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Effects of native probiotic-candidates on the immune status, inflammatory response and disease resistance of turbot (*Scophthalmus maximus, L.*), upon rectal administration

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Master Thesis

Aquaculture and Fisheries Masters Programme

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Declaração de autoria

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

Assinatura: Filipa Beça

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ABSTRACT

Probiotic bacteria have been widely studied in the past few decades as a viable, sustainable and efficient alternative to the existing method to prevent disease outbreak. Most commercial probiotics in animal husbandry are non-native species, which have been reported to fail settlement in the target organism. The aim of this project was to determine if the use of two probiotic-candidates, isolated from healthy wild turbot, could potentially exert beneficial effects on the immune status, inflammatory response and disease resistance of challenged juveniles in turbot farming, assessed upon rectal administration. The isolated bacteria were identified as closely related to *Psychrobacter nivimaris* and *Psychrobacter faecalis*, based on 16S rRNA (100% and 100% identity) and GYRB (96% and 97%) identification, respectively. This was evaluated experimentally by successive administrations of the respective probiotic (Pn, Pf) or, as control, PBS by rectal cannulation. Thereafter, fish were experimentally infected with the pathogen *Tenacibaculum maritimum* using a standardized immersion bath protocol. Virulence of the pathogen strain had previously been determined as LC50 after 7 d, assessing cumulative mortalities at 10^6, 10^7 and 10^8 CFU ml^{-1}. To identify effects on the immune response, groups were assessed in triplicate before, 2 and 5 days post infection (0, 1 and 5 dpi), by determining gene expression of selected marker genes (*mhc II α*, *il-1β*, *tcr*, *tgf β* and *tnf α*) using RT-QPCR. In parallel, cumulative mortality over 14 days was studied at a higher pathogen concentration. Control-treated fish had marginally lower survival rates than fish that received one of the probiotic treatments. At 0 dpi, gene expression between treatments was comparable between treatments and control; only *tgf*, was decreased in the control group. At 1 dpi, gene expression of all genes was lower in the probiotic groups than in control fish. In contrast, at 5 dpi, no significant differences were observed between groups. As a conclusion, even though gene expression of the selected markers was not up-regulated, the native probiotics candidates *P. nivimaris* and *P. faecalis* modulated survival upon experimental infection with *T. maritimum*, thereby illustrating the potential application of native probiotics in disease prophylaxis.

KEYWORDS:

Turbot; *T. maritimum*; probiotics; RT-QPCR; gene expression.

RESUMO

Surtos de doenças transmissíveis são um dos problemas mais preocupantes e dispendiosos em aquacultura, considerando que agentes patogénicos, nomeadamente bactérias, propagam-se muito facilmente neste ambiente. A principal maneira de controlar esta questão, até agora, tem sido através da utilização de substâncias antimicrobianas e químicas, em uso terapêutico e profilático. No entanto, o uso a longo prazo e indiscriminado desses compostos tem sérias consequências no meio ambiente e, inevitavelmente, para a saúde humana. Como tal, é urgente investir e investigar opções sustentáveis para o controlo e prevenção de doenças. As bactérias probióticas têm sido amplamente estudadas nas últimas décadas como uma alternativa viável e eficiente aos métodos de profilaxia existentes. Probióticos definem-se como microorganismos suplementados que beneficiam o seu hospedeiro, através do aumento da resposta imune e resistência a doenças, o complemento da microbiota intestinal, a nutrição do hospedeiro e outros modos de acção. Maioria dos probióticos utilizados comercialmente em aquacultura provém de espécies não nativas ao hospedeiro, no entanto tem-se verificado que muitas vezes estes falham a colonização do organismo-alvo. Recentemente, tem-se recorrido a bactérias nativas como candidatas a probióticos, visto que poderão colonizar o intestino do peixe mais facilmente do que bactérias exógenas, e igualmente exercer efeitos benéficos. Neste âmbito, este projecto procurou determinar se o uso de dois candidatos a probióticos nativos de uma espécie de aquacultura de elevado valor comercial, o pregado, poderia modelar o seu estado imunológico e a resistência à doença quando expostos a uma infecção, após administração rectal dos mesmos. As bactérias em estudo foram isoladas de pregados selvagens saudáveis e identificadas como *Psychrobacter nivimaris* e *Psychrobacter faecalis*, ambas consideradas espécies recentes para a Ciência, e como tal, pouco estudadas com este intuito. Para esta avaliação, realizou-se um estudo experimental, que incluiu um teste com os probióticos putativos durante nove dias, consistindo em dois eventos de inoculação com a bactéria candidata e um controlo de PBS. A administração das bactérias foi efectuada através de uma metodologia inovadora e adaptada a este caso, via canulação rectal, com o intuito de aumentar a probabilidade de colonização do intestino. Seguidamente, efectuou-se um *challenge* de banho de imersão com um patógenio de alta virulência e importância económica, *Tenacibaculum maritimum*. Realizaram-se três eventos de amostragem, um antes da infecção com o patógenio (0 dpi), e os seguintes dois e cinco dias após o *challenge* (1 dpi e 5 dpi). A resistência à doença foi avaliada pela monitorização da mortalidade acumulada

ao longo dos 14 dias do desafio . A resposta inflamatória e o estado imunológico dos peixes foram avaliados por quantificação da expressão de genes marcadores do estado imunitário dos peixes, nomeadamente os genes *mhc II α*, *il-1b*, *tcr*, *tgf β* e *tnf α*, através da técnica RT-QPCR, com SYBR Green. Os peixes do grupo controlo infectado apresentaram as taxas de sobrevivência mais baixas, 80%, enquanto que os peixes inoculados com *P. nivimaris* não obtiveram mortalidade e com *P. faecalis* tiveram uma sobrevivência de 92%. Apenas foi encontrada uma diferença marginal entre as curvas de sobrevivência (teste de tendência Logrank, P = 0,0538). A expressão génica em 0 dpi demonstrou semelhantes níveis de mRNA em todos os tratamentos de todos os genes, excepto no gene *tgf*, onde o controlo demonstrou menor expressão. Às 1 dpi, peixes infectados e inoculados com o controlo demonstraram uma maior expressão génica para todos os genes, enquanto que às 5 dpi a expressão génica foi semelhante para todos os grupos. Considerando que os peixes submetidos à inoculação com as bactérias candidatas obtiveram maior sobrevivência durante a infecção, é provável que estes tenham agido de alguma forma benéfica sob o organismo, mesmo que isto não se reflita na expressão génica dos genes selecionados. Adicionalmente, estes resultados poderão ser reflexo do método de administração dos probióticos putativos, uma vez que esta metodologia é pioneira e ainda não foi testada noutros estudos. Em suma, neste projecto as bactérias nativas *P. nivimaris* e *P. faecalis* afectaram marginalmente a resistência à doença de peixes infectados e significativamente a expressão génica de peixes infectados, quando comparados ao controlo infectado, ainda que tenha diminuído a mesma. Isto pode ser uma indicação de que estas bactérias poderão ser boas candidatas a probióticos comerciais para pregado, já que são capazes de aumentar a resistência à doença *in vivo*, fornecendo uma proteção ampla ao organismo contra o patogénio em questão. Apesar de isto não se reflectir na expressão génica dos marcadores seleccionados, é provável que outros processos fisiológicos sejam modelados por estes candidatos a probióticos. Adicionalmente, a eficácia da inoculação de bactérias por administração rectal foi inconclusiva, pois apesar de ter dido uma técnica rápida e reproduzível, é incerto se este método promoveu a colonização dos probióticos putativos em pregado. Este foi um estudo preliminar para inferir a eficácia de *P. nivimaris* e *P. faecalis* como candidatos a probióticos e serve de base para novos testes para comprovar a sua capacidade como probióticos comerciais.

PALAVRAS-CHAVE:

Pregado; *T. maritimum*; probióticos; RT-QPCR; expressão de genes.

INDEX

LIST OF SYMBOLS AND ABBREVIATIONS

C_T	threshold cycle
cDNA	(first strand) complementary DNA
CFU	colony-forming unit
d	days
dpi	days post-infection
DNA	deoxy-ribonucleic acid
DNAse	deoxy-ribonuclease
dNTP	nucleoside triphosphate, deoxyribonucleotide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
g	centrifugation speed
h	hours
il-1β	interleukin one beta
LC50	lethal concentration required to kill 50% of the tested group
mhc II α	major histocompatibility complex II alpha
MOA	mode of action
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
QPCR	quantitative polymerase chain reaction
RAS	recirculating aquaculture system
RNA	ribonucleic acid
RNAse	ribonuclease
RIN	RNA integrity number
rpl8	ribossomal protein L8
RT	reverse transcriptase
RT-QPCR	reverse transcription quantitative polymerase chain reaction
Ta	annealing temperature
tcr	T-cell receptor
tgf β	transformating growth factor beta
tnf α	tumour necrosis factor alpha

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1. Introduction

1.1. General introduction

As the human population continuously increases and countries achieve higher economic and social development, there is an increasing demand for high quality protein worldwide. Fish, compared to other meat sources, is recognized particularly healthy representing the main source for essential fatty acids (PUFA). Moreover, it is undoubtedly an ideal farming organism regarding animal welfare (farmed species mostly social and can thus be farmed at high densities), environmental sustainability (resource utilization, CO2-, water footprint) as well as reduced risk of disease transmission to humans (Avian influenza). Also, in the view of stagnating fisheries landings over the past 50 years (Moffitt and Cajas-Cano, 2014), aquaculture is the only source for the increasing fish demand (per capita consumption) of the growing human population worldwide. Currently depicting the fastest growth within the food sector, 73.7 million tonnes of farmed aquatic animals' have been harvested in 2014, with an estimated first-value sale of US$160.2 billion (FAO, 2016).

In the scenario of a global population estimated to reach 8.6 billion people in 2030 (UN DESA, 2017), a sustainable growth of the aquaculture sector requires optimization in terms of production efficiency thereby reducing utilization of limited resources. Here, utmost importantly, environmental impacts including nutrient emissions, habitat modification, genetic disturbances caused by escapees and disease transmissions between farmed stocks and wild populations must be reduced (Fernandes et al., 2001, Samuel-Fitwi et al., 2012). Healt management is a prime target for technological innovations, namely in reduction of losses and costs associated with therapy. This, in turn, lessens environmental impact by reducing disease transmission to wild aquatic animals and the use of chemicals. Thereby, food security and disease resilience will be improved, supporting further the expansion of the aquaculture industry.

1.2. Turbot aquaculture

The development of aquaculture was accompanied by a continuous diversification of the cultured species, in particular marine finfish, to meet consumers' demand (Naylor and Burke, 2005). Here, turbot is among the most valuable flatfish species (*Pleuronectiformes, Scophthalmidae*) internationally traded. Its most striking feature is their bilateral asymmetry, allowing them to lay flat on the substrate. As a lurking hunter, turbot is well

camouflaged in the sand and may reach easily above 100 cm in the wild. It is found in the coastal waters of the Northeast Atlantic, the Baltic Sea and the Mediterranean Sea (Fishbase 2017). Usually observed on soft substrates, feeding mainly on other benthic fishes, cephalopods and larger crustaceans and bivalves (Fishbase 2017). In the European Union, extractive fisheries of turbot suffered a dramatic decline in the 1990's from about 10000 t to almost half in the early 2000's, which seems to have stabilized between 4000 to 6000 t annually (Eurostat, 2017).

The first successful pilot-scale farming of turbot was carried out by Purdom et al. (1972). Soon after, farming was introduced in Scotland and quickly adapted by other European countries, e.g. Denmark, Germany, Ireland, France, Norway, Wales, Spain and Portugal. Turbot fry and broodstock were initially obtained by growing out wild-caught animals, however, breeding programmes were established as early as 1993 (Daniels and Watanabe, 2011). During the 1990s, technological advances in larval rearing resulted in a relatively secure supply of turbot juveniles for stocking (Shields, 2001). After the weaning process, intensive juvenile grow-out can carried out in land based tanks and raceways at relatively high-stock densities, or, sometimes, in flat-bottomed cages in coastal areas (Person-Le Ruyet, 2002, Daniels and Watanabe, 2011). Nowadays, European production focuses on recirculating aquaculture systems (RAS). Recently, farming was introduced in China with broodstock imported from Europe, increasing the total global production of turbot to almost 72 000 t in 2014 (FAO 2017). Market-wise, farmed turbot achieve prices over 9 € kg^{-1} (Bjørndal and Guillen, 2016).

Future developments in turbot's production is cost driven and efficient use of resources is required, both from an environmental as well as economic perspective. As with many other piscivorous marine species, expenditure for feed often contributes to more than 50% of the costs, fuelling research in nutrition and dietary requirements of the species (Kroeckel et al., 2012). In the context of disease outbreaks reported in turbot and a paradigm change towards disease prevention, animal welfare and health management are utmost important drivers of aquaculture research and future development (Reiser et al., 2010, Reiser et al., 2011, van Bussel et al., 2012, Winkelbach et al., 2015, Monsees et al., 2016, Azeredo et al., 2017).

1.3.Disease in aquaculture

Disease outbreaks represent one of the most significant setbacks for aquaculture production and comprise the biggest economic losses within the industry (Bondad-Reantaso et al., 2005, Stentiford et al., 2012). Apart from direct cost due to mortalities or therapy, growth retardation, reduced product quality and price volatility reduce seafood's economic revenue substantially (Stentiford et al., 2012, Lafferty et al., 2015). In fact, in 1997, the World bank estimated that global losses due to disease in aquaculture may sum up to over US $3 billion annually (Subasinghe et al., 2001).

