acquired or because it resembled the ideal annealing temperature calculated from the melting temperature of the primer pairs. With the aim of trying to exclude the less intense non-specific bands generated by efficient primer pairs, new PCR reactions were repeated maintaining all PCR conditions except for the annealing temperature, which was raised to 52°C. The results of the above-mentioned specificity tests are shown in **Figures 12** and **13.**

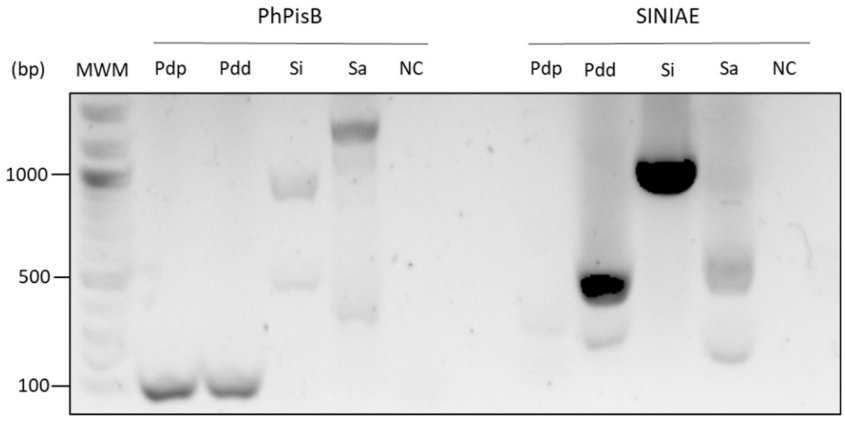

Figure 12. Agarose gel (1%) electrophoresis of the PCR amplification evaluating primer pairs PhPisB, and SINIAE specificity when targeting *Photobacterium damselae* subsp. *piscicida* (Pdp) and *Streptococcus iniae* (Si), respectively. The PCR reactions were carried out at an annealing temperature of 52°C. Water was used as a negative control (NC). MWM, molecular weight marker.

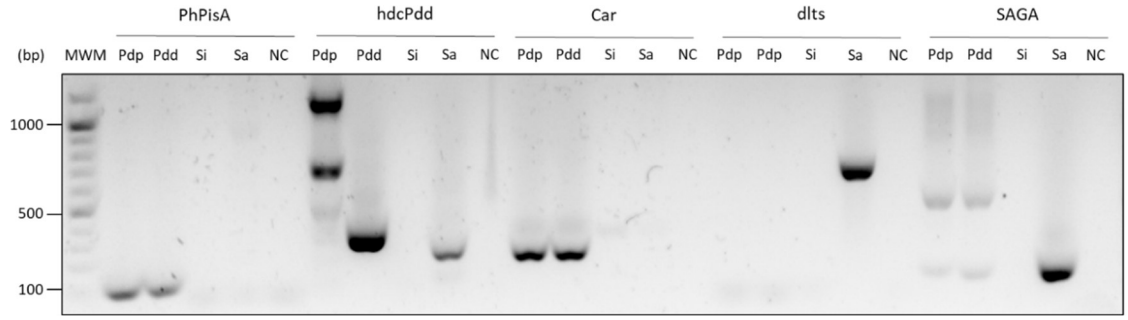

Figure 13. Agarose gel (1%) electrophoresis of the PCR amplification evaluating the specificity of the following primer pairs: primer pair PhPisA when targeting *Photobacterium damselae* subsp. *piscicida* (Pdp), primer pairs hdcPdd and Car when targeting *Photobacterium damselae* subsp. *damselae* (Pdd) and primer pairs dlts and SAGA when targeting *Streptococcus agalactiae* (Sa). The PCR reactions were carried out at an annealing temperature of 52°C. Water was used as a negative control (NC). MWM, molecular weight marker.

Through their observation it is possible to infer that a slight raise in the PCR's annealing temperature improved the specificity of primers PhPisA and PhPisB, targeting *P. damselae* subsp. *piscicida*, as the results show the disappearance and an intensity decrease of the non-specific bands, respectively, except for the band regarding the detection of *P. damselae* subsp. *damselae*. Due to the better results obtained by primers PhPisA, primer pair PhPisB was excluded from the steps involved in the continuation of the present study.

Primer pair Car, targeting *P. damselae* also showed better specificity results. Only primer pair hdcPdd, targeting *P. damselae* subsp. *damselae*, though still resulting in relatively intense non-specific bands, remained able to detect both *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* through different-sized molecular bands. Since three out of four tested primers revealed to be unable to differentiate *P. damselae* subsp. *piscicida* from *P. damselae* subsp. *damselae*, primer pair hdcPdd distinguished itself as an adequate primer for the development of the potential multiplex PCR.

The primer pair targeting *S. iniae*, SINIAE, also showed slightly better specificity results as the annealing temperature was raised, which was equally observed with primers SAGA, targeting *S. agalactiae*. Overall, primer pair dlts maintained the best specificity results only detecting the target species regardless of the annealing temperature used in the PCR reaction.

