

***Bacillus* spp. as source of bioactive compounds for aquaculture bacterial diseases control**

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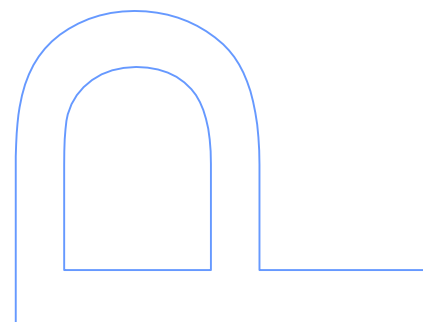
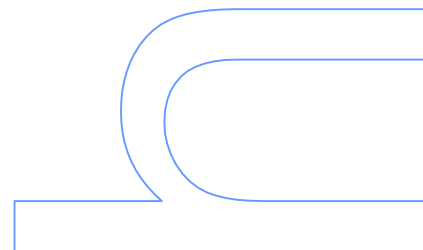
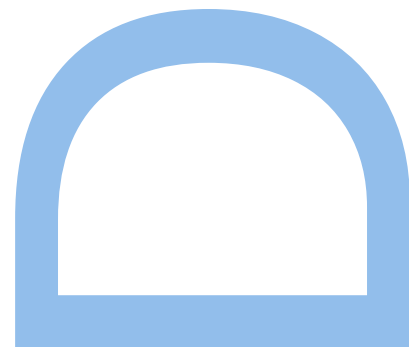
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Previous Note

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To my Family

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Abstract

The continuous intensification and globalization of aquaculture are exacerbating the incidence of aquatic infectious diseases. Aquaculture bacterial outbreaks are responsible for huge annual economic losses worldwide, remaining a major challenge for sustainable aquaculture expansion. Although antibiotics are an important tool for diseases treatment, their damaging effects on the environment and public health have led to increased restrictions to their use in aquaculture. Thus, new biological measures are emerging to control the occurrence of bacterial diseases in aquaculture, assuring advanced and integrated health care for humans, animals, and the environment. Over the last years, fish-gut-associated microorganisms have been exploited as an effective approach to face the challenges of modern aquaculture. Among these, *Bacillus* spp. have recognizable biotechnology applications including the production of Natural Antimicrobial Compounds (NACs) with multiple modes of action to prevent hosts infections. These NACs may constitute a barrier against pathogens proliferation by targeting pathogens growth, biofilm formation, and Quorum-Sensing (QS). QS is a communication mechanism closely related to the expression of virulence factors and biofilm formation that contribute to the pathogenicity of pathogens. Hence, disruption of bacterial QS, known as Quorum-Quenching (QQ) is being proposed as a promising strategy to prevent diseases. Besides other health-promoting characteristics, *Bacillus* spp. are also known for their immunostimulatory properties enhancing the host's innate and adaptive immunity against pathogens. Thus, the overall aim of this Thesis was to isolate *Bacillus* spp. from the gastrointestinal tract of Mediterranean fish species and characterize their health-promoting mechanisms against important aquaculture pathogens.

By harnessing the fish-gut microbial community potential, 172 *Bacillus* spp. representing different colony morphologies were isolated from aquaculture fish species, namely European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), and white seabream (*Diplodus sargus*). All isolates were firstly screened for antimicrobial activity. Of these, 52% antagonized the growth of at least one Gram-negative aquaculture pathogen, including *Aeromonas hydrophila*, *A. salmonicida*, *A. bivalvium*, *A. veronii*, *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Photobacterium damsela*, *Tenacibaculum maritimum*, *Edwardsiella tarda*, and *Shigella sonnei*. Knowing that *Bacillus* spp. metabolites are usually exported to the surrounding environment, the extracellular medium of the most active strains was tested against the growth and biofilm formation of the same pathogens. From these screenings,

three isolates, FI314, FI330, and FI442, all identified as *B. subtilis*, produced extracellular bioactive molecules with a broad spectrum of activity against the growth and biofilm formation of the fourteen tested Gram-negative pathogens.

Since QS mechanisms are important regulators of biofilms, we hypothesized that the observed anti-biofilm activity was caused by the disruption of the pathogens' QS signals. Thus, the QQ potential of the *Bacillus* spp. was evaluated through the degradation of Acyl Homoserine-lactone (AHLs), the QS-signals typically produced by Gram-negative bacteria. The screening was performed using *Chromobacterium violaceum* biosensors, and approximately 12% of the fish-gut *Bacillus* spp. were able to interfere with synthetic AHLs. The isolates were further selected as producers of putative extracellular AHL-lactonase enzymes. By conjugating the bioactivity profile and molecular identification of all positive isolates, FI314 (*B. subtilis*), FI436 (*B. velezensis*), and FI464 (*B. pumilus*) were selected to be further explored. Since the main goal was to characterize isolates capable of antagonizing aquaculture pathogens, the extracellular metabolites of the selected isolates were tested for their QQ capacity against natural AHLs produced by fish pathogens. Of the fourteen pathogens tested, *A. veronii* and *E. tarda* produced AHLs molecules detectable by *Chr. violaceum* biosensor, which were degraded when submitted to the extracellular extracts of the three *Bacillus* spp., indicating the ability of these compounds in silencing pathogens communication.

Quorum-Quenching *Bacillus* spp. strains have already been studied as a strategy for aquaculture diseases mitigation. Nonetheless, *E. tarda* was never the target of such studies. To fulfil this gap, the protective potential of the extracellular metabolites of FI314, FI436, and FI464 against *E. tarda* was evaluated *in vivo*, using zebrafish larvae as a model organism. Larvae treatment with the extracellular metabolites of the three isolates reduced *E. tarda* pathogenicity, increasing zebrafish larvae survival rate up to 50% when compared to the control.

We further hypothesized that the extracellular metabolites of the three *Bacillus* spp. could also have stimulated the fish's innate immune system. Consequently, the putative immunostimulatory potential of the *Bacillus* spp. extracellular metabolites were evaluated *in vitro* using gilthead seabream head-kidney leukocytes. Different parameters were evaluated to access innate immunity, namely viability, reactive oxygen species, phagocytosis, and the expression of immune-relevant genes. *Bacillus* spp. extracellular metabolites demonstrated their immunostimulatory properties by remarkably increasing the engulfment of Gram-negative pathogens. Moreover, FI314 extracellular extract significantly increased the expression of immune-relevant genes associated with inflammation, such as IL-1 β , IL-6, and COX-2.

The outcomes of the present thesis highlight *Bacillus* spp. as a valuable source of bioactive molecules for the control of important aquaculture diseases. *Bacillus* spp. secondary metabolites can act as disease preventive agents through the suppression of pathogens' growth, biofilm formation, and communication, and also through the stimulation of the fish immune system, this way protecting them from infection. Additional studies are required to further confirm the efficacy of *Bacillus* spp. extracellular metabolites in promoting diseases' resistance of aquaculture fish.

Keywords: Fish diseases, *Bacillus* spp., Antimicrobial Compounds, Biofilms, Quorum-Quenching, Immunostimulants

Resumo

A contínua intensificação e globalização da aquacultura tem agravado a incidência de doenças infecciosas aquáticas. Os surtos bacterianos em aquaculturas são responsáveis por enormes perdas económicas anuais a nível mundial, permanecendo, por isso, um grande constrangimento à expansão de uma aquacultura sustentável. Embora os antibióticos sejam um instrumento importante para o tratamento de doenças, os seus efeitos nocivos no ambiente e na saúde pública levaram à implementação de muitas restrições à sua utilização em aquacultura. Deste modo, têm surgido novas estratégias biológicas para controlar a ocorrência de doenças bacterianas em aquacultura, assegurando cuidados de saúde avançados e integrando a saúde humana, nos animal e do ambiente. Nos últimos anos, os microrganismos associados ao trato gastrointestinal de peixes têm sido explorados como uma abordagem eficaz para enfrentar os desafios da aquacultura moderna. Entre estes, os *Bacillus* spp. têm aplicações biotecnológicas reconhecidas, incluindo a produção de Compostos Antimicrobianos Naturais (NACs), que possuem múltiplos modos de ação para prevenir infeções no hospedeiro. Estes NACs podem constituir uma barreira contra a proliferação de agentes patogénicos ao visarem o crescimento dos agentes patogénicos, a formação de biofilmes e o *Quorum-Sensing* (QS). O QS é um mecanismo de comunicação intimamente relacionado com a expressão de fatores de virulência e formação de biofilmes, que contribui para a patogenicidade dos agentes patogénicos dos peixes. Assim, a perturbação do QS bacteriano, conhecida como *Quorum-Quenching* (QQ), tem vindo a ser proposta como uma estratégia promissora para prevenir doenças. Para além de outras características promotoras de saúde, os *Bacillus* spp. são também conhecidos pelas suas propriedades imunoestimuladoras, reforçando a imunidade inata e adquirida do hospedeiro no combate a agentes patogénicos. Assim, o objetivo desta Tese foi isolar *Bacillus* spp. do trato gastrointestinal de espécies de peixes Mediterrânicos e caracterizar os seus mecanismos promotores de saúde contra importantes agentes patogénicos de aquacultura.

Beneficiando do potencial da comunidade microbiana associada ao trato gastrointestinal de peixes, foram obtidas 172 estirpes de *Bacillus* spp., representativas de morfologias coloniais distintas, de espécies de peixes de aquacultura economicamente importantes, nomeadamente robalo Europeu (*Dicentrarchus labrax*), dourada (*Sparus aurata*), e sargo (*Diplodus sargus*). A atividade antimicrobiana foi inicialmente analisada em todos os isolados. Destes, 52% inibiram o crescimento de pelo menos uma bactéria patogénica Gram-negativa de aquacultura, incluindo *Aeromonas hydrophila*,

A. salmonicida, *A. bivalvium*, *A. veronii*, *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Photobacterium damsela*, *Tenacibaculum maritimum*, *Edwardsiella tarda*, e *Shigella sonnei*. Sabendo que os metabolitos de *Bacillus* spp. são geralmente exportados para o ambiente circundante, o meio extracelular das estirpes mais promissoras foi testado contra o crescimento e formação do biofilme dos mesmos agentes patogénicos. Com base nestas análises, três isolados, FI314, FI330 e FI442, identificados como *B. subtilis*, produziram moléculas bioativas extracelulares com um largo espectro de atividade, antagonizando o crescimento e formação de biofilme dos catorze organismos patogénicos Gram-negativos testados. Uma vez que os mecanismos de QS são importantes reguladores de biofilmes, foi especulado que a atividade anti-biofilme observada pudesse ser causada pela perturbação dos sinais de QS dos agentes patogénicos. Assim, o potencial de QQ dos *Bacillus* spp. isolados, foi avaliado através da degradação de *Acyl Homoserine-lactones* (AHLs), que são os sinais de QS tipicamente produzidos por bactérias Gram-negativas. A análise realizou-se utilizando biossensores de *Chromobacterium violaceum*, e aproximadamente 12% dos *Bacillus* spp. isolados do trato gastrointestinal de peixes foram capazes de interferir com as AHLs sintéticas. Os isolados foram ainda selecionados como produtores de possíveis enzimas extracelulares AHL-lactonases. Conjugando o perfil de bioatividade e de identificação molecular de todos os isolados positivos, selecionaram-se as estirpes FI314 (*B. subtilis*), FI436 (*B. velezensis*) e FI464 (*B. pumilus*) para serem exploradas mais a fundo. Uma vez que o principal objetivo era caracterizar isolados capazes de antagonizar os agentes patogénicos da aquacultura, os compostos extracelulares dos isolados selecionados foram testados quanto à sua capacidade QQ contra as AHLs naturais produzidas por agentes patogénicos. Dos catorze agentes patogénicos testados, a *A. veronii* e a *E. tarda* produziram moléculas AHLs detetáveis pelo biossensor *Chr. Violaceum*, que foram degradadas quando submetidas aos extratos extracelulares dos três *Bacillus* spp., demonstrando a capacidade destes compostos em silenciar a comunicação dos agentes patogénicos.

As estirpes de *Bacillus* spp. com atividade QQ têm sido estudadas como uma estratégia de mitigação de doenças em aquacultura. No entanto, a *E. tarda* nunca foi o alvo de tais estudos. Para colmatar esta lacuna, o efeito protetor dos compostos extracelulares de FI314, FI436 e FI464 contra *E. tarda* foi determinado *in vivo* usando larvas de peixe zebra como organismo modelo. O tratamento das larvas com os compostos extracelulares dos três isolados reduziu a patogenidade da *E. tarda*, aumentando a taxa de sobrevivência das larvas de peixe zebra até 50% quando comparado com o grupo controlo.

Assim, colocamos a hipótese de que os compostos extracelulares dos três *Bacillus* spp. pudessem também ter estimulado o sistema imunitário inato dos peixes. Consequentemente, foram avaliadas *in vitro* as possíveis propriedades imunoestimuladoras dos compostos extracelulares de *Bacillus* spp. utilizando leucócitos isolados do rim posterior de dourada. Para avaliar a imunidade inata foram analisados diferentes parâmetros, nomeadamente a viabilidade celular, espécies reativas de oxigénio, fagocitose e a expressão de genes imunes relevantes. Os compostos extracelulares dos *Bacillus* spp. selecionados demonstraram as suas propriedades imunoestimuladoras ao aumentar significativamente a ingestão de patogénicos Gram-negativos. Para além disso, o extrato extracelular do isolado FI314 aumentou significativamente a expressão de genes imunes relevantes associados à inflamação, tais como IL-1 β , IL-6, e COX-2.

Os resultados da presente Tese realçam a contribuição dos *Bacillus* spp. como uma fonte valiosa de moléculas bioativas para o controlo de doenças importantes em aquacultura. Os compostos secundários de *Bacillus* spp. podem atuar como agentes preventivos de doenças através da supressão do crescimento de agentes patogénicos, da formação de biofilmes, e da comunicação de organismos patogénicos, bem como da estimulação do sistema imunitário dos peixes, protegendo-os desta forma contra infeções. São necessários mais estudos para confirmar a eficácia dos compostos extracelulares de *Bacillus* spp. na promoção da resistência a doenças em peixes de aquacultura Mediterrânica.

Palavras-Chave: Doenças de peixes, *Bacillus* spp., Antimicrobianos, Biofilmes, *Quorum-Quenching*, Imunoestimulantes

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Abbreviations

Acyl-ACP – Acylated acyl-carrier protein

AHLs – Acyl-homoserine lactones

AI-2 – Autoinducer-2

ANOVA – Analysis of variance

BCCM/LMG – Belgian Coordinated Collections of Microorganisms Microbiology

BHI – Brain Heart Infusion medium

BLAST – Basic Local Alignment Search Tool

B&W – Bott & Wilson salts

CAI-1 – Cholera autoinducer-1

cDNA – Complementary DNA

CECT – Spanish type culture collection

CFU – Colony-forming unit

CLSI – Clinical & Laboratory Standards Institute

CO₂ – Carbon dioxide

COX-2 – Cyclooxygenase 2

CTR – Control

DC-SIGN – Dendritic cell-specific ICAM-3-grabbing nonintegrin

DCFH-DA – Dichlorodihydrofluorescein diacetate

DNA – Deoxyribonucleic acid

DNAse – Deoxyribonuclease

dNTP – Deoxynucleoside triphosphate

dpf – Days Post Fertilization

DSM – Difco Sporulation Medium

DSMZ/DSM – German Collection of Microorganisms and Cell Cultures

EDTA – Ethylenediamine tetraacetic acid

EEC – European Economic Community

EFSA – European Food Safety Authority

Ef-1 α – Elongation factor 1 α

EPS – Extracellular polymeric substance

EU – European Union

ESB – European seabass

FAO – Food and Agriculture Organization

FBS – Fetal Bovine Serum

FI – Fish Isolate

FITC – Fluorescein isothiocyanate

GR – Granulocytes

GSB – Gilthead seabream

GUT – Gastrointestinal tract

H – Hours

HBSS – Hanks' Balanced Salt Solution

HCL – Hydrochloric acid

HIC – Hydrophobic Interaction Chromatography

Hpi – Hours post-infection

Hsp70 – 70 kilodalton heat shock proteins

H₂O₂ – Hydrogen peroxide

I – Intermediate

IBW – Initial body weight

IEX – Ion exchange chromatography

IgM – Immunoglobulin M

IgT – Immunoglobulin T

IL – Interleukin

IPMA – Instituto Português do Mar e da Atmosfera

L-15 – Leibovitz's L-15 Medium

LB – Luria-Bertani medium

LY – Lymphocytes

MH – Muller-Hinton medium

MNTC – Maximum non-toxic concentration

MO – Monocytes

MOI – Multiplicity of infection

MT – Million tonnes

MTCC – Microbial type culture collection

NACs – Natural Antimicrobial Compounds

NCBMI – National collection of Industrial food and marine bacteria

OD – Optical density

OTUs – Operational taxonomic units

QPS – Qualified presumption of safety

PBS – Phosphate-buffered saline

PCM – Phase contrast microscopy

PCR – Polymerase chain reaction

PAMPs – pathogen-associated molecular patterns

PRRs – Pattern recognition receptors

P/S – 100U mL⁻¹ of penicillin, and 100 µg mL⁻¹ of streptomycin

QS – Quorum-Sensing
 QQ – Quorum-Quenching
 R – Resistant
 RAS – Recirculating Aquaculture System
 RNA – Ribonucleic acid
 RNase – Ribonucleic acid
 RNAseq – RNA sequencing
 ROS – Reactive oxygen species
 Rpm – Rotation per minute
 SAM – S-adenosyl-methionine
 S – Sensitive
 SDS – sodium dodecyl sulfate
 SE – Standard error mean
 SEC – Size Exclusion Chromatography
 SPSS - Statistical Product and Service Solutions
 STE – Sodium Chloride-Tris-EDTA
 TE – Tris-EDTA buffer
 TI – Initial time
 TF – Final Time
 TNF- α – Tumor necrosis factor α
 T3SS – Type three secretion system
 T6SS – Type six secretion system
 US – United States
 WSB – White seabream
 WT – Wild type
 °C – Degrees Celsius
 18S – Ribosomal protein S18

CHAPTER I

General Introduction

1.1. Aquaculture

The continuous increase of the human population raised a challenge on how to ensure the world's food supply requirements while maintaining fish stocks biologically sustainable. In fact, due to the increase in fish consumption, the proportion of stocks fished at unsustainable levels increased from 10% in 1974 to 34.2% in 2017 (FAO, 2020). Although capture fisheries have been relatively stagnated since 1990, a maximum level of 96.4 million tonnes (MT) was reached in 2018 (Figure 1.1). While capture fisheries will remain extremely relevant, aquaculture has already demonstrated its crucial role in global food security and as a source of economic and social development in developing countries (FAO, 2020; Pradeepkiran, 2019).

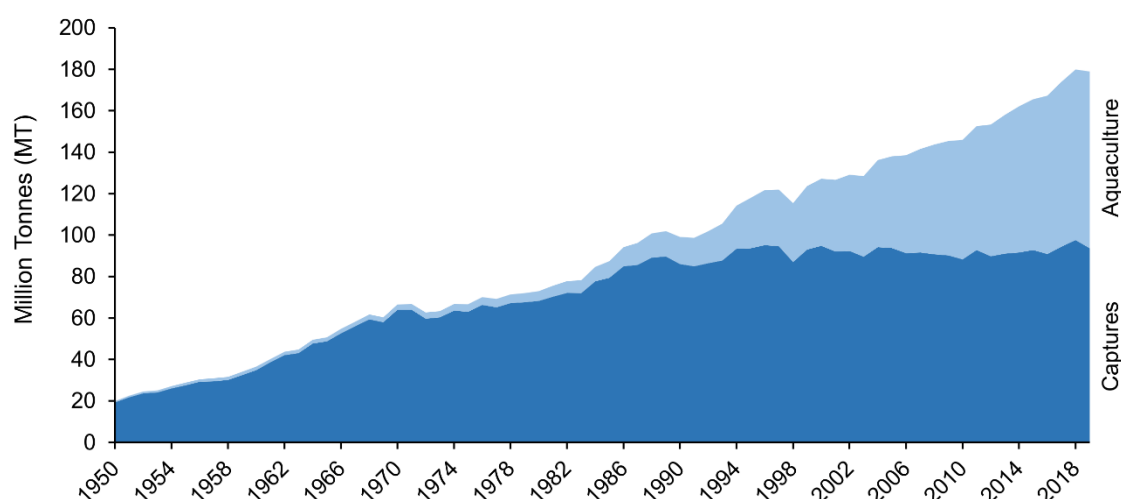


Figure 1.1. World capture fisheries and aquaculture production. Data source: FAO, 2020.

Aquaculture is defined by the Food and Agriculture Organization (FAO) as the “*farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants*”. Farming implies the “*intervention in the rearing process to enhance production, such as feeding and regular stocking*”, and the “*individual or corporate ownership of the production*”. Moreover, aquatic production involves a variety of aquaculture systems, sites, facilities, and techniques. Aquaculture started millennia ago but only significantly contributed to the global food supply over the last 20 years. The technological advances and the increasing demand for aquatic products are the main reasons for the rapid development of the sector as a commercial activity (FAO, 2020).

Nowadays aquaculture is the world's fastest-growing food protein producer (5.3% / year between 2001 and 2018), although currently facing a lower growth rate than in the '80s and '90s (FAO, 2020). Whereas aquaculture provided just 13.4% of the total world fish production in 1990, this share increased to 25.7% in 2000 and 46% in 2016-2018 (FAO, 2020). Global aquaculture production reached out to 82 MT in 2018, with an estimated

value of 250 billion US Dollars (FAO, 2020). Based on time-series data it is expected that aquaculture production continues to grow in all continents, despite an uneven distribution pattern in production contributions. Farmed fish production has been dominated by Asia with an 89% share of the world production in 2018. In the same year, the European Union (EU) only contributed with 1,66% for the total production (FAO, 2020).

Although aquaculture production has experienced a consistent growth worldwide, in the EU the production saw a reversal of the upward trend in the last years. Total aquaculture production volume decreased 4% to 1.32 MT and its value decreased 5% to 4.8 billion € from 2017 to 2018 (EUMOFA, 2020). Although EU countries are the biggest consumers of fish and seafood (24.36 kg per capita consumption in 2019), the majority of this consumption is provided through catches and imported products (EUMOFA, 2020). Portugal is the second country from the EU with the highest rate of fish consumption with 60.92 Kg per capita consumption in 2018, largely driven by the importation of sea products (EUMOFA, 2020). Fish production in Portugal and the Mediterranean area is focused on marine fish, specifically gilthead seabream (*Sparus aurata*), turbot (*Scophthalmus maximus*), and European seabass (*Dicentrarchus labrax*) (EUMOFA, 2020). The Sparidae, gilthead sea bream, and the Moronidae, European seabass, are considered important cultured species along the Mediterranean Sea due to their high market value. Moreover, the production of these species is experiencing a significant expansion and investment in Portugal.

1.2. Infectious diseases: a major constraint in aquaculture

The maintenance of aquatic systems' health is vital for the sustainable growth of the aquaculture sector. Aquatic infectious diseases exacerbated by production intensification (e.g., high-density production systems) and globalization (e.g., increase of aquaculture products' international transactions) are major constraints for the expansion of sustainable aquaculture (Moreira, et al., 2021). Disease outbreaks in aquaculture can have substantial impacts on the environment, economy, and society, including (i) direct costs related to production losses due to mortalities or slow growth rate; (ii) total or partial closure of production facilities with direct impact on workers and downstream industries; and (iii) loss of market sales (exportation or domestic) (FAO, 2020). Diseases outbreaks are the consequence of a complex interaction between the environment and zootechnical aspects (e.g., water quality, poor management, farm location), the produced animal (e.g., animal species, immune system, life stage), and opportunistic pathogenic organisms (e.g., pathogen species, virulence mechanisms, antimicrobial

resistances) (Freitas, et al., 2020; Moreira, et al., 2021; Sitjà-Bobadilla, Oidtmann, 2017). Serious aquatic fish diseases can be caused by viruses, fungus, parasites, and bacteria (Bayliss, et al., 2017). Of these, bacterial infectious diseases are the most prevalent in marine aquaculture (Shefat, 2018). Bacterial pathogens have been reported to cause billions of US dollars of annual economic losses worldwide (Lafferty, et al., 2015). Moreover, there are increasing reports of zoonotic microbes among the major bacterial fish diseases, affecting humans and public health (Gauthier, 2015). Some of the most problematic bacterial diseases occurring in aquaculture are furunculosis, vibriosis, photobacteriosis, edwardsiellosis, and tenacibaculosis (Bayliss, et al., 2017), all caused by Gram-negative pathogens.

1.2.1. Furunculosis

Aeromonas salmonicida is the causative agent of furunculosis, one of the oldest known fish pathogens (Emmerich, Weibel, 1894), characterized by high mortality and morbidity rates (Dallaire-Dufresne, et al., 2014). Although *A. salmonicida* is the most studied as a fish pathogen, other *Aeromonas* species, such as *A. hydrophila* and *A. veronii*, have been associated with significant economic losses (Dallaire-Dufresne, et al., 2014; Janda, Abbott, 2010). *Aeromonas* spp. are globally distributed in both fresh and marine water environments (Menanteau-Ledouble, et al., 2016). The role of *Aeromonas* spp. as a relevant aquaculture pathogen has been recognized for decades, infecting multiple fish and shrimp species, including crucian carp (*Carassius auratus*), tilapia (*Oreochromis niloticus*), eel (*Anguilla japonica*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and whiteleg shrimp (*Litopenaeus vannamei*) (Cai, et al., 2012; Chen, et al., 2019; Doukas, et al., 1998; Gashgari, Selim, 2015; Janda, Abbott, 2010; Menanteau-Ledouble, et al., 2016; Rahman, et al., 2002; Wang, et al., 2021; Yi, et al., 2013; Zhou, et al., 2019). Aeromonad diseases have different presentations depending on the *Aeromonas* species and the infected host (Janda, Abbott, 2010). The clinical signs include darkened skin, exophthalmia, anorexia, external haemorrhages, haemorrhagic septicemia, and furuncle lesions in chronic forms (Menanteau-Ledouble, et al., 2016). *Aeromonas* species are known to be opportunist pathogens and the host skin, wounds, digestive tract, and gills are considered the most vulnerable sites for bacterial adhesion and colonization (Bartkova, et al., 2017; Beaz-Hidalgo, Figueras, 2013).

1.2.2. Vibriosis

Vibriosis is the designation used to refer to a systemic bacterial infection caused by *Vibrio* spp. (Ina-Salwany, et al., 2019). Within the Vibrionaceae family, *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* are responsible for devastating impacts on aquaculture species (Deng, et al., 2020; Mohamad, et al., 2019b). Vibriosis has a wide geographical distribution occurring in both fresh and marine waters, predominantly in warmer months (Ina-Salwany, et al., 2019). *Vibrio* spp. are serious opportunistic pathogens with a broad range of hosts, including European eel (*Anguilla anguilla*), rainbow trout, sole (*Solea senegalensis*), turbot, European seabass, and gilthead seabream (Aydin, et al., 2019; Haldar, et al., 2010; Ina-Salwany, et al., 2019; Mohamad, et al., 2019b; Pujalte, et al., 2003). Vibriosis-like clinical symptoms include lethargy, anorexia, haemorrhages on the ventral and lateral areas, darkened skin pigmentation, ascites, exophthalmia, and ulcerative skin lesions in chronic infections (Frans, et al., 2011; Ina-Salwany, et al., 2019; Mohamad, et al., 2019b). The recognized portal of entry of *Vibrio* spp. are gills, skin lesions, and the gastrointestinal tract (Ina-Salwany, et al., 2019; Mohamad, et al., 2019b; Oisson, et al., 1996).

1.2.3. Photobacteriosis

Photobacteriosis is a septicemia caused by *Photobacterium damsela*. *Ph. damsela* belongs to the Vibrionaceae family and comprises two subspecies, *Ph. damsela* subsp. *damsela* and *Ph. damsela* subsp. *piscicida* (Andreoni, Magnani, 2014). Photobacteriosis is responsible for huge economic losses in farmed marine fish worldwide, in particular in the Mediterranean area (Andreoni, Magnani, 2014; Essam, et al., 2016). *Ph. damsela* subsp. *piscicida* is not host-specific, and its infection potential has been described to be age- and temperature-dependent (Andreoni, Magnani, 2014; Varvarigos, 2020). Photobacteriosis affects a wide range of marine fish, including gilthead seabream, European seabass, and sole (Essam, et al., 2016; Magariños, et al., 2003; Pečur Kazazić, et al., 2019; Toranzo, et al., 2005). Infected fish may develop ulcerative skin lesions on the pectoral fins and caudal peduncle, ascites, and white granulomatous nodules in the haematopoietic tissues such as liver, spleen, and kidney (Pečur Kazazić, et al., 2019). *Ph. damsela* subsp. *damsela*, an autochthonous member of aquatic systems, has been recognized as an emergent pathogen with a wide geographic distribution (Osorio, et al., 2018; Terceti, et al., 2016). *Ph. damsela* subsp. *damsela* cause vibriosis-like symptoms in a broad range of marine animals, including fish (e.g., gilthead seabream, European seabass, turbot *Scophthalmus maximus*, and meagre *Argyrosomus regius*) (Essam, et al., 2016; Ina-Salwany, et al., 2019; Labella, et al., 2010; Osorio, et al., 2018; Terceti, et al., 2016). Despite the significance of *Ph.*

damselfish in the Mediterranean area, the routes of infection of these pathogens are not fully characterized.

1.2.4. Edwardsiellosis

Edwardsiella tarda is the causative agent of edwardsiellosis, a disease affecting freshwater and marine aquacultures and possessing a wide geographical distribution. *E. tarda* is an opportunistic pathogen affecting commercial relevant aquaculture fish, including catfish (*Ictalurus punctatus*), red tilapia (*Oreochromis* spp.), common carp (*Cyprinus carpio*), European eel, Japanese eel, sharpshout sea bream (*Diplodus puntazzo*), sole and turbot (Abo El-Yazeed, Ibrahem, 2009; Alcaide, et al., 2006; Castro, et al., 2006; Iregui, et al., 2012; Katharios, et al., 2015; Miniero Davies, et al., 2018; Mohanty, Sahoo, 2007). Fish clinical signs include loss of skin pigmentation, swelling and abdominal distention, exophthalmia, cutaneous haemorrhages, rectal prolapse ascites, and nodules and abscesses in the spleen and kidney (Katharios, et al., 2015; Miniero Davies, et al., 2018; Park, et al., 2012). Recognized *E. tarda* routes of infection are the gastrointestinal tract, skin, and gills (Ling, et al., 2001).

1.2.5. Tenacibaculosis

Tenacibaculum maritimum is an opportunistic pathogen responsible for an ulcerative disease known as tenacibaculosis (formerly flexibacteriosis) (Avendaño-Herrera, et al., 2006). *T. maritimum* has a wide geographical distribution, being responsible for significant economic losses in the marine aquaculture (Avendaño-Herrera, et al., 2006). Tenacibaculosis lacks host specificity affecting multiple economically important fish such as gilthead seabream, European seabass, Atlantic salmon, sole, turbot, and rainbow trout (Avendaño-Herrera, et al., 2006; Faílde, et al., 2013; Gourzioti, et al., 2016; Kolygas, et al., 2012; Valdes, et al., 2021). Affected fish exhibit ulcerative and haemorrhagic skin lesions, eroded mouth, fins and gills necrosis, and increased mucus (Avendaño-Herrera, et al., 2006; Gourzioti, et al., 2016). The primary sites of *T. maritimum* infection are the mouth, fins, head and body surfaces (Avendaño-Herrera, et al., 2006; Gourzioti, et al., 2016).

1.3. Mechanisms to control aquaculture pathogens

Over the years, antibiotics have been used in the aquaculture industry to control bacterial diseases. However, their massive use as prophylactic and therapeutic agents promoted the selection, persistence, and spread of antibiotic-resistant bacteria among aquaculture

bacterial pathogens (Pérez-Sánchez, et al., 2018). Resistance to different classes of antibiotics including β -lactams, fluoroquinolones, aminoglycosides, quinolones, sulphonamides, and tetracycline have been described in *Aeromonas* spp., *Vibrio* spp., *Edwardsiella* spp., and *T. maritimum* (Borella, et al., 2020; Casas, et al., 2005; Deng, et al., 2020; Gashgari, Selim, 2015; Igbinosa, 2016; Igbinosa, et al., 2015; Mohamad, et al., 2019a).

Despite the lack of regulations in developing countries, in Europe, North America, and Japan, antibiotics use in aquaculture is strictly regulated and only a few substances have been licensed (Defoirdt, et al., 2011; Pérez-Sánchez, et al., 2018). Over the years, vaccination represented an alternative control of infectious diseases in aquaculture; however, the lack of long-term protection, low effectiveness, and side effects have limited their market authorization (Dallaire-Dufresne, et al., 2014; Mohamad, et al., 2019b; Mzula, et al., 2019; Pérez-Sánchez, et al., 2018; Zhao, et al., 2020).

Given this, new biological measures are emerging to prevent and control bacterial diseases in aquaculture, including the use of antimicrobial compounds as disease preventive molecules, the use of anti-quorum-sensing compounds as anti-virulence therapy, and the use of immunostimulants to boost the fish immune system.

1.3.1. Natural Antimicrobial Compounds

Natural products' research has experienced significant progress in the discovery of new compounds with antimicrobial properties (Ali, et al., 2018). Nature offers a valuable source of bioactive compounds with antimicrobial activity. Natural Antimicrobial Compounds (NACs) can be obtained from animals, plants, algae, fungi, and bacteria (Gyawali, Ibrahim, 2014). Plants and plant by-products are a source of valuable secondary compounds with recognized antimicrobial activities. Some of the major plant-derived compounds are phenolics, flavonoids, tannins, and halogenated compounds (Gyawali, Ibrahim, 2014). There is a considerable diversity of plant-derived compounds, indicating different modes of action and consequently multiple antimicrobial actions. For example, the medicinal plant, *Mallotus japonicus*, produces a wide range of compounds, including tannins, flavonoids, terpenoids, and saponins with antimicrobial activities against the fish pathogens *A. hydrophila*, *A. salmonicida*, *E. tarda* and *V. anguillarum* (Appiah, et al., 2021).

Although antimicrobial compounds are widely present in nature, microbes are the major source of NACs (Rončević, et al., 2019). Penicillin, produced by the fungus *Penicillium notatum* was the first discovered NAC (Fleming, 1929). Since then, several fungi species have been reported to secrete different types of bioactive compounds with reported antimicrobial activity against gram-positive and gram-negative bacteria

(Ali, et al., 2018). For example, *Aspergillus* species produce a gliotoxin with antibacterial activity against *S. aureus* (Esteban, et al., 2021; Okutani, 1977). Similarly, pestalachloride D, firstly isolated from a marine fungi *Pestalotiopsis* sp., possesses antibacterial activity against *E. coli*, *V. anguillarum*, and *V. parahaemolyticus* (Wei, et al., 2013). Fungi also produce phenolic compounds, flavonoids, and other antioxidant molecules that inhibit the growth of bacterial pathogens (Gyawali, Ibrahim, 2014).

NACs are believed to be ubiquitous among bacteria, with the majority of bacteria producing at least one type of NACs (Dobson, et al., 2012). Bacteria are known to produce a broad range of antimicrobials. *Streptomyces* spp. are well-studied organisms for the production of several bioactive molecules used as herbicides, antifungals, and antibiotics for the medical and veterinary fields (e.g., streptomycin, erythromycin, and tetracycline) (Quinn, et al., 2020). Over the years, many other bacteria (e.g., *Pseudomonas* spp., *Lactobacillus* spp., and *Bacillus* spp.) have been identified as producers of different NACs, including glycopeptides, lipopeptides, bacteriocins, and cyclic peptides against multiple human and animal pathogens (Ali, et al., 2018). The wide diversity of bacterial NACs implies that their synthesis is mediated by different and complex regulation mechanisms. Nonetheless, the production of the most abundant NACs is usually connected to particular events such as bacterial cell density and stress conditions (Caulier, et al., 2019). Moreover, due to their wide diversity, NACs exhibit different modes of action. Usually, NACs target pathogenic microorganisms through one or more mechanisms, including membrane pore formation and disruption or inhibition of cell-wall biosynthesis (Sumi, et al., 2014). NACs killing ability through the formation of membrane pores is the most studied mechanism, involving three main steps: (i) NACs binding to bacterial membrane; (ii) aggregation within the membrane; and (iii) channels formation (Sumi, et al., 2014). This mechanism leads to leakage of internal cell contents causing cell death. On the other hand, cell-wall biosynthesis can be inhibited through interaction between NACs with lipid II molecules, crucial for the biosynthesis process (Caulier, et al., 2019; Sumi, et al., 2014).

Importantly, the exact ecological function of NACs is not yet totally elucidated. Nonetheless, it is believed that within the gastrointestinal tract, NACs may function as colonizing compounds, facilitating the dominance of NACs' producing bacteria over the community (Dobson, et al., 2012). Additionally, NACs may act by directly killing the pathogens, thus constituting a barrier against pathogen's proliferation (Dobson, et al., 2012; Heilbronner, et al., 2021).

1.3.2. Biofilms disruption

Biofilms refer to the complex three-dimensional communities of microorganisms that may adhere to a surface or form aggregates without adhering to a surface (Roy, et al., 2018). Biofilm formation is a programmed complex mechanism involving different stages. The first stage includes the migration and attachment of planktonic cells to a surface that initiate biofilm production upon advantageous conditions (Barzegari, et al., 2020; Roy, et al., 2018; Verderosa, et al., 2019). After the complete biofilm development and maturation, microcolonies start to detach and disperse, spreading to other locations (Roy, et al., 2018). During biofilm formation, cells secrete an extracellular polymeric substance (EPS) composed of water, polysaccharides, extracellular DNA, proteins, and lipids, that provide enclosure, aggregation, and stability to the bacterial cells within the biofilm (Flemming, et al., 2016). The formation of this matrix confers numerous advantages to the biofilm cells, such as nutrient acquisition, absorption of inorganic and organic particles; it also enables digestion of exogenous macromolecules, promotes the exchange of genetic information, offers protection to antimicrobials, and enables resistance to antibiotics (up to 1000-fold) (Flemming, et al., 2016; Nahar, et al., 2018; Wille, Coenye, 2020). Importantly, during biofilm formation, the communication between bacterial cells through Quorum-Sensing mechanisms (detailed in the following section) is stimulated (up to 1000-fold higher than in planktonic cells) to control population density and to regulate multicellular community behaviour (Flemming, et al., 2016; Verderosa, et al., 2019).

Pathogenic bacteria often require biofilms to establish infection and spread through the host. Biofilms are thus an attractive target to prevent bacterial infections. Development and persistence of bacterial biofilms have been reported in aquaculture bacterial pathogens, including *Vibrio*, *Photobacterium*, *Aeromonas*, and *Edwardsiella* species (Cai, Arias, 2017; Igbinosa, et al., 2015; Ina-Salwany, et al., 2019; Khouadja, et al., 2014; Talagrand-Reboul, et al., 2017; Zhang, et al., 2009). Due to the contribution of biofilms to bacterial pathogenesis and antimicrobial resistance, there is an increasing concern about these biofilm-forming pathogens.

Nowadays, biofilm therapy is a challenge in all fields. Antibiotic treatment, the first line of diseases treatment in aquaculture, is insufficient to face biofilm-related infections (Verderosa, et al., 2019). Thus, efforts have been made to develop new effective strategies to inhibit the formation of new biofilms and to disperse pre-formed biofilms by targeting the biofilm matrix, membrane permeabilization, and cells adhesion, division, survival, and cell-wall composition (Roy, et al., 2018). Enzymatically (e.g., DNase) dispersion of EPS biofilm matrix enables the exposure of biofilm-related cells to antimicrobial agents (Roy, et al., 2018). Despite being a promising biofilm-treatment

mechanism, it requires co-administration with antimicrobial agents to prevent the spreading of such biofilm-forming cells (Verderosa, et al., 2019). Another approach to disrupting the formation of biofilms is through the cleavage of peptidoglycan, an important component of bacterial cell walls (Roy, et al., 2018). Within this approach, tannic acid, a polyphenolic compound, was reported to disrupt *S. aureus* biofilms by increasing the extracellular level of a lysozyme-like enzyme, transglycosylase (IsaA) (Payne, et al., 2013). Additionally, endolysins produced by bacteriophages can hydrolyse peptidoglycans of gram-positive bacteria (Fischetti, 2010). Alteration of cytoplasmatic membrane permeabilization through pore formation or inhibition of cell wall synthesis is another approach of anti-biofilm compounds action (Roy, et al., 2018). Several bacteria and yeast produce this type of anti-biofilm compounds, including *Lactobacillus* spp., *Bacillus* spp., *Staphylococcus* spp., and *Candida* spp. (Chopra, et al., 2015; Diaz De Rienzo, et al., 2015; Kim, et al., 2018b; Saising, et al., 2012). Subtilisin A and sonorensin, both produced by *Bacillus* species, are examples of natural compounds responsible for membrane permeability as anti-biofilm agents (Algburi, et al., 2015; Chopra, et al., 2015; van Kuijk, et al., 2012). Besides these anti-biofilm strategies, in the last decades focus has been given to the bacterial signalling process called Quorum-Sensing, as an anti-biofilm mechanism of action (Roy, et al., 2018).

1.3.3. Shut-down Quorum-Sensing

There is a growing recognition that bacteria exhibit multicellular and complex social behaviours that require cooperation. When engaged at a group level, bacteria evolve competitive strategies to adapt to a wide range of niches, having a better chance to ensure species' survival. Bacteria synchronize collective traits through a cell-cell signalling process or Quorum-Sensing (Hense, et al., 2007; Hibbing, et al., 2010; Mukherjee, Bassler, 2019).

Quorum sensing (QS) is a bacterial communication strategy enabling bacteria to coordinate gene expression, population size, and behaviour. Importantly, QS regulates a wide range of bacterial functions including biofilm formation and virulence-related gene expression (e.g., motility and swarming and the synthesis of extracellular products) (Abisado, et al., 2018; Mukherjee, Bassler, 2019). QS is mediated by small diffusible signals, known as autoinducers, that are produced and exported from the cell, being perceived by specific receptors in the bacterial community, triggering the transcription of target genes (Mukherjee, Bassler, 2019). Up to date, several autoinducers (e.g., acyl-homoserine lactones (AHLs), autoinducer-2 (AI-2), cholera autoinducer-1 (CAI-1),) and their respective QS-systems have been described in numerous pathogens (Wu, Luo, 2021).

AHLs are the most frequent class of autoinducers and are widespread among gram-negative bacteria, including fish pathogens. Depending on the species, the acyl chain varies in length (4-18 carbons), 3-hydroxyl or 3-oxo- substituents, degree of saturation, and methyl branches (Churchill, Chen, 2011; Wu, Luo, 2021). AHLs can freely diffuse across bacterial membranes and accumulate in the extracellular environment. Once reached a certain threshold, AHLs are detected by the cytoplasmic transcriptional regulator LuxR (or LuxR homologous proteins) (Figure 1.2). The stable LuxR-AHL complex binds to DNA triggering gene expression. Importantly, in the absence of AHLs and LuxI-synthases, LuxR can detect AHLs produced by other bacterial species, mediating inter-species communication (Abisado, et al., 2018; Wu, Luo, 2021). The first QS system relied on the identification of AHLs as regulators of bioluminescence production in *Vibrio fischeri* (Fuqua, et al., 1996). Since then, similar QS systems based on the production and detection of AHLs have been described in several Gram-negative bacteria, including *Aeromonas*, *Vibrio*, and *Edwardsiella* species.

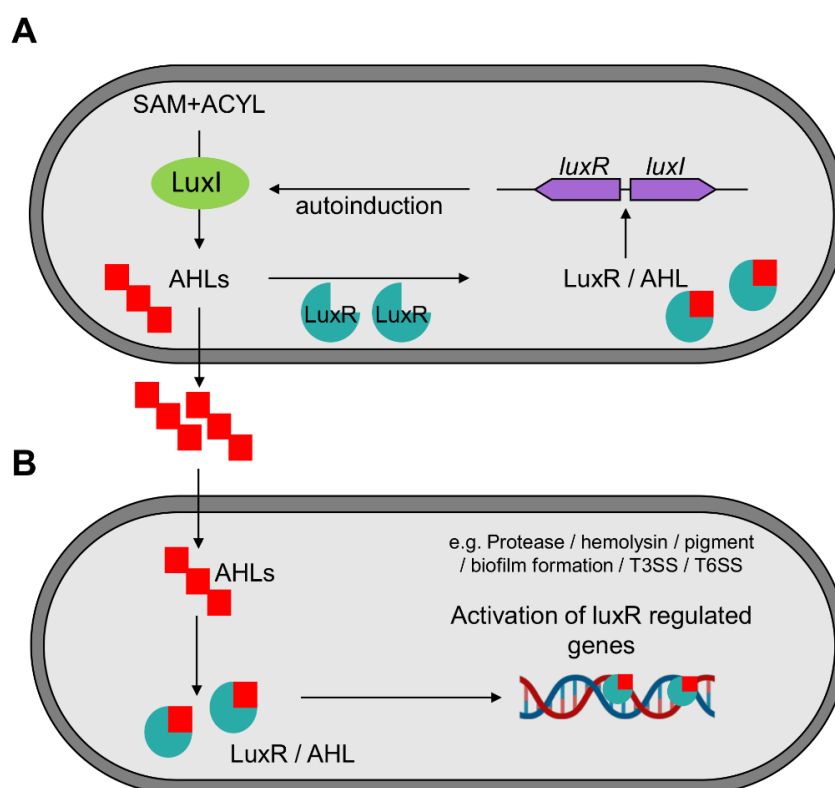


Figure 1.2. Schematic representation of the Acyl-homoserine lactone Quorum-Sensing system in gram-negative bacteria. LuxI family proteins synthesize the acyl-homoserine lactones (AHLs) from S-adenosylmethionine (SAM) and acyl carrier protein (acyl-ACP). LuxR family proteins, the sensor protein, is activated by AHLs molecules. **(A)** At low cell density, the AHL-bound-LuxR type protein positively regulates the expression of the autoinducer synthase gene *luxI*. This positive feedback loop accelerates the synthesis of the AHL autoinducer, leading to synchrony in the QS bacterial behaviours. The AHL molecules freely diffuse across bacterial membranes and accumulate in the extracellular environment. **(B)** Upon certain AHL's threshold, as a result of high bacterial cell density, occurs the intracellular activation. The AHLs bind to the LuxR type protein, and this complex transcriptionally regulates the expression of several genes associated with virulence and biofilm formation. Based on Talagrand-Reboul, et al. (2017).

1.3.3.1. QS and QS-dependent virulence factors in fish pathogens

Over the last years, QS systems, including signalling molecules and regulatory proteins, have been identified in several aquaculture pathogens. Nonetheless, the type of signalling molecules, how are they expressed and what they regulate are yet to be fully understood (Zhao, et al., 2015). Despite that, a major research focus has been the characterization of QS systems and QS-regulated behaviours, such as virulence factors in *Aeromonas* spp., *Vibrio* spp., and *E. tarda*.

QS has been extensively studied in *Aeromonas* species, with a special focus on *A. salmonicida* and *A. hydrophila*. The genes encoding the LuxI/LuxR homologous proteins in *A. salmonicida* and *A. hydrophila* are *asall/asaR* and *ahyl/ahyR*, respectively (Swift, et al., 1997). *Aeromonas* species produce several types of AHLs depending on species and strain. Swift, et al. (1997) were the first authors to report N-butanoyl homoserine lactone (C4-HSL) and N-hexanoyl homoserine lactone (C6-HSL) as major autoinducers of *A. hydrophila* AH-1 and *A. salmonicida* NCIMB 1102. Depending on the strain tested, *A. veronii* was also reported to produce C6-HSL, N-octanoyl-L-Homoserine lactone (C8-HSL), N-3-oxo-octanoyl-L-Homoserine lactone (3-oxo-C8-HSL), N-3-hydroxyoctanoyl-L-Homoserine lactone (3-OH-C8-HSL), 3-Hydroxy-hexanoyl-DL-homoserine lactone (3-OH-C6-HSL), and N-tetradecanoyl-L-Homoserine lactone (C14-HSL), (Gui, et al., 2018; Jangid, et al., 2012). Many authors have demonstrated the role of QS on *Aeromonas* spp. fitness and virulence. LuxI/LuxR system activation in *Aeromonas* has been associated with an enhancement of numerous virulence factors, biofilm formation, and motility (Table 1.1) (Bi, et al., 2007; Gui, et al., 2017; Khajanchi, et al., 2009; Liu, et al., 2018; Swift, et al., 1997). Although only LuxI/LuxR homologous proteins are universally present in *Aeromonas* species (Jangid, et al., 2007), *A. hydrophila* SSU genome contains homologous proteins of the AI-2 system (*luxS* synthetase, *luxU* encoding signal transduction, and *luxO* encoding a response regulator), and the two-component QseB/QseC system (*qseC* sensor kinase and *qseB* transcriptional regulator) (Talagrand-Reboul, et al., 2017). Importantly, deletion of *luxS* and *qseB* in *A. hydrophila* SSU demonstrated their participation in virulence expression (e.g., proteases, biofilm formation, swimming, and swarming motility) (Table 1.1) (Khajanchi, et al., 2012; Kozlova, et al., 2012; Kozlova, et al., 2008). Several *Vibrio* species have been reported to produce and secrete multiple diffusible QS signal molecules, including acyl-homoserine lactones (AHLs), furanosyl borate diester (AI-2), and cholerae autoinducer molecules (CAI-1) (Milton, 2006). Nonetheless, the number and types of circuits are diverse according to the *Vibrio* spp. analysed. The multichannel QS systems of *V. Anguillarum* and *V. harveyi* are the most studied. In general, signal transduction of QS signal molecules in *Vibrio* spp., converges

intracellularly to a shared two-component LuxO/LuxU phosphorelay cascade (Girard, 2019). At low cell density, *luxO* activates the expression of small regulatory RNAs that destabilize mRNA encoding the master regulator (Milton, 2006). On the other hand, at high cell density, *luxO* is inactivated and small regulatory RNAs are not expressed resulting in the translation of the master regulator, responsible for the regulation of QS molecules synthetases and several important functional genes (Milton, 2006). There are two major master regulators in *Vibrio* spp., the LuxR homologous proteins that are AHL-dependent (e.g., *vanR* in *V. anguillarum*), and the AHL-independent ones such as *vanT* (in *V. anguillarum*), *litR* (in *V. fischeri*), *opaR* (in *V. parahaemolyticus*), *smcR* (in *V. vulnificus*) (Croxatto, et al., 2002; Fidopiastis, et al., 2002; Girard, 2019; Kim, et al., 2013; McCarter, 1998; Milton, 2006). Due to *Vibrionaceae* heterogeneity, different *Vibrio* spp. have specific QS signals, master regulators, and circuits. For example, *V. anguillarum* possess three QS circuits (*VanI/VanR*, *VanM/VanN* and *LuxS/LuxP/LuxQ* homologous proteins) and a fourth predicted (*CqsA/CqsS* signal system) (Frans, et al., 2011; Milton, 2006). Both *vanI* and *vanM* synthesize AHLs (C6-HSL, 3-hydroxy-C6-HSL) and are connected, in which *vanM* regulates AHL production by *vanI* (Milton, 2006; Milton, et al., 2001). Importantly, depending on the *Vibrio* species, QS can positively or negatively regulate important virulence factors, including metalloproteases, T3SS, or biofilm formation (Table 1.1).

E. tarda carries multiple QS systems, including an intra-species communication system (*EdwI/EdwR*), an inter-species communication system (*LuxS*), and a putative inter-kingdom communication system (*QseB/QseC*) (Han, et al., 2010; Morohoshi, et al., 2004; Wang, et al., 2011). The *edwI* (*luxI* homolog), responsible for the production of at least 4 different AHLs (N-Butanoyl-L-homoserine lactone, C4-HSL; C6-HSL; N-(3-Oxohexanoyl)-L-homoserine lactone, 3-oxo-C6-HSL; and N-heptanoyl-L-Homoserine Lactone, C7-HSL) (Han, et al., 2010; Morohoshi, et al., 2004), has been demonstrated to negatively regulate the expression of flagellin proteins and *E. tarda*'s motility (Table 1.1) (Morohoshi, et al., 2009). *E. tarda* employs *LuxS* to synthesize AI-2 QS molecules (Han, et al., 2010; Zhang, et al., 2008). *LuxS* inhibition has been associated with positive regulation of biofilm formation and T3SS genes. Additionally, Wang, et al. (2011) described the transcription of a third QS system responding to eukaryotic hormone-like signals (e.g., epinephrine), that also functionally regulate the expression of important virulence factors such as motility and T3SS (Table 1.1).

Table 1.1. Virulence-associated phenotypes controlled by QS regulatory proteins or signalling molecules in different bacterial aquaculture pathogens.

Pathogen	Regulatory protein Signal molecule ^a	QS-regulated phenotypes ^b	Reference
<i>A. hydrophila</i>	AHL	Metalloproteases↑; serine proteases↑; Biofilm formation↑;	Lynch, et al. (2002), Swift, et al. (1997), Swift, et al. (1999)
	AhyR	Metalloproteases↑; Serine proteases; ↑; Amylase↑; DNase↑, Haemolysin↑; S-layer↑	Swift, et al. (1999), Bi, et al. (2007)
	Ahyl/AhyR	Metalloproteases↑; T6SS associated effectors↑; Biofilm formation↑;	Khajanchi, et al. (2009)
	LuxS	Swimming and swarming motility↑	Kozlova, et al. (2008)
	QseBC	Proteases↑; Cytotoxic enterotoxin↑; Swimming and swarming motility↑; Biofilm formation↓	Khajanchi, et al. (2012)
<i>A. salmonicida</i>	AHL	Metalloproteases↑; serine proteases↑	Swift, et al. (1997), Rasch, et al. (2007)
	Asal	Proteases↑↓; Brown pigment↑; protease AsaP1↑; Cytotoxic factor↑; Swimming motility↓; Biofilm formation↑;	Schwenteit, et al. (2011), Liu, et al. (2018)
<i>A. veronii</i>	AHL	Proteases↑; Motility↑	Gui, et al. (2017)
<i>V. anguillarum</i>	RpoS, VanT	Extracellular protease↑, haemolysin coregulated-like protein ↑	Weber, et al. (2009)
<i>V. parahaemolyticus</i>	OpaR	Metalloproteases↓; Serine proteases ↓; T3SS↓; T6SS1↓; T6SS2↑	Chang, Lee (2020), Gode-Potratz, McCarter (2011), Henke, Bassler (2004)
	LuxM	Haemolysin↓; Biofilm Formation↓	Guo, et al. (2018)
	LuxS	Haemolysin↑; Biofilm Formation↓	Guo, et al. (2018)
<i>V. harveyi</i>	LuxM	Biofilm formation↑; T3SS↓; Haemolysin↓; Chitinase↓	Henke, Bassler (2004), Defoirdt, et al. (2010b), Hong, et al. (2016)
	LuxS	T3SS↓; Elastase↑; Caseinase↑; Haemolysin↑; Chitinase↓	Henke, Bassler (2004), Hong, et al. (2016)
	CqsA	Elastase↑; Caseinase↑; Haemolysin↑	Hong, et al. (2016)
	LuxO	Metalloprotease↓; Caseinase↑; gelatinase↑; phospholipase↓	Natrah, et al. (2011)

^a In bold are the signalling molecules, all the others refer to the regulatory proteins involved;

^b↑ indicates that the virulence-associated phenotypes are positively regulated in the presence of the regulatory protein or signalling molecule; ↓ indicates that the virulence-associated phenotypes are negatively regulated in the presence of the regulatory protein or signalling molecule.

Table 1.1. Virulence-associated phenotypes controlled by QS regulatory proteins or signalling molecules in different bacterial aquaculture pathogens (Continued).

Pathogen	Regulatory protein Signal molecule ^a	QS-regulated phenotypes ^b	Reference
<i>V. harveyi</i>	LuxR	T3SS↓	Waters, et al. (2010)
<i>V. vulnificus</i>	SmcR	Metalloproteases↑↓; Protease↑; Cytolysin↑; Extracellular enzymes↑; Biofilm formation↑	Shao, Hor (2001), Kim, et al. (2018a), McDougald, et al. (2001)
	LuxS	Metalloproteases↑↓; Haemolysin↓;	Kim, et al. (2013), Kim, et al. (2018a)
<i>V. alginolyticus</i>	LuxS	Protease↑; Motility↑; Biofilm Formation↓	Ye, et al. (2008), Tian, et al. (2008)
	LuxR	T6SSS1↑; T6SSS2↓	Yang, et al. (2018)
	AHL	Biofilm Formation↑↓	Liu, et al. (2017b)
<i>E. tarda</i>	EdwI	Flagellin↓	Morohoshi, et al. (2009)
	LuxS	T3SS↑; Biofilm Formation↑	Zhang, et al. (2008)
	QseBC	Hemagglutination↓; flagellar motilities↑; T3SS↑	Wang, et al. (2011)

^a In bold are the signalling molecules, all the others refer to the regulatory proteins involved;

^b ↑ indicates that the virulence-associated phenotypes are positively regulated in the presence of the regulatory protein or signalling molecule; ↓ indicates that the virulence-associated phenotypes are negatively regulated in the presence of the regulatory protein or signalling molecule.

Although different QS systems, including signalling molecules and QS-regulatory proteins, were reported over the years, AHLs are the most predominant signalling molecules discovered within aquaculture pathogens (Zhao, et al., 2015). Moreover, AHLs have been described to stimulate pathogens' virulence, resulting in higher mortality rates in prawn and fish larvae (Baruah, et al., 2009; Tinh, et al., 2008). Thus, AHLs disruption will be the focus of the present Thesis.

1.3.3.2. Quorum-Quenching

Interfering with pathogens virulence is emerging as a novel approach to control infections in both humans and animals. The anti-virulence therapy targets specific functions required by pathogens to cause infections, preventing host colonization. By targeting virulence rather than bacterial growth, this approach is unlikely to impose harsh selective pressure for resistance, minimizing the risk of antibiotic resistance among pathogens (Defoirdt, 2018; Defoirdt, et al., 2010a; Fetzner, 2015; Grandclement, et al., 2016). Moreover, when compared to conventional antimicrobials, this strategy will exert less severe side effects towards beneficial bacteria (Grandclement, et al., 2016).

Considering the key role of QS in pathogenesis, one of the most studied anti-virulence strategies is the disruption or disturbance of QS, a process denominated Quorum-Quenching (QQ). QQ can be achieved by (i) inhibiting the signal biosynthesis, (ii) enzymatically degrading/inactivating the signal molecules, or (iii) blocking the signal receptor (Defoirdt, 2018; Fetzner, 2015).

Signal synthesis is by far the less studied and described mechanism to limit signal accumulation. Nonetheless, autoinducer production inhibition is an effective and feasible approach to disrupt QS-mediated virulence factors (Grandclement, et al., 2016; LaSarre, Federle, 2013). AHLs are mainly synthesized by the acyl-HSL synthases (LuxI homologous proteins) from common components of the bacterial metabolism (S-adenosyl-methionine (SAM) and acylated acyl-carrier protein (acyl-ACP)) (Figure 1.2) (Grandclement, et al., 2016). As such, the inhibition of AHLs can be achieved through the inhibition of signal synthase or precursors' synthesis (SAM and acyl-ACP). However, since SAM and acyl-ACP are primary metabolites, complete inhibition of their synthesis, causing bacterial cell death, cannot be considered as an anti-virulence approach (Grandclement, et al., 2016). On the other hand, several reactions end-products and SAM-analogues have been reported for their inhibition effects on LuxI-homologous proteins synthases (Grandclement, et al., 2016; Parsek, et al., 1999). However, up to date, there is no report studying the use of such compounds to disrupt LuxI-homologous protein synthases and consequent AHLs synthesis in fish bacterial pathogens.

The identification of QS-signal analogues that prevent the QS-signal's binding to the

receptor has been reported over the years. Multiple natural or synthetic molecule groups can act as QS-inhibitors by interacting with LuxR-homologous proteins receptors, generating non-productive receptor-signal complexes that block the downstream interactions with QS-regulated genes. Nonetheless, the majority of reports have focused on the compound's chemical and structural characterization (Grandclement, et al., 2016; LaSarre, Federle, 2013).

The most extensively studied QQ approach is the enzymatic degradation or modification of QS signal molecules, a mechanism that appears to be widely distributed in the bacterial kingdom. When studying the enzymatic degradation or inactivation of AHLs, there are three known QQ enzymes: lactonases, acylases, and oxidoreductases. AHL-lactonases are metalloproteins that hydrolase, in a reversible manner, the ester bond of homoserine lactone ring (Chen, et al., 2013). Opening the lactone ring disables the molecule's capacity to bind to the target transcriptional regulator, thus attenuating the effectivity of the signal molecules (LaSarre, Federle, 2013). Nonetheless, hydrolysis of the lactone ring is pH-mediated and, under acidic conditions, the ester bond of AHL-signalling activity is restored (Chen, et al., 2013; LaSarre, Federle, 2013). Importantly, lactonases are known to possess a broad spectrum of AHL specificity since they target the homoserine lactone ring, which is conserved among all AHL molecules (LaSarre, Federle, 2013). On the other hand, AHL-acylases irreversibly hydrolyse the acyl-amide linkage between the acyl chain and the lactone ring (Chen, et al., 2013). This hydrolysis results in the release of a fatty acid chain and a homoserine moiety that does not exhibit any residual signalling activity (Chen, et al., 2013). In contrast with AHL-lactonase, acylases exhibit a narrow specificity depending on the AHL acyl chain length and on the 3-hydroxyl or 3-oxo- substituents (LaSarre, Federle, 2013). The oxidoreductases do not degrade the AHL molecule, but rather modify its chemical structure to an inactive form by oxidizing or/and reducing the acyl chain (Chen, et al., 2013). Such modification causes the non-recognition of the AHL molecule disturbing the signalling pathway. Moreover, up to date, these enzymes are the least abundant and the less studied (LaSarre, Federle, 2013).

The first study on biological AHL inactivation was published in 2000, reporting a lactonase from *Bacillus* sp. 240B1 encoded by the *aiiA* gene (Dong, et al., 2000). Although the *aiiA* gene did not share significant homology to known proteins in databases, it encoded a conserved Zn^{2+} -binding HXHXDH motif of the metallo- β -lactamases superfamily (Dong, et al., 2000). Importantly, the purified AiiA enzyme not only inactivated several AHLs *in vitro*, but also increased plant disease resistance by attenuating *E. carotovora* AHLs accumulation and AHLs-controlled virulence (Dong, et al., 2000). Following the initial report, other QQ-lactonases were

discovered, such as *attM* (*Agrobacterium tumefaciens*) (Zhang, et al., 2002), *aiiM* (*Microbacterium* sp.) (Wang, et al., 2010), and *ytnP* (*B. subtilis*) (Schneider, et al., 2012), confirming the wide distribution of these enzymes within the bacterial kingdom.

1.3.4. Fish Immune system stimulation

Fish are in constant contact with the aquatic environment and therefore in close contact with a complex and dynamic set of microorganisms that may have serious implications on fish health (Perez, et al., 2010; Rauta, et al., 2012). Thus, fish have evolved strong immune responses to recognize pathogenic organisms (distinguishing them from commensals) and triggering pathways for pathogens destruction and elimination (Rauta, et al., 2012; Secombes, Wang, 2012). The fish immune system is composed of stratified defence mechanisms divided into two main components: (i) innate (or non-specific) immune responses, and (ii) adaptive (or specific) immune responses (Rauta, et al., 2012). Both innate and adaptive immune systems are closely related, possessing an interlinking relationship. The non-specific immune responses usually precede the adaptative responses and play an instructive role in the activation and regulation of the adaptive responses (Rauta, et al., 2012; Secombes, Wang, 2012).

Fish are the earliest phylogenetic group of vertebrates to possess developed innate and adaptive immune systems (Rauta, et al., 2012). Nonetheless, fish mostly rely on innate immune mechanisms for survival.

Immune cells are organized into tissues and organs, collectively referred to as the lymphoid system. In teleost fish, the thymus, kidney (anterior/head and middle), and spleen are the main and largest lymphoid organs (Rauta, et al., 2012). The kidney, in particular the head-kidney, is the primary or central lymphoid organ assuming the hematopoietic functions in teleost fish (Kum, Sekkin, 2011). The head-kidney is responsible for leukocytes production, phagocytosis, antigen processing, and formation of IgM cells (Rauta, et al., 2012).

1.3.4.1. Innate immunity

The innate immune system is the earliest immune defence mechanism that responds to the initial infection and does not retain the immunological memory of previous responses to invaders (Rauta, et al., 2012; Secombes, Wang, 2012). Moreover, the innate immune system is responsible for infection recognition, initiation of inflammation, and homeostasis restoration (Rauta, et al., 2012; Secombes, Wang, 2012).

The mucosal barrier of the skin, gills, and gut are equipped with immunocompetent cells and humoral components that act as physical and chemical barriers to prevent bacteria from entering the host (Rauta, et al., 2012; Secombes, Wang, 2012). Besides its primary

role as a physical barrier, the skin is capable of chemically protecting fish from infection through the secretion of mucus. Skin mucus comprises several immune defence components, including antimicrobial peptides, immunoglobulins, proteases, and lysozymes (Dash, et al., 2018; Kiron, 2012; Magnadóttir, 2010; Tiralongo, et al., 2020). Gills, besides their major role in fish respiratory functions, are also involved in immune defence. Gill-associated lymphoid tissue harbours cellular components including macrophages, neutrophils, lymphocytes, and granulocytes (Kiron, 2012). The gastrointestinal tract is a multifunctional organ involved in nutrient absorption and immunity. Although fish gut-associated lymphoid tissue is not as complex and organized as in mammals, it harbours lymphocytes, plasma cells, mast/eosinophilic granulocytes, macrophages, and B and T cells (Kiron, 2012).

Fish depend on the invaders' recognition to trigger inflammatory responses. Pathogen recognition is accomplished through transmembrane or intracellular receptors in different leukocytes (e.g., neutrophils, and monocytes/macrophages) (Li, Wu, 2021). These receptors, known as Pattern Recognition Receptors (PRRs), recognize specific and conserved pathogen-associated molecular patterns (PAMPs) on pathogens surface, apoptotic and damaged cells (Li, Wu, 2021; Palti, 2011; Zhang, et al., 2014). The most known and characterized PRRs in fish are the Toll-Like receptors, which recognize pathogens' structures and molecules, including lipopolysaccharides, peptidoglycans, double-strand RNA, and flagellin. Upon PRRs activation, intracellular signalling cascades initiate, triggering an inflammatory response (Palti, 2011; Zhang, et al., 2014). In the inflammatory process, circulating leukocytes produce and release immune mediators such as cytokines (e.g., IL-1, IL-6, IL-8) that mediate pro-inflammatory responses (Magnadóttir, 2010; Sakai, et al., 2021; Secombes, et al., 2001). On the other hand, anti-inflammatory cytokines (e.g., IL-10) act to promote tissue repair and homeostasis restoration by inhibiting macrophages' recruitment and action (Sakai, et al., 2021). This complex inflammatory network comprises both humoral and cellular mechanisms that protect fish from infectious diseases (Magnadóttir, 2006; Magnadóttir, 2010). In response to inflammation, leukocytes produce and release several bactericidal humoral components, including lysozyme, anti-proteases, proteases, peroxidases, nitric oxide, and reactive oxygen species (ROS) (Kiron, 2012).

Alongside these events, cellular immune responses also play a critical role in the first line of defence against invading pathogens. Immune cells recruitment, in special phagocytes, is crucial when physical barriers are breached to rapidly eliminate the infiltrating organisms or particles during the inflammation and resolution process (Linnerz, Hall, 2020; Rauta, et al., 2012; Wang, et al., 2017).

Phagocytosis is the cellular process of sensing, ingesting, neutralizing/digesting and

eliminating foreign particles larger than 0.5 μm in diameter, including pathogenic microorganisms and cellular debris (Gordon, 2016; Uribe-Querol, Rosales, 2020). Although all cells may perform phagocytosis, monocytes and granulocytes are the professional cells to eliminate invading pathogens (Linnerz, Hall, 2020; Rabinovitch, 1995; Uribe-Querol, Rosales, 2020). Monocytes and granulocytes, generally found in blood and haematopoietic tissues, are highly migratory cells exhibiting strong killing mechanisms (Linnerz, Hall, 2020).

Phagocytosis involves a series of phases: (i) microbial recognition; (ii) phagosome formation; and (iii) phagosome maturation. Microbial recognition by phagocytes is accomplished by PRRs. Although the Toll-Like receptors are the most studied in fish for their role in activating inflammation, they cannot directly induce the phagocytosis process (Uribe-Querol, Rosales, 2020). DC-SIGN (dendritic cell-specific ICAM-3-grabbing nonintegrin) is a recognized bacterial receptor that, by binding to the PAMPs, triggers phagocytosis (Li, Underhill, 2020; Uribe-Querol, Rosales, 2020). The signalling process that initiates phagocytosis leads to important changes in the actin cytoskeleton and cell membrane, to extend and envelop the microorganism in a phagosome (Uribe-Querol, Rosales, 2017). Finally, the new vesicle rapidly transforms its membrane composition and contents to become a microbicidal vesicle, the phagolysosome that degrades the ingested particle (Uribe-Querol, Rosales, 2017; 2020).