Intensive farming often leads to periods of intensified stress and suboptimal rearing conditions, which potentiates the spread of infectious diseases and creates a pathogen-friendly environment. The aquatic environment supports the existence of pathogenic microorganisms that are not as species-specific as those from terrestrial, endothermic vertebrates. Often, densities are much higher in the environment, facilitating infection of healthy fish, either by ingestion or adhesion to skin and epithelial surfaces (Defoirdt et al., 2011). Also, water habitats sustain the rapid propagation and transmission of pathogens from infected fish and pathogens may be introduced via the water exchange (even required in RAS) from the wild. Among the pathologies that may adversely impact fish health such as fungi, parasites and viral diseases, bacterial infections are considered the greatest problem in mariculture (Toranzo et al., 2005, Smith, 2008, Lafferty et al., 2015). Here, prevention and control are by far the most effective strategies to restrict outbreaks. Although very popular, antibiotic treatment is mostly ineffective and considered critical due to the risk of spreading antibiotic resistance. The limitation of antibiotics and increasing multi-drug resistance, together with growing consumer concerns, require alternative prophylax or therapy strategies (Cabello et al., 2013). In turbot farming, the most relevant diseases are vibriosis, tenacibaculosis, furuncolosis and streptococcosis (Devesa et al., 1985, Toranzo and Barja, 1992, Domeénech et al., 1996, Toranzo et al., 2005), FAO 2017).

1.3.1. Tenacibaculosis

Tenacibaculosis is an infectious disease caused by *Tenacibaculum maritimum* which was first described by Masumura and Wakabayashi (1977) in red and black sea bream farming in Japan. Today, it is one of the most important diseases in turbot and other farmed species on a global scale (Avendaño-Herrera et al., 2006, El-Galil and

Hashem, 2012, Frisch et al., 2017). Moreover, among species severely affected, flatfish seem to be relatively susceptible including dover sole (*Solea solea*) (Bernardet et al., 1990) and Senegalese sole (*Solea senegalensis)* (Avendaño-Herrera et al., 2004c, 2005). *Tenacibaculum maritimum* a rod-shaped, Gram-negative, colonial mesophilic bacteria with optimal growth at 30 °C, forming long filaments in aggregates (Santos et al., 1999). A worldwide distribution has been reported with records from Japan, Australia, North-America and several European countries such as France, Italy, Malta, Spain and Portugal (Avendaño-Herrera et al., 2006).

Tenacibaculosis is characterised by an impressive number of external symptoms, which contribute to a lack of consensus in diagnosis. Clinical signs include eroded mouth, ulcerated and shallow skin lesions, darkening of tissue, frayed fins, and tail rot (Magariños et al., 1995). Gills may appear yellowish, with increased mucus and necrotic patches (Mitchell and Rodger, 2011). The primary locations of infection are body surfaces, like the mouth and fins, since the pathogen strongly adheres to the external skin and mucus of fish. *T. maritimum* affects both adults and juveniles, and its prevalence and severity increase with the temperature, as well as with stress factors and hosts' skin surface condition (Toranzo et al., 2005). Pathogenesis and mechanisms of transmission and virulence of this bacterium are still poorly understood (Gourzioti et al., 2016), however, jellyfish are suspected to be a natural host and, consequently, vectors of the disease to fish in open systems (Delannoy et al., 2011). The transmission paths currently suggested involve a direct host to host transmission, via sea-water and, ultimately, uptake by ingestion (Mitchell and Rodger, 2011). The adhesion is supported by extracellular toxins and enzymes (Avendano-Herrera et al., 2006).

1.4.Countermeasures to disease

Several regulations and programs have been implemented to improve health management in aquaculture, including national legislation and international codes, technical guidelines, diagnostic improvement and disease surveillance (Subasinghe et al., 2001). Additionally, good husbandry practices and sanitary measures help minimize the risk of disease. Nevertheless, the major strategy to fight diseases are, in fact, focused on health management and prophylactic measures, such as vaccination and the use of immunostimulants, pro- and prebiotics. Therapeutic treatment, namely the use of

antibiotics, needs to be considered relatively ineffective since it is often referred to as "treatment crisis" in aquaculture.

Therapeutic treatments and prophylaxis

Antibiotics have been used intensely between the 1970's and 2000's, not only for prevention and therapy of disease but also regarding growth promoting effects observed. Since then, in the context of human health concerns, its application in EU has been increasingly regulated by legislative enforcement and governmental food control of traded products, supported by the consumer's perception as well as alternative, antibiotic-free production lines, promoted by biolabels (Alderman 2002). Nowadays, antibiotic therapy may still be applied in case of acute outbreaks. However, follow-on costs related to therapy, losses and reduced value of the product clearly show that antibiotic treatment is an ultimate, and often ineffective, last resort. Here, functional diets represent an alternative if gut health can be improved, providing prophylactic protection.

The main risk of antibiotic treatment is the release of substances into the aquatic environment, by urinary and faecal excretion and also unconsumed food, supplemented with the respective drug (Christensen et al., 2006). Non-biodegradable antibiotics can persist in water for a large amount of time, which promotes the raise of antibiotic resistance (Cabello, 2006). Thus, the indiscriminate use of a wide variety of antimicrobial agents reduces their effectiveness as a treatment procedure. Additionally, it also carries the possibility of transferring antimicrobial-resistant to other organisms, representing a major risk to other animals - farmed and wild - as well as humans (Sørum, 2006, Cabello et al., 2013). In fact, recent studies reported multi-drug resistance (MDR) from aquatic environments and, in particular, from aquaculture activities (Done et al., 2015).

Vaccination provides long-term protection against a specific pathogen, either actively inducing the specific immune response or passively, providing antibodies to counteract the disease for a restricted time. Establishing effective vaccination, though costly and often a difficult, long-term activity, often is highly effective and a major incentive for the development of the industry. For example, introducing vaccination against vibriosis and furunculosis between 1980 and 1990s lead to an erratic increase in salmon farming (Sommerset et al. 2014). One vaccine has been developed to immunize

turbot against *T. maritimum*, increasing survival by 50% upon bath immersion and 85% upon peritoneal injection (Toranzo et al., 2005).

Recently, the importance of the digestive system as an immune related organ has been recognized and improving gut health is widely recognized strategy. Here, stimulation of the immune system by functional feed is a promising strategy, including the supplementation of prebiotics and immunostimulants, as well as probiotics (Newaj-Fyzul and Austin, 2015).

1.4.1. Probiotics

Merrifield et al. (2010) provided a general definition, summarizing probiotics as "any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved by improving the microbial balance of the fish". Probiotics, *sensu strictum*, are living microorganisms, although inactivated compounds and cellular products have been named probiotics (Irianto et al., 2003, Guzmán-Villanueva et al., 2014), but should be adequally called prebiotics. Here, prebiotics are defined as substances that induce the growth or activity of microorganisms that contribute to the health and the fitness of their host.

Selection of bacteria or bacterial compounds is critical regarding unidentified or negative effects on the putative host. Evaluations need to be carried out comprehensively, addressing the safety of the strain and the capability to establish within the gastrointestinal tract. Although colonization is wanted from the administration of non-native bacteria, ultimately the interaction with the endogenous microbiota may impact the host. For the evaluation of beneficial effects, screening strategies are mandatory and always applied, including the production of antimicrobial substances or beneficial compounds, contribution to nutrition, stress resistance and health. From an economic point of view, namely the suitability as a commercial product, upscaled production, viability and storage of probiotics are important issues to consider (Gomez-Gil et al., 2000, Balcázar et al., 2006, Pérez-Sánchez et al., 2014).

Probiotics used in aquaculture

The modes of action (MOA) by which probiotics exert beneficial effects are often incompletely understood. Some possible mechanisms are pathogen exclusion by production of inhibitory compounds or by competition for chemicals, energy and/or adhesion sites in the internal mucosa, improvement of nutrition and subsequent growth

by provision nutrients (e.g. fatty acids and vitamins) or stimulation of enzyme activity, and modulation of the immune response (Verschuere et al., 2000, Balcazar et al., 2006, Pérez-Sánchez et al., 2014, Van Hai, 2015). Moreover, improvement of water quality and positive effects in interaction with phytoplankton have been reported (Verschuere et al., 2000). The easiest way of administrating probiotics to the fish is by dietary supplementation. Inoculation via immersion bath or injection is possible, but large-scale application is costly with such procedures. Many bacteria species have been assessed in the last decades for suitability in aquaculture (Newaj-Fyzul et al., 2014), however the most widely addressed are lactic acid bacteria (LAB) and the genus *Bacillus* (Ringø and Gatesoupe, 1998, Kesarcodi-Watson et al., 2008). LAB are Gram-positive bacteria that do not form endospores and produce lactic acid in fermentation, including *Lactobaccilus* and *Carnobacterium*. Although the abundance of LAB in the microbiome of fishes is considered low (Gatesoupe, 2007), they are frequently used due to their low virulence and pathogen-competition, as well as their ability to improve the hosts' digestion, growth performance and disease resistance. *Bacillus* sp., on the other hand, comprises a group of bacteria that produce endospores and secondary metabolites, including enzymes and antibiotics (Pérez-Sánchez et al., 2014). Here, several species have been studied, including *B. subtilis* (Cutting, 2011). Turbot has been submitted to several studies to find suitable bacteria that can act beneficially against disease and other health improvements. For instance, the survival rate, weight and resistance of larval turbot was increased by rotifer enrichment with LAB, a gut-dominant *Vibrio* sp. strain, *Bacillus* sp. and *Vibrio pelagius* (Gatesoupe 1997, Ringø & Gatesoupe 1998, Ringø & Vadstein 1998). Moreover, in selection and identification of native bacteria trials performed to improve larviculture, it was noted that *V. mediterranei* and *Roseobacter* sp. were beneficial (Huys et al., 2001, Hjelm et al., 2004).

Many commercial probiotics used in aquaculture are obtained from humans or terrestrial animals (Nikoskelainen et al., 2001, Ferguson et al., 2010), as well as bacteria isolated from other fish species (Díaz-Rosales et al., 2009, Sorroza et al., 2012), thus they are non-native microorganisms. Colonization of the gastrointestinal happens early during ontogeny, until the beginning of exogenous feeding, which hinders the settlement of non-native species in the host. So, non-native probiotics mostly survive for a short time upon administration (Balcazar et al., 2006). In fact, the population of probiotics in the gastrointestinal tract of adult animals shows a sharp decrease within a few days after

inoculation (Fuller, 1992). This suggests that non-native bacteria have a lower chance to survive in the host compared to native bacteria. For this matter, native bacteria are often studied as probiotics for their hosts. Native probiotics are microorganisms which are isolated from the target host species organism or rearing environment. Several studies have confirmed that the administration of native putative probiotics is more effective than in non-native bacteria (Verschuere et al., 2000, Balcazar et al., 2006, Merrifield et al., 2010, Pérez-Sánchez et al., 2014).

1.4.2. *Psychrobacter* sp.

Psychrobacter is a genus within the gamma-proteobacteria comprising Gram negative, rod shaped bacteria. Interestingly, they are osmotolerant and cold-adapted, mostly marine species. Moreover, pathogens have not been described yet within this group. High abundance of *Psychrobacter* sp. among culturable bacteria isolated from turbot and other flatfishes (35% of isolates), has been reported within the doctoral thesis of Konrad Wanka (personal communication), suggesting these might be endogenous bacteria.

P. *faecalis* was originally isolated from the bioaerosol after cleaning pidgeon faeces (Kampfer et al., 2002). Isolation of P. faecalis from human have recently been reported, confirming that this species grows at a wide temperature range between 4-36 °C (Deschaght et al., 2012), which is rather unusual for this cold-adapted (psychrophilic) genus. *Psychrobacter nivimaris* was described by Heuchert et al. (2004) after isolation from organic particles of the South Atlantic. Some species of the *Psychrobacter* genus, including *P. faecalis*, colonize the gut microflora of several marine fish species (Jammal et al., 2017). So far, few studies have been carried out on this genus. Still, some experimental evidence suggests a potential use as probiotics in aquaculture (Sun et al., 2011, Yang et al., 2011, Lazado and Caipang, 2014a). Due to the abundance among culturable isolates from flatfish, their adaptation to cold environments, antagonistic effects on *T. maritimum* and reports on probiotic effects on the gut microbiome (Yang et al., 2011), growth and feed conversion (Sun et al., 2011), these are considered viable putative probiotic species.

1.5. Immune response to disease

The immune system of fish comprises the innate and the adaptive immune response. The innate immune system is considered the first line of defence and is non-specific. Thus, pathogen recognition is rather generic and not pathogen specific, providing a fast

response (Secombes & Wang 2012). Unlike, the adaptive (or specific) immune system has a more delayed action mechanism on a first encounter, however, it provides long-term, highly-specific and enhanced response to said pathogen, since it works with antibodies (IbM, IgD and IgT/Z) present in plasma by the means of lymphocytes, B and T cells (Secombes & Wang 2012). The internal organs responsible for the immunity of fish are the thymus, head kidney, intestinal tract, liver, and spleen (Lazado & Caipang 2014a).

RT-QPCR is a sensitive and increasingly used method that conjugates the use of fluorescent labels, such as SYBR Green, to quantify in real time the expression of a desired gene. The use of techniques such as RT-QPCR on immune and inflammatory marker genes on mRNA level can quantify the number of copies of immune-related genes in different key tissues. This allows to understand whether determined genes are being up or down-regulated to cope with the presence of a pathogen or as a response to a certain stimulation. On the use of probiotics to modulate fish immunity, immunological positive effects been recorded on many significant components (Lazado and Caipang, 2014b), thus, it is pertinent to utilize the above-mentioned techniques when assessing the effects of probiotics in the immune status and inflammatory response of fish, as it is done in the context of this project.

