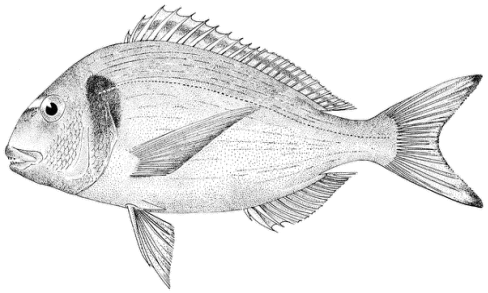

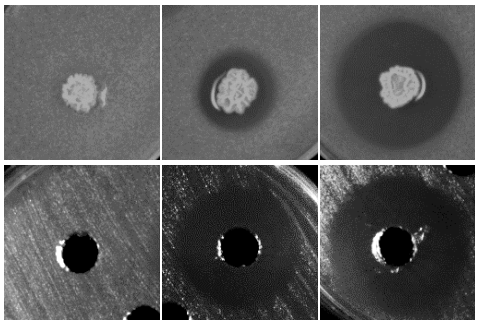

Fighting fish diseases with fish commensals: *Bacillus* and their Natural Antimicrobial Compounds (NACs)

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Mestrado em Recursos Biológicos Aquáticos

Departamento de Biologia

2016

Orientador

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

The work of this dissertation has been presented in one publication, two oral communications and two poster presentations as shown below

Publications

1. **Rafaela A. Santos**, André Couto, Aires Oliva-Teles, Maria J. Saavedra, Paula Enes and Cláudia Serra (2016). Fish Gut Sporeformers to Control Fish Pathogens. Front. Mar. Sci. Conference Abstract: IMMR | International Meeting on Marine Research 2016. doi: 10.3389/conf.FMARS.2016.04.00052

Communications

1. **Rafaela A. Santos**. 2016. *Bacillus* and their Natural Antimicrobial Compounds (NACs) as an approach to control fish diseases. Seminars in Biodiversity and Evolution (Vila do Conde, Portugal). **Oral presentation**

2. **Rafaela A. Santos**, Aires Oliva-Teles, Maria José Saavedra, Paula Enes and Cláudia R. Serra. 2016. Fish gut sporeformers to control fish pathogens: *Bacillus* and their Natural Antimicrobial Compounds. X Jornadas da Biologia 2016 (Vila Real, Portugal). **Oral presentation**

3. **Rafaela A. Santos**, André Couto, Ana Lavrador, Aires Oliva-Teles, Paula Enes and Cláudia R. Serra. 2016. Snack for fish: sporeformers and their natural antimicrobial compounds as a knowledgeable approach to control fish diseases. Aquaculture Europe 2016 Conference (Edinburgh, Scotland). **Poster presentation**

4. **Rafaela A. Santos**, André Couto, Aires Oliva-Teles, Maria José Saavedra, Paula Enes and Cláudia R. Serra. 2016. Fish gut sporeformers to control fish diseases. IMMR-International Meeting on Marine Research 2016 (Peniche, Portugal). **Poster presentation**

The publication of a manuscript in a peer-reviewed journal, gathering all the work and results presented in this dissertation, is currently under preparation.

Acknowledgements

First of all, I would like to express my enormous gratitude to Drª. Cláudia Serra for the great opportunity that gave me by accepting me as her MSc student and sharing with me her immense scientific knowledge. Her guidance, infinite patience and unconditional support along this year were crucial to the achievement of this dissertation. She always had inspired words to help me in every time even in the busiest moments.

I also would like to express my sincere gratitude to Prof. Dr. Aires Oliva-Teles for accepting me in his research group and for giving me an opportunity to start my journey in this field. He always helped me along the two years and shared with me his knowledge and experience.

A very special thanks to Draª. Paula Enes for her constant support whenever I need it and sharing with me her experience and knowledge that were indispensable for the accomplished of this dissertation.

Also, I would like to thank to Prof. Maria José Saavedra for her support along the year, and her help with the *Aeromonas* strains and with the antibiotic susceptibility tests.

My sincere gratitude to Prof. Fernando Tavares and all his team, Cristina Dias and Pedro Albuquerque for always being welcoming at Microbial Diversity and Evolution group of CIBIO.

Thanks to Drª Ana Couto and Filipe Coutinho for counselled and helped me every time I needed it. Also, I'm thankful to all my friends and colleges at NUTRIMU group.

Thanks to the students Ana Sofia Lavrador, André Couto and Bruno Sousa for their help in the laboratory.

A special thanks to Eduarda Almeida for her help in the sampling procedures.

Thanks to Mr. Pedro Correia for the technical assistance and all his sympathy and availability.

I am also grateful to João Pereira for his unconditional support, motivation in every moment along the performance of this dissertation.

At last, I could not achieve this dissertation without the support and dedication of my family. A special thanks to my parents, brother and all my family who always encouraged me through the years.