Phagocytosis is not an isolated cell response, but a large and complex network involving the production of pro-inflammatory mediators and cytokines, and the production and release of large amounts of bactericidal ROS that help to eliminate foreign microbes (Aderem, 2003; Underhill, Ozinsky, 2002; Uribe-Querol, Rosales, 2020). Despite the inhospitable environment for microorganisms inside the phagolysosome, several aquatic pathogenic organisms (e.g., *V. anguillarum*, *E. tarda*, *M. marinum*) have successfully developed strategies to prevent phagocytosis, survive microbicidal responses, and thrive inside phagocytic cells, having a clear advantage to proliferate inside the host (Sepulcre, et al., 2007; Sepulcre, et al., 2010; Uribe-Querol, Rosales, 2017; 2020; Zhou, Sun, 2016).

1.3.4.2. Adaptive immunity

The adaptive immune system in teleost fish is believed to present a more restricted genetic machinery than in mammalian species (Magnadóttir, 2010). The adaptive immune system has a vital role in the protection against recurrent infections, providing a long-lasting, rapid, and efficient response to the pathogens. The primary response to infection often takes days to develop, while in subsequent infections the memory response develops within hours (Secombes, Wang, 2012). The memory immune response is maintained by B lymphocytes that mediate humoral (antibody) responses

and T lymphocytes that mediate the cellular immune responses (Magnadóttir, 2010; Secombes, Wang, 2012).

In adaptive responses, B lymphocytes are activated to induce immunoglobulins production (e.g., IgM and IgT in teleost) (Secombes, Wang, 2012; Zhang, et al., 2010). These soluble forms migrate through the blood and other fluids and bind to specific foreign antigens, blocking them to bind to the host cells' receptors and marking them for destruction by phagocytic cells (Secombes, Wang, 2012). Moreover, T lymphocytes use specific receptors that recognize marked foreign antigens and activate their killing abilities (Magnadóttir, 2010; Secombes, Wang, 2012). Importantly, B lymphocytes may also play a role in intracellular pathogen killing, reinforcing the close relationship between innate and adaptive immune systems (Li, et al., 2006; Wu, et al., 2019).

1.3.4.3. Immunostimulants

Stimulation of the fish immune system using immunostimulants or immunomodulatory compounds is an environmentally friendly strategy to minimize bacterial outbreaks in aquaculture. Immunostimulants are biological (e.g., bacterial, yeast, plant, and algal derivatives) or synthetic compounds that enhance mainly cellular and humoral innate immune responses by interacting and directly activating immune cells (Maqsood, et al., 2011). Immunostimulants may improve phagocytosis, bactericidal, complement, and lysozyme activities and may also stimulate antibody responses (Wang, et al., 2017). Activating the immune system using immunostimulants, increases fish capacity to fight infections. Alongside stimulation of non-specific immune system, immunostimulants are safe for the environment, animal, and human health, lack side effects, are economically effective, and are easy to administrate (injection, immersion, or oral uptake) (Barman, et al., 2013; Maqsood, et al., 2011). Several reports described the potential of immunostimulants (e.g., polysaccharides, plants, vitamins, β -glucans, algae extracts, chitin, and microorganisms) in protecting fish and shrimp against *A. salmonicida*, *A. hydrophila*, *E. tarda*, *V. anguillarum*, *V. vulnificus*, *Yersinia ruckery*, and other bacteria (Ji, et al., 2017; Lu, et al., 2019; Sattanathan, et al., 2020).

1.4. *Bacillus* spp. as eco-friendly microbes for bacterial diseases control in aquaculture

Microorganisms have been exploited over the last years as an effective approach to maintain microbial equilibrium and maximum production in aquaculture (Nayak, 2021). *Bacillus* spp., in particular, exhibit unique features that offer promising solutions to the challenges faced by modern aquaculture (James, et al., 2021; Nayak, 2021; Soltani, et

al., 2019). *Bacillus* spp. are rod-shaped aerobic or facultative anaerobic gram-positive bacteria and are commonly known as soil organisms (Hong, et al., 2009; Nayak, 2021). Nonetheless, in virtue of their physiological and metabolic characteristics, *Bacillus* spp. are commonly found in aquatic environments, food, air, and the gastrointestinal tract of numerous animals and humans (Lee, et al., 2016; Soltani, et al., 2019; Tam, et al., 2006; Zhou, et al., 2014). Multiple studies have isolated and characterized *Bacillus* spp. from the gastrointestinal tract of fish and shrimps, including tilapia, salmon, carp, rohu, European seabass, and others (Askarian, et al., 2012; Mukherjee, et al., 2019b; Serra, et al., 2019; Soltani, et al., 2019; Zhang, et al., 2019b). Fish gut-associated bacteria might be ideal microorganisms for aquaculture applications due to their ability to re-establish within the host intestinal tract. Consequently, several studies have highlighted the potential aquaculture applications of fish- and shrimp- gut commensal *Bacillus* spp. and their metabolites (Li, et al., 2019; Mukherjee, et al., 2019a; Nayak, 2021; Soltani, et al., 2019; Zhang, et al., 2019b).

In comparison with other beneficial microorganisms used in aquaculture, *Bacillus* spp. exhibit critical advantages such as sporulation capacity, which enables them to withstand extreme stress situations, providing advantages for survival in different environments (Kuebutornye, et al., 2020). The sporulation capacity of *Bacillus* spp. allows their dehydration and incorporation in fish feeds without effects on their characteristics and viability (Setlow, 2014). Furthermore, *Bacillus* spp. spores withstand the gastric acidity and bile salts surviving gut transit and eventually germinating and colonizing the host's gut (Nayak, 2021).

The role of *Bacillus* spp. on the nutritional, physiological, immunological, and therapeutic aspects of aquatic organisms are well known (James, et al., 2021; Nayak, 2021). Besides the bioactive aspects of *Bacillus* spp., their general recognition as safe increase their interest over other microbes. Indeed, fourteen *Bacillus* spp. including *B. licheniformis*, *B. amyloliquefaciens*, *B. pumilus*, and *B. subtilis*, are included in the European Food Safety Authority (EFSA) list of qualified presumption of safety (Ricci, et al., 2018). Moreover, commercial products containing *Bacillus* spp. included in this list, are already available for several aquaculture applications, including water bioremediation and growth and feed utilization improvement.

Over the years, *Bacillus* spp. have been explored to produce digestive enzymes, such as chitinase, protease, cellulase, amylase, and lipase, that hydrolyse ingredients and enhance nutrient utilization by fish (James, et al., 2021; Kuebutornye, et al., 2020; Nayak, 2021). Despite being usually linked to the digestive process, these enzymes might also be involved in disease control through the lysis of important components of the pathogens' cell walls (Ramesh, et al., 2015; Soltani, et al., 2019).

Within health-promoting characteristics, *Bacillus* spp. can compete with pathogens for available energy, nutrients, and adhesion receptors in the intestinal epithelial surface (James, et al., 2021). The strong adhesion properties of *Bacillus* spp. cells and spores make them highly apt to compete for occupation of adhesion sites on the intestinal epithelium preventing the adhesion of pathogens (Angioi, et al., 1995; James, et al., 2021; Jung, et al., 2012; Laloo, et al., 2010; Tavares Batista, et al., 2014). Moreover, *Bacillus* spp. use the same sources of energy and nutrients as pathogenic bacteria. Thus, by utilizing available nutrients (e.g., iron) and sources of energy (e.g., carbon), *Bacillus* spp. boost the competitive exclusion of pathogenic bacteria (James, et al., 2021; Laloo, et al., 2010).

Besides competitive exclusion, *Bacillus* spp. can confer fish resistance towards pathogens through the production of antimicrobial compounds, eradication of pathogens biofilms, disruption of pathogens communication, and inducing immunostimulatory mechanisms, as detailed in the following sections.

1.4.1. Antimicrobial activities of *Bacillus* spp.

Bacillus spp. play an important role in controlling infections due to their high metabolic activity (Suva, et al., 2016). The potential of *Bacillus* strains to produce a wide range of NACs is recognized for decades. It has been estimated that 4-5% of the *B. subtilis* genome is solely devoted to the biosynthesis of NACs (Caulier, et al., 2019; Stein, 2005). NACs synthesized by *Bacillus* spp. exhibit a broad range of antimicrobial properties against gram-positive and gram-negative bacteria, fungus, viruses, and parasites. Although the antimicrobial properties of *Bacillus* spp. have been recognized for 50 years (Suva, et al., 2016), their potential application on treating fish diseases has only been studied in the last two decades.

The antagonistic ability of *Bacillus* spp. against different fish and shellfish pathogens such as *Vibrio* (e.g., *V. anguillarum*, *V. harveyi* and *V. parahaemolyticus*), *Aeromonas* (e.g., *A. hydrophila*, *A. salmonicida*, *A. caviae*, and *A. veronii*), *Edwardsiella* (e.g., *E. tarda* and *E. ictaluri*), *Streptococcus* (e.g., *S. agalactiae* and *S. iniae*) and many other pathogens have been demonstrated along the years (Table 1.2).

Table 1.2. *Bacillus* strains with antimicrobial activity against important bacterial aquaculture pathogens.

<i>Bacillus</i> strain	Source	Antimicrobial activity		<i>in vivo</i> protection	Reference
		Whole Cell	Extracellular		
<i>B. subtilis</i> BT23	Shrimp culture pounds	<i>V. harveyi</i>	<i>V. harveyi</i> , <i>V. anguillarum</i> , <i>V. vulnificus</i> , <i>V. damsela</i>	Increase of shrimp survival upon <i>V. harveyi</i> challenge by 60%	Vaseeharan, Ramasamy (2003)
<i>B. subtilis</i> UTM 126	Shrimp culture pounds	n/a	<i>V. alginolyticus</i> , <i>V. harveyi</i> , <i>V. parahaemolyticus</i>	Increase of shrimp survival upon <i>V. harveyi</i> challenge by 30%	Balcazar, Rojas-Luna (2007)
<i>B. subtilis</i> SG4	Mrigal	<i>P. fluorescens</i> , <i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i> , <i>P. aeruginosa</i>	n/a	n/a	Ghosh, et al. (2007)
<i>B. subtilis</i> R3	Rohu	<i>A. hydrophila</i>	<i>A. hydrophila</i>	n/a	Giri, et al. (2011)
<i>B. thuringiensis</i> ; <i>B. cereus</i> ; <i>Bacillus</i> sp.	Salmon	<i>V. anguillarum</i> , <i>A. Salmonicida</i> , <i>M. viscosa</i>	n/a	n/a	Askarian, et al. (2012)
<i>B. subtilis</i> , <i>B. pumilus</i>	Channel Catfish	<i>E. ictaluri</i> , <i>A. Hydrophila</i> , <i>E. tarda</i> , <i>S. inniae</i> , <i>Y.ruckeri</i>	<i>F. columnare</i>	Increase of catfish survival upon <i>E. ictaluri</i> challenge by 60%	Ran, et al. (2012)
<i>B. subtilis</i> NCIMB 3610	Culture collection	n/a	<i>V. anguillarum</i> , <i>Ph. damsela</i> , <i>E. faecalis</i> , <i>L. lactis</i>	n/a	Touraki, et al. (2012)
<i>B. subtilis</i> BHI344	Channel Catfish	<i>E. tarda</i> , <i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i>	n/a	n/a	Luo, et al. (2014)
<i>B. aerius</i> CCH1A, <i>B. sonorensis</i> CCHIPh	Indian major carp	<i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>A. veronii</i>	n/a	n/a	Dutta, Ghosh (2015)
<i>B. amyloliquefaciens</i> CMH1X, <i>B. sonorensis</i> CM2H3L	Mrigal	<i>A. hydrophila</i> , <i>P. putida</i> , <i>A. salmonicida</i> , <i>A. veronii</i> , <i>P. fluorescence</i>	n/a	n/a	Dutta, Ghosh (2015)

Table 1.2. *Bacillus* strains with antimicrobial activity against important bacterial aquaculture pathogens (Continued).

Bacillus strain	Source	Antimicrobial activity		in vivo protection	Reference
		Whole Cell	Extracellular		
<i>B. pumilus</i> B16, <i>B. mojavensis</i> J7	Shrimp gills; Coastal sediment	<i>V. parahaemolyticus</i> ; <i>V. vulnificus</i> , <i>V. harveyi</i> , <i>S. typhimurium</i> ; <i>P. aeruginosa</i> , <i>A. hydrophila</i> , <i>E. coli</i> , <i>L. monocytogenes</i> , <i>S. aureus</i>	<i>V. parahaemolyticus</i> ; <i>V. vulnificus</i> , <i>V. harveyi</i> , <i>S. typhimurium</i> , <i>E. coli</i> , <i>L. monocytogenes</i>	n/a	Liu, et al. (2015)
<i>B. licheniformis</i> KADR5; <i>B. pumilus</i> KADR6	Rohu	<i>A. hydrophila</i> ; <i>Aeromonas</i> sp., <i>A. enteropelogenes</i>	n/a	Increase of rohu survival upon <i>A. hydrophila</i> challenge by 50%	(Ramesh, et al., 2015)
<i>B. subtilis</i> KADR1, <i>B. aerophilus</i> KADR3, <i>B. firmus</i> KADR4	Rohu	<i>A. hydrophila</i> ; <i>Aeromonas</i> sp., <i>A. enteropelogenes</i>	n/a	n/a	Thankappan, et al. (2015)
<i>B. subtilis</i> LR1	Rohu	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i>	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i> ; <i>B. mycoides</i>	n/a	Banerjee, et al. (2016)
<i>B. amyloliquefaciens</i> M001	Olive flounder	<i>V. anguillarum</i> , <i>V. vulnificus</i> , <i>V. campbellii</i> , <i>V. parahaemolyticus</i> , <i>E. tarda</i> , <i>Streptococcus</i> sp.	n/a	Increase of turbot survival upon <i>V. anguillarum</i> challenge by 60%	Chen, et al. (2016)
<i>B. subtilis</i>	Grass carp	<i>A. hydrophila</i> , <i>A. punctata</i> ; <i>E. ictaluri</i> ; <i>V. flurialis</i> ; <i>S. agalactiae</i>	n/a	n/a	Guo, et al. (2016)
<i>B. licheniformis</i> D1	Asian green mussel	n/a	<i>V. harveyi</i> , <i>P. aeruginosa</i>	n/a	Hamza, et al. (2016)
<i>B. methylotrophicus</i> CC1FG2 and CC1FG4, <i>B. subtilis</i> CC1HG5 and CC2HG7	Indian major carp, Java barb	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i> ; <i>P. putida</i> ; <i>A. veronii</i>	n/a	n/a	Mukherjee, Ghosh (2016)

Table 1.2. *Bacillus* strains with antimicrobial activity against important bacterial aquaculture pathogens (Continued).

<i>Bacillus</i> strain	Source	Antimicrobial activity		<i>in vivo</i> protection	Reference
		Whole Cell	Extracellular		
<i>B. stratosphericus</i> CM1HG5, <i>B. aerophilus</i> CM1HG5, <i>B. licheniformis</i> CM3HG10 <i>B. siamensis</i> B44v	Mrigal Fermented foods	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>A. veronii</i> ; <i>P. fluorescens</i>	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>A. veronii</i> ; <i>P. fluorescens</i>	n/a	(Mukherjee, et al., 2016)
<i>Bacillus</i> sp. HGA8B	Climbing perch	n/a	<i>A. hydrophila</i> , <i>P. aeruginosa</i> ; <i>P. fluorescens</i> ; <i>A. sobria</i> ; <i>A. Caviae</i>	Increase of catfish survival upon <i>A. hydrophila</i> challenge by 40% n/a	Meidong, et al. (2017) Midhun, et al. (2017)
<i>B. methylotrophicus</i> LR1FG1; <i>B. amyloliquefaciens</i> LR2HG13; <i>B. licheniformis</i> LR3HG4	Rohu	<i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>A. sobria</i> , <i>A. veronii</i>	<i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>A. sobria</i> , <i>A. veronii</i>	n/a	Mukherjee, et al. (2017)
<i>Bacillus</i> sp. MVF1 and <i>Bacillus</i> sp. MVH7	Striped snakehead, striped dwarf catfish, Labeo calbasu, Mrigal	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i> ; <i>A. sobria</i>	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i> ; <i>A. sobria</i>	n/a	Nandi, et al. (2017a)
<i>Bacillus</i> sp. CCF7, <i>B. amyloliquefaciens</i> CCH9 and PJH1	Indian major carp, Java barb	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i> ; <i>A. sobria</i>	<i>A. hydrophila</i> , <i>A. salmonicida</i> ; <i>P. fluorescens</i> ; <i>A. sobria</i>	n/a	Nandi, et al. (2017b)

Table 1.2. *Bacillus* strains with antimicrobial activity against important bacterial aquaculture pathogens (Continued).

<i>Bacillus</i> strain	Source	Antimicrobial activity		<i>in vivo</i> protection	Reference
		Whole Cell	Extracellular		
<i>B. aerophilus</i> KADR3	Rohu	n/a	<i>A. hydrophila</i> ; <i>Aeromonas</i> sp., <i>A. enteropelogenes</i>	Increase of rohu survival upon <i>A. hydrophila</i> challenge by 50%	Ramesh, et al. (2017)
<i>Bacillus</i> sp. BC01	Soil Sample	<i>A. veronii</i> , <i>P. fluorescens</i> , <i>S. iniae</i>	n/a	n/a	Foysal, Lisa (2018)
<i>B. aerius</i> B81e	fish	<i>A. hydrophila</i> ; <i>S. agalactiae</i>	<i>A. hydrophila</i> ; <i>S. agalactiae</i>	Increase of catfish survival upon <i>A. hydrophila</i> challenge by 30%	Meidong, et al. (2018)
<i>B. coagulans</i> MTCC 9872	Culture collection	<i>P. fluorescens</i> , <i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i> , <i>P. aeruginosa</i>	n/a	n/a	Midhun, et al. (2018)
<i>B. pumilus</i> , <i>Bacillus</i> sp.	Trout	<i>A. hydrophila</i> , <i>E. sakazakii</i>	n/a	n/a	Ramírez-Torrez, et al. (2018)
<i>B. velezensis</i> JW	Carp	<i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>V. parahaemolyticus</i> , <i>L. garviae</i> , <i>S. agalactiae</i>	n/a	Increase of goldfish survival upon <i>A. hydrophila</i> challenge by 50%	Yi, et al. (2018)
<i>Bacillus</i> sp. YB1701	sediment, fish pounds	<i>A. hydrophila</i> , <i>V. parahaemolyticus</i>	n/a	Increase of goldfish survival upon <i>A. hydrophila</i> challenge by 50%	Zhou, et al. (2018)
<i>B. velezensis</i> BvL03	Soil Sample	n/a	<i>A. hydrophila</i> , <i>A. veronii</i> , <i>A. caviae</i> , <i>A. sobria</i>	Increase of grass carp survival upon <i>A. hydrophila</i> challenge by 80%	Cao, et al. (2019)
<i>B. subtilis</i> BStH-5 and BStH-19	Dabry's sturgeon	<i>A. hydrophila</i> , <i>A. veronii</i> , <i>A. media</i> , <i>S. iniae</i>	n/a	Increase of dabry's sturgeon survival upon <i>A. hydrophila</i> challenge by 30%	Di, et al. (2019)
<i>B. velezensis</i> TPS3N, <i>B. amyloliquefaciens</i> TPS17	Nile tilapia	n/a	<i>S. agalactiae</i> , <i>V. harveyi</i> , <i>A. hydrophila</i>	n/a	Kuebutornye, et al. (2019)

Table 1.2. *Bacillus* strains with antimicrobial activity against important bacterial aquaculture pathogens (Continued).

<i>Bacillus</i> strain	Source	Antimicrobial activity		<i>in vivo</i> protection	Reference
		Whole Cell	Extracellular		
<i>B. methylotrophicus</i> LR1FG1; <i>B. amyloliquefaciens</i> LR2HG13; <i>B. licheniformis</i> LR3HG4 <i>B. subtilis</i> MBTDCMFRI Ba37	Rohu Soil Sample	n/a <i>A. hydrophila</i> , <i>A. veronii</i> , <i>V. anguillarum</i> , <i>V. alginolyticus</i> , <i>V. harveyi</i> , <i>V. parahaemolyticus</i>	<i>A. hydrophila</i> <i>A. hydrophila</i> , <i>A. veronii</i> , <i>V. anguillarum</i> , <i>V. alginolyticus</i> , <i>V. harveyi</i> , <i>V. parahaemolyticus</i>	n/a Increase of green chromide survival upon <i>V. anguillarum</i> challenge by 30%	Mukherjee, et al. (2019b) Nair, et al. (2021)
<i>B. subtilis</i> ; <i>B. licheniformis</i>	European seabass	<i>S. aureus</i> , <i>Ph. damseale</i> , <i>V. harveyi</i> , <i>T. maritimum</i>	<i>T. maritimum</i>	n/a	Serra, et al. (2019)
<i>B. velezensis</i> LF01	Tilapia	n/a	<i>S. agalactiae</i> , <i>S. iniae</i> , <i>A. hydrophila</i> , <i>E. tarda</i> , <i>E. ictaluri</i> , <i>A. schuberii</i> , <i>A. veronii</i> , <i>A. jandaei</i> , <i>V. harveyi</i>	Increase of tilapia survival upon <i>S. agalactiae</i> challenge by 50%	Zhang, et al. (2019b)
<i>B. velezensis</i> K2	Grouper	<i>V. alginolyticus</i> , <i>V. harveyi</i> , <i>A. hydrophila</i> , <i>A. veronii</i> , <i>A. caviae</i> , <i>E. casseliflavus</i> , <i>L. garviae</i>	n/a	Increase of grouper survival upon <i>V. harveyi</i> challenge by 30%	Li, et al. (2019)
<i>Bacillus</i> spp.	Rohu, catla; mrigal	<i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>P. fluorescens</i> , <i>P. putida</i> , <i>A. veronii</i>	n/a	n/a	Ghosh, et al. (2021)
<i>B. subtilis</i> GPSAK9	Brown-marbled grouper	n/a	<i>S. agalactiae</i> , <i>S. iniae</i> , <i>V. harveyi</i> , <i>V. alginolyticus</i>	n/a	Monzón-Atienza, et al. (2021)

Two *B. subtilis* strains isolated from shrimp ponds exhibit antagonistic activity against pathogens belonging to *Vibrio* species such as *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus*. Alongside the antimicrobial capacity, these *B. subtilis* strains demonstrated strong protection of shrimp upon *V. harveyi* challenge, increasing survival rate by 30 and 60% (Balcázar, et al., 2006; Vaseeharan, Ramasamy, 2003). Similarly, *B. licheniformis*, *B. pumilus*, *B. subtilis*, *B. aerophilus*, *B. firmus*, *B. methylotrophicus*, and *B. amyloliquefaciens* strains isolated from the gastrointestinal tract of rohu, showed antagonistic abilities against a broad range of pathogens belonging to *Aeromonas* and *Pseudomonas* (Banerjee, et al., 2016; Mukherjee, et al., 2019b; Ramesh, et al., 2015; Thankappan, et al., 2015). Moreover, by administering *B. licheniformis* KADR5, *B. pumilus* KADR6, *B. aerophilus* KADR3 to fish, Ramesh, et al. (2015) observed an increase of rohu survival up to 50% upon challenge with *A. hydrophila*. Also, *B. velezensis* strains, isolated from soil samples and different fish species, demonstrated a broad range of activity by inhibiting *Aeromonas*, *Edwardsiella*, *Lactococcus*, *Streptococcus*, and *Vibrio* species (Cao, et al., 2019; Li, et al., 2019; Yi, et al., 2018; Zhang, et al., 2019b). Importantly, these bioactive *B. velezensis* strains increased up to 80% the survival of different fish species upon infection with *A. hydrophila*, *S. agalactiae*, and *V. harveyi* (Cao, et al., 2019; Li, et al., 2019; Zhang, et al., 2019b). The majority of studies have focused on the *Bacillus* spp. inhibition profile against *Aeromonas*, *Edwardsiella*, and *Vibrio* species. Nonetheless, two studies have reported the inhibition of other important bacterial pathogens affecting Mediterranean aquaculture, *Ph. damsela* and *T. maritimum*, by *B. subtilis* and *B. licheniformis* strains isolated from a culture collection and from the gastrointestinal tract of European seabass (Serra, et al., 2019; Touraki, et al., 2012). The antimicrobial compounds produced by *Bacillus* spp. have been extensively studied in the medicinal, veterinary, and food industries to control important bacterial diseases. Members of the *Bacillus* genus produce more than 167 antimicrobial compounds with a wide range of activity (Caulier, et al., 2019; Kuebutornye, et al., 2020). Major NACs produced by *Bacillus* include bacitracin, difficin, lipopeptides (bacillomycin, iturin, and fengycin), surfactin, subtilin, subtilosin A, sonorensin, ericin and amylolysin (Caulier, et al., 2019; James, et al., 2021; Nayak, 2021). Although several studies have demonstrated the antimicrobial potential of *Bacillus* strains, only a few studies identified and characterized the bioactive molecules responsible for the antimicrobial activities exhibited by *Bacillus* spp. against fish pathogens (Table 1.3).

Table 1.3. Antimicrobial compounds produced by *Bacillus* strains with antimicrobial activity against important bacterial aquaculture pathogens.

<i>Bacillus</i> strain	Source	Antimicrobial compounds or genes identified	Antimicrobial activity	Reference
<i>B. amyloliquefaciens</i> GA1	Strawberry	Amylolysin	<i>S. agalactiae</i> , <i>S. aureus</i> , <i>E. faecalis</i> , <i>L. monocytogenes</i> , <i>L. innocua</i>	Arguelles Arias, et al. (2013)
<i>B. amyloliquefaciens</i> DSM7	Surgical room	Surfactin, Fengycin, Iturin A, Macrolactin, Difficidin, Bacillaene	<i>A. hydrophila</i> , <i>P. aeruginosa</i>	Nastro, et al. (2013)
<i>B. subtilis</i> MMA7	Marine sponge	Subtilomycin	<i>L. monocytogenes</i> , <i>L. innocua</i> , <i>E. faecium</i> , <i>S. aureus</i> , <i>A. hydrophila</i> , <i>V. anguillarum</i> , <i>Alteromonas</i> sp.	Phelan, et al. (2013)
<i>B. sonorensis</i> MT93	Marine soil	Sonorensin	<i>S. aureus</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>V. vulnificus</i> and <i>L. monocytogenes</i>	Chopra, et al. (2014)
<i>B. amyloliquefaciens</i> M1	MCCC	Cyclic lipopeptide N3	<i>V. anguillarum</i> , <i>V. septicus</i> , <i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>S. aquimarina</i> , <i>V. salmonicida</i> , <i>V. splendidus</i> , <i>V. ichthyenteri</i> , <i>V. fischeri</i> , <i>V. vulnificus</i>	Xu, et al. (2014)
<i>B. amyloliquefaciens</i> ZJHD3-06	Areolate grouper	Bacteriocin CAMT2	<i>L. monocytogenes</i> , <i>S. aureus</i> , <i>V. parahaemolyticus</i> , <i>E. coli</i>	An, et al. (2015)
<i>B. velezensis</i> V4	Marine RAS	Iturin A, bacillomycin D, macrolactin A, oxydifficidin	<i>A. hydrophila</i> , <i>A. salmonicida</i>	Gao, et al. (2017)
<i>B. siaensis</i> JFL15	Hairtail	Bacillomycin, fengycin, iturin and surfactin families	<i>A. hydrophila</i> , <i>Ph. damsela</i> , <i>V. harveyi</i> , <i>V. alginolyticus</i> , <i>E. coli</i> , <i>E. tarda</i> , <i>P. aeruginosa</i> , <i>V. parahaemolyticus</i> , <i>V. chagassi</i>	Xu, et al. (2018)

B. amyloliquefaciens GA1, isolated from a strawberry, produces an antimicrobial peptide, amylolysin, active against several pathogens, including *S. agalactiae* and *S. aureus* (Arguelles Arias, et al., 2013). Another strain, *B. amyloliquefaciens* M1 produces a cyclic lipopeptide N3, with antibacterial activity against several *Vibrio* species, including *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* (Xu, et al., 2014). Similarly, An, et al. (2015) described the production of a bacteriocin, CAMT2, by *B. amyloliquefaciens* ZJHD3-06 responsible for the inhibition of *L. monocytogenes*, *S. aureus*, *V. parahaemolyticus*, and *E. coli*. *B. subtilis* MMA7, isolated from marine sponges, was also reported to produce a subtilomycin with a broad range of activity against, among others, *S. aureus*, *A. hydrophila*, and *V. anguillarum* (Phelan, et al., 2013). Chopra, et al. (2014) isolated from marine soil a *B. sonorensis* MT93 with a broad spectrum of activity against *S. aureus*, *V. vulnificus*, *E. coli*, *P. aeruginosa*, and *L. monocytogenes*. These authors also purified a new bacteriocin, a sonorensin. *B. velezensis* V4 isolated from a marine recirculating aquaculture system (RAS), produced different antimicrobial compounds such as iturin A, bacillomycin D, macrolactin A, and oxyflicidin responsible for the inhibition of *A. hydrophila* and *A. salmonicida* (Gao, et al., 2017). Similarly, Xu, et al. (2014) isolated a *B. siaensis* JFL15 that produced several compounds belonging to the bacillomycin, fengycin, iturin, and surfactin families capable of inhibiting the growth of *A. hydrophila*, *Ph. damsela*, *V. harveyi*, *V. alginolyticus*, *E. tarda*, and *V. parahaemolyticus*.

The use of purified NACs in aquaculture is still a controversial issue, as the main apprehension is the efficiency and economic feasibility of administration these compounds to farmed animals. Indeed, some reports suggest the direct application of NACs-producing bacterial cells as the more reasonable and practical approach (Rather, et al., 2017). Nonetheless, since these NACs exhibit critical advantages such as low toxicity, high biodegradability, and eco-friendly nature (Nayak, 2021), further research on their identification, purification, and characterization is required to elucidate and explore their practical application as disease prevention agents in aquaculture (Soltani, et al., 2019).

1.4.2. Anti-biofilm activities of *Bacillus* spp.

The use of *Bacillus* spp. as anti-biofilm agents is an increasingly explored research area and holds considerable promise for diseases control. *Bacillus* spp. and their NACs have been extensively studied against planktonic cells, but only a few studies have characterized their potential targeting biofilm-associated cells, and even fewer studies have addressed fish bacterial diseases (Table 1.4).

Table 1.4. *Bacillus* strains with reported bioactivity against biofilm formation of important bacterial aquaculture pathogens. In bold are the anti-biofilm activity and *in vivo* protection due to the isolated bioactive compounds, all the others refer to the organism itself.

Bacillus strain	Source	Anti-biofilm activity	Bioactive compounds or genes identified	<i>in vivo</i> protection	Reference
<i>B. pumilus</i> S8-07, <i>B. indicus</i> S6-01	Soil samples	<i>V. vulnificus</i> , <i>V. alginolyticus</i> , <i>V. parahaemolyticus</i>	n/a	n/a	Nithya, Pandian (2010)
<i>B. pumilus</i> MTCC 5560	MTCC	<i>V. alginolyticus</i>, <i>V. parahaemolyticus</i>, <i>S. aureus</i>, <i>P. aeruginosa</i>, <i>E. coli</i>, <i>Chr. violaceum</i>, <i>P. mirabilis</i>, <i>S. boydii</i>, <i>S. flexneri</i>, <i>S. salvarius</i>	4-phenylbutanoic acid	n/a	Nithya, et al. (2011)
<i>B. amyloliquefaciens</i> DSM7	Surgical room	<i>A. hydrophila</i>	Surfactin, Fengycin, Iturin A, Macrolactin, Difficidin, Bacillaene	n/a	Nastro, et al. (2013)
<i>Bacillus</i> sp.	Prussian carp	<i>A. hydrophila</i>	n/a	<i>Bacillus</i> sp. increased zebrafish survival upon <i>A. hydrophila</i> challenge by 70%	Chu, et al. (2014)
<i>B. licheniformis</i> DAHB1	Indian white shrimp	<i>V. parahaemolyticus</i>	<i>aiiA</i> AHL-Lactonase	<i>aiiA</i> increased Indian white shrimp survival upon <i>V. parahaemolyticus</i> challenge by 55%	Vinoj, et al. (2014)
<i>B. sonorensis</i> MT93	Marine soil	<i>S. aureus</i>	Sonorensin	n/a	Chopra, et al. (2015)
<i>B. licheniformis</i> Dahb1	Asian catfish	<i>V. parahaemolyticus</i>	n/a	n/a	Gobi, et al. (2016)
<i>B. licheniformis</i> D1	Asian green mussel	<i>V. harveyi</i> , <i>P. gessardi</i> .	n/a	n/a	Hamza, et al. (2016)
<i>Bacillus</i> sp. 176	Marine sediment/water	<i>V. anguillarum</i> , <i>P. aeruginosa</i>	Pumilacidin-like lipopeptides	Increase of zebrafish survival by 70% upon challenge with <i>V. alginolyticus</i>	Xiu, et al. (2017)

Table 1.4. *Bacillus* strains with reported bioactivity against biofilm formation of important bacterial aquaculture pathogens. In bold are the anti-biofilm activity and *in vivo* protection due to the isolated bioactive compounds, all the others refer to the organism itself (Continued).

Bacillus strain	Source	Anti-biofilm activity	Bioactive compounds or genes identified	<i>in vivo</i> protection	Reference
<i>B. licheniformis</i> Dahb1	Indian white shrimp	<i>V. parahaemolyticus</i>	n/a	Increase of zebrafish survival upon <i>V. parahaemolyticus</i> challenge by 18%	Girija, et al. (2018)
<i>B. licheniformis</i> D1	Asian green mussel	<i>V. alginolyticus</i> ; <i>P. gessardi</i>	n/a	Increase of artemia survival upon <i>V. alginolyticus</i> challenge by 60%	Hamza, et al. (2018)
<i>B. cereus</i> 24Q, <i>B. thuringiensis</i> 7Q	rainbow trout	<i>Y. ruckeri</i>	n/a	Increase of rainbow trout survival by 80% upon challenge with <i>Y. ruckeri</i>	Torabi Delshad, et al. (2018a)
<i>Bacillus</i> sp. QSI-1	Prussian carp	<i>A. hydrophila</i>	aiiA_{QSI-1} AHL-Lactonase	Increase of crucian carp survival by 26% upon challenge with <i>A. hydrophila</i>	Zhang, et al. (2019a)
<i>Bacillus</i> spp.	Water and soil samples	<i>P. aeruginosa</i> , <i>A. caviae</i> , <i>V. parahaemolyticus</i> , <i>V. alginolyticus</i> , <i>Virgibacillus</i> sp.		n/a	Gutiérrez-Almada, et al. (2020)
<i>Bacillus</i> spp.	Soft Corals	<i>V. alginolyticus</i> , <i>A. hydrophila</i> , <i>P. aeruginosa</i>	<i>aiiA</i> AHL-Lactonase	n/a	El-Kurdi, et al. (2021)
<i>B. licheniformis</i> T-1	Sediment samples	<i>A. hydrophila</i>	<i>ytnP</i> AHL-lactonase	Increase of goldfish survival by 50% upon infection with <i>A. hydrophila</i>	Peng, et al. (2021)

Nithya, Pandian (2010) demonstrated the extracellular activity of *B. pumilus* and *B. indicus* against the attachment, biofilm formation, and dispersal of *V. vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus*. Similarly, the extracellular compounds of *B. licheniformis* D1 were shown to disrupt biofilm formation and pre-formed biofilms of *V. harveyi* and *P. gessardii* (Hamza, et al., 2016). Importantly, some studies demonstrated the *in vivo* potential of *Bacillus* spp. with anti-biofilm activity in protecting fish and crustaceans. *B. licheniformis* Bahb1 isolated from white shrimp not only disrupted biofilm formation of *V. parahaemolyticus*, but also protected zebrafish from infection, increasing its survival by 18% (Girija, et al., 2018). Other *B. licheniformis* produced extracellular compounds with anti-biofilm and anti-adhesion properties against *V. alginolyticus* and *P. gessardii* and were capable of protecting artemia from *V. parahaemolyticus* infection (Hamza, et al., 2018). Although the capacity of *Bacillus* spp. to disrupt fish pathogens' biofilms has been demonstrated in the last years, only three studies characterized the specific compounds responsible for the activity. Nithya, et al. (2011) described a carboxylic acid derivative (4-phenylbutanoic acid) produced by *B. pumilus* as responsible for the disruption of biofilms and EPS production of multiple pathogens including *V. alginolyticus*, *V. parahaemolyticus*, and *S. aureus*. On the other hand, sonorensin, a bacteriocin produced by *B. sonorensis*, was shown to inhibit *S. aureus* biofilm (Chopra, et al., 2015). Similarly, *Bacillus* sp. 176 produced pumilacidin-like lipopeptides that suppressed motility, adherence, biofilm formation, and virulence of *V. parahaemolyticus*, reducing zebrafish mortality by 70% (Xiu, et al., 2017).

1.4.3. Quorum-Quenching activities of *Bacillus* spp.

Over the years lactonases were reported in numerous *Bacillus* spp., and different studies highlighted their potential in diseases prevention both in animals and plants (Chen, et al., 2010; Dong, et al., 2002; LaSarre, Federle, 2013; Pan, et al., 2008; Vinoj, et al., 2014; Yin, et al., 2012). Besides the commonly reported *aiiA* lactonase gene within *Bacillus* spp., another QQ gene encoding a lactonase-homologous protein, the *ytnP* from *B. subtilis* NCIB3610, was described by Schneider, et al. (2012). YtnP was responsible for the interruption of the signalling pathway required for streptomycin production and aerial mycelium development in *Streptomyces griseus*. Moreover, small amounts of YtnP could inhibit biofilm formation in a QS-dependent manner, and two recent studies described the potential of YtnP to attenuate *Aeromonas* species infections (Chen, et al., 2020; Peng, et al., 2021). Adding to AHL-lactonases (produced by the majority of *Bacillus* spp.) Chowdhary, et al. (2007) described the synthesis of an oxidoreductase, the cytochrome P450 monooxygenase CYP102A1 from *B. megaterium*, that oxidizes long-chain AHLs (C12-HSL). Also, Nithya, et al. (2010) reported a putative acylase from *B.*

pumilus that reduced biofilm formation and attenuated several QS-regulated virulence factors, including proteases and haemolytic activity.

The biocontrol activity of QQ *Bacillus* spp. against QS-mediated infections in plants is well-established (Dong, et al., 2000; Dong, et al., 2002; Lee, et al., 2002). More recently, QQ *Bacillus* spp. and their enzymes have been characterized for their protective effects against fish bacterial diseases. Intraperitoneal injections or feed supplementation of purified lactonases or *Bacillus* spp. cells attenuated the infection of *A. hydrophila* (Cao, et al., 2012; Chen, et al., 2010; Chu, et al., 2011; Chu, et al., 2014; Peng, et al., 2021; Zhang, et al., 2019a; Zhou, et al., 2018), *V. campbellii* (Pande, et al., 2015), *V. parahaemolyticus* (Vinoj, et al., 2014), and *Y. ruckeri* (Torabi Delshad, et al., 2018a) by regulating QS-dependent virulence factors (Table 1.5). The purified lactonase AiiA_{AI96} degraded AHLs produced by *A. veronii* QS-system and reduced the pathogens proteases and motility activities (Gui, et al., 2017). Moreover, oral administration of the same thermostable lactonase produced by the *Bacillus* sp. AI96, increased zebrafish survival by 40% when challenged with *A. hydrophila* (Cao, et al., 2012). In another study, an intraperitoneal co-injection of lactonase AiiA_{B546} (produced by *Bacillus* sp. B546) with *A. hydrophila* protected common carp from infection, increasing its survival rate by 25% (Chen, et al., 2010). *Bacillus* sp. strain QSI-1 isolated from Prussian carp gastrointestinal tract, encoding the *aiiA* lactonase gene, reduced the accumulation of *A. hydrophila* AHLs-signals and significantly reduced virulence factors (extracellular proteases and haemolysin) and biofilm formation (Chu, et al., 2011). Oral administration of *Bacillus* sp. QSI-1 reduced pathogenicity (skin lesions and haemorrhages) of *A. hydrophila*, increasing zebrafish and carp survival by 70% and 60%, respectively (Chu, et al., 2011; Chu, et al., 2014). Similarly, AiiA_{QSI-1} degraded C6-HSL produced by *A. hydrophila*, resulting in a decrease in bacterial motility, virulence factors (extracellular proteases and haemolysin), and biofilm formation (Zhang, et al., 2019a). Further, oral administration of the purified enzyme through feed supplementation increased crucian carp survival by 26% when challenged with *A. hydrophila* (Zhang, et al., 2019a). The QQ *B. licheniformis* T-1, isolated from freshwater culture pond sediment, increased zebrafish survival by 50% when co-injected with *A. hydrophila* (Chen, et al., 2020). QQ gene *ytnP* was identified through genome sequencing as responsible for the activities observed in *B. licheniformis* T-1. Further, purified lactonase YtnP reduced *A. hydrophila* biofilm formation and virulence factors. Intraperitoneal co-injection of purified YtnP with *A. hydrophila* attenuated *A. hydrophila* pathogenicity and increased goldfish survival by 50% (Peng, et al., 2021).

Table 1.5. QQ *Bacillus* strains and corresponding QQ enzymes known to degrade the QS signals of important aquaculture pathogens. In bold are the QQ activity and *in vivo* protection due to the isolated QQ enzymes, all the others refer to the organism itself.

<i>Bacillus</i> strain	Source	AHLs degraded	QQ genes or enzymes	<i>in vivo</i> protection	Reference
<i>Bacillus</i> spp.	Culture and lab collections	3-Oxo-C6-HSL	AiiA	n/a	Dong, et al. (2002)
<i>Bacillus</i> sp. B546	Mud	3-oxo-C8-HSL	AiiAB₅₄₆ AHL-lactonase	Increase of common carp survival by 25% upon <i>A. hydrophila</i> challenge	Chen, et al. (2010)
<i>Bacillus</i> sp. QSI-1	Prussian carp	C6-HSL; AHL signals from <i>A. hydrophila</i>	<i>aiaA</i> AHL-lactonase gene	Increase of carp survival by 60% upon <i>A. hydrophila</i> challenge	Chu, et al. (2011)
<i>B. circulans</i> X3	Soil sample	C4-HSL, C6-HSL, C10-HSL, C12-HSL	n/a	n/a	Romero, et al. (2011)
<i>Bacillus</i> sp. AI96	Soil samples	C4-HSL, C6-HSL, C7-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL, 3-Oxo-C6-HSL, 3-Oxo-C8-HSL, 3-Oxo-C10-HSL, 3-Oxo-C12-HSL, 3-Oxo-3-Hydroxy-C8-HSL, 3-Hydroxy-C14-HSL	AiiA_{AI96} AHL-lactonase	Increase of zebrafish survival by 40% upon <i>A. hydrophila</i> challenge	Cao, et al. (2012)
<i>Bacillus</i> sp. QSI-1	Prussian carp	C6-HSL; AHL signals from <i>A. hydrophila</i>	n/a	Increase of zebrafish survival by 70% upon <i>A. hydrophila</i> challenge	Chu, et al. (2014)
<i>B. licheniformis</i> DAHB1	Indian white shrimp	C6-HSL	AiiA AHL-lactonase	Increase of Indian white shrimp survival by 55% upon <i>V. parahaemolyticus</i> challenge	Vinoj, et al. (2014)
<i>Bacillus</i> sp. TSAWB	Soil samples	AHLs produced by <i>Chr. violaceum</i> ATCC 12472	AiiA _{TSAWB}	n/a	Easwaran, et al. (2015)

Table 1.5. QQ *Bacillus* strains and corresponding QQ enzymes known to degrade the QS signals of important aquaculture pathogens. In bold are the QQ activity and *in vivo* protection due to the isolated QQ enzymes, all the others refer to the organism itself (Continued).

<i>Bacillus</i> strain	Source	AHLs degraded	QQ genes or enzymes	<i>in vivo</i> protection	Reference
<i>Bacillus</i> sp. NFMI-C	Microalgae	C6-HSL; AHLs produced by <i>V. campbellii</i>	n/a	Increase of giant prawn larvae survival by 25% upon <i>V. campbellii</i> challenge	Pande, et al. (2015)
<i>B. subtilis</i> KATMIRA	Unknown	AHLs produced by <i>Chr. violaceum</i> ATCC 12472	Subtilisin	n/a	Algburi, et al. (2016)
<i>Bacillus</i> sp. AI96	Soil samples	AHLs produced by <i>A. veronii</i>	AiiA_{AI96} AHL-lactonase	n/a	Gui, et al. (2017)
<i>B. cereus</i> N26.2	Water samples	C6-HSL; 3-Oxo-C10-HSL	AiiA_{N26.2} AHL-lactonase	No effect on seabass larvae and artemia upon infection with <i>V. anguillarum</i>	Das, et al. (2018)
<i>B. subtilis</i> R-18	Soil samples	AHLs produced by <i>Chr. violaceum</i> ATCC 12472	n/a	n/a	Devi 2018
<i>B. cereus</i> 24QT, <i>B. thuringiensis</i> 7QT	Rainbow trout	C6-HSL; AHLs produced by <i>Y. ruckeri</i>	<i>aiaA</i> genes	Increase of rainbow trout survival by 80% upon challenge with <i>Y. ruckeri</i>	Torabi Delshad, et al. (2018a), Torabi Delshad, et al. (2018b)
<i>Bacillus</i> sp. YB1701	Sediment, fish pounds	AHLs produced by <i>Chr. violaceum</i> ATCC 12472	n/a	Increase of goldfish survival upon <i>A. hydrophila</i> challenge by 50%	Zhou, et al. (2018)
<i>B. thuringiensis</i> KMCL07	Plant samples	AHLs produced by <i>Chr. violaceum</i> ATCC 12472, C4-HSL, C6-HSL, 3-OXO-C12-HSL	AiiA AHL-lactonase	n/a	Anandan, Vittal (2019)

Table 1.5. QQ *Bacillus* strains and corresponding QQ enzymes known to degrade the QS signals of important aquaculture pathogens. In bold are the QQ activity and *in vivo* protection due to the isolated QQ enzymes, all the others refer to the organism itself (Continued).

<i>Bacillus</i> strain	Source	AHLs degraded	QQ genes or enzymes	<i>in vivo</i> protection	Reference
<i>B. thuringiensis</i> QQ1, <i>B. cereus</i> QQ2, <i>Bacillus</i> sp. QQ4	Barramundi	3-oxo-C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C14-HSL; AHLs produced by <i>V. harveyi</i> and <i>V. alginolyticus</i>	<i>aiiA</i> and <i>ytnP</i> genes	n/a	Ghanei-Motlagh, et al. (2019)
<i>Bacillus</i> sp. QSI-1	Prussian carp	C6-HSL;	AiiA_{QSI-1} AHL-lactonase	Increased of crucian carp survival by 26% upon challenge with <i>A. hydrophila</i>	Zhang, et al. (2019a)
<i>B. licheniformis</i> T-1	Sediment samples	AHLs produced by <i>Chr. violaceum</i> ATCC 12472, C6-HSL	<i>ytnP</i> gene	Increase of zebrafish survival by 50% upon <i>A. hydrophila</i> challenge	Chen, et al. (2020)
<i>Bacillus</i> spp.	Water and soil samples	C6-HSL	n/a	n/a	Gutiérrez-Almada, et al. (2020)
<i>Bacillus</i> spp.	Soft Corals	AHLs produced by <i>Chr. violaceum</i> ATCC 12472	AiiA AHL-lactonase	n/a	El-Kurdi, et al. (2021)
<i>B. licheniformis</i> T-1	Sediment samples	C6-HSL	YtnP AHL-lactonase	Increase of goldfish survival by 50% upon infection with <i>A. hydrophila</i>	Peng, et al. (2021)

While most studies focused on *A. hydrophila*, QQ *Bacillus* spp. can also induce protection against *Vibrio* spp. and *Y. ruckeri* infections. Two *Bacillus* strains isolated from the rainbow trout gastrointestinal tract were shown to degrade AHLs produced by *Y. ruckeri* QS-system and reduced pathogens motility and biofilm formation. Oral administration of *Bacillus* spp. through feed supplementation, increased rainbow trout survival by 80% upon challenge with *Y. ruckeri* (Torabi Delshad, et al., 2018a). Three *Bacillus* strains isolated from the barramundi gastrointestinal tract showed an impressive *in vitro* AHL degradation activity against numerous synthetic AHLs and the naturally produced AHLs from *V. harveyi* and *V. alginolyticus* (Ghanei-Motlagh, et al., 2019). *Bacillus* sp. NFMI-C isolated from microalgae open cultures interfered with AHLs produced by *V. campbellii*. Administration of *Bacillus* sp. NFMI-C by immersion improved giant prawn larvae survival by 25% upon infection with *V. campbellii* (Pande, et al., 2015). *B. licheniformis* DAHB1 obtained from the Indian white shrimp gastrointestinal tract and encoding an AiiA lactonase enzyme reduced *V. parahaemolyticus* biofilm formation. Co-injection of purified lactonase AiiA with *V. parahaemolyticus* increased shrimp survival by 25% (Vinoj, et al., 2014). Differently, in another study, the recombinant lactonase AiA_{N26.2} produced by *B. cereus* N26.2 did not affect *V. anguillarum* virulence factors (lipase, phospholipase, and caseinase) nor protected artemia and seabass larvae from *V. anguillarum* infection (Das, et al., 2018).

Since *Bacillus* spp. produce QQ enzymes that interfere with pathogens' signalling processes and virulence towards fish and shrimps, their application on aquaculture systems as therapeutic/prophylactic agents is of most importance.

1.4.4. Immunostimulatory properties of *Bacillus* spp.

The health-protective effects of *Bacillus* spp. in several aquatic animals can also be achieved through the enhancement of the host's innate and adaptive immunity (James, et al., 2021). *Bacillus* spp. produce bioactive substances that trigger both local and systemic immunity of the host (Nayak, 2021). Nonetheless, the exact mechanisms by which *Bacillus* spp. exert their immunostimulant effects and offer protection against certain diseases remains to be elucidated. Importantly, not only vegetative cells but also spores and extracellular metabolites can trigger the host's immune response (Kuebutornye, et al., 2020; Nayak, 2021).

Several reports have demonstrated that the administration of *Bacillus* can increase (i) leukocytes, monocytes, or granulocytes population; (ii) phagocytic activity; (iii) respiratory burst activity; (iv) lysozyme activity; (v) plasma bactericidal activity; (vi) complement activity; (vii) immunoglobulins; (viii) cytokine secretion; and other immune parameters (Table 1.6) (Galagarza, et al., 2018; Kumar, et al., 2008; Meidong,

et al., 2018; Nguafack, et al., 2020; Sookchaiyaporn, et al., 2020; Truong Thy, et al., 2017; Van Doan, et al., 2018; Wu, et al., 2021).

Nayak, et al. (2007) and Brunt, et al. (2007) were the first to describe the immunostimulatory properties of *Bacillus* spp. Newaj-Fyzul, et al. (2007) observed that rainbow trout fed with *B. subtilis* AB1 cells had a significant increase of important immune parameters, including respiratory burst, phagocytosis, and lymphocyte populations. Moreover, rainbow trout fed with *B. subtilis* AB1 cells or cell-free extract had a higher survival rate upon *Aeromonas* sp. infection (Newaj-Fyzul, et al., 2007). In a similar study, Brunt, et al. (2007) used another *B. subtilis* strain isolated from the rainbow trout gastrointestinal tract and observed an impressive fish immune stimulation and responses. Fish were fed for 14 days with *B. subtilis* JB-1 (2×10^8 cfu kg⁻¹) before challenge with several fish pathogens including *A. salmonicida*, *S. iniae*, *V. anguillarum*, *V. ordalli*, and *Y. ruckeri*, and survival remarkably increased, specifically through the production of antimicrobial substances and the stimulation of important innate immune parameters (leukocytes population, anti-protease activity, phagocytosis and lysozyme). The effect of two *Bacillus* strains (*B. coagulans* MTCC 9872 and *B. licheniformis* MTCC 6824) on the innate immune system of common carp and disease protection against *A. hydrophila* was evaluated by Gupta, et al. (2014). Fish fed for 80 days with 10^9 cfu kg⁻¹ of each *Bacillus* strain increased lysozyme, myeloperoxidase and respiratory burst activities when compared to the control group. This increased innate immune parameters resulted in a high protection against *A. hydrophila* in the case of *B. coagulans* (Gupta, et al., 2014). In a similar study, Giri, et al. (2014) reported that oral administration of *B. subtilis* VSG1 as a single organism or in combination with *P. aeruginosa* and *L. plantarum*, increased non-specific and specific immune parameters (serum lysozyme, alternative complement, phagocytic, and respiratory burst activities, and IgM) while also increasing disease resistance against *A. hydrophila*. The administration of *B. aerophilus* KADR3 enhanced some immune responses (lysozyme, phagocytosis, respiratory burst, and serum IgM levels) and disease resistance of rohu against *A. hydrophila*. In two separate studies, *B. velezensis* JW and *B. amyloliquefaciens* R8 administrated to goldfish and zebrafish, respectively, enhanced the secretion of several pro- and anti-inflammatory cytokines and also induced strong protection against *A. hydrophila* (Lin, et al., 2019; Yi, et al., 2018). A recent study demonstrated the immunostimulatory properties of *B. velezensis* B8 when fed to grass carp, by enhancing serum alkaline phosphatase and superoxide dismutase activities, and IgM and TNF- α expression levels, and by increasing fish survival upon infection with *A. veronii* (Wu, et al., 2021).

Table 1.6. Effect of *Bacillus* spp. on the immune responses and disease resistance in fish. In bold are the dose of spores' supplementation, all the others refer to supplementation with *Bacillus* cells.

<i>Bacillus</i> strain	Fish host	Dose of Cells/Spores (CFU/g diet)	Duration	Route	Immune response*	Pathogen resistance	Reference
<i>B. subtilis</i> CECT 35	Gilthead seabream	n/a	30 min of incubation	n/a	Respiratory burst↑; phagocytosis↑; cytotoxic activity↑	n/a	Salinas, et al. (2006)
<i>B. subtilis</i> JB-1	Rainbow trout	2x10 ⁸	14 days	oral	Leukocytes population↑; anti-protease activity↑; phagocytosis↑; lysozyme↑	<i>A. salmonicida</i> ; <i>L. garvieae</i> ; <i>S. iniae</i> ; <i>V. anguillarum</i> ; <i>V. ordalii</i> ; <i>Y. ruckery</i>	Brunt, et al. (2007)
<i>B. subtilis</i>	Carp	10 ⁸	60 days	oral	Lymphocytes population↑; total serum protein↑; antibody response against <i>E. tarda</i> bacterin↑	n/a	Nayak, et al. (2007)
<i>B. subtilis</i> AB1	Rainbow trout	10 ⁷	14 days	oral	Respiratory burst↑; serum and gut lysozyme↑; peroxidase↑; phagocytosis↑; total antiprotease↑; lymphocyte population↑	<i>Aeromonas</i> sp.	Newaj-Fyzul, et al. (2007)
<i>B. subtilis</i>	Carp	0.5-1.5x10 ⁷	14 days	oral	Granulocytes population↑; monocytes population↑; lymphocytes population↓; respiratory burst↑; serum bactericidal↑	n/a	Kumar, et al. (2008)
<i>B. clausii</i> DE5, <i>B. pumilus</i> SE5	Grouper	10 ⁸	30-60 days	oral	Lysozyme↑; phagocytosis of <i>S. aureus</i> ↑; SOD=; complement activity=; IgM↑	n/a	Sun, et al. (2010)
<i>B. subtilis</i> B10; <i>B. coagulans</i> B16	Tilapia	10 ⁷	40 days	bath	Respiratory burst↑; SOD↑; CAT↑; myeloperoxidase↑; total serum protein↑	n/a	Zhou, et al. (2010)

* ↑ indicates an increase on the parameter; ↓ indicates a decrease on the parameter; = indicates no effect on the parameter

Table 1.6. Effect of *Bacillus* spp. on the immune responses and disease resistance in fish. In bold are the dose of spores' supplementation, all the others refer to supplementation with *Bacillus* cells (Continued).

<i>Bacillus</i> strain	Fish host	Dose of Cells/Spores (CFU/g diet)	Duration	Route	Immune response	Pathogen resistance	Reference
<i>B. subtilis</i> CECT 35	Gilthead seabream	10 ⁷ , 10 ⁸	2 weeks	oral	IgM level↑; complement activity↑; respiratory burst↑; phagocytosis↑	<i>Ph. damsela</i> <i>piscicida</i>	Cerezuela, et al. (2012b)
<i>B. subtilis</i> E20	Grouper	10 ⁴ , 10 ⁶ , and 10 ⁸	28 days	oral	Phagocytosis↑; respiratory burst↑; SOD↑; lysozyme↑; complement activity↑	<i>Streptococcus</i> <i>sp.</i>	Liu, et al. (2012)
<i>B. subtilis</i>, <i>B. pumilus</i>, <i>B. licheniformis</i>	Olive flounder	10¹⁰	8 weeks	oral	Respiratory burst↑; lysozyme=; SOD=; myeloperoxidase=	<i>S. iniae</i>	Cha, et al. (2013)
<i>B. amyloliquefaciens</i> FPTB16	Rohu	10 ⁷ , 10 ⁸ , 10 ⁹	4-8 weeks	oral	Respiratory burst↑; lysozyme↑; myeloperoxidase↑	<i>E. tarda</i>	Das, et al. (2013)
<i>B. subtilis</i> VSG1	Rohu	10 ⁸	60 days	oral	Respiratory burst↑; lysozyme↑; complement activity↑; phagocytosis↑; IgM↑	<i>A. hydrophila</i>	Giri, et al. (2014)
<i>B. coagulans</i> MTCC 9872, <i>B. licheniformis</i> MTCC 6824	Common Carp	10 ⁹	80 days	oral	Respiratory burst↑; lysozyme↑; myeloperoxidase↑	<i>A. hydrophila</i>	Gupta, et al. (2014)
<i>B. subtilis</i>	Tilapia	5x10 ⁶	84 days	oral	Lysozyme↑; phagocytosis↑	n/a	Telli, et al. (2014)
<i>B. licheniformis</i> XY-52 metabolites	Common Carp	n/a	3 weeks	oral	Lysozyme↑; phagocytosis↑; serum bactericidal activity↑; IL-1β, IL-10; TNF-α↑; hsp70↓	<i>A. hydrophila</i>	Chen, et al. (2015)
<i>B. subtilis</i> FPTB13	Catla	10 ⁷ , 10 ⁸ , 10 ⁹	2 weeks / in vitro	oral	Respiratory burst↑; myeloperoxidase↑; lysozyme↑	<i>E. tarda</i>	Sangma, Kamilya (2015)

* ↑ indicates an increase on the parameter; ↓ indicates a decrease on the parameter; = indicates no effect on the parameter

Table 1.6. Effect of *Bacillus* spp. on the immune responses and disease resistance in fish. In bold are the dose of spores' supplementation, all the others refer to supplementation with *Bacillus* cells (Continued).

<i>Bacillus</i> strain	Fish host	Dose of Cells/Spores (CFU/g diet)	Duration	Route	Immune response	Pathogen resistance	Reference
<i>B.amyloliquefaciens</i>	Tilapia	10 ⁴ , 10 ⁶	30 days	oral	Lysozyme↑; phagocytosis of <i>Candida albicans</i>↑; blood serum bactericidal activity↑; TNF-α, IL-1β↑	<i>Y. ruckeri</i> , <i>C. perfringers</i>	Selim, Reda (2015)
<i>B. amyloliquefaciens</i> M001	Turbot	10 ⁸	42 days	oral	Total protein↑; SOD↑	<i>V. anguillarum</i>	Chen, et al. (2016)
<i>B. licheniformis</i> DAHB1	Asian catfish	10 ⁵	24 days	bath	Respiratory burst↑; Lysozyme↑; myeloperoxidase↑; complement activity↑; total and reduced glutathione↑	n/a	Gobi, et al. (2016)
<i>B. subtilis</i> HAINUP40	Tilapia	10 ⁸	8 weeks	oral	SOD↑; respiratory burst↑; lysozyme↑	<i>S. agalactiae</i>	Liu, et al. (2017a)
<i>B. aerophilus</i> KADR3	Rohu	10 ⁷ , 10 ⁸ , 10 ⁹	6 weeks	oral	Lysozyme↑; phagocytosis↑; respiratory burst↑; SOD↑; serum IgM levels↑; complement↑	<i>A. hydrophila</i>	Ramesh, et al. (2017)
<i>B. pumilus</i> 47B, <i>B.amyloliquefaciens</i> 54A	Striped catfish	10 ⁸ , 3x10 ⁸ , 5x10 ⁸	90 days	oral	Respiratory burst↑; phagocytosis↑; lysozyme↑	<i>E. ictaluri</i>	Truong Thy, et al. (2017)
<i>B. velezensis</i> H3.1	Tilapia	10 ⁷	30 days	oral	Respiratory burst↑; lysozyme↑; peroxidase↑; complement↑	<i>S. agalactiae</i>	Van Doan, et al. (2018)
<i>B. subtilis</i> NZ86 and O14VRQ	Tilapia	10 ⁷	51 days	oral	Respiratory burst↑; phagocytosis↑; lysozyme↑; complement↑; TNF-α, IL-1β↑	n/a	Galagarza, et al. (2018)
<i>B. aerius</i> B81	Pla-mong	10 ⁷	8 weeks	oral	Lysozyme↑; phagocytosis of <i>A. hydrophila</i> ↑; complement↑	<i>A. hydrophila</i>	Meidong, et al. (2018)

* ↑ indicates an increase on the parameter; ↓ indicates a decrease on the parameter; = indicates no effect on the parameter

Table 1.6. Effect of *Bacillus* spp. on the immune responses and disease resistance in fish. In bold are the dose of spores' supplementation, all the others refer to supplementation with *Bacillus* cells (Continued).

<i>Bacillus</i> strain	Fish host	Dose of Cells/Spores (CFU/g diet)	Duration	Route	Immune response	Pathogen resistance	Reference
<i>B. velezensis</i> JW	Goldfish	10 ⁷ , 10 ⁹	7-28 days	oral	Glutathione peroxidase↑; phosphatase↑; Lysozyme=; TNF-α, IL-1β, IL-10, IL-4↑; IL-2↓	<i>A. hydrophila</i>	Yi, et al. (2018)
<i>B. subtilis</i> BStH-5 and BStH-19	Dabry's sturgeon	2x10 ⁸	8 weeks	oral	SOD↑; malondialdehyde↓; lysozyme↑; IgM↑	<i>A. hydrophila</i>	Di, et al. (2019)
<i>B. velezensis</i> K2	Grouper	10 ⁷	28 days	oral	Phosphatase↑; IL-8 ↓; lysozyme↑; IgM↓; MyD88↑; TLR-3↓	<i>V. harveyi</i>	Li, et al. (2019)
<i>B. amyloliquefaciens</i> R8	Zebrafish	2x10 ⁶	30 days	oral	IL-1B, IL-6, IL-21, TNF-α↑	<i>A. hydrophila</i> ; <i>S. agalactiae</i>	Lin, et al. (2019)
<i>B. methylotrophicus</i> KU556164, <i>B. amyloliquefaciens</i> KU556165, <i>B. licheniformis</i> KU556167	Indian major carp	10 ⁷	60 days	oral	Lysozyme↑; phagocytosis↑; serum peroxidase↑; complement activity↑; respiratory burst↑; anti-proteases↑; superoxide anion production↑	<i>A. hydrophila</i>	Mukherjee, et al. (2019a)
<i>Bacillus</i> sp. KUAQ1 and KUAQ2	Tilapia	10⁸, 3x10⁸, 5x10⁸	4 weeks	oral	Lysozyme=; complement activity=; superoxide dismutase=; phagocytosis↑; superoxide anion↑; respiratory burst↑	No	Sookchaiyaporn, et al. (2020)
<i>B. velezensis</i> LF01	Tilapia	10 ⁹	9 weeks	oral	Lysozyme↑; SOD↑	<i>S. agalactiae</i>	Zhang, et al. (2019b)
<i>Bacillus</i> sp. SJ-10	Olive flounder	10 ⁸	8 weeks	oral	Lysozyme↑; respiratory burst↑; SOD↑; myeloperoxidase↑; IL-1β, IL-6↑	<i>S. iniae</i>	Nguafack, et al. (2020)
<i>B. velezensis</i> B8	Grass carp	10 ⁷ , 10 ⁹	4 weeks	oral	Alkaline phosphatase↑; SOD↑; IgM↑; TNF-α↑	<i>A. veronii</i>	Wu, et al. (2021)

* ↑ indicates an increase on the parameter; ↓ indicates a decrease on the parameter; = indicates no effect on the parameter

Turbot fed with *B. amyloliquefaciens* M001, presented increased serum superoxide dismutase, and higher survival after being challenged with *V. anguillarum* (Chen, et al., 2016). Two other studies showed that two *Bacillus* sp. (*B. amyloliquefaciens* FPTB16, and *B. subtilis* FPTB13) enhanced respiratory burst, lysozyme and myeloperoxidase activities, and consequent fish protection against *E. tarda* infection (Das, et al., 2013; Sangma, Kamilya, 2015). Cerezuela, et al. (2012a) showed that oral administration of *B. subtilis* CECT 35 as a single organism or in combination with microalgae and inulin to gilthead seabream, increased non-specific and specific immune parameters (complement, phagocytic and respiratory burst activities and IgM) and disease resistance against *Ph. damsela* subsp. *piscicida*.

Although several studies have demonstrated the immunostimulatory properties of *Bacillus* spp. cells and spores, only one report has evaluated the immunomodulation potential of *Bacillus* spp. secondary metabolites (Chen, et al., 2015). Chen, et al. (2015) reported that common carp fed for 3 weeks with two purified metabolites produced by *B. licheniformis* XY-52 had a significant increase of important immune parameters, including lysozyme, phagocytic and bactericidal activities, and an up-regulation of important immune-related genes. Moreover, when compared to the control, fish had a higher survival rate against *A. hydrophila*.

1.5. Objectives of this thesis

The use of environmentally friendly solutions to improve fish health and growth is in line with the Sustainable Development Goals established by the United Nations to be achieved by 2030. By enhancing the fish immune system and resistance against infectious diseases, *Bacillus* spp. and their NACs are important for the development of sustainable and environmentally friendly aquaculture (in frame with Goal 2 - End hunger, achieve food security and improved nutrition and promote sustainable agriculture and Goal 12 - Ensure sustainable consumption and production patterns).

Bacillus spp. are recognized producers of NACs capable of inhibiting bacterial pathogens growth, biofilm formation, and communication signals, while also enhancing the host immune responses. Overall, in aquaculture, *Bacillus* spp. health-promoting effects were mainly studied against infections caused by *Aeromonas* and *Vibrio* species. Thus, to fill this gap, the overall aim of this thesis was to isolate, identify and characterize the health-promoting mechanism of sporeforming *Bacillus* spp. from the gastrointestinal tract of important Mediterranean fish species (*Sparus aurata*, *Dicentrarchus labrax*, and *Diplodus sargus*). *Bacillus* spp. cells and their extracellular NACs were tested *in vitro* for their anti-growth and anti-biofilm activities against fourteen different fish pathogens, including *Aeromonas* spp., *Vibrio* spp., *Photobacterium damsela*, *Edwardsiella tarda*, and *Shigella sonnei* (Chapter II). By recognizing the potential of *Bacillus* spp. in suppressing pathogens communication, the extracellular NACs of the isolated *Bacillus* spp. were characterized for their potential of enzymatic disruption of AHLs produced by important fish pathogens. Moreover, the best three QQ isolates were tested for their protective effects in zebrafish larvae challenged with *E. tarda*, an important fish pathogen (Chapter III). Finally, the immune-modulatory properties of the extracellular NACs produced by these three *Bacillus* spp. strains were evaluated *in vitro* using gilthead seabream leukocytes upon infection with *V. anguillarum* and *E. tarda* (Chapter IV).