Since most primers remained non-specifically detecting non-target species, showing slight improvements following an increment of 2°C in the annealing temperature of the original PCR conditions, a second optimization step consisted in testing another raise in annealing temperature, this time, to 54°C. Results are shown in **Figures 14** and **15**.

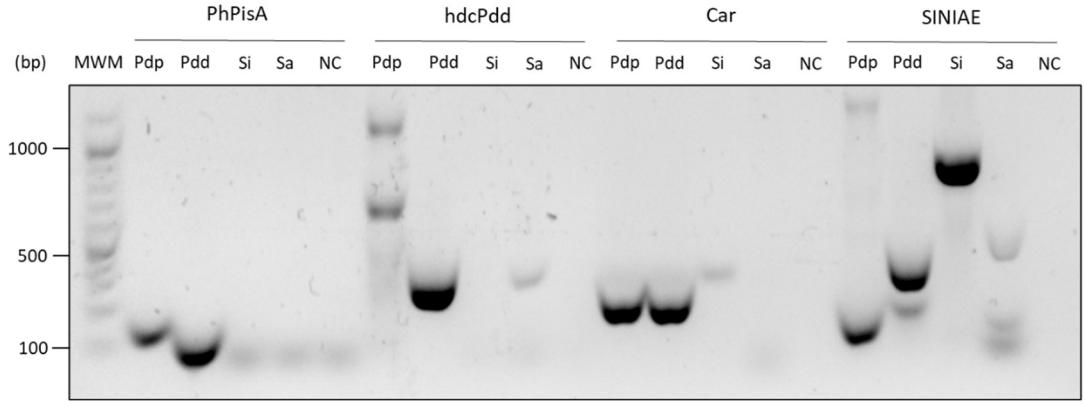

Figure 14. Agarose gel (1%) electrophoresis of the PCR amplification evaluating the specificity of the following primer pairs: primer pair PhPisA when targeting *Photobacterium damselae* subsp. *piscicida* (Pdp), primer pairs hdcPdd and Car when targeting *Photobacterium damselae* subsp. *damselae* (Pdd) and primer pair SINIAE when targeting *Streptococcus iniae* (Si). The PCR reactions were carried out at an annealing temperature of 54°C. Water was used as a negative control (NC). MWM, molecular weight marker.

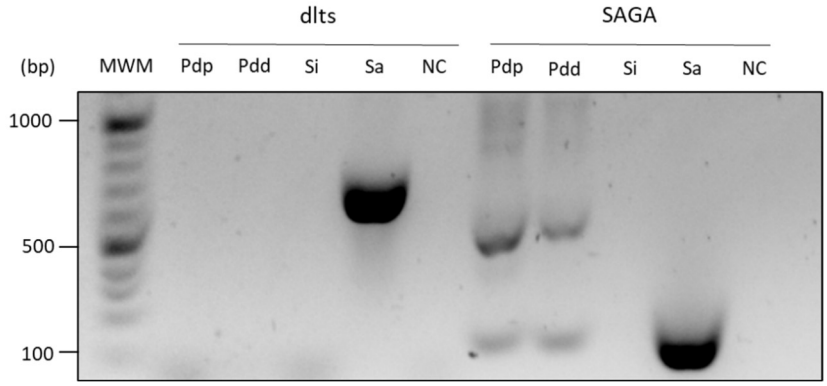

Figure 15. Agarose gel (1%) electrophoresis of the PCR amplification evaluating primer pairs dlts and SAGA specificity when targeting *Streptococcus agalactiae* (Sa). The PCR reactions were carried out at an annealing temperature of 54°C. Water was used as a negative control (NC). MWM, molecular weight marker.

Primer pair PhPisA, targeting *P. damselae* subsp. *piscicida*, showed worst results than those obtained with a lower annealing temperature, reflected in the loss of definition of bands detecting *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* and the reappearance of non-specific bands detecting *S. iniae* and *S. agalactiae*.

Primers hdcPdd and Car achieved better results by the loss of intensity of non-specific molecular bands detecting species beyond *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*. At this point of the present work, in general, better results were obtained using higher annealing temperatures except for the performance of primers targeting *P. damselae* subsp. *piscicida* (PhPisA). Notwithstanding, primer pairs targeting *P. damselae* and *P. damselae* subsp. *damselae*, Car and hdcPdd, demonstrated the ability of simultaneously detecting *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* with the latter being able to differentiate the two species. Considering this and the purpose of establishing a multiplex PCR, the present work was continued taking into account that using the above-mentioned primers simultaneously guaranteed the detection and distinction of *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* in the same PCR reaction and retrieving primer pair PhPisA from this study.