1.5.1. Immunity-related genes

T-cells and the major histocompatibility complex (MHC) are crucial factors to an acquired immune response to any foreign bodies, thus a reliable biomarker for immunology studies. T-cells are thymus-derived lymphocytes, with functions in cell-mediated immunity response that contain T-cell receptors (*tcr*) in their surface, which can recognize self or non-self peptides bound to MHC molecules (Davis and Bjorkman, 1988). The MHC complex is a set of cell surface proteins, whose main function is to bind to antigens, and present them for recognition of T-cells and tissue compatibility determination, well described in fish (Flajnik et al., 1999). MHC peptides from class II (*mhc II α*) usually occur in professional antigen-presenting cells (APCs), namely macrophages, B-cells and dendritic cells. Previous studies show that both these components can be consider good biomarkers for gene expression regulation of teleost fish (Boudinot et al., 2001, Yang et al., 2016, Li et al., 2017).

Cytokines are small signalling proteins that play relevant a role in the regulation of the immune and inflammatory responses to infections (Callard and Gearing, 1994).

These are produced by a large range of immunity-related cells and can be functionally divided into type 1, which enhance cellular immune responses, and type 2, that favour antibody responses.

Interleukin-1 beta (*il-1β*) is a signalling cytokine, also known as leukocytic pyrogen, and a pivotal early response pro-inflammatory substance that induces an inflammatory cascade, along with other defensive responses in case of pathogen invasion (Huising et al., 2004). Overall, studies involving this gene recorded responses to bacterial infection by enhancing leukocyte phagocytosis and bactericidal activity; as well as induce leukocyte proliferation, differentiation, and maturation; activate lymphocytes or inducing cytokines that activate macrophages, NK cells and lymphocytes (Low et al., 2003, Chai et al., 2006).

The transforming growth factor beta (*tgf β*) superfamily of cytokines is a large group of multifunctioning cell factors with diverse activities, including cell growth, differentiation, and morphogenesis (Massague, 1990). For instance, a *tgf β* related has been produced as a recombinant protein in several fish species, and shown to have an immunosuppressive function, while enhancement of the proliferation and viability of peripheral blood leucocytes have also been described (Zou and Secombes, 2016). Other studies demonstrate that *tgf β* might be up-regulated, in different fish species, in the context of bacterial infection (Lindenstrøm et al., 2004, Purcell et al., 2004, Mulder et al., 2007).

Tumour necrosis factor alfa (*tnf α*) or cachectin is another inflammatory cytokine which is involved in the acute phase reaction response to a stimulus. This cytokine, initially described as causing necrosis in tumours by Carswell et al. (1975), has been described to play a role in resistance to bacterial and viral infections (Czarniecki, 1993, Steinshamn et al., 1996). Since these cytokines have active roles in immune response, their gene expression is often assessed in infection trials.

1.6. MAIN GOALS

The mandatory economical interest in solving one of the biggest obstacles for the industry of aquaculture, and the parallel interest of ensuring farmed fish health and well-being are strong reasons for the importance on the study of probiotics and disease resistance. Considering the problematic around the subject in focus of this project, there are three main goals:

- Study whether native probiotic candidates isolated from the digestive system of wild turbot can modulate innate immune response;

- Evaluate the effects of probiotics on the inflammatory response and disease resistance of turbot exposed to pathogen *T. maritimum*;

- Assess rectal administration as a faster, more efficient method to test probiotic effects upon administration.

To achieve this, healthy juvenile turbots were exposed to the pathogen *T. maritimum*, after being inoculated with two potential probiotics, isolated from the GIT of wild-caught turbot, by rectal administration. The inflammatory response and immune status of the fish stimulated with the probiotic candidates were evaluated by analysing:

- Gene expression: quantification by RT-QPCR for *mhc II α* (major histocompatibility complex II alfa); *tgf β* (transforming growth factor beta); *il-1β* (interleukin one beta); *tcr* (T-cell receptor); *rpl8* (Ribosomal protein L8 as housekeeping gene);

Disease resistance against the pathogen was measured by assessing:

- Deceased fish throughout the challenge period, when comparing fish stimulated with probiotics and non-stimulated.

2. Methodology

2. METHODOLOGY

2.1.Ethical Statement

The aim of this project could only be assessed in an animal experiment since alternative methods such as cell cultures or bioassays cannot sufficiently reflect the complexity of the immune system, nor the administration and uptake of a probiotics via the digestive system. Consequently, alternative methods cannot **replace** the animal experiment. Nevertheless, to **reduce** the number of fish involved in this study, all candidate isolates were taxonomically identified as well as screened using specifically developed *in vitro* assays to select interesting candidates for this study and reduce the number of experimental groups. A G-power analysis referring to the variation observed in other studies was carried out to plan the actual sample size. As an alternative to a combined feeding and challenge trial, rectal administration by cannulation (Winkelbach et al., 2015) was used to reduce the experimental period of the trial, **refining** the distress of the animals. The pathogen challenge was carried out according to Avendaño-Herrera et al. (2006), applying an immersion bath for the challenge, which is considered less stressful as, for example, intraperitoneal infection. All fish were euthanized prior to dissection, making use of a state-of-the art humane protocol including an overexposure to the anaesthesia and cutting of vertebrae close to the brain (Close et al., 1997). The current study was carried out in compliance with the Portuguese legislation on animal welfare in animal experiments and the EU Directive 2010/63/EU.

2.2. Fish rearing

Healthy, one-year-old juvenile turbot (mean weight: 30.93 ± 0.89 g, total length: 12.24 ± 0.18 cm) were obtained from Acuinova S.A. (Mira, Portugal) in October 2016 and transferred to the facilities of the CIIMAR, (Porto, Portugal). 162 fish were randomly distributed to 18 rectangular flat-bottomed 8 L tanks (9 fish per tank), with individual aeration and arranged as three separate recirculating systems (water turnover 2 volumes/h), each with a mechanical filter unit of glass wool and a moving bed biofilter (Figure 6.1 in Annexes), providing two probiotic treatment groups and a control (Figure 2.3). In addition, 30 fish were distributed to a fourth recirculating aquaculture system (5

fish per tank) to assess LC50 (2.3.1). Temperature was controlled with a thermostat and maintained at 19±1°C, the photoperiod was set to the natural cycle. Water quality parameters were monitored daily: salinity and oxygen levels (O_2 > 95%, salinity 35 ppm) were measured with a HQ40d multimeter, total ammonia-nitrogen (TAN) and nitrite (NO_2^--N) were measured spectrophotometrically (Palintest 7000SE photometer, Portugal) using the respective kits according to the manufacturer's instructions. Faeces and uneaten feed were removed daily by siphoning. Fish were fed a commercial diet (R-3 EUROPA 22%, Skretting) twice a day at 1% of the initial biomass to avoid build-up of waste. The acclimation period lasted for 36 days until the start of the probiotic cannulation trial (as described in Figure 2.2).

2.3. Experimental design

Upon acclimatization (Figure 2.2), fish were infected with a dilution series of a *Tenacibaculum maritimum* suspension to experimentally assess the LC50 of the pathogen strain (2.3.1). In the subsequent experiment on the immunomodulation of the two probiotic candidates *Psychrobacter nivimaris* (Pn) and *Psychrobacter faecalis* (Pf), pathogen concentration was adjusted considering the LC50 (2.3.2). Here, fish received two successive administrations (0 d, 5 d) of the respective probiotic by rectal cannulation according to Winkelbach et al. (2015). In the control, fish received a saline solution. Thereafter, on 10 d (0 dpi), an experimental infection with *T. maritimum* was performed by bath immersion to study the immunomodulation of the probiotic treatment. Sampling was carried out before (0 dpi), 1 and 5 days post infection (dpi) and cumulative mortalities were recorded until 14 dpi.

2.3.1. Determination of the LC50 of *T. maritimum*

The experimental infection with *T. maritimum* was carried out according to Avendano-Herrera et al. (2006) using the strain ACC6.1, which was originally isolated from diseased sole and kindly provided by B. Costas (CIIMAR, Universty of Porto, Portugal).

For the infection with T. maritimum, pathogen suspensions were freshly prepared. Briefly, after streaking on marine agar 2216 [55.1 $g.L^{-1}$, pH=7.6 ± 0.2, Laboratorios Conda, Spain] an individual colony was picked to inoculate in marine broth [40.1 $g.L^{-1}$, pH=7.6 ± 0.2, Laboratorios Conda, Spain]. After incubation at 25 °C, for approximately 48 h,

pathogens were harvested with a Heraeus Multifuge 1 S-R (4500*g, 30 min, 4 °C), including two washing steps with 0.9% NaCl and resuspended in saline solution. The optical density of 1 mL (OD_{600}) was determined at 600 nm to calculate the pathogen concentration from a dilution series of *T. maritimum* (data not shown).

The determination of the LC50 was performed and given by another workgroup in CIIMAR, and the determined concentration was $1x10^8$ CFU mL^{-1}. For the determination of the LC50, fish were infected by immersion bath according to Avendano-Herrera et al. (2006), using a pathogen concentration of 10^6, 10^7 and 10^8 CFU mL^{-1}. Therefore, pumps were switched off and water in each tank was discharged until 2 L remaining in the tank. Aeration was intensified with additional air stones, enhancing mixing and exposure to the pathogen. Adding 50 mL of pathogen suspension, the respective concentration was established, and fish were exposed in these conditions for 18 h. Afterwards, the water was renewed three times to flush out pathogens and pumps were switched on again. Subsequently, cumulated mortalities were recorded over 7 d and statistically analysed by calculating LC50 value and its 95 % confidence limit after 7 d according to the Spearman–Karber method (Hamilton et al., 1977) (data not shown).

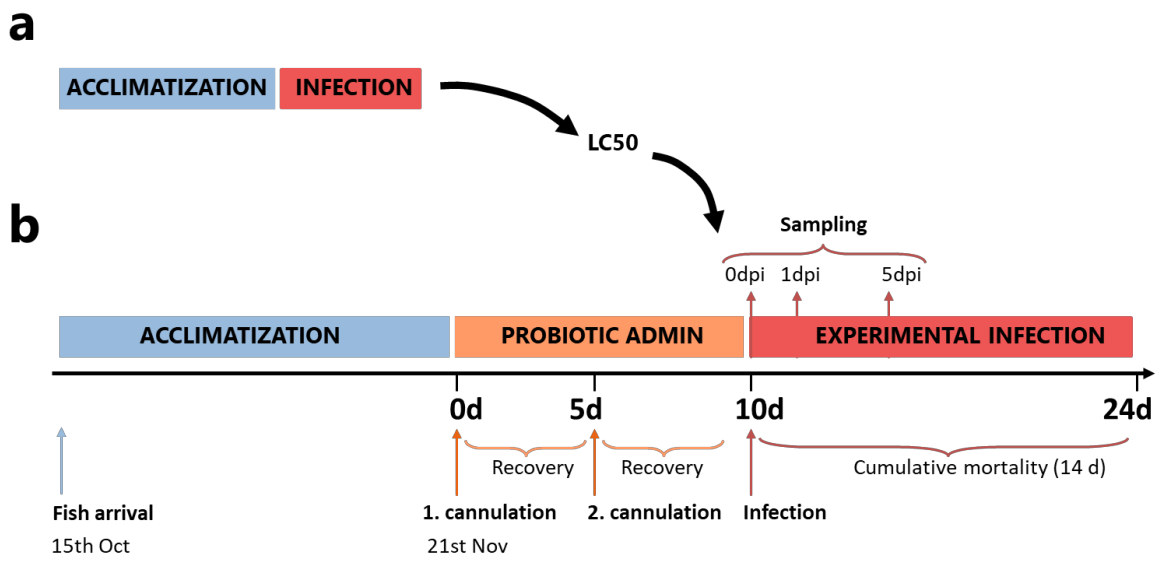

Figure 2.1 Time course of the study comprising (a) the experimental assessment of the LC50 of the *Tenacibalulum maritimum* ACC6.1 strain subsequently used to study (b) the immune-modulation of two probiotics administered by rectal cannulation before (0 dpi), 1 d post infection (1 dpi) and 5 dpi as well as the cumulative mortality over 14 dpi.