1.6. References

- Abisado, R.G., Benomar, S., Klaus, J.R., Dandekar, A.A., Chandler, J.R., 2018. Bacterial Quorum Sensing and Microbial Community Interactions. *mBio*. 9, e02331-02317. doi: <https://doi.org/10.1128/mBio.02331-17>.
- Abo El-Yazeed, H., Ibrahim, M.D., 2009. Studies on *Edwardsiella tarda* infection in catfish and *Tilapia nilotica*. *Journal of Veterinary Medical Research*. 19, 44-50. doi: <https://doi.org/10.21608/jvmr.2009.77808>.
- Aderem, A., 2003. Phagocytosis and the Inflammatory Response. *The Journal of Infectious Diseases*. 187, S340-345. doi: <https://doi.org/10.1086/374747>.
- Alcaide, E., Herraiz, S., Esteve, C., 2006. Occurrence of *Edwardsiella tarda* in wild European eels *Anguilla anguilla* from Mediterranean Spain. *Dis Aquat Organ*. 73, 77-81. doi: <https://doi.org/10.3354/dao073077>.
- Algburi, A., Volski, A., Chikindas, M.L., 2015. Natural antimicrobials subtilisin and lauramide arginine ethyl ester synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli. *Pathogens and Disease*. 73. doi: <https://doi.org/10.1093/femspd/ftv018>.
- Algburi, A., Zehm, S., Netrebov, V., Bren, A.B., Chistyakov, V., Chikindas, M.L., 2016. Subtilisin Prevents Biofilm Formation by Inhibiting Bacterial Quorum Sensing. *Probiotics Antimicrob Proteins*. doi: <https://doi.org/10.1007/s12602-016-9242-x>.
- Ali, S.M., Siddiqui, R., Khan, N.A., 2018. Antimicrobial discovery from natural and unusual sources. *Journal of Pharmacy and Pharmacology*. 70, 1287-1300. doi: <https://doi.org/https://doi.org/10.1111/jphp.12976>.
- An, J., Zhu, W., Liu, Y., Zhang, X., Sun, L., Hong, P., Wang, Y., Xu, C., Xu, D., Liu, H., 2015. Purification and characterization of a novel bacteriocin CAMT2 produced by *Bacillus amyloliquefaciens* isolated from marine fish *Epinephelus areolatus*. *Food Control*. 51, 278-282. doi: <https://doi.org/10.1016/j.foodcont.2014.11.038>.
- Anandan, K., Vittal, R.R., 2019. Quorum quenching activity of AiiA lactonase KMMI17 from endophytic *Bacillus thuringiensis* KMCL07 on AHL- mediated pathogenic phenotype in *Pseudomonas aeruginosa*. *Microb Pathog*. 132, 230-242. doi: <https://doi.org/10.1016/j.micpath.2019.05.015>.
- Andreoni, F., Magnani, M., 2014. Photobacteriosis: prevention and diagnosis. *J Immunol Res*. 2014, 793817. doi: <https://doi.org/10.1155/2014/793817>.

- Angioi, A., Zanetti, S., Sanna, A., Delogu, G., Fadda, G., 1995. Adhesiveness of *Bacillus subtilis* Strains to Epithelial Cells Cultured in vitro. *Microb Ecol Health Dis.* 8, 71-77. doi: <https://doi.org/10.3109/08910609509141385>.
- Appiah, E.K., Hashem, S., Fatsi, P.S.K., Tettey, P.A., Saito, H., Omura, M., Kawai, K., 2021. Antibacterial activity of *Mallotus japonicus* (L.F.) Müller Argoviensis on growth of *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda* and *Vibrio anguillarum*. *J Appl Microbiol.* doi: <https://doi.org/10.1111/jam.15198>.
- Arguelles Arias, A., Ongena, M., Devreese, B., Terrak, M., Joris, B., Fickers, P., 2013. Characterization of amylolysin, a novel lantibiotic from *Bacillus amyloliquefaciens* GA1. *PLoS ONE.* 8, e83037-e83037. doi: <https://doi.org/10.1371/journal.pone.0083037>.
- Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S., Ringø, E., 2012. Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. *Aquaculture.* 326-329, 1-8. doi: <https://doi.org/10.1016/j.aquaculture.2011.10.016>.
- Avendaño-Herrera, R., Toranzo, A.E., Magariños, B., 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review, *Diseases of aquatic organisms*, pp. 255-266. doi: <https://doi.org/10.3354/dao071255>.
- Aydin, B., Akayli, T., Ürkü, Ç., Kayalar, O., 2019. Diagnosis of *Vibrio anguillarum* in Cultured Rainbow Trout (*Oncorhynchus mykiss*) by Different Methods. *European Journal of Biology.* 77, 26-31. doi: <https://doi.org/10.26650/EuroJBiol.2018.404499>.
- Balcazar, J.L., Rojas-Luna, T., 2007. Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against vibrio species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). *Curr Microbiol.* 55, 409-412. doi: <https://doi.org/10.1007/s00284-007-9000-0>.
- Balcázar, J.L., Blas, I.d., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Múzquiz, J.L., 2006. The role of probiotics in aquaculture. *Vet Microbiol.* 114, 173-186. doi: <https://doi.org/http://dx.doi.org/10.1016/j.vetmic.2006.01.009>.
- Banerjee, G., Nandi, A., Ray, A.K., 2016. Assessment of hemolytic activity, enzyme production and bacteriocin characterization of *Bacillus subtilis* LR1 isolated from the gastrointestinal tract of fish. *Arch Microbiol.* 30;71(33):255-266. doi: <https://doi.org/10.1007/s00203-016-1283-8>.
- Barman, D., Nen, P., Mandal, S., Kumar, V., 2013. Immunostimulants for aquaculture health management. *J Marine Sci Res Dev.* 3, 1-11.

- Bartkova, S., Kokotovic, B., Dalsgaard, I., 2017. Infection routes of *Aeromonas salmonicida* in rainbow trout monitored in vivo by real-time bioluminescence imaging. J Fish Dis. 40, 73-82. doi: <https://doi.org/10.1111/jfd.12491>.
- Baruah, K., Cam, D.T.V., Dierckens, K., Wille, M., Defoirdt, T., Sorgeloos, P., Bossier, P., 2009. In vivo effects of single or combined N-acyl homoserine lactone quorum sensing signals on the performance of *Macrobrachium rosenbergii* larvae. Aquaculture. 288, 233-238. doi: <https://doi.org/https://doi.org/10.1016/j.aquaculture.2008.11.034>.
- Barzegari, A., Kheyrolahzadeh, K., Hosseiniyan Khatibi, S.M., Sharifi, S., Memar, M.Y., Zununi Vahed, S., 2020. The Battle of Probiotics and Their Derivatives Against Biofilms. Infect Drug Resist. 13, 659-672. doi: <https://doi.org/10.2147/IDR.S232982>.
- Bayliss, S.C., Verner-Jeffreys, D.W., Bartie, K.L., Aanensen, D.M., Sheppard, S.K., Adams, A., Feil, E.J., 2017. The Promise of Whole Genome Pathogen Sequencing for the Molecular Epidemiology of Emerging Aquaculture Pathogens. Front Microbiol. 8. doi: <https://doi.org/10.3389/fmicb.2017.00121>.
- Beaz-Hidalgo, R., Figueras, M.J., 2013. *Aeromonas* spp. whole genomes and virulence factors implicated in fish disease. J Fish Dis. 36, 371-388. doi: <https://doi.org/10.1111/jfd.12025>.
- Bi, Z.X., Liu, Y.J., Lu, C.P., 2007. Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res Vet Sci. 83, 150-156. doi: <https://doi.org/10.1016/j.rvsc.2007.01.003>.
- Borella, L., Salogni, C., Vitale, N., Scali, F., Moretti, V.M., Pasquali, P., Alborali, G.L., 2020. Motile aeromonads from farmed and wild freshwater fish in northern Italy: an evaluation of antimicrobial activity and multidrug resistance during 2013 and 2016. Acta Vet Scand. 62, 6. doi: <https://doi.org/10.1186/s13028-020-0504-y>.
- Brunt, J., Newaj-Fyzul, A., Austin, B., 2007. The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis. 30, 573-579. doi: <https://doi.org/https://doi.org/10.1111/j.1365-2761.2007.00836.x>.
- Cai, S.H., Wu, Z.H., Jian, J.C., Lu, Y.S., Tang, J.F., 2012. Characterization of pathogenic *Aeromonas veronii* bv. *veronii* associated with ulcerative syndrome from chinese longsnout catfish (*Leiocassis longirostris* Gunther). Braz J Microbiol. 43, 382-388. doi: <https://doi.org/10.1590/s1517-838220120001000046>.
- Cai, W., Arias, C.R., 2017. Biofilm Formation on Aquaculture Substrates by Selected Bacterial Fish Pathogens. J Aquat Anim Health. 29, 95-104. doi: <https://doi.org/https://doi.org/10.1080/08997659.2017.1290711>.

- Cao, L., Pan, L., Gong, L., Yang, Y., He, H., Li, Y., Peng, Y., Li, D., Yan, L., Ding, X., Hu, S., Yu, Z., Sun, Y., Huang, W., Hu, Y., Yi, G., Xia, L., 2019. Interaction of a novel *Bacillus velezensis* (BvL03) against *Aeromonas hydrophila* in vitro and in vivo in grass carp. *Appl Microbiol Biotechnol.* 103, 8987-8999. doi: <https://doi.org/10.1007/s00253-019-10096-7>.
- Cao, Y., He, S., Zhou, Z., Zhang, M., Mao, W., Zhang, H., Yao, B., 2012. Orally administered thermostable N-acyl homoserine lactonase from *Bacillus* sp. strain AI96 attenuates *Aeromonas hydrophila* infection in zebrafish. *Appl Environ Microbiol.* 78, 1899-1908. doi: <https://doi.org/10.1128/aem.06139-11>.
- Casas, C., Anderson, E.C., Ojo, K.K., Keith, I., Whelan, D., Rainnie, D., Roberts, M.C., 2005. Characterization of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. *FEMS Microbiol Lett.* 242, 59-63. doi: <https://doi.org/10.1016/j.femsle.2004.10.039>.
- Castro, N., Toranzo, A.E., Barja, J.L., Núñez, S., Magariños, B., 2006. Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.). *J Fish Dis.* 29, 541-547. doi: <https://doi.org/10.1111/j.1365-2761.2006.00750.x>.
- Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Front Microbiol.* 10. doi: <https://doi.org/10.3389/fmicb.2019.00302>.
- Cerezuela, R., Guardiola, F.A., Meseguer, J., Esteban, M.Á., 2012a. Increases in immune parameters by inulin and *Bacillus subtilis* dietary administration to gilthead seabream (*Sparus aurata* L.) did not correlate with disease resistance to *Photobacterium damsela*. *Fish Shellfish Immunol.* 32, 1032-1040. doi: <https://doi.org/10.1016/j.fsi.2012.02.025>.
- Cerezuela, R., Guardiola, F.A., González, P., Meseguer, J., Esteban, M.Á., 2012b. Effects of dietary *Bacillus subtilis*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 33, 342-349. doi: <https://doi.org/10.1016/j.fsi.2012.05.004>.
- Cha, J.-H., Rahimnejad, S., Yang, S.-Y., Kim, K.-W., Lee, K.-J., 2013. Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. *Aquaculture.* 402-403, 50-57. doi: <https://doi.org/10.1016/j.aquaculture.2013.03.030>.

Chang, S.C., Lee, C.Y., 2020. Quorum-Sensing Regulator OpaR Directly Represses Seven Protease Genes in *Vibrio parahaemolyticus*. *Front Microbiol.* 11, 534692. doi: <https://doi.org/10.3389/fmicb.2020.534692>.

Chen, B., Peng, M., Tong, W., Zhang, Q., Song, Z., 2020. The Quorum Quenching Bacterium *Bacillus licheniformis* T-1 Protects Zebrafish against *Aeromonas hydrophila* Infection. *Probiotics Antimicrob Proteins*. doi: <https://doi.org/10.1007/s12602-018-9495-7>.

Chen, F., Gao, Y., Chen, X., Yu, Z., Li, X., 2013. Quorum Quenching Enzymes and Their Application in Degrading Signal Molecules to Block Quorum Sensing-Dependent Infection. *Int J Mol Sci.* 14, 17477-17500. doi: <https://doi.org/10.3390/ijms140917477>.

Chen, F., Sun, J., Han, Z., Yang, X., Xian, J.-a., Lv, A., Hu, X., Shi, H., 2019. Isolation, Identification and Characteristics of *Aeromonas veronii* From Diseased Crucian Carp (*Carassius auratus gibelio*). *Front Microbiol.* 10. doi: <https://doi.org/10.3389/fmicb.2019.02742>.

Chen, R., Zhou, Z., Cao, Y., Bai, Y., Yao, B., 2010. High yield expression of an AHL-lactonase from *Bacillus* sp. B546 in *Pichia pastoris* and its application to reduce *Aeromonas hydrophila* mortality in aquaculture. *Microb Cell Fact.* 9, 39-39. doi: <https://doi.org/10.1186/1475-2859-9-39>.

Chen, X.M., Lu, H.M., Niu, X.T., Wang, G.Q., Zhang, D.M., 2015. Enhancement of secondary metabolites from *Bacillus Licheniformis* XY-52 on immune response and expression of some immune-related genes in common carp, *Cyprinus carpio*. *Fish Shellfish Immunol.* 45, 124-131. doi: <https://doi.org/10.1016/j.fsi.2015.02.019>.

Chen, Y., Li, J., Xiao, P., Li, G.Y., Yue, S., Huang, J., Zhu, W.Y., Mo, Z.L., 2016. Isolation and characterization of *Bacillus* spp. M001 for potential application in turbot (*Scophthalmus maximus* L.) against *Vibrio anguillarum*. *Aquacult Nutr.* 22, 374-381. doi: <https://doi.org/https://doi.org/10.1111/anu.12259>.

Chopra, L., Singh, G., Choudhary, V., Sahoo, D.K., 2014. Sonorensin: an antimicrobial peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new marine isolate, *Bacillus sonorensis* MT93. *Appl Environ Microbiol.* 80, 2981-2990. doi: <https://doi.org/10.1128/AEM.04259-13>.

Chopra, L., Singh, G., Kumar Jena, K., Sahoo, D.K., 2015. Sonorensin: A new bacteriocin with potential of an anti-biofilm agent and a food biopreservative. *Sci Rep.* 5, 13412. doi: <https://doi.org/10.1038/srep13412>.

- Chowdhary, P.K., Keshavan, N., Nguyen, H.Q., Peterson, J.A., Gonzalez, J.E., Haines, D.C., 2007. *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. *Biochemistry*. 46, 14429-14437. doi: <https://doi.org/10.1021/bi701945j>.
- Chu, W., Lu, F., Zhu, W., Kang, C., 2011. Isolation and characterization of new potential probiotic bacteria based on quorum-sensing system. *J Appl Microbiol*. 110, 202-208. doi: <https://doi.org/10.1111/j.1365-2672.2010.04872.x>.
- Chu, W., Zhou, S., Zhu, W., Zhuang, X., 2014. Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. *Sci Rep*. 4, 5446. doi: <https://doi.org/10.1038/srep05446>.
- Churchill, M.E.A., Chen, L., 2011. Structural basis of acyl-homoserine lactone-dependent signaling. *Chem Rev*. 111, 68-85. doi: <https://doi.org/10.1021/cr1000817>.
- Croxatto, A., Chalker, V.J., Lauritz, J., Jass, J., Hardman, A., Williams, P., Camara, M., Milton, D.L., 2002. VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum*. *J Bacteriol*. 184, 1617-1629.
- Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A., Charette, S.J., 2014. Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol*. 169, 1-7. doi: <https://doi.org/10.1016/j.vetmic.2013.06.025>.
- Das, A., Nakhro, K., Chowdhury, S., Kamilya, D., 2013. Effects of potential probiotic *Bacillus amyloliquefaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). *Fish Shellfish Immunol*. 35, 1547-1553. doi: <https://doi.org/10.1016/j.fsi.2013.08.022>.
- Das, M., Li, X., Dung, N.V., Bossier, P., Tinh, N.T.N., 2018. No effect of N-acyl homoserine lactones disruption by lactonase enzyme on the virulence of *Vibrio anguillarum* towards sea bass (*Dicentrarchus labrax*) and brine shrimp (*Artemia franciscana*). *Aquac Int*. 26, 363-374. doi: <https://doi.org/10.1007/s10499-017-0214-2>.
- Dash, S., Das, S.K., Samal, J., Thatoi, H.N., 2018. Epidermal mucus, a major determinant in fish health: a review. *Iran J Vet Res*. 19, 72-81.
- Defoirdt, T., 2018. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends Microbiol*. 26, 313-328. doi: <https://doi.org/10.1016/j.tim.2017.10.005>.

- Defoirdt, T., Boon, N., Bossier, P., 2010a. Can bacteria evolve resistance to quorum sensing disruption? PLoS Pathog. 6, e1000989-e1000989. doi: <https://doi.org/10.1371/journal.ppat.1000989>.
- Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr Opin Microbiol. 14, 251-258. doi: <https://doi.org/10.1016/j.mib.2011.03.004>.
- Defoirdt, T., Darshanee Ruwandeepika, H.A., Karunasagar, I., Boon, N., Bossier, P., 2010b. Quorum sensing negatively regulates chitinase in *Vibrio harveyi*. Environmental Microbiology Reports. 2, 44-49. doi: <https://doi.org/10.1111/j.1758-2229.2009.00043.x>.
- Deng, Y., Xu, L., Chen, H., Liu, S., Guo, Z., Cheng, C., Ma, H., Feng, J., 2020. Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China. Sci Rep. 10, 14329. doi: <https://doi.org/10.1038/s41598-020-71288-0>.
- Di, J., Chu, Z., Zhang, S., Huang, J., Du, H., Wei, Q., 2019. Evaluation of the potential probiotic *Bacillus subtilis* isolated from two ancient sturgeons on growth performance, serum immunity and disease resistance of *Acipenser dabryanus*. Fish Shellfish Immunol. 93, 711-719. doi: <https://doi.org/10.1016/j.fsi.2019.08.020>.
- Diaz De Rienzo, M.A., Banat, I., Dolman, B., Winterburn, J., Martin, P., 2015. Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. New biotechnology. 32. doi: <https://doi.org/10.1016/j.nbt.2015.02.009>.
- Dobson, A., Cotter, P.D., Ross, R.P., Hill, C., 2012. Bacteriocin Production: a Probiotic Trait? Appl Environ Microbiol. 78, 1-6. doi: <https://doi.org/10.1128/AEM.05576-11>.
- Dong, Y.-H., Xu, J.-L., Li, X.-Z., Zhang, L.-H., 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc Natl Acad Sci. 97, 3526. doi: <https://doi.org/10.1073/pnas.060023897>.
- Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L., Zhang, L.H., 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol. 68, 1754-1759.
- Doukas, Athanassopoulou, Karagouni, Dotsika, 1998. Short communication *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* L., and *Puntazzo puntazzo* Cuvier from the Aegean Sea. J Fish Dis. 21, 317-320. doi: <https://doi.org/https://doi.org/10.1046/j.1365-2761.1998.00105.x>.

Dutta, D., Ghosh, K., 2015. Screening of extracellular enzyme-producing and pathogen inhibitory gut bacteria as putative probiotics in mrigal, *Cirrhinus mrigala* (Hamilton, 1822). International Journal of Fisheries and Aquatic Studies. 2, 310-318.

Easwaran, N., Karthikeyan, S., Sridharan, B., Gothandam, K.M., 2015. Identification and analysis of the salt tolerant property of AHL lactonase (AiiATSAWB) of *Bacillus* species. J Basic Microbiol. 55, 579-590. doi: <https://doi.org/10.1002/jobm.201400013>.

El-Kurdi, N., Abdulla, H., Hanora, A., 2021. Anti-quorum sensing activity of some marine bacteria isolated from different marine resources in Egypt. Biotechnol Lett. 43, 455-468. doi: <https://doi.org/10.1007/s10529-020-03020-x>.

Emmerich, R., Weibel, E., 1894. Ueber eine durch Bakterien erengte Seuche unter den Forellen. Archives fur Hygiene und Bakteriologie. 21:1-21.

Essam, H.M., Abdellrazeq, G.S., Tayel, S.I., Torky, H.A., Fadel, A.H., 2016. Pathogenesis of *Photobacterium damsela* subspecies infections in sea bass and sea bream. Microb Pathog. 99, 41-50. doi: <https://doi.org/https://doi.org/10.1016/j.micpath.2016.08.003>.

Esteban, P., Redrado, S., Comas, L., Domingo, M.P., Millán-Lou, M.I., Seral, C., Algarate, S., Lopez, C., Rezusta, A., Pardo, J., Arias, M., Galvez, E.M., 2021. *In Vitro* and *In Vivo* Antibacterial Activity of Gliotoxin Alone and in Combination with Antibiotics against *Staphylococcus aureus*. Toxins (Basel). 13. doi: <https://doi.org/10.3390/toxins13020085>.

EUMOFA, 2020. The UE Fish Market, European Market Observatory for Fisheries and Aquaculture Products, Brussels.

Faílde, L.D., Losada, A.P., Bermúdez, R., Santos, Y., Quiroga, M.I., 2013. *Tenacibaculum maritimum* infection: pathology and immunohistochemistry in experimentally challenged turbot (*Psetta maxima* L.). Microb Pathog. 65, 82-88. doi: <https://doi.org/10.1016/j.micpath.2013.09.003>.

FAO, 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action., Rome.

Fetzner, S., 2015. Quorum quenching enzymes. J Biotechnol. 201, 2-14. doi: <https://doi.org/10.1016/j.jbiotec.2014.09.001>.

Fidopiastis, P.M., Miyamoto, C.M., Jobling, M.G., Meighen, E.A., Ruby, E.G., 2002. LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic

light organ colonization. *Mol Microbiol.* 45, 131-143. doi: <https://doi.org/10.1046/j.1365-2958.2002.02996.x>.

Fischetti, V.A., 2010. Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. *International journal of medical microbiology : IJMM.* 300, 357-362. doi: <https://doi.org/10.1016/j.ijmm.2010.04.002>.

Fleming, A., 1929. On the Antibacterial Action of Cultures of a *Penicillium*, with Special Reference to their Use in the Isolation of *B. influenzae*. *Br J Exp Pathol.* 10, 226-236.

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. *Nat Rev Micro.* 14, 563-575. doi: <https://doi.org/10.1038/nrmicro.2016.94>.

Foyosal, M.J., Lisa, A.K., 2018. Isolation and characterization of *Bacillus* sp. strain BC01 from soil displaying potent antagonistic activity against plant and fish pathogenic fungi and bacteria. *J Genet Eng Biotechnol.* 16, 387-392. doi: <https://doi.org/10.1016/j.jgeb.2018.01.005>.

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B., Rediers, H., 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *J Fish Dis.* 34, 643-661. doi: <https://doi.org/10.1111/j.1365-2761.2011.01279.x>.

Freitas, J., Vaz-Pires, P., Câmara, J.S., 2020. From aquaculture production to consumption: Freshness, safety, traceability and authentication, the four pillars of quality. *Aquaculture.* 518, 734857. doi: <https://doi.org/https://doi.org/10.1016/j.aquaculture.2019.734857>.

Fuqua, C., Winans, S.C., Greenberg, E.P., 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol.* 50, 727-751. doi: <https://doi.org/10.1146/annurev.micro.50.1.727>.

Galagarza, O.A., Smith, S.A., Drahos, D.J., Eifert, J.D., Williams, R.C., Kuhn, D.D., 2018. Modulation of innate immunity in Nile tilapia (*Oreochromis niloticus*) by dietary supplementation of *Bacillus subtilis* endospores. *Fish Shellfish Immunol.* 83, 171-179. doi: <https://doi.org/10.1016/j.fsi.2018.08.062>.

Gao, X.-Y., Liu, Y., Miao, L.-L., Li, E.-W., Sun, G.-x., Liu, Y., Liu, Z.-P., 2017. Characterization and mechanism of anti-*Aeromonas salmonicida* activity of a marine probiotic strain, *Bacillus velezensis* V4. *Appl Microbiol Biotechnol.* 101, 3759-3768. doi: <https://doi.org/10.1007/s00253-017-8095-x>.

- Gashgari, R.M., Selim, S.A., 2015. Detection and Characterization of Antimicrobial Resistance and Putative Virulence Genes in *Aeromonas veronii* Biovar Sobria Isolated from Gilthead Sea Bream (*Sparus aurata* L.). Foodborne Pathog Dis. 12, 806-811. doi: <https://doi.org/10.1089/fpd.2015.1976>.
- Gauthier, D.T., 2015. Bacterial zoonoses of fishes: a review and appraisal of evidence for linkages between fish and human infections. Vet J. 203, 27-35. doi: <https://doi.org/10.1016/j.tvjl.2014.10.028>.
- Ghanei-Motlagh, R., Mohammadian, T., Gharibi, D., Menanteau-Ledouble, S., Mahmoudi, E., Khosravi, M., Zarea, M., El-Matbouli, M., 2019. Quorum Quenching Properties and Probiotic Potentials of Intestinal Associated Bacteria in Asian Sea Bass *Lates calcarifer*. Mar Drugs. 18. doi: <https://doi.org/10.3390/md18010023>.
- Ghosh, K., Mukherjee, A., Dutta, D., Banerjee, S., Breines, E.M., Hareide, E., Ringø, E., 2021. Endosymbiotic pathogen-inhibitory gut bacteria in three Indian Major Carps under polyculture system: A step toward making a probiotics consortium. Aquaculture and Fisheries. 6, 192-204. doi: <https://doi.org/https://doi.org/10.1016/j.aaf.2020.03.009>.
- Ghosh, S., Sinha, A., Sahu, C., 2007. Isolation of putative probionts from the intestines of Indian major carps.
- Girard, L., 2019. Quorum sensing in *Vibrio* spp.: the complexity of multiple signalling molecules in marine and aquatic environments. Crit Rev Microbiol. 45, 451-471. doi: <https://doi.org/10.1080/1040841X.2019.1624499>.
- Giri, S.S., Sukumaran, V., Sen, S.S., Jena, P.K., 2014. Effects of dietary supplementation of potential probiotic *Bacillus subtilis* VSG1 singularly or in combination with *Lactobacillus plantarum* VSG3 or/and *Pseudomonas aeruginosa* VSG2 on the growth, immunity and disease resistance of *Labeo rohita*. Aquacult Nutr. 20, 163-171. doi: <https://doi.org/https://doi.org/10.1111/anu.12062>.
- Giri, S.S., Sukumaran, V., Sen, S.S., Vinumonia, J., Banu, B.N., Jena, P.K., 2011. Antagonistic Activity of Cellular Components of Potential Probiotic Bacteria, Isolated from the Gut of *Labeo rohita*, Against *Aeromonas hydrophila*. Probiotics Antimicrob Proteins. 3, 214-222. doi: <https://doi.org/10.1007/s12602-011-9078-3>.
- Girija, V., Malaikozhundan, B., Vaseeharan, B., Vijayakumar, S., Gobi, N., Del Valle Herrera, M., Chen, J.C., Santhanam, P., 2018. *In vitro* antagonistic activity and the protective effect of probiotic *Bacillus licheniformis* Dahb1 in zebrafish challenged with GFP tagged *Vibrio parahaemolyticus* Dahv2. Microb Pathog. 114, 274-280. doi: <https://doi.org/10.1016/j.micpath.2017.11.058>.

Gobi, N., Malaikozhundan, B., Sekar, V., Shanthi, S., Vaseeharan, B., Jayakumar, R., Khudus Nazar, A., 2016. GFP tagged *Vibrio parahaemolyticus* Dahv2 infection and the protective effects of the probiotic *Bacillus licheniformis* Dahb1 on the growth, immune and antioxidant responses in *Pangasius hypophthalmus*. Fish Shellfish Immunol. 52, 230-238. doi: <https://doi.org/10.1016/j.fsi.2016.03.006>.

Gode-Potratz, C.J., McCarter, L.L., 2011. Quorum Sensing and Silencing in *Vibrio parahaemolyticus*. J Bacteriol. 193, 4224-4237. doi: <https://doi.org/doi:10.1128/JB.00432-11>.

Gordon, S., 2016. Phagocytosis: An Immunobiologic Process. Immunity. 44, 463-475. doi: <https://doi.org/10.1016/j.immuni.2016.02.026>.

Gourzioti, E., Kolygas, M., Athanassopoulou, F., Babili, V., 2016. Tenacibaculosis in aquaculture farmed marine fish. Journal of the Hellenic Veterinary Medical Society. 67, 21-32.

Grandclement, C., Tannieres, M., Morera, S., Dessaux, Y., Faure, D., 2016. Quorum quenching: role in nature and applied developments. FEMS Microbiol Rev. 40, 86-116. doi: <https://doi.org/10.1093/femsre/fuv038>.

Gui, M., Wu, R., Liu, L., Wang, S., Zhang, L., Li, P., 2017. Effects of quorum quenching by AHL lactonase on AHLs, protease, motility and proteome patterns in *Aeromonas veronii* LP-11. Int J Food Microbiol. 252, 61-68. doi: <https://doi.org/10.1016/j.ijfoodmicro.2017.04.005>.

Gui, M., Liu, L., Wu, R., Hu, J., Wang, S., Li, P., 2018. Detection of New Quorum Sensing N-Acyl Homoserine Lactones From *Aeromonas veronii*. Front Microbiol. 9, 1712. doi: <https://doi.org/10.3389/fmicb.2018.01712>.

Guo, M., Fang, Z., Sun, L., Sun, D., Wang, Y., Li, C., Wang, R., Liu, Y., Hu, H., Liu, Y., Xu, D., Gooneratne, R., 2018. Regulation of Thermostable Direct Hemolysin and Biofilm Formation of *Vibrio parahaemolyticus* by Quorum-Sensing Genes luxM and luxS. Curr Microbiol. 75, 1190-1197. doi: <https://doi.org/10.1007/s00284-018-1508-y>.

Guo, X., Chen, D.-D., Peng, K.-S., Cui, Z.-W., Zhang, X.-J., Li, S., Zhang, Y.-A., 2016. Identification and characterization of *Bacillus subtilis* from grass carp (*Ctenopharyngodon idellus*) for use as probiotic additives in aquatic feed. Fish Shellfish Immunol. 52, 74-84. doi: <https://doi.org/10.1016/j.fsi.2016.03.017>.

Gupta, A., Gupta, P., Dhawan, A., 2014. Dietary supplementation of probiotics affects growth, immune response and disease resistance of *Cyprinus carpio* fry. Fish Shellfish Immunol. 41, 113-119. doi: <https://doi.org/10.1016/j.fsi.2014.08.023>.

- Gutiérrez-Almada, K., González-Acosta, B., Borges-Souza, J.M., Aguila-Ramírez, R.N., 2020. Marine bacteria associated with shallow hydrothermal systems in the Gulf of California with the capacity to produce biofilm inhibiting compounds. *Arch Microbiol.* 202, 1477-1488. doi: <https://doi.org/10.1007/s00203-020-01851-w>.
- Gyawali, R., Ibrahim, S.A., 2014. Natural products as antimicrobial agents. *Food Control.* 46, 412-429. doi: <https://doi.org/https://doi.org/10.1016/j.foodcont.2014.05.047>.
- Haldar, S., Maharajan, A., Chatterjee, S., Hunter, S.A., Chowdhury, N., Hinenoya, A., Asakura, M., Yamasaki, S., 2010. Identification of *Vibrio harveyi* as a causative bacterium for a tail rot disease of sea bream *Sparus aurata* from research hatchery in Malta. *Microbiol Res.* 165, 639-648. doi: <https://doi.org/10.1016/j.micres.2009.12.001>.
- Hamza, F., Kumar, A.R., Zinjarde, S., 2016. Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. *Aquac Res.* 47, 2661-2669. doi: <https://doi.org/10.1111/are.12716>.
- Hamza, F., Kumar, A.R., Zinjarde, S., 2018. Efficacy of cell free supernatant from *Bacillus licheniformis* in protecting *Artemia salina* against *Vibrio alginolyticus* and *Pseudomonas gessardii*. *Microb Pathog.* 116, 335-344. doi: <https://doi.org/10.1016/j.micpath.2018.02.003>.
- Han, Y., Li, X., Qi, Z., Zhang, X.H., Bossier, P., 2010. Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda* strain LTB-4. *J Appl Microbiol.* 108, 139-147. doi: <https://doi.org/10.1111/j.1365-2672.2009.04405.x>.
- Heilbronner, S., Krismer, B., Brötz-Oesterhelt, H., Peschel, A., 2021. The microbiome-shaping roles of bacteriocins. *Nat Rev Micro.* 19, 726-739. doi: <https://doi.org/10.1038/s41579-021-00569-w>.
- Henke, J.M., Bassler, B.L., 2004. Quorum Sensing Regulates Type III Secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J Bacteriol.* 186, 3794-3805. doi: <https://doi.org/doi:10.1128/JB.186.12.3794-3805.2004>.
- Hense, B.A., Kuttler, C., Müller, J., Rothballer, M., Hartmann, A., Kreft, J.-U., 2007. Does efficiency sensing unify diffusion and quorum sensing? *Nat Rev Micro.* 5, 230-239. doi: <https://doi.org/10.1038/nrmicro1600>.
- Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature reviews. Microbiology.* 8, 15-25. doi: <https://doi.org/10.1038/nrmicro2259>.

- Hong, H.A., To, E., Fakhry, S., Baccigalupi, L., Ricca, E., Cutting, S.M., 2009. Defining the natural habitat of *Bacillus* spore-formers. *Res Microbiol.* 160, 375-379. doi: <https://doi.org/10.1016/j.resmic.2009.06.006>.
- Hong, N.T., Baruah, K., Vanrompay, D., Bossier, P., 2016. Characterization of phenotype variations of luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants. *J Fish Dis.* 39, 317-327. doi: <https://doi.org/10.1111/jfd.12365>.
- Igbinosa, E.O., 2016. Detection and Antimicrobial Resistance of *Vibrio* Isolates in Aquaculture Environments: Implications for Public Health. *Microb Drug Resist.* 22, 238-245. doi: <https://doi.org/10.1089/mdr.2015.0169>.
- Igbinosa, I.H., Igbinosa, E.O., Okoh, A.I., 2015. Detection of antibiotic resistance, virulence gene determinants and biofilm formation in *Aeromonas* species isolated from cattle. *Environ Sci Pollut Res Int.* 22, 17596-17605. doi: <https://doi.org/10.1007/s11356-015-4934-4>.
- Ina-Salwany, M.Y., Al-Saari, N., Mohamad, A., Mursidi, F.A., Mohd-Aris, A., Amal, M.N.A., Kasai, H., Mino, S., Sawabe, T., Zamri-Saad, M., 2019. Vibriosis in Fish: A Review on Disease Development and Prevention. *J Aquat Anim Health.* 31, 3-22. doi: <https://doi.org/10.1002/aah.10045>.
- Iregui, C.A., Guarín, M., Tibatá, V.M., Ferguson, H.W., 2012. Novel brain lesions caused by *Edwardsiella tarda* in a red tilapia (*Oreochromis* spp.). *Journal of Veterinary Diagnostic Investigation.* 24, 446-449. doi: <https://doi.org/10.1177/1040638711435232>.
- James, G., Das, B.C., Jose, S., V.J., R.K., 2021. *Bacillus* as an aquaculture friendly microbe. *Aquac Int.* 29, 323-353. doi: <https://doi.org/10.1007/s10499-020-00630-0>.
- Janda, J.M., Abbott, S.L., 2010. The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. *Clin Microbiol Rev.* 23, 35-73. doi: <https://doi.org/10.1128/cmr.00039-09>.
- Jangid, K., Parameswaran, P.S., Shouche, Y.S., 2012. A variant quorum sensing system in *Aeromonas veronii* MTCC 3249. *Sensors (Basel).* 12, 3814-3830. doi: <https://doi.org/10.3390/s120403814>.
- Jangid, K., Kong, R., Patole, M.S., Shouche, Y.S., 2007. luxRI homologs are universally present in the genus *Aeromonas*. *BMC Microbiol.* 7, 93. doi: <https://doi.org/10.1186/1471-2180-7-93>.
- Ji, L., Sun, G., Li, J., Wang, Y., Du, Y., Li, X., Liu, Y., 2017. Effect of dietary β -glucan on growth, survival and regulation of immune processes in rainbow trout

(*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida*. Fish Shellfish Immunol. 64, 56-67. doi: <https://doi.org/10.1016/j.fsi.2017.03.015>.

Jung, J.H., Lee, M.Y., Chang, H.C., 2012. Evaluation of the probiotic potential of *Bacillus polyfermenticus* CJ6 isolated from Meju, a Korean soybean fermentation starter. J Microbiol Biotechnol. 22, 1510-1517. doi: <https://doi.org/10.4014/jmb.1205.05049>.

Katharios, P., Kokkari, C., Dourala, N., Smyrli, M., 2015. First report of Edwardsiellosis in cage-cultured sharpsnout sea bream, *Diplodus puntazzo* from the Mediterranean. BMC Vet Res. 11, 155. doi: <https://doi.org/10.1186/s12917-015-0482-x>.

Khajanchi, B.K., Kozlova, E.V., Sha, J., Popov, V.L., Chopra, A.K., 2012. The two-component QseBC signalling system regulates *in vitro* and *in vivo* virulence of *Aeromonas hydrophila*. Microbiology (Reading). 158, 259-271. doi: <https://doi.org/10.1099/mic.0.051805-0>.

Khajanchi, B.K., Sha, J., Kozlova, E.V., Erova, T.E., Suarez, G., Sierra, J.C., Popov, V.L., Horneman, A.J., Chopra, A.K., 2009. N-acylhomoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and *in vivo* virulence in a clinical isolate of *Aeromonas hydrophila*. Microbiology (Reading). 155, 3518-3531. doi: <https://doi.org/10.1099/mic.0.031575-0>.

Khouadja, S., Lamari, F., Bakhrouf, A., Gaddour, K., 2014. Virulence properties, biofilm formation and random amplified polymorphic DNA analysis of *Photobacterium damsela* subsp. *damsela* isolates from cultured sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). Microb Pathog. 69-70, 13-19. doi: <https://doi.org/10.1016/j.micpath.2014.03.007>.

Kim, J.A., Lee, M.A., Jung, Y.C., Jang, B.R., Lee, K.H., 2018a. Repression of VvpM Protease Expression by Quorum Sensing and the cAMP-cAMP Receptor Protein Complex in *Vibrio vulnificus*. J Bacteriol. 200. doi: <https://doi.org/10.1128/jb.00526-17>.

Kim, N.-N., Kim, W., Kang, S.-S., 2018b. Anti-biofilm effect of crude bacteriocin derived from *Lactobacillus brevis* DF01 on *Escherichia coli* and *Salmonella Typhimurium*. Food Control. 98. doi: <https://doi.org/10.1016/j.foodcont.2018.11.004>.

Kim, S.M., Park, J.H., Lee, H.S., Kim, W.B., Ryu, J.M., Han, H.J., Choi, S.H., Blanke, S.R., 2013. LuxR Homologue SmcR Is Essential for *Vibrio vulnificus* Pathogenesis and Biofilm Detachment, and Its Expression is Induced by Host Cells. Infect Immun. 81, 3721-3730. doi: <https://doi.org/doi:10.1128/IAI.00561-13>.

- Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology*. 173, 111-133. doi: <https://doi.org/https://doi.org/10.1016/j.anifeedsci.2011.12.015>.
- Kolygas, M.N., Gourzioti, E., Vatsos, I.N., Athanassopoulou, F., 2012. Identification of *Tenacibaculum maritimum* strains from marine farmed fish in Greece. *Vet Rec*. 170, 623. doi: <https://doi.org/10.1136/vr.100778>.
- Kozlova, E.V., Khajanchi, B.K., Popov, V.L., Wen, J., Chopra, A.K., 2012. Impact of QseBC system in c-di-GMP-dependent quorum sensing regulatory network in a clinical isolate SSU of *Aeromonas hydrophila*. *Microb Pathog*. 53, 115-124. doi: <https://doi.org/10.1016/j.micpath.2012.05.008>.
- Kozlova, E.V., Popov, V.L., Sha, J., Foltz, S.M., Erova, T.E., Agar, S.L., Horneman, A.J., Chopra, A.K., 2008. Mutation in the S-ribosylhomocysteinase (luxS) gene involved in quorum sensing affects biofilm formation and virulence in a clinical isolate of *Aeromonas hydrophila*. *Microb Pathog*. 45, 343-354. doi: <https://doi.org/10.1016/j.micpath.2008.08.007>.
- Kuebutornye, F.K.A., Abarike, E.D., Lu, Y., 2019. A review on the application of *Bacillus* as probiotics in aquaculture. *Fish Shellfish Immunol*. 87, 820-828. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2019.02.010>.
- Kuebutornye, F.K.A., Abarike, E.D., Lu, Y., Hlordzi, V., Sakyi, M.E., Afriyie, G., Wang, Z., Li, Y., Xie, C.X., 2020. Mechanisms and the role of probiotic *Bacillus* in mitigating fish pathogens in aquaculture. *Fish Physiology and Biochemistry*. 46, 819-841. doi: <https://doi.org/10.1007/s10695-019-00754-y>.
- Kum, C., Sekkin, S., 2011. The immune system drugs in fish: immune function, immunoassay, drugs. *Recent advances in fish farms*, 169-210.
- Kumar, R., Mukherjee, S.C., Ranjan, R., Nayak, S.K., 2008. Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish Shellfish Immunol*. 24, 168-172. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2007.10.008>.
- Labella, A., Sanchez-Montes, N., Berbel, C., Aparicio, M., Castro, D., Manchado, M., Borrego, J.J., 2010. Toxicity of *Photobacterium damsela* subsp. *damsela* strains isolated from new cultured marine fish. *Dis Aquat Organ*. 92, 31-40. doi: <https://doi.org/10.3354/dao02275>.
- Lafferty, K.D., Harvell, C.D., Conrad, J.M., Friedman, C.S., Kent, M.L., Kuris, A.M., Powell, E.N., Rondeau, D., Saksida, S.M., 2015. Infectious diseases affect marine

fisheries and aquaculture economics. *Ann Rev Mar Sci.* 7, 471-496. doi: <https://doi.org/10.1146/annurev-marine-010814-015646>.

Laloo, R., Moonsamy, G., Ramchuran, S., Görgens, J., Gardiner, N., 2010. Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent. *Lett Appl Microbiol.* 50, 563-570. doi: <https://doi.org/10.1111/j.1472-765X.2010.02829.x>.

LaSarre, B., Federle, M.J., 2013. Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol Mol Biol Rev.* 77, 73-111. doi: <https://doi.org/10.1128/MMBR.00046-12>.

Lee, M.H., Lee, J., Nam, Y.D., Lee, J.S., Seo, M.J., Yi, S.H., 2016. Characterization of antimicrobial lipopeptides produced by *Bacillus* sp. LM7 isolated from chungkookjang, a Korean traditional fermented soybean food. *Int J Food Microbiol.* 221, 12-18. doi: <https://doi.org/10.1016/j.ijfoodmicro.2015.12.010>.

Lee, S.J., Park, S.Y., Lee, J.J., Yum, D.Y., Koo, B.T., Lee, J.K., 2002. Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl Environ Microbiol.* 68, 3919-3924.

Li, D., Wu, M., 2021. Pattern recognition receptors in health and diseases. *Signal Transduction and Targeted Therapy.* 6, 291. doi: <https://doi.org/10.1038/s41392-021-00687-0>.

Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol.* 7, 1116-1124. doi: <https://doi.org/10.1038/ni1389>.

Li, J., Wu, Z.B., Zhang, Z., Zha, J.W., Qu, S.Y., Qi, X.Z., Wang, G.X., Ling, F., 2019. Effects of potential probiotic *Bacillus velezensis* K2 on growth, immunity and resistance to *Vibrio harveyi* infection of hybrid grouper (*Epinephelus lanceolatus* ♂ × *E. fuscoguttatus* ♀). *Fish Shellfish Immunol.* 93, 1047-1055. doi: <https://doi.org/10.1016/j.fsi.2019.08.047>.

Li, K., Underhill, D.M., 2020. C-Type Lectin Receptors in Phagocytosis. *Curr Top Microbiol Immunol.* 429, 1-18. doi: https://doi.org/10.1007/82_2020_198.

Lin, Y.S., Saputra, F., Chen, Y.C., Hu, S.Y., 2019. Dietary administration of *Bacillus amyloliquefaciens* R8 reduces hepatic oxidative stress and enhances nutrient metabolism and immunity against *Aeromonas hydrophila* and *Streptococcus agalactiae* in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 86, 410-419. doi: <https://doi.org/10.1016/j.fsi.2018.11.047>.

- Ling, S.H.M., Wang, X.H., Lim, T.M., Leung, K.Y., 2001. Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. FEMS Microbiol Lett. 194, 239-243. doi: [https://doi.org/10.1016/S0378-1097\(00\)00498-5](https://doi.org/10.1016/S0378-1097(00)00498-5).
- Linnerz, T., Hall, C.J., 2020. The Diverse Roles of Phagocytes During Bacterial and Fungal Infections and Sterile Inflammation: Lessons From Zebrafish. Front Immunol. 11, 1094-1094. doi: <https://doi.org/10.3389/fimmu.2020.01094>.
- Liu, C.-H., Chiu, C.-H., Wang, S.-W., Cheng, W., 2012. Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. Fish Shellfish Immunol. 33, 699-706. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2012.06.012>.
- Liu, H., Wang, S., Cai, Y., Guo, X., Cao, Z., Zhang, Y., Liu, S., Yuan, W., Zhu, W., Zheng, Y., Xie, Z., Guo, W., Zhou, Y., 2017a. Dietary administration of *Bacillus subtilis* HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus*. Fish Shellfish Immunol. 60, 326-333. doi: <https://doi.org/10.1016/j.fsi.2016.12.003>.
- Liu, J., Fu, K., Wang, Y., Wu, C., Li, F., Shi, L., Ge, Y., Zhou, L., 2017b. Detection of Diverse N-Acyl-Homoserine Lactones in *Vibrio alginolyticus* and Regulation of Biofilm Formation by N-(3-Oxodecanoyl) Homoserine Lactone *In vitro*. Front Microbiol. 8, 1097. doi: <https://doi.org/10.3389/fmicb.2017.01097>.
- Liu, L., Yan, Y., Feng, L., Zhu, J., 2018. Quorum sensing asal mutants affect spoilage phenotypes, motility, and biofilm formation in a marine fish isolate of *Aeromonas salmonicida*. Food Microbiol. 76, 40-51. doi: <https://doi.org/10.1016/j.fm.2018.04.009>.
- Liu, X.F., Li, Y., Li, J.R., Cai, L.Y., Li, X.X., Chen, J.R., Lyu, S.X., 2015. Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahaemolyticus* for use as probiotics in aquaculture. World J Microbiol Biotechnol. 31, 795-803. doi: <https://doi.org/10.1007/s11274-015-1833-2>.
- Lu, D.L., Limbu, S.M., Lv, H.B., Ma, Q., Chen, L.Q., Zhang, M.L., Du, Z.Y., 2019. The comparisons in protective mechanisms and efficiencies among dietary α -lipoic acid, β -glucan and l-carnitine on Nile tilapia infected by *Aeromonas hydrophila*. Fish Shellfish Immunol. 86, 785-793. doi: <https://doi.org/10.1016/j.fsi.2018.12.023>.
- Luo, Z., Bai, X., Chen, C., 2014. Integrated application of two different screening strategies to select potential probiotics from the gut of channel catfish

Ictalurus punctatus. Fisheries Sci. 80, 1269-1275. doi: <https://doi.org/10.1007/s12562-014-0794-y>.

Lynch, M.J., Swift, S., Kirke, D.F., Keevil, C.W., Dodd, C.E., Williams, P., 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ Microbiol. 4, 18-28. doi: <https://doi.org/10.1046/j.1462-2920.2002.00264.x>.

Magariños, B., Romalde, J.L., López-Romalde, S., Moriño, M.A., Toranzo, A.E., 2003. Pathobiological characterisation of *Photobacterium damsela* subsp. *piscicida* isolated from cultured sole (*Solea senegalensis*). BULLETIN-EUROPEAN ASSOCIATION OF FISH PATHOLOGISTS. 23, 183-190.

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. 20, 137-151. doi: <https://doi.org/10.1016/j.fsi.2004.09.006>.

Magnadóttir, B., 2010. Immunological Control of Fish Diseases. Mar Biotechnol. 12, 361-379. doi: <https://doi.org/10.1007/s10126-010-9279-x>.

Maqsood, S., Singh, P., Samoon, M.H., Munir, K., 2011. Emerging role of immunostimulants in combating the disease outbreak in aquaculture. International Aquatic Research (Islamic Azad University, Tonekabon Branch). 3.

McCarter, L.L., 1998. OpaR, a homolog of *Vibrio harveyi* LuxR, controls opacity of *Vibrio parahaemolyticus*. J Bacteriol. 180, 3166-3173. doi: <https://doi.org/10.1128/JB.180.12.3166-3173.1998>.

McDougald, D., Rice, S.A., Kjelleberg, S., 2001. SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus*. J Bacteriol. 183, 758-762. doi: <https://doi.org/10.1128/JB.183.2.758-762.2001>.

Meidong, R., Doolgindachbaporn, S., Jamjan, W., Sakai, K., Tashiro, Y., Okugawa, Y., Tongpim, S., 2017. A novel probiotic *Bacillus siamensis* B44v isolated from Thai pickled vegetables (Phak-dong) for potential use as a feed supplement in aquaculture. J Gen Appl Microbiol. 63, 246-253. doi: <https://doi.org/10.2323/jgam.2016.12.002>.

Meidong, R., Khotchanalekha, K., Doolgindachbaporn, S., Nagasawa, T., Nakao, M., Sakai, K., Tongpim, S., 2018. Evaluation of probiotic *Bacillus aerius* B81e isolated from healthy hybrid catfish on growth, disease resistance and innate immunity of Pla-mong *Pangasius bocourti*. Fish Shellfish Immunol. 73, 1-10. doi: <https://doi.org/10.1016/j.fsi.2017.11.032>.

- Menanteau-Ledouble, S., Kumar, G., Saleh, M., El-Matbouli, M., 2016. *Aeromonas salmonicida*: updates on an old acquaintance. Dis Aquat Organ. 120, 49-68. doi: <https://doi.org/10.3354/dao03006>.
- Midhun, S.J., Neethu, S., Vysakh, A., Radhakrishnan, E.K., Jyothis, M., 2018. Antagonism Against Fish Pathogens by Cellular Components/Preparations of *Bacillus coagulans* (MTCC-9872) and It's *In Vitro* Probiotic Characterisation. Curr Microbiol. 75, 1174-1181. doi: <https://doi.org/10.1007/s00284-018-1506-0>.
- Midhun, S.J., Neethu, S., Vysakh, A., Sunil, M.A., Radhakrishnan, E.K., Jyothis, M., 2017. Antibacterial activity of autochthonous bacteria isolated from *Anabas testudineus* (Bloch, 1792) and it's in vitro probiotic characterization. Microb Pathog. 113, 312-320. doi: <https://doi.org/10.1016/j.micpath.2017.10.058>.
- Milton, D.L., 2006. Quorum sensing in vibrios: Complexity for diversification. Int J Med Microbiol. 296, 61-71. doi: <https://doi.org/10.1016/j.ijmm.2006.01.044>.
- Milton, D.L., Chalker, V.J., Kirke, D., Hardman, A., Camara, M., Williams, P., 2001. The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of N-(3-hydroxyhexanoyl)homoserine lactone and N-hexanoylhomoserine lactone. J Bacteriol. 183, 3537-3547. doi: <https://doi.org/10.1128/jb.183.12.3537-3547.2001>.
- Miniero Davies, Y., Xavier de Oliveira, M.G., Paulo Vieira Cunha, M., Soares Franco, L., Pulecio Santos, S.L., Zanolli Moreno, L., Túlio de Moura Gomes, V., Zanolli Sato, M.I., Schiavo Nardi, M., Micke Moreno, A., Becker Saidenberg, A., Rose Marques de Sá, L., Knöbl, T., 2018. *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. Vet Q. 38, 99-105. doi: <https://doi.org/10.1080/01652176.2018.1540070>.
- Mohamad, N., Amal, M.N.A., Saad, M.Z., Yasin, I.S.M., Zulkipli, N.A., Mustafa, M., Nasruddin, N.S., 2019a. Virulence-associated genes and antibiotic resistance patterns of *Vibrio* spp. isolated from cultured marine fishes in Malaysia. BMC Veterinary Research. 15, 176. doi: <https://doi.org/10.1186/s12917-019-1907-8>.
- Mohamad, N., Amal, M.N.A., Yasin, I.S.M., Zamri Saad, M., Nasruddin, N.S., Al-saari, N., Mino, S., Sawabe, T., 2019b. Vibriosis in cultured marine fishes: a review. Aquaculture. 512, 734289. doi: <https://doi.org/10.1016/j.aquaculture.2019.734289>.
- Mohanty, B.R., Sahoo, P.K., 2007. Edwardsiellosis in fish: a brief review. J Biosci. 32, 1331-1344. doi: <https://doi.org/10.1007/s12038-007-0143-8>.
- Monzón-Atienza, L., Bravo, J., Torrecillas, S., Montero, D., Canales, A.F.G., de la Banda, I.G., Galindo-Villegas, J., Ramos-Vivas, J., Acosta, F., 2021. Isolation and

Characterization of a *Bacillus velezensis* D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture. Probiotics Antimicrob Proteins. 13, 1404-1412. doi: <https://doi.org/10.1007/s12602-021-09782-8>.

Moreira, M., Schrama, D., Farinha, A.P., Cerqueira, M., Raposo de Magalhães, C., Carrilho, R., Rodrigues, P., 2021. Fish Pathology Research and Diagnosis in Aquaculture of Farmed Fish; a Proteomics Perspective. Animals : an open access journal from MDPI. 11, 125. doi: <https://doi.org/10.3390/ani11010125>.

Morohoshi, T., Inaba, T., Kato, N., Kanai, K., Ikeda, T., 2004. Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. Journal of Bioscience and Bioengineering. 98, 274-281. doi: [https://doi.org/10.1016/S1389-1723\(04\)00281-6](https://doi.org/10.1016/S1389-1723(04)00281-6).

Morohoshi, T., Yokoyama, Y., Ouchi, M., Kato, N., Ikeda, T., 2009. Motility and the expression of the flagellin protein FliC are negatively regulated by quorum sensing in *Edwardsiella tarda*. J Biosci Bioeng. 108, 314-318. doi: <https://doi.org/10.1016/j.jbiosc.2009.04.006>.

Mukherjee, A., Ghosh, K., 2016. Antagonism against fish pathogens by cellular components and verification of probiotic properties in autochthonous bacteria isolated from the gut of an Indian major carp, *Catla catla* (Hamilton). Aquac Res. 47, 2243-2255. doi: <https://doi.org/https://doi.org/10.1111/are.12676>.

Mukherjee, A., Chandra, G., Ghosh, K., 2019a. Single or conjoint application of autochthonous *Bacillus* strains as potential probiotics: Effects on growth, feed utilization, immunity and disease resistance in Rohu, *Labeo rohita* (Hamilton). Aquaculture. 512, 734302. doi: <https://doi.org/https://doi.org/10.1016/j.aquaculture.2019.734302>.

Mukherjee, A., Banerjee, G., Mukherjee, P., Ray, A.K., Chandra, G., Ghosh, K., 2019b. Antibacterial substances produced by pathogen inhibitory gut bacteria in *Labeo rohita*: Physico-chemical characterization, purification and identification through MALDI-TOF mass spectrometry. Microb Pathog. doi: <https://doi.org/10.1016/j.micpath.2019.02.028>.

Mukherjee, A., Dutta, D., Banerjee, S., Ringø, E., Breines, E.M., Hareide, E., Chandra, G., Ghosh, K., 2016. Potential probiotics from Indian major carp, *Cirrhinus mrigala*. Characterization, pathogen inhibitory activity, partial characterization of bacteriocin and production of exoenzymes. Res Vet Sci. 108, 76-84. doi: <https://doi.org/10.1016/j.rvsc.2016.08.011>.

Mukherjee, A., Dutta, D., Banerjee, S., Ringø, E., Breines, E.M., Hareide, E., Chandra, G., Ghosh, K., 2017. Culturable autochthonous gut bacteria in rohu, *Labeo rohita*. In vitro

growth inhibition against pathogenic *Aeromonas* spp., stability in gut, bio-safety and identification by 16S rRNA gene sequencing. *Symbiosis*. 73, 165-177. doi: <https://doi.org/10.1007/s13199-017-0474-7>.

Mukherjee, S., Bassler, B.L., 2019. Bacterial quorum sensing in complex and dynamically changing environments. *Nat Rev Micro*. 17, 371-382. doi: <https://doi.org/10.1038/s41579-019-0186-5>.

Mzula, A., Wambura, P.N., Mdegela, R.H., Shirima, G.M., 2019. Current State of Modern Biotechnological-Based *Aeromonas hydrophila* Vaccines for Aquaculture: A Systematic Review. *Biomed Res Int*. 2019, 3768948-3768948. doi: <https://doi.org/10.1155/2019/3768948>.

Nahar, S., Mizan, M.F.R., Ha, A.J.-w., Ha, S.-D., 2018. Advances and Future Prospects of Enzyme-Based Biofilm Prevention Approaches in the Food Industry. *Compr Rev Food Sci Food Saf*. 17, 1484-1502. doi: <https://doi.org/10.1111/1541-4337.12382>.

Nair, A.V., Leo Antony, M., Praveen, N.K., Sayooj, P., Raja Swaminathan, T., Vijayan, K.K., 2021. Evaluation of in vitro and in vivo potential of *Bacillus subtilis* MBTDCMFRI Ba37 as a candidate probiont in fish health management. *Microb Pathog*. 152, 104610. doi: <https://doi.org/10.1016/j.micpath.2020.104610>.

Nandi, A., Banerjee, G., Dan, S.K., Ghosh, P., Ghosh, K., Ray, A.K., 2017a. Screening of autochthonous intestinal microbiota as candidate probiotics isolated from four freshwater teleosts. *Current Science*. 113, 767-773.

Nandi, A., Dan, S.K., Banerjee, G., Ghosh, P., Ghosh, K., Ringø, E., Ray, A.K., 2017b. Probiotic Potential of Autochthonous Bacteria Isolated from the Gastrointestinal Tract of Four Freshwater Teleosts. *Probiotics Antimicrob Proteins*. 9, 12-21. doi: <https://doi.org/10.1007/s12602-016-9228-8>.

Nastro, R.A., Arguelles-Arias, A., Ongena, M., Di Costanzo, A., Trifuoggi, M., Guida, M., Fickers, P., 2013. Antimicrobial Activity of *Bacillus amyloliquefaciens* ANT1 Toward Pathogenic Bacteria and Mold: Effects on Biofilm Formation. *Probiotics Antimicrob Proteins*. 5, 252-258. doi: <https://doi.org/10.1007/s12602-013-9143-1>.

Natrah, F.M.I., Ruwandeepika, H.A.D., Pawar, S., Karunasagar, I., Sorgeloos, P., Bossier, P., Defoirdt, T., 2011. Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Vet Microbiol*. 154, 124-129. doi: <https://doi.org/https://doi.org/10.1016/j.vetmic.2011.06.024>.

Nayak, S.K., 2021. Multifaceted applications of probiotic *Bacillus* species in aquaculture with special reference to *Bacillus subtilis*. Rev Aquacult. 13, 862-906. doi: <https://doi.org/https://doi.org/10.1111/raq.12503>.

Nayak, S.K., Swain, P., Mukherjee, S.C., 2007. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). Fish Shellfish Immunol. 23, 892-896. doi: <https://doi.org/10.1016/j.fsi.2007.02.008>.

Newaj-Fyzul, A., Adesiyun, A.A., Mutani, A., Ramsubhag, A., Brunt, J., Austin, B., 2007. *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J Appl Microbiol. 103, 1699-1706. doi: <https://doi.org/10.1111/j.1365-2672.2007.03402.x>.

Nguafack, T.T., Jang, W.J., Hasan, M.T., Choi, Y.H., Bai, S.C., Lee, E.W., Lee, B.J., Hur, S.W., Lee, S., Kong, I.S., 2020. Effects of dietary non-viable *Bacillus* sp. SJ-10, *Lactobacillus plantarum*, and their combination on growth, humoral and cellular immunity, and streptococcosis resistance in olive flounder (*Paralichthys olivaceus*). Res Vet Sci. 131, 177-185. doi: <https://doi.org/10.1016/j.rvsc.2020.04.026>.

Nithya, C., Pandian, S.K., 2010. The *in vitro* antibiofilm activity of selected marine bacterial culture supernatants against *Vibrio* spp. Arch Microbiol. 192, 843-854. doi: <https://doi.org/10.1007/s00203-010-0612-6>.

Nithya, C., Aravindraja, C., Pandian, S.K., 2010. *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. Res Microbiol. 161, 293-304. doi: <https://doi.org/10.1016/j.resmic.2010.03.002>.

Nithya, C., Devi, M.G., Karutha Pandian, S., 2011. A novel compound from the marine bacterium *Bacillus pumilus* S6-15 inhibits biofilm formation in gram-positive and gram-negative species. Biofouling. 27, 519-528. doi: <https://doi.org/10.1080/08927014.2011.586127>.

Oisson, J.C., Jöborn, A., Westerdahl, A., Blomberg, L., Kjelleberg, S., Conway, P.L., 1996. Is the turbot, *Scophthalmus maximus* (L.), intestine a portal of entry for the fish pathogen *Vibrio anguillarum*? J Fish Dis. 19, 225-234. doi: <https://doi.org/10.1111/j.1365-2761.1996.tb00129.x>.

Okutani, K., 1977. Gliotoxin Produced by a strain of *Aspergillus* isolated from marine mud. NIPPON SUISAN GAKKAISHI. 43, 995-1000. doi: <https://doi.org/10.2331/suisan.43.995>.

- Osorio, C.R., Vences, A., Matanza, X.M., Terceti, M.S., 2018. *Photobacterium damsela* subsp. *damsela*, a generalist pathogen with unique virulence factors and high genetic diversity. J Bacteriol. 200, e00002-00018. doi: <https://doi.org/10.1128/jb.00002-18>.
- Palti, Y., 2011. Toll-like receptors in bony fish: from genomics to function. Dev Comp Immunol. 35, 1263-1272. doi: <https://doi.org/10.1016/j.dci.2011.03.006>.
- Pan, J., Huang, T., Yao, F., Huang, Z., Powell, C.A., Qiu, S., Guan, X., 2008. Expression and characterization of *aiiA* gene from *Bacillus subtilis* BS-1. Microbiol Res. 163, 711-716. doi: <https://doi.org/10.1016/j.micres.2007.12.002>.
- Pande, G.S., Natrah, F.M., Flandez, A.V., Kumar, U., Niu, Y., Bossier, P., Defoirdt, T., 2015. Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae. Appl Microbiol Biotechnol. 99, 10805-10813. doi: <https://doi.org/10.1007/s00253-015-6918-1>.
- Park, S.B., Aoki, T., Jung, T.S., 2012. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Vet Res. 43, 67. doi: <https://doi.org/10.1186/1297-9716-43-67>.
- Parsek, M.R., Val, D.L., Hanzelka, B.L., Cronan, J.E., Greenberg, E.P., 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci. 96, 4360-4365. doi: <https://doi.org/10.1073/pnas.96.8.4360>.
- Payne, D.E., Martin, N.R., Parzych, K.R., Rickard, A.H., Underwood, A., Boles, B.R., 2013. Tannic acid inhibits *Staphylococcus aureus* surface colonization in an IsaA-dependent manner. Infect Immun. 81, 496-504. doi: <https://doi.org/10.1128/IAI.00877-12>.
- Pečur Kazazić, S., Topić Popović, N., Strunjak-Perović, I., Florio, D., Fioravanti, M., Babić, S., Čož-Rakovac, R., 2019. Fish photobacteriosis-The importance of rapid and accurate identification of *Photobacterium damsela* subsp. *piscicida*. J Fish Dis. 42, 1201-1209. doi: <https://doi.org/10.1111/jfd.13022>.
- Peng, M., Tong, W., Zhao, Z., Xiao, L., Wang, Z., Liu, X., He, X., Song, Z., 2021. Attenuation of *Aeromonas hydrophila* Infection in *Carassius auratus* by YtnP, a N-acyl Homoserine Lactonase from *Bacillus licheniformis* T-1. Antibiotics (Basel). 10. doi: <https://doi.org/10.3390/antibiotics10060631>.
- Pérez-Sánchez, T., Mora-Sánchez, B., Balcázar, J.L., 2018. Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. Trends Microbiol. 26, 896-903. doi: <https://doi.org/https://doi.org/10.1016/j.tim.2018.05.002>.

- Perez, T., Balcazar, J.L., Ruiz-Zarzuela, I., Halaihel, N., Vendrell, D., de Blas, I., Muzquiz, J.L., 2010. Host-microbiota interactions within the fish intestinal ecosystem. *Mucosal Immunol.* 3, 355-360. doi: <https://doi.org/10.1038/mi.2010.12>.
- Phelan, R.W., Barret, M., Cotter, P.D., O'Connor, P.M., Chen, R., Morrissey, J.P., Dobson, A.D.W., O'Gara, F., Barbosa, T.M., 2013. Subtilomycin: A New Lantibiotic from *Bacillus subtilis* Strain MMA7 Isolated from the Marine Sponge *Haliclona simulans*. *Mar Drugs.* 11, 1878-1898. doi: <https://doi.org/10.3390/md11061878>.
- Pradeepkiran, J.A., 2019. Aquaculture role in global food security with nutritional value: a review. *Translational Animal Science.* 3, 903-910. doi: <https://doi.org/10.1093/tas/txz012>.
- Pujalte, M.J., Sitjà-Bobadilla, A., Macián, M.C., Belloch, C., Álvarez-Pellitero, P., Pérez-Sánchez, J., Uruburu, F., Garay, E., 2003. Virulence and Molecular Typing of *Vibrio harveyi* Strains Isolated from Cultured Dentex, Gilthead Sea Bream and European Sea Bass. *Syst Appl Microbiol.* 26, 284-292. doi: <https://doi.org/http://dx.doi.org/10.1078/072320203322346146>.
- Quinn, G.A., Banat, A.M., Abdelhameed, A.M., Banat, I.M., 2020. Streptomyces from traditional medicine: sources of new innovations in antibiotic discovery. *J Med Microbiol.* 69, 1040-1048. doi: <https://doi.org/10.1099/jmm.0.001232>.
- Rabinovitch, M., 1995. Professional and non-professional phagocytes: an introduction. *Trends Cell Biol.* 5, 85-87. doi: [https://doi.org/10.1016/s0962-8924\(00\)88955-2](https://doi.org/10.1016/s0962-8924(00)88955-2).
- Rahman, M., Colque-Navarro, P., Kuhn, I., Huys, G., Swings, J., Mollby, R., 2002. Identification and characterization of pathogenic *Aeromonas veronii* biovar *sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. *Appl Environ Microbiol.* 68, 650-655.
- Ramesh, D., Souissi, S., Ahamed, T.S., 2017. Effects of the potential probiotics *Bacillus aerophilus* KADR3 in inducing immunity and disease resistance in *Labeo rohita*. *Fish Shellfish Immunol.* 70, 408-415. doi: <https://doi.org/10.1016/j.fsi.2017.09.037>.
- Ramesh, D., Vinothkanna, A., Rai, A.K., Vignesh, V.S., 2015. Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in *Labeo rohita* against *Aeromonas hydrophila*. *Fish Shellfish Immunol.* 45, 268-276. doi: <https://doi.org/10.1016/j.fsi.2015.04.018>.
- Ramírez-Torrez, J.A., Monroy-Dosta, M.d.C., Hernández-Hernández, L.H., Castro-Mejía, J., Bustos-Martínez, J.A., Hamdan-Partida, A., 2018. Presumptive probiotic

isolated from *Oncorhynchus mykiss* (Walbaum, 1792), cultivated in Mexico. Int. J. of Aquatic Science. 9, 3-12.

Ran, C., Carrias, A., Williams, M.A., Capps, N., Dan, B.C.T., Newton, J.C., Kloepper, J.W., Ooi, E.L., Browdy, C.L., Terhune, J.S., Liles, M.R., 2012. Identification of *Bacillus* Strains for Biological Control of Catfish Pathogens. PLoS ONE. 7, e45793. doi: <https://doi.org/10.1371/journal.pone.0045793>.

Rasch, M., Kastbjerg, V.G., Bruhn, J.B., Dalsgaard, I., Givskov, M., Gram, L., 2007. Quorum sensing signals are produced by *Aeromonas salmonicida* and quorum sensing inhibitors can reduce production of a potential virulence factor. Dis Aquat Organ. 78, 105-113. doi: <https://doi.org/10.3354/dao01865>.

Rather, I.A., Galope, R., Bajpai, V.K., Lim, J., Paek, W.K., Park, Y.-H., 2017. Diversity of Marine Bacteria and Their Bacteriocins: Applications in Aquaculture. Rev Fish Sci Aquac. 25, 257-269. doi: <https://doi.org/10.1080/23308249.2017.1282417>.

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. Immunology Letters. 148, 23-33. doi: <https://doi.org/https://doi.org/10.1016/j.imlet.2012.08.003>.

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Fernández Escámez, P.S., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlström, H., Cocconcelli, P.S., Peixe, L., Maradona, M.P., Querol, A., Suarez, J.E., Sundh, I., Vlask, J., Barizzone, F., Correia, S., Herman, L., 2018. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. EFSA Journal. 16, e05131. doi: <https://doi.org/doi:10.2903/j.efsa.2018.5131>.

Romero, M., Martin-Cuadrado, A.B., Roca-Rivada, A., Cabello, A.M., Otero, A., 2011. Quorum quenching in cultivable bacteria from dense marine coastal microbial communities. FEMS Microbiol Ecol. 75, 205-217. doi: <https://doi.org/10.1111/j.1574-6941.2010.01011.x>.

Rončević, T., Puizina, J., Tossi, A., 2019. Antimicrobial Peptides as Anti-Infective Agents in Pre-Post-Antibiotic Era? Int J Mol Sci. 20, 5713. doi: <https://doi.org/10.3390/ijms20225713>.

Roy, R., Tiwari, M., Donelli, G., Tiwari, V., 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 9, 522-554. doi: <https://doi.org/10.1080/21505594.2017.1313372>.