Abstract

Fish diseases caused by bacterial pathogens have been increasing around the world, limiting the development of aquaculture due to the economic losses associated. The conventional approach to avoid bacterial diseases is the massive use of antibiotics, exacerbating the potential for antimicrobial resistance development among fish pathogens. Nowadays, new disease-preventive measures are emerging, such as the modulation of the gut microbiota through dietary changes or by using probiotics. Knowing the benefits of the gut microbiota on host health (e.g. exclusion of potential pathogens), one aim of this work was to evaluate if dietary regimes are enough to modulate the gut microbiota composition of two species with distinct feeding habits. To do so, the omnivorous white sea bream and the carnivorous gilthead sea bream were fed with the same commercial diet for five weeks and the corresponding gut microbiota dynamics were evaluated by Denaturing Gradient Gel Electrophoresis (DGGE). An increase of the similarity between replicates in the two species was observed from the beginning to the end of the experiment, with gilthead sea bream showing significant results. This suggests that the carnivorous fish gut microbiota might be more susceptible to diet manipulation when using a carnivorous diet, than the omnivorous fish gut microbiota. Nevertheless, and according to previous studies, the microbiota of the omnivorous species (white sea bream) tends to be richer and more diverse, even when fed with a carnivorous diet, thus indicating that fish feeding habits and fish genetics may play a role with greater importance than feed itself, on defining fish gut microbiota.

An efficient way of modulating the gut microbiota is by using probiotics, in particular *Bacillus* species that are known to produce Natural Antimicrobial Compounds (NACs) able to antagonize different pathogens. Taking advantage of the *Bacillus* ubiquitous nature, we also aimed with this work to isolate, identify and characterize from the gut of aquaculture fish species, several *Bacillus* strains able to antagonize important fish pathogens. 176 isolates representing different colony morphologies and samples were obtained, and an identification based on the 16S rRNA gene sequencing revealed a clear abundance of *B. subtilis.* Screening the entire collection of sporeformers for NACs production allowed the selection of three most promising isolates that were capable to produce and release to the extra-cellular environment active NACs capable of suppressing pathogens bacterial growth and biofilm formation. These three fish-gut isolates, identified as *B. subtilis,* also shown to be sensitive to the antibiotic classes required by the European Union, and therefore are considered putatively safe to be used as future probiotics or as source of bioactive molecules in aquaculture.

Keywords: Aquaculture; Gut microbiota; Sporeformers; Natural Antimicrobial Compounds; Fish Pathogens;

Resumo

As doenças de peixes causadas por bactérias patogénicas têm aumentado em todas as zonas do globo, limitando o desenvolvimento da aquacultura devido às perdas económicas associadas. O método convencional para evitar a ocorrência de infeções bacterianas é o uso massivo de antibióticos, aumentando o potencial de desenvolvimento de resistência aos antimicrobianos por parte dos agentes patogénicos. Ultimamente, têm emergido novas medidas preventivas para limitar a ocorrência de doenças bacterianas, como por exemplo a modulação do microbiota intestinal através da manipulação da composição da dieta ou do uso de probióticos. Conhecendo as ações benéficas do microbiota intestinal na saúde do hospedeiro (como por exemplo através da exclusão de potenciais agentes patogénicos), um dos objetivos do presente trabalho foi avaliar se o regime alimentar, por si só, é suficiente para modular a composição do microbiota intestinal de duas espécies de peixes com hábitos alimentares distintos. Para tal, alimentaram-se durante 5 semanas, exemplares da espécie omnívora sargo e da espécie carnívora dourada, com a mesma dieta comercial, avaliando-se as respetivas dinâmicas do microbiota intestinal através da técnica de Electroforése em Gel de Gradiente Desnaturante (DGGE). Neste estudo, observou-se um aumento na similaridade entre as réplicas das amostras de ambas as espécies do inicio para o fim da experiencia, tendo este aumento sido significativo na espécie dourada. Este resultado sugere que o microbiota intestinal de peixes carnívoros pode ser mais suscetível a manipulações através da dieta, quando se utiliza uma dieta carnívora, do que o microbiota de espécies omnívoras. No entanto, e de acordo com estudos anteriores, o microbiota da espécie omnívora (sargo) tende a ser mais rico e diversificado, mesmo quando alimentada com uma dieta carnívora, indicando que os hábitos alimentares e a genética dos peixes podem ter uma maior influência do que a dieta em si, na definição do microbiota intestinal.