Primer pair SINIAE, targeting *S. iniae*, maintained its inability to specifically detect Si, which equally resulted in its retrieval from the present work. Considering the fact that none of the other primer pairs showed the ability to detect *S. iniae*, this retrieval ceased the attempt to detect this species with the development of a multiplex PCR. Notwithstanding, it was decided to continue using *S. iniae* DNA as a negative control in the attempt to develop the desired molecular tool.

Between primers dlts and SAGA, targeting *S. agalactiae,* dlts showed the best specificity results using a higher annealing temperature. As a result, primers SAGA were also withdrawn from the development of the present work.

3.2.3. Multiplex PCR development

Having chosen three efficient and specific primer pairs to detect the target species, Car, hdcPdd and dlts, different combinations of these primers were used in the same reaction aiming to achieve a successful multiplex PCR reaction. For that purpose, each primer combination was tested against each target species, individually, i.e., *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae*, *S. iniae* and *S. agalactiae* and against a mix of the latter. Since primers Car showed a better performance at 52°C and hdcPdd at 54°C, both pairs were individually combined with dlts in a PCR reaction using the most adequate annealing temperature, accordingly. Results are shown in **Figure 16**.

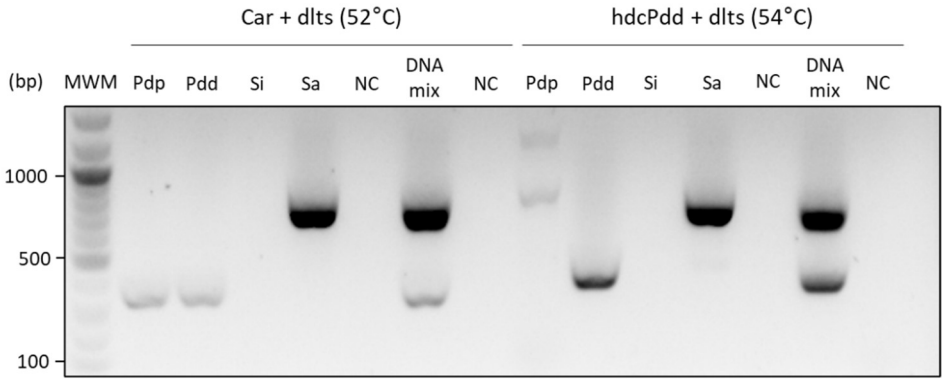

Figure 16. Agarose gel (1%) electrophoresis of the Multiplex PCR using primer pairs Car + dlts (annealing temperature of 52°C) that target *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd) and *Streptococcus agalactiae* (Sa) and primer pairs hdcPdd + dlts (annealing temperature of 54°C) that target *Photobacterium damselae* subsp. *damselae* (Pdd) and *Streptococcus agalactiae* (Sa). Multiplex PCR was done using individual DNA of each pathogen species under study, *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si) and *Streptococcus agalactiae* (Sa), or a mix of all DNAs in the same reaction (DNA mix). Water was used as a negative control (NC). MWM, molecular weight marker.

Using the primer pairs simultaneously made it possible to efficiently and specifically detect target species *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae* and *S. agalactiae* both individually and when the target DNA was mixed.

Since the development of multiplex PCR requires using a single PCR reaction following certain conditions, the next step consisted in the attempt to combine all primer pairs in one reaction, testing two distinct annealing temperatures, 52 and 54°C. This time, the combination of the three primer pairs, Car, hdcPdd and dlts, was tested against each target species individually and in a mix, using 52 or 54°C as the annealing temperature. Results are shown in **Figure 17**.

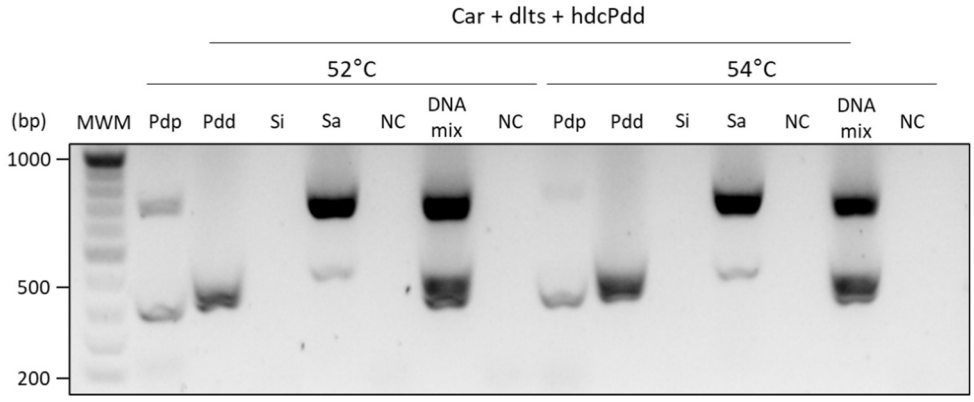

Figure 17. Agarose gel (1%) electrophoresis of the Multiplex PCR using primer pairs Car + dlts + hdcPdd, (at two annealing temperatures of 52°C or 54 °C) that target *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd) and *Streptococcus agalactiae* (Sa). The Multiplex PCR was done using individual DNA of each pathogen species under study, *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si) and *Streptococcus agalactiae* (Sa), or a mix of all DNAs in the same reaction (DNA mix). Water was used as a negative control (NC). MWM, molecular weight marker.