- Saising, J., Dube, L., Ziebandt, A.-K., Voravuthikunchai, S.P., Nega, M., Götz, F., 2012. Activity of gallidermin on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother.* 56, 5804-5810. doi: <https://doi.org/10.1128/AAC.01296-12>.
- Sakai, M., Hikima, J.-i., Kono, T., 2021. Fish cytokines: current research and applications. *Fisheries Sci.* 87, 1-9. doi: <https://doi.org/10.1007/s12562-020-01476-4>.
- Salinas, I., Díaz-Rosales, P., Cuesta, A., Meseguer, J., Chabrilón, M., Moriño, M.Á., Esteban, M.Á., 2006. Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). *Vet Immunol Immunopathol.* 111, 279-286. doi: <https://doi.org/10.1016/j.vetimm.2006.01.020>.
- Sangma, T., Kamilya, D., 2015. Dietary *Bacillus subtilis* FPTB13 and chitin, single or combined, modulate systemic and cutaneous mucosal immunity and resistance of catla, *Catla catla* (Hamilton) against edwardsiellosis. *Comp Immunol Microbiol Infect Dis.* 43, 8-15. doi: <https://doi.org/10.1016/j.cimid.2015.09.003>.
- Sattanathan, G., Tamizhazhagan, V., Padmapriya, S., Liu, W.-C., Balasubramanian, B., 2020. Effect of Green Algae Chaetomorpha antennina Extract on Growth, Modulate Immunity, and Defenses against *Edwardsiella tarda* Infection in Labeo rohita. *Animals : an open access journal from MDPI.* 10, 2033. doi: <https://doi.org/10.3390/ani10112033>.
- Schneider, J., Yepes, A., Garcia-Betancur, J.C., Westedt, I., Mielich, B., López, D., 2012. Streptomycin-induced expression in *Bacillus subtilis* of YtnP, a lactonase-homologous protein that inhibits development and streptomycin production in *Streptomyces griseus*. *Appl Environ Microbiol.* 78, 599-603. doi: <https://doi.org/10.1128/aem.06992-11>.
- Schwenteit, J., Gram, L., Nielsen, K.F., Fridjonsson, O.H., Bornscheuer, U.T., Givskov, M., Gudmundsdottir, B.K., 2011. Quorum sensing in *Aeromonas salmonicida* subsp. *achromogenes* and the effect of the autoinducer synthase AsaI on bacterial virulence. *Vet Microbiol.* 147, 389-397. doi: <https://doi.org/10.1016/j.vetmic.2010.07.020>.
- Secombes, C.J., Wang, T., 2012. 1 - The innate and adaptive immune system of fish. in: Austin, B. (Ed.), *Infectious Disease in Aquaculture*. Woodhead Publishing, pp. 3-68.
- Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C., Zou, J., 2001. Cytokines and innate immunity of fish. *Dev Comp Immunol.* 25, 713-723. doi: [https://doi.org/10.1016/s0145-305x\(01\)00032-5](https://doi.org/10.1016/s0145-305x(01)00032-5).
- Selim, K.M., Reda, R.M., 2015. Improvement of immunity and disease resistance in the Nile tilapia, *Oreochromis niloticus*, by dietary supplementation with

Bacillus amyloliquefaciens. Fish Shellfish Immunol. 44, 496-503. doi: <https://doi.org/10.1016/j.fsi.2015.03.004>.

Sepulcre, M.P., Sarropoulou, E., Kotoulas, G., Meseguer, J., Mulero, V., 2007. *Vibrio anguillarum* evades the immune response of the bony fish sea bass (*Dicentrarchus labrax* L.) through the inhibition of Leukocyte respiratory burst and down-regulation of apoptotic caspases. Molecular Immunology. 44, 3751-3757. doi: <https://doi.org/https://doi.org/10.1016/j.molimm.2007.03.021>.

Sepulcre, M.P., Muñoz, I., Roca, F.J., López-Muñoz, A., Mulero, V., 2010. Molecular strategies used by fish pathogens to interfere with host-programmed cell death. Dev Comp Immunol. 34, 603-610. doi: <https://doi.org/10.1016/j.dci.2010.01.010>.

Serra, C.R., Almeida, E.M., Guerreiro, I., Santos, R., Merrifield, D.L., Tavares, F., Oliva-Teles, A., Enes, P., 2019. Selection of carbohydrate-active probiotics from the gut of carnivorous fish fed plant-based diets. Sci Rep. 9, 6384. doi: <https://doi.org/10.1038/s41598-019-42716-7>.

Setlow, P., 2014. Spore Resistance Properties. Microbiol Spectr. 2. doi: <https://doi.org/10.1128/microbiolspec.TBS-0003-2012>.

Shao, C.P., Hor, L.I., 2001. Regulation of metalloprotease gene expression in *Vibrio vulnificus* by a *Vibrio harveyi* LuxR homologue. J Bacteriol. 183, 1369-1375. doi: <https://doi.org/10.1128/JB.183.4.1369-1375.2001>.

Shefat, S., 2018. Vaccines for infectious bacterial and viral diseases of fish. J Bacteriol Infec Dis. 2018; 2 (2): 1. 5.

Sitjà-Bobadilla, A., Oidtmann, B., 2017. Chapter 5 - Integrated Pathogen Management Strategies in Fish Farming. in: Jeney, G. (Ed.), Fish Diseases. Academic Press, pp. 119-144.

Soltani, M., Ghosh, K., Hoseinifar, S.H., Kumar, V., Lymbery, A.J., Roy, S., Ringø, E., 2019. Genus *Bacillus*, promising probiotics in aquaculture: Aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. Rev Fish Sci Aquac. 27, 331-379. doi: <https://doi.org/10.1080/23308249.2019.1597010>.

Sookchaiyaporn, N., Srisapoome, P., Unajak, S., Areechon, N., 2020. Efficacy of *Bacillus* spp. isolated from Nile tilapia *Oreochromis niloticus* Linn. on its growth and immunity, and control of pathogenic bacteria. Fisheries Sci. 86, 353-365. doi: <https://doi.org/10.1007/s12562-019-01394-0>.

- Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol. 56, 845-857. doi: <https://doi.org/10.1111/j.1365-2958.2005.04587.x>.
- Sumi, C.D., Yang, B.W., Yeo, I.-C., Hahm, Y.T., 2014. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Can J Microbiol. 61, 93-103. doi: <https://doi.org/10.1139/cjm-2014-0613>.
- Sun, Y.Z., Yang, H.L., Ma, R.L., Lin, W.Y., 2010. Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*. Fish Shellfish Immunol. 29, 803-809. doi: <https://doi.org/10.1016/j.fsi.2010.07.018>.
- Suva, M., Sureja, V., Kheni, D., 2016. Novel insight on probiotic *Bacillus subtilis*: Mechanism of action and clinical applications. Journal of Current Research in Scientific Medicine. 2, 65-72. doi: <https://doi.org/10.4103/2455-3069.198381>.
- Swift, S., Lynch, M.J., Fish, L., Kirke, D.F., Tomás, J.M., Stewart, G.S., Williams, P., 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect Immun. 67, 5192-5199. doi: <https://doi.org/10.1128/IAI.67.10.5192-5199.1999>.
- Swift, S., Karlyshev, A.V., Fish, L., Durant, E.L., Winson, M.K., Chhabra, S.R., Williams, P., Macintyre, S., Stewart, G.S., 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. J Bacteriol. 179, 5271-5281. doi: <https://doi.org/10.1128/jb.179.17.5271-5281.1997>.
- Talagrand-Reboul, E., Jumas-Bilak, E., Lamy, B., 2017. The Social Life of *Aeromonas* through Biofilm and Quorum Sensing Systems. Front Microbiol. 8. doi: <https://doi.org/10.3389/fmicb.2017.00037>.
- Tam, N.K., Uyen, N.Q., Hong, H.A., Duc le, H., Hoa, T.T., Serra, C.R., Henriques, A.O., Cutting, S.M., 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J Bacteriol. 188, 2692-2700. doi: <https://doi.org/10.1128/jb.188.7.2692-2700.2006>.
- Tavares Batista, M., Souza, R.D., Paccez, J.D., Luiz, W.B., Ferreira, E.L., Cavalcante, R.C.M., Ferreira, R.C.C., Ferreira, L.C.S., 2014. Gut adhesive *Bacillus subtilis* spores as a platform for mucosal delivery of antigens. Infect Immun. 82, 1414-1423. doi: <https://doi.org/10.1128/IAI.01255-13>.
- Telli, G.S., Ranzani-Paiva, M.J., Dias Dde, C., Sussel, F.R., Ishikawa, C.M., Tachibana, L., 2014. Dietary administration of *Bacillus subtilis* on hematology and non-specific

immunity of Nile tilapia *Oreochromis niloticus* raised at different stocking densities. Fish Shellfish Immunol. 39, 305-311. doi: <https://doi.org/10.1016/j.fsi.2014.05.025>.

Terceti, M.S., Ogut, H., Osorio, C.R., 2016. *Photobacterium damsela* subsp. *damsela*, an Emerging Fish Pathogen in the Black Sea: Evidence of a Multiclonal Origin. Appl Environ Microbiol. 82, 3736-3745. doi: <https://doi.org/10.1128/aem.00781-16>.

Thankappan, B., Ramesh, D., Ramkumar, S., Natarajaseenivasan, K., Anbarasu, K., 2015. Characterization of *Bacillus* spp. from the gastrointestinal tract of *Labeo rohita*: towards to identify novel probiotics against fish pathogens. Appl Biochem Biotechnol. 175, 340-353. doi: <https://doi.org/10.1007/s12010-014-1270-y>.

Tian, Y., Wang, Q., Liu, Q., Ma, Y., Cao, X., Guan, L., Zhang, Y., 2008. Involvement of LuxS in the regulation of motility and flagella biogenesis in *Vibrio alginolyticus*. Biosci Biotechnol Biochem. 72, 1063-1071. doi: <https://doi.org/10.1271/bbb.70812>.

Tinh, N.T.N., Yen, V.H.N., Dierckens, K., Sorgeloos, P., Bossier, P., 2008. An acyl homoserine lactone-degrading microbial community improves the survival of first-feeding turbot larvae (*Scophthalmus maximus* L.). Aquaculture. 285, 56-62. doi: <https://doi.org/https://doi.org/10.1016/j.aquaculture.2008.08.018>.

Tiralongo, F., Messina, G., Lombardo, B.M., Longhitano, L., Li Volti, G., Tibullo, D., 2020. Skin Mucus of Marine Fish as a Source for the Development of Antimicrobial Agents. Frontiers in Marine Science. 7. doi: <https://doi.org/10.3389/fmars.2020.541853>.

Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H., Bossier, P., 2018a. Effect of quorum quenching bacteria on growth, virulence factors and biofilm formation of *Yersinia ruckeri* in vitro and an in vivo evaluation of their probiotic effect in rainbow trout. J Fish Dis. 41, 1429-1438. doi: <https://doi.org/10.1111/jfd.12840>.

Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H., Haghkhah, M., Bossier, P., 2018b. Identification of N-acyl homoserine lactone-degrading bacteria isolated from rainbow trout (*Oncorhynchus mykiss*). J Appl Microbiol. 125, 356-369. doi: <https://doi.org/10.1111/jam.13891>.

Toranzo, A.E., Magariños, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61. doi: <https://doi.org/http://dx.doi.org/10.1016/j.aquaculture.2005.01.002>.

Touraki, M., Frydas, I., Karamanlidou, G., Mamara, A., 2012. Partial purification and characterization of a bacteriocin produced by *Bacillus subtilis* NCIMB 3610 that exhibits antimicrobial activity against fish pathogens.

- Truong Thy, H.T., Tri, N.N., Quy, O.M., Fotedar, R., Kannika, K., Unajak, S., Areechon, N., 2017. Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (*Pangasianodon hypophthalmus*). *Fish Shellfish Immunol.* 60, 391-399. doi: <https://doi.org/10.1016/j.fsi.2016.11.016>.
- Underhill, D.M., Ozinsky, A., 2002. Phagocytosis of Microbes: Complexity in Action. *Annual Review of Immunology.* 20, 825-852. doi: <https://doi.org/10.1146/annurev.immunol.20.103001.114744>.
- Uribe-Querol, E., Rosales, C., 2017. Control of Phagocytosis by Microbial Pathogens. *Front Immunol.* 8. doi: <https://doi.org/10.3389/fimmu.2017.01368>.
- Uribe-Querol, E., Rosales, C., 2020. Phagocytosis: Our Current Understanding of a Universal Biological Process. *Front Immunol.* 11. doi: <https://doi.org/10.3389/fimmu.2020.01066>.
- Valdes, S., Irgang, R., Barros, M.C., Ilardi, P., Saldarriaga-Córdoba, M., Rivera-Bohle, J., Madrid, E., Gajardo-Córdova, J., Avendaño-Herrera, R., 2021. First report and characterization of *Tenacibaculum maritimum* isolates recovered from rainbow trout (*Oncorhynchus mykiss*) farmed in Chile. *J Fish Dis.* 44, 1481-1490. doi: <https://doi.org/10.1111/jfd.13466>.
- Van Doan, H., Hoseinifar, S.H., Khanongnuch, C., Kanpiengjai, A., Unban, K., Van Kim, V., Srichaiyo, S., 2018. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture.* 491, 94-100. doi: <https://doi.org/10.1016/j.aquaculture.2018.03.019>.
- van Kuijk, S., Noll, K.S., Chikindas, M.L., 2012. The species-specific mode of action of the antimicrobial peptide subtilisin against *Listeria monocytogenes* Scott A. *Lett Appl Microbiol.* 54, 52-58. doi: <https://doi.org/10.1111/j.1472-765X.2011.03170.x>.
- Varvarigos, P., 2020. 10. *Photobacterium damsela* subsp. *piscicida*. *Options Méditerranéennes: Série B. Etudes et Recherches*, 83-96.
- Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett Appl Microbiol.* 36, 83-87. doi: <https://doi.org/10.1046/j.1472-765X.2003.01255.x>.

- Verderosa, A.D., Totsika, M., Fairfull-Smith, K.E., 2019. Bacterial Biofilm Eradication Agents: A Current Review. *Frontiers in Chemistry*. 7. doi: <https://doi.org/10.3389/fchem.2019.00824>.
- Vinoj, G., Vaseeharan, B., Thomas, S., Spiers, A.J., Shanthi, S., 2014. Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits *Vibrio* biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. *Mar Biotechnol* (NY). 16, 707-715. doi: <https://doi.org/10.1007/s10126-014-9585-9>.
- Wang, B., Mao, C., Feng, J., Li, Y., Hu, J., Jiang, B., Gu, Q., Su, Y., 2021. A First Report of *Aeromonas veronii* Infection of the Sea Bass, *Lateolabrax maculatus* in China. *Frontiers in Veterinary Science*. 7. doi: <https://doi.org/10.3389/fvets.2020.600587>.
- Wang, W.-Z., Morohoshi, T., Ikenoya, M., Someya, N., Ikeda, T., 2010. AiiM, a novel class of N-acylhomoserine lactonase from the leaf-associated bacterium *Microbacterium testaceum*. *Appl Environ Microbiol*. 76, 2524-2530. doi: <https://doi.org/10.1128/AEM.02738-09>.
- Wang, W., Sun, J., Liu, C., Xue, Z., 2017. Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquac Res*. 48, 1-23. doi: <https://doi.org/https://doi.org/10.1111/are.13161>.
- Wang, X., Wang, Q., Yang, M., Xiao, J., Liu, Q., Wu, H., Zhang, Y., 2011. QseBC controls flagellar motility, fimbrial hemagglutination and intracellular virulence in fish pathogen *Edwardsiella tarda*. *Fish Shellfish Immunol*. 30, 944-953. doi: <https://doi.org/10.1016/j.fsi.2011.01.019>.
- Waters, C.M., Wu, J.T., Ramsey, M.E., Harris, R.C., Bassler, B.L., 2010. Control of the Type 3 Secretion System in *Vibrio harveyi* by Quorum Sensing through Repression of ExsA. *Appl Environ Microbiol*. 76, 4996-5004. doi: <https://doi.org/doi:10.1128/AEM.00886-10>.
- Weber, B., Hasic, M., Chen, C., Wai, S.N., Milton, D.L., 2009. Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. *Environ Microbiol*. 11, 3018-3028. doi: <https://doi.org/10.1111/j.1462-2920.2009.02005.x>.
- Wei, M.Y., Li, D., Shao, C.L., Deng, D.S., Wang, C.Y., 2013. (±)-Pestalachloride D, an antibacterial racemate of chlorinated benzophenone derivative from a soft coral-derived fungus *Pestalotiopsis* sp. *Mar Drugs*. 11, 1050-1060. doi: <https://doi.org/10.3390/md11041050>.

- Wille, J., Coenye, T., 2020. Biofilm dispersion: The key to biofilm eradication or opening Pandora's box? *Biofilm.* 2, 100027. doi: <https://doi.org/https://doi.org/10.1016/j.biofilm.2020.100027>.
- Wu, L., Luo, Y., 2021. Bacterial Quorum-Sensing Systems and Their Role in Intestinal Bacteria-Host Crosstalk. *Front Microbiol.* 12. doi: <https://doi.org/10.3389/fmicb.2021.611413>.
- Wu, L., Kong, L., Yang, Y., Bian, X., Wu, S., Li, B., Yin, X., Mu, L., Li, J., Ye, J., 2019. Effects of Cell Differentiation on the Phagocytic Activities of IgM(+) B Cells in a Teleost Fish. *Front Immunol.* 10, 2225-2225. doi: <https://doi.org/10.3389/fimmu.2019.02225>.
- Wu, Z., Qi, X., Qu, S., Ling, F., Wang, G., 2021. Dietary supplementation of *Bacillus velezensis* B8 enhances immune response and resistance against *Aeromonas veronii* in grass carp. *Fish Shellfish Immunol.* doi: <https://doi.org/10.1016/j.fsi.2021.05.012>.
- Xiu, P., Liu, R., Zhang, D., Sun, C., 2017. Pumilacidin-Like Lipopeptides Derived from Marine Bacterium *Bacillus* sp. Strain 176 Suppress the Motility of *Vibrio alginolyticus*. *Appl Environ Microbiol.* 83, e00450-00417. doi: <https://doi.org/10.1128/AEM.00450-17>.
- Xu, B.H., Ye, Z.W., Zheng, Q.W., Wei, T., Lin, J.F., Guo, L.Q., 2018. Isolation and characterization of cyclic lipopeptides with broad-spectrum antimicrobial activity from *Bacillus siamensis* JFL15. *3 Biotech.* 8, 444. doi: <https://doi.org/10.1007/s13205-018-1443-4>.
- Xu, H.M., Rong, Y.J., Zhao, M.X., Song, B., Chi, Z.M., 2014. Antibacterial activity of the lipopeptides produced by *Bacillus amyloliquefaciens* M1 against multidrug-resistant *Vibrio* spp. isolated from diseased marine animals. *Appl Microbiol Biotechnol.* 98, 127-136. doi: <https://doi.org/10.1007/s00253-013-5291-1>.
- Yang, Z., Zhou, X., Ma, Y., Zhou, M., Waldor, M.K., Zhang, Y., Wang, Q., 2018. Serine/threonine kinase PpkA coordinates the interplay between T6SS2 activation and quorum sensing in the marine pathogen *Vibrio alginolyticus*. *Environ Microbiol.* 20, 903-919. doi: <https://doi.org/https://doi.org/10.1111/1462-2920.14039>.
- Ye, J., Ma, Y., Liu, Q., Zhao, D.L., Wang, Q.Y., Zhang, Y.X., 2008. Regulation of *Vibrio alginolyticus* virulence by the LuxS quorum-sensing system. *J Fish Dis.* 31, 161-169. doi: <https://doi.org/10.1111/j.1365-2761.2007.00882.x>.
- Yi, S.W., You, M.J., Cho, H.S., Lee, C.S., Kwon, J.K., Shin, G.W., 2013. Molecular characterization of *Aeromonas* species isolated from farmed eels (*Anguilla japonica*). *Vet Microbiol.* 164, 195-200. doi: <https://doi.org/10.1016/j.vetmic.2013.02.006>.

- Yi, Y., Zhang, Z., Zhao, F., Liu, H., Yu, L., Zha, J., Wang, G., 2018. Probiotic potential of *Bacillus velezensis* JW: Antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. *Fish Shellfish Immunol.* 78, 322-330. doi: <https://doi.org/10.1016/j.fsi.2018.04.055>.
- Yin, W.-F., Tung, H.-J., Sam, C.-K., Koh, C.-L., Chan, K.-G., 2012. Quorum Quenching *Bacillus sonorensis* Isolated from Soya Sauce Fermentation Brine. *Sensors* (Basel, Switzerland). 12, 4065-4073. doi: <https://doi.org/10.3390/s120404065>.
- Zhang, B., Zhuang, X., Guo, L., McLean, R.J.C., Chu, W., 2019a. Recombinant N-acyl homoserine lactone-Lactonase AiiA(QSI-1) Attenuates *Aeromonas hydrophila* Virulence Factors, Biofilm Formation and Reduces Mortality in Crucian Carp. *Mar Drugs.* 17. doi: <https://doi.org/10.3390/md17090499>.
- Zhang, D., Gao, Y., Ke, X., Yi, M., Liu, Z., Han, X., Shi, C., Lu, M., 2019b. *Bacillus velezensis* LF01: in vitro antimicrobial activity against fish pathogens, growth performance enhancement, and disease resistance against streptococcosis in Nile tilapia (*Oreochromis niloticus*). *Appl Microbiol Biotechnol.* 103, 9023-9035. doi: <https://doi.org/10.1007/s00253-019-10176-8>.
- Zhang, H.-B., Wang, L.-H., Zhang, L.-H., 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci.* 99, 4638-4643. doi: <https://doi.org/10.1073/pnas.022056699>.
- Zhang, J., Kong, X., Zhou, C., Li, L., Nie, G., Li, X., 2014. Toll-like receptor recognition of bacteria in fish: Ligand specificity and signal pathways. *Fish Shellfish Immunol.* 41, 380-388. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2014.09.022>.
- Zhang, M., Sun, K., Sun, L., 2008. Regulation of autoinducer 2 production and luxS expression in a pathogenic *Edwardsiella tarda* strain. *Microbiology.* 154, 2060-2069. doi: <https://doi.org/https://doi.org/10.1099/mic.0.2008/017343-0>.
- Zhang, M., Jiao, X.D., Hu, Y.H., Sun, L., 2009. Attenuation of *Edwardsiella tarda* virulence by small peptides that interfere with LuxS/autoinducer type 2 quorum sensing. *Appl Environ Microbiol.* 75, 3882-3890. doi: <https://doi.org/10.1128/aem.02690-08>.
- Zhang, Y.-A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J., Sunyer, J.O., 2010. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol.* 11, 827-835. doi: <https://doi.org/10.1038/ni.1913>.
- Zhao, J., Chen, M., Quan, C.S., Fan, S.D., 2015. Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. *J Fish Dis.* 38, 771-786. doi: <https://doi.org/10.1111/jfd.12299>.

- Zhao, X.L., Wu, G., Chen, H., Li, L., Kong, X.H., 2020. Analysis of virulence and immunogenic factors in *Aeromonas hydrophila*: Towards the development of live vaccines. J Fish Dis. 43, 747-755. doi: <https://doi.org/10.1111/jfd.13174>.
- Zhou, H., Gai, C., Ye, G., An, J., Liu, K., Xu, L., Cao, H., 2019. *Aeromonas hydrophila*, an Emerging Causative Agent of Freshwater-Farmed Whiteleg shrimp *Litopenaeus vannamei*. Microorganisms. 7, 450. doi: <https://doi.org/10.3390/microorganisms7100450>.
- Zhou, S., Xia, Y., Zhu, C., Chu, W., 2018. Isolation of Marine *Bacillus* sp. with Antagonistic and Organic-Substances-Degrading Activities and Its Potential Application as a Fish Probiotic. Mar Drugs. 16. doi: <https://doi.org/10.3390/md16060196>.
- Zhou, X., Tian, Z., Wang, Y., Li, W., 2010. Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. Fish Physiol Biochem. 36, 501-509. doi: <https://doi.org/10.1007/s10695-009-9320-z>.
- Zhou, Z.-j., Sun, L., 2016. *Edwardsiella tarda* - Induced Inhibition of Apoptosis: A Strategy for Intracellular Survival. Front Cell Infect Microbiol. 6. doi: <https://doi.org/10.3389/fcimb.2016.00076>.
- Zhou, Z., Zhou, X., Zhong, Z., Wang, C., Zhang, H., Li, D., He, T., Li, C., Liu, X., Yuan, H., Ji, H., Luo, Y., Gu, W., Fu, H., Peng, G., 2014. Investigation of antibacterial activity of *Bacillus* spp. isolated from the feces of Giant Panda and characterization of their antimicrobial gene distributions. World J Microbiol Biotechnol. 30, 3129-3136. doi: <https://doi.org/10.1007/s11274-014-1740-y>.

CHAPTER II

Isolation and characterization of fish-gut *Bacillus* spp. as source of Natural Antimicrobial Compounds to fight aquaculture bacterial diseases

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Isolation and characterization of fish-gut *Bacillus* spp. as source of Natural Antimicrobial Compounds to fight aquaculture bacterial diseases

Abstract

Aquaculture is responsible for more than 50% of global seafood consumption. Bacterial diseases are a major constraint to this sector and associated with misuse of antibiotics, pose serious threats to public health. Fish-symbionts, co-inhabitants of fish pathogens, might be a promising source of Natural Antimicrobial Compounds (NACs) alternative to antibiotics, limiting bacterial diseases occurrence in aquafarms. In particular, sporeforming *Bacillus* spp. are known for their probiotic potential and production of NACs antagonistic of bacterial pathogens and are abundant in aquaculture fish guts. Harnessing the fish-gut microbial community potential, 172 sporeforming strains producing NACs were isolated from economically important aquaculture fish species, namely European seabass, gilthead seabream, and white seabream. We demonstrated that they possess anti-growth, anti-biofilm, or anti-quorum-sensing activities, to control bacterial infections and 52% of these isolates effectively antagonized important fish pathogens, including *Aeromonas hydrophila*, *A. salmonicida*, *A. bivalvium*, *A. veronii*, *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Photobacterium damsela*, *Tenacibaculum maritimum*, *Edwardsiella tarda* and *Shigella sonnei*. By *in vitro* quantification of sporeformers' capacity to suppress growth and biofilm formation of fish pathogens, and by assessing their potential to interfere with pathogens communication, we identified three promising candidates to become probiotics or source of bioactive molecules to be used in aquaculture against bacterial aquaculture diseases.

Keywords

Fish diseases; Sporeformers; Bioactivity; Biofilms; Quorum-Quenching

2.1. Introduction

Aquaculture industry is the world's fastest growing animal protein producer. It supplies already 50% of the global seafood consumption, and it is indispensable to satisfy the world's growing fish demand. However, frequent bacterial diseases occurring during fish farming can limit the worldwide growth of this sector (Lafferty, et al., 2015; Munn, 2005; Plumb, Hanson, 2011). In fact, according to the United Nations, bacterial diseases are a major constraint to the economical profitability of the aquaculture industry (Lafferty, et al., 2015; WHO, 2014).

Vibriosis (*Vibrio* spp.), photobacteriosis (*Photobacterium damsela* sp.), tenacibaculosis (*Tenacibaculum maritimum*), edwardsiellosis (*Edwardsiella tarda*), and furunculosis (*Aeromonas salmonicida*) are among the most common bacterial diseases known to affect important marine aquaculture fish species, such as turbot (*Scophthalmus maximus*), European seabass (*Dicentrarchus labrax*), Senegalese sole (*Solea senegalensis*), and gilthead seabream (*Sparus aurata*) (Andreoni, Magnani, 2014; Avendaño-Herrera, et al., 2006; Frans, et al., 2011; Janda, Abbott, 2010; Lafferty, et al., 2015; Menanteau-Ledouble, et al., 2016; Park, et al., 2012; Plumb, Hanson, 2011; Rivas, et al., 2013). The symptomatology of these diseases include haemorrhagic septicemia, ulcers, gill inflammation, exophthalmia, multifocal necrosis and white granulomas in the hematopoietic tissues (Andreoni, Magnani, 2014; Avendaño-Herrera, et al., 2006; Frans, et al., 2011; Janda, Abbott, 2010; Menanteau-Ledouble, et al., 2016; Park, et al., 2012). The exact pathogenetic mechanisms are not fully understood, but virulence factors of the reported Gram-negative bacteria, both extracellular (e.g., cytotoxins, proteases) and cell-wall associated (e.g., adhesins, invasins), are thought to play a prominent role in the development of diseases (Dallaire-Dufresne, et al., 2014; Defoirdt, 2013; Rasmussen-Ivey, et al., 2016). Additionally, most of these pathogens form biofilms (mono- or multi- species communities attached to a surface and embedded in a polysaccharide matrix) that appear determinant for colonization and persistence inside the host (Andreoni, Magnani, 2014; Avendaño-Herrera, et al., 2006; Defoirdt, 2013; Frans, et al., 2011; Janda, Abbott, 2010; Jones, Oliver, 2009; Park, et al., 2012; Wang, et al., 2015). When associated in biofilms, bacteria are generally more resistant to antibiotics and innate immune defences, and also express more virulence factors as result of gene activation by quorum-sensing (Defoirdt, 2013; Fey, 2010; Moons, et al., 2009). Quorum-Sensing (QS) is the molecular strategy used by bacteria to monitor and regulate the size and density of their own population (Abisado, et al., 2018; Miller, Bassler, 2001). QS systems are mediated by small diffusible signalling molecules (e.g., acyl homoserine lactone, autoinducer-2) that regulate virulence, biofilm formation, inter- and intra-specific interactions or antibiotic resistance (Abisado, et

al., 2018; Chu, McLean, 2016; Miller, Bassler, 2001).

Importantly, there is increasing evidence that *Aeromonas* spp., *Vibrio* spp., *Photobacterium damsela* sp., and *Edwardsiella* spp. might be responsible for emerging zoonoses which are dangerous for public health (Cabello, et al., 2016; Gauthier, 2015; Igbinosa, 2016; Khajanchi, et al., 2010). The routes of human infection are considered to be water (swimming or drinking) and water-associated food like fish, shellfish, and raw-edible products contaminated via irrigation. Aquaculture farmers are frequently exposed to fish therefore, they are at higher risk of infection through open wounds, bites, or pincer injuries (Weir, et al., 2012). Furthermore, the susceptibility of the above-mentioned bacteria to traditional antibiotic treatments is not well documented and their resistance to different classes of antibiotics has been reported (Cabello, et al., 2016; Gauthier, 2015; Igbinosa, 2016; Khajanchi, et al., 2010). In this context, it is urgent to find alternative solutions to antibiotics that assure an advanced and integrated health care for humans, animals, and environment.

One of the most promising health-promoting strategies to improve fish resistance to diseases is the use of probiotics, defined as “live organisms which when administrated in adequate amounts confer a health benefit on the host” (Food and Agriculture Organization of the United Nations, 2001). Probiotic bacteria may contribute to the gut microbial balance, enhance the host immune responses, compete with pathogens for adhesion sites and nutrients, or produce natural antimicrobial compounds (NACs). NACs can be metabolites, peptides, or proteins and might inhibit pathogen growth, biofilm formation, and QS (Hai, 2015; Hong, et al., 2005; Verschuere, et al., 2000). Probiotic bacteria have also been implicated in bioremediation and water quality improvement, and have been reported to reduce antibiotic residues in the environment, contributing to the sustainability of aquaculture (Verschuere, et al., 2000).

Among bacteria currently used as probiotics, sporeforming species such as *Bacillus* spp. exhibit critical advantages for application in aquafeeds, as their spores are easily produced in large scale and can be dehydrated, thus facilitating feed incorporation and long-term storage without losing characteristics (Cutting, 2011; Hong, et al., 2005). Moreover, *Bacillus* spp. spores survive and transit through the gut since they are acid and bile tolerant (Tam, et al., 2006). Further, *Bacillus* spp. are recognized to be NAC producers and are capable to antagonize important human and animal pathogens (Abriouel, et al., 2011; Sahoo, et al., 2016; Sumi, et al., 2014). Indeed, we have recently isolated from the gut of European seabass sporeforming *Bacillus* spp. with promising probiotic characteristics, including a broad capacity to inhibit the growth of different fish pathogens (Serra, et al., 2019). Thus, *Bacillus* spp. might be an ideal source of new NACs with anti-growth, anti-biofilm, or anti-QS activities, and thus a useful weapon

against fish pathogens.

Here, we describe the isolation, identification, and characterization of sporeforming *Bacillus* spp. from the gut of fish with different feeding habits, and thus different gastrointestinal structures: the European seabass (*Dicentrarchus labrax*), white seabream (*Diplodus sargus*), and gilthead seabream (*Sparus aurata*). The bacterial isolates and their NACs were tested *in vitro* for their capacity to suppress growth and biofilm formation of fish pathogens, their potential to interfere with pathogens QS, and their suitability to become probiotics for aquaculture.

2.2. Material and Methods

2.2.1. Experimental conditions

The trial was performed at the experimental facilities of the Marine Zoological Station, Faculty of Sciences, Porto University. White sea bream (*Diplodus sargus*) and European sea bass (*Dicentrarchus labrax*) juveniles were obtained from IPMA, Olhão, Portugal, and gilthead sea bream (*Sparus aurata*) juveniles were obtained from Atlantik Fish, Algarve, Portugal. After 15 days of quarantine, fish were transferred to the experimental tanks and acclimatized to the rearing conditions for 15 days. The experiment was performed in a recirculating water system equipped with 9 cylindrical fiberglass tanks of 100 L capacity and thermo-regulated to 22.0 ± 1.0 °C. Tanks were supplied with continuous flow of filtered seawater ($2.5\text{--}3.5$ l min⁻¹), salinity average 34.0 ± 1.0 g l⁻¹ and dissolved oxygen was kept near saturation (7 mg l⁻¹). Seawater was filtered by passing through a biological filter followed by a sand filter and an UV light. Triplicate groups of 10 gilthead seabream (initial body weight (IBW) of 62 g), European seabass (IBW of 30 g), and white seabream (IBW of 40 g) were randomly distributed to each tank. The trial lasted 6 weeks, and during that period, fish were fed by hand twice daily, 6 days a week, until apparent visual satiation with a commercial diet (Skretting, Stavanger, Norway) containing 16% lipids and 47% protein. Fish handling and procedures were performed in compliance with the recommendations of the EEC Directive (2010/63/EU) for care and use of laboratory animals.

2.2.2. Sampling

At the end of the trial, nine fish of each species (3 fish per tank) were randomly captured 4 h after the morning meal (to guarantee that intestines were filled with digesta) and euthanized by a lethal dose (1 mL L⁻¹) of anaesthetic (ethylene glycol monophenyl ether). Digesta contents were collected under aseptic conditions by squeezing the entire gut (without pyloric caeca), previously excised. To reduce inter-fish variation, each sample

was composed of a pool of gut digesta contents from the 3 fish per tank.

2.2.3. Isolation, selection, and characterization of sporeforming bacteria

To select the aerobic bacterial sporeforming isolates, around 150 mg of each digesta were diluted in buffered peptone water (0.9%) and homogenized by vigorous vortexing. Then, serial dilutions (10^{-0} , 10^{-1} , 10^{-2}) of homogenate were prepared in Bott and Wilson (B&W) salts (1.24% K_2HPO_4 , 0.76% H_2PO_4 , 0.1% trisodium citrate, 0.6% $[NH_4]_2SO_4$, pH6.7) and 100 μ L aliquots were spread onto Luria Bertani (LB) agar plates (Becton, Dickinson and Company, USA) after 20-min heat treatment at 65 °C (Barbosa, et al., 2005).

Following incubation at 37 °C for 48 h, colonies obtained from each digesta were counted and randomly selected by their different morphologies. All selected isolates were purified, numbered, and stored at -80 °C in 30% glycerol until used. Spore production was evaluated by phase-contrast microscopy observation of isolates grown for 48 h in solid Difco Sporulation Medium or DSM (Becton Dickinson and Company, USA) (Barbosa, et al., 2005), using a Nikon Eclipse Ci -L microscope, equipped with a CoolLED's pE 300 lite illumination system, and images were obtained with a DS-Ri 2 camera. All isolates were further investigated for catalase activity by suspending a fresh colony in 3% hydrogen peroxide (H_2O_2) solution; the production of air bubbles was considered a positive response (Barbosa, et al., 2005).

2.2.4. Molecular identification and phylogenetic analysis

Genomic DNA of sporeformers was extracted from overnight LB liquid cultures, based on the method of Pitcher, et al. (1989) with few modifications. Briefly, each bacterial pellet was homogenized with 50 mg/mL of lysozyme (Sigma-Aldrich, Germany), in TE buffer (10 mM Tris HCl, 1 mM EDTA) followed the addition of 2 mg/mL of RNase (Sigma-Aldrich, Germany). After incubation at 37 °C for 1 h, 10% SDS, and 0.2 mg/mL of proteinase K (Sigma-Aldrich, Germany) were added, and tubes incubated for 30 min at 55 °C. Then, GES solution (5 M guanidine thiocyanate, 0.5 M EDTA, 10% N-lauroylsarcosine) and 7.5 M ammonium acetate precipitated the remaining proteins prior to nucleic acids extraction with of phenol:chloroform:isoamyl alcohol (25:24:1). After the aqueous phase collection, the addition of chloroform:isoamyl-alcohol (24:1) allowed a re-extraction of the nucleic acids. The DNA was precipitated with 0.6 volumes of isopropanol washed with ice-cold 70% ethanol and dissolved in ultrapure water.

PCR amplification of the 16S rRNA gene was accomplished using universal primers 16S-27F (Weisburg, et al., 1991) and 16S-1492R (Weisburg, et al., 1991), at an

annealing temperature of 55 °C. Each 50 µL reaction contained 1 x DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 0.2 mM of each dNTP (Thermo Scientific, Vilnius, Lithuania), 0.2 µM of each primer (STAB Vida, Lisboa, Portugal), 1.25 U of DreamTaq DNA polymerase enzyme (Thermo Scientific, Vilnius, Lithuania), and 25 ng DNA template. The amplified products were sequenced with the primer 16S-27F (Weisburg, et al., 1991) at STABVIDA (Caparica, Portugal).

Phylogenetic analysis was accomplished online, using the Sequence Match software package through the GenBank non-redundant nucleotide database with BLAST (<http://www.ncbi.nlm.nih.gov>) or by comparison with sequences in the Ribosomal Database Project 10 (<http://rdp.cme.msu.edu/>).

2.2.5. Bacterial strains and culture conditions

The sporeforming bacterial strains isolated from fish gut were denominated “producer strains” and were routinely grown in LB medium at 37 °C, with constant agitation (120 rpm). The laboratory strain *Bacillus subtilis* 168 (Zeigler, et al., 2008) (Table 2.1) was used as control for “producer strains”, and the Gram-positive *Staphylococcus aureus* LMG 2884, was used as control for “indicator strains” due to its inhibition by *B. subtilis* 168 which has been previously described (Serra, et al., 2019). These controls were grown in the same conditions as the “producer strains” and “indicator strains”, respectively. Target fish pathogens presented in Table 2.1 were used as “indicator strains” and were grown aerobically in BHI medium (except for *T. maritimum*, which was grown in Marine medium) at 25 °C (*A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Photobacterium damsela* subsp. *damsela*, *Photobacterium damsela* subsp. *piscicida*, and *T. maritimum*) or 37 °C (for *E. tarda*, *S. sonnei*, and *S. aureus*).

A strain of *Chromobacterium violaceum* (CECT 494) and the *cvil::mini-Tn5* mutant of *Chr. violaceum* CV026 (CECT 5999) (Table 2.1) were used as biosensors for Quorum-Quenching (QQ) detection. Biosensors were routinely cultivated aerobically in LB media at 30 °C for 48 h. *Pseudomonas aeruginosa* PAO-1 was used as positive control for QQ activity and was grown aerobically at 37 °C for 24 h.

Table 2.1. Bacterial strains used in the present study.

Bacterial species	Strain	Origin / Source
Fish pathogens		
<i>Aeromonas salmonicida</i>	LMG 3780	BCCM/LMG
<i>Aeromonas veronii</i>	Fish isolate	NUTRIMU collection
<i>Aeromonas bivalvium</i>	Fish isolate	NUTRIMU collection
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>	LMG 2844	BCCM/LMG
<i>Vibrio anguillarum</i>	DSM 21597	DSMZ
<i>Vibrio harveyi</i>	Fish isolate	NUTRIMU collection
<i>Vibrio parahaemolyticus</i>	LMG 2850	BCCM/LMG
<i>Vibrio vulnificus</i>	LMG 13545	BCCM/LMG
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	LMG 7892	BCCM/LMG
<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	<i>Lg_{h41/01}</i>	Diaz-Rosales, et al. (2003)
<i>Tenacibaculum maritimum</i>	LMG 11612	BCCM/LMG
<i>Edwardsiella tarda</i>	LMG 2793	BCCM/LMG
<i>Shigella sonnei</i>	LMG 10473	BCCM/LMG
Other		
<i>Staphylococcus aureus</i>	LMG 8224	F. Tavares
<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	168 (BGSC1A1)	A.O. Henriques
<i>Chromobacterium violaceum</i> WT	CECT 494	CECT
<i>Chromobacterium violaceum</i> CV026	CECT 5999	CECT
<i>Pseudomonas aeruginosa</i> PAO-1	LMG 12228	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; CECT, Spanish Type Culture Collection, Valencia, Spain), from our laboratory stocks (NUTRIMU collection) or kindly supplied by F. Tavares (Centro de Investigação em Biodiversidade e Recursos Genéticos), M.A. Morinigo (Universidad Málaga), and A. O. Henriques (Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal).

2.2.6. Anti-growth activity screening

The antimicrobial activity of all producer strains was first evaluated by colony overlay assay, as described by Barbosa, et al. (2005). Briefly, producer strains were inoculated as 5 µL spots on LB plates, grown for 24 h and then killed with chloroform vapors. Plates were overlaid with soft BHI agar inoculated with the indicator strains ($OD_{600} \sim 0.1$) and incubated for 24 h at 25 °C or 37 °C (for *S. sonnei* and *E. tarda*). Growth inhibition zones around the colonies, after 24 h incubation at 25 °C or 37 °C as described above, were considered positive and the corresponding growth inhibition halos were measured (mm). All plates were photographed in Gel-Doc™ XR+ System, using Image Lab™ Software (Bio-Rad, EU).

The antimicrobial activity of cell-free supernatant containing NACs of the most promising producer strains (those exhibited a stronger antimicrobial activity against fish pathogens or “indicator strains”) was detected by two independent growth inhibition assays. First, a well-diffusion assay was performed as described by Serra, et al. (2019). The indicator

strains were inoculated ($OD_{600} \sim 0.1$) on BHI agar plates, assuring uniform coverage. Then, 9-mm-diameter wells were punched and filled with 100 μ L cell-free supernatant of each producer strain, previously grown overnight at 37 °C and 150 rpm, centrifuged for 10 min at 11800g, and sterilized by filtration with 0.22 μ m cellulose acetate filter (VWR, Portugal). Growth inhibition zones around the wells after 24 h incubation at 25 °C or 37 °C were considered positive, and the corresponding growth inhibition halos were measured (mm). Plates were photographed as described above. For the second growth inhibition assay (microplate assay), 100 μ L cell-free supernatant of each producer strain (obtained as described above) were added to 96-well flat-bottomed polystyrene plates and inoculated with 100 μ L of indicator bacterial cultures ($OD_{600} \sim 0.1$). A positive control containing 100 μ L of LB medium and 100 μ L of the appropriate bacterial cultures ($OD_{600} \sim 0.1$) and a negative control containing only 200 μ L of LB medium were also prepared. The 96-well microplate was aerobically incubated (120 rpm) at 25 °C, and the optical density at 600 nm (OD_{600}) was measured every hour for 24 h in a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc.). The data is composed by three independent experiments.

2.2.7. Anti-biofilm formation screening

The ability of sporeformers to inhibit biofilm formation of each pathogenic strain was tested by a modification of Papa, et al. (2015) method. In brief, 100 μ L of indicator cultures ($OD_{600} \sim 0.1$) were added to 96-well flat-bottomed polystyrene plates. Each well was filled with 100 μ L of cell-free supernatant of each sporeforming strain. Moreover, 100 μ L of LB medium and 100 μ L of the appropriate bacterial cultures ($OD_{600} \sim 0.1$) was used as positive control, and 200 μ L of LB medium was used as negative control. After 24 h of aerobic incubation at 25 °C, bacterial cells were removed, and the wells were washed 3 times with 250 μ L of Phosphate-Buffered Saline (PBS) and allowed to dry in inverted position. The wells were stained with 250 μ L of 0.1% crystal violet for 15 min at room temperature and rinsed twice with 250 μ L of double-distilled water. After wells' drying (in inverted position), the dye bound to adherent cells was solubilized in 250 μ L of 20% glacial acetic acid and 80% ethanol for 30 min at room temperature. The final quantification of biomass was accessed by measuring the optical density at 590 nm in a Microplate Spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific Inc.). The data is composed by three independent experiments.

2.2.8. Anti-Quorum Sensing (anti-QS) screening (Quorum-Quenching-Potential)

Sporeformers QQ-potential was evaluated as described by McLean, et al. (2004) with few modifications. Briefly, producer strains were inoculated as 5 μ L spots on LB plates

and grown at 37 °C. After overnight incubation, bacterial spots were killed with chloroform vapours for 30 min, followed by plate covers replacement and plates aeration for 20 min. Then, producer strains were overlaid with LB soft agar (0.8% agar) previously inoculated with 100 µL of biosensor strains ($OD_{600} \sim 0.1$), followed by plates incubation for 48 h at 30 °C. In the case of *Chr. violaceum* CV026, the LB soft agar was supplemented with 25 µg mL⁻¹ of kanamycin (Nzytech, Lisboa, Portugal) and N-(β-ketocaproyl)-L-Homoserine Lactone (3-Oxo-C6-HSL) (Sigma-Aldrich, Germany) to 5 µM of final concentration (McClean, et al., 1997), prior to bacterial culture inoculation. A positive QQ result was represented by inhibition of purple pigmentation around the producer strains. The cell-free supernatant screening was performed by overlaying LB plates with LB soft agar (0.8% agar) previously inoculated with 100 µL of biosensor strains ($OD_{600} \sim 0.1$) in the same experimental conditions described above. Once the plates solidified, 9-mm-diameter wells were punched and filled with 100 µL cell-free supernatant of each producer strain (obtained as described above). Zones of purple pigmentation inhibition around the wells after incubation for 48 h at 30 °C were considered a positive result. *P. aeruginosa* PAO-1 and laboratory strain *B. subtilis* 168 were used as positive control for QQ activity and for bacterial growth, respectively. All digital photos were taken with a Sony IMX240 camera and zones (in mm) of pigment inhibition recorded.

2.2.9. Sporulation capacity

Sporulation of each “producer strain” was induced by nutrient exhaustion in DSM (Difco Sporulation Medium) for 24 h at 37 °C in an orbital shaker at 150 rpm. Grown cultures were serial diluted in B&W isotonic buffer (Bott and Wilson salts: 1.24% K₂HPO₄, 0.76% H₂PO₄, 0.1% trisodium citrate, 0.6% [NH₄]₂SO₄, pH6.7), and plated in LB agar before and after 20 min heat treatment at 80 °C, to determinate heat-resistant spores. After incubation at 37 °C for 24 h, visible colonies were counted and the sporulation efficiency determined by quantifying colony forming units (CFU/mL) titer before and after the heat treatment (Barbosa, et al., 2005; Nicholson, Setlow, 1990). The data is composed of three independent experiments.

2.2.10. Antibiotic susceptibility test

Antibiotic susceptibility of “producer strains” was determined by the disk diffusion method of Kirby-Bauer (Bauer, et al., 1966), using antimicrobial susceptibility discs (Oxoid Limited, Thermo Fisher Scientific Inc.) containing teicoplanin 30 µg/disc (TEC₃₀), vancomycin 30 µg/disc (VA₃₀), chloramphenicol 30 µg/disc (C₃₀), tetracycline 30 µg/disc (TE₃₀), erythromycin 15 µg/disc (E₁₅), gentamycin 10 µg/disc (CN₁₀), kanamycin

30 µg/disc (K_{30}), and streptomycin 10 µg/disc (S_{10}). The antibiotics were tested followed the recommendations of the European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed (FEEDAP, 2012). Bacterial inoculums were prepared in sterile saline solution, at an optical density adjusted to 0.5 McFarland standard units ($OD_{600} \sim 0.1$) and were spread with cotton swab onto 20 mL MH (Muller-Hinton, Oxoid) agar plates, assuring full coverage, and the antibiotics were distributed on the plates with a disk dispenser (Oxoid Limited, Thermo Fisher Scientific Inc.). After 24 h of incubation at 37 °C, bacteria were classified as sensitive (S), intermediate (I), and resistant (R), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013).

2.2.11. Statistical analysis

The statistical analysis was done using the SPSS 23.0 software package for Windows. Before any test, data were checked for normal distribution and subject to Levene's test to ensure homogeneity of variances that complies the requirements of ANOVA.

Repeated measures ANOVA and one-way ANOVA were performed to evaluate differences in the ability of sporeforming isolates to inhibit pathogens growth and biofilm formation, respectively. When p -values were significant ($p < 0.05$), means were compared with Dunnet's test.

2.3. Results

2.3.1. Culturable aerobic sporeformers are abundant in the gut of marine fish

The heat-treated gut content of each fish species allowed selection, isolation, and purification of 176 bacterial isolates (61 from gilthead seabream, 51 from white seabream, and 64 from European seabass). Strain's selection was based on macroscopic differences in colony morphologies and also on the collection of samples from different animal groups (tanks). Spore production analysis, induced by nutrient exhaustion in DSM, revealed that 172 strains (98%) produced endospores of different sizes and shapes (Table 2.2 and Figure 2.1), and were catalase positive, indicating that these were probably *Bacillus* spp. and not aero-tolerant strains of *Clostridium* spp. (catalase negative) (Table 2.2).

Table 2.2. Identification and characterization of 41 sporeformers isolated from the intestinal contents of white seabream (WSB), gilthead seabream (GSB) and European seabass (ESB).

Isolate ^a	Source	Tank	16S rRNA gene analysis ^b		GenBank accession	Sporulation test ^c	Catalase test ^d
			Closest known species	% ID			
FI300	GSB	1	<i>Bacillus</i> sp.	98	MT081444	+	+
FI302	GSB	1	<i>B. subtilis</i>	98	MT081445	+	+
FI304	GSB	1	<i>B. subtilis</i>	99	MT081446	+	+
FI307	GSB	2	<i>B. licheniformis</i>	98	MT081447	+	+
FI314	GSB	2	<i>B. subtilis</i>	97	MT081448	+	++
FI321	GSB	2	<i>Bacillus</i> sp.	99	MT081449	+	++
FI324	GSB	2	<i>B. cereus</i>	99	MT081450	+	++
FI326	GSB	2	<i>B. subtilis</i> ; <i>B. cereus</i>	99	MT081451	+	++
FI330	GSB	3	<i>B. subtilis</i>	98	MT081452	+	++
FI333	GSB	3	<i>B. methylotrophicus</i>	97	MT081453	+	+
FI335	GSB	3	<i>B. methylotrophicus</i>	98	MT081454	+	++
FI338	GSB	3	<i>B. subtilis</i>	98	MT081455	+	++
FI342	GSB	3	<i>B. licheniformis</i>	98	MT081456	+	++
FI347	GSB	3	<i>Bacillus</i> sp.	98	MT081457	+	+
FI348	GSB	3	<i>B. licheniformis</i>	99	MT081458	+	+
FI353	WSB	1	<i>B. subtilis</i>	96	MT081460	+	+
FI354	WSB	1	<i>B. subtilis</i>	98	MT081461	+	+
FI355	WSB	1	<i>B. subtilis</i>	98	MT081462	+	+
FI359	WSB	1	<i>B. subtilis</i>	98	MT081463	+	++
FI361	WSB	1	<i>B. licheniformis</i>	99	MT081464	+	++
FI367	WSB	2	<i>B. amyloliquefaciens</i> ; <i>B. methylotrophicus</i>	99	MT081465	+/-	+
FI368	WSB	2	<i>B. subtilis</i>	98	MT081466	+	+
FI373	WSB	2	<i>B. subtilis</i>	97	MT081467	+	+
FI375	WSB	3	<i>B. pumilus</i>	97	MT081468	+	+
FI376	WSB	3	<i>B. subtilis</i>	97	MT081469	+	++
FI377	WSB	3	<i>B. subtilis</i>	98	MT081470	+	++
FI378	WSB	3	<i>B. subtilis</i>	99	MT081471	+/-	+
FI387	WSB	3	<i>B. subtilis</i>	98	MT081472	+	++
FI390	WSB	3	<i>Bacillus</i> sp.	99	MT081473	+	+
FI401	ESB	1	<i>B. subtilis</i>	97	MT081474	+	+
FI414	ESB	1	<i>B. methylotrophicus</i>	99	MT081475	+	+
FI423	ESB	2	<i>B. amyloliquefaciens</i>	99	MT081476	+	++
FI424	ESB	2	<i>Bacillus</i> sp.	99	MT081477	+	+
FI429	ESB	2	<i>B. amyloliquefaciens</i>	100	MT081478	+	+
FI436	ESB	3	<i>B. subtilis</i>	98	MT081479	+	++
FI442	ESB	3	<i>B. subtilis</i>	98	MT081480	+	++
FI455	ESB	3	<i>B. subtilis</i>	97	MT081481	+	+
FI456	ESB	3	<i>B. subtilis</i>	99	MT081482	+	+
FI464	ESB	3	<i>B. safensis</i>	99	MT081483	+	+
FI469	ESB	3	<i>B. subtilis</i>	98	MT081484	+	+
FI480	ESB	3	<i>B. subtilis</i>	99	MT081485	+	+

^a In bold are the 8 isolates with the most promising antimicrobial activities. ^b Closest known species using BLAST based on partial sequences of 16S rRNA gene (~700nt); ^c Spores detected by phase-contrast microscopy of 24 h cultures in DSM agar; + extensive formation of spores; +/- limited formation of spores. ^d Catalase activity tested by resuspending a colony in a 3% solution of hydrogen peroxide (Sigma); ++ Fast positive reaction; + Slow positive reaction

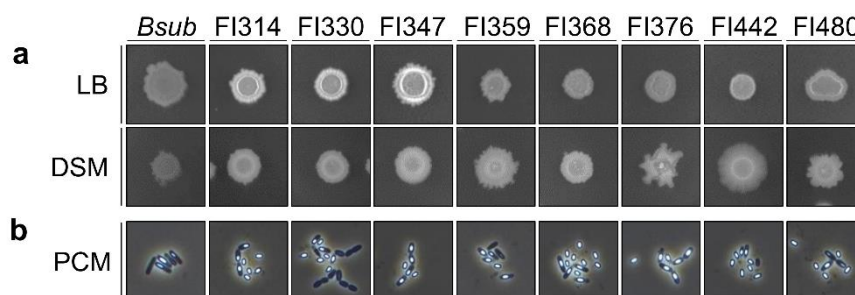


Figure 2.1 (a) Morphological diversity of representative sporeforming isolates (FI numbers on top) obtained from fish intestinal contents. Photographs of colonies grown for 24h at 37 °C in LB (Luria-Bertani) and DSM (Difco Sporulation Medium) agar medium were taken in a Gel-DocTM XR+ System, using the Image LabTM Software (Bio-Rad, EU) and are at the same scale. **(b)** Sporulation capacity of the fish isolates, illustrating refractile sporulating cells and free spores. Spore production was evaluated by phase-contrast microscopy (PCM) using a Nikon Eclipse Ci -L microscope, equipped with a CoolLED's pE 300 lite illumination system, of isolates cultured for 48h in solid DSM. Images were obtained with a DS-Ri 2 camera, all in the same conditions and at the same scale. The laboratory strain *B. subtilis* 168 (*Bsub*, on top) was used as a control.

2.3.2. *Bacillus* sporeformers from fish-gut are promising antagonists of fish-pathogens

Among the 172 endospore-forming Fish-Isolates (FIs) evaluated for their antimicrobial activity against a wide range of fish pathogens, 52% (90 isolates) were able to inhibit at least one of the tested pathogenic strains, and 23% (41 isolates) inhibited 2 or more pathogens. The later 41 isolates were identified by partial sequencing of the 16S rRNA gene (~ 700 kb), revealing a clear abundance of *B. subtilis* (54%) among the sporeformers isolated from the gut of gilthead seabream, white seabream, and European seabass (Table 2.2). *B. licheniformis* and *B. methylotrophicus* represented 9% and *B. amyloliquefaciens* represented 7% of the identified strains, whereas other bacterial species were present in smaller quantities. The identification of bacterial species was inconclusive for 10% of the strains (Table 2.2).

The colony-overlay assay allowed a progressive selection of the 8 most promising fish-isolates according to the size of the inhibitory halos as well as the number of fish pathogens that they inhibited. The 8 FIs with the most promising antimicrobial activities, namely FI314, FI330, FI347, FI359, FI368, FI376, FI442, and FI480, were then tested simultaneously (via colony-overlay assay), in the exact same conditions (Figure 2.2). Strains FI314, FI330, and FI442 showed antagonistic capacity against the growth of all pathogenic strains, except for *S. sonnei*. FI347 was active against *A. hydrophila* and *A. veronii*. FI359 inhibited the growth of *A. hydrophila*, *A. veronii*, *V. harveyi*, *S. aureus* and, in a smaller extent, of *Ph. damsela* subsp. *piscicida*. FI368, FI376, and FI480 inhibited the growth of *S. aureus*, *V. harveyi*, and *A. veronii* and, less markedly, that of *P. damsela* subsp. *piscicida*.

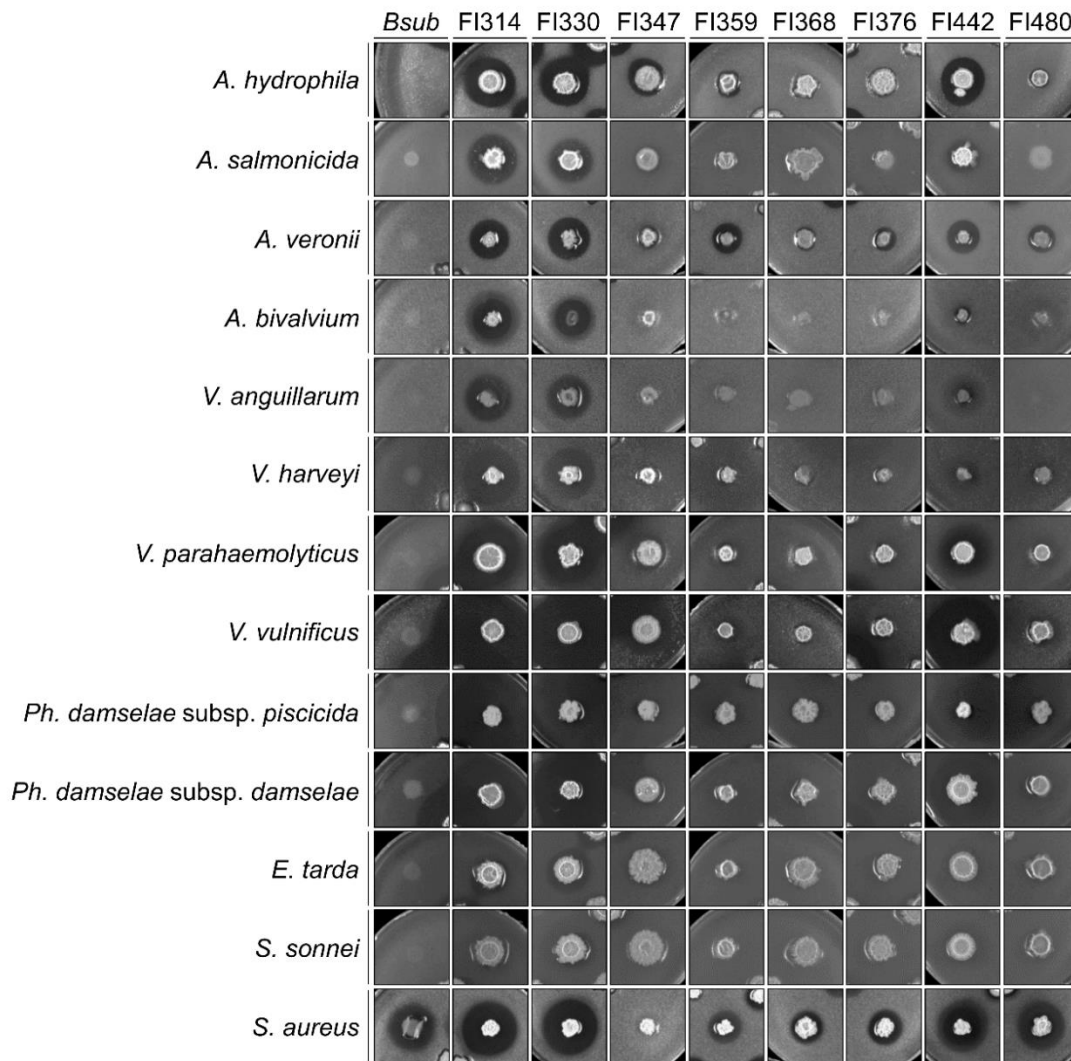


Figure 2.2 Growth inhibition zones for the indicator strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela* subsp. *piscicida*, *Ph. damsela* subsp. *damsela*, *E. tarda*, *S. sonnei* and *S. aureus* around colonies of sporeforming fish isolates (FI numbers on top). The laboratory strain *B. subtilis* 168 was used as a control. All photos were taken in a Gel-DocTM XR+ System, using the Image LabTM Software (Bio-Rad, EU) and are at the same scale.

2.3.3 Antimicrobial activity of *Bacillus* sporeformers is mediated by extracellular NACs

The antimicrobial activity of cell-free supernatant of the most promising *Bacillus* sporeformers (FI314, FI330, FI347, FI359, FI368, FI376, FI442, and FI480) is illustrated in Figure 2.3. The extracellular compounds (hereinafter referred as extracellular NACs) of FI314 and FI330 produced clear inhibitory halos against *A. salmonicida*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela* subsp. *piscicida*, *Ph. damsela* subsp. *damsela*, *T. maritimum*, and *S. aureus*, and small inhibitory halos (2 mm, not clearly visible in Figure 2.3) against *A. bivalvium*. FI442 extracellular NACs inhibited *V. vulnificus* and *Ph. damsela* subsp. *damsela*. The remaining strains (FI347, FI359, FI368, FI376, and FI480) did not inhibit the growth of the tested fish pathogens

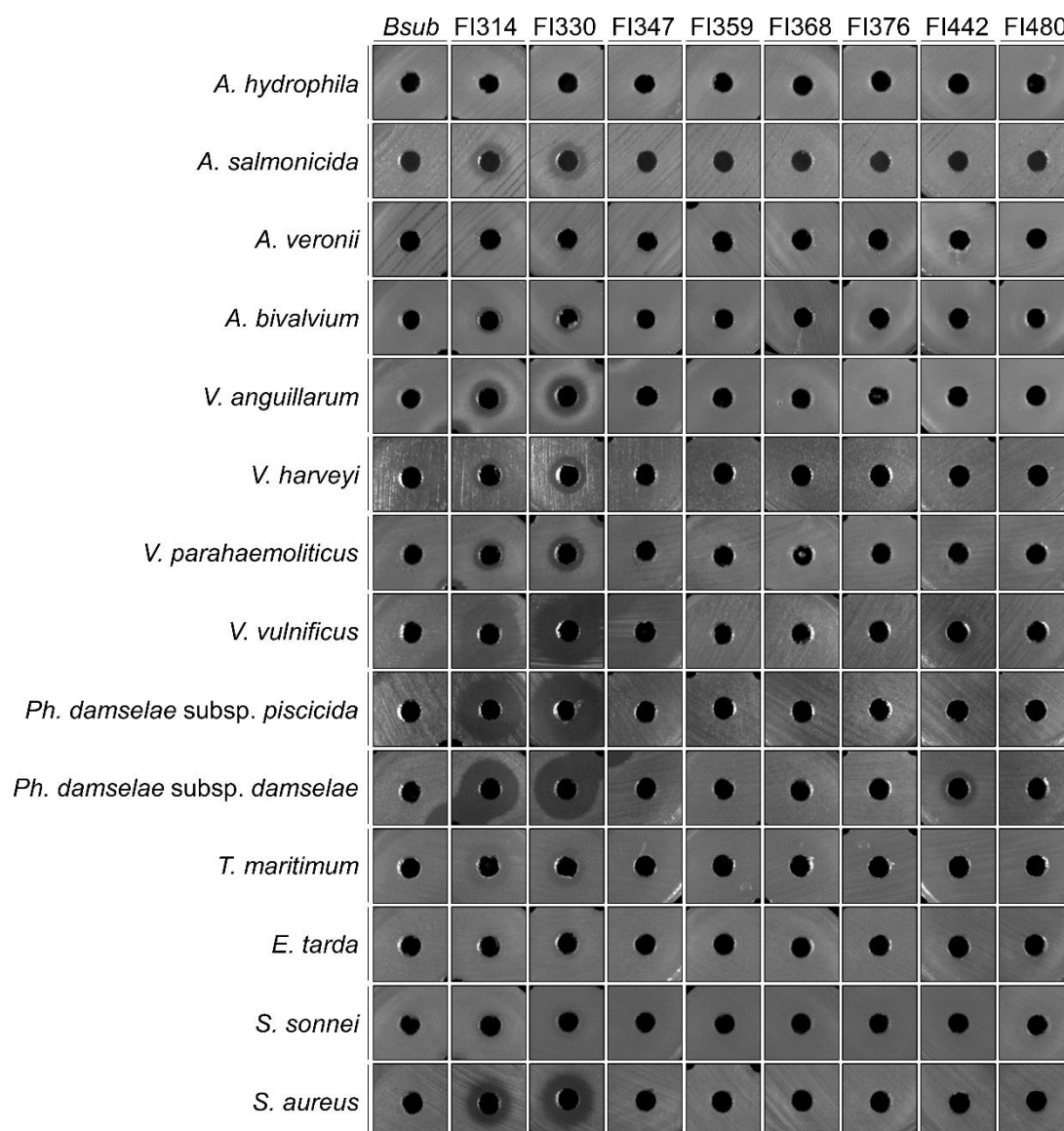


Figure 2.3 Growth inhibition zones for the indicator strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela* subsp. *piscicida*, *Ph. damsela* subsp. *damsela*, *T. maritimum*, *E. tarda*, *S. sonnei* and *S. aureus* around the wells with cell-free supernatant of sporeforming fish isolates (FI numbers on top). All photos were taken in a Gel-Doc™ XR+ System, using the Image Lab™ Software (Bio-Rad, EU) and are at the same scale. The laboratory strain *B. subtilis* 168 (*Bsub* on top) was used as a control.

Based on the results of these experiments, six producer strains were selected having the best antimicrobial potential: FI314, FI330, FI359, FI376, FI442, FI480. The antimicrobial pattern of their extracellular NACs against the same fish pathogens was quantified via a growth-inhibition assay in liquid medium (microplate assay). FI314, FI330, and FI442 extracellular NACs were the most efficient in inhibiting bacterial growth (Figure 2.4). In particular, the extracellular NACs of strains FI314 and FI330 reduced the growth of all the pathogens ($p < 0.001$). Furthermore, these two strains completely inhibited the growth of *A. salmonicida*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela* subsp. *piscicida*, *Ph. damsela* subsp. *damsela*, *T.*

maritimum, and *S. aureus* for the entire duration of the assay (24 h). The extracellular NACs of FI442 extended the lag phase duration of *V. anguillarum* and *Ph. damsela* subsp. *piscicida* growth curves, and completely inhibited the growth of *V. vulnificus* and *Ph. damsela* subsp. *damsela* for 24 h. In general, the NACs of this isolate reduced the growth of *A. salmonicida*, *V. anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela* subsp. *piscicida*, *T. maritimum*, *S. aureus*, and *Ph. damsela* subsp. *damsela* ($p < 0.01$). The extracellular NACs of FI376 reduced the growth of *T. maritimum* and *S. aureus* ($p < 0.01$), whereas those of FI480 reduced the growth of *Ph. damsela* subsp. *damsela* and *T. maritimum* ($p < 0.01$). Interestingly, the growth of *A. salmonicida* and *V. harveyi* was higher in the presence of FI359 and FI376 ($p < 0.05$) cell-free supernatants when compared to the control. FI480 cell-free supernatant also increased *A. salmonicida* growth ($p < 0.05$).

Overall, only the fish isolates FI314, FI330, and FI442 were capable of suppressing fish pathogen's growth for 24 h in microplate (Figure 2.4) produce clear inhibitory halos against the bacteria in the well agar diffusion assay (Figure 2.3).

2.3.4. Extracellular NACs of *Bacillus* sporeformers inhibit fish-pathogen biofilms

Cell-free supernatants of the six isolates tested above (FI314, FI330, FI359, FI376, FI442, FI480) were also evaluated for their ability to interfere with biofilm formation of each fish pathogen (Figure 2.5). The extracellular NACs of FI314 and FI330 decreased the biofilm formation of *A. salmonicida*, *V. anguillarum*, *V. parahaemolyticus*, *Ph. damsela* subsp. *damsela*, *T. maritimum*, and *S. sonnei* ($p < 0.01$). FI330 also reduced *A. hydrophila* biofilm ($p < 0.05$). The extracellular NACs of FI359 and FI376 were active on biofilms of *A. hydrophila*, *A. salmonicida* and *S. sonnei*, whereas those of FI442 and FI480 reduced the biofilm synthesis of *A. salmonicida* and *S. sonnei* ($p < 0.05$). The control *B. subtilis* 168 was also able to decrease the biofilm synthesis of *T. maritimum* ($p < 0.05$).