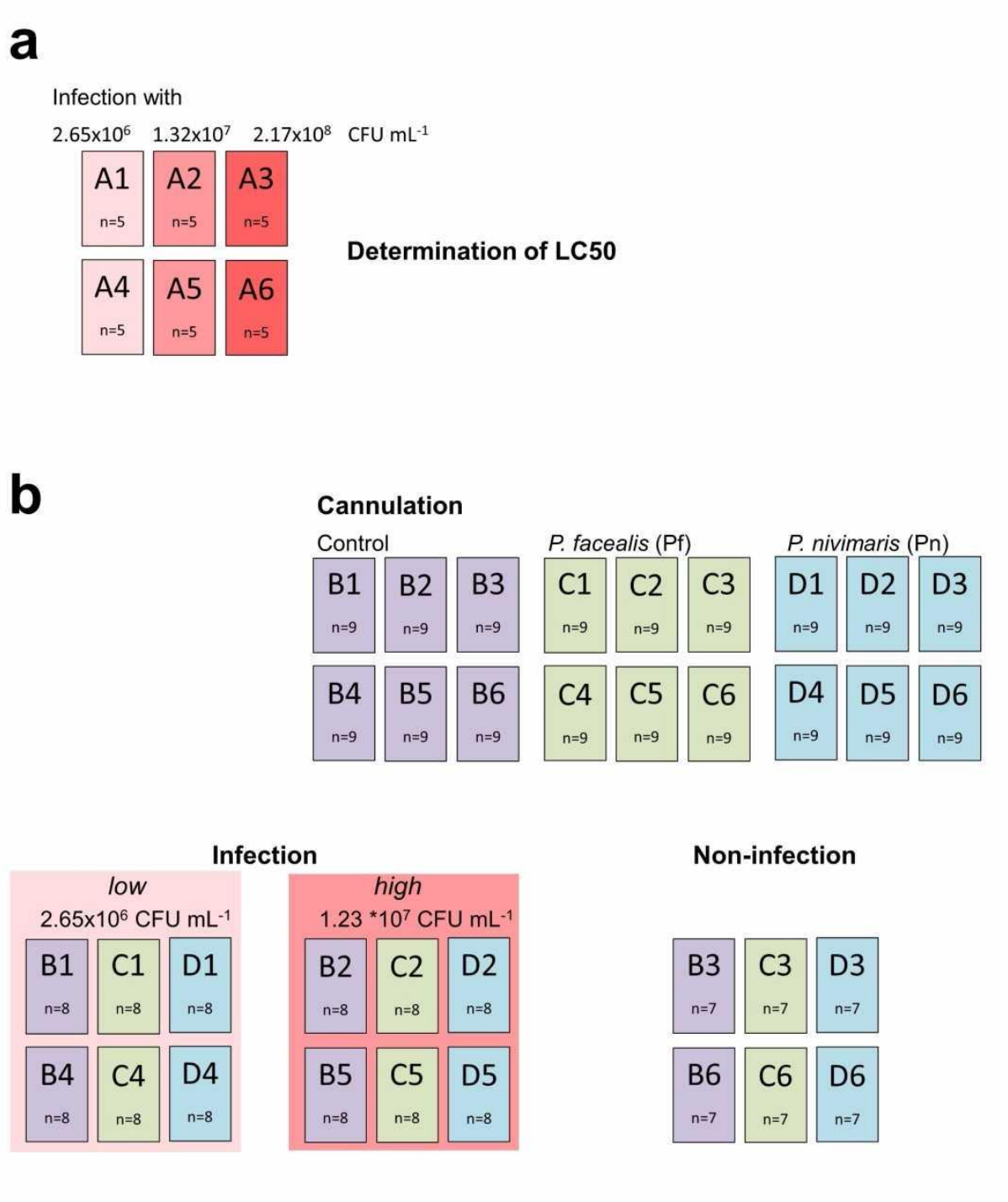

Figure 2.2 Experimental setup comprising 4 recirculation systems for a) the determination of the lethal concentration (LC50) after 7 d (2.3.1) and b) the immunemodulation of two probiotics, *Psychrobacter facealis* and *P. nivimaris* compared to a saline control administered twice by rectal cannulation after experimental infection with *Tenacibaculum maritimum* at a low pathogen concentration ($2.65x10^7$ CFU mL^{-1}) and cumulated mortalities at a high pathogen concentration ($1.23x10^8$ CFU mL^{-1}) 14 d post infection (2.3.2, 2.3.3).

2.3.2. Administration of probiotics by rectal cannulation

The present study is integrated into the project "Development of probiotics for the production of turbot in recirculating aquaculture systems" (BLE 2817303710). The two probiotic-candidates used in this experiment were previously isolated from healthy wild turbot, characterised by sequencing partial 16S rRNA and, subsequently, sequencing of the GYRB gene with diagnostic primers for the genus *Psychrobacter*. Screening for interesting traits among cultivable isolates identified antagonistic effects towards *T. maritimum* in a well diffusion assay.

Probiotics were freshly cultured from a cryoculture after streaking on marine agar 2216 and transfer of an individual colony to marine broth. After incubation at 18 °C for approximately 48 h, bacteria were centrifuged (4500*g, 30 min, 4 °C), washed in 0.9 % NaCl (w:V) and harvested by centrifugation, providing approximately 10^9 CFU g^{-1} as experimentally estimated by plate counting. Bacteria were kept on ice until used for cannulation.

For cannulation, fish were anaesthetized with 2-phenoxyethanol at 250 ppm. When a slower operculum movement was observed, the cannula (0.7 mm diameter, 19 mm length, Braun) was inserted via the anal pore and 1 mL of probiotic suspension (10^8 CFU ml^{-1}) were injected slowly into the hind gut with a 3-mL syringe. When the cannula was removed, the anal pore was immediately blocked with a finger and kept closed for 10 to 15 s to avoid excessive loss of the inoculum, while the body cavity was gently massaged to spread the inoculum over the gastrointestinal tract. Thereafter, fish were transferred to an intermediate rearing tank to wash off excess probiotic inoculum, before being returned to the original rearing system. The day before cannulation, feeding was suspended and only restarted the day after handling. Consequently, after each cannulation (0 d, 5 d), fish were allowed to recover for 3 and 4 days, respectively. The experimental infection (Figure 2.3.2) was carried out 4 days after the second rectal cannulation, directly after the first sampling (0 dpi).

Table 2.1 Molecular characterization of the probiotic candidates isolated from the intestine of a wild turbot, using sequence information of the 16 S rRNA and the GyrB gene.

Origin	Gastro-intestinal segment	Association to host gut cells surface	Isolate no.	ID	target 16 S rRNA gene sequencing	Consensus length [bp]	BLAST analysis 16 S rRNA gene sequencing: closest relative	first hit with	Accession no.	Similarity
Turbot wild *(Psetta maxima)*	stomach	moderate	S1"	002	*Psychrobacter* sp. Cluster 2	866	*Psychrobacter* sp. BB83	genus name	FR693355.1	100%
							Ps. nivimaris strain CJ-S-NA3	species name	HM584287.1	100%
	midgut	strong	S39"	077	*Psychrobacter* sp. Cluster 7	837	*Ps. pulmonis* strain PIGB186	species name	KU364058.1	100%

Origin	Gastro-intestinal segment	Association to host gut cells surface	Isolate no.	ID	target gyrB gene sequencing	Consensus length [bp]	BLAST analysis gyrB gene sequencing: closest relative	first hit with	Accession no.	Similarity
Turbot wild *(Psetta maxima)*	see above		(S1")	(002)	*Psychrobacter nivimaris* representative of cluster 2 ID=013 used for sequencing	1145	*Ps. nivimaris* gyrB gene	species name	AB490499.1	96%
	see above		(S39")	(077)	*Psychrobacter faecalis* representative of cluster 7 ID=082 used for sequencing	1146	*Ps. faecalis* strain Iso-46	species name	DQ143921.1	97%

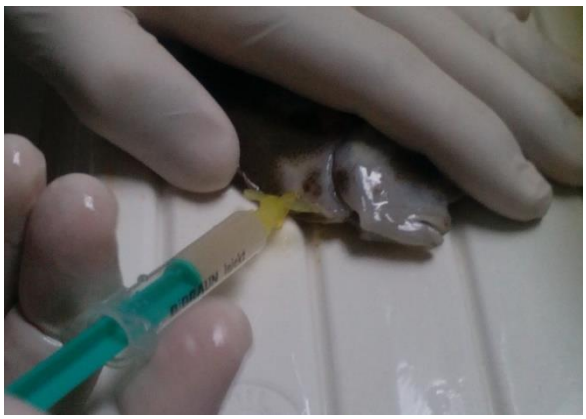

Figure 2.3 Administration of probiotic candidates by rectal cannulation in a juvenile turbot (details refer to 2.5.1).

2.3.3. Experimental infection with *T. maritimum*

After the administration of the probiotics, for the experimental infection on 10 d (corresponding to 0 dpi), a fresh suspension of T. maritimum ACC6.1 was prepared and fish were infected in an immersion bath as described above (2.4.1), using a concentration of 2.65×10^7 CFU mL^{-1} to study the immune modulation of the probiotic administration compared to the control (Figure 2.3).

For each treatment (Pf, Pn, control), a non-infected control group was additionally assessed in triplicate. These fish were kept in a separate system and, instead of the pathogen suspension, marine broth was added to the remaining rearing water.

In addition, for each treatment, cumulative mortalities at approximately a five-fold higher pathogen concentration ($1.23x10^8$ CFU mL^{-1}) were assessed over 14 d. Therefore, lethal and moribund fish were recorded twice a day and cumulative mortality was calculated accordingly:

$$Cumulative\ mortality = \frac{di}{ni}, \quad (1)$$

where di is the number of deceased fish and ni the total number of fish assessed (Figure 3.1).

2.4. Sampling

Sampling was carried out directly before the experimental infection on 10 d, subsequently referred to as 0 dpi (days post infection). For each group of infected and non-infected fish, further sampling was carried out on 1 dpi and 5 dpi (Figure 2.1). For RT-QPCR analysis, tissue samples (20-100 mg) of spleen, were transferred to 500 µl RNAlater (Qiagen), incubated at 4 °C for 24 h and stored at -20 °C.

2.5. Gene expression

RNA extraction

Total RNA was extracted with TRIzol as described by Reiser et al. (2011), including a DNase I digestion. In this project, all spleen samples were extracted and analysed. In brief, RNAlater was removed, 700 µL TRIzol reagent was added and tissue was homogenized with a Qiagen Tissue Lyzer and a metal bead (2 cycles at 18 rpm, 90 s). Thereafter, 600 µL TRizol were added and samples were mixed intensively. After centrifugation (10 min, 12000*g, 4 °C), 500 µL of the supernatant were transferred into a new 2 mL vial filled with 750 µL TRizol. After mixing, 250 µL chloroform were added, carefully vortexed (15 s) and incubated for 10 min at room temperature to allow phase separation. After centrifugation (15 min, 12000*g, 4 °C), 300 µL of the aqueous phase were collected in a new 2 mL vial and RNA was precipitated with 300 µL isopropanol after vigorous shaking (by hand, 10 s) and incubated for one hour at -20 °C, followed by 3 min at room temperature. After centrifugation (15 min, 12000*g, 4 °C), the solvent was discarded and the RNA pellet was washed with 300 µL ice cold ethanol (70 % V:V). Upon centrifugation (6 min, 12000*g, 4 °C) and discharge of the solvent, the pellet was dried with a Speed Vac for 4 min to remove excess ethanol completely. The RNA pellet was

dissolved in 20 µL of RNAse-free water (Sigma). Total RNA concentration and purity was determined with a Nanodrop® ND-1000 UV–Vis spectrophotometer, in duplicates. Purity was determined as the ratio of the absorbance at 260 and 280 nm (A260/280) ranging between 1.8 to 2.0. Moreover, integrity of the total RNA was checked by gel electrophoresis of all samples (Figure 6.3, in Annexes). Therefore, 500 ng of each sample were denaturated at 70 °C for 2 min in a thermal cycler and assessed in a TAE buffer 1.5% agarose gel with 1% ethidium bromide under UV light using a Gel Doc XR (Biorad). Additionally, 10% of all samples were analysed on RNA 6000 Nano chip with an Agilent 2100 Bioanalyzer, confirming high RNA integrity (RIN > 7) for all samples (Figure 6.4 in Annexes). Subsequently, samples were diluted to 25 ng μL^{-1} and final concentration was confirmed (± 20%) by RiboGreen RNA quantitation (Invitrogen) in 20% of the samples in an Infinite 200 microplate reader (Tecan).

Transcription (cDNA synthesis)

To eliminate any potential DNA contamination, DNAse I digestion was performed in all samples prior to transcription. Therefore, 1 µg of sample was diluted with pureH_20 to 8 µL, followed by the addition of 1 µL 10x DNAse buffer and 1 µL DNAse and, after mixing, incubation for 15 min at room temperature. Then, 1 µL 25 mM EDTA solution was added to stop the reaction. Finally, the enzyme was denatured in a thermal cycler for 10 min at 65 °C. Subsequently, reverse transcription (RT) was carried out after addition of 1.5 µL of Oligo(dt) primers (100 $ng.\mu L^{-1}$) and 1.5 µL of H_20 in the first thermal cycling program for primer annealing [65°C at 5 min, 40 °C at 3 min, 35 °C at 3 min, 30 °C at 3 min and 25 °C for 3 min]. Next, 2 µL of Affinity Script buffer, 2 µL of DTT, 1 µL dNTPs (10 mM of each) and 1 µL of Affinity Script reverse transcriptase enzyme were added to each sample for a second thermal cycling [42 °C for 60 min and 70 °C for 15 min]. In 10% of the samples, the enzyme was substituted by pure H_20, serving as a control (-RT) to monitor DNA contamination. Samples were stored at -20 °C until analysed by RT-QPCR.