Uma maneira eficiente de modular o microbiota intestinal é através do uso de probióticos, com especial interesse nas espécies de *Bacillus* que são conhecidas por produzir Compostos Antimicrobianos Naturais (NACs) capazes de antagonizar diferentes agentes patogénicos. Tendo em consideração a natureza ubíqua dos *Bacillus*, o outro objetivo deste trabalho baseou-se em isolar, identificar e caracterizar espécies de *Bacillus* presentes no trato gastrointestinal e capazes de antagonizar

bactérias patogénicas conhecidas por afetar importantes espécies de peixes usadas em aquacultura. Obtiveram-se 176 isolados representativos de morfologias coloniais e origens distintas e, a identificação baseada na sequenciação do gene 16S rRNA, revelou uma nítida abundancia da espécie *B. subtilis.* A análise de toda a coleção de isolados esporulantes, permitiu a seleção dos três isolados mais promissores, que demonstraram ser capazes de produzir e libertar para o meio extracelular, importantes compostos antagonistas do crescimento e da formação de biofilmes dos agentes patogénicos testados. Os três esporulantes, identificados como *B. subtilis,* demonstraram também um perfil de suscetibilidade às classes de antibióticos requeridos pela União Europeia, sendo, portanto, considerados como potencialmente seguros para incorporar futuros probióticos ou como fonte de moléculas bioativas para a aquacultura.

Palavras-chave: Aquacultura; Bactérias esporulantes; Compostos Naturais Antimicrobianos; Doenças de peixes; Microbiota intestinal.

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Abbreviations List

ANOVA: Analysis of variance

BHI: Brain Heart Infusion medium

BLAST: Basic Local Alignment Search Tool

B & W: Bott & Wilson salts

CFU: Colony-forming unit

CLSI: Clinical & Laboratory Standards Institute

CO2: Carbon dioxide

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleoside triphosphate

DSM: Difco Sporulation Medium

DGGE: Denaturating Gradient Gel Electrophoresis

EEC committee: European Economic Community committee

EFSA: European Food Safety Authority

EtOH: Ethanol

EU: European Union

ESB: European sea bass

GSB: Gilthead sea bream

GUT: Gastrointestinal tract

I: Intermediate

IPMA: Instituto Português do Mar e da Atmosfera

LB: Luria-Bertani medium

MH: Muller-Hinton medium

MT: Million Tonnes

NACs: Natural Antimicrobial Compounds

NCBMI: National Collection of Industrial Food and Marine Bacteria

OD: Optical Density

OTUs: Operational taxonomic units

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

R: Resistant

RNA: Ribonucleic acid

S: Sensitive

STE: Sodium Chloride-Tris-EDTA

TE: Tris-EDTA buffer

TI: Initial time

TF: Final Time

WSB: White sea bream

1. Introduction

1.1 Past, Present and Future of Aquaculture

The continuous increase of human population (1.6% / year) together with the intrinsic growing need for food, have been putting a pressure in natural resources including fish, causing doubts about how to feed the planet while still maintaining natural fish stocks for generations to come (FAO 2016d). The increase of fish consumption per capita (from 9.9 kg in 1960 to 20 kg in 2014), led to an expansion in the production of aquaculture products, since capture alone is and will not be able to meet the expected demand of seafood in the future. In fact, the percentage of stocks fished at an unsustainable level has been increasing since the 1970's, and in 2013 31.4% of fish stocks were believed to be overexploited. Although capture of fishery products has reached a high level of 93.4 million tonnes (MT) (Figure 1), marine captures have slightly decreased from 2011 (82.6 MT) to 2014 (81.5MT) (FAO 2016d).

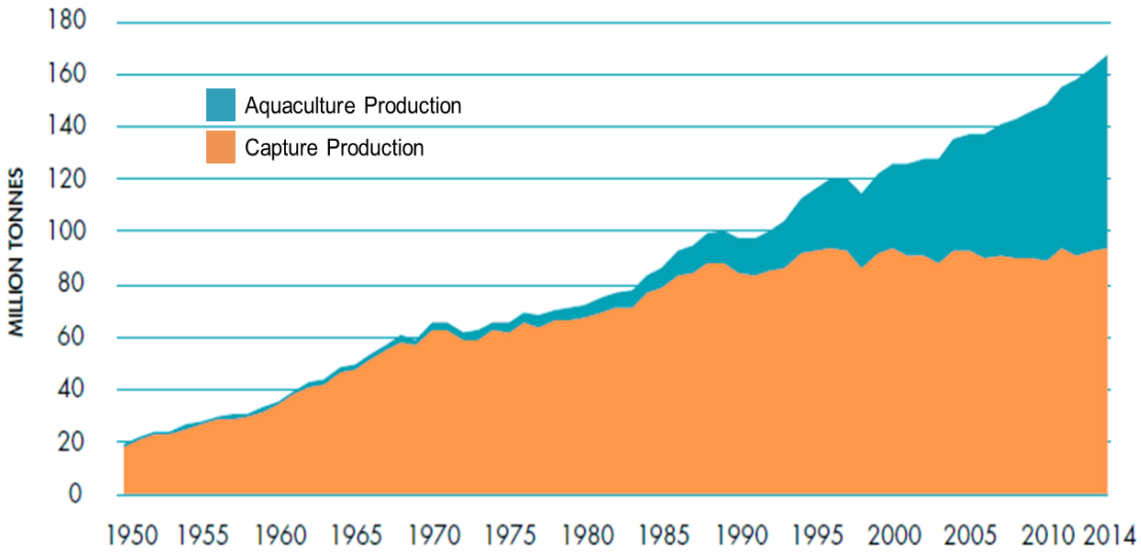

Figure 1. World capture fisheries and aquaculture production. Source: FAO (2016)

Due the relative stagnation of fisheries (Figure 1), aquaculture has been playing an important role in providing aquatic organisms in the required amounts, thus decreasing the pressure on fish stocks. Aquaculture is the cultivation of aquatic organisms in inland and coastal areas, including the intervention on their rearing process, such as regular stocking, feeding or protections from predators, with the objective to improve production. This process also implies individual or corporal ownership of the cultivated stock (Martínez Cruz *et al.* 2012, FAO 2016d). The production includes an

immense variety of culture systems, techniques, where currently about 600 different aquatic species are cultivated all around the globe (FAO 2016d).