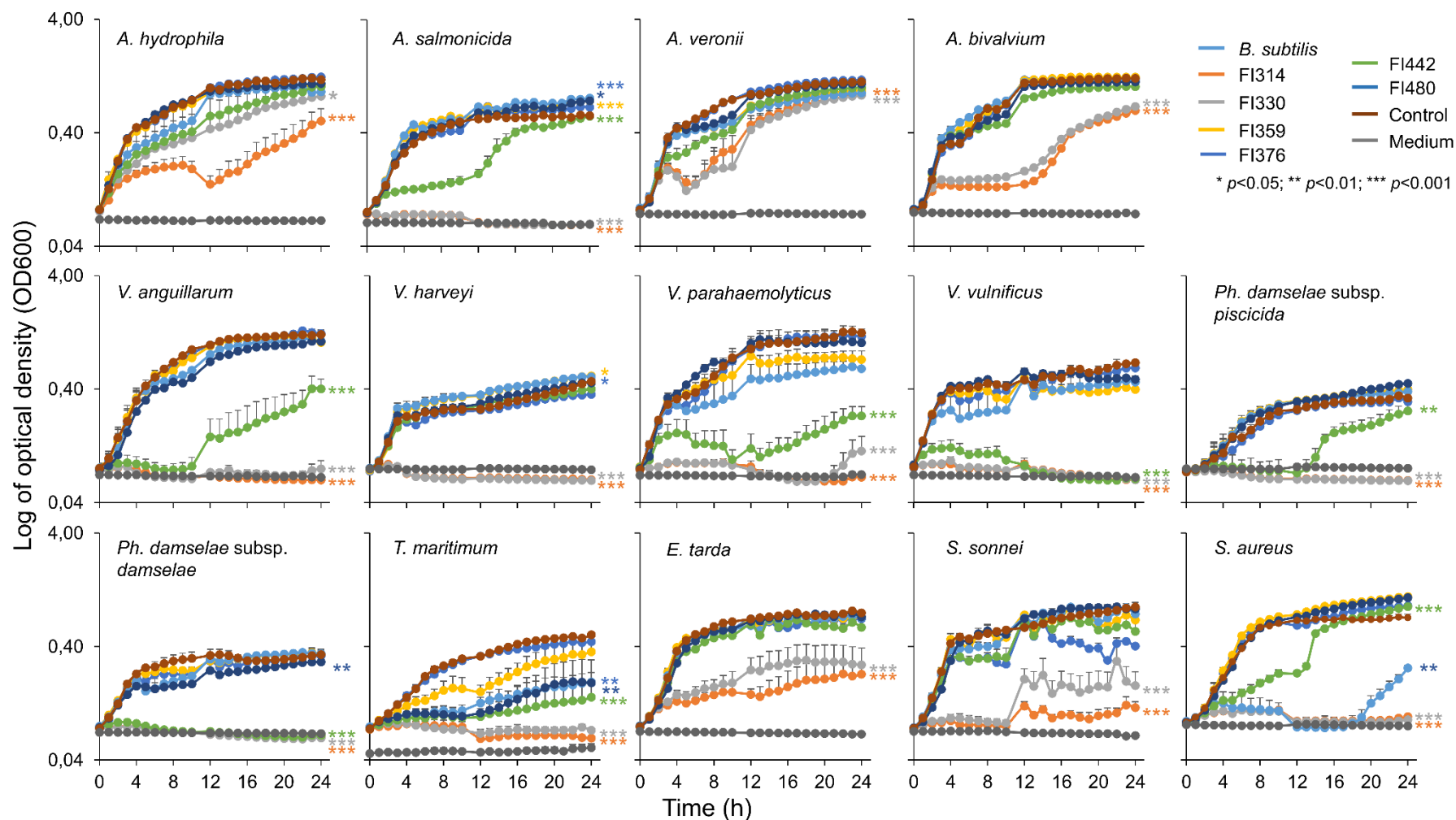


Figure 2.4 Growth inhibition assays of the indicator strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela subsp. piscicida*, *Ph. damsela subsp. damsela*, *T. maritimum*, *E. tarda*, *S. sonnei* and *S. aureus* when cultured alone in Brain Heart Infusion (BHI) or Marine (MA) medium (in the case of *T. maritimum*), or supplemented with cell-free supernatant of the sporeforming isolates (FI numbers). Pathogenic strains growing without cell-free supernatant's supplementation were used as positive control (Control), and BHI or MA medium alone used as negative control (medium). The laboratory strain *B. subtilis* 168 was used as a control for bacterial growth. The data are obtained from three independent experiments. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

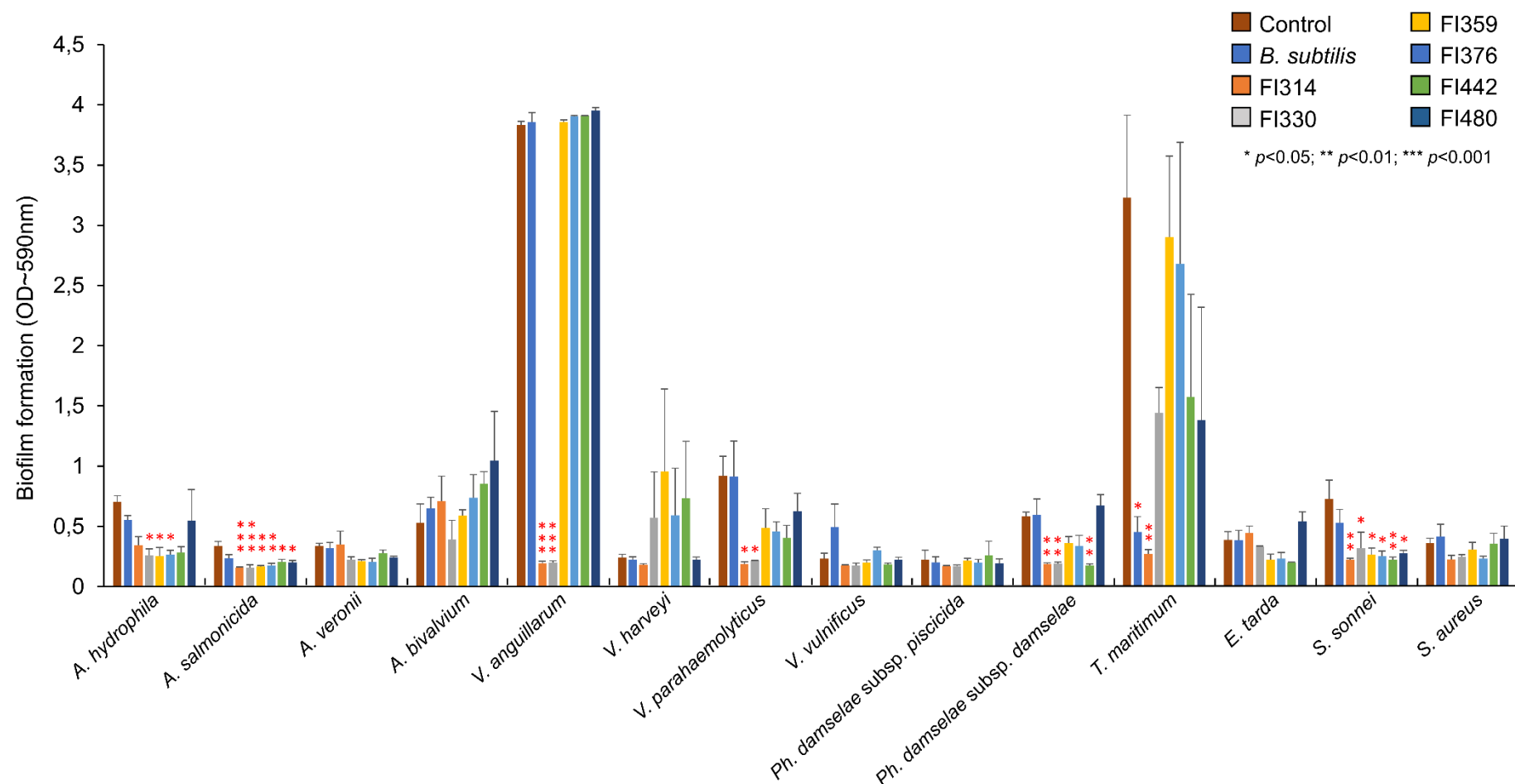


Figure 2.5 Biofilm formation of the fish pathogens *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela subsp. piscicida*, *Ph. damsela subsp. damsela*, *T. maritimum*, *E. tarda*, *S. sonnei* and *S. aureus* when cultured alone in Brain Heart Infusion (BHI) or Marine (MA) medium (in the case of *T. maritimum*), or supplemented with cell-free supernatant of the sporeforming isolates (FI numbers). The laboratory strain *B. subtilis* 168 was used as a control for bacterial growth. The data are obtained from three independent experiments. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

2.3.5. Extracellular NACs of *Bacillus* sporeformers have QQ activity

Chr. violaceum WT CECT 494 produces and senses several AHLs (e.g., N-(3-hydroxydecanoyl)-L-homoserine lactone and N-octanoyl-L-Homoserine lactone), whereas *Chr. violaceum* CV026 is unable to synthesize AHL, but responds to exogenous AHLs supplementation.

QQ was confirmed by the loss of purple violacein pigmentation around the sporeforming strains FI314, FI330, and FI442, suggesting the inactivation of AHLs produced by *Chr. violaceum* WT CECT 494 and of the 3-oxo-C6-HSL signal molecule externally added to *Chr. violaceum* CV026 biosensor (Figure 2.6a). Additionally, the observed QQ activity of FI314 and FI330 was due to extracellular NACs, as their cell-free supernatants displayed activity against 3-oxo-C6-HSL signal molecule and interfered with the AHLs produced by *Chr. violaceum* WT CECT 494 to a lower extent (Figure 2.6b). On the contrary, FI442 cell-free supernatant did not shown any QQ activity. The control *P. aeruginosa* PAO-1 effectively degraded the AHLs tested in all the experiments performed during the present study.

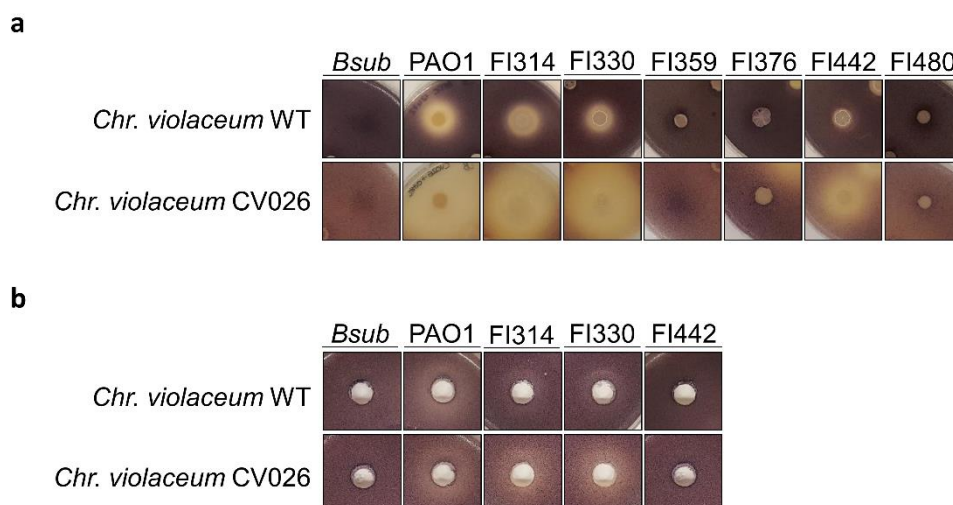


Figure 2.6 Degradation of the violacein pigment produced by *Chr. violaceum* biosensors around colonies of sporeforming fish isolates **(a)** or around the wells with cell-free supernatant of sporeforming fish isolates **(b)** The laboratory strain *B. subtilis* 168 (*Bsub*) was used as a control for bacterial growth and *P. aeruginosa* PAO-1 as control for quorum-quenching (QQ) activity. All photos were taken with a Sony IMX240 camera and are at the same scale.

2.3.6. Most promising *Bacillus* fish-gut sporeformers have probiotic potential

Sporulation efficiency of the most promising strains (FI314, FI330, FI442) was determined due to their potential industrial application as probiotics. By comparison with the laboratory strain *B. subtilis* 168 (Zeigler, et al., 2008), all the selected strains showed good spore titres (around 10^8 CFU mL⁻¹), and sporulating efficiency $\geq 79\%$ (Table 2.3). All “producer strains” were also sensitive to all the antibiotics required by EFSA (2012), with the exception of FI314 isolate that presented an intermediate susceptibility to streptomycin (S₁₀) (Table 2.3)

Table 2.3. Sporulating efficiency and antibiotic susceptibility profile to antibiotics recommended by the European Food Safety Authority (EFSA) of the three most promising strains.

	<i>B. subtilis</i>	FI314	FI330	FI442
Sporulation efficiency ^a				
Viable cells (CFU/mL)	2.6x10 ⁸	2.1x10 ⁸	4.2x10 ⁸	5.0x10 ⁸
Heat-resistant cells (CFU/mL)	1.3x10 ⁸	2.4x10 ⁸	3.8 x10 ⁸	3.9x10 ⁸
Sporulation efficiency (%)	51	100	89	79
Antibiotic susceptibility profile ^b				
TEC ₃₀	S	S	S	S
VA ₃₀	S	S	S	S
C ₃₀	S	S	S	S
TE ₃₀	S	S	S	S
E ₁₅	S	S	S	S
CN ₁₀	S	S	S	S
K ₃₀	S	S	S	S
S ₁₀	S	I	S	S

^a Sporulation was induced by nutrient exhaustion in DSM (Difco Sporulating medium). At 24h after inoculation, viable and heat-treated cells were measured by plating serial dilutions before and after 20 minutes heat treatment at 80°C, respectively. Sporulation efficiency was calculated as the quotient between the heat-resistant cells and the total viable cells. ^b Strain's susceptibility to teicoplanin 30ug/disc (TEC₃₀), vancomycin 30ug/disc (VAN₃₀), chloramphenicol 30ug/disc (C₃₀), tetracycline 30ug/disc (TE₃₀), erythromycin 15ug/disc (E₁₅), gentamycin 10ug/disc (CN₁₀), kanamycin 30ug/disc (K₃₀), streptomycin 10ug/disc (S₁₀). Each strain was classified as sensitive (S) or intermediate (I) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). No resistant (R) strains were found.

2.4. Discussion

It is well established that gastrointestinal microbiota is a key-player in human and animal health (Wang, et al., 2018a; Wang, et al., 2018b), and the use of probiotics represents an excellent approach to improve gut microbial balance and host health (Dimitroglou, et al., 2011). One important criterion for probiotic selection is the capacity to minimize pathogens' growth by competitive exclusion or through the production of antimicrobial molecules (Banerjee, Ray, 2017; Dobson, et al., 2012; Hai, 2015; Verschuere, et al., 2000). The aquaculture sector growth is limited by frequent infection episodes (Lafferty, et al., 2015; WHO, 2014), but no specifically dedicated probiotics have so far been developed.

This study assumed that probiotics would be potentially more effective if originated from the gut of target animals and from the target pathogens ecological niche. Focus was on *Bacillus* species, which are natural habitants of the animal's gut, including fish (Barbosa, et al., 2005; Guo, et al., 2016; Hong, et al., 2005; Midhun, et al., 2017; Tam, et al., 2006; Zhou, et al., 2014) and are known producers of NACs capable of inhibiting pathogen's growth and proliferation (Abriouel, et al., 2011; Caulier, et al., 2019). In addition, different *Bacillus* spp. are commercially available probiotic products for human and animal use. Specifically, *B. subtilis*, *B. vallismortis*, *B. mojavensis*, *B. atrophaeus*, *B. amyloliquefaciens*, and *B. licheniformis* are included in the EFSA list of Qualified

Presumption of Safety (QPS). However, no *Bacillus* probiotic is yet available for fish in the EU. To fulfil this market gap, we isolated several putative probiotic sporeforming *Bacillus* spp. from the gut of three marine aquaculture species, including gilthead seabream and European seabass (the most important fish species reared in the Mediterranean), and white seabream (a species with potential for aquaculture).

More than 50% of the *Bacillus* spp. isolates were active against at least one of the 14 tested bacterial fish pathogens, reinforcing their recognition, as producers of important bioactive metabolites (Caulier, et al., 2019). Previous research reported that *Bacillus* spp. isolated from fish gut exhibited inhibitory activity against fish pathogens, including *Aeromonas* (Askarian, et al., 2012; Banerjee, et al., 2016; Gao, et al., 2017; Guo, et al., 2016; Kuebutornye, et al., 2020; Meidong, et al., 2018; Midhun, et al., 2017; Mukherjee, et al., 2019; Nandi, et al., 2017; Ramesh, et al., 2015; Ran, et al., 2012; Serra, et al., 2019; Thankappan, et al., 2015) or *Vibrio* species (Askarian, et al., 2012; Chen, et al., 2016; Kuebutornye, et al., 2020; Serra, et al., 2019). The extent of the inhibitory actions observed in our study, enlarges the potential role of fish-gut *Bacillus* community against furunculosis, vibriosis, photobacteriosis, tenacibaculosis and edwardsiellosis diseases, protecting the hosts against opportunistic bacteria.

Because *Bacillus* antimicrobial activity is usually a result of molecules produced and released to the surrounding environment (Abriouel, et al., 2011; Caulier, et al., 2019), the cell-free supernatants of each fish isolate were also tested for their antimicrobial activity. As expected, the anti-growth activity of *Bacillus* fish-gut isolates was mediated by extracellular NACs. In particular, the cell-free supernatants of strains FI314 and FI330 were highly effective in reducing the growth of all the tested fish pathogens. Unexpectedly, an increased growth of *A. salmonicida* and *V. harveyi* in the presence of FI359, FI376, and FI480 cell-free supernatants has been observed. A potential explanation of this evidence could be that cell-free supernatants might harbour substances that serve as nutrients for some bacteria (Hibbing, et al., 2010; Moons, et al., 2009). However, such hypothesis would need to be confirmed for our strains, under our experimental conditions. Another intriguing observation was the variation of results obtained when using two different antimicrobial assays for cell-free supernatant: only the extracellular NACs suppressing pathogen's growth for 24 h in liquid medium produced a strong inhibitory halo against the same pathogen onto agar medium. For example, the FI314 and FI330 extracellular NACs inhibited the growth of *S. sonnei*, in liquid medium without a visible inhibitory halo onto agar medium. Such differences may reflect technical limitations, since agar-based tests can be influenced by compound's diffusion into the agar, whereas the liquid-based assays allow full contact between the compounds and the target pathogenic strain. Thus, agar-based assays allow quick and less laborious

screenings, but liquid-based assays are preferable when a quantitative analysis is to be done (Balouiri, et al., 2016).

Bacterial biofilms are important key-parameters for bacterial proliferation and persistence, through the promotion of resistance and tolerance to external assaults, including antimicrobials (Flemming, et al., 2016). The capacity of *Bacillus* spp. to regulate or inhibit pathogens biofilm formation has been already studied with the objective of controlling bacterial infections in medical and industrial fields (Kalpana, et al., 2012; Nahar, et al., 2018), including aquaculture (Hamza, et al., 2016). Previous studies demonstrated *Bacillus* spp. potential in biofilm control of a broad range of aquatic pathogens, including *Aeromonas*, *Pseudomonas*, and *Vibrio* species (Chu, et al., 2014; Hamza, et al., 2016; Hamza, et al., 2018; Kalpana, et al., 2012; Vinoj, et al., 2014; Yatip, et al., 2018). Accordingly, fish-gut isolates in the present study produced extracellular NACs capable of reducing biofilm formation of at least one of the tested pathogens, emphasizing the potential use of fish-gut *Bacillus* spp. in preventing the colonization and persistence of fish-pathogenic bacteria. Interestingly, and contrary to the anti-growth results, some fish-gut *Bacillus* extracellular NACs could reduce *A. salmonicida* biofilm formation. This was the case of isolates FI359, FI376, and FI480. Similarly, FI359, FI376, and FI442 extracellular NACs also inhibited the biofilm production by *A. hydrophila*, and *S. sonnei* without interfering with their growth. These observations open the possibility of developing new prophylactic or therapeutic approaches, to deal with problematic fish pathogens and zoonotic agents, such as *A. salmonicida*. Although not directly inhibiting the growth of fish bacterial pathogens, *Bacillus* spp. might control their proliferation by reducing their defense mechanisms, such as biofilm (Grassi, et al., 2017; Pletzer, et al., 2016).

Another promising pathogen-controlling strategy is interfering with their communication or quorum-sensing, in a process called quorum-quenching (QQ) (Grandclement, et al., 2016). Indeed, members of the *Bacillus* genus are known as prominent producers of QQ molecules, such as AHL-lactonases (*AiiA* and homologues) and one AHL-oxidoreductase (CYP102A1, P450BM-3) (Chowdhary, et al., 2007; Chu, et al., 2014; Dong, et al., 2000; Tinh, et al., 2013; Torabi Delshad, et al., 2018; Vinoj, et al., 2014; Zhou, et al., 2016). The present results showed that FI314, FI330, and FI442 produce compounds capable of interfering with externally added 3-oxo-C6-HSL (in the case of *Chr. violaceum* CV026 biosensor) and with AHLs produced by the wild type *Chr. violaceum* CECT 494, namely N-(3-hydroxydecanoyl)-L-homoserine lactone (3-hydroxy-C10-HSL), N-octanoyl-L-Homoserine lactone (C8-HSL) and other several minor AHLs (Morohoshi, et al., 2008). The cell-free supernatant QQ-activity of these 3 promising *Bacillus* strains suggests an extracellular nature of the QQ molecules from FI314 and

FI330. On the contrary, FI442 cell-free supernatant did not show any QQ activity, which indicates that its corresponding QQ molecules might possess an intracellular or cell-wall location, as reported in literature (Cao, et al., 2012; Chu, et al., 2014; Pande, et al., 2015; Romero, et al., 2011; Torabi Delshad, et al., 2018; Zhou, et al., 2018) or were degraded/inactivated under our experimental conditions.

Based on the results of the *in vitro* tests described in this study, the fish-gut isolates FI314, FI330, and FI442, identified as *B. subtilis*, were considered the most promising to be used as probiotics to incorporate in aquafeeds, or to become source of bioactive molecules able to antagonize important bacterial fish pathogens. Despite belonging to the same species (*B. subtilis*), the three selected isolates correspond to different strains. FI314 and FI330 were isolated from gilthead seabream (*Sparus aurata*), but from different digesta samples (different fish from different tanks) and FI442 was isolated from European seabass (*Dicentrarchus labrax*). Adding to the different origin and different colony morphologies, all three strains possess different phenotypes (e.g., results on antimicrobial activities or sporulation efficiency), an indication of own genotypes.

Fish pathogens' infection routes are skin, gills, and gastrointestinal tract. In fact, some authors report the gastrointestinal tract as the main route of colonization in *Aeromonas* sp. (Lødemel, et al., 2001; Ringø, et al., 2004), *Vibrio* sp. (Caruffo, et al., 2015; Oisson, et al., 1996), and *Edwardsiella* sp. (Baldwin, Newton, 1993), and imaging tools are being developed to visualize the exact dissemination of several pathogens inside the fish host (Bartkova, et al., 2017; O'Toole, et al., 2004). Thus, the selected *Bacillus* isolates and their NACs are expected to constitute a barrier against pathogens proliferation inside the animal's gut. This may be achieved indirectly through the regulation of inflammatory pathways by enhancing the host immune system (Canny, McCormick, 2008) or directly either by killing the pathogens, by preventing pathogens' biofilm formation and subsequent gut colonizing, or by acting as quenching molecules shutting down pathogens communication or QS (Abriouel, et al., 2011; Dobson, et al., 2012; Sahoo, et al., 2016; Wang, et al., 2008). Importantly, NACs are directed to non-essential functions like biofilms or QS, and thus are less likely to induce resistance than common antibiotics (Sumi, et al., 2014).

To confirm their potential probiotic application, the *Bacillus* fish-gut isolates were also evaluated for their capacity to produce spores. This feature is not only important from the production point of view, as the best sporeformers are the most attractive for high-yield fermentations, but also because spores allow bacteria to survive passage through the acidic stomach conditions, acting as a form of propagation inside the animal (Tam, et al., 2006). All the isolated *Bacillus* strains tested showed a good sporulation efficiency, equivalent to the control laboratory strain *B. subtilis* 168. More importantly, all *Bacillus*

isolates were susceptible to a series of antibiotic classes, including those demanded by EFSA as mandatory to comply with minimal safety requirements (FEEDAP, 2012; Cabello, et al., 2016) to be considered a probiotic.

The NACs produced by these *B. subtilis* strains, which are responsible for the observed anti-growth, anti-biofilm and, anti-QS activities, are currently being further studied for their isolation, identification, and full characterization. This is a challenging task, as *Bacillus* spp. produce a wide variety of antimicrobial molecules, from ribosomal peptides (RPs), volatile compounds, polyketides (PKs) and non-ribosomal peptides (NRPs) to hybrids between PKs and NRPs (Caulier, et al., 2019). Interestingly, studies reporting the antimicrobial activity of *B. subtilis* against Gram-negative bacteria (the ones we target in this work) are scarce, when compared to the investigations on Gram-positive targets (Caulier, et al., 2019; Olishchevska, et al., 2019). For example, seven compounds produced by *B. subtilis sensu lato* exhibited activity against gram-negative bacteria [Subtilosin A (Shelburne, et al., 2007), Sonorensin (Chopra, et al., 2014), CAMT2 (An, et al., 2015), Subtulene A (Thasana, et al., 2010), Lichenysin (Jenny, et al., 1991), Lichenysin A (Yakimov, et al., 1995), and Pumilacidin (Xiu, et al., 2017)]. Among these compounds, only Subtilosin A and Subtulene A were isolated from *B. subtilis sensu strictu*, and their antimicrobial activity against fish pathogens is underexplored. Taking into account that 4-5% of the *B. subtilis* genome is dedicated to the production of antimicrobial compounds (Caulier, et al., 2019) and the lack of studies on fish-gut associated *Bacillus* as producers of antimicrobial compounds (Soltani, et al., 2019), the present investigation emphasizes the importance of described screening and bacterial collection, for the identification of new NACs with disease -preventive potential in aquaculture.

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Data Availability Statement: 16S rRNA gene sequences of fish isolates described in this manuscript have been deposited in GenBank with the accession numbers provided in Table 2.2. Authors confirm that all relevant data are included in the article.

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Competing interests: RJ is an employee of Epicore Inc., a subsidiary of Archer Daniels Midland, that supported this research by completing a commercial licensing agreement for the potential utilization of the probiotic bacteria FI314, FI330, FI442 identified in this research, in commercial products for aquaculture. The opinions expressed in the manuscript are those of the authors and do not necessarily reflect company policies. All other authors declare no competing interests.

2.5 References

- Abisado, R.G., Benomar, S., Klaus, J.R., Dandekar, A.A., Chandler, J.R., 2018. Bacterial Quorum Sensing and Microbial Community Interactions. *mBio*. 9, e02331-02317. doi: <https://doi.org/10.1128/mBio.02331-17>.
- Abriouel, H., Franz, C.M., Ben Omar, N., Galvez, A., 2011. Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiol Rev.* 35, 201-232. doi: <https://doi.org/10.1111/j.1574-6976.2010.00244.x>.
- An, J., Zhu, W., Liu, Y., Zhang, X., Sun, L., Hong, P., Wang, Y., Xu, C., Xu, D., Liu, H., 2015. Purification and characterization of a novel bacteriocin CAMT2 produced by *Bacillus amyloliquefaciens* isolated from marine fish *Epinephelus areolatus*. *Food Control*. 51, 278-282. doi: <https://doi.org/10.1016/j.foodcont.2014.11.038>.
- Andreoni, F., Magnani, M., 2014. Photobacteriosis: prevention and diagnosis. *J Immunol Res*. 2014, 793817. doi: <https://doi.org/10.1155/2014/793817>.
- Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S., Ringø, E., 2012. Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. *Aquaculture*. 326-329, 1-8. doi: <https://doi.org/10.1016/j.aquaculture.2011.10.016>.
- Avendaño-Herrera, R., Toranzo, A.E., Magariños, B., 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review, *Diseases of aquatic organisms*, pp. 255-266. doi: <https://doi.org/10.3354/dao071255>.
- Baldwin, T.J., Newton, J.C., 1993. Pathogenesis of Enteric Septicemia of Channel Catfish, Caused by *Edwardsiella ictaluri*: Bacteriologic and Light and Electron Microscopic Findings. *J Aquat Anim Health*. 5, 189-198. doi: [https://doi.org/10.1577/1548-8667\(1993\)005<0189:POESOC>2.3.CO;2](https://doi.org/10.1577/1548-8667(1993)005<0189:POESOC>2.3.CO;2).
- Balouiri, M., Sadiki, M., Ibnsouda, S.K., 2016. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal*. 6, 71-79. doi: <https://doi.org/10.1016/j.jpha.2015.11.005>.
- Banerjee, G., Ray, A.K., 2017. The advancement of probiotics research and its application in fish farming industries. *Res Vet Sci*. 115, 66-77. doi: <https://doi.org/10.1016/j.rvsc.2017.01.016>.
- Banerjee, G., Nandi, A., Ray, A.K., 2016. Assessment of hemolytic activity, enzyme production and bacteriocin characterization of *Bacillus subtilis* LR1 isolated from the

gastrointestinal tract of fish. Arch Microbiol, 30;71(33):255-266. doi: <https://doi.org/10.1007/s00203-016-1283-8>.

Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J., Henriques, A.O., 2005. Screening for *Bacillus* Isolates in the Broiler Gastrointestinal Tract. Appl Environ Microbiol. 71, 968-978. doi: <https://doi.org/10.1128/AEM.71.2.968-978.2005>.

Bartkova, S., Kokotovic, B., Dalsgaard, I., 2017. Infection routes of *Aeromonas salmonicida* in rainbow trout monitored in vivo by real-time bioluminescence imaging. J Fish Dis. 40, 73-82. doi: <https://doi.org/10.1111/jfd.12491>.

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 45, 493-496.

Cabello, F.C., Godfrey, H.P., Buschmann, A.H., Dölz, H.J., 2016. Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. Lancet Infect Dis. 16, 127-133. doi: [https://doi.org/10.1016/S1473-3099\(16\)00100-6](https://doi.org/10.1016/S1473-3099(16)00100-6).

Canny, G.O., McCormick, B.A., 2008. Bacteria in the Intestine, Helpful Residents or Enemies from Within? Infect Immun. 76, 3360-3373. doi: <https://doi.org/10.1128/iai.00187-08>.

Cao, Y., He, S., Zhou, Z., Zhang, M., Mao, W., Zhang, H., Yao, B., 2012. Orally administered thermostable N-acyl homoserine lactonase from *Bacillus* sp. strain AI96 attenuates *Aeromonas hydrophila* infection in zebrafish. Appl Environ Microbiol. 78, 1899-1908. doi: <https://doi.org/10.1128/aem.06139-11>.

Caruffo, M., Navarrete, N., Salgado, O., Díaz, A., López, P., García, K., Feijóo, C.G., Navarrete, P., 2015. Potential probiotic yeasts isolated from the fish gut protect zebrafish (*Danio rerio*) from a *Vibrio anguillarum* challenge. Front Microbiol. 6. doi: <https://doi.org/10.3389/fmicb.2015.01093>.

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. Front Microbiol. 10. doi: <https://doi.org/10.3389/fmicb.2019.00302>.

Chen, Y., Li, J., Xiao, P., Li, G.Y., Yue, S., Huang, J., Zhu, W.Y., Mo, Z.L., 2016. Isolation and characterization of *Bacillus* spp. M001 for potential application in turbot (*Scophthalmus maximus* L.) against *Vibrio anguillarum*. Aquacult Nutr. 22, 374, 378 p. doi: <https://doi.org/10.1111/anu.12259>.

- Chopra, L., Singh, G., Choudhary, V., Sahoo, D.K., 2014. Sonorensin: an antimicrobial peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new marine isolate, *Bacillus sonorensis* MT93. *Appl Environ Microbiol.* 80, 2981-2990. doi: <https://doi.org/10.1128/AEM.04259-13>.
- Chowdhary, P.K., Keshavan, N., Nguyen, H.Q., Peterson, J.A., Gonzalez, J.E., Haines, D.C., 2007. *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. *Biochemistry.* 46, 14429-14437. doi: <https://doi.org/10.1021/bi701945j>.
- Chu, W., McLean, R.J., 2016. Quorum Signal Inhibitors and Their Potential Use against Fish Diseases. *J Aquat Anim Health.* 28, 91-96. doi: <https://doi.org/10.1080/08997659.2016.1150907>.
- Chu, W., Zhou, S., Zhu, W., Zhuang, X., 2014. Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. *Sci Rep.* 4, 5446. doi: <https://doi.org/10.1038/srep05446>.
- CLSI, 2013. Clinical and Laboratory Standards Institute.
- Cutting, S.M., 2011. *Bacillus* probiotics. *Food Microbiol.* 28, 214-220. doi: <https://doi.org/10.1016/j.fm.2010.03.007>.
- Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A., Charette, S.J., 2014. Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol.* 169, 1-7. doi: <https://doi.org/10.1016/j.vetmic.2013.06.025>.
- Defoirdt, T., 2013. Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. *Rev Aquacult.* 6, 100-114. doi: <https://doi.org/10.1111/raq.12030>.
- Diaz-Rosales, P., Chabrillon, M., Morinigo, M.A., Balebona, M.C., 2003. Survival against exogenous hydrogen peroxide of *Photobacterium damsela* subsp. *piscicida* under different culture conditions. *J Fish Dis.* 26, 305-308. doi: <https://doi.org/10.1046/j.1365-2761.2003.00455.x>.
- Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Guroy, D., Davies, S.J., 2011. Microbial manipulations to improve fish health and production--a Mediterranean perspective. *Fish Shellfish Immunol.* 30, 1-16. doi: <https://doi.org/10.1016/j.fsi.2010.08.009>.

Dobson, A., Cotter, P.D., Ross, R.P., Hill, C., 2012. Bacteriocin Production: a Probiotic Trait? *Appl Environ Microbiol.* 78, 1-6. doi: <https://doi.org/10.1128/AEM.05576-11>.

Dong, Y.-H., Xu, J.-L., Li, X.-Z., Zhang, L.-H., 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc Natl Acad Sci.* 97, 3526. doi: <https://doi.org/10.1073/pnas.060023897>.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed (EFSA-FEEDAP). *EFSA J* 10:2740

Fey, P.D., 2010. Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections? *Curr Opin Microbiol.* 13, 610-615. doi: <https://doi.org/10.1016/j.mib.2010.09.007>.

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. *Nat Rev Micro.* 14, 563-575. doi: <https://doi.org/10.1038/nrmicro.2016.94>.

Food and Agriculture Organization of the United Nations, W.H.O., 2001. Report of the Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina. doi: <https://doi.org/10.1201/9781420009613.ch16>.

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B., Rediers, H., 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *J Fish Dis.* 34, 643-661. doi: <https://doi.org/10.1111/j.1365-2761.2011.01279.x>.

Gao, X.-Y., Liu, Y., Miao, L.-L., Li, E.-W., Sun, G.-x., Liu, Y., Liu, Z.-P., 2017. Characterization and mechanism of anti-*Aeromonas salmonicida* activity of a marine probiotic strain, *Bacillus velezensis* V4. *Appl Microbiol Biotechnol.* 101, 3759-3768. doi: <https://doi.org/10.1007/s00253-017-8095-x>.

Gauthier, D.T., 2015. Bacterial zoonoses of fishes: a review and appraisal of evidence for linkages between fish and human infections. *Vet J.* 203, 27-35. doi: <https://doi.org/10.1016/j.tvjl.2014.10.028>.

Grandclement, C., Tannieres, M., Morera, S., Dessaux, Y., Faure, D., 2016. Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev.* 40, 86-116. doi: <https://doi.org/10.1093/femsre/fuv038>.

- Grassi, L., Maisetta, G., Esin, S., Batoni, G., 2017. Combination Strategies to Enhance the Efficacy of Antimicrobial Peptides against Bacterial Biofilms. *Front Microbiol.* 8, 2409-2409. doi: <https://doi.org/10.3389/fmicb.2017.02409>.
- Guo, X., Chen, D.-D., Peng, K.-S., Cui, Z.-W., Zhang, X.-J., Li, S., Zhang, Y.-A., 2016. Identification and characterization of *Bacillus subtilis* from grass carp (*Ctenopharyodon idellus*) for use as probiotic additives in aquatic feed. *Fish Shellfish Immunol.* 52, 74-84. doi: <https://doi.org/10.1016/j.fsi.2016.03.017>.
- Hai, N.V., 2015. The use of probiotics in aquaculture. *J Appl Microbiol.* 119, 917-935. doi: <https://doi.org/10.1111/jam.12886>.
- Hamza, F., Kumar, A.R., Zinjarde, S., 2016. Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. *Aquac Res.* 47, 2661-2669. doi: <https://doi.org/10.1111/are.12716>.
- Hamza, F., Kumar, A.R., Zinjarde, S., 2018. Efficacy of cell free supernatant from *Bacillus licheniformis* in protecting *Artemia salina* against *Vibrio alginolyticus* and *Pseudomonas gessardii*. *Microb Pathog.* 116, 335-344. doi: <https://doi.org/10.1016/j.micpath.2018.02.003>.
- Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature reviews. Microbiology.* 8, 15-25. doi: <https://doi.org/10.1038/nrmicro2259>.
- Hong, H.A., Duc, L.H., Cutting, S.M., 2005. The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev.* 29, 813-835. doi: <https://doi.org/10.1016/j.femsre.2004.12.001>.
- Igbinosa, E.O., 2016. Detection and Antimicrobial Resistance of *Vibrio* Isolates in Aquaculture Environments: Implications for Public Health. *Microb Drug Resist.* 22, 238-245. doi: <https://doi.org/10.1089/mdr.2015.0169>.
- Janda, J.M., Abbott, S.L., 2010. The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. *Clin Microbiol Rev.* 23, 35-73. doi: <https://doi.org/10.1128/cmr.00039-09>.
- Jenny, K., Käppeli, O., Fiechter, A., 1991. Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Appl Microbiol Biotechnol.* 36, 5-13. doi: <https://doi.org/10.1007/BF00164690>.
- Jones, M.K., Oliver, J.D., 2009. *Vibrio vulnificus*: Disease and Pathogenesis. *Infect Immun.* 77, 1723. doi: <https://doi.org/10.1128/IAI.01046-08>.

- Kalpana, B.J., Aarthy, S., Pandian, S.K., 2012. Antibiofilm Activity of α -Amylase from *Bacillus subtilis* S8-18 Against Biofilm Forming Human Bacterial Pathogens. *Appl Biochem Biotechnol.* 167, 1778-1794. doi: <https://doi.org/10.1007/s12010-011-9526-2>.
- Khajanchi, B.K., Fadl, A.A., Borchardt, M.A., Berg, R.L., Horneman, A.J., Stemper, M.E., Joseph, S.W., Moyer, N.P., Sha, J., Chopra, A.K., 2010. Distribution of Virulence Factors and Molecular Fingerprinting of *Aeromonas* Species Isolates from Water and Clinical Samples: Suggestive Evidence of Water-to-Human Transmission. *Appl Environ Microbiol.* 76, 2313-2325. doi: <https://doi.org/10.1128/AEM.02535-09>.
- Kuebutornye, F.K.A., Lu, Y., Abarike, E.D., Wang, Z., Li, Y., Sakyi, M.E., 2020. In vitro Assessment of the Probiotic Characteristics of Three *Bacillus* Species from the Gut of Nile Tilapia, *Oreochromis niloticus*. *Probiotics Antimicrob Proteins.* 12, 412-424. doi: <https://doi.org/10.1007/s12602-019-09562-5>.
- Lafferty, K.D., Harvell, C.D., Conrad, J.M., Friedman, C.S., Kent, M.L., Kuris, A.M., Powell, E.N., Rondeau, D., Saksida, S.M., 2015. Infectious diseases affect marine fisheries and aquaculture economics. *Ann Rev Mar Sci.* 7, 471-496. doi: <https://doi.org/10.1146/annurev-marine-010814-015646>.
- Lødemel, J.B., Mayhew, T.M., Myklebust, R., Olsen, R.E., Espelid, S., Ringø, E., 2001. Effect of three dietary oils on disease susceptibility in Arctic charr (*Salvelinus alpinus* L.) during cohabitant challenge with *Aeromonas salmonicida* ssp. *salmonicida*. *Aquac Res.* 32, 935-945. doi: <https://doi.org/10.1046/j.1365-2109.2001.00621.x>.
- McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S., Williams, P., 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology.* 143 (Pt 12), 3703-3711. doi: <https://doi.org/10.1099/00221287-143-12-3703>.
- McLean, R.J., Pierson, L.S., 3rd, Fuqua, C., 2004. A simple screening protocol for the identification of quorum signal antagonists. *J Microbiol Methods.* 58, 351-360. doi: <https://doi.org/10.1016/j.mimet.2004.04.016>.
- Meidong, R., Khotchanalekha, K., Doolgindachbaporn, S., Nagasawa, T., Nakao, M., Sakai, K., Tongpim, S., 2018. Evaluation of probiotic *Bacillus aerius* B81e isolated from healthy hybrid catfish on growth, disease resistance and innate immunity of Pla-mong *Pangasius bocourti*. *Fish Shellfish Immunol.* 73, 1-10. doi: <https://doi.org/10.1016/j.fsi.2017.11.032>.

- Menanteau-Ledouble, S., Kumar, G., Saleh, M., El-Matbouli, M., 2016. *Aeromonas salmonicida*: updates on an old acquaintance. Dis Aquat Organ. 120, 49-68. doi: <https://doi.org/10.3354/dao03006>.
- Midhun, S.J., Neethu, S., Vysakh, A., Sunil, M.A., Radhakrishnan, E.K., Jyothis, M., 2017. Antibacterial activity of autochthonous bacteria isolated from *Anabas testudineus* (Bloch, 1792) and it's in vitro probiotic characterization. Microb Pathog. 113, 312-320. doi: <https://doi.org/10.1016/j.micpath.2017.10.058>.
- Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu Rev Microbiol. 55, 165-199. doi: <https://doi.org/10.1146/annurev.micro.55.1.165>.
- Moons, P., Michiels, C.W., Aertsen, A., 2009. Bacterial interactions in biofilms. Crit Rev Microbiol. 35, 157-168. doi: <https://doi.org/10.1080/10408410902809431>.
- Morohoshi, T., Kato, M., Fukamachi, K., Kato, N., Ikeda, T., 2008. N-acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472. FEMS Microbiol Lett. 279, 124-130. doi: <https://doi.org/10.1111/j.1574-6968.2007.01016.x>.
- Mukherjee, A., Banerjee, G., Mukherjee, P., Ray, A.K., Chandra, G., Ghosh, K., 2019. Antibacterial substances produced by pathogen inhibitory gut bacteria in *Labeo rohita*: Physico-chemical characterization, purification and identification through MALDI-TOF mass spectrometry. Microb Pathog. doi: <https://doi.org/10.1016/j.micpath.2019.02.028>.
- Munn, C., 2005. Pathogens in the Sea: An Overview. in: Belkin, S., Colwell, R. (Eds.), Oceans and Health: Pathogens in the Marine Environment. Springer US, pp. 1-28.
- Nahar, S., Mizan, M.F.R., Ha, A.J.-w., Ha, S.-D., 2018. Advances and Future Prospects of Enzyme-Based Biofilm Prevention Approaches in the Food Industry. Compr Rev Food Sci Food Saf. 17, 1484-1502. doi: <https://doi.org/10.1111/1541-4337.12382>.
- Nandi, A., Dan, S.K., Banerjee, G., Ghosh, P., Ghosh, K., Ringø, E., Ray, A.K., 2017. Probiotic Potential of Autochthonous Bacteria Isolated from the Gastrointestinal Tract of Four Freshwater Teleosts. Probiotics Antimicrob Proteins. 9, 12-21. doi: <https://doi.org/10.1007/s12602-016-9228-8>.
- Nicholson, W., Setlow, P., 1990. Sporulation, germination and outgrowth, Harwood CR, Cutting SM, editors. Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, UK, pp. 391-450.

- O'Toole, R., Von Hofsten, J., Rosqvist, R., Olsson, P.E., Wolf-Watz, H., 2004. Visualisation of zebrafish infection by GFP-labelled *Vibrio anguillarum*. *Microb Pathog.* 37, 41-46. doi: <https://doi.org/10.1016/j.micpath.2004.03.001>.
- Oisson, J.C., Jöborn, A., Westerdahl, A., Blomberg, L., Kjelleberg, S., Conway, P.L., 1996. Is the turbot, *Scophthalmus maximus* (L.), intestine a portal of entry for the fish pathogen *Vibrio anguillarum*? *J Fish Dis.* 19, 225-234. doi: <https://doi.org/10.1111/j.1365-2761.1996.tb00129.x>.
- Olishevskaya, S., Nickzad, A., Déziel, E., 2019. *Bacillus* and *Paenibacillus* secreted polyketides and peptides involved in controlling human and plant pathogens. *Appl Microbiol Biotechnol.* 103, 1189-1215. doi: <https://doi.org/10.1007/s00253-018-9541-0>.
- Pande, G.S., Natrah, F.M., Flandez, A.V., Kumar, U., Niu, Y., Bossier, P., Defoirdt, T., 2015. Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae. *Appl Microbiol Biotechnol.* 99, 10805-10813. doi: <https://doi.org/10.1007/s00253-015-6918-1>.
- Papa, R., Selan, L., Parrilli, E., Tilotta, M., Sannino, F., Feller, G., Tutino, M.L., Artini, M., 2015. Anti-Biofilm Activities from Marine Cold Adapted Bacteria Against *Staphylococci* and *Pseudomonas aeruginosa*. *Front Microbiol.* 6, 1333. doi: <https://doi.org/10.3389/fmicb.2015.01333>.
- Park, S.B., Aoki, T., Jung, T.S., 2012. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Vet Res.* 43, 67-67. doi: <https://doi.org/10.1186/1297-9716-43-67>.
- Pitcher, D.G., Saunders, N.A., Owen, R.J., 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol.* 8, 151-156. doi: <https://doi.org/10.1111/j.1472-765X.1989.tb00262.x>.
- Pletzer, D., Coleman, S.R., Hancock, R.E., 2016. Anti-biofilm peptides as a new weapon in antimicrobial warfare. *Curr Opin Microbiol.* 33, 35-40. doi: <https://doi.org/10.1016/j.mib.2016.05.016>.
- Plumb, J.A., Hanson, L.A., 2011. Health maintenance and principal microbial diseases of cultured fishes. 3th ed. Blackwell Publishing Ltd., Ames, Iowa, USA.
- Ramesh, D., Vinokhanna, A., Rai, A.K., Vignesh, V.S., 2015. Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in *Labeo rohita* against *Aeromonas hydrophila*. *Fish Shellfish Immunol.* 45, 268-276. doi: <https://doi.org/10.1016/j.fsi.2015.04.018>.
- Ran, C., Carrias, A., Williams, M.A., Capps, N., Dan, B.C.T., Newton, J.C., Kloepper, J.W., Ooi, E.L., Browdy, C.L., Terhune, J.S., Liles, M.R., 2012. Identification of *Bacillus*

Strains for Biological Control of Catfish Pathogens. PLoS ONE. 7, e45793. doi: <https://doi.org/10.1371/journal.pone.0045793>.

Rasmussen-Ivey, C.R., Figueras, M.J., McGarey, D., Liles, M.R., 2016. Virulence Factors of *Aeromonas hydrophila*: In the Wake of Reclassification. Front Microbiol. 7, 1337. doi: <https://doi.org/10.3389/fmicb.2016.01337>.

Ringø, E., Jutfelt, F., Kanapathippillai, P., Bakken, Y., Sundell, K., Glette, J., Mayhew, T.M., Myklebust, R., Olsen, R.E., 2004. Damaging effect of the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida* on intestinal enterocytes of Atlantic salmon (*Salmo salar* L.). Cell Tissue Res. 318, 305-311. doi: <https://doi.org/10.1007/s00441-004-0934-2>.

Rivas, A.J., Lemos, M.L., Osorio, C.R., 2013. *Photobacterium damsela* subsp. *damsela*, a bacterium pathogenic for marine animals and humans. Front Microbiol. 4, 283. doi: <https://doi.org/10.3389/fmicb.2013.00283>.

Romero, M., Martin-Cuadrado, A.B., Roca-Rivada, A., Cabello, A.M., Otero, A., 2011. Quorum quenching in cultivable bacteria from dense marine coastal microbial communities. FEMS Microbiol Ecol. 75, 205-217. doi: <https://doi.org/10.1111/j.1574-6941.2010.01011.x>.

Sahoo, T.K., Jena, P.K., Patel, A.K., Seshadri, S., 2016. Bacteriocins and their applications for the treatment of bacterial diseases in aquaculture: a review. Aquac Res. 47, 1013-1027. doi: <https://doi.org/10.1111/are.12556>.

Serra, C.R., Almeida, E.M., Guerreiro, I., Santos, R., Merrifield, D.L., Tavares, F., Oliva-Teles, A., Enes, P., 2019. Selection of carbohydrate-active probiotics from the gut of carnivorous fish fed plant-based diets. Sci Rep. 9, 6384. doi: <https://doi.org/10.1038/s41598-019-42716-7>.

Shelburne, C.E., An, F.Y., Dholpe, V., Ramamoorthy, A., Lopatin, D.E., Lantz, M.S., 2007. The spectrum of antimicrobial activity of the bacteriocin subtilisin A. J Antimicrob Chemother. 59, 297-300. doi: <https://doi.org/10.1093/jac/dkl495>.

Soltani, M., Ghosh, K., Hoseinifar, S.H., Kumar, V., Lymbery, A.J., Roy, S., Ringø, E., 2019. Genus *Bacillus*, promising probiotics in aquaculture: Aquatic animal origin, bioactive components, bioremediation and efficacy in fish and shellfish. Rev Fish Sci Aquac. 27, 331-379. doi: <https://doi.org/10.1080/23308249.2019.1597010>.

Sumi, C.D., Yang, B.W., Yeo, I.-C., Hahm, Y.T., 2014. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Can J Microbiol. 61, 93-103. doi: <https://doi.org/10.1139/cjm-2014-0613>.

- Tam, N.K., Uyen, N.Q., Hong, H.A., Duc le, H., Hoa, T.T., Serra, C.R., Henriques, A.O., Cutting, S.M., 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J Bacteriol. 188, 2692-2700. doi: <https://doi.org/10.1128/jb.188.7.2692-2700.2006>.
- Thankappan, B., Ramesh, D., Ramkumar, S., Natarajaseenivasan, K., Anbarasu, K., 2015. Characterization of *Bacillus* spp. from the gastrointestinal tract of *Labeo rohita*: towards to identify novel probiotics against fish pathogens. Appl Biochem Biotechnol. 175, 340-353. doi: <https://doi.org/10.1007/s12010-014-1270-y>.
- Thasana, N., Prapagdee, B., Rangkadilok, N., Sallabhan, R., Aye, S.L., Ruchirawat, S., Loprasert, S., 2010. *Bacillus subtilis* SSE4 produces subtilene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated beta-amino acid. FEBS Lett. 584, 3209-3214. doi: <https://doi.org/10.1016/j.febslet.2010.06.005>.
- Tinh, N.T.N., Dung, N.V., Trung, C.T., Thuy, V.T., 2013. In Vitro Characterization of a Recombinant AHL-Lactonase from *Bacillus cereus* Isolated from a Striped Catfish (*Pangasianodon hypophthalmus*) Pond. Indian J Microbiol. 53, 485-487. doi: <https://doi.org/10.1007/s12088-013-0415-y>.
- Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H., Haghkhah, M., Bossier, P., 2018. Identification of N-acyl homoserine lactone-degrading bacteria isolated from rainbow trout (*Oncorhynchus mykiss*). J Appl Microbiol. 125, 356-369. doi: <https://doi.org/10.1111/jam.13891>.
- Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol R. 64, 655-671.
- Vinoj, G., Vaseeharan, B., Thomas, S., Spiers, A.J., Shanthi, S., 2014. Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits *Vibrio* biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. Mar Biotechnol (NY). 16, 707-715. doi: <https://doi.org/10.1007/s10126-014-9585-9>.
- Wang, A.R., Ran, C., Ringø, E., Zhou, Z.G., 2018a. Progress in fish gastrointestinal microbiota research. Rev Aquacult. 10, 626-640. doi: <https://doi.org/10.1111/raq.12191>.
- Wang, H., Wei, C.-X., Min, L., Zhu, L.-Y., 2018b. Good or bad: gut bacteria in human health and diseases. Biotechnol Biotechnol Equip. 32, 1075-1080. doi: <https://doi.org/10.1080/13102818.2018.1481350>.
- Wang, R., Zhong, Y., Gu, X., Yuan, J., Saeed, A.F., Wang, S., 2015. The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. Front Microbiol. 6, 144. doi: <https://doi.org/10.3389/fmicb.2015.00144>.

- Wang, Y.-B., Li, J.-R., Lin, J., 2008. Probiotics in aquaculture: Challenges and outlook. *Aquaculture*. 281, 1-4. doi: <https://doi.org/10.1016/j.aquaculture.2008.06.002>.
- Weir, M., Rajić, A., Dutil, L., Uhland, C., Bruneau, N., 2012. Zoonotic bacteria and antimicrobial resistance in aquaculture: opportunities for surveillance in Canada. *Can Vet J*. 53, 619-622.
- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. 173, 697-703. doi: <https://doi.org/10.1128/jb.173.2.697-703.1991>.
- WHO, 2014. Antimicrobial Resistance: Global Report on Surveillance
- Xiu, P., Liu, R., Zhang, D., Sun, C., 2017. Pumilacidin-Like Lipopeptides Derived from Marine Bacterium *Bacillus* sp. Strain 176 Suppress the Motility of *Vibrio alginolyticus*. *Appl Environ Microbiol*. 83, e00450-00417. doi: <https://doi.org/10.1128/AEM.00450-17>.
- Yakimov, M.M., Timmis, K.N., Wray, V., Fredrickson, H.L., 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl Environ Microbiol*. 61, 1706-1713.
- Yatip, P., Nitin Chandra Teja, D., Flegel, T.W., Soowannayan, C., 2018. Extract from the fermented soybean product Natto inhibits *Vibrio* biofilm formation and reduces shrimp mortality from *Vibrio harveyi* infection. *Fish Shellfish Immunol*. 72, 348-355. doi: <https://doi.org/10.1016/j.fsi.2017.11.008>.
- Zeigler, D.R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., Perkins, J.B., 2008. The Origins of 168, W23, and Other *Bacillus subtilis* Legacy Strains. *J Bacteriol*. 190, 6983-6995. doi: <https://doi.org/10.1128/jb.00722-08>.
- Zhou, S., Zhang, A., Yin, H., Chu, W., 2016. *Bacillus* sp. QSI-1 Modulate Quorum Sensing Signals Reduce *Aeromonas hydrophila* Level and Alter Gut Microbial Community Structure in Fish. *Front Cell Infect Microbiol*. 6, 184. doi: <https://doi.org/10.3389/fcimb.2016.00184>.
- Zhou, S., Xia, Y., Zhu, C., Chu, W., 2018. Isolation of Marine *Bacillus* sp. with Antagonistic and Organic-Substances-Degrading Activities and Its Potential Application as a Fish Probiotic. *Mar Drugs*. 16. doi: <https://doi.org/10.3390/md16060196>.
- Zhou, Z., Zhou, X., Zhong, Z., Wang, C., Zhang, H., Li, D., He, T., Li, C., Liu, X., Yuan, H., Ji, H., Luo, Y., Gu, W., Fu, H., Peng, G., 2014. Investigation of antibacterial activity of *Bacillus* spp. isolated from the feces of Giant Panda and characterization of their antimicrobial gene distributions. *World J Microbiol Biotechnol*. 30, 3129-3136. doi: <https://doi.org/10.1007/s11274-014-1740-y>.

CHAPTER III

Bacillus spp. Inhibit *Edwardsiella tarda* Quorum-Sensing and Fish Infection

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***Bacillus* spp. inhibit *Edwardsiella tarda* quorum-sensing and fish infection**

Abstract:

The disruption of pathogen communication or quorum-sensing (QS) via quorum-quenching (QQ) molecules, has been proposed as a promising strategy to fight bacterial infections. *Bacillus* spp. have recognizable biotechnology applications, namely as probiotic health-promoting agents or as a source of natural antimicrobial molecules, including QQ molecules. This study characterized the QQ potential of 200 *Bacillus* spp., isolated from the gut of different aquaculture fish species, to suppress fish pathogens QS. Approximately 14% of the tested *Bacillus* spp. fish isolates (FI) were able to interfere with synthetic QS molecules. Ten isolates were further selected as producers of extracellular QQ-molecules and their QQ capacity was evaluated against QS of important aquaculture bacterial pathogens, namely *Aeromonas* spp., *Vibrio* spp., *Photobacterium damsela*, *Edwardsiella tarda*, and *Shigella sonnei*. The results revealed that *A. veronii* and *E. tarda* produce QS molecules that are detectable by the *Chr. violaceum* biosensor, which were degraded when exposed to the extracellular extracts of three FI isolates. Moreover, the same isolates, identified as *B. subtilis*, *B. velezensis*, and *B. pumilus*, significantly reduced the pathogenicity of *E. tarda* in zebrafish larvae, increasing its survival by 50%. Taken together, these results identified three *Bacillus* spp. capable of extracellularly quenching aquaculture pathogens communication and thus become a promising source of bioactive molecules for use in the biocontrol of aquaculture bacterial diseases.

Keywords: Fish diseases; Quorum-Quenching, *Bacillus* spp., Zebrafish larv

3.1. Introduction

Despite experiencing continuous growth, the development of aquaculture remains highly vulnerable to the occurrence of infectious bacterial diseases. Furunculosis, edwardsielosis, vibriosis, and photobacteriosis are among the most prevalent diseases, with serious effects on marine fish species (Bayliss, et al., 2017). Although antibiotics are an important tool for disease treatment, their damaging effects on the environment and public health have led to increased restrictions on their use in aquaculture (Jayaprakashvel, Subramani, 2019). An alternative to antibiotics relies on attenuating pathogen virulence, a process called anti-virulence therapy (Defoirdt, 2013).

Virulence factors (e.g., motility, extracellular polysaccharides, biofilms, lytic enzymes, or secretion systems) enable bacterial pathogens to colonize and damage their host. Their expression can be regulated through complex regulatory networks, including Quorum-Sensing (QS) (Defoirdt, 2018). QS is a process of cell-to-cell communication in which bacteria coordinate their population size and behaviour (Abisado, et al., 2018; Miller, Bassler, 2001). It is mediated by small diffusible signals, known as autoinducers, that are produced and exported from the cell, and then perceived by specific receptors in the bacterial community, improving their global fitness (Abisado, et al., 2018). QS can occur both within and between bacterial species. The first QS system model relied on the identification of acyl-homoserine lactones (AHLs) as regulators of bioluminescence production in *Vibrio fischeri* (Fuqua, et al., 1996). In brief, AHLs are synthesized by LuxI homologs proteins (AhlI, AsaI, EdwI in *A. hydrophila*, *A. salmonicida* and *E. tarda*, respectively) that diffuse out/into the cell, where the LuxR homolog (AhlR, AsaR, EdwR in *A. hydrophila*, *A. salmonicida* and *E. tarda*, respectively) detects these molecules and binds to the promoter of QS-dependent target genes (Abisado, et al., 2018).

Although AHLs are the most common signal molecules in Gram-negative bacteria (including fish pathogens), bacteria can produce and detect other signal molecules involved in gene-virulence regulation, such as autoinducer-2 (AI-2) and cholera autoinducer-1 (CAI-1) (Zhao, et al., 2015). In *Vibrio anguillarum*, *V. harveyi*, and *V. parahaemolyticus*, these three types of signal molecules were identified to regulate the expression of genes encoding proteases, toxins, and other virulence-associated factors (Croxatto, et al., 2002; Milton, 2006). *Aeromonas hydrophila*, *V. vulnificus*, and *Edwardsiella tarda* use a combination of several AHLs with AI-2 to regulate motility, biofilm formation, proteases, haemolysin, and secretion systems (Defoirdt, 2014; Zhao, et al., 2015), whereas *A. salmonicida* and *Tenacibaculum maritimum* were reported to only use AHLs as signal molecules (Romero, et al., 2010; Swift, et al., 1997).

Considering the key role of QS in bacterial communities' fitness, one of the most studied anti-virulence therapy strategies is the disruption of QS, called Quorum-Quenching (QQ). Compared to bactericidal/bacteriostatic approaches, QQ is unlikely to promote strong selective pressures, minimizing the development of resistance among pathogens, since it does not directly target bacterial growth (Defoirdt, 2018; Fetzner, 2015). QQ can be achieved by (i) inhibiting the signal biosynthesis, (ii) enzymatically degrading/modifying the signal molecules, or (iii) inhibiting signal detection by blocking the signal receptor (Grandclement, et al., 2016). Enzymatic degradation of the signal is the most-studied approach in QQ and appears to be widely distributed in the bacterial kingdom. Thus, the majority of QQ enzymes were discovered in bacteria, targeting AHLs signal molecules (Fetzner, 2015), namely, AHL lactonases, acylases, or oxidoreductases, depending on the molecule modification. By degrading the QS signal, these enzymes can decrease the production of virulence factors in pathogenic bacteria. The first identified QQ enzyme was an AHL-lactonase (encoded by *aiiA*) from *Bacillus* sp. 240B1 that, when tested *in vivo* increased plant disease resistance by attenuating *Erwinia carotovora* virulence (Dong, et al., 2000). Other QQ enzymes-encoding genes were discovered such as *attM* (*Agrobacterium tumefaciens*) (Zhang, et al., 2002), *ahID* (*Arthrobacter* sp.) (Park, et al., 2003), *aiiM* (*Microbacterium* sp.) (Wang, et al., 2010), and *ytnP* (*B. subtilis*) (Schneider, et al., 2012).

Bacillus spp. are sporeforming Gram-positive bacteria with recognizable biotechnology applications, including as probiotic-health-promoting agents or as a source of antimicrobial molecules with the capacity to inhibit fish pathogens (Caulier, et al., 2019; Kuebutornye, et al., 2020; Meidong, et al., 2017; Meidong, et al., 2018; Mukherjee, et al., 2019; Santos, et al., 2021; Serra, et al., 2019). Indeed, from the gut of marine fish species, we recently isolated several *Bacillus* spp. strains capable of inhibiting the growth and biofilm formation of 14 different fish pathogens and with *in vitro* QQ capacity (Santos, et al., 2021). Recent reports suggest that *Bacillus* spp. with QQ activity can reduce pathogens' QS and virulence, increasing fish protection against *A. hydrophila* and *Vibrio* spp.: (i) *B. licheniformis* T1 and *Bacillus* sp. AI96 QQ increased zebrafish resistance to *A. hydrophila* by 50% and 40%, respectively (Cao, et al., 2012; Chen, et al., 2020); (ii) *Bacillus* sp. QSI-1 increased zebrafish survival by 70% when challenged with *A. hydrophila* (Chu, et al., 2014); (iii) The QQ strain *Bacillus* sp. YB1701 improved *Carassius auratus gibelio* resistance to *A. hydrophila* by 25% (Zhou, et al., 2018); iv) *B. licheniformis* DAHB1 AiiA enzyme and *Bacillus* sp. NFMI-C protected shrimp and prawn larvae against *V. parahaemolyticus* and *V. campbellii* infection, respectively (Pande, et al., 2015; Vinoj, et al., 2014); v) *B. thuringiensis* and *B. cereus* strains showed

QQ and protective activities against *Y. ruckeri* in rainbow trout (Torabi Delshad, et al., 2018).

This study screened the QQ capacity of the extracellular components of a collection of *Bacillus* spp., which were previously isolated from the gut of different marine fish species, and tested their ability to degrade the AHLs signals produced by important fish pathogenic strains. After validation of fish isolates (FIs) with QQ capacity, their potential protective effects were determined in an *in vivo* model (zebrafish larvae) when challenged with *E. tarda*.

3.2. Materials and Methods

3.2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 3.1. Fish-Isolates (FI) from our collection obtained from the gut of different fish species (*Sparus aurata*, *Dicentrarchus labrax*, and *Diplodus sargus*) (Santos, et al., 2021; Serra, et al., 2019), and the laboratory strain *Bacillus subtilis* 168 (Zeigler, et al., 2008) used as control for FI growth, were routinely and aerobically grown in Luria-Bertani (LB; Fisher BioReagents, Waltham, MA, U.S.A) medium at 37 °C. QQ biosensor strains, *Chromobacterium violaceum* (CECT 494, ATCC 12472) and *Chr. violaceum* CV026 (CECT 5999) (Table 3.1), were grown aerobically in LB medium at 30 °C. *Chr. violaceum* CV026 is a *cvil::mini-Tn5* mutant requiring kanamycin (25 µg mL⁻¹) (Nzytech, Lisboa, Portugal) supplementation. Fish pathogens were grown aerobically in Brain Heart Infusion (BHI, BD Difco) medium (except for *T. maritimum*, which was grown in marine medium (BD Difco)) at 25 °C or 37 °C (for *E. tarda*, *S. sonnei*).

Table 3.1. Bacterial strains used in this study.

Bacterial species	Strain	Origin / Source ^a
Fish pathogens		
<i>Aeromonas salmonicida</i>	LMG 3780	BCCM/LMG
<i>Aeromonas veronii</i>	Fish isolate	NUTRIMU collection
<i>Aeromonas bivalvium</i>	Fish isolate	NUTRIMU collection
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>	LMG 2844	BCCM/LMG
<i>Vibrio anguillarum</i>	DSM 21597	DSMZ
<i>Vibrio harveyi</i>	Fish isolate	NUTRIMU collection
<i>Vibrio parahaemolyticus</i>	LMG 2850	BCCM/LMG
<i>Vibrio vulnificus</i>	LMG 13545	BCCM/LMG
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	LMG 7892	BCCM/LMG
<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	<i>Lg_{h41/01}</i>	Diaz-Rosales, et al. (2003)
<i>Tenacibaculum maritimum</i>	LMG 11612	BCCM/LMG
<i>Edwardsiella tarda</i>	LMG 2793	BCCM/LMG
<i>Shigella sonnei</i>	LMG 10473	BCCM/LMG
<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	168 (BGSC1A1)	A.O. Henriques
<i>Chromobacterium violaceum</i> WT	CECT 494	CECT
<i>Chromobacterium violaceum</i> CV026	CECT 5999	CECT

^a 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; CECT, Spanish Type Culture Collection, Valencia, Spain), from our laboratory stocks (NUTRIMU collection) or kindly supplied by M.A. Morinigo (Universidad Málaga), and A. O. Henriques (Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal).

3.2.2. Evaluation of isolates QQ activity

A flow diagram with the methodology used for evaluating QQ activity is presented in Supplementary Figure S1 (Appendix I).

QQ potential of 200 FI was first evaluated with overnight cultures of each FI inoculated as 5 µL spot on LB agar plates and incubated for 24 h at 37 °C before killing with chloroform vapours. Plates were aerated before overlaying with LB soft agar (0.8% agar) inoculated with *Chr. violaceum* WT (OD₆₀₀ ~ 0.1), or with *Chr. violaceum* CV026 (OD₆₀₀ ~ 0.1) supplemented with 5 µM of N-(β-Ketocaproyl)-L-Homoserine Lactone (3-Oxo-C6-HSL) (Sigma-Aldrich, Darmstadt, Germany). Zones of violacein pigmentation inhibition around the *Bacillus* sp. colonies (without interference on the biosensor growth) after 48 h at 30 °C were considered a positive result for QQ activity.

The extracellular QQ activity was measured in all positive strains from the previous assay. For that, cell-free supernatant of each FI was prepared from overnight cultures grown at 37 °C and 140 rpm, followed by centrifugation for 10 min at 13000 g and sterilization by filtration with 0.22 µm cellulose acetate filter. LB agar plates were overlaid with LB soft agar (0.8% agar) inoculated with *Chr. violaceum* WT (OD₆₀₀ ~ 0.1), or with *Chr. violaceum* CV026 (OD₆₀₀ ~ 0.1) supplemented with 5 µM of 3-Oxo-C6-HSL. Once the plates solidified, 9 mm diameter wells were punched and filled with 100 µL of the cell-

free supernatant of each FI. As the cell-free supernatants of all fish isolates tested, do not interfere with the biosensor's growth, the zones of violacein pigmentation inhibition around the wells after 48 h at 30 °C were considered a positive result for QQ extracellular activity. The laboratory strain *B. subtilis* 168 was used as a control for FIs bacterial growth and strains FI314, FI330, and FI442 were used as a positive control for QQ activity, as described earlier (Santos, et al., 2021). All digital photos were taken with a Sony IMX240 camera and zones (in mm) of pigment inhibition recorded

3.2.3. AHLs enzymatic inactivation by isolates' extracellular compounds

Fish isolates' cell-free supernatant was tested for AHL enzymatic degradation of 30 μ M of 3-Oxo-C6-HSL, using a well-diffusion method, as follows: LB plates were overlaid with 8 mL of LB soft agar (0.8% agar) previously inoculated with *Chr. violaceum* CV026 (OD600 ~ 0.1). After plate solidification, 9 mm diameter wells were punched and filled with 100 μ L of AHL-SUP reaction mixtures, (cell-free supernatant of each FI strain mixed with 30 μ M of 3-Oxo-C6-HSL. To evaluate enzymatic activity, the AHL-SUP reaction mixtures were incubated at room temperature, with agitation at 120 rpm, for 0 h and 24 h, before using in the bioassays with *Chr. violaceum* CV026. LB media with and without the same concentration of 3-Oxo-C6-HSL were used as negative controls. An intensity and size reduction of the violacein pigment halo on the biosensor submitted to both reactions mixtures 0 h and 24 h was considered a positive result for enzymatic degradation.