RT-QPCR

Gene-specific primers for QPCR were designed based on reference sequences (GeneBank, NCBI) and confirmed by direct sequencing (Table 2). QPCR was carried out with a Mx3005p Cycler (Stratagene), monitoring specificity (e.g. formation of primer dimers or unspecific products) by analysing the melting curve. Briefly, 2 µL of a fivefold

diluted cDNA sample was used as template in a 25 µL PCR mix [1x PCR buffer, 3 mM $MgCl_2$, 0.2 mM of dNTPs (Qbiogene), 0.3 fold SYBR-Green I (Invitrogen), 0.4 µM each primer, 2 U Platinum Taq polymerase (Invitrogen)]. PCR conditions comprised an initial denaturation at 96 °C for 5 min, followed by 40 cycles of denaturation at 96 °C for 20 s, primer annealing (Ta for each primer in Table 2) for 20 s and elongation at 72 °C for 20 s. For each gene, -RT samples were used to monitor DNA contamination during RT as described above and a non-template negative control with water was assessed to exclude contamination during QPCR. Samples, controls and calibrator were assessed in duplicate. PCR primer efficiencies were determined experimentally with a dilution series of a calibrator (pooled sample of 20 ng μL^{-1} cDNA, only TNF 1µg μL^{-1}) and ranged between 92% - 116%, with a coefficient of determination R^2 = >0.9811. Expression of target genes were corrected for the assay efficiencies, normalised to *rpl8* as a housekeeping gene and calculated by the comparative C_T method (ΔΔCT), as described by Pfaffl (2001) according to the following equation (2):

$$Relative\ gene\ expression = \frac{(E_{target})^{\Delta CP target(control-sample)}}{(E_{ref})^{\Delta CP ref(control-sample)}} \quad (2)$$

where E_{target} is the QPCR efficiency of the target gene; E_{ref} is the QPCR efficiency of housekeeping gene *rpl8*; ΔCP_{target} is the difference in C_T between the calibrator and the sample regarding the target gene and ΔCP_{ref} is the difference in C_T between the calibrator and the sample referring to the housekeeping gene *rpl8*.

2.6.Data statistical analysis

All data are presented as mean ± standard deviation (SD). The comparison of survival curves resulting from the cumulative mortality trial was assessed by a Logrank-trend test, considering a significance value of $P<0.05$.

Gene expression data were log-transformed and checked for normality using the Shapiro-Wilk test and for homoscedasticity using Bartlett's test. Data were submitted to either a one-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) to compare treatments at a timepoint, for both infected and non-infected fish, and the same treatment between timepoints for infected fish. When significant differences were observed, either a Tukey's HSD (parametric) or a Dunn's (non-parametric) multiple

comparison test was performed to identify differences between groups. Moreover, non-infected and infected groups were compared by a Welch Two Sample t-test (parametric) or a Wilcoxon test (non-parametric). All statistical tests were carried out considering $P<0.05$ and were performed using R software (R Core Team, 2017).

Table 2: Specifications of QPCR assays including primer sequences, annealing temperature (Ta), amplicon length [bp], PCR efficiency (Eff) and NCBI accession number of the respective target genes: *rpl8* (housekeeping gene), *tcr*, *tgf β*, *tnf α*, *mhc II α*, *il-1β*.

gene	primer	5'-3' sequence	Ta [°C]	Length [bp]	Eff. [%]	GeneBank #
*rpl8**	F	CTCCgCCACATTgACTTC	64	197	94	DQ848874
	R	gCCTTCTTgCCACAgTAg				
tcr	F	ACgCCAATCACACggTCA	63	129	116	AY303762
	R	ATCCgAACTgCTCTCgTgg				
tgf β	F	gCTACCATgCCAACTACTgC	64	101	109	AJ276709
	R	TCCCgggTTgTgATgCTT				
tnf α	F	ATCCCCACTCCACgCTgA	65	224	95	FJ654645
	R	CgTCCTTgCTgTCATCgTC				
mhc II α	F	gATCCTCCTTCCAgTCCgAT	63	140	105	DQ094170
	R	AATgTTgAgACTCgCTCCC				
il-1β	F	CAgAAATCgCACCATgTCg	62	191	98	AJ295836
	R	gACAACCgCAAAgTTAACCTg				

* primers from Urbatzka et al. (2013)

3. Results

3. RESULTS

3.1.Cumulative mortality

Cumulative mortality was determined to assess effects of the administrated probiotic-candidates on disease resistance of fish. Compared to the assessment of the ideal concentration to use for the cumulative mortality trial (as described in section 2.5.2), the pathogen concentration of 1.23 x 10^8 CFU.mL^{-1} resulted in lower cumulative mortalities than expected. Fish that previously received probiotic suspension revealed a higher survival upon infection with *T. maritimum* than the control fish that received saline solution. These control fish revealed the lowest survival rate (80%). Supplementation with probiotic candidate Pf increased survival to 92% and, in probiotic candidate Pn, no mortalities were observed after 14 days post infection. Interestingly, all mortalities were observed two days post-infection (1 dpi). Still, there were no significant differences between the survival curves (Logrank trend test, P = 0.0538).

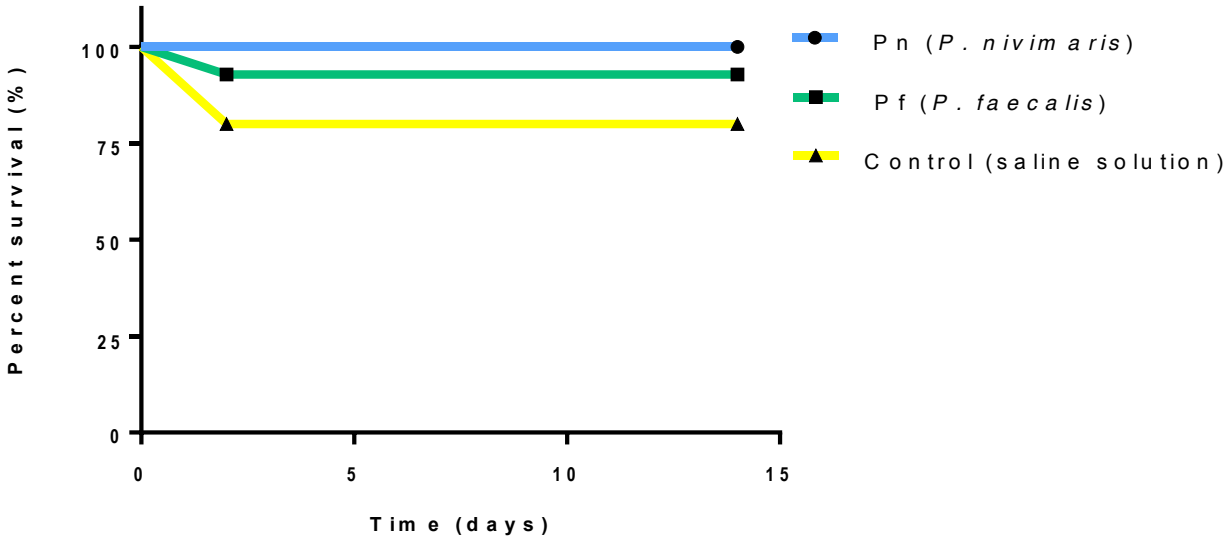

Figure 3.1. Kaplan-Meier survival curves (Kaplan and Meier, 1958) upon infection trial with *T. maritimum* (1.23 x 10^8 CFU.mL^{-1}) in juvenile turbot that previously received repeatedly probiotic suspensions of *Psychrobacter nivimaris* (Pn) or *Psychrobacter faecalis* (Pf) compared to a saline solution control. Logrank trend test, p-value<0.05, n=16, 15 and14 each group, respectively.

3.2.Gene expression

Immune-related gene expression was quantified in the spleen by RT-QPCR and normalized to the housekeeping gene *rpl8* to assess any modulation by the probiotic-candidates. Differences in gene expression were evaluated between infected fish that received probiotics via rectal cannulation and a saline control group at 0 dpi, 1 dpi and 5 dpi. Additionally, at each timepoint, infected fish were compared with an internal control of unchallenged fish. In general, gene expression of the selected markers revealed a similar pattern over time, with a few exceptions. The detailed results, specific of each gene, are presented below.

T-cell receptor (*tcr*)

After infection, at 1 dpi, treatments were considered significantly different (one-way ANOVA, P = 0.0083), namely Pn compared to the control (Tukey multiple comparisons, P = 0.0064), which coincidently, shows the lowest expression values at this timepoint. Also, *tcr* expression was affected in all treatments (Welch Two Sample t-test, P = 0.0003) comparing to the internal unchallenged control.

At 5 dpi, all treatments exhibited similar levels of expression and, accordingly, there were no significant differences between treatments (one-way ANOVA, P = 0.5910). Moreover, there were no differences in gene regulation of infected fish to the internal unchallenged control (Welch Two Sample t-test, P = 0.9656). Expression values in 5 dpi were slightly higher than in 1 dpi for treatments Pn and Pf (Figure 3.4).

Over time, *tcr* expression was relatively constant for fish submitted to the probiotic-candidate treatments, however control fish had a significantly higher gene expression right after infection, comparing to 0 dpi and 5 dpi (Dunn's test, P = 0.0136 and 0.0096, respectively).

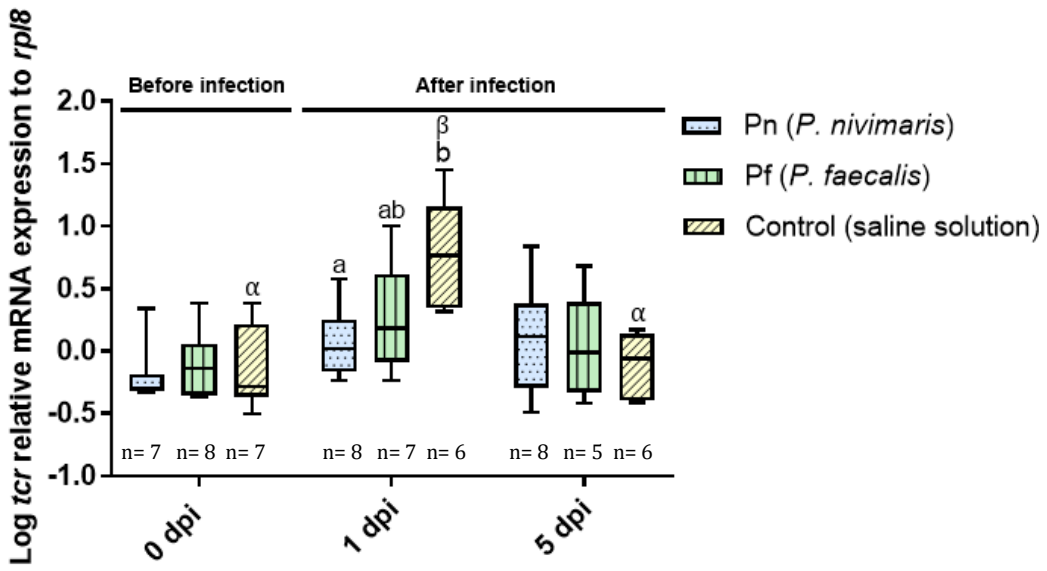

Figure 3.2 Relative mRNA T-cell receptor (*tcr*) in the spleen of juvenile turbot 0 dpi, 1 dpi and 5 dpi with *T. martimum*, previously exposed via rectal cannulation with to *P. nivimaris* (Pn), *P. faecalis* (Pf) and a saline solution control. Gene expression was normalized to ribosomal protein L8 (*rpl8*) and is presented as fold increase of the respective control. Error bars represent 5th and 95th percentiles and the box identify the median. Differences were found by parametric Tukey's HSD test or non-parametric Dunn's test. Number of samples used is given above the respective box. At the same timepoint, boxes with different letters indicate significant differences among treatments. Within the same treatment, different symbols indicate significant differences between timepoints. In the absence of a signifier, there were no significant differences. Text contains levels of significance. Number of samples for each treatment is described under the respective bar of the figure.

Major histocompatibility complex II alpha (*mhc II α*)

Comparing to the internal non-infected group, *mhc II α* expression was upregulated in all treatments (Welch Two Sample t-test, P = 0.0002) at 1 dpi. Here, treatments were found significantly different (one-way ANOVA, P = 0.00822), with the control group having the highest gene expression when compared with *P. nivimaris* treatment (Tukey multiple comparison test, P = 0.0084) and *P. faecalis* (Tukey multiple comparison test, P = 0.0330), which were showed no differences. At 5 dpi, treatment C expression dropped to the lowest values, while treatments Pn and Pf remained with similar levels of expression (Figure 3.2), thus, there were no significant differences between treatments (one-way ANOVA, P = 0.4470). Moreover, there were no differences in gene regulation of infected fish to the internal unchallenged control (Welch Two Sample t-test, P = 0.5532).

Throughout the challenge, fish from the control group treatment exhibited a high peak in expression values, significantly different from 0 dpi to 1 dpi (Dunn's test, P = 0.0028) and to 5 dpi (Dunn's test, P = 0.0279), but fish cannulated with the probiotic-candidates maintained similar expression levels, with a solid increase when comparing initial T0 values to the other sampling events.