Aquaculture production was firstly documented in 2000 B.C. in China with the production of freshwater common carp (Martínez Cruz *et al.* 2012, FAO 2016d), and with scientific and technological advances became more efficient, increasing the intensification of production and arising worldwide as a commercial activity.

Currently, aquaculture is considered the fastest growing of all animal production sectors (5.8% / year) (Subasinghe 2001, Campbell and Pauly 2013, FAO 2016d), however it is currently growing at a slower rate than the one verified in the 80's and 90's. The contribution of aquaculture in total worldwide fish production has been continuously growing in the past few years. 1990 saw aquaculture take 13,4 percent of the world's fish production; 2000 saw this value increasing to 25.7 and 2014 saw a record of 44,1 percent of the total 167.2 MT of fish produced (captures and aquaculture). However, it's important to highlight that these values are result of irregular contributions, since Asia is the only continent that has been producing more farmed fish than wild catch, and in 2014 accounted, alone, for 88.9% of the worldwide production. In the same year, the contribution of the European Union in the world fish supply represented a small percentage, corresponding to 3.97% (FAO 2016d).

In European countries, fish and other aquatic animals are an important food resource, with 25.53 kg per capita consumption in 2014. However, this consumption is mostly supplied by imported seafood and not by EU production. In 2014, EU was the third producer worldwide (aquaculture and fisheries) with a total volume of 6.15 MT, in which the aquaculture contribution was higher than 20% (1.282 MT). Between 2013 and 2014 the production increased 8%, mostly due the higher production rates of bivalves, other molluscs and other aquatic invertebrates, and also to salmonids. Spain and United Kingdom were the Member States with higher volumes of farmed products followed by France, Italy and Greece (European Commission 2014, EUMOFA 2016, FAO 2016d).

Portugal has been the European country with the highest rate of fish consumption (55.3 kg per capita) (EUMOFA 2016), however the aquaculture remains remarkably underdeveloped. Besides the high population density in coastal areas, a large portion of that land is now under protection, limiting the establishment of aquaculture facilities. Also, the complicated bureaucratic system precluded the development of this sector (Afonso 2008). In this country, the production in 2014 has surpassed 10 thousand tonnes corresponding to an increase of 7.3% in relation to the previous year. However the economic value obtained represented a decrease of 8.3% in the same period, due to the inferior commercial price (INE 2016).

With the expected 2 billion demographic growth over the next 30 years, and assuming continuous technological improvements in fish production, world aquaculture is expected to expand and remain as one of the fastest-growing sectors, reaching a new record in 2025 and surpassing the capture fisheries.

1.2 Aquaculture species

In UE countries, the major farmed products produced in 2014 were salmon, Trout, oysters, gilthead sea bream and mussels. In Portugal, fish production is mostly focused on marine species and specifically in gilthead sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*) and European sea bass (*Dicentrarchus labrax*). (European Commission 2014, INE 2016). White sea bream (*Diplodus sargus*) has also been documented as a good candidate for aquaculture regardless its slower growth rate.

1.2.1 White sea bream (*Diplodus sargus*, Linnaeus, 1758)

White sea bream belongs to Actinopterygii class, Perciformes order and Sparidae family (Pollard 2014). This species has an oval, compressed and grey body with dark vertical bands that disappear with age and the characteristic dark saddle on the caudal peduncle (Abellán and Basurco 1999, FAO 2016b, Fishbase 2016b). The mouth is in a terminal position and slightly protusible with thick lips. Relatively to fins, the caudal is forked and the dorsal one has 11 or 12 spines and 12 to 16 soft rays while the anal one only has 3 spines and 12 to 14 soft rays (FAO 2016b, Fishbase 2016b).