The method described by Yates, et al. (2002) was used to elucidate whether the enzymatic degradation of the 3-Oxo-C6-HSL could be due to a lactonase. Reaction mixtures were acidified with 10 N HCL to pH 2.0, and then used again in a new bioassay with *Chr. violaceum* CV026, as described. Since lactonase enzymatic reaction is pH-mediated and reversible by acidification, the restoration of the violacein pigment halo on *Chr. violaceum* CV026 is considered positive for lactonase activity. All digital photos were taken with a Sony IMX240 camera and zones (in mm) of pigment inhibition were recorded.

3.2.4. Design of QQ primers

To obtain a set of primers specific to genes encoding putative QQ enzymes, an initial search was conducted at the Protein Knowledgebase – UniProtKB for “Bacillus Quorum Quenching lactonase”. Selected enzymes included YtnP (probable QQ lactonase) (<https://www.uniprot.org/uniprot/O34760>, accessed on 9 June 2019) and AiiA (N-acyl homoserine lactonase) (<https://www.uniprot.org/uniprot/Q9L8R8>, accessed on 9 June 2019). The protein sequence of each enzyme was used to search for similar

proteins in the translated nucleotide database using NCBI (<https://www.ncbi.nlm.nih.gov>, accessed on 9 June 2019). Nucleotide sequence alignments with ClustalW (GenomeNet, Kyoto University, Japan) allowed the detection of regions of sequence conservation used to design a pair of primers for each enzyme encoding gene (*ytnP* and *aiiA*) with the SnapGene software version 5.2.3 (GSL Biotech LLC, San Diego, CA, USA) (Table 3.2).

3.2.5. PCR Amplification of 16S rRNA Genes and Genes Coding for Putative QQ Enzymes

Fish isolates' genomic DNA (gDNA) was extracted using ZymoBIOMICS DNA Miniprep kit (Zymoresearch, Irvine, CA, USA). For strain identification, a DNA fragment containing almost the complete sequence of 16S rRNA gene (~1465 bp) was amplified using primers 16S-27F and 16S-1492R (Table 3.2). Similarly, putative QQ-genes were amplified using primers *ytnP*-149F and *ytnP*-708R and, *aiiA*-1F and *aiiA*-584R (Table 3.2). A putative *aiiA* gene was also amplified using a set of primers described by Kalia, et al. (2011) for Firmicutes (267 bp) (Table 3.2).

Table 3.2. Oligonucleotide primers used in this study.

Name	Sequence (5' - 3')	Amplicon (bp)	Reference
16S rRNA			
27F	AGAGTTTGATCMTGGCTCAG	1465	Weisburg, et al. (1991)
1492R	GGYTTACCTTGTTAYGACTT		Weisburg, et al. (1991)
N-acyl homoserine lactonase / <i>aiiA</i> ^a			
<i>aiiA</i> –309F	TCACTTACATTTTGATCATGCAGGAGGAAA	267	Kalia, et al. (2011)
<i>aiiA</i> –576R	TCCGGTTCAGTTTTATTAACGATTGATGCA		Kalia, et al. (2011)
<i>aiiA</i> –1F	ATGACAGTAAAGAAGCTTTATT	584	This study
<i>aiiA</i> –584R	CATCTTCAAATTCTCTTTTCG		This study
Probable quorum-quenching lactonase / <i>ytnP</i> ^b			
<i>ytnP</i> –149F	ATCGGATAATCATCGTAAGC	559	This study
<i>ytnP</i> –708R	ATTGAACTAAGAACAGACCC		This study

^a gene name in *B. subtilis* strain BS2 genome, whose sequence was used to design the oligonucleotide primers for *aiiA*.

^b gene name in *B. subtilis* strain 168 genome, whose sequence was used to design the oligonucleotide primers for *ytnP*.

Each Polymerase Chain Reaction (PCR) contained 5 x MyTaq Reaction Buffer (Bioline, London, United Kingdom), 10 μ M of each primer (STAB Vida, Lisboa, Portugal), 1 U of Mytaq DNA polymerase enzyme (Bioline, United Kingdom), and DNA template. The program consisted of an initial denaturation step at 95 °C for 60 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C (for 16S rRNA), 49 °C (for *ytnP*), 45 °C (for *aiiA*) or 50 °C (for *aiiA* using Kalia, et al. (2011) primers) for 30 s and, a final extension at 72 °C for 10 s. All PCR reactions were performed using a T100™ Thermal Cycler (Bio-Rad, Algés, Portugal).

Phylogenetic analysis was performed with the GenBank non-redundant nucleotide database (Blastn) and the GenBank protein database using a translated nucleotide sequence (Blastx) with BLAST (<http://www.ncbi.nlm.nih.gov>, accessed on 15 January 2020). The amino acid sequences of the putative AHL-lactonase enzymes were aligned with ClustalW software (<https://www.genome.jp/tools-bin/clustalw>, accessed on 15 January 2020) and the phylogenetic tree was built using the neighbor-joining method available in ClustalW software.

3.2.6. AHL production profile of fish pathogens

AHLs production by fish pathogens was explored using a cross-feeding method, in which *Aeromonas bivalvium*, *A. hydrophila* LMG 2844, *A. salmonicida* LMG 3780, *A. veronii*, *Edwardsiella tarda* LMG 2793, *Photobacterium damsela* subsp. *piscicida*, *Ph. damsela* subsp. *damsela* LMG 7892, *Shigella sonnei* LMG 10473, 2, *Vibrio anguillarum* DSM 21597, *V. harveyi*, *V. parahaemolyticus* LMG 2850 and *V. vulnificus* LMG 13545 were cross-fed alongside (1 cm apart) the biosensor *Chr. violaceum* CV026 on an LB plate. Plates were incubated for 48 h at 30 °C and the induction of violacein production on the biosensor was considered a positive result for AHLs production by the tested fish pathogens.

Pathogens' AHLs-production kinetics were evaluated in the supernatant of *A. salmonicida*, *A. veronii*, *A. bivalvium* and *E. tarda*. In brief, an inoculum ($OD_{600} \sim 0.05$) of *A. bivalvium*, *A. salmonicida*, *A. veronii* and *E. tarda* was prepared in BHI medium and grown for 48 h at 25 °C (or 37 °C in the case of *E. tarda*), 140 rpm. Every 2 hours (or every 4 hours after the first 24 h of growth), the optical density (OD_{600}) was measured and the pathogens' supernatant was obtained by centrifugation for 10 min at 16000 g, and filtration with 0.22 μ m cellulose acetate filter. The revelation of AHLs in the pathogen's supernatant was performed by overlaying a LB agar plate with 8 mL of LB soft agar (0.8% agar) inoculated with *Chr. violaceum* CV026 ($OD_{600} \sim 0.1$). After plate solidification, 9 mm diameter wells were punched and filled with 100 μ L of each pathogen's supernatant. Plates were incubated for 48 h at 30 °C and violacein pigment

halos around the wells were considered a positive result. All digital photos were taken with a Sony IMX240 camera (Sony, Tokyo, Japan) and zones (in mm) of pigment inhibition were recorded.

3.2.7. Extraction of fish pathogens' AHLs

The AHLs produced by *A. salmonicida*, *A. veronii*, *A. bivalvium*, and *E. tarda* were extracted as described in Shaw, et al., (1997), with some modifications. In brief, an inoculum of each bacterial strain ($OD_{600} \sim 0.05$) was prepared in 25 mL of BHI medium from overnight cultures. *A. salmonicida*, *A. veronii*, *A. bivalvium* were grown for 6 h at 25 °C, and *E. tarda* was grown for 14 h at 37 °C, 140 rpm. Pathogens' supernatant was obtained from centrifugation for 10 min at 16000 g, and filtration with 0.22 µm cellulose acetate filter and mixed with an equal volume of acidified ethyl acetate (0.1% of acetic acid). Mixtures were shaken for 30 min, followed by phase separation. The organic phases were pooled and stored at 4 °C. The extraction procedure was repeated three times to improve AHLs extraction. The pooled fraction was concentrated in a rotary evaporator at room temperature. Finally, the dried extract was dissolved in 500 µL of ethyl acetate and stored at 4 °C until use.

3.2.8. Fish isolates QQ activity on fish pathogens' AHLs

Fish isolates QQ activity on *A. salmonicida*, *A. veronii*, *A. bivalvium*, and *E. tarda* AHL's was evaluated by overlaying LB plates with 8 mL of LB soft agar inoculated with *Chr. violaceum* CV026 ($OD_{600} \sim 0.05$) supplemented the natural AHLs extracted from the fish pathogens (10, 20, 40, 60, 80 and 100 µL). Once the plates solidified, 9 mm diameter wells were punched and filled with 100 µL of cell-free supernatant of each fish isolate strain. As in previous bioassays, zones of violacein inhibition around the wells (without interference on biosensor growth) after 48 h at 30 °C were considered a positive result for QQ activity. All digital photos were taken with a Sony IMX240 camera and zones (in mm) of pigment inhibition were recorded.

3.2.9. Ethics statement

Zebrafish experiments and handling were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), performed by trained scientists (with FELASA category C), carried out in a registered installation (N16091.UDER), and in compliance with the European directive 2010/63/EU for the care and use of laboratory animals.

3.2.10. Zebrafish larvae general care

Zebrafish embryos were obtained from a wild-type zebrafish broodstock, and incubated in egg water at 28 °C under a photoperiod of 14 h of light:10 h of darkness until hatching. After hatching, larvae were kept in the same conditions and from 6 dpf were fed twice a day (diet containing 36.7% of total crude protein and 15% of total lipids). After each experiment, the surviving larvae were euthanized with a lethal dose of tricaine methanesulfonate (MS-222, 300mg L⁻¹).

3.2.11. Isolates extracts preparation and testing for toxicity in zebrafish larvae

The extracts of the 3 most promising QQ fish isolates were obtained by freeze-drying the filtered cell-free supernatant of 8 h cultures and resuspending it in sterile 1x PBS.

The evaluation of their *in vivo* toxicity was performed using zebrafish larvae (*Danio rerio*) as a model, following the Organisation for Economic Co-operation and Development (OECD) Guidelines for Fish Embryo Toxicity Tests (OECD, 2013).

Zebrafish larvae at 4 days post-fertilization (dpf) were distributed into 6-well plates containing 10 larvae / well in 5 mL of egg water (26.4 mg L⁻¹ of Instant Ocean® Salt) and exposed to fish isolates' extracts with concentrations ranging from 67.5 ug mL⁻¹ to 1 mg mL⁻¹. Larval mortality was recorded at 4, 5, 6, and 7 dpf, and the dead larvae were removed and discarded. Larvae kept in egg water (without treatment) were used as a negative control. The experiment was performed in triplicate to determine the maximum non-toxic extract concentration (MNTC) to be used in subsequent assays.

3.2.12. *E. tarda* infection model

Zebrafish larvae were used to establish an infection model of *E. tarda* by bath immersion. *E. tarda* was cultured for 24 h in BHI at 37 °C with 140 rpm, pelleted by centrifugation (6000 g) at room temperature, washed twice with sterile 1x PBS and then diluted to the correct concentration in 1x PBS. Before the establishment of the infection model, bacterial cell densities ranging from 10⁴ to 10⁹ were tested to evaluate their virulence in zebrafish larvae during 24 h and determine the lowest lethal concentration causing 100% mortalities (5x10⁸ CFUs mL⁻¹) and the non-lethal dose (1x10⁷ CFUs mL⁻¹) (data not shown).

Zebrafish larvae at 10 dpf were distributed into 6-well plates containing 10 larvae / well in 5 mL of Egg water and inoculated with 5x10⁷, 1x10⁸, and 3x10⁸ CFUs mL⁻¹ of *E. tarda*. After inoculation, larvae were fed, and the plate was incubated at 28 °C. The *E. tarda* inoculum was kept in the water throughout the whole experiment (24 h). Cumulative mortalities were registered for 24 h and dead larvae found during the assay were removed and discarded. Control groups were included: (i) non-inoculated larvae, with

egg-water only; (ii) larvae inoculated with 1x PBS. The experiment was independently performed 3 times.

3.2.13. Fish isolates' protection assay against *E. tarda* infection in zebrafish larvae

The fish isolates' protection of zebrafish larvae against *E. tarda* infection was performed by testing the lyophilized extracts at a final concentration of 250 µg mL⁻¹. Before the assay, a pre-treatment experiment was performed where zebrafish larvae were treated with the extracts once, twice, or thrice for 2 or 24 h before challenge with *E. tarda*, allowing the establishment of the best protection method. Thus, at 4 dpf, 10 healthy larvae were distributed into each well of a 6-well plate, containing 5 mL of Egg water. Larvae were treated with the extracts once after mouth's opening (7 dpf), for 2 h at 28 °C and then transferred to new 5 mL egg water. The treated 10 dpf larvae were challenged by immersion with *E. tarda* at 1x10⁸ CFU mL⁻¹. After inoculation, larvae were fed, and the plate was incubated at 28 °C for 24 h. Cumulative mortalities were registered between 16 and 24 h and the dead larvae were removed and discarded. Control groups were included: (i) non-treated larvae inoculated with *E. tarda*; (ii) non-inoculated larvae; (iii) larvae inoculated with 1x PBS. The experiment was independently performed 3 times.

3.2.14. Statistical analysis

Survival data were analysed using Kaplan–Meier, and group differences were analysed by the log-rank, using the GraphPad Prism 9 software.

The one-way ANOVA was performed to compare between treatments and the control, using the SPSS 26.0 software (IBM Corp., New York, NY, USA) package for Windows. When *p*-values were significant (*p* < 0.05), means were compared with Dunnett's test.

3.3. Results

3.3.1 Fish-Isolates produce extracellular compounds with QQ capacity

Around 200 fish-isolates (FI) were tested for their quorum-quenching activity (QQ) of the broad range AHLs (N-(3hydroxydecanoyl)-L-homoserine lactone (3-hydroxy-C10HSL), N-octanoyl-L-Homoserine lactone (C8-HSL), and other minor AHLs) produced by the wild-type biosensor *Chromobacterium violaceum* (CECT 494, ATCC 12472) (Morohoshi, et al., 2008) or of the N-(3-Oxohexanoyl)-L-homoserine lactone (3-Oxo-C6-HSL), supplemented to the CV026 biosensor, a *Chr. violaceum* *Cvil*-negative mutant in which violacein production can be restored in the presence of exogenous AHLs (McClean, et al., 1997). The inactivation of AHL compounds was confirmed by the loss of purple

violacein pigmentation in the QQ biosensors around FI colonies (Figure 3.1A). The screenings revealed that out of the 200 FI tested, 11 (5.5%) displayed activity against AHLs produced by the wild-type biosensor, and 29 (14.5%) degraded the 3-oxo-C6-HSL signalling molecule. When using the CV026 biosensor supplemented with 3-Oxo-C6-HSL, 18 FI produced a clear pigment inhibition halo (Figure 3.1A), indicating that all isolates could inhibit 3-Oxo-C6-HSL-like molecules, but when using the wild-type biosensor, only isolates FI314, FI330, FI335, FI346, FI383, FI414, FI423, FI424, FI432, FI436, and FI442 could interfere with the several AHLs that were produced (Figure 3.1A).

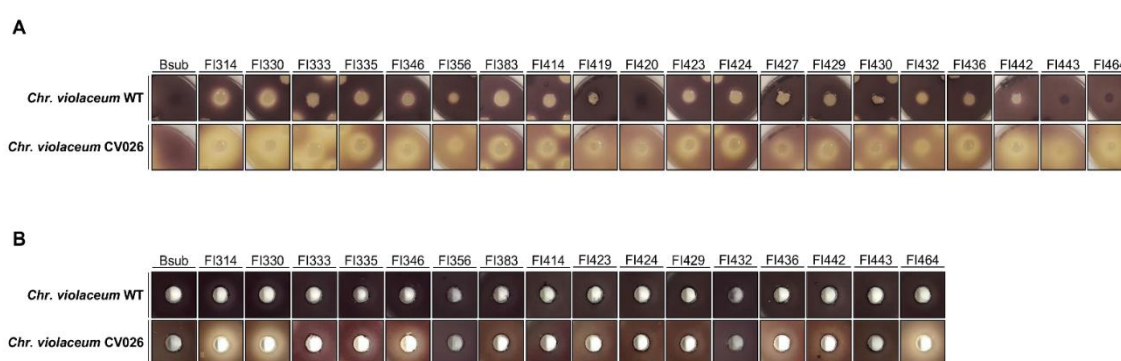


Figure 3.1. Degradation of the violacein pigment produced by *Chr. violaceum* biosensors of sporeforming fish isolates. **(A)** Biosensor's violacein pigment degradation by FI isolates (FI numbers on top) inoculated as 5 μ L spots on Luria-Bertani plates. **(B)** Biosensor's violacein pigment inhibition around wells containing cell-free supernatant of sporeforming fish isolates (FI numbers on top). The laboratory strain *B. subtilis* 168 (*Bsub*) was used as a control for FI bacterial growth and strains FI314, FI330 and FI442 were used as a positive control for QQ activity. All photos were taken with a Sony IMX240 camera and are at the same scale

When testing their cell-free supernatants (to establish the intra- or extracellular localization of the QQ compounds), it became clear that FI did not produce extracellular compounds capable of interfering with all AHLs of the wild-type biosensor (Figure 3.1B). In contrast, the inhibition of violacein pigment production was observed in the CV026 biosensor supplemented with 5 μ M of 3-Oxo-C6-HSL, and was particularly evident in FI314, FI330, and FI464, which showed a stronger activity when compared to the other isolates (Figure 3.1B). These results indicate that FI isolates produce extracellular compounds with QQ capacity against at least 3-Oxo-C6-HSL QS molecules. The laboratory strain *B. subtilis* 168 could not inhibit pigment production of the biosensors in both bioassays.

3.3.2. Isolates QQ activity is mediated through AHLs enzymatic inactivation

To evaluate the catalyst nature of the QQ activity observed in the previous experiment, the extracellular compounds of all positive strains (FI314, FI330, FI333, FI335, FI346,

FI383, FI423, FI436, FI442, and FI464) were mixed for 24 h with 30 μ M of synthetic 3-Oxo-C6-HSL, before a bioassay with CV026 biosensor. In this, the reduction of the violacein halo when submitted to the H24 reaction mixture, when compared to a fresh reaction mixture (H0), indicates lactone degradation by the extracellular compounds present on the FI supernatants, and thus an enzymatic reaction. The FI extracellular compounds mixed with the 3-Oxo-C6-HSL led to a reduction in pigment production by the biosensor in all combinations, with strains FI314, FI330, FI346, and FI464 showing the highest activity (Figure 3.2A). A reduction of the pigment production was also noticed when using the fresh (0H) reactions of strains FI314, FI330, and FI464, which might indicate the presence of signal blockers (Figure 3.2A) on their supernatant.

To clarify if the degradation of the 3-Oxo-C6-HSL could be due to lactonase, FI extracellular compounds were simultaneously tested for lactonase enzymatic activity, through acidification, since lactonase enzymatic reaction is pH-mediated and reversible by acidification. After the acidification process, the enzymatic reaction could be reversed as confirmed by the regain of purple pigmentation intensity, suggesting a lactonase-type FI QQ activity (Figure 3.2A). As expected, the negative control (LB medium mixed with 30 μ M of 3-Oxo-C6-HSL) did not reduce pigment production during the bioassay (Figure 3.2A).

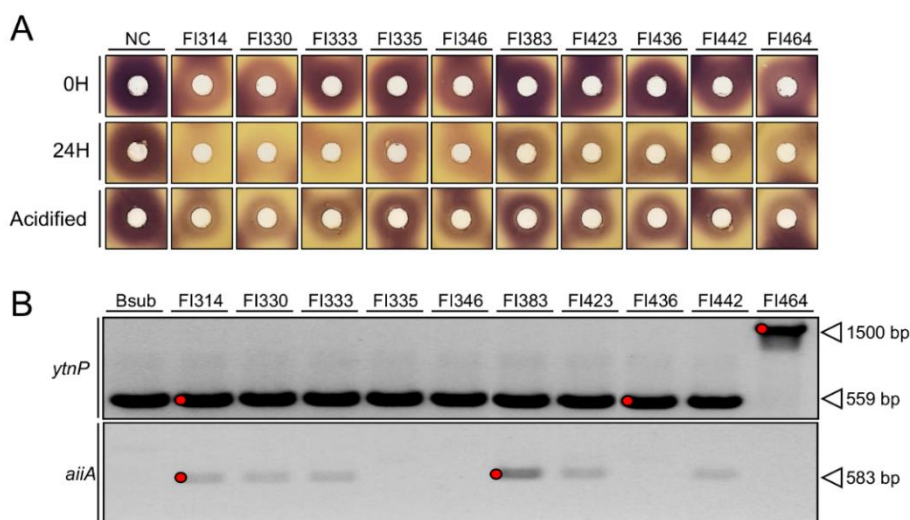


Figure 3.2. Activity and genomic detection of putative QQ lactonases. **(A)** Enzymatic degradation of 3-Oxo-C6-HSL by the fish isolates' extracellular compounds (FIs on the top), revealed by the loss or reduction of the violacein pigment production by the biosensor *Chr. violaceum* CV026. Since lactonase enzymatic reaction is pH-mediated and reversible by acidification, mixtures were acidified to pH 2 to restore the violacein pigment induction on the biosensor. LB supplemented with 30 μ M 3-Oxo-C6-HSL was used as negative control (NC). All photos were taken with a Sony IMX240 camera and are at the same scale. **(B)** PCR detection of genes coding for a putative QQ lactonase (*ytnP*) and N-acyl homoserine lactonase (*aiiA*) in the genomes of *B. subtilis* 168 (Bsub) and fish isolates (FI numbers on top of the figure). The amplicon size, in base pairs (bp), is shown on the right. The figure was constructed using different zones of the agarose gel. PCR products marked with a red circle in the figure were sequenced using the corresponding forward and reverse primers (Table 3.2).

To elucidate the lactonases behind the observed QQ activities, all 10 FI isolates were first identified based on partial sequencing of the 16S rRNA gene (~1000 bp), as part of the *Bacillus* genera. FI314, FI330, FI346, and FI442 were identified as *B. subtilis*; FI333, and FI423 as *B. amyloliquefaciens*; FI335, FI383, and FI436 as *B. velezensis*; and FI464 as a *B. pumilus* (Supplementary Table 1 – Appendix I) The presence of genes that were previously correlated with QQ activity in *Bacillus* species was first investigated by using oligonucleotide primers described by Kalia, et al. (2011) to amplify the Firmicutes AHL-lactonase gene *aiiA* (Table 3.2). Since no amplification could be obtained in the target FI (data not shown), novel oligonucleotide primers were specifically designed in highly conserved genomic regions, to target the genes of interest: *aiiA* (N-acyl homoserine lactonase) and *ytnP* (probable quorum-quenching lactonase). As shown in Figure 3.2B, all strains except FI464 showed a PCR band with the expected size (559 bp) of *ytnP* gene. In isolate FI464, a strong PCR band was observed at a higher molecular weight (~1500 bp). However, only faint bands with the expected size (583bp) of *aiiA* could be detected in FI314, FI330, FI333, FI383, FI423, and FI442.

Conjugating the previously observed genomic profile and QQ bioactivities, five PCR products were sequenced to confirm if they matched the targeted gene (bands marked in red in Figure 3.2B). All DNA sequences could be assigned to a protein family using BLASTx (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; accessed on 15th January 2020) (Table 3.3). The amino acid sequences of putative AHL-lactonases were aligned with other known AHL-lactonases, and the conserved motif “HXHDXH” present in the metallo- β -lactamase superfamily was searched for, as illustrated in Supplementary Figure S2A (Appendix I). Putative *ytnP*-like AHL-lactonases from FI314 and FI436 have the zinc-binding motif “HXHDXH” (Supplementary Figure S2A - Appendix I) and cluster with the *B. subtilis* 168 *ytnP* (Supplementary Figure S2B - Appendix I), being identified through BlastX as *ytnP*-like metallo-hydrolase and MBL fold metallo-hydrolase, respectively. On the other hand, the high molecular weight band of FI464 did not correspond to a putative QQ lactonase but to a DAK2 domain-containing protein (Table 3.3) and did not cluster to any QQ enzymes known in *Bacillus* spp. (Supplementary Figure S2B - Appendix I). Putative AHL-lactonase *aiiA*, from strains FI314 and FI383, clustered separately from the other AHL-lactonases in the metallo- β -lactamase family, and were identified as containing a rhodanese-like homology domain (Table 3.3).

Table 3.3. Identification of putative QQ related genes amplified from gDNA of fish isolates (FI).

Gene	FI nº	Closest known Protein ^a	QC (%) ^b	Identity (%) ^c
<i>ytnP</i>	314	ytnP-like metallo-hydrolase	99	100
	436	MBL fold metallo-hydrolase	100	100
	464	DAK2 domain-containing protein	88	99.2
<i>aiiA</i>	314	MBL fold metallo-hydrolase – Rhodanese Homology Domain	96	100
	383	MBL fold metallo-hydrolase – Rhodanese Homology Domain	95	100

^a Closest known protein using BLASTx based on partial sequences of QQ genes (~300–400 nt).

^b Query Cover - the percentage of the query sequence covered by the reference sequence.

^c Percent Identity - the percentage of similarity between the query sequence and the reference sequence.

3.3.3. Isolates QQ compounds can interfere with fish pathogens AHLs

After establishing the putative lactonase type of FI QQ activity, FI QQ compounds were tested for interference with fish pathogens AHLs. For this, 13 different fish pathogens (Table 3.1) were studied for the production of AHLs detectable by *Chr. violaceum* CV026 biosensor. From the 13 tested pathogens, only *Aeromonas salmonicida*, *A. veronii*, *A. bivalvium*, and *Edwardsiella tarda* could induce the production of a violacein pigment on the biosensor, by cross-feeding (Supplementary Figure S3 - Appendix I).

As AHLs can diffuse through the cell, their production kinetics and cell-release were investigated on those four pathogens. By using pathogens extracellular medium in a well-diffusion method, it was observed that the production of AHLs by *A. veronii* started at the beginning of its growth curve, lasting for 12 h, with a maximum peak at the transition from the late exponential to the early stationary phase of growth (Figure 3.3A,B). In *E. tarda*, AHLs detection started at the transition from the exponential to stationary growth phase and was extended until 36 h of growth (Figure 3.3A,B). Although observable by cross-feeding (Supplementary Figure S3 - Appendix I), the use of an extracellular medium in the well-diffusion method did not allow the detection of AHLs produced by *A. salmonicida* and *A. bivalvium* by the CV026 biosensor during the 48h of the assay (Figure 3.3B).

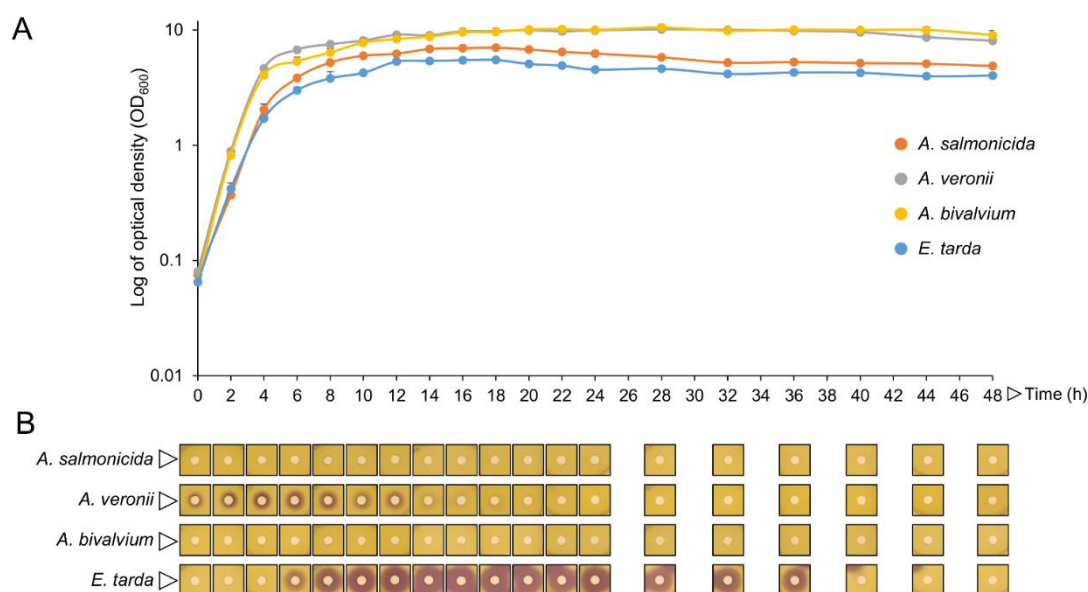


Figure 3.3. Growth curves and AHLs production kinetics of *A. salmonicida*, *A. veronii*, *A. bivalvium* and *E. tarda*. **(A)** Optical density of bacterial pathogens grown for 48h at 25 °C (or 37 °C in the case of *E. tarda*), 140 rpm. **(B)** Violacein pigmentation halos of *Chr. violaceum* CV026 around the wells filled with filtered cell-free supernatant from the pathogens' bacterial cultures at different time points. The data is composed by three independent experiments. All photos were taken with a Sony IMX240 camera and are at the same scale.

Before ascertaining the FI QQ capacity against natural AHLs from fish pathogens, the QQ kinetics of FI314, FI436, and FI464 (selected by conjugating the previous QQ bioassays, molecular identification, and the QQ genomic profile) was assessed using synthetic 3-oxo-C6-HSL. For all strains, the maximum production of QQ compounds was during the early stationary growth phase (~8h) (Supplementary Figure S4 - Appendix I). Fish pathogens AHLs were extracted from the cell-free supernatants of 6 h and 14 h cultures of *A. veronii* and *E. tarda*, respectively, and analysed through agar well-diffusion assay using different supplementation amounts (10-100 μ L in 8 mL of soft-agar) (Figure 3.4). *A. veronii* crude AHL extracts induced a slight violacein pigmentation on CV026 biosensor with 100 μ L of supplementation. On the other hand, the AHL crude extract from *E. tarda* induced the purple pigmentation on the biosensor CV026 from 10-100 μ L of supplementation. As illustrated in Figure 3.4, all three selected FI strains could inactivate the signals produced by *A. veronii* and *E. tarda*, with FI314 and FI464 showing a higher QQ potential. Additionally, a decrease in QQ activity could also be observed with an increase of *E. tarda* AHLs supplementation (Figure 3.4), indicating a concentration-dependent activity.

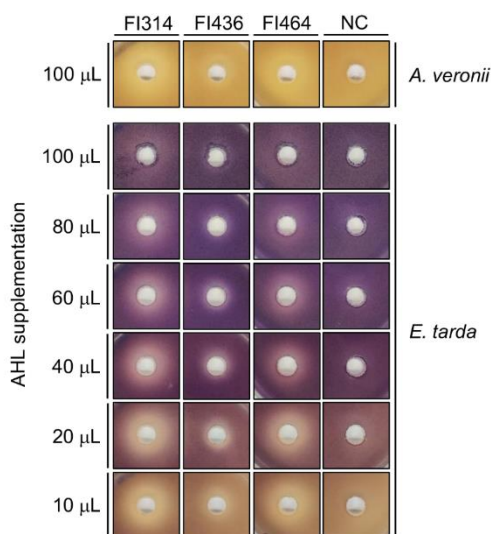


Figure 3.4. Inhibition of *A. veronii* and *E. tarda* AHLs' detection by the *Chr. violaceum* CV026 biosensor. Biosensor's violacein pigment production when supplemented with natural AHLs extracted from *A. veronii* and *E. tarda* (10, 20, 40, 60, 80 and 100 μ L) around wells containing cell-free supernatant of sporeforming fish isolates (FI numbers on top). All photos were taken with a Sony IMX240 camera and are at the same scale.

3.3.4. Isolates QQ Compounds protect zebrafish larvae upon *E. tarda* challenge

The next question was whether the *in vitro* FI QQ activity against *E. tarda* AHLs could originate from an *in vivo* protection of FI strains to zebrafish larvae challenged with *E. tarda*.

For this, the maximum non-toxic extract concentration (MNTC) of each FI to be employed in protective assays, was first established using 4dpf zebrafish larvae (Supplementary Figure S5A - Appendix I). The results showed that extracts from all strains induced toxicity in zebrafish larvae when administrated at concentrations above 250 μ g mL⁻¹ and thus, FI extracts were used at 250 μ g mL⁻¹ (MNTC). This concentration did not influence *E. tarda* growth (data not shown).

Secondly, an *E. tarda* infection model was established by exposing 10 dpf zebrafish larvae by immersion with 5x10⁷, 1x10⁸ and 3x10⁸ CFU mL⁻¹ of *E. tarda* for 24h. Larvae exposed to 3x10⁸ CFU *E. tarda* mL⁻¹ started to show mortality at 10 hours post-infection (hpi) and rapidly progressed until 18hpi with 100% mortality (data not shown and Supplementary Figure S5 - Appendix I). Mortalities in larvae exposed to 1x10⁸ CFU *E. tarda* mL⁻¹ began at 17 hpi and progressed through time, reaching ~ 60% at 24 hpi. On the other hand, 5x10⁷ CFU *E. tarda* mL⁻¹ started to induce mortalities only at 23 hpi (Supplementary Figure S5B – Appendix I). Control larvae did not exhibit any mortality throughout the experimental trial. From the overall results, 1x10⁸ CFU *E. tarda* mL⁻¹ was selected as the bacterial concentration for the challenge experiment.

Finally, the protective effect of FI extracellular compounds was evaluated using the *E. tarda* infection model. As illustrated in Figure 3.5, FI extracellular compounds from FI314, FI436, and FI464 were able to protect zebrafish larvae from *E. tarda* infection, significantly increasing larvae survival rate when compared to the control (non-treated zebrafish larvae infected with *E. tarda*). In the control group, at 24 hpi only 30% of zebrafish larvae survived which resulted in 100% mortality at 48 hpi. By comparing treatments with the control group, after 48 hpi FI314 increased the average survival rate of challenged larvae by 43% ($p < 0.01$), and strains FI436 and FI464 increased the survival rate upon challenge by 50% ($p < 0.001$).

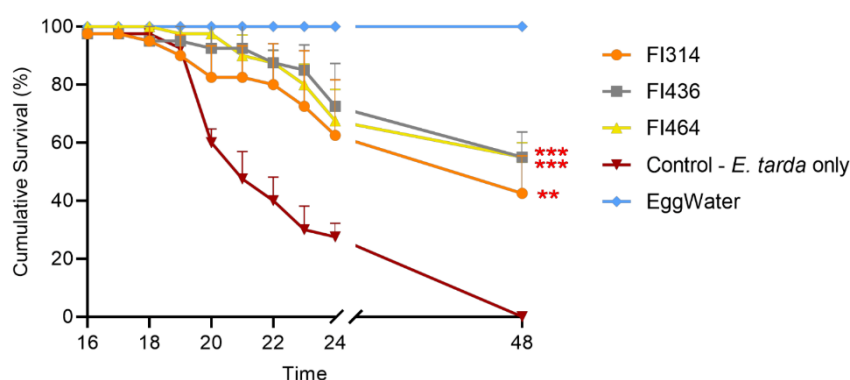


Figure 3.5. FI extracts protection of zebrafish larvae against infection with *E. tarda*. 7dpf zebrafish larvae were immersed for 2h with $250 \mu\text{g mL}^{-1}$ of each FI extract and three days later, challenged with *E. tarda* at a final concentration of 1×10^8 CFUs mL^{-1} for 24h. Untreated larvae challenged with *E. tarda* and, untreated and unchallenged larvae were used as positive and negative control, respectively. Data is composed by three independent experiments. Significant differences ($p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (**, ***, respectively).

3.4. Discussion

Bacillus spp. have been extensively studied for their use in aquaculture due to their probiotic attributes which include the production of bioactive compounds and the modulation of the host immune response (James, et al., 2021). In addition to their well-known antimicrobial activity, *Bacillus* spp. are also producers of quorum-quenching (QQ) molecules (Chen, et al., 2020; Ghanei-Motlagh, et al., 2019; Santos, et al., 2021). The disruption of pathogens' communication or quorum-sensing via QQ molecules has been proposed as a promising alternative to fighting bacterial infections in aquaculture (Grandclement, et al., 2016), which remains a major constraint to the sustainable development of the sector. We recently described the potential of fish-gut *Bacillus* spp. isolates (FI) as a source of QQ molecules (Santos, et al., 2021). In this study, we took advantage of 200 FI isolated from the gut of different fish species (*Sparus aurata*, *Dicentrarchus labrax*, and *Diplodus sargus*) (Santos, et al., 2021; Serra, et al., 2019) to

further explore their QQ potential, by testing their ability to partially or completely degrade AHLs' QS molecules used by gram-negative fish pathogens. Consistent with the literature, the results indicate that fish gut *Bacillus* spp. have QQ potential and, by testing their extracellular compounds, the majority of these *Bacillus* spp. produce and release QQ compounds to the extracellular environment. This observation contradicts previous reports that described AHL-degrading activities in *Bacillus* spp. as cytoplasmatic or cell-wall-associated (Dong, et al., 2000; Dong, et al., 2002; Fetzner, 2015) and might represent a technological advantage for QQ diseases control since compounds that work extracellularly are believed to exert less selective pressure in evolving resistance among pathogens (Defoirdt, 2013; Fetzner, 2015; García-Contreras, et al., 2013).

AHLs can be degraded through the enzymatic activity of lactonases, acylases, or oxidoreductases. To date, mainly lactonases, but also one oxidoreductase and one putative acylase, have been described in *Bacillus* species (Chowdhary, et al., 2007; Dong, et al., 2002; Nithya, et al., 2010; Tinh, et al., 2013). When an AHL-lactonase is present, cleavage of the homoserine lactone ring of the AHL molecule occurs. The opening of the lactone ring makes the AHL molecule incapable of binding to the target transcriptional regulators (e.g VanT, AhyR, and LuxR homologs), attenuating its effectiveness and detection by the biosensor (Chen, et al., 2013). This hydrolysis is pH-mediated and can be reversed by acidification. Taking this into consideration, the extracellular enzymatic activity of the positive FI isolates was tested, and all 10 strains enzymatically degraded the AHLs after 24 h of incubation, with a partial restoration of the AHL molecule, inferred by the recovery of the violacein pigment on the biosensor, upon the acidification process. The restoration of the violacein pigment on the biosensor after the acidification process indicated the putative presence of an AHL-lactonase. However, pigment regain was only partial, which may be explained by the lack of pH control during incubation, which is essential to the hydrolysis reversion of the lactone ring (Yates, et al., 2002). Although the presence of an acylase, an oxidoreductase, or small molecules that blocked the biosensor signal receptors cannot be ruled out, AHLs degradation seen by the smaller colour formation on the biosensor submitted to the 24 h AHL-SUP mixture when compared to the colour observed on the biosensor submitted to fresh (H0) AHL-SUP mixture, followed by colour restoration upon acidification, indicate that the observable QQ activity is probably due to a lactonase-like enzyme.

In the lactonase-like enzyme family, the *aiiA* gene and AiiA enzyme were the first to be discovered and characterized for their QQ activity in *Bacillus* spp. (Dong, et al., 2000). Over the years, different QQ studies highlighted the potential of the AiiA enzyme in the

prevention of plant and animal bacterial infections (Cao, et al., 2012; Dong, et al., 2000; Pan, et al., 2008). In the present study, attempts to amplify the *aiiA* gene, both using the literature-described primers and primers designed by us, were unsuccessful. Despite the presence of light PCR bands in the agarose gel (using the primers designed in this study), the analysis of their nucleotide and translated sequences did not correspond to the *aiiA* gene or contain the AHL-lactonases' conserved zinc-binding motif "HxHxDH" (Dong, et al., 2002). Although the Metallo- β -lactamase family members possess the same folding and conserved sequences, the group englobes proteins with divergent sequences and biological functions (Daiyasu, et al., 2001). Thus, it was hypothesized that, by using the primers designed in homologous sequences, other Metallo- β -lactamase proteins were amplified, with no similarity to the *aiiA* gene. In fact, by considering the taxonomic identification of the QQ strains, only a few studies have reported the presence of the *aiiA* gene in *B. subtilis* and *B. amyloliquefaciens* (Pan, et al., 2008; Yin, et al., 2010), and none in *B. velezensis* and *B. pumilus*.

Meanwhile, Schneider, et al. (2012) reported the expression of another QQ gene, the *ytnP* from *B. subtilis*, capable of interfering with the signalling pathways of biofilm formation and streptomycin production in *Pseudomonas aeruginosa* and *Streptomyces griseus*. Here, the *ytnP* gene was successfully amplified in 9 out of the 10 QQ strains. Protein sequence analysis of FI314 and FI436 showed similarity with *ytnP*-like and MBL fold proteins, respectively, and revealed the presence of the AHL-lactonase zinc-binding motif containing the key histidine residues "HxHxDH" of the lactonase architecture (Dong, et al., 2002). The YtnP enzyme has been described to accumulate in the cytoplasm, and only upon the presence of other antimicrobials and stress factors (Schneider, et al., 2012). In the strains used in this study, the observed QQ activity was extracellular, and is thus unlikely to only be due to the action of YtnP. An additional undescribed QQ lactonase, or at least a differently regulated *ytnP*, must be present. The deletion of *ytnP* in the FI isolates background could provide a glimpse into their QQ mechanism, but transformation of these strains (both by chemical methods and by electroporation) has been unsuccessful to date (data not shown).

Additionally, despite showing a putative AHL-lactonase activity in the biochemical test, none of the tested QQ genes nor the conserved motif of AHL-lactonase were amplified from FI464, identified as *B. pumilus*. In fact, to the authors' knowledge, there is only one report describing *B. pumilus* QQ potential. Nithya, et al. (2010) reported a *B. pumilus* strain with a putative acylase activity, which effectively reduced biofilm formation and other virulence factors in *P. aeruginosa*. However, the authors did not characterize the protein in detail nor provided the gene sequence. Thus, the FI464 genome is now

undergoing further investigation as it is assumed that this strain possesses a completely different lactonase activity to the one described in the literature for the other *Bacillus* species.

The ability of the FI to interfere with fish pathogens QS was the core of this study. Thus, the three strains with the best QQ profile, FI314, FI436, and FI464, were evaluated for their ability to degrade the natural signals produced by different species of problematic fish pathogens. QS systems based on AHLs are well-described and reviewed in fish bacterial pathogens (Bruhn, et al., 2005; Defoirdt, et al., 2008; Gui, et al., 2017; Han, et al., 2010; Milton, et al., 2001; Milton, et al., 1997; Morohoshi, et al., 2004; Romero, et al., 2010; Swift, et al., 1997; Zhao, et al., 2015). Here, AHLs' production was evaluated by cross-feeding fourteen different fish pathogens, including species from *Aeromonas*, *Vibrio*, *Photobacterium*, *Edwardsiella*, and *Shigella* genera, with the biosensor *Chr. violaceum* CV026. Of the tested pathogens, only *A. salmonicida*, *A. veronii*, *A. bivalvium*, and *E. tarda* had the capacity to induce violacein pigmentation on the biosensor *Chr. violaceum* CV026. This biosensor has the limitation of being stimulated by AHLs with acyl chains ranging from C4 to C8 and, when these are present alongside with the long-acyl-chain molecules (C8-C14), inhibition of violacein production occurs (McClean, et al., 1997). This might explain the lack of violacein induction on the biosensor by the tested *Vibrio* spp., which are known to produce several QS molecules, including AHLs with different lengths and derivatives, such as C4-HSL, C6-HSL, 3-oxo-C10-HSL, 3-oxo-C14-HSL (Milton, 2006; Milton, et al., 2001; Milton, et al., 1997; Valiente, et al., 2009). Bruhn, et al. (2005) also reported a lack of violacein induction on the CV026 biosensor when testing *V. anguillarum*, *V. vulnificus*, and *Photobacterium damsela* subsp. *damsela* strains, but demonstrated AHLs production in *Vibrio* spp. using another biosensor.

E. tarda and *Aeromonas* spp. have previously been reported to induce the violacein pigment on the CV026 biosensor (Han, et al., 2010; Jangid, et al., 2007; Morohoshi, et al., 2004; Swift, et al., 1997). The exception in these results, as anticipated, was the *A. hydrophila* LMG 2844 strain, as it was unable to induce an AHL-mediating response detectable by different biosensors, including CV026 (dos Reis Ponce-Rossi, et al., 2016; Jangid, et al., 2007). An unexpected result was the lack of violacein pigment when testing *T. maritimum*, since Romero, et al. (2010) reported that this species produces C4-HSL. Although the CV026 biosensor detects C4-HSL molecules, no activity was reported in the Flavobacteriaceae family using this biosensor (Bruhn, et al., 2005), and to the authors' knowledge, there is no study reporting violacein induction on the CV026 biosensor by *T. maritimum* AHLs. Moreover, genome sequencing of the *T. maritimum*

strain NCIMB 2154^T revealed the absence of homologous genes for AHLs synthesis (Pérez-Pascual, et al., 2017).

Next, the pathogens that induced violacein pigmentation on the *Chr. Violaceum* biosensor, i.e., *A. salmonicida*, *A. veronii*, *A. bivalvium*, and *E. tarda*, were tested for the maximum peak in AHLs production and extracellular accumulation. Although *A. salmonicida* and *A. bivalvium* induced the violacein pigment on the cross-feeding bioassays, they failed to induce it when testing the extracellular accumulation for 48h. This might be due to low AHLs concentration or stability outside the cell. *A. veronii* accumulated AHLs during the whole exponential phase with a maximum peak at the time of transition to the stationary phase, followed by a rapid decline, as described by Jangid, et al. (2012). However, AHLs accumulation in *E. tarda* was only detected when the cells were entering the stationary phase, and was stable throughout this phase, with the maximum peak detected at 16 h of growth. Accordingly, Han, et al. (2010) also detected AHLs production during the *E. tarda* growth phase (1-21 h) using the *Ag. tumefaciens* KYC55 biosensor and, since this biosensor is considered to be ultra-sensitive in AHLs detection, the accumulation of AHLs was detected 5 h earlier.

A. veronii is known to produce natural short and medium acyl-chains, such as C6-HSL, C8-HSL, 3-oxo-C8-HSL, and 3-hydro-C8-HSL (Gui, et al., 2018). In CV026, C8 acyl chains led to an inhibition of violacein induction by other molecules such as C6-HSL (McClean, et al., 1997). This might explain the minor induction of violacein pigment on the biosensor observed when the crude and concentrated *A. veronii* extracts were used. Nonetheless, FI314 and FI464 were able to interfere with these natural AHLs, either through enzymatical degradation or by interrupting their detection by bacterial receptors. When testing different quantities of *E. tarda* AHLs supplementation, it could be observed that an increase in supplementation led to a decrease in QQ activity, which might indicate an enzymatic degradation of C4-HSL, C6-HSL, 3-oxo-C6-HSL, and C7-HSL (Han, et al., 2010; Morohoshi, et al., 2004) by all the tested FIs. Recent studies have highlighted the QQ potential of *Bacillus* spp. against natural AHLs produced by fish pathogens such as *A. hydrophila* (Chu, et al., 2011; Chu, et al., 2014; Zhou, et al., 2019), *Yersinia ruckeri* (Torabi Delshad, et al., 2018), and *V. harveyi* and *V. alginolyticus* (Ghanei-Motlagh, et al., 2019). Similarly, Gui, et al. (2017) demonstrated that a purified lactonase AiiA_{AI96} from *Bacillus* sp. enzymatically inactivated the AHLs produced by *A. veronii*, as well as other QS-controlled behaviors. To the authors' knowledge, to date, only Romero, et al. (2014) have described an *in vitro* quenching of *E. tarda* AHLs (C6-HSL and 3-oxo-C6-HSL) using *Tenacibaculum* sp. strain 20J cell extracts. Thus, this is the first report

demonstrating the potential of QQ *Bacillus* spp. in inhibiting the natural AHLs produced by *E. tarda*.

E. tarda is an important bacterial pathogen that causes haemorrhagic septicemia, edwardsielosis, affecting economically important aquaculture fish species such as turbot (Castro, et al., 2006), Senegalese sole (Castro, et al., 2012), and tilapia (Abo El-Yazeed, Ibrahem, 2009; Iregui, et al., 2012; Miniero Davies, et al., 2018), and has been associated with gastro- and extraintestinal infections in humans (Hirai, et al., 2015; Slaven, et al., 2001).

Bacillus QQ strains have been used in several *in vivo* studies for fish disease mitigation, but to date none has addressed *E. tarda* infections. For example, oral administration of purified QQ enzyme AiiA increased zebrafish survival by 40% when challenged with *A. hydrophila* (Cao, et al., 2012), and increased shrimp survival by 50% when challenged with *V. parahaemolyticus* (Vinoj, et al., 2014). Similarly, when cells of QQ *B. cereus* and *B. thuringiensis* strains were fed to rainbow trout, fish survival upon infection with *Y. ruckeri* increased by 80% (Torabi Delshad, et al., 2018). Additionally, Chen, et al. (2010) co-injected a purified QQ enzyme (AiiA B546) with *A. hydrophila*, reducing common carp mortality by 25% and, in a similar experiment, cells of the QQ *B. licheniformis* T-1 strains decreased zebrafish mortality by 50% when co-injected with *A. hydrophila* (Chen, et al., 2020).

In the present study, the treatment of zebrafish larvae with FI314, FI436, and FI464 extracts significantly reduced their mortality when challenged with *E. tarda* by immersion. In *E. tarda*, the routes of infection are believed to be the gut, the skin, and the gills (Ling, et al., 2001). The gills and the skin are in constant contact with the environment, and consequently are accessible to pathogen entry. Thus, the presence of QQ molecules at these sites may help prevent or delay fish infection. On the other hand, in the present study, there was no physical contact between the FIs extracts and *E. tarda*, suggesting that these molecules can circulate through the host, exerting their protective effect. Although FIs extracts did not inhibit *E. tarda* growth (data not shown), a possible direct stimulus in the larvae immune system, facilitating disease resistance, cannot be ruled out. This interpretation is only speculative and requires further investigation, including the determination of a putative stimulus in the fish immune system using such extracts. Importantly, the extracts showed good stability (up to 12 months) under no specific storage conditions (room temperature). Moreover, a small bacterial culture volume (200 mL) allowed to obtain lyophilized extract to make up to 40L of treating water. These characteristics reinforce the practical application of these extracts in the aquaculture industry.

As a conclusion, this work describes three *Bacillus* spp. capable of extracellular quenching *E. tarda* AHLs while protecting the model zebrafish larvae from infection. The lack of studies regarding *E. tarda* infection and its crescent impact in aquaculture emphasizes the novelty and importance of this study. Nonetheless, further studies are required to fully characterize the QQ molecules responsible for the bioactivities described and to clarify the QQ mechanism involved. QS plays an important role in bacterial pathogenesis and virulence; thus, the QQ molecules from FI314 (*B. subtilis*), FI436 (*B. velezensis*), and FI464 (*B. pumilus*) may be promising tools for disease control in aquaculture.

Supplementary Materials: The following are available in Appendix I. Table S1: Identification of the 10 fish-gut isolates with QQ activity, using 16S rRNA gene analysis. Figure S1: Flow diagram of the methodology used for evaluating FI isolates QQ activity. Figure S2: Amino acid sequence comparison of the putative AHL-lactonases with known AHL-lactonase enzymes. Figure S3: Detection of AHLs production by gram-negative fish pathogens using *Chr. violaceum* CV026 as a biosensor. Figure S4: Growth curves and QQ kinetics of FI314, FI436 and FI464. Figure S5: Toxicity of fish isolates' extracellular compounds and establishment of *E. tarda* infection model in zebrafish larvae.

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Institutional Review Board Statement: Animal experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) and carried out in a registered installation (N16091.UDER) and were performed by trained scientists (following FELASA category C recommendations) in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Data Availability Statement: 16S rRNA gene sequences of fish isolates described in this manuscript have been deposited in GenBank with the accession numbers provided in Table 3.2. Authors confirm that all relevant data are included in the article.

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Conflicts of Interest: RJ is an employee of Epicore Inc., a subsidiary of Archer Daniels Midland, that supported this research by completing a commercial licensing agreement for the potential utilization of the probiotic bacteria FI314, FI330, FI442 used in this research, in commercial products for aquaculture. The opinions expressed in the manuscript are those of the authors and do not necessarily reflect company policies. All other authors declare no competing interests.

3.5. References

- Abisado, R.G., Benomar, S., Klaus, J.R., Dandekar, A.A., Chandler, J.R., 2018. Bacterial Quorum Sensing and Microbial Community Interactions. *mBio*. 9, e02331-02317. doi: <https://doi.org/10.1128/mBio.02331-17>.
- Abo El-Yazeed, H., Ibrahim, M.D., 2009. Studies on *Edwardsiella tarda* infection in catfish and *Tilapia nilotica*. *Journal of Veterinary Medical Research*. 19, 44-50. doi: <https://doi.org/10.21608/jvmr.2009.77808>.
- Bayliss, S.C., Verner-Jeffreys, D.W., Bartie, K.L., Aanensen, D.M., Sheppard, S.K., Adams, A., Feil, E.J., 2017. The Promise of Whole Genome Pathogen Sequencing for the Molecular Epidemiology of Emerging Aquaculture Pathogens. *Front Microbiol*. 8, 121-121. doi: <https://doi.org/10.3389/fmicb.2017.00121>.
- Bruhn, J.B., Dalsgaard, I., Nielsen, K.F., Buchholtz, C., Larsen, J.L., Gram, L., 2005. Quorum sensing signal molecules (acylated homoserine lactones) in gram-negative fish pathogenic bacteria. *Dis Aquat Organ*. 65, 43-52. doi: <https://doi.org/10.3354/dao065043>.
- Cao, Y., He, S., Zhou, Z., Zhang, M., Mao, W., Zhang, H., Yao, B., 2012. Orally administered thermostable N-acyl homoserine lactonase from *Bacillus* sp. strain AI96 attenuates *Aeromonas hydrophila* infection in zebrafish. *Appl Environ Microbiol*. 78, 1899-1908. doi: <https://doi.org/10.1128/aem.06139-11>.
- Castro, N., Toranzo, A.E., Barja, J.L., Núñez, S., Magariños, B., 2006. Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.). *J Fish Dis*. 29, 541-547. doi: <https://doi.org/10.1111/j.1365-2761.2006.00750.x>.
- Castro, N., Toranzo, A.E., Devesa, S., González, A., Nuñez, S., Magariños, B., 2012. First description of *Edwardsiella tarda* in Senegalese sole, *Solea senegalensis* (Kaup). *J Fish Dis*. 35, 79-82. doi: <https://doi.org/10.1111/j.1365-2761.2011.01325.x>.
- Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Front Microbiol*. 10. doi: <https://doi.org/10.3389/fmicb.2019.00302>.
- Chen, B., Peng, M., Tong, W., Zhang, Q., Song, Z., 2020. The Quorum Quenching Bacterium *Bacillus licheniformis* T-1 Protects Zebrafish against *Aeromonas hydrophila* Infection. *Probiotics Antimicrob Proteins*. doi: <https://doi.org/10.1007/s12602-018-9495-7>.

- Chen, F., Gao, Y., Chen, X., Yu, Z., Li, X., 2013. Quorum Quenching Enzymes and Their Application in Degrading Signal Molecules to Block Quorum Sensing-Dependent Infection. *Int J Mol Sci.* 14, 17477-17500. doi: <https://doi.org/10.3390/ijms140917477>.
- Chen, R., Zhou, Z., Cao, Y., Bai, Y., Yao, B., 2010. High yield expression of an AHL-lactonase from *Bacillus* sp. B546 in *Pichia pastoris* and its application to reduce *Aeromonas hydrophila* mortality in aquaculture. *Microb Cell Fact.* 9, 39-39. doi: <https://doi.org/10.1186/1475-2859-9-39>.
- Chowdhary, P.K., Keshavan, N., Nguyen, H.Q., Peterson, J.A., Gonzalez, J.E., Haines, D.C., 2007. *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. *Biochemistry.* 46, 14429-14437. doi: <https://doi.org/10.1021/bi701945j>.
- Chu, W., Lu, F., Zhu, W., Kang, C., 2011. Isolation and characterization of new potential probiotic bacteria based on quorum-sensing system. *J Appl Microbiol.* 110, 202-208. doi: <https://doi.org/10.1111/j.1365-2672.2010.04872.x>.
- Chu, W., Zhou, S., Zhu, W., Zhuang, X., 2014. Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. *Sci Rep.* 4, 5446. doi: <https://doi.org/10.1038/srep05446>.
- Croxatto, A., Chalker, V.J., Lauritz, J., Jass, J., Hardman, A., Williams, P., Camara, M., Milton, D.L., 2002. VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum*. *J Bacteriol.* 184, 1617-1629.
- Daiyasu, H., Osaka, K., Ishino, Y., Toh, H., 2001. Expansion of the zinc metallo-hydrolase family of the β -lactamase fold. *FEBS Letters.* 503, 1-6. doi: [https://doi.org/10.1016/S0014-5793\(01\)02686-2](https://doi.org/10.1016/S0014-5793(01)02686-2).
- Defoirdt, T., 2013. Antivirulence Therapy for Animal Production: Filling an Arsenal with Novel Weapons for Sustainable Disease Control. *PLoS Pathog.* 9, e1003603. doi: <https://doi.org/10.1371/journal.ppat.1003603>.
- Defoirdt, T., 2014. Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. *Rev Aquacult.* 6, 100-114. doi: <https://doi.org/10.1111/raq.12030>.
- Defoirdt, T., 2018. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends Microbiol.* 26, 313-328. doi: <https://doi.org/10.1016/j.tim.2017.10.005>.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2008. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. *Isme j.* 2, 19-26. doi: <https://doi.org/10.1038/ismej.2007.92>.

Diaz-Rosales, P., Chabrillon, M., Morinigo, M.A., Balebona, M.C., 2003. Survival against exogenous hydrogen peroxide of *Photobacterium damsela* subsp. *piscicida* under different culture conditions. *J Fish Dis.* 26, 305-308. doi: <https://doi.org/10.1046/j.1365-2761.2003.00455.x>.

Dong, Y.-H., Xu, J.-L., Li, X.-Z., Zhang, L.-H., 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc Natl Acad Sci.* 97, 3526. doi: <https://doi.org/10.1073/pnas.060023897>.

Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L., Zhang, L.H., 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. *Appl Environ Microbiol.* 68, 1754-1759.

dos Reis Ponce-Rossi, A., Pinto, U.M., de Oliveira Barros Ribon, A., Bazzolli, D.M.S., Vanetti, M.C.D., 2016. Quorum sensing regulated phenotypes in *Aeromonas hydrophila* ATCC 7966 deficient in AHL production. *Annals of Microbiology.* 66, 1117-1126. doi: <https://doi.org/10.1007/s13213-016-1196-4>.

Fetzner, S., 2015. Quorum quenching enzymes. *J Biotechnol.* 201, 2-14. doi: <https://doi.org/10.1016/j.jbiotec.2014.09.001>.

Fuqua, C., Winans, S.C., Greenberg, E.P., 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol.* 50, 727-751. doi: <https://doi.org/10.1146/annurev.micro.50.1.727>.

García-Contreras, R., Maeda, T., Wood, T.K., 2013. Resistance to Quorum-Quenching Compounds. *Appl Environ Microbiol.* 79, 6840. doi: <https://doi.org/10.1128/AEM.02378-13>.

Ghanei-Motlagh, R., Mohammadian, T., Gharibi, D., Menanteau-Ledouble, S., Mahmoudi, E., Khosravi, M., Zarea, M., El-Matbouli, M., 2019. Quorum Quenching Properties and Probiotic Potentials of Intestinal Associated Bacteria in Asian Sea Bass *Lates calcarifer*. *Mar Drugs.* 18. doi: <https://doi.org/10.3390/md18010023>.

Grandclement, C., Tannieres, M., Morera, S., Dessaux, Y., Faure, D., 2016. Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev.* 40, 86-116. doi: <https://doi.org/10.1093/femsre/fuv038>.

- Gui, M., Wu, R., Liu, L., Wang, S., Zhang, L., Li, P., 2017. Effects of quorum quenching by AHL lactonase on AHLs, protease, motility and proteome patterns in *Aeromonas veronii* LP-11. *Int J Food Microbiol.* 252, 61-68. doi: <https://doi.org/10.1016/j.ijfoodmicro.2017.04.005>.
- Gui, M., Liu, L., Wu, R., Hu, J., Wang, S., Li, P., 2018. Detection of New Quorum Sensing N-Acyl Homoserine Lactones From *Aeromonas veronii*. *Front Microbiol.* 9, 1712. doi: <https://doi.org/10.3389/fmicb.2018.01712>.
- Han, Y., Li, X., Qi, Z., Zhang, X.H., Bossier, P., 2010. Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda* strain LTB-4. *J Appl Microbiol.* 108, 139-147. doi: <https://doi.org/10.1111/j.1365-2672.2009.04405.x>.
- Hirai, Y., Asahata-Tago, S., Ainoda, Y., Fujita, T., Kikuchi, K., 2015. *Edwardsiella tarda* bacteremia. A rare but fatal water- and foodborne infection: Review of the literature and clinical cases from a single centre. *Can J Infect Dis Med Microbiol.* 26, 313-318. doi: <https://doi.org/10.1155/2015/702615>.
- Iregui, C.A., Guarín, M., Tibatá, V.M., Ferguson, H.W., 2012. Novel brain lesions caused by *Edwardsiella tarda* in a red tilapia (*Oreochromis* spp.). *Journal of Veterinary Diagnostic Investigation.* 24, 446-449. doi: <https://doi.org/10.1177/1040638711435232>.
- James, G., Das, B.C., Jose, S., V.J, R.K., 2021. *Bacillus* as an aquaculture friendly microbe. *Aquac Int.* 29, 323-353. doi: <https://doi.org/10.1007/s10499-020-00630-0>.
- Jangid, K., Parameswaran, P.S., Shouche, Y.S., 2012. A variant quorum sensing system in *Aeromonas veronii* MTCC 3249. *Sensors (Basel).* 12, 3814-3830. doi: <https://doi.org/10.3390/s120403814>.
- Jangid, K., Kong, R., Patole, M.S., Shouche, Y.S., 2007. luxRI homologs are universally present in the genus *Aeromonas*. *BMC Microbiol.* 7, 93. doi: <https://doi.org/10.1186/1471-2180-7-93>.
- Jayaprakashvel, M., Subramani, R., 2019. Implications of Quorum Sensing and Quorum Quenching in Aquaculture Health Management. in: Bramhachari, P.V. (Ed.), *Implication of Quorum Sensing and Biofilm Formation in Medicine, Agriculture and Food Industry*. Springer Singapore, Singapore, pp. 299-312.
- Kalia, V.C., Raju, S.C., Purohit, H.J., 2011. Genomic analysis reveals versatile organisms for quorum quenching enzymes: acyl-homoserine lactone-acylase and -lactonase. *Open Microbiol J.* 5, 1-13. doi: <https://doi.org/10.2174/1874285801105010001>.

- Kuebutornye, F.K.A., Lu, Y., Abarike, E.D., Wang, Z., Li, Y., Sakyi, M.E., 2020. *In vitro* Assessment of the Probiotic Characteristics of Three *Bacillus* Species from the Gut of Nile Tilapia, *Oreochromis niloticus*. Probiotics Antimicrob Proteins. 12, 412-424. doi: <https://doi.org/10.1007/s12602-019-09562-5>.
- Ling, S.H.M., Wang, X.H., Lim, T.M., Leung, K.Y., 2001. Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. FEMS Microbiol Lett. 194, 239-243. doi: [https://doi.org/10.1016/S0378-1097\(00\)00498-5](https://doi.org/10.1016/S0378-1097(00)00498-5).
- McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S., Williams, P., 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology. 143 (Pt 12), 3703-3711. doi: <https://doi.org/10.1099/00221287-143-12-3703>.
- Meidong, R., Doolgindachbaporn, S., Jamjan, W., Sakai, K., Tashiro, Y., Okugawa, Y., Tongpim, S., 2017. A novel probiotic *Bacillus siamensis* B44v isolated from Thai pickled vegetables (Phak-dong) for potential use as a feed supplement in aquaculture. J Gen Appl Microbiol. 63, 246-253. doi: <https://doi.org/10.2323/jgam.2016.12.002>.
- Meidong, R., Khotchanalekha, K., Doolgindachbaporn, S., Nagasawa, T., Nakao, M., Sakai, K., Tongpim, S., 2018. Evaluation of probiotic *Bacillus aerius* B81e isolated from healthy hybrid catfish on growth, disease resistance and innate immunity of Pla-mong *Pangasius bocourti*. Fish Shellfish Immunol. 73, 1-10. doi: <https://doi.org/10.1016/j.fsi.2017.11.032>.
- Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu Rev Microbiol. 55, 165-199. doi: <https://doi.org/10.1146/annurev.micro.55.1.165>.
- Milton, D.L., 2006. Quorum sensing in vibrios: Complexity for diversification. Int J Med Microbiol. 296, 61-71. doi: <https://doi.org/10.1016/j.ijmm.2006.01.044>.
- Milton, D.L., Chalker, V.J., Kirke, D., Hardman, A., Camara, M., Williams, P., 2001. The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of N-(3-hydroxyhexanoyl)homoserine lactone and N-hexanoylhomoserine lactone. J Bacteriol. 183, 3537-3547. doi: <https://doi.org/10.1128/jb.183.12.3537-3547.2001>.
- Milton, D.L., Hardman, A., Camara, M., Chhabra, S.R., Bycroft, B.W., Stewart, G.S., Williams, P., 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/vanR* locus and identification of the autoinducer N-(3-oxodecanoyl)-L-homoserine lactone. J Bacteriol. 179, 3004-3012. doi: <https://doi.org/10.1128/jb.179.9.3004-3012.1997>.

Miniero Davies, Y., Xavier de Oliveira, M.G., Paulo Vieira Cunha, M., Soares Franco, L., Pulecio Santos, S.L., Zanolli Moreno, L., Túlio de Moura Gomes, V., Zanolli Sato, M.I., Schiavo Nardi, M., Micke Moreno, A., Becker Saidenberg, A., Rose Marques de Sá, L., Knöbl, T., 2018. *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. Vet Q. 38, 99-105. doi: <https://doi.org/10.1080/01652176.2018.1540070>.

Morohoshi, T., Inaba, T., Kato, N., Kanai, K., Ikeda, T., 2004. Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. Journal of Bioscience and Bioengineering. 98, 274-281. doi: [https://doi.org/10.1016/S1389-1723\(04\)00281-6](https://doi.org/10.1016/S1389-1723(04)00281-6).

Morohoshi, T., Kato, M., Fukamachi, K., Kato, N., Ikeda, T., 2008. N-acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472. FEMS Microbiol Lett. 279, 124-130. doi: <https://doi.org/10.1111/j.1574-6968.2007.01016.x>.

Mukherjee, A., Banerjee, G., Mukherjee, P., Ray, A.K., Chandra, G., Ghosh, K., 2019. Antibacterial substances produced by pathogen inhibitory gut bacteria in *Labeo rohita*: Physico-chemical characterization, purification and identification through MALDI-TOF mass spectrometry. Microb Pathog. 130, 146-155. doi: <https://doi.org/10.1016/j.micpath.2019.02.028>.

Nithya, C., Aravindraja, C., Pandian, S.K., 2010. *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. Res Microbiol. 161, 293-304. doi: <https://doi.org/10.1016/j.resmic.2010.03.002>.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET); Test OECD: Paris, France.

Pan, J., Huang, T., Yao, F., Huang, Z., Powell, C.A., Qiu, S., Guan, X., 2008. Expression and characterization of *aiiA* gene from *Bacillus subtilis* BS-1. Microbiol Res. 163, 711-716. doi: <https://doi.org/10.1016/j.micres.2007.12.002>.

Pande, G.S., Natrah, F.M., Flandez, A.V., Kumar, U., Niu, Y., Bossier, P., Defoirdt, T., 2015. Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae. Appl Microbiol Biotechnol. 99, 10805-10813. doi: <https://doi.org/10.1007/s00253-015-6918-1>.

Park, S.Y., Lee, S.J., Oh, T.K., Oh, J.W., Koo, B.T., Yum, D.Y., Lee, J.K., 2003. AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. Microbiology (Reading). 149, 1541-1550. doi: <https://doi.org/10.1099/mic.0.26269-0>.