The primer combination revealed to be able to detect each target species regardless of the annealing temperature, although using an annealing temperature of 54°C revealed slightly better results. Nevertheless, the lack of definition of some of the expected molecular bands encouraged the repetition of the results' electrophoresis, this time, preparing a gel containing 1.2% agarose, in an attempt to promote a well-defined separation of the expected molecular bands. The new set of obtained results, shown in **Figure 18**, was well-succeeded, confirming that the combination of primers Car, hdcPdd and dlts, following a PCR reaction using an annealing temperature of 54°C is able to efficiently and specifically detect *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae* and *S. agalactiae*, originating well-demarcated molecular bands of the expected molecular size.

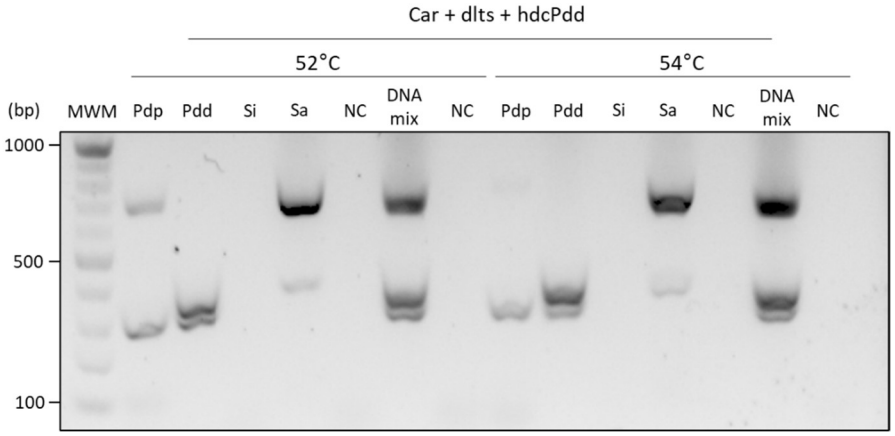

Figure 18. Agarose gel (1.2%) electrophoresis of the Multiplex PCR using primer pairs Car + dlts + hdcPdd, (at two annealing temperatures of 52°C or 54 °C) that target *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd) and *Streptococcus agalactiae* (Sa). The Multiplex PCR was done using individual DNA of each pathogen species under study, *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si) and *Streptococcus agalactiae* (Sa), or a mix of all DNAs in the same reaction (DNA mix). Water was used as a negative control (NC). MWM, molecular weight marker.

Another multiplex PCR, established in a previous work (Nunes, 2022), was used for the detection of *Aeromonas* spp., *Vibrio* spp. and species *Edwardsiella tarda* using primers AgryB, VrpoA and etfD, respectively. The PCR reaction in question was carried out under the same PCR conditions as the ones used in the present study, except for the annealing temperature, which was 56°C.

Considering the importance of developing a robust multiplex PCR to efficiently detect pathogens affecting aquaculture productions, an attempt to incorporate the established multiplex PCR in the one being developed was in the best interest of this work. If successful, such addition would allow an even more complete screening for pathogens. In order to achieve this goal, the first step consisted in testing the multiplex PCR being developed using a 56°C annealing temperature. With the aim of maintaining the quality

of the results, a 1.2% agarose percentage was kept in the preparation of the electrophoresis gel. The results are shown in **Figure 19** and reveal that the temperature raise did not interfere with the primers ability to efficiently and specifically detect the target species, a good sign towards the development of a more robust multiplex PCR.

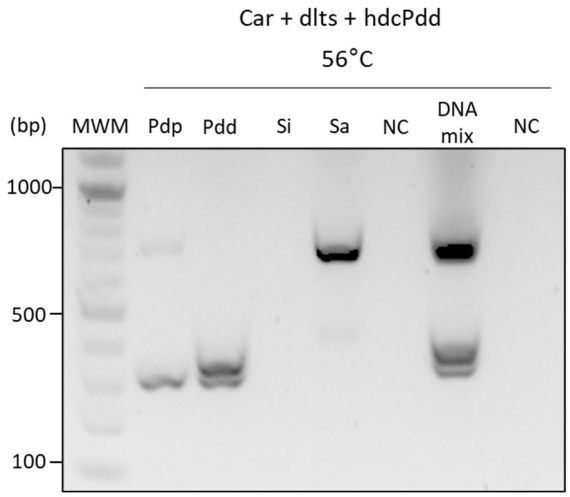

Figure 19. Agarose gel (1.2%) electrophoresis of the Multiplex PCR using primer pairs Car + dlts + hdcPdd, (at annealing temperature of 56°C) that target *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd) and *Streptococcus agalactiae* (Sa). The Multiplex PCR was done using individual DNA of each pathogen species under study, *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd), *Streptococcus iniae* (Si) and *Streptococcus agalactiae* (Sa), or a mix of all DNAs in the same reaction (DNA mix). Water was used as a negative control (NC). MWM, molecular weight marker.