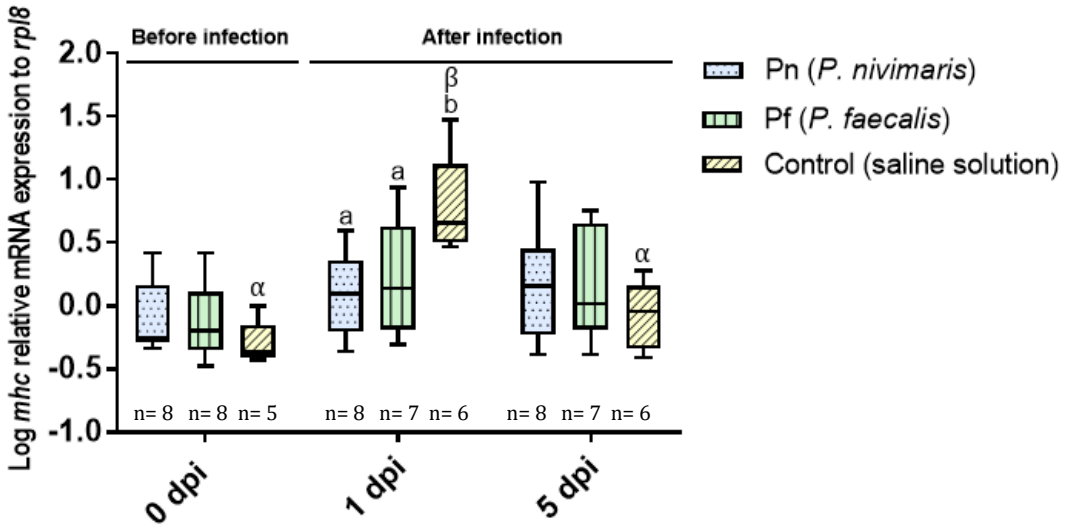

Figure 3.3 Relative mRNA expression of the major histocompatibility complex II alpha (*mhc II α*) in the spleen of juvenile turbot 0 dpi, 1 dpi and 5 dpi post infection with *T. maritimum*, previously exposed via rectal cannulation with to *P. nivimaris* (Pn), *P. faecalis* (Pf) and a saline solution control. Gene expression was normalized to ribosomal protein L8 (*rpl8*) and is presented as fold increase of the respective control. Error bars represent 5th and 95th percentiles and the box identifies the median. Differences were found by parametric Tukey's HSD test or non-parametric Dunn's test. Number of samples used is given above the respective box. At the same timepoint, boxes with different letters indicate significant differences among treatments. Within the same treatment, different symbols indicate significant differences between timepoints. In the absence of a signifier, there were no significant differences. Text contains levels of significance. Number of samples for each treatment is described under the respective bar of the figure.

Interleukin one beta *(il-1β)*

At 1 dpi, *il-1β* gene expression was regulated in fish exposed to all treatments, when comparing to an internal control of non-infected fish (Wilcoxon rank sum test, P = 0.0029). There were significant differences in RNA expression between treatments (Kruskal-Wallis rank sum test, P = 0.0053): specifically, the control group showed a significantly higher gene expression than Pn (Dunn's test, P = 0.0082) and Pf (Dunn's test, P = 0.0162). Notably, the control group at timepoint 1 dpi presented the highest *il-1β* relative expression (Figure 3.3). Later in the challenge, at 5 dpi, there were no statistical significant differences (one-way ANOVA, P = 0.2990) between fish exposed to different

treatments. Moreover, when comparing with a non-challenged fish group, no treatments appear to have affected transcripts expression significantly (Welch Two Sample t-test, P = 0.2451). Over time, *il-1β* expression was higher (not statistically significant) in fish treated with *P. nivimaris* than as with *P. faecalis* or the control at 5 dpi, whereas fish of Pf had a relatively constant expression of this gene throughout sampling points. Only the control fish showed significant differences in gene expression, between 1 dpi and 0 dpi (Dunn's test, P = 0.0087) and 1 dpi and 5 dpi (Dunn's test, P = 0.0121).

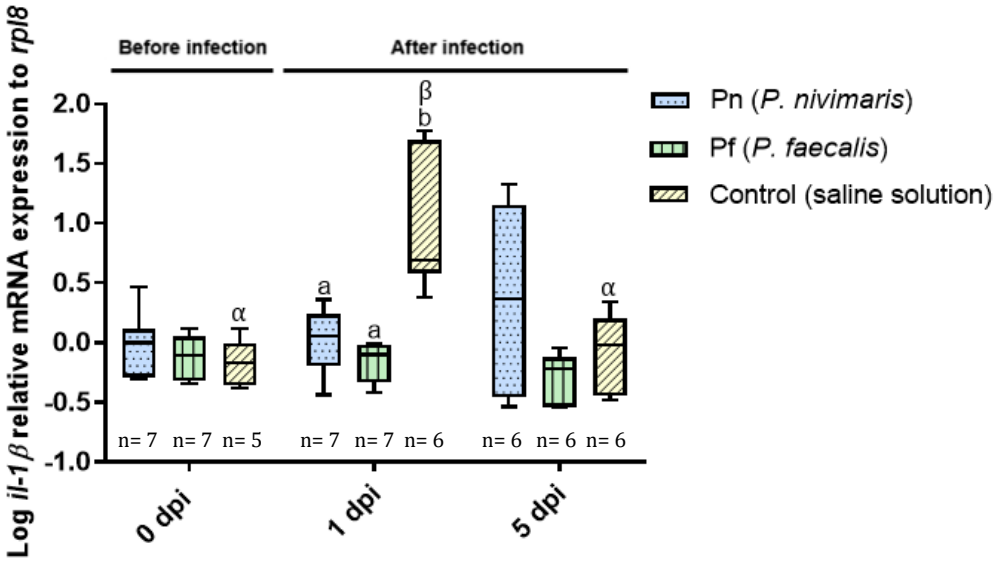

Figure 3.4 Relative mRNA expression of the interleukin beta one (*il-1β*) in the spleen of juvenile turbot 0 dpi, 1 dpi and 5 dpi post infection with *T. martimum*, previously exposed via rectal cannulation with to *P. nivimaris* (Pn), *P. faecalis* (Pf) and a saline solution control. Gene expression was normalized to ribosomal protein L8 (*rpl8*) and is presented as fold increase of the respective control. Error bars represent 5th and 95th percentiles and the box identifies the median. Differences were found by parametric Tukey's HSD test or non-parametric Dunn's test. Number of samples used is given above the respective box. At the same timepoint, boxes with different letters indicate significant differences among treatments. Within the same treatment, different symbols indicate significant differences between timepoints. In the absence of a signifier, there were no significant differences. Text contains levels of significance. Number of samples for each treatment is described under the respective bar of the figure.

Transforming growth factor beta *(tgf β)*

Unlike the other target genes, *tgf β* had significant differences (P = 0.0200) at the first sampling event, prior to infection: both treatments Pn and Pf presented higher expression than the control (P = 0.0292 and 0.0468, respectively). The differences showcased at T0 lead to the belief that cannulation provoked an additional immune response (Figure 3.5).

At 1 dpi, gene expression was influenced by the exposure to the pathogen (P = 0.0125). Control-treated fish suffered a rise in the expression values, resulting in no significant differences between treatments (one-way ANOVA, P = 0.0714), however there was a marginal difference between *P. nivimaris* and the control (Tukey multiple comparison test, P = 0.0590). At 5 dpi, expression values decreased and there were no more differences between treatments (one-way ANOVA, P = 0.7830), and no differences in expression when comparing to the non-infected fish (Welch Two Sample t-test, P = 0.9132). Over time, control-treated fish had a significantly higher gene expression in 1 dpi when comparing to 0 dpi and 5 dpi (Dunn's test, P = 0.0003 and 0.0175, respectively).

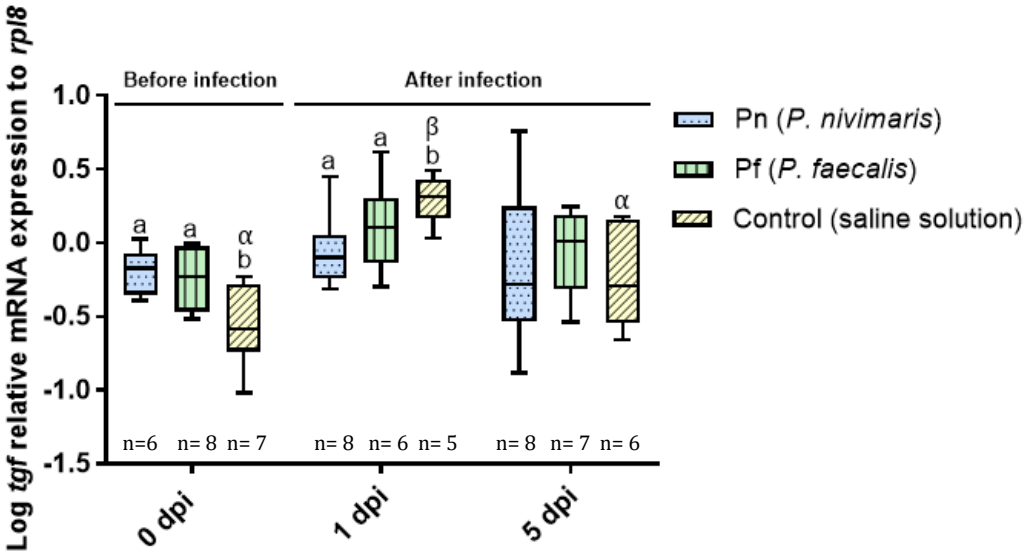

Figure 3.5 Relative mRNA transforming growth factor beta (*tgf β*) in the spleen of juvenile turbot 0 dpi, 1 dpi and 5 dpi post infection with *T. martimum*, previously exposed via rectal cannulation with to *P. nivimaris* (Pn), *P. faecalis* (Pf) and a saline solution control. Gene expression was normalized to ribosomal protein L8 (*rpl8*) and is presented as fold increase of the respective control. Error bars represent 5th and 95th percentiles and the box identifies the median. Differences were found by parametric Tukey's HSD test or non-parametric Dunn's test. Number of samples used is given above the respective box. At the same timepoint, boxes with different letters indicate significant differences among treatments. Within the same treatment, different symbols indicate significant differences between timepoints. In the absence of a signifier, there were no significant differences. Text contains levels of significance. Number of samples for each treatment is described under the respective bar of the figure.

Tumour necrosis factor alpha *(tnf α)*

The expression of *tnf α* was only marginally different at 1 dpi (one-way ANOVA, P = 0.0520). Specifically, *P. nivimaris* presented lower expression values than the control group (Tukey multiple comparison test, P = 0.0425). Comparing to the internal non-infected group, expression was different for all treatments (Welch Two Sample t-test, P =

0.0001). At 5 dpi, *tnf α* expression was similar for all treatments (one-way ANOVA, P = 0.5100). Moreover, these did not show significant differences towards the internal unchallenged group (Welch Two Sample t-test, P = 0.2043).

The pattern of *tnf α* expression again suggested that fish affected with the probiotic-candidates reacted differently than the control shortly after the infection point, increasing slightly over T0. Control-treated fish at 1 dpi exhibited higher gene expression when comparing with 0 dpi and 5 dpi (P = 0.0102 and 0.0131, respectively).

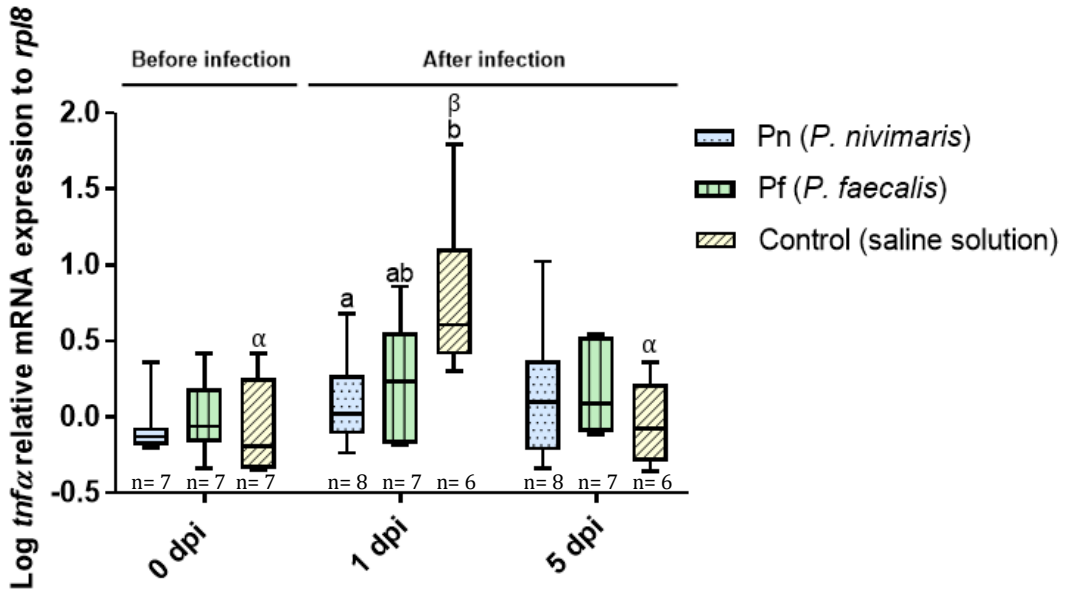

Figure 3.6 Relative mRNA tumor necrosis factor (*tnf α*) in the spleen of juvenile turbot 0 dpi, 1 dpi and 5 dpi with *T. martimum*, previously exposed via rectal cannulation with to *P. nivimaris* (Pn), *P. faecalis* (Pf) and a saline solution control. Gene expression was normalized to ribosomal protein L8 (*rpl8*) and is presented as fold increase of the respective control. Error bars represent 5th and 95th percentiles and the box identifies the median. Differences were found by parametric Tukey's HSD test or non-parametric Dunn's test. At the same timepoint, boxes with different letters indicate significant differences among treatments. Within the same treatment, different symbols indicate significant differences between timepoints. In the absence of a signifier, there were no significant differences. Text contains levels of significance. Number of samples for each treatment is described under the respective bar of the figure.

In addition, fish not infected with *T. maritimum* that were exposed to the same treatments of the probiotic trial (Pn, Pf and control) were also compared within timepoints, to assess immunomodulation in unchallenged fish. There were no statistical differences in gene expression between treatments Pn, Pf and control for genes *tcr*, *mhc II α*, *il-1β, tgf* and *tnf* in all timepoints. However, there is an increase in gene expression, represented in all genes in fish exposed to treatment Pn later in the infection trial, at 5 dpi, which is displayed in Figure 3.7.