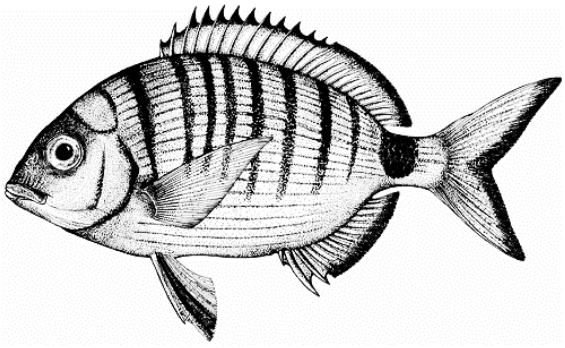

Figure 2. White sea bream (*Diplodus sargus*, L. 1758). Source: FAO (2016)

White sea bream express a demersal behaviour being very common in the Mediterranean Sea and widespread from the Bay of Biscay to the west coast of Africa and the Persian Gulf. It inhabits shallow coastal waters and the juveniles prefer sandy bottoms (enter in the lagoons on the spring and return to the ocean in autumn), while adults are present in rocky areas covered by seaweed usually until 50m depth, but can

reach 150m. The species is omnivore, feeding specially on algae, worms, molluscs, crustaceans, echinoderms and hydrozoans (FAO 2016b, Fishbase 2016b, Pollard 2014).

White sea bream is considered as a potential species for Mediterranean aquaculture diversification due to its high market value and flesh quality (Ozorio *et al.* 2006, Sa *et al.* 2007, Sa *et al.* 2008a, Sa *et al.* 2008b). The production technologies are similar to other *Sparidae* such as gilthead sea bream, however the tonnes produced around the world had reached a maximum level in 2010 with 174 tonnes, and declined significantly until 2014, with just 13 tonnes produced (FAO 2016b).

In larval and juvenile stages the growth is identical, or even better than gilthead sea bream, but in later stages the growth rate decreases which difficult the production of white sea bream. Genetic selection and the formulation of adequate diets have been proposed has the major improvements needed in the culture of this species (Sa *et al.* 2007, Sa *et al.* 2008a, Sa *et al.* 2008b).

1.2.2 Gilthead sea bream (*Sparus aurata*, Linnaeus, 1758)

Gilthead sea bream belongs to the same family classification as white sea bream, (Fishbase 2016c). This species possesses a grey, oval and tall body with the characteristic black spot on the gill cover. The head is curved and the mouth is in a low position. The dorsal fin of these fishes has 11 spines and 13/14 soft rays, while the anal fin just has spines and 11/12 soft rays (FAO 2016c, Fishbase 2016c).

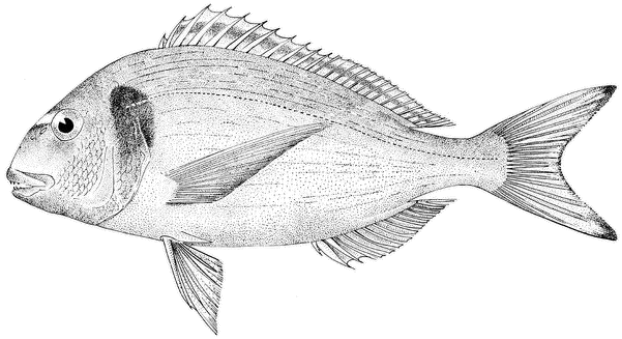

Figure 3. Gilthead sea bream (*Sparus aurata*, L. 1758). Source: FAO (2016)

Gilthead sea bream is an euryhaline species, been commonly found in the Mediterranean Sea (FAO 2016c). The geographic distribution also extends along the Eastern Atlantic coast from Great Britain to Senegal, and in the Black Sea, although rarely found. Gilthead sea bream inhabits coastal marine and estuarine zones with seagrass or sandy grounds. It is a sedentary fish being normally isolated or in small aggregations and commonly swim at 30m in deep, although adults can reach 150m (Russell 2014). This species is principally carnivorous, feeding on fish, mussels and

crustaceans. Additionally it can also be an accessory herbivorous (Russell 2014, Fishbase 2016c).

The production of gilthead sea bream has begun in an extended form in coastal lagoons until the development of the intensive systems in the 80's. This species is the most important aquaculture production in the Mediterranean Sea due to its high survival rate, feeding habits and commercial price, although this one is decreasing over the years. The production of gilthead sea bream has raised along the years and in 2014 has surpassed 158 tonnes in the Mediterranean Sea, with the main four producers being Greece (49%), Turkey (15%), Spain (14%) and Italy (6%) (FAO 2016c).

1.2.3 European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758)

European sea bass belongs to Actinopterygii class, Teleostea superorder, Perciformes order and Moronidae family. It is characterized by an elongated body with two separate dorsal fins (one with 8-10 spines and the other with 12-13 soft rays), and a lateral line with 62-74 scales until the anal fin (3 spines and 10-12 soft rays). It presents a terminal and moderate protactile mouth. Juveniles have black spots along the body while adults present a silver grey body colour (Fishbase 2016a).