3.2.4. Detection of bacterial pathogens by multiplex PCR in infected zebrafish samples

Having established the ideal PCR conditions and in order to make the most of the limited time for this study, it was decided to proceed with the testing of zebrafish larvae's extracted DNA, previously infected with the target species *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* and species belonging to the genus *Vibrio*, namely *Vibrio anguillarum, V. harveyi, V. vulnificus and V. parahaemolyticus.*

An optimization step regarding the DNA extraction of zebrafish infected larvae consisted in conducting two separate extraction protocols, A and B (details in the Materials & Methods section). Only zebrafish larvae infected with *P. damselae* subsp. *piscicida* and larvae unsubmitted to any treatment (serving as a negative control for the detection of pathogens) were used in the extraction's optimization step. The extracted DNA samples were subsequently used in a touchdown PCR reaction, following a previously established protocol (Serra et al., 2018) capable of detecting minimal amounts of target DNA to determine which of the extraction protocols achieved better results. The results, presented in **Figure 20**, show that the extraction protocol B revealed better results by obtaining slightly more intense bands in gel electrophoresis.

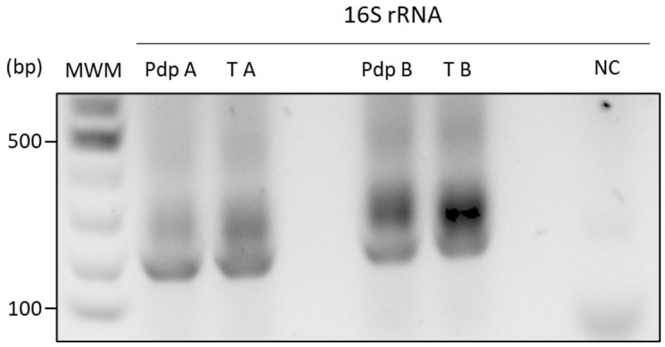

Figure 20. Agarose gel (1.2%) electrophoresis of the PCR amplification using primers 16S-GC-358F and 16S-517R, targeting *P. damselae* subsp. *piscicida* (Pdp A and Pdp B) performed with the aim of evaluating the DNA extraction protocols' (A and B) quality. DNA samples from uninfected zebrafish larvae extracted following extraction protocols A and B were used as negative controls (T A and T B) as well as water (NC). MWM, molecular weight marker.

Besides the touchdown PCR reaction, the extracted samples were also used in a uniplex PCR reaction against primer pair Car, targeting *P. damselae*, following the previously established conditions for the multiplex PCR being developed. Results are shown in **Figure 21**.

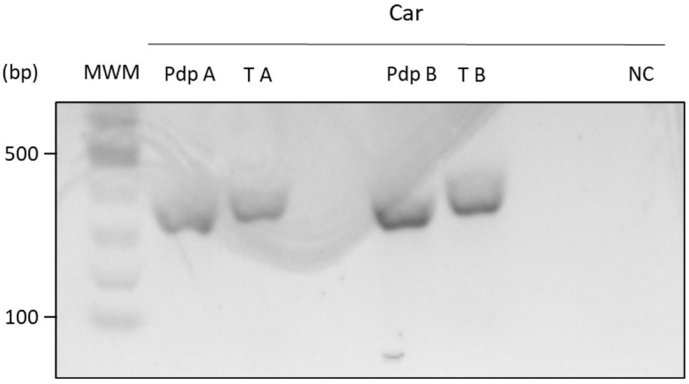

Figure 21. Agarose gel (1.2%) electrophoresis of the PCR amplification using primer pair Car, targeting *Photobacterium damselae* subsp. *piscicida* (Pdp), to target this species in DNA samples extracted from zebrafish larvae previously infected with Pdp. DNA samples from uninfected zebrafish larvae extracted following extraction protocols A and B were used as negative controls (T A and T B) as well as water (NC). MWM, molecular weight marker.

The primer pair remained able to detect a molecular band of the expected molecular size in the sample corresponding to the DNA extracted from larvae infected with *P. damselae* subsp. *piscicida*. However, the appearance of an intense similar-sized molecular band in the samples corresponding to the negative control and the lack of a positive control of *P. damselae* subsp. *piscicida* DNA due to a human laboratory error in the preparation of the reaction led to question the actual presence of *P. damselae* subsp. *piscicida*. Notwithstanding, the samples extracted using protocol B continued revealing better results in gel electrophoresis reflected in well-defined molecular bands, thus determining the use of protocol B to perform the extraction of the remaining zebrafish larvae's DNA.