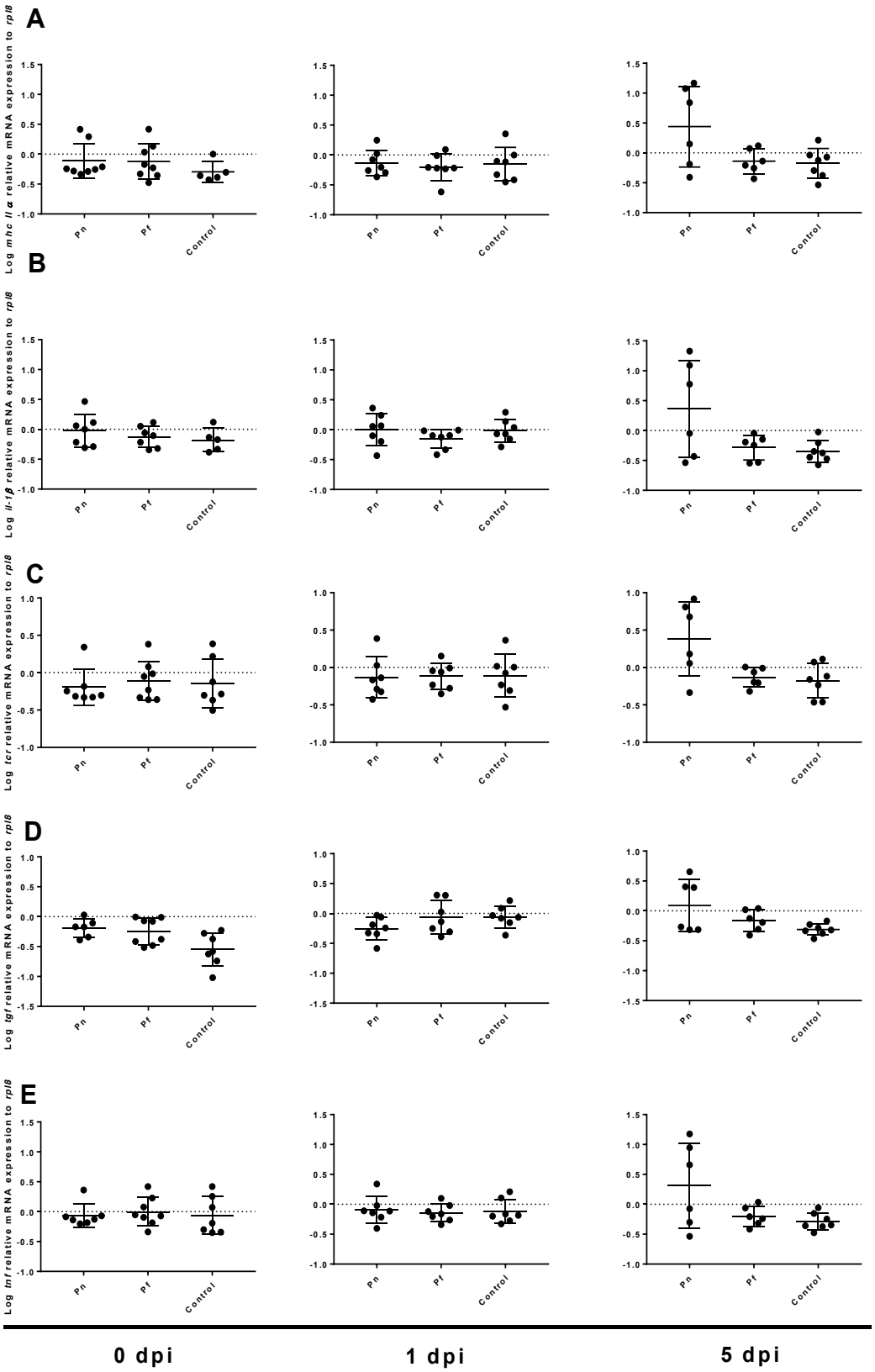

Figure 3.7 Relative mRNA expression of (A) *mhc II α* (B) *il-1β* (C) *tcr* (D) *tgf* and (E) *tnf* in the spleen of non-infected juvenile turbot at 0 dpi (days post infection), 1 dpi and 5 dpi. Pn and Pf were administered previously probiotic candidates *Psychrobacter nivimaris* and, *P. faecalis* (Pf), control fish received PBS via rectal administration. Gene expression was normalized to ribosomal

protein L8 (rpl8) and is presented as fold increase of the respective control. Data are presented as mean, whiskers represent 5th and 95th percentiles. Significant differences were not detected (Tukey's HSD test, Dunn's test, $p<0.05$). Individual number of samples per treatment is shown by the dots in each graph, in the respective treatment bar.

4. Discussion

4. DISCUSSION

Probiotic testing has been a focal point for aquaculture research over the last years. In this study, the beneficial effects of probiotic candidates on the immune status were assessed experimentally, aiming at the application as feed supplement to improve health of farmed turbot and, thereby, improve disease resistance in practice. This was evaluated by inoculating healthy juvenile turbot with native bacteria and then challenging them with an important pathogen, *T. maritimum*. In addition, this project attempted an innovative method of bacterial inoculation for probiotic gut colonization. To our knowledge, this was the first time the *in vivo* probiotic effects of *Psychrobacter faecalis* and *Psychrobacter nivimaris* were assessed in turbot.

There are no current published studies regarding the *in vivo* effects of rectally-injected *P. nivimaris* or *P. faecalis* in gene expression of challenged turbot, which does not allow a direct comparison of these results with similar studies. Additionally, *Psychrobacter* spp. were described only recently, thus there are few studies regarding their use as probiotics, although they have been described as beneficial for seafood in several cases. For instance, diet supplementation of *Psychrobacter* sp. improved feed utilization, digestion (activity of digestive enzymes) and innate immunity (Sun et al., 2011) and also the microbial diversity along the gastrointestinal tract (Ling Yang et al., 2011) in *Epinephelus coioides*. In shrimp post-larvae, a native *Psychrobacter* sp. improved growth performance, immunity and disease resistance when used as a water-additive (Franco et al., 2016). Recently, the supplementation in diet of *P. namhaensis* improved growth rates, feed utilization efficiency and the immune response of Nile tilapia (Makled et al., 2017). As *P. nivimaris* and *P. faecalis* were directly isolated from the host, they are assumed to withstand typical internal conditions of turbot and attach to the intestinal tissue surfaces. Moreover, they have previously displayed a growth inhibition effect on *T. maritimum* but not *Vibrio* sp., while not displaying cross-inhibition. Other authors have described *in vitro* antagonistic effects of *Psychrobacter* sp. against *Vibrio* spp. (Sun et al., 2009) and *Aeromonas* sp. (Franco et al., 2016). Likewise, as host-derived bacteria, *Psychrobacter* spp. have modulated *in vitro* immune responses against a pathogen (*Vibrio anguillarium*) in epidermal cells of Atlantic cod (Lazado & Caipang 2014b). Subsequently, in the context of this study, P. *nivimaris* and P. *faecalis*

were considered strong candidates for probiotic bacteria in turbot, especially when challenged by the economically-relevant pathogen for aquaculture, *T. maritimum*.

Disease resistance of challenged turbot was assessed as cumulative mortality upon infection. The group inoculated with *P. nivimaris* had no mortalities, whereas fish treated with *P. faecalis* had 92% of survival, compared to 80% in the control. This suggests that both probiotic-candidates boosted disease resistance against *T. maritimum* during the infection challenge. Other studies described probiotics as increasing fish survival by enhancing their immune response, digestion and nutrition (Verschuere et al., 2000, Balcazar et al., 2006). Particularly, *Psychrobacter* sp. has been shown to enhance the survival of white shrimp post-larvae when challenged with *Aeromonas* sp. (Franco et al., 2016).

The pathogen strain used in the present trial has been described to successfully infect turbot with tenacibaculosis (Avendaño-Herrera et al., 2006, Costas et al., 2014, Mabrok et al., 2016), exemplified as severe mortalities and outbreak of the disease. In our experiment, however, the control group suffered just 20 % of mortality during the challenge, which is considered an unusually low when compared to other infection studies. For instance, Avendaño-Herrera et al. (2006) obtained full mortality in juvenile turbot, between 1 and 10 dpi, at a similar pathogen concentration (10^6 to 10^8 CFU.mL^{-1}). Still, fish were smaller, weighing between 4 to 6 g, which may justify the disparate results here, considering the higher sensitivity of smaller fish towards infection. In a similar study with juvenile turbot, mortality reached 100% at 14 dpi in a bath challenge with the less virulent strain *T. maritimum* LL01.8.3.8 applying 10^9 cfu.ml^{-1} (Munoz-Atienza et al., 2014). In fact, in our own pre-trial, the control group mortality (Table 6.1 in Annexes), was 100% at the same concentration used to assess cumulative mortality (10^8 cfu.mL^{-1}).

T. maritimum's pathogenesis has been poorly understood thus far (Avendaño-Herrera et al., 2006, Gourzioti et al., 2016). Several strategies have been explored to determine a liable infection methodology, observing variable mortalities: documented infection techniques include bath immersion challenge, subcutaneous and intraperitoneal inoculation (Wakabayashi et al., 1984, Baxa et al., 1987, Alsina & Blanch 1993, Powell et al., 2004, 2005, Failde et al., 2013). In this experiment, pathogen infection was performed by a bath-immersion challenge. This is a common and successfully used strategy for inoculation of bacterial pathogens to aquatic organisms (Roque et al., 1998, Aoki et al., 2005, Avendaño-Herrera et al., 2006, Nishioka et al., 2009). Avendaño-Herrera

et al., (2006) have developed the reproducible bath-immersion for experimental infection with *T. maritimum*, applied here in juvenile turbot. This method was chosen because it is a standardized protocol designed specifically for studying the host-pathogen interaction (Avendaño-Herrera et al., 2006). Although studies have demonstrated subcutaneous and intraperitoneal injection as a reliable method for experimental infection with *T. maritimum* (Failde et al., 2013, Faílde et al., 2014), these require individual handling of the fish, which is a time-consuming process that causes substantial stress. Furthermore, during bath immersion, close contact with the fish's body surface enables bacteria adhesion to primary infection sites such as mouth, fins and gills, thus improving the success and liability of the infection (Nordmo, 1997). Finally, bath immersion simulates a realistic scenario for fish infection, likely to occur in the wild or farming context (Nordmo, 1997), although actual concentrations of *T. maritimum* in the wild are presumably lower.

Since the pre-trial was conducted in a separate recirculation system than the rest, with all safety precautions to avoid any contamination with the pathogen, an improved immunity (e.g. adaptive immunity) of the fish can be excluded. It is possible that these low mortalities reflect a less severe *T. maritimum* outbreak. Despite the low mortalities recorded, sampled fish did appear to have elicited an immune response after infection, since results on the control group demonstrated an up-regulation in gene expression, which also corroborates the efficacy of the infection.

At the end of the probiotic trial, at 0 dpi, results showed that *mhc II α*, *il-1β*, *tcr*, and *tnf α* gene expression of fish treated with *P. nivimaris* and *P. faecalis* did not show any differences from the control. Thus, probiotic treatment did not result in an immunomodulation before the actual challenge. Considering that the bacteria candidates used were part of the native microbiota of turbot, it seems plausible that an application did not alter or induce the immune system. There were no signs of stress observed after rectal cannulation following a short period of recovery, thus the immunomodulation of probiotics will be discussed in comparison to the PBS treated control. Upon experimental infection, *tgf* gene expression was up-regulated in both probiotic groups compared with the control. This cytokine is activated by regulatory T-cells and inhibits the action of other T-cells, thereby acting as an immunosuppressor, (Massague, 1990). Shortly after infection, at 1 dpi, expression of *mhc II α*, *il-1β*, *tcr*, *tnf α* and *tgf* was comparable between treatments with *P. nivimaris* and *P. faecalis*. However, control fish revealed an up-

regulation in gene expression, for all genes, comparing with the first and last sampling points (0 dpi and 5 dpi). The up-regulation of these genes has been described in finfish before and it is considered as an immune response (Newaj-Fyzul and Austin, 2015). More specifically, in turbot, Millán et al. (2011) described an up-regulation of interleukin-1 (IL-1) and *tnf α* related genes in spleen, as well as upregulation of *mhc II α* in liver of turbot exposed to a pathogen *Aeromonas salmonicida*. On the other hand, a recent study demonstrated that genes *mhc II*, *il-1b*, *tcr*, and *tnf α* were significantly up-regulated under the effect of formalin-killed cells against *E. tarda* with flagellin, as an adjuvant in turbot (Liu et al., 2017).

The results of this study suggest that turbot stimulated with native bacteria exhibit lower immunomodulation, since the control group exhibited the highest levels of gene expression in all cases, thus a stronger immune response. The marginal survival difference between putative probiotic-treated fish and the control group suggests these bacteria did improve disease resistance in turbot, even though this is not reflected in gene expression. Other studies have demonstrated that *in vivo* administration *Psychrobacter* sp. can modulate the immune system. Sun et al. (2011) described that dietary administration of *Psychrobacter* sp. increased phagocytic activity, phagocytic index, superoxide dismutase activity and serum complement component 3 (C3) and 4 (C4) levels in *E. coioides*. Moreover, addition of *Psychrobacter* sp. to the rearing water of *P. vannamei* post-larvae increased its superoxidase dismutase activity, peroxidase activity and nitric oxide formation (Franco et al., 2016). Finally, Makled et al. (2017) described that dietary administration of *P. namhaensis* increased immunoglobulin M (IgM), alternative complement haemolysis (ACH50), phagocytic and lysozyme activities in *O. niloticus*. Even though gene expression of the selected immune markers was low, turbot immunomodulation by the probiotic-candidates might have been detected in other physiological processes. Alternatively, this suggests that *P. nivimaris* and *P. faecalis* might have reduced the pathogenesis of *T. maritimum* without directly involving the immune system of the host. Although immunomodulation is a well-known MOA described for several probiotics (Balcazar et al., 2006, Newaj-Fyzul et al., 2014, Newaj-Fyzul and Austin, 2015), other mechanisms can benefit the host and affect fish survival. Other MOA described for probiotics, not directly related to immunoregulation, include improved digestibility and enzymatic contribution to nutrition, improvement of water quality, competitive exclusion and production of inhibitory compounds (Verschuere et al., 2000,

Balcazar et al., 2006, Pérez-Sánchez et al., 2014). These are common characteristics for native gut microflora. Since *Psychrobacter* sp. was described to inhibit *T. maritimum* growth, it is possible they might produce antagonist substances towards this pathogen or compete for substantial factors, like energy, chemicals or adhesion sites.