Pérez-Pascual, D., Lunazzi, A., Magdelenat, G., Rouy, Z., Roulet, A., Lopez-Roques, C., Larocque, R., Barbeyron, T., Gobet, A., Michel, G., Bernardet, J.-F., Duchaud, E., 2017. The Complete Genome Sequence of the Fish Pathogen *Tenacibaculum maritimum* Provides Insights into Virulence Mechanisms. *Front Microbiol.* 8. doi: <https://doi.org/10.3389/fmicb.2017.01542>.

Romero, M., Avendano-Herrera, R., Magarinos, B., Camara, M., Otero, A., 2010. Acylhomoserine lactone production and degradation by the fish pathogen *Tenacibaculum maritimum*, a member of the Cytophaga-Flavobacterium-Bacteroides (CFB) group. *FEMS Microbiol Lett.* 304, 131-139. doi: <https://doi.org/10.1111/j.1574-6968.2009.01889.x>.

Romero, M., Muras, A., Mayer, C., Buján, N., Magariños, B., Otero, A., 2014. In vitro quenching of fish pathogen *Edwardsiella tarda* AHL production using marine bacterium *Tenacibaculum* sp. strain 20J cell extracts. *Dis Aquat Organ.* 108, 217-225. doi: <https://doi.org/10.3354/dao02697>.

Santos, R.A., Oliva-Teles, A., Pousão-Ferreira, P., Jerusik, R., Saavedra, M.J., Enes, P., Serra, C.R., 2021. Isolation and Characterization of Fish-Gut *Bacillus* spp. as Source of Natural Antimicrobial Compounds to Fight Aquaculture Bacterial Diseases. *Mar Biotechnol (NY)*. 23, 276-293. doi: <https://doi.org/10.1007/s10126-021-10022-x>.

Schneider, J., Yepes, A., Garcia-Betancur, J.C., Westedt, I., Mielich, B., López, D., 2012. Streptomycin-induced expression in *Bacillus subtilis* of YtnP, a lactonase-homologous protein that inhibits development and streptomycin production in *Streptomyces griseus*. *Appl Environ Microbiol.* 78, 599-603. doi: <https://doi.org/10.1128/aem.06992-11>.

Serra, C.R., Almeida, E.M., Guerreiro, I., Santos, R., Merrifield, D.L., Tavares, F., Oliva-Teles, A., Enes, P., 2019. Selection of carbohydrate-active probiotics from the gut of carnivorous fish fed plant-based diets. *Sci Rep.* 9, 6384. doi: <https://doi.org/10.1038/s41598-019-42716-7>.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Rinehart, K.L., Farrand, S.K., 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc Natl Acad Sci.* 94, 6036-6041. doi: <https://doi.org/10.1073/pnas.94.12.6036>.

Slaven, E.M., Lopez, F.A., Hart, S.M., Sanders, C.V., 2001. Myonecrosis Caused by *Edwardsiella tarda*: A Case Report and Case Series of Extraintestinal *E. tarda* Infections. *Clinical Infectious Diseases.* 32, 1430-1433. doi: <https://doi.org/10.1086/320152>.

- Swift, S., Karlyshev, A.V., Fish, L., Durant, E.L., Winson, M.K., Chhabra, S.R., Williams, P., Macintyre, S., Stewart, G.S., 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. *J Bacteriol.* 179, 5271-5281. doi: <https://doi.org/10.1128/jb.179.17.5271-5281.1997>.
- Tinh, N.T.N., Dung, N.V., Trung, C.T., Thuy, V.T., 2013. *In Vitro* Characterization of a Recombinant AHL-Lactonase from *Bacillus cereus* Isolated from a Striped Catfish (*Pangasianodon hypophthalmus*) Pond. *Indian J Microbiol.* 53, 485-487. doi: <https://doi.org/10.1007/s12088-013-0415-y>.
- Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H., Bossier, P., 2018. Effect of quorum quenching bacteria on growth, virulence factors and biofilm formation of *Yersinia ruckeri* in vitro and an in vivo evaluation of their probiotic effect in rainbow trout. *J Fish Dis.* 41, 1429-1438. doi: <https://doi.org/10.1111/jfd.12840>.
- Valiente, E., Bruhn, J.B., Nielsen, K.F., Larsen, J.L., Roig, F.J., Gram, L., Amaro, C., 2009. *Vibrio vulnificus* produces quorum sensing signals of the AHL-class. *FEMS Microbiol Ecol.* 69, 16-26. doi: <https://doi.org/10.1111/j.1574-6941.2009.00691.x>.
- Vinoj, G., Vaseeharan, B., Thomas, S., Spiers, A.J., Shanthi, S., 2014. Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits *Vibrio* biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. *Mar Biotechnol (NY)*. 16, 707-715. doi: <https://doi.org/10.1007/s10126-014-9585-9>.
- Wang, W.-Z., Morohoshi, T., Ikenoya, M., Someya, N., Ikeda, T., 2010. AiiM, a novel class of N-acylhomoserine lactonase from the leaf-associated bacterium *Microbacterium testaceum*. *Appl Environ Microbiol.* 76, 2524-2530. doi: <https://doi.org/10.1128/AEM.02738-09>.
- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* 173, 697-703. doi: <https://doi.org/10.1128/jb.173.2.697-703.1991>.
- Yates, E.A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S.R., Sockett, R.E., Goldner, M., Dessaux, Y., Cámara, M., Smith, H., Williams, P., 2002. N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun.* 70, 5635-5646. doi: <https://doi.org/10.1128/iai.70.10.5635-5646.2002>.
- Yin, X.-T., Xu, L., Fan, S.-S., Xu, L.-N., Li, D.-C., Liu, Z.-Y., 2010. Isolation and characterization of an AHL lactonase gene from *Bacillus amyloliquefaciens*. *World*

Journal of Microbiology and Biotechnology. 26, 1361-1367. doi: <https://doi.org/10.1007/s11274-010-0308-8>.

Zeigler, D.R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., Perkins, J.B., 2008. The Origins of 168, W23, and Other *Bacillus subtilis* Legacy Strains. J Bacteriol. 190, 6983-6995. doi: <https://doi.org/10.1128/jb.00722-08>.

Zhang, H.-B., Wang, L.-H., Zhang, L.-H., 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc Natl Acad Sci. 99, 4638-4643. doi: <https://doi.org/10.1073/pnas.022056699>.

Zhao, J., Chen, M., Quan, C.S., Fan, S.D., 2015. Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. J Fish Dis. 38, 771-786. doi: <https://doi.org/10.1111/jfd.12299>.

Zhou, S., Yu, Z., Chu, W., 2019. Effect of quorum-quenching bacterium *Bacillus* sp. QSI-1 on protein profiles and extracellular enzymatic activities of *Aeromonas hydrophila* YJ-1. BMC Microbiol. 19, 135. doi: <https://doi.org/10.1186/s12866-019-1515-6>.

Zhou, S., Xia, Y., Zhu, C., Chu, W., 2018. Isolation of Marine *Bacillus* sp. with Antagonistic and Organic-Substances-Degrading Activities and Its Potential Application as a Fish Probiotic. Mar Drugs. 16. doi: <https://doi.org/10.3390/md16060196>.

CHAPTER IV

In vitro modulation of gilthead seabream (*Sparus aurata* L.) leukocytes by *Bacillus* spp. extracellular molecules upon bacterial challenge

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***In vitro* modulation of gilthead seabream (*Sparus aurata* L.) leukocytes by *Bacillus* spp. extracellular molecules upon bacterial challenge**

Abstract

Stimulation of the fish immune system using immunostimulants is an environmentally friendly strategy to minimize bacterial outbreaks in aquaculture. Different biological and synthetic immunostimulants can enhance non-specific innate immune responses by directly activating immune cells. An example are *Bacillus* spp., known for their immunostimulatory effects, although the exact mechanisms by which *Bacillus* spp. offer protection against diseases remains to be elucidated. Furthermore, most studies have focused on *Bacillus* spp. cells, while the immunostimulant effect of their extracellular metabolome, known to harbour biologically important metabolites, including antimicrobial molecules, has been scarcely evaluated.

Here, we evaluated the *in vitro* immune-modulatory properties of extracellular extracts of three *Bacillus* spp. strains (*B. subtilis* FI314, *B. velezensis* FI436 and *B. pumilus* FI464), previously isolated from fish-guts and characterized for their *in vitro* and *in vivo* antimicrobial activity against a wide range of fish pathogens.

Bacillus spp. extracellular extracts did not affect immune cells viability, but remarkably increased pathogens' phagocytosis when seabream head-kidney leukocytes were challenged with *Vibrio anguillarum* and *Edwardsiella tarda*. All extracts significantly increased the engulfment of bacterial pathogens 1h post-infection. Cells stimulated with the extracellular extracts showed an up-regulation of the expression of immune-relevant genes associated with inflammation, including IL-1 β , IL-6, and COX-2. In cells challenged with *E. tarda*, FI314 extracellular extract significantly increased the expression of IL-1 β , IL-6, and COX-2, while FI436 and FI464 significantly increased IL-6 expression. The results of this study revealed that the extracellular molecules from *Bacillus* spp. fish isolates improved the *in vitro* response of gilthead seabream immune cells and are thus promising candidates to act as immunostimulants, helping fish fight diseases.

Keywords: Fish diseases; *Bacillus* spp., Immunostimulants; Gilthead seabream (*Sparus aurata* L.), *Vibrio anguillarum*, *Edwardsiella tarda*

4.1. Introduction

Bacterial diseases are a major cause of economic losses in aquaculture. Antibiotics are the traditional strategy for the treatment of bacterial infections but the deleterious effects on the environment and public health, have led to strict regulations on their use in fish production (Pérez-Sánchez, et al., 2018). Disease prevention is thus fundamental for sustainable aquaculture development and a substantial effort is being made on the establishment of disease preventive measures, including best farming practices, and strategies to strengthen the fish immune system. In recent years, immunological control of fish diseases has gained attention, becoming one of the most studied areas in aquaculture (Wang, et al., 2017).

The fish immune system is composed of stratified defence mechanisms with increased specificity to fight infections (Rauta, et al., 2012). Physical barriers (e.g., scales, skin) prevent the entrance of bacterial pathogens, and once breached, the innate immune system provides non-specific immune responses. Innate immunity is the first and strongest immune response in fish, comprising humoral (e.g., lysozyme, lectin, transferrin, and anti-protease activities) and cellular (leukocytes and non-specific cytotoxic cells) mechanisms that scout foreign invaders (e.g., bacteria) and initiate the inflammatory process (Kiron, 2012). Importantly, leukocytes play a critical role in the first line of defence against invading pathogens through the recruitment of phagocytic cells (granulocytes and monocytes/macrophages). These initiate the pathogen's engulfment and consequent release of large amounts of bactericidal reactive oxygen species (ROS), that help to eliminate foreign microbes (Magnadóttir, 2006; Magnadóttir, 2010). The activation of the adaptive immune system is the last defence mechanism and may be turned-on to further improve the pathogen's recognition and immunological memory (Rauta, et al., 2012; Wang, et al., 2017).

Bacterial invaders can also stimulate cytokines production. The release of cytokines is involved in different regulatory processes especially important in the innate immune system (Magnadóttir, 2010; Sakai, et al., 2021). Several cytokines were already identified in fish through gene expression or functional activity analysis. Interleukin 1 (IL-1 α), interleukin 6 (IL-6), and tumour necrosis factor α (TNF- α) promote an inflammatory response by activating lymphocytes and phagocytic cells (Sakai, et al., 2021). On the other hand, interleukin 10 (IL-10) is involved in anti-inflammatory responses and acts by inhibiting the secretion of cytokines and macrophages action (Sakai, et al., 2021).

One strategy to strengthen the fish immune system and help prevent disease outbreaks in aquaculture is by immunomodulation with immunostimulant compounds. Immunostimulants are biological (e.g., bacterial, yeast, plant, and algal derivatives) or

synthetic compounds that enhance the non-specific cellular innate immune responses by interacting and directly activating immune cells (Maqsood, et al., 2011). By activating the immune system using immunostimulants, fish increase their capacity to fight infections. Alongside stimulation of the non-specific immune system to protect fish from infections, immunostimulants are safe for the environment and human health, lack side effects, are economically effective, and are easy to administrate (injection, immersion, or oral uptake) (Barman, et al., 2013; Maqsood, et al., 2011). Several reports described the potential of immunostimulants in protecting fish and shrimp against *Aeromonas salmonicida*, *A. hydrophila*, *Edwardsiella tarda*, *Vibrio anguillarum*, *V. vulnificus*, *Yersinia ruckery*, and other bacteria (Ji, et al., 2017; Lu, et al., 2019; Reyes-Becerril, et al., 2016; Sattanathan, et al., 2020).

Bacillus spp. have gained attention as important microorganisms to maintain the health and well-being of aquaculture animals (James, et al., 2021). *Bacillus* spp. are recognized for the production of natural antimicrobial compounds (Caulier, et al., 2019; James, et al., 2021; Santos, et al., 2021a; Serra, et al., 2019) and quorum quenching enzymes (Chen, et al., 2020; Santos, et al., 2021a; Santos, et al., 2021b) capable of controlling important fish bacterial pathogens. Alongside with other probiotic health-promoting characteristics, *Bacillus* spp. are also known for their immunostimulatory effects enhancing the host's innate and adaptive immunity against fish pathogens. Several reports have demonstrated that the administration of *Bacillus* can increase (i) leukocytes, monocytes, or granulocytes population; (ii) phagocytic activity; (iii) respiratory burst activity; (iv) lysozyme activity; (v) plasma bactericidal activity; (vi) complement activity; (vii) immunoglobulins; (viii) cytokine secretion; and other immune parameters (Galagarza, et al., 2018; Kumar, et al., 2008; Meidong, et al., 2018; Nguafack, et al., 2020; Sookchaiyaporn, et al., 2020; Truong Thy, et al., 2017; Van Doan, et al., 2018; Wu, et al., 2021). Nonetheless, only one report has evaluated the immunomodulation potential of *Bacillus* spp. secondary metabolites (Chen, et al., 2015).

Here we investigated the *in vitro* immunomodulation of gilthead seabream (*Sparus aurata*) innate immune responses by extracts of *B. subtilis*, *B. velezensis*, and *B. pumilus* strains isolated from the gut of marine fish. The *Bacillus* spp. used are well-characterized for their production of antimicrobial and quorum-quenching compounds active against a wide range of fish pathogens (Santos, et al., 2021a; Santos, et al., 2021b), and for their protective effects in zebrafish when challenged with *E. tarda* (Santos, et al., 2021b). Here we go further on the characterization of the extracellular metabolome of these putative fish probiotic strains, establishing a relationship between the antimicrobial effect observed both *in vitro* and *in vivo* with the capacity to modulate the fish immune system.

4.2. Material and Methods

4.2.1. Ethics statement

Fish experiments and handling were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR; reference ORBEA_CIIMAR_27_2019), performed by accredited scientists (following FELASA category C recommendations), and in compliance with the European directive 2010/63/EU for the care and use of laboratory animals.

4.2.2. Fish

The trial was conducted at the experimental facilities of CIIMAR, Porto, Portugal. Gilthead seabream (*Sparus aurata*) juveniles, with an average weight of 50 g, were acquired from a certified hatchery (SONRÍONANSA; Cantabria, Spain). After arrival at the experimental unit, fish were held in quarantine for 15 days, in a recirculation aquaculture system (RAS) with a 2000 L tank, and hand-fed with a commercial diet (NEO diet, AQUASOJA, Portugal).

Fish were then moved to an experimental RAS system, equipped with six tanks of 500L, and kept in these conditions until reaching an average weight of 222 ± 20 g. Fish were fed twice a day, six days a week, with a commercial diet (NEO diet, AQUASOJA, Portugal). During the quarantine and this period, water parameters were maintained within the recommended ranges for the species (Temperature: 22 ± 2 °C, Salinity: 33 ± 2 ‰; Oxygen: 7.5 mg L^{-1} ; and $\text{NH}_4^+ \leq 0.05 \text{ mg l}^{-1}$; $\text{NO}_2^- \leq 0.5 \text{ mg l}^{-1}$).

4.2.3. Bacteria

The three Fish Isolates (FI) used in this study (*B. subtilis* FI314, *B. velezensis* FI436, and *B. pumilus* FI464) were selected based on their antimicrobial and Quorum-Quenching properties previously described by Santos, et al. (2021a,b). FI314 was isolated from the gut of gilthead seabream and FI436 and FI464 were isolated from the gut of European seabass (Santos, et al., 2021a). FI were routinely grown aerobically in Luria-Bertani (LB; Fisher BioReagents) medium at 37 °C.

FI extracts used in this study were obtained from freeze-drying the filtered (0.22 µm cellulose acetate filter) cell-free supernatant of 8 h cultures and resuspending it in sterile Leibovitz's L-15 medium (L-15, Gibco) supplemented with 10% of fetal bovine serum (FBS, Gibco), 100 U mL^{-1} of penicillin (Gibco), 100 µg mL^{-1} of streptomycin (Gibco).

Vibrio anguillarum DSMZ 21597 and *Edwardsiella tarda* LMG 2793 were grown aerobically for 24 h in Brain Heart Infusion (BHI, BD Difco) medium at 25 °C or 37 °C, respectively. Before the assays, bacteria were washed twice and resuspended on HBSS without Ca^{2+} and Mg^{2+} (Gibco) to obtain $1 \times 10^8 \text{ CFU mL}^{-1}$, followed by heat inactivation at

60 °C for 30 min and storage at -80 °C until use (Esteban, et al., 1998). After the heat-inactivation, an aliquot of each bacteria (1×10^8 CFU mL⁻¹) was quantified using the flow-cytometer and plated to ensure the success of the heat-inactivation procedure.

4.2.4. Leukocyte's isolation

Gilthead seabream head–kidney leukocytes were isolated from fish under sterile conditions as described in Salinas, et al. (2006), with few modifications. Briefly, head-kidney from 6 fish was excised and transferred to 10 mL of ice-cold Leibovitz's L-15 medium supplemented with 5% of FBS, 100U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin, and 10U of heparin (Braun). Tissue fragments were forced through a 100 µm nylon mesh (Pluriselect) and homogenized in the ice-cold medium. Leukocytes were purified from the cell suspension by layering 5 mL of suspension onto 8 mL of Percoll (Sigma) gradient (30-51%) followed by centrifugation at 400 g for 30 min at 4 °C. Leukocytes were collected from Percoll interface, washed twice in ice-cold L-15 medium supplemented with 5% FBS, 100U mL⁻¹ of penicillin, and 100 µg mL⁻¹ of streptomycin and resuspended in 5 mL of L-15 medium supplemented with 10% FBS, 100U mL⁻¹ of penicillin, and 100 µg mL⁻¹ of streptomycin (L-15+10%FBS+P/S). Head–kidney leukocytes were counted on a Neubauer chamber, adjusted to 10^6 cells mL⁻¹ in L-15+10%FBS+P/S, and distributed in sterile cell culture 24-well plates (5×10^5 cells/well). Cells' viability was analysed immediately after isolation and before stimulation, using flow cytometry (described in section 2.7).

4.2.5. Flow-cytometry analysis and leukocyte populations distribution parameters

All cytometric cell measurements were performed with an Accuri™ C6 flow cytometer (Becton Dickinson) and analysed with the Accuri™ C6 software. Cell cytograms were analysed from a total of 10000 events recorded with an acquired rate of 10000 events/second. Partial differentiation of leukocytes subpopulations was done according to the forward and side scatter parameters as described by Samaï, et al. (2018) and Inoue, et al. (2002). Three cell populations were established: (i) lymphocytes + thrombocytes, (ii) monocytes, and (iii) granulocytes (Supplementary Figure S1 - Appendix II). Before the cytometric analysis, samples were washed twice and resuspended in HBSS without Ca²⁺ and Mg²⁺. After impurities exclusion, the total events recorded from 10 µL of cell suspension in HBSS allowed determining leukocytes concentration (cells/mL) in each sample.

4.2.6. Leukocyte's stimulation and challenge with pathogenic bacteria

Before leukocytes stimulation, a preliminary *in vitro* toxicity test was conducted to determine the maximum non-toxic concentration (MNTC) of each FI extract, to be used in the cells' stimulation assays. Cells were exposed to FI's extracts homogenized in sterile L-15+10%FBS+P/S with concentrations ranging from 0.5 $\mu\text{g mL}^{-1}$ to 1 mg mL^{-1} . Cell mortality and viability were evaluated after 24 h of incubation using microscopy (Nikon Eclipse Ti inverted microscope, Tokyo, Japan) and flow cytometry (data not shown).

FI's stimulation of head-kidney leukocytes was performed by using the MNTC of the lyophilized extracts of FI314, FI436, and FI464 (final concentrations of 30, 10, and 0.8 $\mu\text{g mL}^{-1}$, respectively). Cells were incubated with the extracts for 24 h at 20 °C before collection to determine the basal stimulation of the FI extracts. The control group consisted of leukocytes without any treatment. After FI stimulation for 24 h, leukocytes were challenged with heat-inactivated *V. anguillarum* or *E. tarda* at a Multiplicity of Infection (MOI) of 10:1 (bacteria: leukocytes). Non-stimulated leukocytes incubated with *V. anguillarum* or *E. tarda* were used as a positive control, and leukocytes incubated with culture medium alone with HBSS supplementation were used as a negative control.

Before all cytometric analysis, samples were washed twice and resuspended in HBSS without Ca^{2+} and Mg^{2+} .

4.2.7. Leukocyte's viability

Leukocyte's viability was evaluated in non-stimulated and FI-stimulated cells, and in cells challenged with *V. anguillarum* and *E. tarda* for 60 min, using propidium iodide staining. Cells were pelleted, washed in HBSS without Ca^{2+} and Mg^{2+} , and stained with 10 $\mu\text{g mL}^{-1}$ of propidium iodide (Sigma). After incubation for 5 min in the dark, cells were analysed by flow cytometry using a 488 nm excitation laser. Positive reaction for dead or injured cells was detected by measuring the red fluorescence signal emitted by the propidium iodide (PI) bound to the DNA in a 670LP filter. PI-positive cells were expressed as cell death/injury percentage (or the number of dead/injury cells).

4.2.8. Leukocyte's respiratory burst activity

The generation of intracellular reactive oxygen species (ROS) was quantified using dichlorodihydrofluorescein diacetate (DCFH-DA) probe using a modification of Samaï, et al. (2018) protocol. Briefly, in a light-protected environment cells were incubated with 1 μM of DCFH-DA followed by challenge with heat-inactivated bacteria at 20 °C (no challenge in control samples). The mean fluorescence of oxidized DCFH-DA (using APC filter, 675/25 nm) was measured 15, 30, and 60 min after the challenge. Positive

ROS production was expressed as the percentage of cells emitting fluorescence over time.

4.2.9. Leukocyte's phagocytosis

Leukocyte's phagocytic activity was measured using FITC-labelled bacteria, based on a modification of the method described by Esteban, et al. (1998). To label bacteria with fluorescein isothiocyanate (FITC) (Sigma), 24 h cultures of *V. anguillarum* and *E. tarda* were washed twice and resuspended in 0.1 M of sodium bicarbonate supplemented with 25 μ M of FITC. Labelling occurred in a light-protected environment for 2 h at 300 rpm. FITC was removed from bacterial cells by washing thrice and resuspending in HBSS without Ca^{2+} and Mg^{2+} to obtain 1×10^8 CFU mL^{-1} . Bacteria were heat-inactivated at 60 °C for 30 min followed by wash and homogenization (Esteban, et al., 1998). FITC-labelling bacteria were analysed for staining uniformity using flow cytometry and stored at -80 °C until use.

FITC-labelled bacteria were added to stimulated leukocytes (MOI of 10:1), well mixed, and incubated at 20 °C. At the end of the reaction (60 min), samples were incubated on ice to stop phagocytosis and added ice-cold HBSS. Fluorescence of non-ingested bacteria was quenched using trypan blue. Phagocytosis analysis was carried in the flow-cytometer using an excitation laser of 488 nm and measuring the fluorescence with a 533/30 nm filter. Samples incubated at 4 °C were used as a negative control for phagocytosis. Phagocytosis ability was expressed as the percentage of leukocytes with ingested FITC-labelled bacteria.

4.2.10. Gene expression

Immunity-related gene expression was evaluated in stimulated and 4 h post-challenged cells (Sepulcre, et al., 2007). Cells were pelleted and resuspended in 500 μ L of TRIzol reagent (Zymo Research) and stored at -80 °C until RNA extraction. Total RNA was extracted from the cell's pellet using Direct-zol™ RNA Miniprep (Zymo Research) following manufacturer instructions. RNA quality and quantity were accessed by electrophoresis (on 1% agarose gel) and spectrophotometry (μ Drop™ plate, ThermoScientific). RNA concentration was adjusted to 0.5 μ g/8 μ L H_2O before the synthesis of complementary DNA, using the NZY First-Strand cDNA Synthesis Kit (NZYTech. MB12502, Lisbon, Portugal).

Gene expression was determined by real-time quantitative PCR analysis. (CFX Connect™ Real-Time System, Bio-Rad, California, USA). Reaction mixtures were carried out using 0.4 μ L of diluted cDNA (1:2), 0.2 μ L of each primer (10 μ M), 5 μ L SsoAdvanced Universal SYBR® Green supermix (Bio-Rad), and 4.2 μ L

DNase/RNase/Protease-free water, in a total volume of 10 μ l. Sequences of the forward and reverse primers used are presented in Table 4.1. Primer efficiency was validated with serial two-fold dilutions of cDNA and calculated from the slope of the regression line of the quantification cycle (Ct) versus the relative concentration of cDNA (Pfaffl, 2001). Primer efficiency was between 90 and 110%, except 18S with an efficiency of 87%. Reaction mixtures were incubated at 95 °C for 30s hot-start iTaq™ DNA polymerase activation, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C (for EF-1 α and IL-6) or 60 °C (for 18S, Hsp70, COX-2, IL-1 β , IL-10, and TNF- α). Each PCR run included duplicates of reverse transcription for each sample and negative control (RNA-free sample). Results were normalized using previously validated EF-1 α and 18S as reference genes. Results are expressed as mean normalized values $\pm$ standard error mean (SE), corresponding to the ratio between the number of copies of the target genes transcripts and the geometric mean of copy numbers of reference genes ef1 α and 18s as described by Vandesompele, et al. (2002).

Table 4.1. Oligonucleotide primers used for gene expression analysis.

Gene	Sequence (5' - 3')	Accession number	Reference
Interleukin 1- β (IL-1 β)	F: GGGCTGAACAACAGCACTCTC R: TTAACACTCTCCACCCTCCA	AJ277166	Chaves-Pozo, et al. (2008)
Interleukin 6 (IL-6)	F: GCTCTGCTGCGGTGTGCTCC R: GTCTCCCACTCCTCACCTG	EU244588	Castillo, et al. (2009)
Interleukin 10 (IL-10)	F: TGGAGGGCTTTCCTGTCAGA R: TGCTTCGTAGAAGTCTCGGATGT	FG261948	Angosto, et al. (2014)
70 kilodalton heat shock proteins (Hsp70)	F: AATGTTCTGCGCATCATCAA R: GCCTCCACCAAGATCAAAGA	EU805481	Benhamed, et al. (2016)
Cyclooxygenase 2 (COX-2)	F: GAGTACTGGAAGCCGAGCAC R: GATATCACTGCCGCCTGAGT	AM296029	Chaves-Pozo, et al. (2008)
Tumor necrosis factor- α (TNF- α)	F: TCGTTCAGAGTCTCCTGCAG R: CATGGACTCTGAGTAGCGCGA	AJ413189	Chaves-Pozo, et al. (2008)
Elongation factor 1 α (Ef-1 α)	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG	AF184170	Cerezuela, et al. (2013)
Ribosomal protein S18 (18S)	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC	AM490061	Chaves-Pozo, et al. (2008)

4.2.11. Statistical analysis

Statistical analysis was performed using SPSS 27.0 software package for Windows. All data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). All data were analysed by one-way ANOVA, except ROS production. When p -values were significant ($p < 0.05$), means were compared with Dunnet's test. ROS production was analysed by two-way ANOVA with time (15, 30, 60) and extracts (FI314, FI436, and FI464) as independent variables. Significant differences ($p < 0.05$) between means were determined by Tukey's multiple range test. GraphPad Prism 9 software was used for graphics design.

4.3. Results

4.3.1. Basal concentration and viability of seabream leukocytes

The basal activity of FI extracts on head kidney leukocytes was established by exposing cells to FI extracts for 24 h before the determination of leukocytes concentration, viability, and immunity-related gene expression.

Total leukocytes concentration was not affected by any FI extract upon 24 h exposure (Figure 4.1, top panel, grey bars). A similar result was observed for the percentage of granulocytes, monocytes, and lymphocytes (Figure 4.1, bottom panel, grey bars).

FI extracts also did not affect total leukocytes viability (Figure 4.1, top panel, white bars). Monocytes and lymphocytes viability was also not different from that of the control group, while a decrease in granulocytes viability was observed in cells treated with FI314 and FI436 (Figure 4.1, bottom panel, white bars).

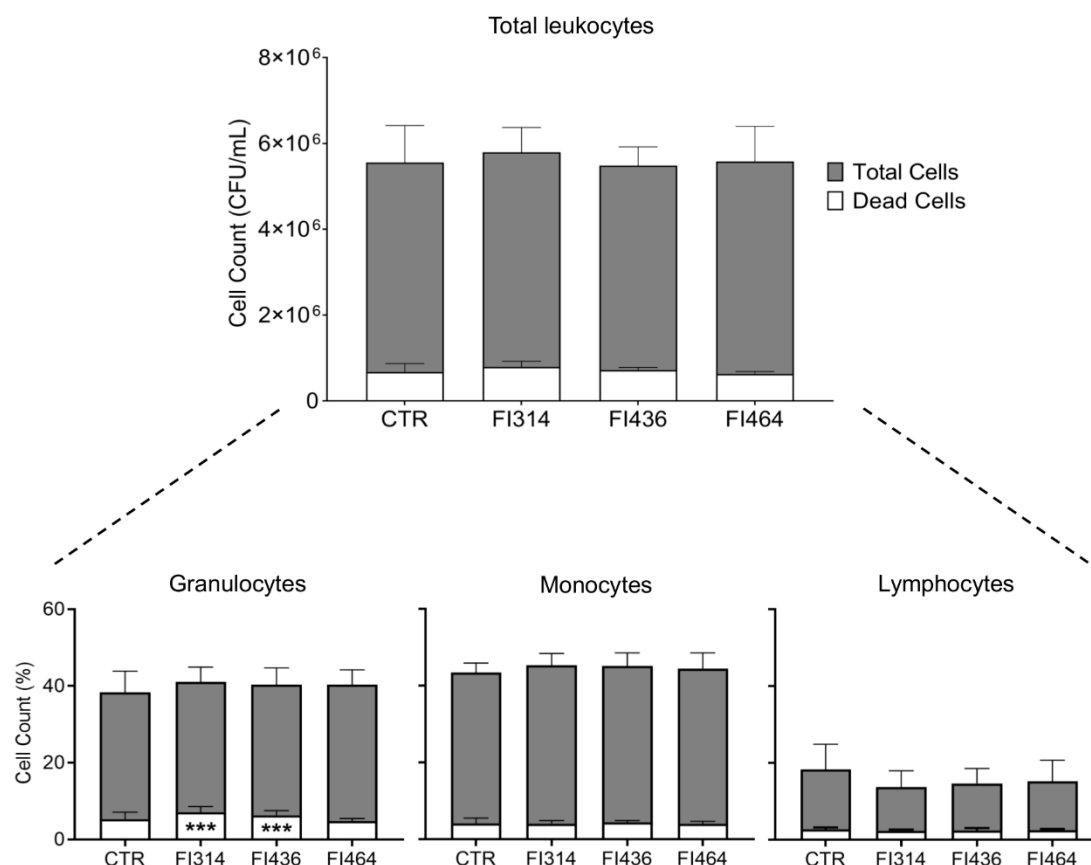


Figure 4.1. Abundance (grey bars) and viability (white bars) of gilthead seabream head-kidney whole leukocytes (top panel) and subpopulations (granulocytes, monocytes and lymphocytes; bottom panel) after 24 h exposure to lyophilized extracts of FI314 (30 $\mu\text{g mL}^{-1}$), FI436 (10 $\mu\text{g mL}^{-1}$) and FI464 (0.8 $\mu\text{g mL}^{-1}$). Cells were washed and resuspended in HBSS before analysis using flow-cytometry. The control (CTR) corresponds to unstimulated leukocytes. The gates used for cell populations' analysis are shown in supplementary figure S1 (Appendix I). Data is composed by 6 biological replicates and is represented as mean $\pm$ SD. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

The expression of Hsp70, COX-2, IL-6, and TNF- α was not modified in response to FI extracts stimulation, while IL-1 β expression was up-regulated by FI314 and FI464 (Figure 4.2). Although not significant, a tendency to down-regulation of IL-10 was noticed with all FI extracts.

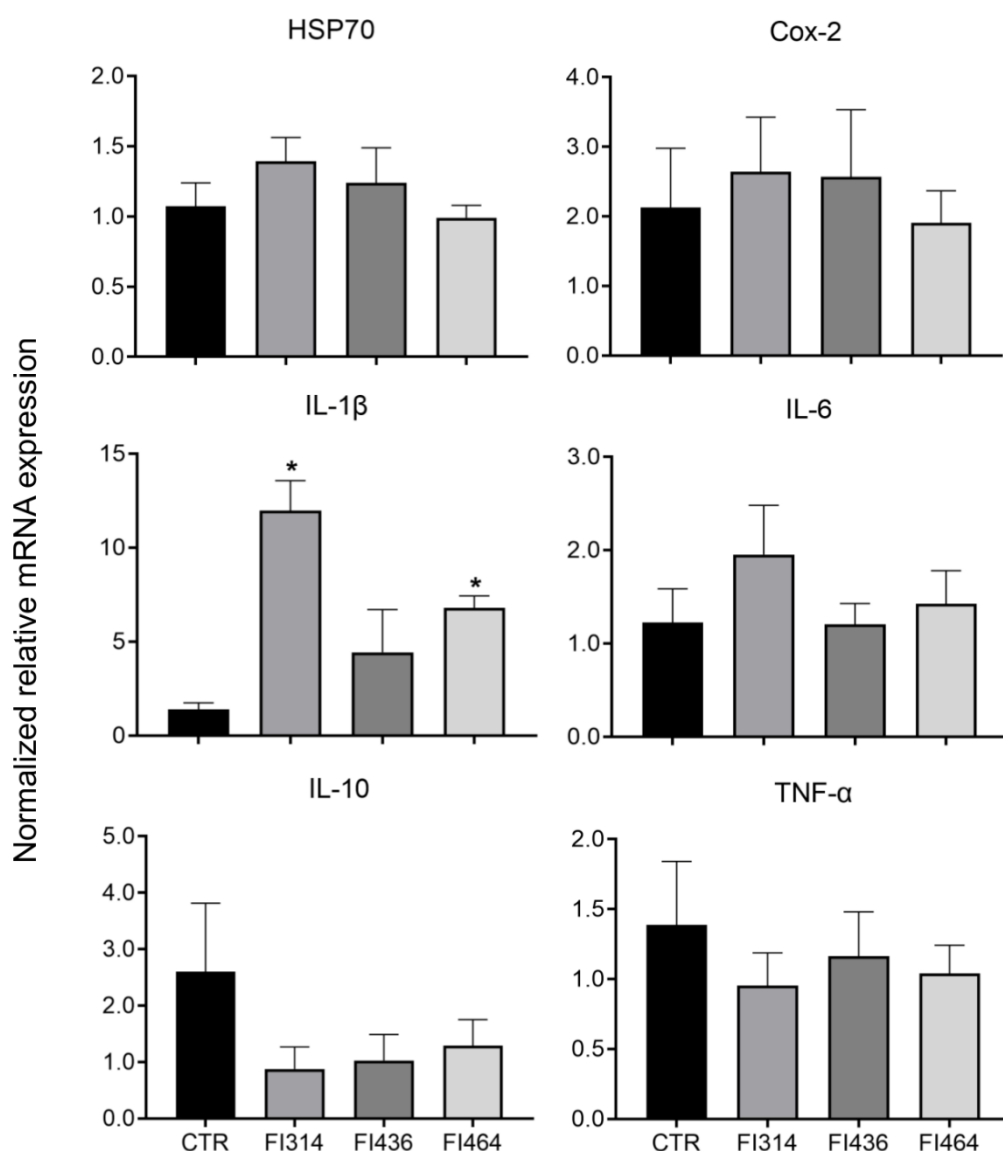


Figure 4.2. Relative expression (measured by RT-PCR) of stress-related (Hsp70), cytokine-related (COX-2, IL-1 β , IL-6, IL-10) and apoptosis-related (TNF- α) genes in gilthead seabream head-kidney leukocytes exposed to lyophilized extracts of FI314 (30 $\mu\text{g mL}^{-1}$), FI436 (10 $\mu\text{g mL}^{-1}$) and FI464 (0.8 $\mu\text{g mL}^{-1}$) for 24 h. The control (CTR) corresponds to unstimulated leukocytes. RNA was isolated and converted to cDNA before gene amplification and quantification. Data are expressed as normalized gene expression. Data is composed by 6 biological replicates and is represented as mean $\pm$ SEM. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

4.3.2. Leukocytes' concentration and viability upon challenge with *V. anguillarum* and *E. tarda*

Head-kidney leukocytes stimulated with FI extracts for 24 h showed no differences in cells concentration nor the percentage of granulocytes, monocytes, and lymphocytes upon challenge with *V. anguillarum* or *E. tarda* (Figure 4.3, grey bars).

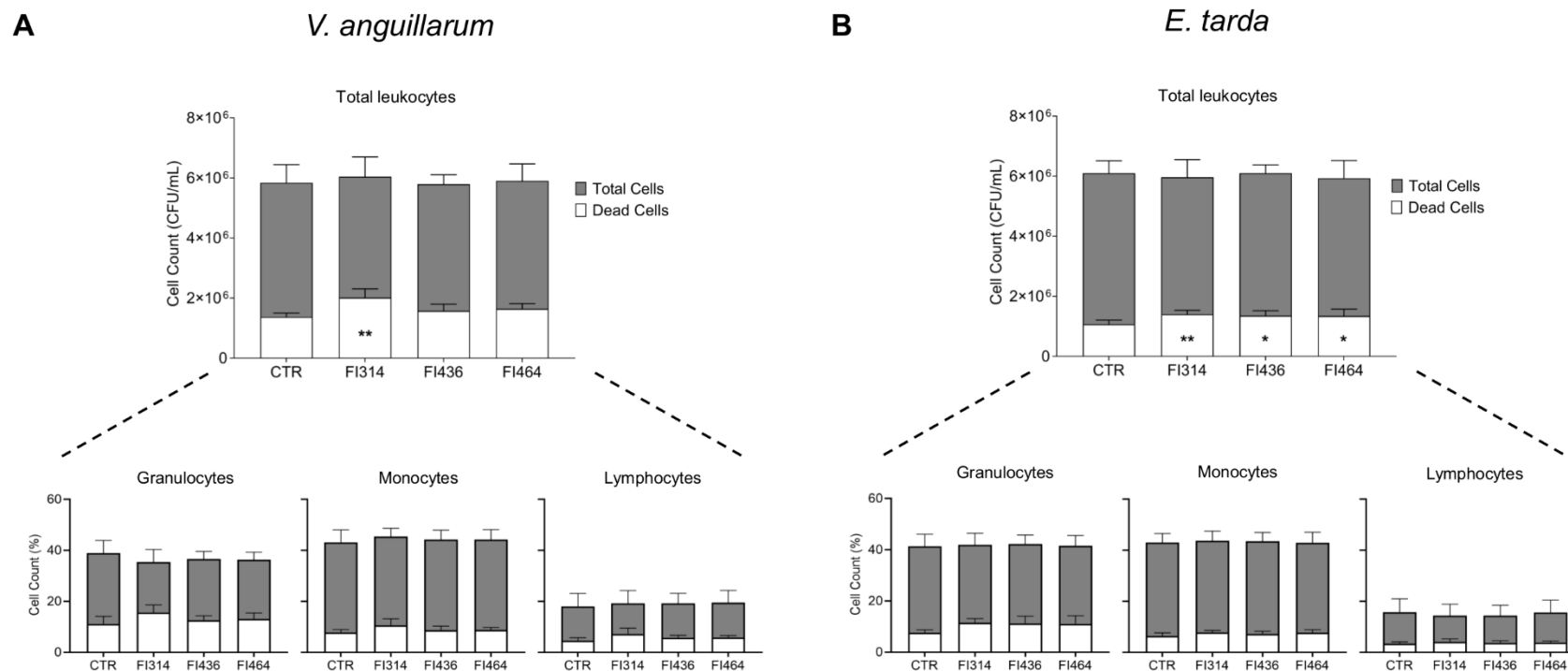


Figure 4.3. Abundance (grey bars) and viability (white bars) of gilthead seabream head-kidney whole leukocytes (top panel) and subpopulations (granulocytes, monocytes and lymphocytes; bottom panel) after 24 h stimulation with lyophilized extracts of FI314 ($30 \mu\text{g mL}^{-1}$), FI436 ($10 \mu\text{g mL}^{-1}$) and FI464 ($0.8 \mu\text{g mL}^{-1}$) followed by 6 h challenge with heat-inactivated *V. anguillarum* (A) or *E. tarda* (B). Leukocytes were washed and resuspended in HBSS before analysis using flow-cytometry. Control (CTR) corresponds to unstimulated leukocytes challenged with *V. anguillarum* or *E. tarda*. The gates used for cell populations' analysis are shown in supplementary figure S1 (Appendix I). Data is composed by 6 biological replicates and is represented as mean $\pm$ SD. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

Previous stimulation with FI314 extract decreased the viability of total leukocytes upon challenge with *V. anguillarum*, whereas previous stimulation with FI436 and FI464 did not affect cell viability (Figure 4.3A, top panel, white bars). FI extracts did not change leukocytes subpopulations after exposition to *V. anguillarum* (Figure 4.3A, bottom panel, white bars).

Previous stimulation with all FI decreased the viability of total leukocytes upon challenge with *E. tarda* (Figure 4.3B, top panel, white bars). FI extracts did not change leukocytes subpopulations after exposition to *E. tarda* (Figure 4.3B, bottom Panel, white bars).

4.3.3. ROS production upon challenge with *V. anguillarum* and *E. tarda*

Total leukocytes ROS production upon *V. anguillarum* challenge was lower in FI464-stimulated cells than in non-stimulated cells (Figure 4.4A, top and bottom panels), while cell-stimulation with FI314 and FI436 did not affect ROS production. Moreover, ROS production increased with time, independently of cell stimulation. No differences between stimulated and non-stimulated cells were observed in granulocytes and monocytes, whereas in lymphocytes a decrease in ROS production was observed upon FI314 stimulation (Figure 4.4A, middle and bottom panels). Additionally, a decrease in ROS production with time was observed in granulocytes and monocytes, whereas lymphocytes increased ROS production with time.

Stimulation with FI314, FI436, and FI464 extracts did not affect total leukocytes ROS production upon *E. tarda* challenge, while time increased ROS production, independently of cell stimulation (Figure 4.4B, top and bottom panels). Compared to the control, granulocytes and monocytes ROS production was not affected by FI extracts stimulation, whereas a decreased ROS production by lymphocytes was observed upon treatment with FI314 extract (Figure 4.4B, bottom panel). Granulocytes and monocytes decreased ROS production over time, while the opposite was observed in lymphocytes.

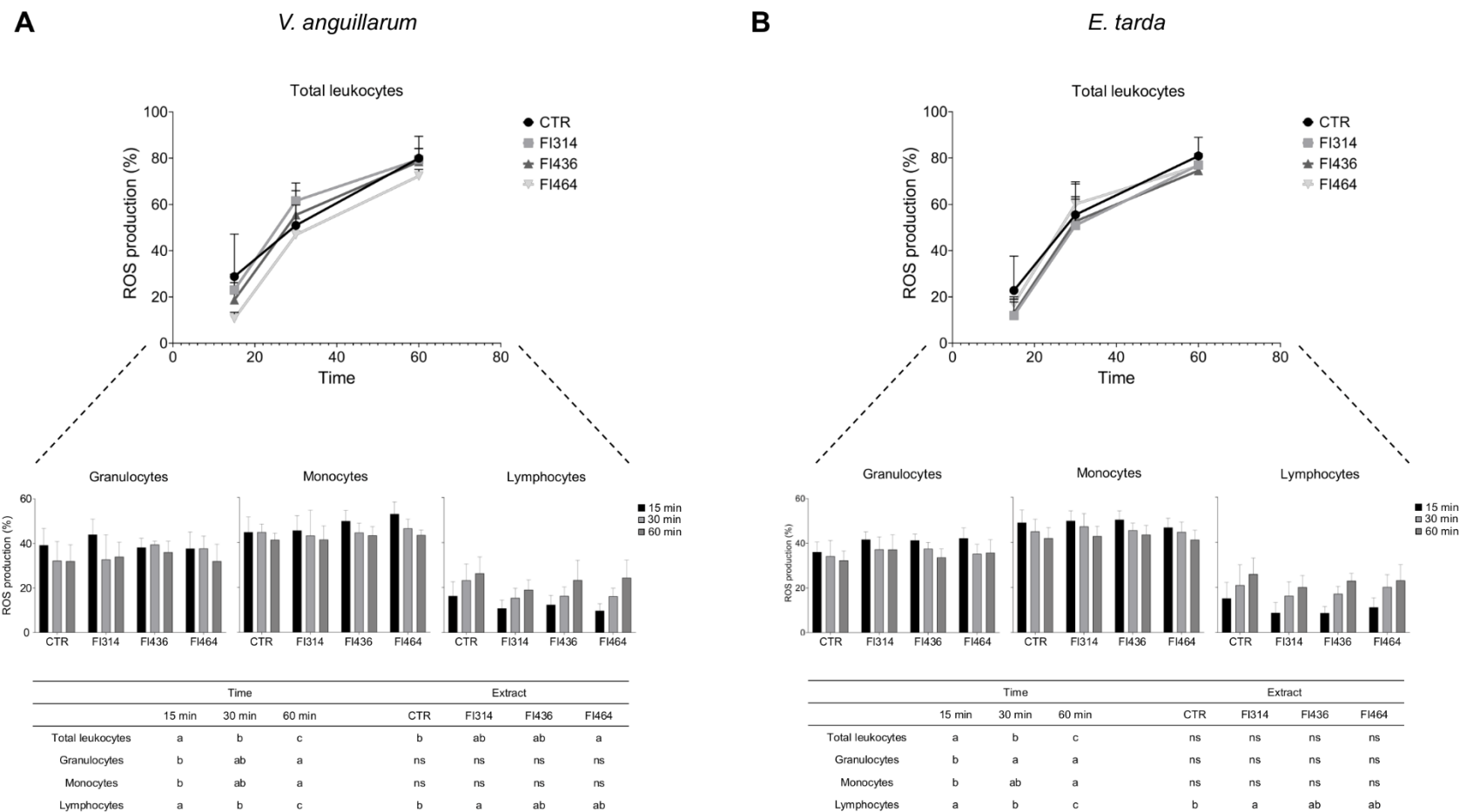


Figure 4.4. Cellular oxidative stress kinetics, measured as the production of reactive oxygen species (ROS) of gilthead seabream head-kidney whole leukocytes (top panel) and cell subpopulations (granulocytes, monocytes and lymphocytes; middle panel) after 24 h stimulation with lyophilized extracts of FI314 ($30 \mu\text{g mL}^{-1}$), FI436 ($10 \mu\text{g mL}^{-1}$) and FI464 ($0.8 \mu\text{g mL}^{-1}$) followed by 6 h challenge with heat-inactivated *V. anguillarum* (A) or *E. tarda* (B). Leukocytes were washed and resuspended in HBSS before analysis using flow-cytometry. Control (CTR) corresponds to unstimulated leukocytes challenged with *V. anguillarum* or *E. tarda*. Mean fluorescence of DCFH-DA was used to determine the percentage of ROS production. The gates used for cell populations' analysis are shown in Supplementary Figure S1 (Appendix I). Data is composed by 6 biological replicates and is represented as mean $\pm$ SD. Significant differences between extracts or times are represented by the different lower cases on the tables (bottom panel); ns: non-significant differences.

4.3.4. Leukocytes' phagocytosis of *V. anguillarum* and *E. tarda*

Leukocytes stimulated with all FI extracts showed a much higher phagocytic capacity of both *V. anguillarum* (CTR: 11.01%, FI314: 24.26%, FI436: 21.78%, FI464: 21.20%) and *E. tarda* (CTR: 15.23%, FI314: 25.07%, FI436: 23.55%, FI464: 24.58%) then non-stimulated cells (Figure 4.5A,B).

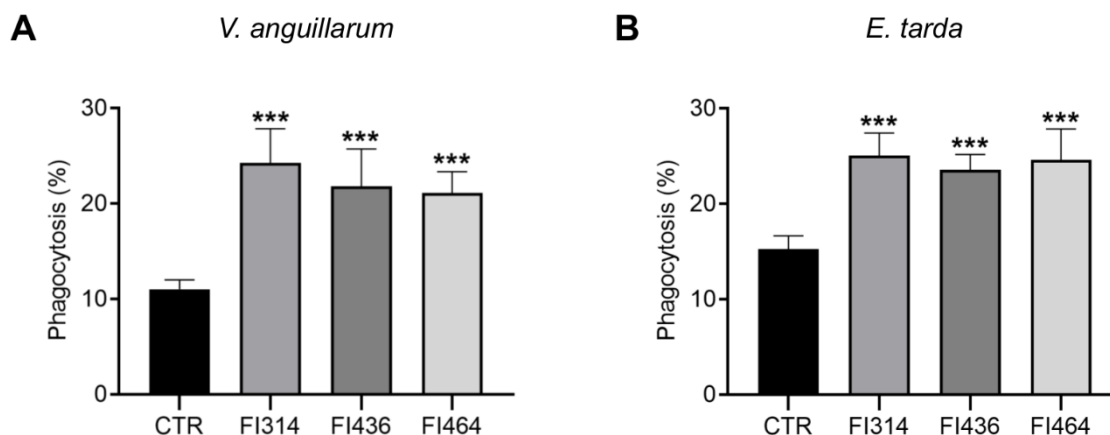


Figure 4.5. Phagocytic activity of gilthead seabream head-kidney whole leukocytes after 24 h stimulation with lyophilized extracts of FI314 (30 $\mu\text{g mL}^{-1}$), FI436 (10 $\mu\text{g mL}^{-1}$) and FI464 (0.8 $\mu\text{g mL}^{-1}$) followed by 6 h challenge with heat-inactivated and FITC-labelled (25 μM) *V. anguillarum* (A) and *E. tarda* (B). Leukocytes were washed and resuspended in HBSS before analysis using flow-cytometry. FITC fluorescence signal was used to determine the percentage of phagocytic cells. Control (CTR) corresponds to unstimulated leukocytes challenged with *V. anguillarum* or *E. tarda*. Data is composed by 6 biological replicates and is represented as mean $\pm$ SD. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

4.3.5. Gene expression of selected immunity-related genes upon challenge with *V. anguillarum* and *E. tarda*

No significant effects on the expression of genes related to stress (Hsp70), inflammation (COX-2, IL-1 β , IL-6, IL-10), and apoptosis (TNF- α) were observed on FI extracts stimulated leukocytes upon challenge with *V. anguillarum* (Figure 4.6A). There was, however, a trend for an increase of the expression levels of COX-2, IL-1 β , and IL-6 in all FI extracts stimulated cells.

The expression of Hsp70, IL-10, and TNF- α was also not affected in FI extracts stimulated cells upon challenge with *E. tarda* (Figure 4.6B). Differently, IL-6 expression was significantly increased by all FI extracts, and FI314 extract also up-regulated COX-2 and IL-1 β expression.

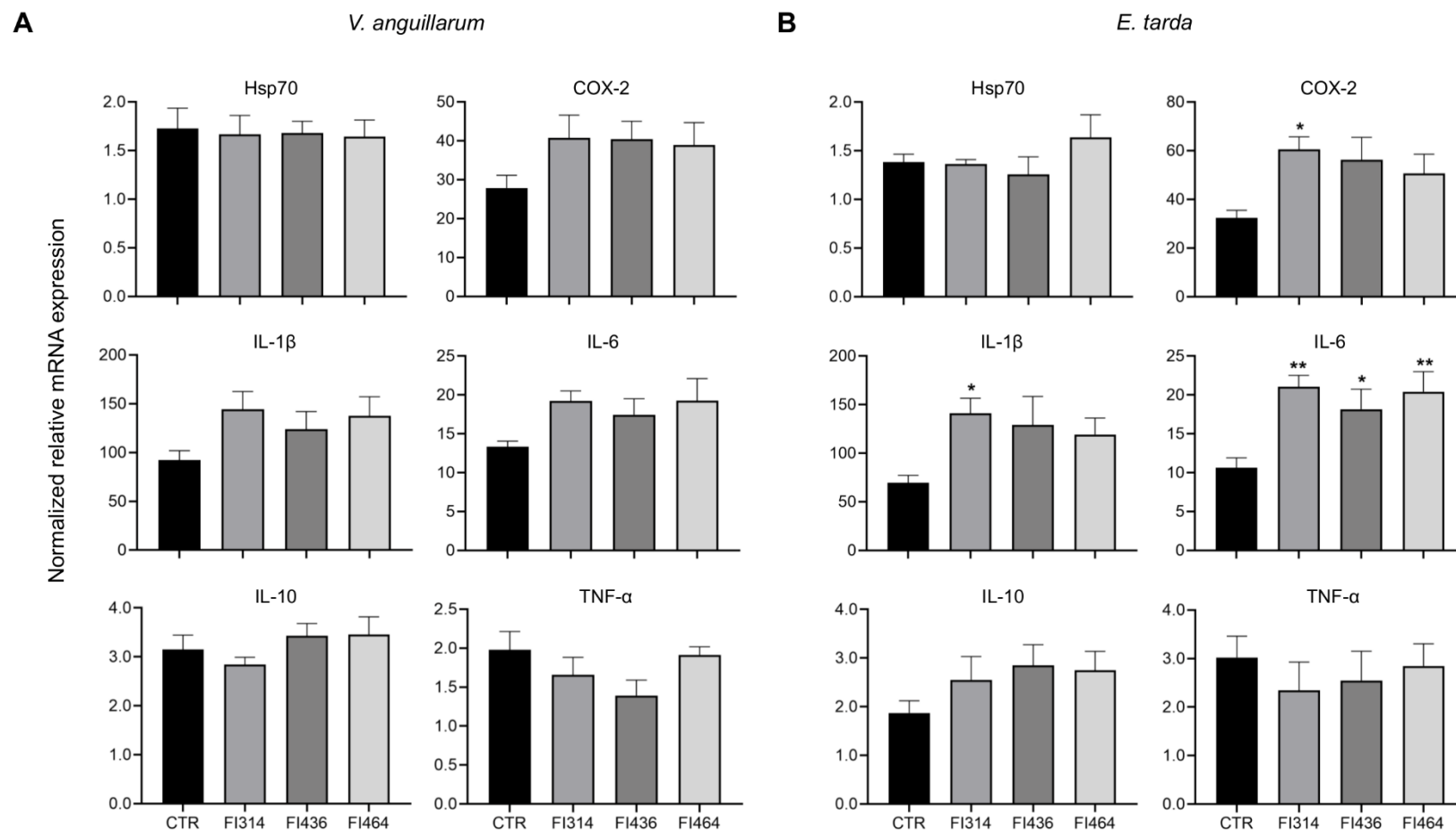


Figure 4.6. Expression of genes related to stress (Hsp70), cytokine related genes (COX-2, IL-1 β , IL-6, IL-10) and apoptosis (TNF- α) in gilthead seabream head-kidney leukocytes stimulated for 24h with lyophilized extracts of FI314 (30 $\mu\text{g mL}^{-1}$), FI436 (10 $\mu\text{g mL}^{-1}$) and FI464 (0.8 $\mu\text{g mL}^{-1}$) followed by a 6 h challenge with heat-inactivated *V. anguillarum* (**A**) and *E. tarda* (**B**). RNA was isolated and converted to cDNA before gene amplification and quantification. Control (CTR) corresponds to unstimulated leukocytes challenged with *V. anguillarum* or *E. tarda*. Data are expressed as normalized gene expression. Data is composed by 6 biological replicates and is represented as mean $\pm$ SEM. Significant differences ($p<0.05$; $p<0.01$; $p<0.001$) in relation to control are represented by asterisks (*, **, ***, respectively).

4.4. Discussion

Besides numerous health-promoting attributes, *Bacillus* spp. enhance fish growth, promote disease resistance by producing natural antimicrobial compounds, and stimulate the immune system (James, et al., 2021). In fish, several studies showed that dietary administration of *Bacillus* spp. cells or spores enhanced the innate and adaptive immune responses and increased disease resistance against different pathogens, namely *Aeromonas hydrophila*, *E. tarda*, *E. ictaluri*, *Streptococcus agalactiae*, *S. iniae*, *V. anguillarum*, and *Yersinia ruckery* (Chen, et al., 2016; Lin, et al., 2019; Nguafack, et al., 2020; Sangma, Kamilya, 2015; Selim, Reda, 2015; Sookchaiyaporn, et al., 2020; Truong Thy, et al., 2017; Van Doan, et al., 2018; Yi, et al., 2018).

The exact mechanisms by which *Bacillus* spp. act as immunostimulants and convey protection against certain diseases when fed to fish remains to be elucidated. Nonetheless, it is assumed that *Bacillus* spp. enhance the immune responses by interacting with different fish immune cells (granulocytes, monocytes, lymphocytes, and natural killer cells) (Daniella de Carla, et al., 2020; Nayak, et al., 2007; Newaj-Fyzul, et al., 2007). Further, despite the potential direct effect of *Bacillus* spp. cells or spores on fish innate immune response, the *Bacillus* spp. extracellular metabolome, known to contain several biologically important metabolites, including antimicrobial and quorum-quenching molecules may also have a relevant effect. However, to our knowledge, there is only one study that focused on the immune-stimulation potential of *Bacillus* spp. extracellular metabolome (Chen, et al., 2015).

To fulfil this gap, in the present study, we tested *in vitro* the potential of extracellular extracts of three *Bacillus* spp. strains (*B. subtilis* FI314, *B. velezensis* FI436, and *B. pumilus* FI464), previously isolated from fish-guts and characterized for their antimicrobial activity against fish pathogens (Santos, et al., 2021a), to induce cellular innate immune responses in gilthead seabream head-kidney leukocytes. Leukocytes were exposed for 24 h to *Bacillus* spp. extracts, and although whole leukocytes viability was not affected, an increase in granulocytes apoptosis was noticed. Granulocytes are sophisticated cells, critical in host defense and maintenance of homeostasis. Previous studies have demonstrated the potential of putative probiotic bacteria to help control inflammation by modulating immune cells numbers. However, only one study has reported the *in vitro* influence of probiotic bacteria on granulocytes' numbers. Sustrova, et al. (2017) incubated porcine and human neutrophils with *Bifidobacterium bifidum*, *Lactobacillus rhamnosus*, and *Enterococcus faecium* and observed a significant increase in apoptotic neutrophils, indicating a more efficient efferocytosis and a fastest inflammation resolution by probiotic strains. Apoptotic granulocytes death can exert anti-inflammatory effects that are crucial in the resolution of inflammation (Dransfield, Rossi,

2004; Fox, et al., 2010). Thus, it can be speculated that the FI extracts activated the immune cells to respond to an infection, that in the absence of an intruder regulated the inflammatory process and homeostasis by activating granulocytes' specific apoptosis. Moreover, the FI extracts that decreased granulocytes viability also showed a tendency to promote an up-regulation of cyclooxygenase-2 (COX-2), a typical oxidative stress marker regulated by inflammatory proteins such as IL-1 β , IL-6, TNF- α , that plays a dual role in inflammation by generating pro- and anti-inflammatory mediators (Chen, 2010). Further, we also observed an up-regulation of the pro-inflammatory cytokine IL-1 β , which is important for triggering an inflammatory activity by activating immune responses such as phagocytosis. Previously, *Bacillus* spp. were also described to up-regulate the expression of pro-inflammatory cytokines (e.g., IL-1 β), resulting in a lower fish mortality rate upon challenge (Chen, et al., 2015; Galagarza, et al., 2018; Lin, et al., 2019; Nguafack, et al., 2020; Sakai, et al., 2021; Yi, et al., 2018).

After establishing the immunostimulatory properties of FI extracts, their contribution to immune protection was tested upon challenge with aquaculture bacterial pathogens. *V. anguillarum* and *E. tarda* cause particularly problematic diseases affecting a range of economically important aquaculture species such as rainbow trout, gilthead seabream, turbot, Senegalese sole, and tilapia (Castro, et al., 2006; Castro, et al., 2012; Frans, et al., 2011; Miniero Davies, et al., 2018). In this study, gilthead seabream head-kidney leukocytes challenged with heat-inactivated *V. anguillarum* and *E. tarda*, presented a decrease in viability in cells previously stimulated with FI extracts, comparatively to non-stimulated cells. A tendency for an increase of granulocytes' apoptosis was also observed on these treatments, which might explain the decrease of total leukocytes viability. Immune cells' apoptosis is usually observed in response to an infection, leading to the removal of the intracellular microorganisms, the release of microbicidal components, and an increase of inflammatory responses propagation to neighbour cells (Stephenson, et al., 2016). Thus, an increase of cells apoptosis upon stimulation with FI extracts might represent a critical advantage to control the proliferation of these pathogens.

Another important pathogen control mechanism is phagocytosis. Cells use phagocytosis to capture and ingest foreign particles including pathogenic microorganisms and cellular debris (Gordon, 2016; Uribe-Querol, Rosales, 2020). Although all cells may perform phagocytosis, monocytes and granulocytes are the professional cells to eliminate invading pathogens (Rabinovitch, 1995). Several studies demonstrated that the oral administration of *Bacillus* spp. enhance phagocytosis of foreign particles (Chen, et al., 2015; Salinas, et al., 2006; Selim, Reda, 2015; Sookchaiyaporn, et al., 2020; Truong Thy, et al., 2017). Brunt, et al. (2007) showed that the oral administration of *Bacillus* sp.

JB-1 increased the phagocytic killing ability of rainbow trout macrophages of several important bacteria such as *A. salmonicida*, *Lactococcus garviae*, *S. iniae*, *V. anguillarum* and *Y. ruckeri*. Similarly, oral administration of *B. aerophilus* KADR3 to *L. rohita* and *Bacillus* sp. B81e to *P. bocourti* increased the phagocytosis of *A. hydrophila* by peripheral blood leukocytes (Abellán, Basurco, 1999; Meidong, et al., 2018; Ramesh, et al., 2017). Here we also observed that FI-extracts stimulated cells also showed a significant increase of *V. anguillarum* and *E. tarda* phagocytosis, thus also demonstrating the immunostimulatory action of *Bacillus* spp. extracts, adding to the already establish immunostimulatory effect of *Bacillus* spp. cells and spores.

Fish phagocytic cells are notable in rapidly killing infiltrated pathogens by the production of reactive oxygen species (ROS) and nitrogen compounds with antimicrobial properties, in a process called oxidative or respiratory burst. Nonetheless, several pathogens, including *V. anguillarum* and *E. tarda*, have developed strategies to survive microbicidal responses and to thrive inside phagocytic cells, having a clear advantage to proliferate infection inside the host (Grayfer, et al., 2014; Grayfer, et al., 2018; Uribe-Querol, Rosales, 2017). Further, Sepulcre, et al. (2007) showed that *V. anguillarum* can survive inside European sea bass phagocytic cells by inhibiting ROS production. Moreover, *V. anguillarum* downregulated the apoptotic caspases, indicating that this organism can manipulate the host killing mechanisms and apoptotic programs to eliminate pathogens. Similarly, Ishibe, et al. (2008) demonstrated the ability of a virulent *E. tarda* strain (NUF251) to survive and multiply inside olive flounder macrophages, whereas the non-virulent strain (NUF194) had no capacity. Additionally, the virulent strain inhibited ROS production during phagocytosis demonstrating the ability of *E. tarda* to disseminate inside the host. More recently, it was demonstrated that *E. tarda* not only replicates inside macrophages but also utilizes these cells to increase virulence factors (type III secretion system T3SS/T6SS-related genes) that upon release from macrophages enhance infectivity in the host cells and increase resistance to the multiple host defences (Zhang, et al., 2016). Although in the present study a significant increase in *V. anguillarum* and *E. tarda* phagocytosis was observed, no differences were found in the respiratory burst activity (vital for the subsequent degradation of internalized bacteria) of stimulated leukocytes. The exception was extract FI464 that led to a decrease in the respiratory burst activity which might represent an advantage for *V. anguillarum* proliferation inside the host (Sepulcre, et al., 2007). Thus, further investigation is required to ascertain if the increased phagocytosis seen in our experiments corresponds to a decrease of bacterial load. Moreover, upon challenge, an increase in lymphocytes ROS production over time was also observed, indicating that under our experimental conditions lymphocytes may also play a role in intracellular pathogen's killing, as previously observed by others

(Li, et al., 2006; Wu, et al., 2019).

Adding to phagocytosis and ROS production, cytokines release is another important innate immune system response for the early control of infections. Secretion of pro-inflammatory cytokines, such as TNF- α , IL-1, and IL6, is crucial to the recruitment of immune cells upon infection sites and consequent pathogen's elimination (Zhu, et al., 2013; Zou, Secombes, 2016). Nonetheless, some pathogens have developed strategies to manipulate cytokines response, by inducing anti-inflammatory cytokines production, which reduces the host immune response (Condotta, Richer, 2017; Hornef, et al., 2002). Previous reports have shown that *Bacillus* spp. up-regulated crucial cytokines for pathogens elimination, such as TNF- α , IL-1 β , IL-6, and COX-2 (Cerezuela, et al., 2013; Chen, et al., 2015; Galagarza, et al., 2018; Lin, et al., 2019; Nguafack, et al., 2020; Selim, Reda, 2015; Yi, et al., 2018). However, so far there is only one study that evaluated the *in vitro* induction of cell immunity markers, including cytokines, by *Bacillus* spp. secondary metabolites (Chen, et al., 2015), and none upon bacterial challenge. Under our experimental conditions, cells submitted to an *E. tarda* challenge showed a significant up-regulation of the pro-inflammatory genes IL-1 β , IL-6, and COX-2 when previously stimulated with FI extracts. This may represent a clear advantage in the early immune response. Although not statistically different, there was also a tendency for an up-regulation of the same pro-inflammatory genes in FI-stimulated cells upon challenge with *V. anguillarum*. These results indicate that the tested extracts of *Bacillus* spp. may induce a stronger early resolution of infection upon challenge with bacterial pathogens. Unexpectedly, TNF- α expression, involved in the activation of phagocytic cells and microbicidal activities (Zou, Secombes, 2016), was barely affected by *Bacillus* spp. extracts. Yet, the maximum ROS and phagocytic activity was observed at 1 h post-infection, and the TNF- α expression was measured at 4 h post-infection, which might contribute to explain this lack of response. Further studies should quantify these parameters over time to characterize their evolution during infection. An over-time evaluation of the effects of FI extracts on the resolution of inflammation and homeostasis restoration by the activation of anti-inflammatory cytokines (e.g., IL-10), also merits further investigation.

Overall, the results of this study complement and reinforce the importance of *Bacillus* spp. in controlling fish bacterial diseases, by establishing the *in vitro* immunostimulant potential of their extracellular compounds. Targeting *Bacillus* spp. isolated from aquaculture fish and with proven *in vitro* and *in vivo* antimicrobial activities against fish pathogens, should be further pursued as a valuable approach to find potential immunostimulant compounds to control fish diseases.

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4.5. References

- Abellán, E., Basurco, B., 1999. Marine Finfish Species Diversification: Current Situation and Prospects in Mediterranean Aquaculture. in: Abellán, E., Basurco, B. (Eds.), Marine Finfish Species Diversification: Current Situation and Prospects in Mediterranean Aquaculture. Zaragoza : CIHEAM, pp. 1-139.
- Angosto, D., Montero, J., López-Muñoz, A., Alcaraz-Pérez, F., Bird, S., Sarropoulou, E., Abellán, E., Meseguer, J., Sepulcre, M.P., Mulero, V., 2014. Identification and functional characterization of a new IL-1 family member, IL-1Fm2, in most evolutionarily advanced fish. *Innate Immun.* 20, 487-500. doi: <https://doi.org/10.1177/1753425913501011>.
- Barman, D., Nen, P., Mandal, S., Kumar, V., 2013. Immunostimulants for aquaculture health management. *J Marine Sci Res Dev.* 3, 1-11.
- Benhamed, S., Guardiola, F.A., Martínez, S., Martínez-Sánchez, M.J., Pérez-Sirvent, C., Mars, M., Esteban, M.A., 2016. Exposure of the gilthead seabream (*Sparus aurata*) to sediments contaminated with heavy metals down-regulates the gene expression of stress biomarkers. *Toxicology Reports.* 3, 364-372. doi: <https://doi.org/https://doi.org/10.1016/j.toxrep.2016.02.006>.
- Brunt, J., Newaj-Fyzul, A., Austin, B., 2007. The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis.* 30, 573-579. doi: <https://doi.org/https://doi.org/10.1111/j.1365-2761.2007.00836.x>.
- Castillo, J., Teles, M., Mackenzie, S., Tort, L., 2009. Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (*Sparus aurata*). *Fish Shellfish Immunol.* 27 3, 493-499.
- Castro, N., Toranzo, A.E., Barja, J.L., Núñez, S., Magariños, B., 2006. Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.). *J Fish Dis.* 29, 541-547. doi: <https://doi.org/10.1111/j.1365-2761.2006.00750.x>.
- Castro, N., Toranzo, A.E., Devesa, S., González, A., Nuñez, S., Magariños, B., 2012. First description of *Edwardsiella tarda* in Senegalese sole, *Solea senegalensis* (Kaup). *J Fish Dis.* 35, 79-82. doi: <https://doi.org/10.1111/j.1365-2761.2011.01325.x>.
- Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Front Microbiol.* 10. doi: <https://doi.org/10.3389/fmicb.2019.00302>.
- Cerezuela, R., Meseguer, J., Esteban, M.Á., 2013. Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream

(*Sparus aurata* L.). Fish Shellfish Immunol. 34, 843-848. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2012.12.026>.