Such extraction was followed by a PCR reaction using primers targeting the 16S rRNA gene to guarantee its success. The electrophoresis results are shown in **Figure 22** and the appearance of molecular bands with a 500 – 600 bp molecular weight confirmed the protocol's success.

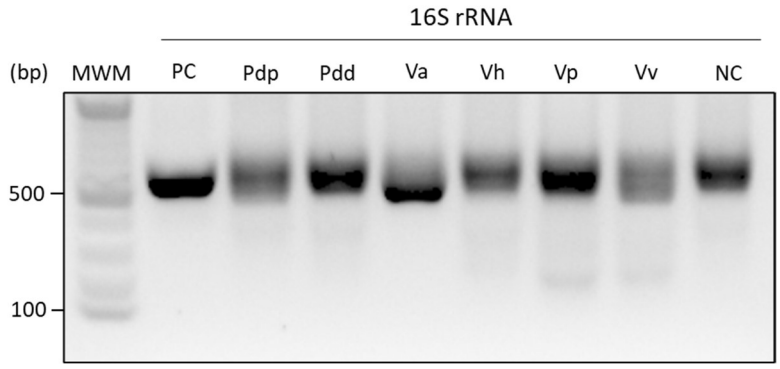

Figure 22. Agarose gel (1.2%) electrophoresis of the PCR amplification using primer pair 16S-27F and 16S-517R targeting the 16S rRNA gene, in DNA extracted from zebrafish larvae infected with *P. damselae* subsp. *piscicida (*Pdp*)*, *P. damselae* subsp. *damselae (*Pdd*)*, *V. anguillarum (*Va*)*, *V. harveyi (*Vh*), V. parahaemolyticus* (Vp)*, or V.vulnificus (*Vv*)*. A DNA sample from *S. iniae* was used as a positive control (PC). A DNA sample from uninfected zebrafish larvae was used as a negative control (NC). MWM, molecular weight marker.

After confirming the success of the infected zebrafish larvae's DNA extraction, a PCR reaction was carried out using primers hdcPdd to test the ability of such primers to specifically detect *P. damselae* subsp. *damselae* from infected zebrafish larvae's DNA samples. The results are shown in **Figure 23**.

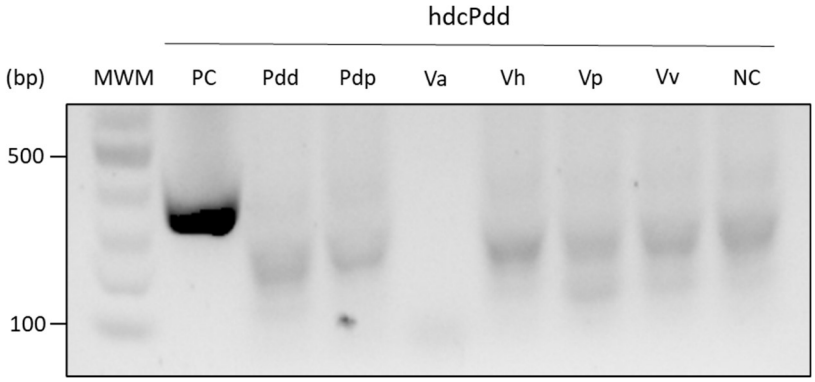

Figure 23. Agarose gel (1.2%) electrophoresis of the PCR amplification using primer pair hdcPdd to target *P. damselae* subsp. *damselae (Pdd)* in DNA samples extracted from zebrafish larvae previously infected with *P. damselae* subsp. *damselae (*Pdd*)*, *P. damselae* subsp. *piscicida (*Pdp*), V. anguillarum (*Va*)*, *V. harveyi (*Vh*), V. parahaemolyticus (*Vp*), or V.vulnificus (*Vv*)*. A DNA sample extracted from a pure culture of *P. damselae* subsp. *damselae* was used as a positive control (PC) and a DNA sample extracted from uninfected zebrafish larvae was used as a negative control (NC). MWM, molecular weight marker.

Although it is possible to identify light molecular weight bands in the DNA samples extracted from infected zebrafish larvae, the lack of correspondence between the positive control and the DNA sample extracted from larvae infected with *P. damselae* subsp. *damselae* and the PCR bands' lack of definition and similar molecular weight,

point toward the hypothesis that the infection method used in this study did not prove to be the most suitable for the validation of the developed mPCR. Moreover, the ability of primers hdcPdd to amplify a PCR product in each PCR reaction, except the one using the DNA of larvae infected with *V. anguillarum*, lead to the suspicion that the low number of *V. anguillarum* infected survivors suitable for DNA extraction were not enough to allow the pathogen's detection.