No studies have yet described the effects in fish immune system of probiotics which are rectally administrated. Considering other species of this genus have been able to regulate the immune system of fish, when used as a feeding supplement, it is possible that these results reflect the administration method. For example, (Munoz-Atienza et al., 2014) reported an up-regulation of gene expression in the skin of turbot by bath administration of LAB, however there was a down-regulation of these genes in spleen and other internal organs. The main probiotic inoculation methods currently used include oral administration, addition to water and injection. One of the candidates' main challenges is the ability to endure the pathway of oral administration until the adherence to the intestine. Since the stomach is a very harsh environment for living organisms, due to the low pH and the presence of proteases, bacteria do not survive long enough to allow colonization of the gut (Goldin and Gorbach, 1992, Bezkorovainy, 2001). In this study, it was decided to inoculate the fish via rectal cannulation, increasing their presence in the gut. Several treatments in human medicine have been tested to be administrated rectally, including probiotic bacteria, because it is a fast and successful way of retaining these compounds (De Boer et al., 1982, Stocker and Montgomery, 2001, D'Incà et al., 2011). This procedure could be considered more invasive than other methods, given fish had to be anesthetized and directly manipulated. However, recovery was noticeably fast – fish were responsive to stimuli and food after one-hour post-trial, which was considered to be an empiric sign of low stress. Besides, turbot is described as being stress-tolerant, with fast recovery to rearing activities and handling (Waring et al., 1996).

The rectal cannulation events proved to be time efficient, even considering each fish was individually inoculated with the probiotic-candidates. It was noticed, however, that when fish were returned to an intermediate rearing tank, a part of this solution would be lost in the water. In general, less volume was lost when it was slowly injected and when the fish was less massaged. Although this was a simple, easily reproducible, and fast inoculation method when compared to oral administration, it is inconclusive whether rectal cannulation was effective in supplying the bacteria for colonization of the

gut. Nonetheless, the differences in survival and gene expression, comparing to the control group, point to a somewhat successful methodology. As mentioned before, previous studies underline that few days after ceasing the supply of probiotics, their amount decreases sharply (Fuller, 1992). So, in the context of this project, application of the probiotic-candidates should be intensified in future trials, using either higher dosage of inoculum or more cannulation events. To verify the efficacy of this practice, the presence of the target bacteria in several gut regions should be confirmed using techniques such as RT-qPCR (Nadkarni et al., 2002) with species-specific primers to determine *Psychrobacter sp.* bacterial load. Further testing must be performed to validate this methodology as bacterial administration.

CONCLUSIONS AND OUTLOOKS

In the context of this study, although there were positive effects on fish inoculated with *Psychrobacter* sp., expression of the selected target genes appeared to be somewhat supressed. Moreover, the lack of studies of rectally administrated bacteria leads to inconclusive results on immunomodulation of turbot. This study suggests that *P. nivimaris* and *P. faecalis* might be solid candidates to native commercial probiotics for turbot, since they could marginally improve disease resistance against a high-virulence pathogen. However, it is unclear how they acted in the immune system in a global manner, since they mainly did not up-regulate gene expression. Possible benefits provided by *P. nivimaris* and *P. faecalis* could be manifested in other physiological processes. Further analyses, including humoral parameters and full blood analyses, should be performed to accurately understand their effects against pathogens. Additionally, histology studies could be performed to assess if *P. nivimaris* and *P. faecalis* possess *in vivo* antagonist effects against *T. maritimum*, as ithas been observed in *in vitro* tests. Inoculation by rectal administration proved to be an easy and time-efficient technique, however, it is inconclusive whether it is capable of promoting gut colonization by cannulated native bacteria. In the future, to truly assess the suitability of *P. nivimaris* and *P. faecalis*, a diet supplementation via oral administration should be performed to assess their capacity to survival in turbot's digestive tract and their effects in the immune system. This project served as a preliminary trial to test the efficacy of these probiotic candidates, and provides a basis for further tests on their suitability as commercial probiotics.

5. References

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http://www.fishbase.org/summary/1348

6. Annexes

6. ANNEXES

Figure 6.1 Individual recirculation systems set-up used for all stages of the experimental trial.

Figure 6.2 Deceased juvenile turbot post cannulation trial.

Table 6.1 Cumulative mortality assessment of the pre-trial *T. maritimum* challenge in probiotic-unchallenged juvenile turbot (n=10 in each group, N=30), expressed in numbers (A) and percentage (B).

(A)

		Trial days							
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
T. maritimum concentration (cfu.mL^{-1})	**10^6**	10	10	10	10	10	10	10	10
	10^7	10	10	9	9	9	9	9	9
	10^8	10	8	4	2	1	1	0	0

(B) Cumulative mortality of Pre-challenge trial

		Trial days							
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
T. maritimum concentration (cfu.mL^{-1})	**10^6**	0%	0%	0%	0%	0%	0%	0%	0%
	10^7	0%	0%	10%	10%	10%	10%	10%	10%
	10^8	0%	20%	60%	80%	90%	90%	100%	100%

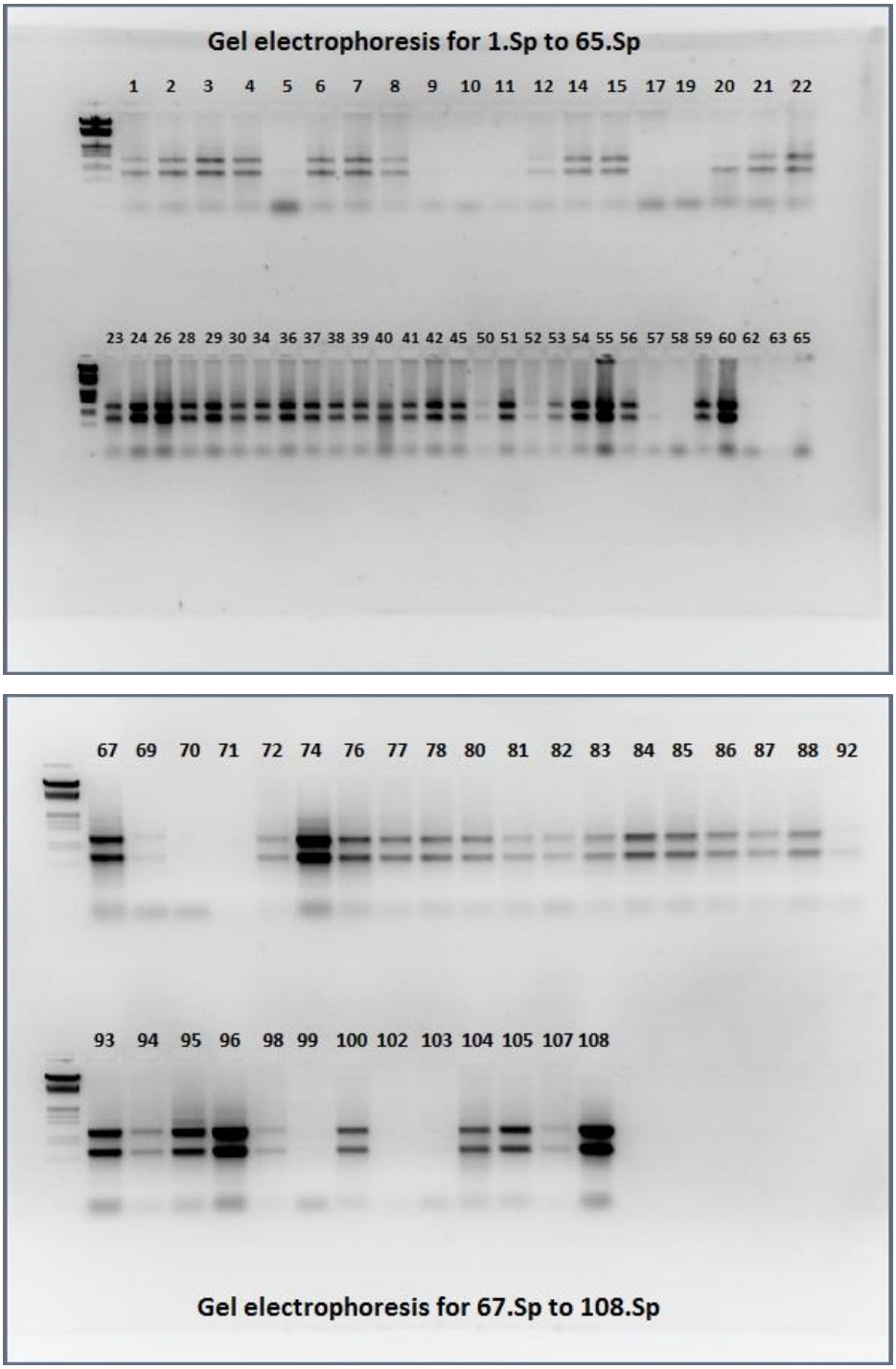

Figure 6.3 Electrophoresis gel of juvenile turbot spleen RNA samples.

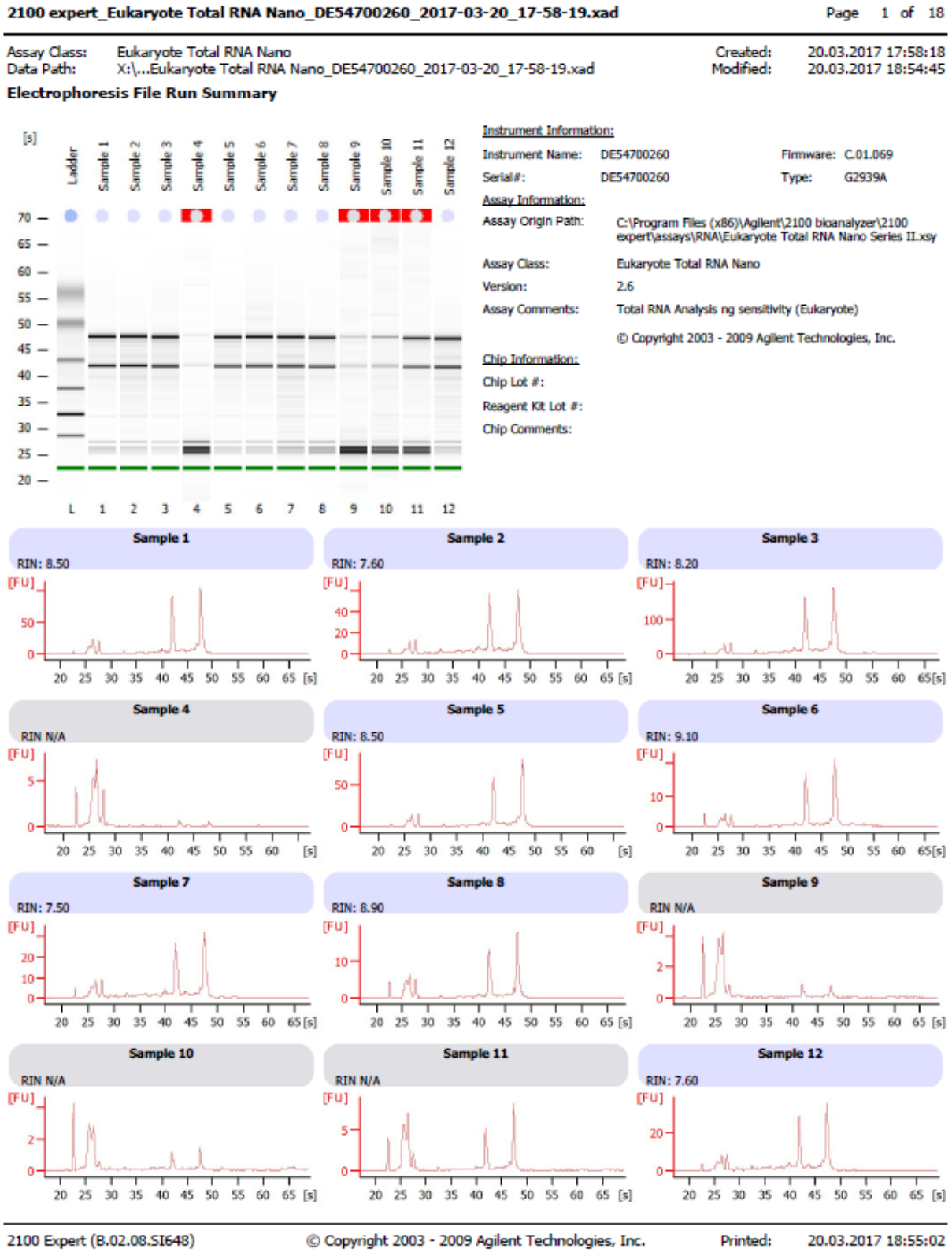

Figure 6.4 Juvenile turbot spleen RNA Nano chip summary.