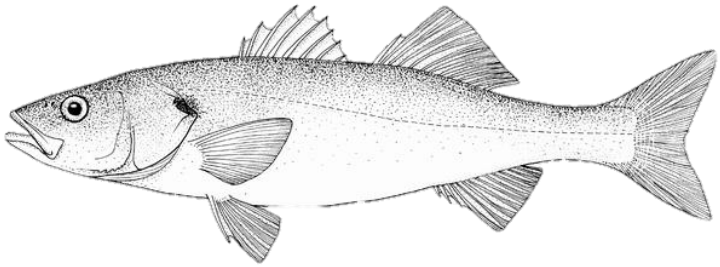

Figure 4. European sea bass (*Dicentrarchus labrax*, L. 1758). Source: FAO (2016)

European sea bass is a eurythermic (8-25 ^{o}C) and euryhaline marine species. The species has a demersal behaviour inhabiting coastal waters, estuaries with different types of bottoms lagoons and rivers. In the summer months it enters on river mouths, and when the water temperature drops it migrates to offshore and deep waters. The geographic distribution extents from Eastern Atlantic to Morocco, Canary Islands and Senegal, to Black and Mediterranean Sea (FAO 2016a).

European sea bass is a carnivorous species, with juveniles feeding on invertebrates and adults on shrimp, molluscs, copepods, crabs and fish. The predation on fish species increases with age (Fishbase 2016a).

European sea bass is one of the most important species cultured along the Mediterranean Sea and was one of the first non-salmonids species being commercialized and produced in Europe. The production is mostly made in sea cages, but it can also be cultivated in lagoons and pounds. The production has increased along the years and in 2014 reached a total of 156.449 tonnes. The main producers are Greece, Turkey, Italy and Spain (FAO 2016a).

1.3 Aquaculture health constraints: bacterial diseases

The continuous intensification of aquaculture, requesting high densities of cultivation and consequently high-stress levels, has been responsible for damages in the surrounding environment in result of organic waste dumping and toxic compounds (like methane, ammonia and nitrites), but also for the emergence of several bacterial outbreaks (Munn 2005, Martínez Cruz *et al.* 2012). Fish bacterial diseases have assumed a huge significance following the widespread expansion of aquaculture, and are considered one of the biggest constraints to the sector development in result of the economic negative impacts (Larsen *et al.* 2014, Hai 2015, Verschuere, 2000).

A wide range of bacterial pathogens has been described in marine fish and some have affected and limited the development of marine culture (Subasinghe 2001, Munn 2005, Sihag and Sharma 2012). There are also increasing evidences that some of these pathogens might be responsible for emerging zoonosis affecting humans and the public health (Gauthier 2015). Some of the bacterial diseases known to cause huge losses in marine aquaculture are Furunculosis, Vibriosis and Pasteurellosis (Toranzo 2004), whose main affected species and infection consequences are summarized in Table 1.

1.3.1 Furunculosis

The aetiological agent of furunculosis is *Aeromonas salmonicida,* a non-motile, Gram-negative bacteria characterized as short bacillus or coccobacillus. This disease causes huge losses in salmonids, but can also be found in a variety of non-salmonids fish such as gilthead sea bream, European sea bass and turbot (Toranzo 2004, Austin 2005). Furunculosis outbreaks typically occur at temperatures above 10°C, and are reported in many parts of the world, occurring in wild and cultured species causing furuncles in chronical infections. The acute form is mostly common in juveniles and adults and it is responsible for huge mortalities in short time with no evident clinical sights (Austin 2005, Janda and Abbott 2010, Roberts 2012). The routes of infection are the contact with infected fish, contaminated water or poor husbandry conditions (Austin and

Austin 2012). Several virulence factors are known in this species like the production of extracellular molecules (like proteases), and the presence of three secretion systems (Dallaire-Dufresne *et al.* 2014). Additionally, the establishment of biofilms and the presence of quorum-sensing molecules that can induce higher virulence, have also been detected (Janda and Abbott 2010). Along the years, *A. salmonicida* strains have been usually associated with antibiotic resistance genes to some of the common antibiotics used worldwide such as tetracycline, chloramphenicol, streptomycin and others, which difficult the treatment of this disease (Dallaire-Dufresne *et al.* 2014). Therefore, many vaccines have been developed since 1980, to be used by injection, immersion or orally, however their efficacy has been questioned because of the lack of results and the short period of protection against the disease (Dallaire-Dufresne *et al.* 2014). New vaccines are being investigated to improve the efficacy of the furunculosis immunization (Toranzo 2004, Austin and Austin 2012).

Besides *A. salmonicida,* another species with severe impact on fish production is *Aeromonas veronii* (a rod-shaped motile Gram-negative bacterium). *A. veronii* was first reported in Catfish, where fish presented a haemorrhagic septicaemia and severe dermal ulcers on the body, head and dorsal regions (Rahman *et al.* 2002, Cai *et al.* 2012). With these lesions, fish died within a week, leading to an economic and public health problem (Rahman *et al.* 2002, Austin and Austin 2012). More recent, this bacteria was also isolated from gilthead sea bream, revealing possible resistance genes against some antimicrobials (Gashgari and Selim 2015). *Aeromonas veronii* can be isolated from aquatic systems such as freshwater, marine animals, soil and non-faecal material, being frequently reported in humans and other vertebrates (Roberts *et al.* 2006).