Facing the latter results and due to the lack of time to complete the present study, in order to make the most of it, the last phase regarding the validation of the mPCR consisted in performing two multiplex PCR reactions using, in the first one, primers Car, hdcPdd and dlts and in the second, primers AgryB, VrpoA and etfD to ascertain their ability to identify the pathogens with which zebrafish larvae had been infected. Such reactions were carried out in the exact same conditions, except for the set of primers and corresponding DNA samples used as the reactions' positive control. For the first PCR reaction, the positive control consisted in a DNA mix of *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae* and *S. agalactiae*, as for the second, it consisted in a DNA mix of *Aeromonas hydrophila*, *V. harveyi* and *Edwardsiella tarda*. The reaction's results are shown in **Figure 24.**

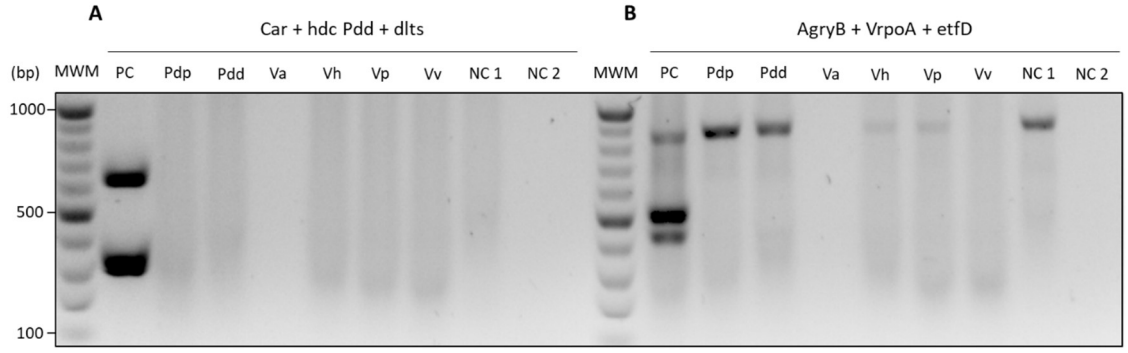

Figure 24. Agarose gel (1.2%) electrophoresis of the Multiplex PCR using different primer pairs **(A)** Car + hdcPdd + dlts that target *Photobacterium damselae* subsp. *piscicida* (Pdp), *Photobacterium damselae* subsp. *damselae* (Pdd) and *Streptococcus agalactiae* (Sa) or **(B)** AgryB + VrpoA + etfD that target *Aeromonas* spp., *Vibrio* spp. and *Edwardsiella tarda,* in DNA samples extracted from zebrafish larvae previously infected with *P. damselae* subsp. *piscicida (*Pdp*)*, *P. damselae* subsp. *damselae (*Pdd*)*, *V. anguillarum (*Va*)*, *V. harveyi (*Vh*), V. parahaemolyticus (*Vp*), or V.vulnificus (*Vv*)*. A mix of DNA samples extracted from pure cultures of *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae* and *S. agalactiae* was used as a positive control (PC) in (A) while a mix of DNA samples extracted from pure cultures of *A. hydrophila*, *V. harveyi* and *Edwardsiella tarda* was used as a positive control (PC) in (B). A DNA sample extracted from uninfected zebrafish larvae (NC 1) and water (NC 2) were used as negative controls in both mPCR reactions. MWM, molecular weight marker.

The gel electrophoresis results confirm that the infection method used in this study was unsuitable for the validation of the developed mPCR using primers Car, hdcPdd and dlts since they were unable to identify the target species (*P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*) in DNA samples extracted from *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* infected zebrafish larvae. A series of

optimization steps would have had to be performed regarding both the infection method and zebrafish larvae DNA extraction to increase the robustness of the mPCR validation results.

On the other hand, the previously developed mPCR using primers AgryB, VrpoA and etfD obtained unexpected results as a molecular band corresponding to the detection of *Aeromonas hydrophila* subsp. *hydrophila* appeared in DNA samples known to be infected with *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae*, *V. harveyi* and *V. parahaemolyticus*, as well as in the DNA sample extracted from non-infected zebrafish larvae.

Research shows that bacteria belonging to the genus *Aeromonas* spp. are usually found in the GI tract of zebrafish, being the only group of bacteria present throughout this species entire life cycle due to their important role in immune defense, gut cell growth and the development of the pancreas (Burns & Guillemin, 2017; Hossain et al., 2019; Matos & Leulier, 2018; Mocho et al., 2017).

Considering this, as well as the obtained results, arose the suspicion that the zebrafish larvae used in the challenge might already been infected with *Aeromonas hydrophila* subsp. *hydrophila*. That being the case, sequencing of the PCR bands obtained in this mPCR became essential to better understand the obtained results.

Firstly, sequencing of the molecular bands obtained in the electrophoresis analysis of PCR reactions using zebrafish larvae DNA previously infected with *P. damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae*, *V. harveyi*, *V. parahaemolyticus* and larvae used as a negative control with primer AgryB (**Figure 20 – B**), confirmed the identification of *Aeromonas* spp. **(Supplementary material Table S1)**, reinforcing the hypothesis presented above.