Chaves-Pozo, E., Liarte, S., Fernandez-Alacid, L., Abellan, E., Meseguer, J., Mulero, V., Garcia-Ayala, A., 2008. Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream (*Sparus aurata* L.). Mol Immunol. 45, 2998-3011. doi: <https://doi.org/10.1016/j.molimm.2008.01.018>.

Chen, B., Peng, M., Tong, W., Zhang, Q., Song, Z., 2020. The Quorum Quenching Bacterium *Bacillus licheniformis* T-1 Protects Zebrafish against *Aeromonas hydrophila* Infection. Probiotics Antimicrob Proteins. doi: <https://doi.org/10.1007/s12602-018-9495-7>.

Chen, C., 2010. COX-2's new role in inflammation. Nat Chem Biol. 6, 401-402. doi: <https://doi.org/10.1038/nchembio.375>.

Chen, X.M., Lu, H.M., Niu, X.T., Wang, G.Q., Zhang, D.M., 2015. Enhancement of secondary metabolites from *Bacillus Licheniformis* XY-52 on immune response and expression of some immune-related genes in common carp, *Cyprinus carpio*. Fish Shellfish Immunol. 45, 124-131. doi: <https://doi.org/10.1016/j.fsi.2015.02.019>.

Chen, Y., Li, J., Xiao, P., Li, G.Y., Yue, S., Huang, J., Zhu, W.Y., Mo, Z.L., 2016. Isolation and characterization of *Bacillus* spp. M001 for potential application in turbot (*Scophthalmus maximus* L.) against *Vibrio anguillarum*. Aquacult Nutr. 22, 374-381. doi: <https://doi.org/https://doi.org/10.1111/anu.12259>.

Condotta, S.A., Richer, M.J., 2017. The immune battlefield: The impact of inflammatory cytokines on CD8+ T-cell immunity. PLoS Pathog. 13, e1006618-e1006618. doi: <https://doi.org/10.1371/journal.ppat.1006618>.

Daniella de Carla, D., Leonardo, T., Marina Keiko Pieroni, I., Ivan Bernardoni, N., Elizabeth, R., Robson, S., Maria José Tavares, R.-P., 2020. Probiotic supplementation causes hematological changes and improves non-specific immunity in *Brycon amazonicus*. Acta Scientiarum. Biological Sciences. 42. doi: <https://doi.org/10.4025/actasciobiolsci.v42i1.52473>.

Dransfield, I., Rossi, A.G., 2004. Granulocyte apoptosis: who would work with a 'real' inflammatory cell? Biochem Soc Trans. 32, 447-451. doi: <https://doi.org/10.1042/bst0320447>.

Esteban, M.A., Mulero, V., Muñoz, J., Meseguer, J., 1998. Methodological aspects of assessing phagocytosis of *Vibrio anguillarum* by leukocytes of gilthead seabream

(*Sparus aurata* L.) by flow cytometry and electron microscopy. Cell and Tissue Research. 293, 133-141. doi: <https://doi.org/10.1007/s004410051105>.

Fox, S., Leitch, A.E., Duffin, R., Haslett, C., Rossi, A.G., 2010. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. J Innate Immun. 2, 216-227. doi: <https://doi.org/10.1159/000284367>.

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B., Rediers, H., 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. J Fish Dis. 34, 643-661. doi: <https://doi.org/10.1111/j.1365-2761.2011.01279.x>.

Galagarza, O.A., Smith, S.A., Drahos, D.J., Eifert, J.D., Williams, R.C., Kuhn, D.D., 2018. Modulation of innate immunity in Nile tilapia (*Oreochromis niloticus*) by dietary supplementation of *Bacillus subtilis* endospores. Fish Shellfish Immunol. 83, 171-179. doi: <https://doi.org/10.1016/j.fsi.2018.08.062>.

Gordon, S., 2016. Phagocytosis: An Immunobiologic Process. Immunity. 44, 463-475. doi: <https://doi.org/10.1016/j.immuni.2016.02.026>.

Grayfer, L., Hodgkinson, J.W., Belosevic, M., 2014. Antimicrobial responses of teleost phagocytes and innate immune evasion strategies of intracellular bacteria. Developmental & Comparative Immunology. 43, 223-242. doi: <https://doi.org/https://doi.org/10.1016/j.dci.2013.08.003>.

Grayfer, L., Kerimoglu, B., Yaparla, A., Hodgkinson, J.W., Xie, J., Belosevic, M., 2018. Mechanisms of Fish Macrophage Antimicrobial Immunity. Front Immunol. 9, 1105. doi: <https://doi.org/10.3389/fimmu.2018.01105>.

Hornef, M.W., Wick, M.J., Rhen, M., Normark, S., 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. Nat Immunol. 3, 1033-1040. doi: <https://doi.org/10.1038/ni1102-1033>.

Inoue, T., Moritomo, T., Tamura, Y., Mamiya, S., Fujino, H., Nakanishi, T., 2002. A new method for fish leukocyte counting and partial differentiation by flow cytometry. Fish Shellfish Immunol. 13, 379-390. doi: <https://doi.org/https://doi.org/10.1006/fsim.2002.0413>.

Ishibe, K., Osatomi, K., Hara, K., Kanai, K., Yamaguchi, K., Oda, T., 2008. Comparison of the responses of peritoneal macrophages from Japanese flounder (*Paralichthys olivaceus*) against high virulent and low virulent strains of *Edwardsiella tarda*. Fish Shellfish Immunol. 24, 243-251. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2007.11.001>.

- James, G., Das, B.C., Jose, S., V.J., R.K., 2021. *Bacillus* as an aquaculture friendly microbe. *Aquac Int.* 29, 323-353. doi: <https://doi.org/10.1007/s10499-020-00630-0>.
- Ji, L., Sun, G., Li, J., Wang, Y., Du, Y., Li, X., Liu, Y., 2017. Effect of dietary β -glucan on growth, survival and regulation of immune processes in rainbow trout (*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida*. *Fish Shellfish Immunol.* 64, 56-67. doi: <https://doi.org/10.1016/j.fsi.2017.03.015>.
- Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology.* 173, 111-133. doi: <https://doi.org/https://doi.org/10.1016/j.anifeedsci.2011.12.015>.
- Kumar, R., Mukherjee, S.C., Ranjan, R., Nayak, S.K., 2008. Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish Shellfish Immunol.* 24, 168-172. doi: <https://doi.org/https://doi.org/10.1016/j.fsi.2007.10.008>.
- Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol.* 7, 1116-1124. doi: <https://doi.org/10.1038/ni1389>.
- Lin, Y.S., Saputra, F., Chen, Y.C., Hu, S.Y., 2019. Dietary administration of *Bacillus amyloliquefaciens* R8 reduces hepatic oxidative stress and enhances nutrient metabolism and immunity against *Aeromonas hydrophila* and *Streptococcus agalactiae* in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 86, 410-419. doi: <https://doi.org/10.1016/j.fsi.2018.11.047>.
- Lu, D.L., Limbu, S.M., Lv, H.B., Ma, Q., Chen, L.Q., Zhang, M.L., Du, Z.Y., 2019. The comparisons in protective mechanisms and efficiencies among dietary α -lipoic acid, β -glucan and l-carnitine on Nile tilapia infected by *Aeromonas hydrophila*. *Fish Shellfish Immunol.* 86, 785-793. doi: <https://doi.org/10.1016/j.fsi.2018.12.023>.
- Magnadóttir, B., 2006. Innate immunity of fish (overview). *Fish Shellfish Immunol.* 20, 137-151. doi: <https://doi.org/10.1016/j.fsi.2004.09.006>.
- Magnadóttir, B., 2010. Immunological Control of Fish Diseases. *Mar Biotechnol.* 12, 361-379. doi: <https://doi.org/10.1007/s10126-010-9279-x>.
- Maqsood, S., Singh, P., Samoon, M.H., Munir, K., 2011. Emerging role of immunostimulants in combating the disease outbreak in aquaculture. *International Aquatic Research* (Islamic Azad University, Tonekabon Branch).

- Meidong, R., Khotchanalekha, K., Doolgindachbaporn, S., Nagasawa, T., Nakao, M., Sakai, K., Tongpim, S., 2018. Evaluation of probiotic *Bacillus aerius* B81e isolated from healthy hybrid catfish on growth, disease resistance and innate immunity of Pla-mong *Pangasius bocourti*. Fish Shellfish Immunol. 73, 1-10. doi: <https://doi.org/10.1016/j.fsi.2017.11.032>.
- Miniero Davies, Y., Xavier de Oliveira, M.G., Paulo Vieira Cunha, M., Soares Franco, L., Pulecio Santos, S.L., Zanolli Moreno, L., Túlio de Moura Gomes, V., Zanolli Sato, M.I., Schiavo Nardi, M., Micke Moreno, A., Becker Saidenberg, A., Rose Marques de Sá, L., Knöbl, T., 2018. *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. Vet Q. 38, 99-105. doi: <https://doi.org/10.1080/01652176.2018.1540070>.
- Nayak, S.K., Swain, P., Mukherjee, S.C., 2007. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). Fish Shellfish Immunol. 23, 892-896. doi: <https://doi.org/10.1016/j.fsi.2007.02.008>.
- Newaj-Fyzul, A., Adesiyun, A.A., Mutani, A., Ramsubhag, A., Brunt, J., Austin, B., 2007. *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J Appl Microbiol. 103, 1699-1706. doi: <https://doi.org/10.1111/j.1365-2672.2007.03402.x>.
- Nguafack, T.T., Jang, W.J., Hasan, M.T., Choi, Y.H., Bai, S.C., Lee, E.W., Lee, B.J., Hur, S.W., Lee, S., Kong, I.S., 2020. Effects of dietary non-viable *Bacillus* sp. SJ-10, *Lactobacillus plantarum*, and their combination on growth, humoral and cellular immunity, and streptococcosis resistance in olive flounder (*Paralichthys olivaceus*). Res Vet Sci. 131, 177-185. doi: <https://doi.org/10.1016/j.rvsc.2020.04.026>.
- Pérez-Sánchez, T., Mora-Sánchez, B., Balcázar, J.L., 2018. Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. Trends Microbiol. 26, 896-903. doi: <https://doi.org/https://doi.org/10.1016/j.tim.2018.05.002>.
- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research. 29, e45-e45. doi: <https://doi.org/10.1093/nar/29.9.e45>.
- Rabinovitch, M., 1995. Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 5, 85-87. doi: [https://doi.org/10.1016/s0962-8924\(00\)88955-2](https://doi.org/10.1016/s0962-8924(00)88955-2).
- Ramesh, D., Souissi, S., Ahamed, T.S., 2017. Effects of the potential probiotics *Bacillus aerophilus* KADR3 in inducing immunity and disease resistance in *Labeo rohita*. Fish Shellfish Immunol. 70, 408-415. doi: <https://doi.org/10.1016/j.fsi.2017.09.037>.

- Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. *Immunology Letters*. 148, 23-33. doi: <https://doi.org/10.1016/j.imlet.2012.08.003>.
- Reyes-Becerril, M., Alamillo, E., Sánchez-Torres, L., Ascencio-Valle, F., Perez-Urbiola, J.C., Angulo, C., 2016. Leukocyte susceptibility and immune response against *Vibrio parahaemolyticus* in *Totoaba macdonaldi*. *Dev Comp Immunol*. 65, 258-267. doi: <https://doi.org/10.1016/j.dci.2016.07.016>.
- Sakai, M., Hikima, J.-i., Kono, T., 2021. Fish cytokines: current research and applications. *Fisheries Sci*. 87, 1-9. doi: <https://doi.org/10.1007/s12562-020-01476-4>.
- Salinas, I., Díaz-Rosales, P., Cuesta, A., Meseguer, J., Chabrállón, M., Moriñigo, M.Á., Esteban, M.Á., 2006. Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). *Vet Immunol Immunopathol*. 111, 279-286. doi: <https://doi.org/10.1016/j.vetimm.2006.01.020>.
- Samaï, H.C., Rioult, D., Bado-Nilles, A., Delahaut, L., Jubréaux, J., Geffard, A., Porcher, J.-M., Betoulle, S., 2018. Procedures for leukocytes isolation from lymphoid tissues and consequences on immune endpoints used to evaluate fish immune status: A case study on roach (*Rutilus rutilus*). *Fish Shellfish Immunol*. 74, 190-204. doi: <https://doi.org/10.1016/j.fsi.2017.12.040>.
- Sangma, T., Kamilya, D., 2015. Dietary *Bacillus subtilis* FPTB13 and chitin, single or combined, modulate systemic and cutaneous mucosal immunity and resistance of catla, *Catla catla* (Hamilton) against edwardsiellosis. *Comp Immunol Microbiol Infect Dis*. 43, 8-15. doi: <https://doi.org/10.1016/j.cimid.2015.09.003>.
- Santos, R.A., Oliva-Teles, A., Pousão-Ferreira, P., Jerusik, R., Saavedra, M.J., Enes, P., Serra, C.R., 2021a. Isolation and Characterization of Fish-Gut *Bacillus* spp. as Source of Natural Antimicrobial Compounds to Fight Aquaculture Bacterial Diseases. *Mar Biotechnol (NY)*. 23, 276-293. doi: <https://doi.org/10.1007/s10126-021-10022-x>.
- Santos, R.A., Monteiro, M., Rangel, F., Jerusik, R., Saavedra, M.J., Carvalho, A.P., Oliva-Teles, A., Serra, C.R., 2021b. *Bacillus* spp. Inhibit *Edwardsiella tarda* Quorum-Sensing and Fish Infection. *Mar Drugs*. 19, 602.
- Sattanathan, G., Tamizhazhagan, V., Padmapriya, S., Liu, W.-C., Balasubramanian, B., 2020. Effect of Green Algae *Chaetomorpha antennina* Extract on Growth, Modulate Immunity, and Defenses against *Edwardsiella tarda* Infection in *Labeo rohita*. *Animals* : an open access journal from MDPI. 10, 2033. doi: <https://doi.org/10.3390/ani10112033>.

- Selim, K.M., Reda, R.M., 2015. Improvement of immunity and disease resistance in the Nile tilapia, *Oreochromis niloticus*, by dietary supplementation with *Bacillus amyloliquefaciens*. Fish Shellfish Immunol. 44, 496-503. doi: <https://doi.org/10.1016/j.fsi.2015.03.004>.
- Sepulcre, M.P., Sarropoulou, E., Kotoulas, G., Meseguer, J., Mulero, V., 2007. *Vibrio anguillarum* evades the immune response of the bony fish sea bass (*Dicentrarchus labrax* L.) through the inhibition of Leukocyte respiratory burst and down-regulation of apoptotic caspases. Molecular Immunology. 44, 3751-3757. doi: <https://doi.org/https://doi.org/10.1016/j.molimm.2007.03.021>.
- Serra, C.R., Almeida, E.M., Guerreiro, I., Santos, R., Merrifield, D.L., Tavares, F., Oliva-Teles, A., Enes, P., 2019. Selection of carbohydrate-active probiotics from the gut of carnivorous fish fed plant-based diets. Sci Rep. 9, 6384. doi: <https://doi.org/10.1038/s41598-019-42716-7>.
- Sookchaiyaporn, N., Srisapoome, P., Unajak, S., Areechon, N., 2020. Efficacy of *Bacillus* spp. isolated from Nile tilapia *Oreochromis niloticus* Linn. on its growth and immunity, and control of pathogenic bacteria. Fisheries Sci. 86, 353-365. doi: <https://doi.org/10.1007/s12562-019-01394-0>.
- Stephenson, H.N., Herzig, A., Zychlinsky, A., 2016. Beyond the grave: When is cell death critical for immunity to infection? Current Opinion in Immunology. 38, 59-66. doi: <https://doi.org/https://doi.org/10.1016/j.coi.2015.11.004>.
- Sustrova, T., Ondrackova, P., Leva, L., Kolarova, M., Kulich, P., Sladek, Z., 2017. Effect of probiotics on the viability of porcine and human neutrophils in vitro. Veterinární medicína. 62, 637-646.
- Truong Thy, H.T., Tri, N.N., Quy, O.M., Fotedar, R., Kannika, K., Unajak, S., Areechon, N., 2017. Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (*Pangasianodon hypophthalmus*). Fish Shellfish Immunol. 60, 391-399. doi: <https://doi.org/10.1016/j.fsi.2016.11.016>.
- Uribe-Querol, E., Rosales, C., 2017. Control of Phagocytosis by Microbial Pathogens. Front Immunol. 8. doi: <https://doi.org/10.3389/fimmu.2017.01368>.
- Uribe-Querol, E., Rosales, C., 2020. Phagocytosis: Our Current Understanding of a Universal Biological Process. Front Immunol. 11. doi: <https://doi.org/10.3389/fimmu.2020.01066>.

- Van Doan, H., Hoseinifar, S.H., Khanongnuch, C., Kanpiengjai, A., Unban, K., Van Kim, V., Srichaiyo, S., 2018. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*. 491, 94-100. doi: <https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.03.019>.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 3, research0034.0031. doi: <https://doi.org/10.1186/gb-2002-3-7-research0034>.
- Wang, W., Sun, J., Liu, C., Xue, Z., 2017. Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquac Res*. 48, 1-23. doi: <https://doi.org/https://doi.org/10.1111/are.13161>.
- Wu, L., Kong, L., Yang, Y., Bian, X., Wu, S., Li, B., Yin, X., Mu, L., Li, J., Ye, J., 2019. Effects of Cell Differentiation on the Phagocytic Activities of IgM(+) B Cells in a Teleost Fish. *Front Immunol*. 10, 2225-2225. doi: <https://doi.org/10.3389/fimmu.2019.02225>.
- Wu, Z., Qi, X., Qu, S., Ling, F., Wang, G., 2021. Dietary supplementation of *Bacillus velezensis* B8 enhances immune response and resistance against *Aeromonas veronii* in grass carp. *Fish Shellfish Immunol*. doi: <https://doi.org/10.1016/j.fsi.2021.05.012>.
- Yi, Y., Zhang, Z., Zhao, F., Liu, H., Yu, L., Zha, J., Wang, G., 2018. Probiotic potential of *Bacillus velezensis* JW: Antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. *Fish Shellfish Immunol*. 78, 322-330. doi: <https://doi.org/10.1016/j.fsi.2018.04.055>.
- Zhang, L., Ni, C., Xu, W., Dai, T., Yang, D., Wang, Q., Zhang, Y., Liu, Q., Mullineaux, C.W., 2016. Intramacrophage Infection Reinforces the Virulence of *Edwardsiella tarda*. *J Bacteriol*. 198, 1534-1542. doi: <https://doi.org/doi:10.1128/JB.00978-15>.
- Zhu, L.Y., Nie, L., Zhu, G., Xiang, L.X., Shao, J.Z., 2013. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. *Dev Comp Immunol*. 39, 39-62. doi: <https://doi.org/10.1016/j.dci.2012.04.001>.
- Zou, J., Secombes, C.J., 2016. The Function of Fish Cytokines. *Biology (Basel)*. 5, 23. doi: <https://doi.org/10.3390/biology5020023>.

CHAPTER V

General discussion, Conclusion and Future Perspectives

5.1. General discussion

The concept of One Health characterized as the collaborative and transdisciplinary approach to achieve human, animal, and environmental health is inevitable to ensure a sustainable food production system (Stentiford, et al., 2020). The emergence and globalization of antimicrobial-resistant bacteria and zoonotic diseases raised the need to reinforce One Health principles' implementation (Huntington, et al., 2021). Under the One Health framework, efficient production of aquaculture fish is enhanced, while also following sustainable environmental metrics (Stentiford, et al., 2020). Importantly, this notion provides an important boost for the development of novel measures to prevent aquaculture diseases, one of the biggest constraints for aquaculture development (Cabello, et al., 2016). Moreover, aquaculture industries are constantly looking for sustainable and innovative solutions that ensure the safety of their products. Concomitantly, different sustainable strategies have been studied to control aquatic pathogenic and opportunistic bacteria (Pérez-Sánchez, et al., 2018). Besides direct pathogen growth inhibition, strategies to overcome bacterial persistence and virulence through anti-biofilm and QQ molecules have also been considered innovative weapons to control animal infections (Defoirdt, 2018).

Over the years, *Bacillus* spp. have been described as a treasure of several secondary metabolites that suppress the growth and virulence of harmful microbes of aquatic animals (James, et al., 2021). *Bacillus* spp. have also been studied for their capability of activation fish immune responses, thus having a role in fish defence mechanisms against pathogens (James, et al., 2021; Nayak, 2021). Despite the increasing knowledge on pathogens control using *Bacillus* spp. compounds, there is a lack of research targeting pathogens affecting highly valuable fish species.

To gain further insights on disease control in Mediterranean fish, we adopted a tripartite approach, targeting pathogens growth, biofilm formation, and communication or quorum-sensing (QS). Thus, the main objective of this PhD project was to isolate *Bacillus* spp. from the gastrointestinal tract of fish and characterize these isolates for the production of secondary metabolites capable to suppress growth (Chapter II), biofilm formation (Chapter II), and quorum-sensing (Chapter III) of important aquaculture pathogens. Moreover, extracellular secondary metabolites were used to strengthen the fish immune system (Chapter IV), ultimately increasing fish survival rates (Chapter III). The overall results of this thesis are expected to provide important knowledge on the use of *Bacillus* spp. extracellular compounds as biocontrol measures of Mediterranean fish pathogens.

5.1.1. *Bacillus* spp. bioactivities

The intestinal microbiota is considered a pool of potential probiotics with important biological functions for the animal industry sector (Elshagabee, et al., 2017). Many of these gut-associated probiotics are strains of *Bacillus* spp. Thus, it was not surprising that a great variety of *Bacillus* spp. strains (based on morphological differences) could be isolated from the gastrointestinal tract of fish, further suggesting that these organisms may play an important role in aquatic animals' health. Nonetheless, when this project started, there was no available study reporting the isolation of *Bacillus* spp. from the gastrointestinal tract of Mediterranean fish capable of inhibiting bacterial fish pathogens. Additionally, although the majority of fish bacterial pathogens are gram-negative bacteria, studies describing *Bacillus* spp. NACs against gram-negative bacteria were limited when compared to gram-positive pathogens (Caulier, et al., 2019). Gram-positive bacteria exhibit a porous peptidoglycan layer in the cell wall that facilitates the penetration of antimicrobial compounds (Soltani, et al., 2020). On the other hand, the outer membrane of Gram-negative bacteria is a strong and effective barrier against cationic bioactive compounds produced by Gram-positive bacteria, including *Bacillus* spp. (Soltani, et al., 2020). Thus, Gram-negative bacteria might be more resistant to compounds produced by *Bacillus* spp. Nonetheless, before starting this work, we had already isolated from the gastrointestinal tract of European seabass carbohydrate-active sporeforming isolates and characterized their probiotic activities, including the production of antimicrobial molecules against Gram-negative fish pathogens (Serra, et al., 2019).

Thus, the first ambition of this Thesis was to isolate, from the gut of three aquaculture fish species in the Mediterranean, the community of culturable *Bacillus* spp. and characterize their antimicrobial properties (Chapter II). Since *Bacillus* spp. are recognized for the production of numerous bioactive secondary metabolites, the isolates were screened against a broad range of fish pathogens including *Aeromonas* spp., *Vibrio* spp., *Photobacterium damsela*, *E. tarda*, *T. maritimum*, *S. sonnei*, and *S. aureus*. More than 50% of the isolated community showed antagonistic activities against at least one of the thirteen pathogens tested, highlighting the capacity of *Bacillus* spp. to produce bioactive compounds as disease preventive molecules for aquaculture.

It is known that several bioactive secondary compounds produced by *Bacillus* spp. are released to the surrounding environment (Abriouel, et al., 2011; Caulier, et al., 2019). When this project started there were already some reports on *Bacillus* spp. as producers of extracellular compounds capable to inhibit specific fish pathogens (Mukherjee, et al., 2016; Touraki, et al., 2012; Vaseeharan, Ramasamy, 2003), but the extent of their

antimicrobial activity against a broad range of fish pathogens remained unknown. Thus, in Chapter II, the extracellular medium of *Bacillus* spp. isolates showing stronger antimicrobial activity in the initial screening were tested against the growth of fourteen important aquaculture pathogens. Although all *Bacillus* spp. isolates produced extracellular compounds with some degree of inhibition, 3 isolates were shown to release to the surrounding environment molecules with a broad spectrum of antimicrobial activity, affecting the growth of the majority of pathogen tested. Even though, it cannot be ruled out that the other *Bacillus* spp. isolates might produce unstable extracellular molecules that were degraded or altered when centrifugation or filtration procedures for isolation of extracellular medium was applied.

Adding to the direct bacterial growth inhibition, the interference with important characteristics known to promote pathogenicity and treatments resilience can be important for diseases control. Biofilms are a microbial fortress in which bacteria exhibit resilience to conventional antibiotics and host immune responses, complicating their eradication (Vestby, et al., 2020). Moreover, biofilms can be a reservoir of persistent infections and can contribute to the pathogenesis of chronic infections (Vestby, et al., 2020). Through the reduction or dispersion of biofilm structures, cells become more susceptible to antimicrobials than when associated with biofilms, increasing the efficiency of infections control (Wille, Coenye, 2020). Thus, biofilm inhibition is an attractive approach to reduce pathogenesis and re-emerging of bacterial pathogens. Results present in this Thesis revealed that extracellular compounds from *Bacillus* spp. strains can inhibit biofilms formation (Chapter II). Moreover, NACs of some *Bacillus* spp. presented different modes of action that targeted pathogens' biofilms rather than their growth. By reducing pathogens' colonization, persistence, and defence mechanisms, *Bacillus* compounds increased the possibilities of controlling pathogens' proliferation. Knowing that bacterial communication through QS mechanisms is an important regulator of biofilm formation, we hypothesized that the observed biofilm inhibition could be due to the disruption of pathogens' communication signals. Moreover, *Bacillus* spp. were reported to be involved in anti-virulence therapy through the secretion of anti-QS (or Quorum-Quenching – QQ - enzymes) (Caulier, et al., 2019; Soltani, et al., 2019). Indeed, after recognising the QQ ability of the three fish-gut *Bacillus* spp. (Chapter II), the QQ potential of the fish-gut isolates collection was further explored, by screening their ability to partially or completely degrade the major QS molecules used by Gram-negative bacteria, Acyl-Homoserine Lactones (AHLs) (Chapter III). Only 5% of the tested isolates could interfere with several AHLs produced by the wild-type biosensor, and 14% disrupted the detection of 3-oxo-C6-HSL. Despite the lack of studies comparing the anti-

growth and QQ activities of *Bacillus* spp., from the results obtained within this project it seems that FI *Bacillus* spp. produce more antimicrobial (Chapter II) than QQ (Chapter III) compounds. Nonetheless, the screening process only targeted the multiple AHLs molecules produced by the wild-type biosensor or the 3-oxo-C6-HSL as a single supplementation molecule in the CV026 biosensor. Consequently, we cannot rule out a possible QQ activity against other AHLs molecules. QQ extracellular compounds are believed to induce less pressure to the development of resistance among pathogens (García-Contreras, et al., 2013). Thus, *Bacillus* spp. with stronger QQ activity were selected as producers of extracellular QQ-molecules active against QS of the same aquaculture bacterial pathogens.

Upon characterization of *Bacillus* spp. bioactivities, 3 isolates were selected to be further explored. The extracellular compounds of FI314 (*B. subtilis*) showed remarkable bioactivities, inhibiting the growth and biofilm formation of fourteen pathogens, and enzymatically disrupting AHLs signal molecules produced by *A. veronii* and *E. tarda*. On the other hand, the extracellular molecules of FI436 (*B. velezensis*) and FI464 (*B. pumilus*) showed effective QQ activities against *A. veronii* and *E. tarda* but did not produce significant anti-growth and anti-biofilm phenotypes.

5.1.2. Purification of *Bacillus* spp. bioactive compounds

One of the major ambitions of this project was to isolate, purify and characterize bioactive molecules responsible for the anti-growth, anti-biofilm, and QQ activities. This was also the biggest challenge of this PhD project.

Isolation of the extracellular molecules responsible for the bioactivities was attempted using biochemical techniques. First, an ammonium sulphate precipitation (80%) of the cell-free supernatant of the isolates was performed, which retained the anti-growth and QQ bioactivities. This fraction was dialysed against 50 mM phosphate buffer but resulted in the loss of bioactivity. Thus, the precipitated fraction was directly loaded to a Size Exclusion Chromatography (SEC) column - SEC HiPrep 16/60 Sephacryl S300 HR (GE Healthcare, USA) that was used to separate small molecules (e.g. salts) from the larger molecules, allowing desalting and buffer exchange. Twenty pools were obtained from the column's elution, seven of which retained bioactivity. These active fractions were further purified using Ion Exchange Chromatography (IEX), a method based on the electrostatic interaction between the charged molecules in the samples and the chromatography matrix. Each diluted fraction was loaded to a cation exchange chromatography column - HiTrap SP (GE Healthcare, USA) for the elution of cationic compounds. Pools were fractioned, but bioactivity was lost in all fractions (Figure 5.1).

Other attempts were performed to discard potential errors during the procedure but without success. After the confirmed lack of positive results, an anion exchange chromatography column – IEX HiTrap Q (GE Healthcare, USA) was tested but, again, no bioactivity was observed. A Hydrophobic Interaction Chromatography-HIC Phenyl HP (GE Healthcare, USA), that separates molecules based on their hydrophobic interaction to the column resin, also revealed to be unsuccessful. Due to the lack of positive results, a polar solvent extraction method was performed. Thus, an ethyl acetate extraction with and without cell-free supernatant precipitation (using ammonium sulphate, trichloroacetic acid, or hydrochloric acid) was tried, but again no antimicrobial or QQ activity was observed.

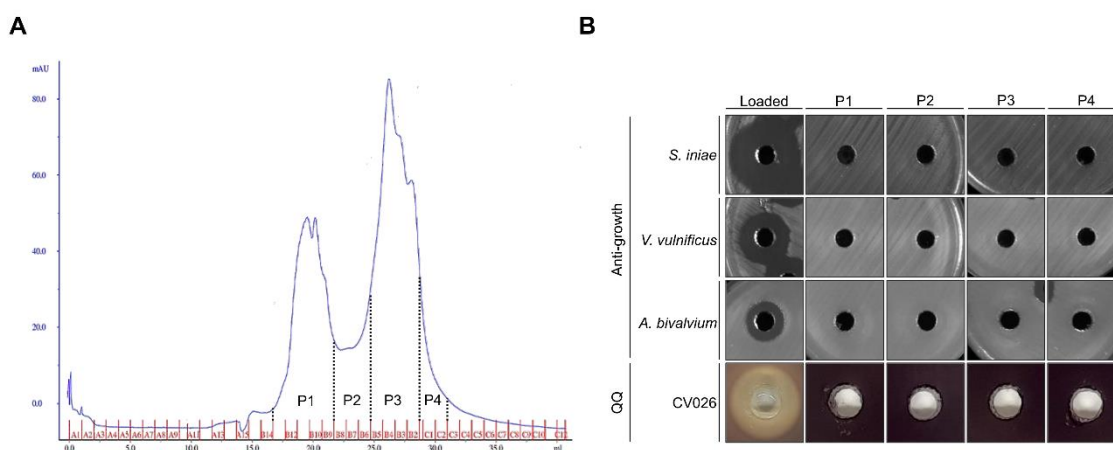


Figure 5.1. (A) Ion Exchange chromatography (HiTrap Q) elution profile of FI314 putative bioactive compounds. Elution was performed with 0-1M NaCl-50 mM phosphate buffer, pH 7. Peaks were pooled (P1-P4) and tested for bioactivities. **(B)** QQ and antimicrobial profile of the loaded sample into the column (Loaded) and eluted samples (P1-P4) against *S. iniae*, *V. vulnificus* and *A. bivalvium*.

Biochemical techniques are the traditional way to identify new bioactive molecules based on their affinity to the small-molecules ligands (Zheng, et al., 2004). However, this approach presents some limitations, including weak, unstable, and reversible affinity, low abundance, and high background that disturb the affinity (Serrano, et al., 2015; Yoshida, 2019). Although biochemical approaches have been used for years, many bioactive secondary metabolites proved to be impossible to extract in the laboratory becoming a bottleneck for their application in drug discovery (Tran, et al., 2019). New sequencing and bioinformatic technologies facilitated the process of bioactive molecules identification, allowing gene mapping and gene identification in a short period (Tran, et al., 2019). Thus, genetic approaches were planned to identify the bioactive compounds produced by the FI strains selected in this project, as detailed in section 5.3- Future Perspectives.

5.1.3. Zebrafish larvae protection by *Bacillus* spp. extracellular NACs upon challenge

Due to the unsuccessful purification of the *Bacillus* spp. extracellular bioactive compounds, this project continued with the characterization of the crude extracellular NACs produced by the three selected *Bacillus* spp. and their *in vivo* protective effects upon challenge. Among the bacterial pathogenic species targeted in this Thesis, *E. tarda* is by far the least studied. Although affecting economically important aquaculture fish species such as turbot (Castro, et al., 2006), Senegalese sole (Castro, et al., 2012), and European seabass (Blanch, et al., 1990), *E. tarda* has never been the target of studies regarding QQ *Bacillus* spp. as protective agents. To fulfil this gap, in Chapter III the potential protective effects of the crude extracellular metabolites produced by FI314, FI436, and FI464 were determined in an *in vivo* model (zebrafish larvae) challenged with *E. tarda*.

Zebrafish (*Danio rerio*) has many advantages as a model organism, including the optical transparency of embryos and larvae, easy maintenance, low housing costs, and the embryo's permeability to small compounds (Ali, et al., 2011). Zebrafish larvae are vulnerable to bacteria (including pathogens not associated with zebrafish infections) and are frequently used to study bacterial pathogenesis (Rasheed, et al., 2021). Moreover, zebrafish larvae are an important tool in drug discovery screening, allowing the study of drug toxicity, delivery system, and efficacy (MacRae, Peterson, 2015; Rasheed, et al., 2021). Thus, in this Thesis, zebrafish larvae were used to determine the toxicity of the secondary metabolites of the FI314, FI436, and FI464 isolates, to establish the *E. tarda* infection model, and to determine the efficacy of those metabolites against *E. tarda* infection. After determining the Maximum Non-Toxic Concentration (MNTC) of each extract to zebrafish larvae, this MNTC was tested *in vitro* against *E. tarda* to ensure that any protective effect upon challenge was not due to growth inhibition. Since FI436 and FI464 did not demonstrated any antimicrobial activity against *E. tarda* in Chapter II, the MNTC of these extracts also did not affect *E. tarda* growth. On the contrary, as shown in Chapter II, the FI314 extracellular medium significantly inhibited *E. tarda* growth. Despite that, the MNTC of this extract did not affect *E. tarda* growth, discarding any possible direct growth inhibition when zebrafish was challenged with *E. tarda* (Chapter III). As detailed in Chapter III, zebrafish larvae treatment with FI314, FI436, and FI464 crude extracts significantly increased their survival by 50% upon *E. tarda* challenge. Importantly, FI extracts were not in contact with *E. tarda*, indicating that aside from bioactivities, the secondary metabolites of FI314, FI436, and FI464 may also exert a stimulation of zebrafish larvae immune system, enhancing disease resistance.

5.1.4. Fish immune system stimulation by *Bacillus* spp. extracellular NACs

As a teleost fish, zebrafish possess a complete and mature immune system (Yoshida, et al., 2017). However, in the early stages of development, embryos and larvae rely only on their innate immune system to respond to injuries and pathogens (Novoa, Figueras, 2012). In zebrafish, the innate immunity, mainly including macrophages and granulocytes, is fully functional after 2 days post-fertilization (dpf), whereas the adaptive immune system takes 4-6 weeks to become functionally mature (Keij, et al., 2021; Novoa, Figueras, 2012). Due to this temporal separation of innate and adaptive immune systems maturation, the protective effects of the crude extracellular metabolites produced by FI314, FI436, and FI464 to zebrafish larvae upon *E. tarda* challenge (Chapter III) could be induced by a stimulation of the innate immune system. Thus, this project continued with the characterization of the immunomodulatory properties of the crude extracts on fish innate immunity (Chapter IV). For that purpose, gilthead seabream, one of the main marine species farmed in the Mediterranean aquaculture, was chosen as the target organism. Moreover, gilthead seabream production in Portugal is experiencing a significant expansion, especially in Madeira Island.

Gilthead seabream head-kidney leukocytes were isolated and used in an *in vitro* study. The European regulations (Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes) require the implementation of the 3Rs principle (animal replacement, refinement, and reduction) (Commission, 2010). Within this framework, the implementation of *in vitro* studies in drug discovery and development is a useful tool for the screening and selection of the best candidate to be later tested *in vivo*. Moreover, through the *in vitro* pre-selection of the best candidate, the success of an *in vivo* approach will be potentiated while minimizing the number of animals to be used.

In chapter IV, the extracellular NACs of the selected three *Bacillus* spp. were tested for their immune-modulatory properties upon infection with two important fish pathogens, *E. tarda* and *V. anguillarum*. *Bacillus* spp. extracts did not affect immune cells viability, but remarkably increased the engulfment of bacterial pathogens. Moreover, cells stimulation up-regulated the expression of immune-relevant genes associated with inflammation, including IL-1 β , IL-6, and Cox-2. Overall, these results reinforced the hypothesized idea that extracellular NACs might have stimulated the fish innate-immune system, minimizing their susceptibility to infection. Moreover, these results provided new insights on the use of *Bacillus* spp. secondary metabolites as immunomodulatory agents. An *in vivo* follow-up study will be useful to confirm these immunostimulant properties.

5.2. Main Conclusions

The main conclusions that can be highlighted from the current thesis are:

- Aerobic sporulating *Bacillus* spp. can be found in association with the gastrointestinal tract of important Mediterranean fish species, and more than 50% of this fish-gut community produces Natural Antimicrobial Compounds (NACs) active against the growth of aquaculture bacterial pathogens known to constraint the sustainable growth of the aquaculture sector.
- Three fish-gut isolates, FI314, FI330, and FI442, identified as *B. subtilis*, produce and release to the extracellular medium antimicrobial and anti-biofilm compounds with a broad range of activities. Based on these characteristics, these strains can be important sources of bioactive compounds with multiple modes of action to control fish infectious diseases.
- Fish-gut *Bacillus* spp. can produce Quorum-Quenching (QQ) compounds capable of enzymatically disrupting the most common signalling molecules used in gram-negative bacterial communication - Acyl-Homoserine Lactones (AHLs). Isolates FI314, FI436, and FI464, identified as *B. subtilis*, *B. velezensis*, and *B. pumilus*, produce extracellular QQ compounds that disrupt AHLs produced by *A. veronii* and *E. tarda*. By targeting bacterial communities' fitness, the extracellular compounds produced by these three strains might reduce pathogens' virulence and pathogenesis.
- Extracellular secondary metabolites of FI314, FI436, and FI464 have *in vitro* immunostimulatory properties in gilthead seabream leukocytes, particularly upon challenge, by increasing the engulfment of bacterial pathogens, including *E. tarda*, and by up-regulating inflammatory biomarkers.
- Extracellular secondary metabolites of FI314, FI436, and FI464 reduce *E. tarda* pathogenesis, by increasing zebrafish larvae survival up to 50% upon challenge, reinforcing their potential application in aquaculture, as diseases-preventive molecules.

5.3. Future Perspectives

The work gathered in this PhD thesis highlights the relevant contribution of *Bacillus* spp. and their extracellular secondary metabolites as disease preventive agents for the aquaculture sector. Through inhibition of bacterial pathogens growth and biofilm, disruption of pathogens communication or quorum-sensing, stimulation of fish immune system, and protection of zebrafish upon challenge, the extracellular Natural Antimicrobial Compounds produced by fish-gut *Bacillus* spp. may have an important role in aquaculture. Importantly, aquafeed companies are looking for new and innovative solutions to promote aquaculture sustainable growth and food safety. Indeed, three of the most promising fish-gut *Bacillus* spp. described in this work, were licensed to one of the top-world animal nutrition and health companies to develop an innovative product for diseases control in fish and shrimp. Nonetheless, to deepen the current knowledge of the *Bacillus* spp. bioactive applications, further research is needed.

As described in the general discussion (Section 5.1.2), purification of bioactive compounds from the selected strains was an impossible task during the course of this PhD. Nonetheless, identification of the bioactive compounds responsible for anti-growth, anti-biofilm, Quorum-Quenching, and immunostimulant activities would allow further insights into their mode of action and genetic regulatory circuits. For that, two genomic approaches - RNAseq and Whole Genome Sequencing - were applied and the corresponding transcripts and genomes are currently under bioinformatics analysis to discover the biosynthetic gene clusters and bioactive genes responsible for the phenotypes observed.

To validate the protective effects observed in Chapter III and a possible application in the aquaculture sector, an *in vivo* study is required. Gilthead seabream or other important European aquaculture fish (e.g. European seabass or turbot) should be used to evaluate the best delivery system (feed or bath) of the crude extracts or purified compounds, as well as their efficacy in promoting fish disease resistance by increasing their survival upon live bacterial challenge.

5.4. References

- Abriouel, H., Franz, C.M., Ben Omar, N., Galvez, A., 2011. Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiol Rev.* 35, 201-232. doi: <https://doi.org/10.1111/j.1574-6976.2010.00244.x>.
- Ali, S., Champagne, D.L., Spaink, H.P., Richardson, M.K., 2011. Zebrafish embryos and larvae: a new generation of disease models and drug screens. *Birth Defects Res C Embryo Today.* 93, 115-133. doi: <https://doi.org/10.1002/bdrc.20206>.
- Blanch, A.R., Pintó, R.M., Jofre, J.T., 1990. Isolation and characterization of an *Edwardsiella* sp. strain, causative agent of mortalities in sea bass (*Dicentrarchus labrax*). *Aquaculture.* 88, 213-222. doi: [https://doi.org/https://doi.org/10.1016/0044-8486\(90\)90149-H](https://doi.org/https://doi.org/10.1016/0044-8486(90)90149-H).
- Cabello, F.C., Godfrey, H.P., Buschmann, A.H., Dölz, H.J., 2016. Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. *Lancet Infect Dis.* 16, 127-133. doi: [https://doi.org/10.1016/S1473-3099\(16\)00100-6](https://doi.org/10.1016/S1473-3099(16)00100-6).
- Castro, N., Toranzo, A.E., Barja, J.L., Núñez, S., Magariños, B., 2006. Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.). *J Fish Dis.* 29, 541-547. doi: <https://doi.org/10.1111/j.1365-2761.2006.00750.x>.
- Castro, N., Toranzo, A.E., Devesa, S., González, A., Nuñez, S., Magariños, B., 2012. First description of *Edwardsiella tarda* in Senegalese sole, *Solea senegalensis* (Kaup). *J Fish Dis.* 35, 79-82. doi: <https://doi.org/10.1111/j.1365-2761.2011.01325.x>.
- Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Front Microbiol.* 10. doi: <https://doi.org/10.3389/fmicb.2019.00302>.
- Commission, E., 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off. J. Eur. Union.* 53, 33-79.
- Defoirdt, T., 2018. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends Microbiol.* 26, 313-328. doi: <https://doi.org/10.1016/j.tim.2017.10.005>.
- Elshaghabee, F.M.F., Rokana, N., Gulhane, R.D., Sharma, C., Panwar, H., 2017. *Bacillus* As Potential Probiotics: Status, Concerns, and Future Perspectives. *Front Microbiol.* 8. doi: <https://doi.org/10.3389/fmicb.2017.01490>.

- García-Contreras, R., Maeda, T., Wood, T.K., 2013. Resistance to Quorum-Quenching Compounds. *Appl Environ Microbiol.* 79, 6840. doi: <https://doi.org/10.1128/AEM.02378-13>.
- Huntington, B., Bernardo, T., Bruce, M., Devleesschauwer, B., Gilbert, W., Havelaar, A., Herrero, M., Marsh, T., Mesenhowski, S., Pendell, D., 2021. Global Burden of Animal Diseases: A novel approach to understanding and managing disease in livestock and aquaculture. *Scientific and Technical Review*.
- James, G., Das, B.C., Jose, S., V.J., R.K., 2021. *Bacillus* as an aquaculture friendly microbe. *Aquac Int.* 29, 323-353. doi: <https://doi.org/10.1007/s10499-020-00630-0>.
- Keij, F.M., Koch, B.E.V., Lozano Vigario, F., Simons, S.H.P., van Hasselt, J.G.C., Taal, H.R., Knibbe, C.A.J., Spaink, H.P., Reiss, I.K.M., Krekels, E.H.J., 2021. Zebrafish larvae as experimental model to expedite the search for new biomarkers and treatments for neonatal sepsis. *J Clin Transl Sci.* 5, e140-e140. doi: <https://doi.org/10.1017/cts.2021.803>.
- MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. *Nature Reviews Drug Discovery.* 14, 721-731. doi: <https://doi.org/10.1038/nrd4627>.
- Mukherjee, A., Dutta, D., Banerjee, S., Ringø, E., Breines, E.M., Hareide, E., Chandra, G., Ghosh, K., 2016. Potential probiotics from Indian major carp, *Cirrhinus mrigala*. Characterization, pathogen inhibitory activity, partial characterization of bacteriocin and production of exoenzymes. *Res Vet Sci.* 108, 76-84. doi: <https://doi.org/10.1016/j.rvsc.2016.08.011>.
- Nayak, S.K., 2021. Multifaceted applications of probiotic *Bacillus* species in aquaculture with special reference to *Bacillus subtilis*. *Rev Aquacult.* 13, 862-906. doi: <https://doi.org/https://doi.org/10.1111/raq.12503>.
- Novoa, B., Figueras, A., 2012. Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. *Adv Exp Med Biol.* 946, 253-275. doi: https://doi.org/10.1007/978-1-4614-0106-3_15.
- Pérez-Sánchez, T., Mora-Sánchez, B., Balcázar, J.L., 2018. Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends Microbiol.* 26, 896-903. doi: <https://doi.org/https://doi.org/10.1016/j.tim.2018.05.002>.
- Rasheed, S., Fries, F., Müller, R., Herrmann, J., 2021. Zebrafish: An Attractive Model to Study *Staphylococcus aureus* Infection and Its Use as a Drug Discovery Tool. *Pharmaceuticals (Basel).* 14, 594. doi: <https://doi.org/10.3390/ph14060594>.

- Serra, C.R., Almeida, E.M., Guerreiro, I., Santos, R., Merrifield, D.L., Tavares, F., Oliva-Teles, A., Enes, P., 2019. Selection of carbohydrate-active probiotics from the gut of carnivorous fish fed plant-based diets. *Sci Rep.* 9, 6384. doi: <https://doi.org/10.1038/s41598-019-42716-7>.
- Serrano, M., Kombrink, E., Meesters, C., 2015. Considerations for designing chemical screening strategies in plant biology. *Frontiers in Plant Science.* 6. doi: <https://doi.org/10.3389/fpls.2015.00131>.
- Soltani, M., Ghosh, K., Hoseinifar, S.H., Kumar, V., Lymbery, A.J., Roy, S., Ringø, E., 2019. Genus *Bacillus*, promising probiotics in aquaculture: Aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. *Rev Fish Sci Aquac.* 27, 331-379. doi: <https://doi.org/10.1080/23308249.2019.1597010>.
- Soltani, S., Hammami, R., Cotter, P.D., Rebuffat, S., Said, L.B., Gaudreau, H., Bédard, F., Biron, E., Drider, D., Fliss, I., 2020. Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. *FEMS Microbiol Rev.* 45. doi: <https://doi.org/10.1093/femsre/fuaa039>.
- Stentiford, G.D., Bateman, I.J., Hinchliffe, S.J., Bass, D., Hartnell, R., Santos, E.M., Devlin, M.J., Feist, S.W., Taylor, N.G.H., Verner-Jeffreys, D.W., van Aerle, R., Peeler, E.J., Higman, W.A., Smith, L., Baines, R., Behringer, D.C., Katsiadaki, I., Froehlich, H.E., Tyler, C.R., 2020. Sustainable aquaculture through the One Health lens. *Nature Food.* 1, 468-474. doi: <https://doi.org/10.1038/s43016-020-0127-5>.
- Touraki, M., Frydas, I., Karamanlidou, G., Mamara, A., 2012. Partial purification and characterization of a bacteriocin produced by *Bacillus subtilis* NCIMB 3610 that exhibits antimicrobial activity against fish pathogens.
- Tran, P.N., Yen, M.-R., Chiang, C.-Y., Lin, H.-C., Chen, P.-Y., 2019. Detecting and prioritizing biosynthetic gene clusters for bioactive compounds in bacteria and fungi. *Appl Microbiol Biotechnol.* 103, 3277-3287. doi: <https://doi.org/10.1007/s00253-019-09708-z>.
- Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett Appl Microbiol.* 36, 83-87. doi: <https://doi.org/10.1046/j.1472-765X.2003.01255.x>.
- Vestby, L.K., Grønseth, T., Simm, R., Nesse, L.L., 2020. Bacterial Biofilm and its Role in the Pathogenesis of Disease. *Antibiotics* (Basel, Switzerland). 9, 59. doi: <https://doi.org/10.3390/antibiotics9020059>.

Wille, J., Coenye, T., 2020. Biofilm dispersion: The key to biofilm eradication or opening Pandora's box? *Biofilm.* 2, 100027. doi: <https://doi.org/https://doi.org/10.1016/j.biofilm.2020.100027>.

Yoshida, M., 2019. Recent advances in target identification of bioactive natural products. *Bioscience, Biotechnology, and Biochemistry.* 83, 1-9. doi: <https://doi.org/10.1080/09168451.2018.1533804>.

Yoshida, N., Frickel, E.-M., Mostowy, S., 2017. Macrophage-Microbe Interactions: Lessons from the Zebrafish Model. *Front Immunol.* 8, 1703-1703. doi: <https://doi.org/10.3389/fimmu.2017.01703>.

Zheng, X.S., Chan, T.-F., Zhou, H.H., 2004. Genetic and Genomic Approaches to Identify and Study the Targets of Bioactive Small Molecules. *Chemistry & Biology.* 11, 609-618. doi: <https://doi.org/https://doi.org/10.1016/j.chembiol.2003.08.011>.

Appendices

Appendix I

Bacillus spp. Inhibit *Edwardsiella tarda* Quorum-Sensing and Fish Infection

Supplementary Tables

Supplementary Table 1. Identification of the 10 fish-gut isolates with QQ activity, using 16S rRNA gene analysis.

Isolate n°	Closest known species ^a	% Q.Cover ^b	% Identity ^c	GenBank accession
FI314	<i>Bacillus subtilis</i>	100	99.9	MT081448
FI330	<i>Bacillus subtilis</i>	100	100	MT081452
FI333	<i>Bacillus amyloliquefaciens</i>	100	99.7	MT081453
FI335	<i>Bacillus velezensis</i>	100	100	MT081454
FI346	<i>Bacillus subtilis</i>	100	99.7	OK094928
FI383	<i>Bacillus velezensis</i>	99	99.4	OK094929
FI423	<i>Bacillus amyloliquefaciens</i>	100	99.7	MT081476
FI436	<i>Bacillus velezensis</i>	100	99.4	MT081479
FI442	<i>Bacillus subtilis</i>	100	100	MT081480
FI464	<i>Bacillus pumilus</i>	99	98.9	MT081483

^a Closest known species using BLASTn based on partial sequences of 16S rRNA gene (~1000nt)

^b Query Cover - the percentage of the query sequence covered by the reference sequence

^c Percent Identity - the percentage of similarity between the query sequence and the reference sequence

Supplementary Figures

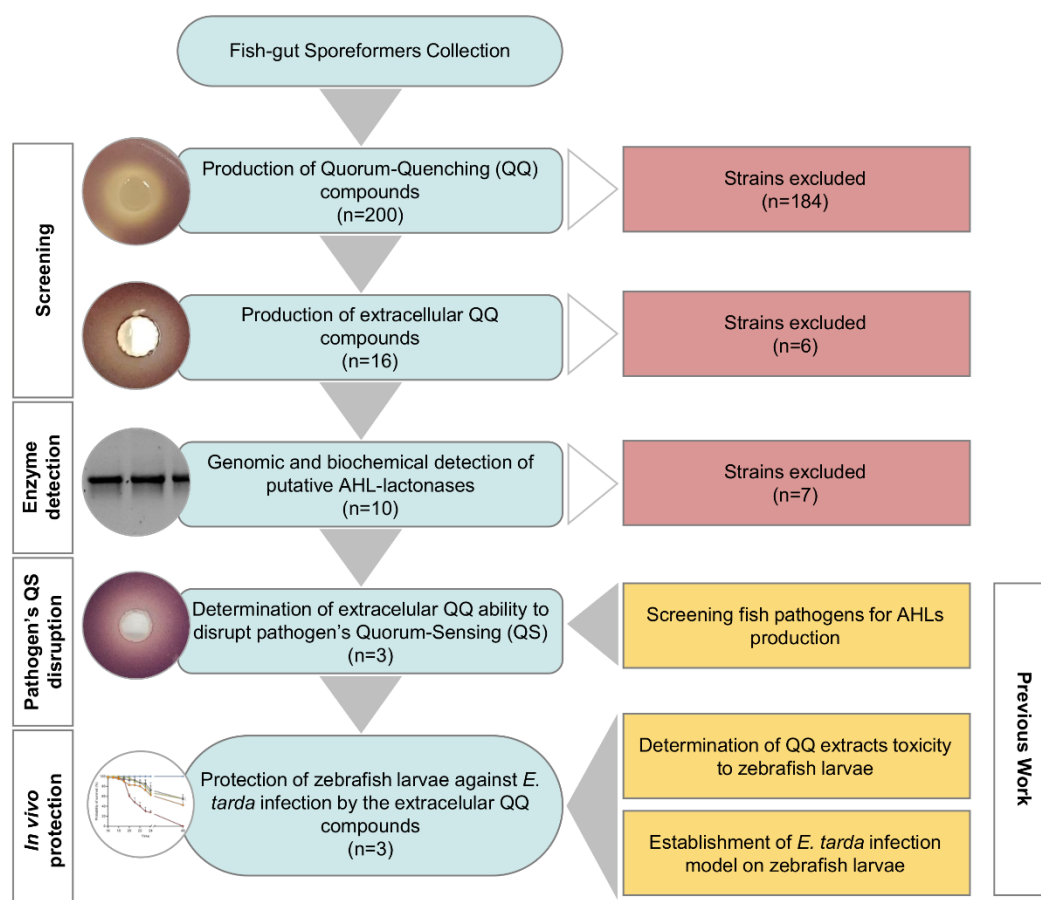


Figure S1. Flow diagram of the methodology used for evaluating FI isolates QQ activity.

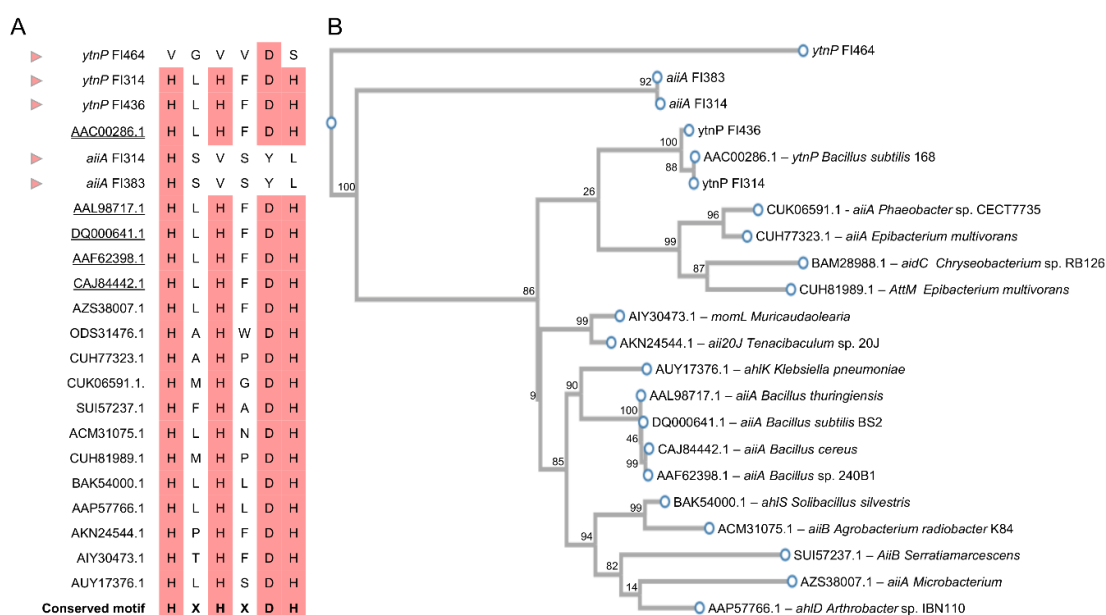


Figure S2. Amino acid sequence's comparison of the putative AHL-lactonases with known AHL-lactonase enzymes. **(A)** Amino acid sequences corresponding to putative AHL-lactonases from *aiiA* (*aiiA* FI314 and *aiiA* FI383) and *ytnP* (*ytnP* FI314, *ytnP* FI436 and *ytnP* FI464) were aligned with known AHL-lactonases enzymes from MBL fold metallohydrolase family, and the conserved motif "HXHXDH" is represented in red. Columns with the same colour represent the amino acid or motif conservation between the sequences. In underlined and double underlined lettering are the known *aiiA* and *ytnP* enzymes from *Bacillus* species, respectively. **(B)** Phylogenetic analysis based on the amino acid sequences of the putative AHL-lactonases (*aiiA* FI314, *aiiA* FI383, *ytnP* FI314 and *ytnP* FI464) and known homologous AHL-lactonases enzymes from different species including *B. subtilis*, *B. thuringiensis*, *B. cereus*, *Microbacterium*, *Epibacterium multivorans*, *Phaeobacter* sp., *Chryseobacterium* sp., *Serratia marcescens*, *Arthrobacter* sp., *Tenacibaculum* sp., *Muricaudaolearia*, *Klebsiella pneumoniae*, *Agrobacterium radiobacter* and *Solibacillus silvestris*. Phylogenetic analysis and dendrogram construction were performed using ClustalW software.



Figure S3. Detection of AHLs production by gram-negative fish pathogens using *Chr. violaceum* CV026 as a biosensor. *Aeromonas bivalvium*, *A. hydrophila*, *A. salmonicida*, *A. veronii*, *Edwardsiella tarda*, *Photobacterium damselae* subsp. *piscicida* (PDP), *Ph. damselae* subsp. *damselae* (PDD), *Shigella sonnei*, *Tenacibaculum maritimum*, *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* were streaked in parallel (1 cm apart) to the biosensor on LB agar plates. Induction of violacein pigment production by the biosensor was considered a positive result for AHLs production. All photos were taken with a Sony IMX240 camera and are at the same scale.

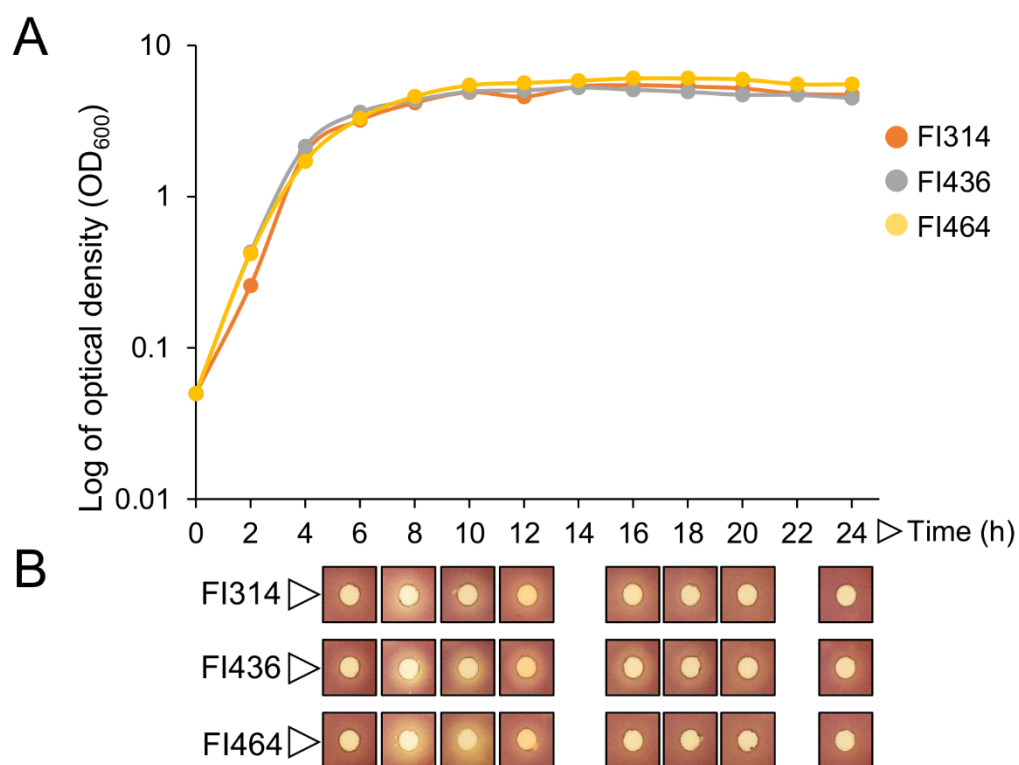


Figure S4. Growth curves and QQ kinetics of FI314, FI436 and FI464. **(A)** Optical density of FI isolates grown in LB medium at 37 °C, 140 rpm. **(B)** *Chr. violaceum* CV026 biosensor violacein pigment production inhibition around the wells containing the cell-free supernatant of sporeforming fish isolates (FI) at different time points (6, 8, 10, 12, 16, 18, 20, 24H). All photos were taken with a Sony IMX240 camera and are at the same scale.

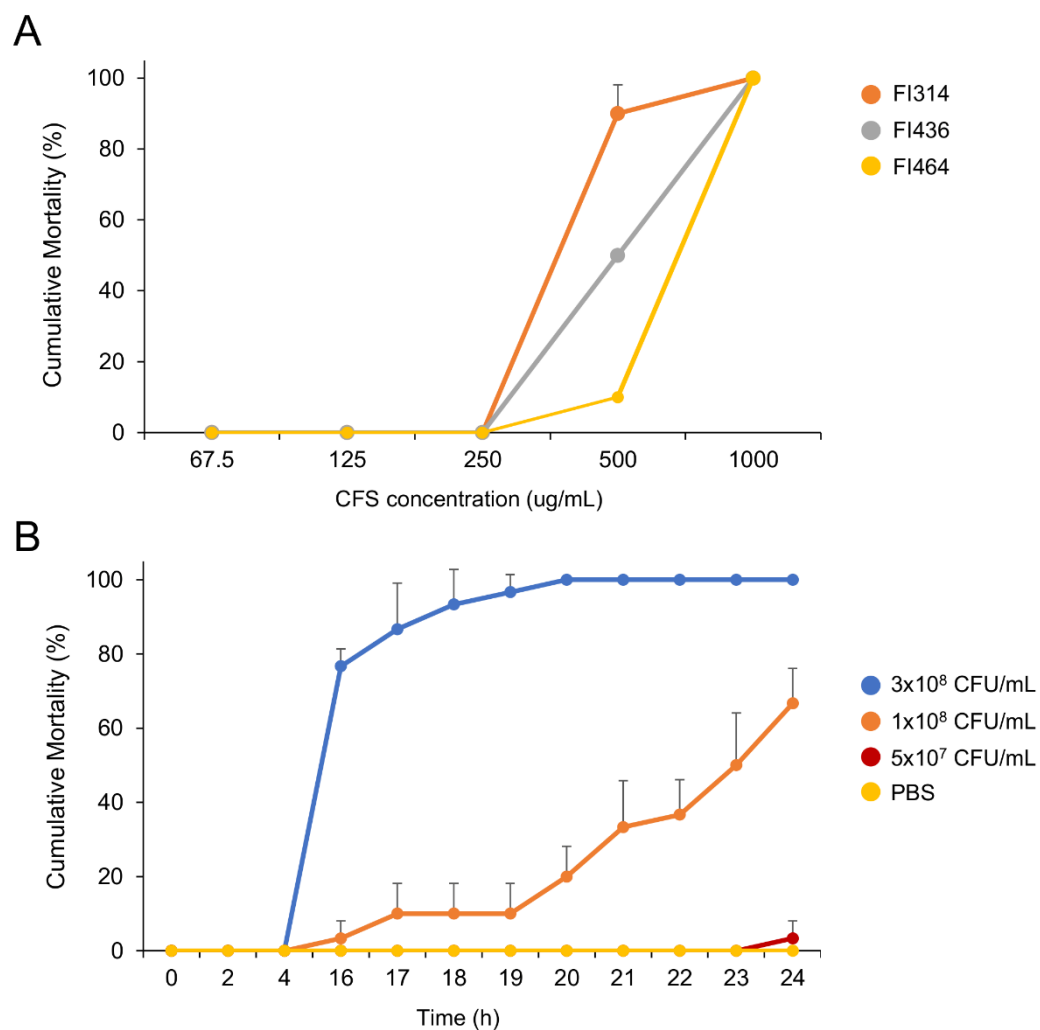


Figure S5. Toxicity of fish isolates (FI) extracellular compounds and establishment of *E. tarda* infection model in zebrafish larvae. **(A)** Toxicity of zebrafish larvae when exposed to several concentrations (67.5, 125, 250, 500 and 1000 $\mu\text{g mL}^{-1}$) of the FI cell free supernatant extracts. Mortalities were recorded each 24h for 3 days. Data is composed by three independent experiments using 10 larvae per concentration in each experiment. **(B)** Evolution of cumulative mortality (for 24h) of zebrafish larvae infected with *E. tarda* at three concentrations (5×10^7 , 1×10^8 and 3×10^8 CFUs mL^{-1}). Data is composed by three independent experiments using 10 larvae per treatment in each experiment.

Appendix II

In vitro modulation of gilthead seabream (*Sparus aurata* L.)
leukocytes by *Bacillus* spp. extracellular molecules upon bacterial
challenge

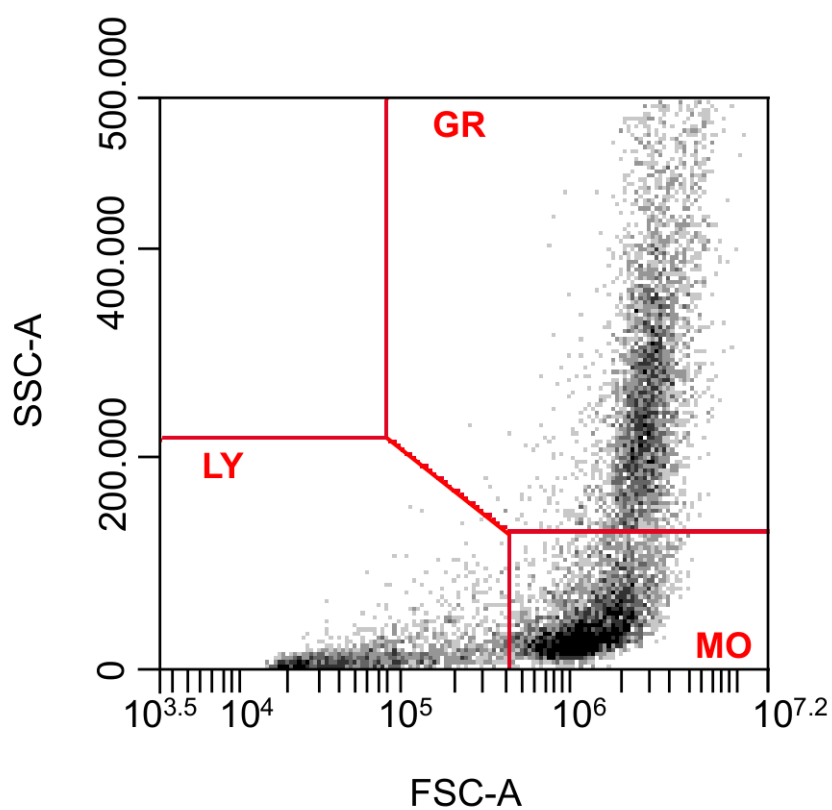


Figure S1. Flow cytometry gating strategy used to determine leukocytes subpopulation distribution in the whole leukocytes' suspension. Cell subpopulations were separated according to their forward and side scatter parameters (FSC-A and SSC-A dotplots). Three cell populations were established: (i) lymphocytes (LY) gated as the population of lymphocytes + thrombocytes, (ii) monocytes (MO), and (iii) granulocytes (GR.)