Recently, a new *Aeromonas* species, was isolated from cockles (*Cardium* spp.) and razor shells (*Ensis* spp.) in Spain by Minana-Galbis *et al.* (2007). It is characterized as a motile, Gram-negative with coccoid or rod shape. The authors studied the phylogenetic relationships with other *Aeromonas* species and found a 99.7% of similarity with *A. popoffii*, known to cause urinary tract infections in humans (Hua *et al.* 2004) Although no pathologies in fish have been identified so far due to *A. bivalvium,* its pathogenicity to bivalve mollusc and its potential to cause human diseases makes this an important species to be studied (Minana-Galbis *et al.* 2007, Buller 2014).

Table 1. Bacterial fish pathogens described in this work

Fish pathogen	Disease	Affected species	Reference
Aeromonas salmonicida	Furunculosis	Gilthead sea bream; European sea bass; Turbot	Toranzo *et al.* (2005), Austin and Austin (2012), Roberts (2012), Dallaire-Dufresne *et al.* (2014)
Aeromonas veronii	Haemorrhagic septicaemia; Dermal ulcers	Catfish; Gilthead sea bream; Humans	Rahman *et al.* (2002), Roberts *et al.* (2006), Cai *et al.* (2012), Gashgari and Selim (2015)
Aeromonas bivalvium	Unknown	Cockles; Razor shells	Minana-Galbis *et al.* (2007)
Vibrio anguillarum	Vibrio septicaemia	Gilthead sea bream; European sea bass; White seabream	Toranzo (2004), Toranzo *et al.* (2005), Golomazou *et al.* (2006), Frans *et al.* (2011)
Vibrio harveyi	Vasculitis; Eye disease; Gastroenteritis	Penaeid shrimp; Sole, Atlantic salmon; Humans	Zhang and Austin (2000), Pujalte *et al.* (2003), Austin and Zhang (2006), Haldar *et al.* (2010), Akram *et al.* (2015)
Photobacterium damselae	Pasteurellosis; Multifocal necrosis	Gilthead sea bream; European sea bass; White sea bream; Humans	Romalde (2002), Andreoni and Magnani (2014), Akram *et al.* (2015)
Staphylococcus aureus	Degeneration of eye tissues; Melanosis; Brain diseases	Carp; Humans	del Mar Lleo *et al.* (2005), Austin and Austin (2012)

1.3.2 Vibriosis

Vibriosis is one of the oldest diseases discovered in fish that constrain the production of marine species (Austin 2005). It is caused by halophilic, motile, Gram-negative curved-rod shape (comma shape) bacterium called *Vibrio anguillarum* which cause deadly widespread haemorrhages and ulcers in chronical fish. In acute epizooties, fish die without any visible signs (Frans *et al.* 2011, Austin and Austin 2012, Roberts 2012). The disease is described in numerous species of fish, such as European sea bass, gilthead sea bream, white sea bream, sole, rainbow trout and salmon (Toranzo 2004, Toranzo *et al.* 2005, Golomazou *et al.* 2006). *V. anguillarum* is found in marine, estuarine and freshwater habitats and possesses a wide distribution around the world, affecting more than 14 countries (Toranzo 2004, Frans *et al.* 2011, Austin and Austin 2012, Roberts 2012). The epizooties of this disease occur mostly in the warmer months (water temperature exceeding 15°C) with the association of stress and depletion of oxygen. The organism comprises part of the normal alimentary microbiota of the aquatic environment, being available for fish through the ingestion of rotifers and others invertebrates, occurring more frequently in summer (Austin 2005, Austin and Austin 2012, Roberts 2012). This route of infection works through the ingestion of contaminated live food, where the bacteria can survive the gastric pH in the stomach, colonizing the GUT and proliferating. Consequently, enters into the blood, resulting in the septicaemia that affects the other internal organs (Frans *et al.* 2011). The other mode of infection involves the colonization of the skin and consequent penetration in the tissues and organs, leading to the death of the fish (Austin and Austin 2012). The bacterial survival and proliferation inside the host is mostly accomplished by their capacity to form biofilms and their reported quorum-sensing system that can control the virulence gene expression. There are 23 serotypes of this bacterium, but only 3 (O_1, O_2, O_3) can cause damage to fish. There are many commercial vaccines against this disease, mostly used by bath or injection, but their efficacy is greatly dependent on the knowledge of the serotype causing the disease (Austin and Austin 2012).

Another *Vibrio* bacteria with high impact in aquaculture is *V. harveyi,* a motile, Gram-negative bacterium with rod shape which cause vasculitis, eye disease and gastroenteritis (Austin 2005, Austin and Zhang 2006). The development of aquaculture has recognized this bacteria has an important disease affecting mostly finfish and penaeid shrimp, but also marine vertebrates such as sole, rainbow trout and Atlantic salmon (Zhang and Austin 2000, Austin and Zhang 2006), gilthead sea bream (Haldar *et al.* 2010), and also European sea bass (Pujalte *et al.* 2003). The pathogenic mechanism is not completely elucidated, however outbreaks occur in summer when the

water temperature exceeds 25°C. Austin and Zhang (2006) reported that the biofilm structure is responsible for the persistence and the survival of this bacteria in shrimps. *V. harveyi* is known as a fish pathogen, however it was reported in humans after exposure to contaminated water, raising concerns about public health (Akram *et al.* 2015). The development of vaccines for this bacteria has not been successful so far, although some recent studies have become effective in a demonstration of some protection in turbot (Zhang and Austin 2000, Austin and Austin 2012).