In addition, sequencing of the above-mentioned molecular bands with primer VrpoA, targeting *Vibrio* spp., failed regarding molecular bands corresponding to the DNA of zebrafish larvae previously infected with *P. damselae* subsp. *piscicida*, *V. parahaemolyticus* and larvae used as a negative control. Notwithstanding, sequencing results also showed that primer VrpoA was able to amplify the DNA of the zebrafish larvae in the DNA sample resulting from their infection with *P. damselae* subsp. *damselae* and to amplify the DNA of *Aeromonas hydrophila* in the DNA sample resulting from the infection of the larvae with *V. harveyi* **(Supplementary material Table S1)**. That being the case, the primer pair VrpoA, used to amplify the DNA of *Vibrio* spp., may not be as specific for its detection as indicated in the work from which it was taken.

Lastly, as expected, sequencing of the above-mentioned resulting molecular bands with primer etfD, targeting *Edwardsiella tarda*, failed.

4. Conclusions and future work

With this work it was possible to develop a multiplex PCR capable of simultaneously identify the bacterial species *Photobacterium damselae* subsp. *damselae*, *Photobacterium damselae* subsp. *piscicida* and *Streptococcus agalactiae* in *in vitro* conditions using DNA samples extracted from these bacterial species cultures.

However, the attempt to validate the developed mPCR using experimental samples failed. Consequently, the obtained results show that this methodology needs validation of the developed molecular tool *in vivo*. To do so, there is a clear need to carry out several optimization steps.

First of all, infecting the zebrafish with some *Vibrio* spp. species employing the infection model used in the zebrafish larvae challenge assay, resulted in a lack or reduced number of infected survivors at the end of the challenge assay. Consequently, the DNA extraction was jeopardized as later PCR amplification of such DNA did not work. As such, there is a clear need to optimize this infection model, for example, using lower, effective, infective concentrations of each bacterial species, guaranteeing the possibility to validate and, eventually, apply the developed molecular tool in experimental samples. Moreover, it would also be important to ensure that the experimental model used in the challenge assay was free of microorganisms or, at least, that their presence was known in advance preventing any conflict regarding the expected results.

It would also be necessary to take a few more optimization steps regarding the DNA extraction from infected organisms, such as targeting this extraction only to the organs previously known to be the most affected. This would also require the use of a larger experimental model rather than using zebrafish. In addition, it would also be relevant to test a greater number of different homogenization procedures during the extraction of DNA from the respective isolated organs while promoting a simultaneous quality analysis of the products resulting from their DNA amplification to determine the best possible procedure in the present context.

Finally, one of the most important optimization steps in the development of a molecular tool in this context could be the need to design new primers and establish the efficiency and specificity of such primers, particularly when used to detect their target species in experimentally infected fish samples with each bacterial species (individually and in co-infection). Only by establishing a suitable multiplex PCR using efficient and specific primers and an effective infection model to validate such multiplex PCR could an adequate analysis of complex environmental samples be guaranteed.

Despite the imperative need to optimize the methodology developed throughout this work, the approach taken not only achieved promising results towards filling an existing gap regarding the need of detecting pathogens of great relevance to the aquaculture sector using fast and reliable molecular tools as also made it possible to identify critical points in the process of developing such molecular tools.

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Supplementary Material

Table S1. Sequencing results of the molecular bands obtained in agarose gel electrophoresis of multiplex PCR reactions depicted in Figures 7 and 24(B).

Figure	Primer/Tested DNA	BLAST result	%ID	LINK
7	PhPisA/Pdp	*Photobacterium damselae* subsp. *piscicida*	96.9	AP018045.1
	PhPisB/Pdp	*Photobacterium damselae* subsp. *piscicida*	98.0	AP018045.1
	Car/Pdd	*Photobacterium damselae*	100.0	OQ240203.1
	hdcPdd/Pdd	*Photobacterium damselae* subsp. *damselae*	99.6	CP035780.1
	sim/Si	*Streptococcus iniae*	99.4	MK959355.1
	SINIAE/Si	*Streptococcus iniae*	99.7	CP032401.1
	SAGA/Sa	*Streptococcus agalactiae*	100.0	KP729641.2
	dlts/Sa	*Streptococcus agalactiae*	99.6	CP053027.1
24(B)	AgryB/Pdp	*Aeromonas caviae*	100.0	MK512359.1
	AgryB/Pdd	*Aeromonas caviae*	99.7	MK512359.1
	VrpoA/Pdd	*Danio rerio*	98.9	LR812082.1
	AgryB/Vh	*Aeromonas hydrophila*	99.8	CP083944.1
	VrpoA/Vh	*Aeromonas hydrophila*	99.5	N711794.1
	AgryB/Vp	*Aeromonas hydrophila*	99.8	CP083944.1
	AgryB/NC 1	*Aeromonas caviae*	100.0	MK512359.1