1.3.3 Photobacteriosis

The motile, Gram-negative rod-shaped *Photobacterium damselae* is the agent that causes pasteurellosis or pseudo-tuberculosis, a disease that occurs in chronically infected fish (Roberts 2012). This disease affects populations in wild and cultured conditions of species like gilthead sea bream, European sea bass and sole, causing significant economic losses in the sector of aquaculture due to white granulomas in haematopoietic tissues in advanced stages, and multifocal necrosis in acute forms (Romalde 2002, Toranzo 2004, Håstein *et al.* 2005, Austin and Austin 2012, Andreoni and Magnani 2014). This bacteria also affects humans, as reported in United States, Australia and Japan, after the exposure of wounds to infected water (Akram *et al.* 2015). The geographical distribution of *P. damselae* comprises Europe, Japan and USA, causing epizooties in summer with 40-50% of mortalities and being transmitted from fish to fish. Gills are considered as the development key of the disease (Austin 2005, Austin and Austin 2012, Roberts 2012). These bacteria do not seem to have host specificity, however show differences in the susceptibility based on the fish age. Larvae and juveniles of sea bream are very affected by this pathogen with mortalities up to 90-100%, while fish above 50g become more resistant due to the efficiency of neutrophils and macrophages in killing the bacteria. The pathogenicity of pasteurellosis is not totally known, however the virulence factors are identified has been the polysaccharide capsular material and the high affinity siderophore mediated the iron-request system (Romalde 2002, Austin and Austin 2012, Andreoni and Magnani 2014). Antibiotics were effective in controlling *P. damselae* outbreaks, however after a few years the bacteria had developed a resistance to the major chemicals. In addition, it is known that the intracellular parasitism period in the macrophages explains the ineffectiveness of chemotherapy. So, in the past few years, many researches have been conducted to prevent the outbreaks by vaccination. Vaccines have been formulated along the years being under experimental stages or already commercialized against

P. damselae subsp*. piscicida,* however the efficacy depends on the species, fish size and vaccine formulation (Romalde 2002, Toranzo 2004, Andreoni and Magnani 2014).

1.3.4 Staphylococcal infection

The Gram-positive cocci *Staphylococcus aureus,* is commonly present in human's skin and mucous being an important reservoir for infections such as pneumonia, bacteraemia and skin infections in immunocompromised patients. But it can also be isolated from the eye and brain tissue of fish and from water samples. For example in India it is responsible for mortalities in carp causing degeneration of eye tissues, melanosis and affecting the brain and the optic nerves (Austin and Austin 2012). However, it is not certain if water is a vector for the transmission of the pathogen to humans or if the water creates opportunities for the bacteria present in human skin to self-infect (del Mar Lleo *et al.* 2005). Due the huge effect of *Staphylococcus aureus* on the public health the virulence factors are already well establish, as well as the biofilm formation capacity in order to protect bacterial cells and improve their virulence (Archer *et al.* 2011).

1.4 Health-promoting strategies

1.4.1 Balanced gut microbiota

Animal health in general, and fish health in particular, is dependent on the surrounding environment, due to the constant contact with a wide range of pathogenic and opportunistic microorganisms that are capable of infecting when conditions become favourable (Gomez and Balcazar 2008). It is proved that gut microbiota interactions are essential to exclude potential pathogens and maintaining the host health status, to supply essential nutrients to the host and contribute to the development of the intestinal architecture (Round and Mazmanian 2009, Perez *et al.* 2010, Larsen *et al.* 2014). Gut microbiota is the entire microbial community that inhabits the intestinal tract while gut microbiome is the genomic content that in humans is estimated to be 100 times more numerous than the human genome itself (Round and Mazmanian 2009). Studies regarding the function and structure of intestinal bacteria have become more prevalent in humans and mammals (Sullam *et al.* 2012, Wong *et al.* 2013) showing that the composition of commensal microbiota is dependent on genetic, nutritional and environmental factors (Gomez and Balcazar 2008, Perez *et al.* 2010, Maslowski and Mackay 2011). However, studies regarding the fish endogenous microbiota have been

mostly focused on the role and influence of probiotics on the immune system. More recent research, emphasizes the influence of seasoning, development stage (Sullam *et al.* 2012), diet composition, ingredient origin (Larsen *et al.* 2014), rearing system and density, (Roeselers *et al.* 2011, Wong *et al.* 2013), and also, fish origin (salt or fresh water) in fish gut microbiota (Perez *et al.* 2010).

Up to this point, the major knowledge provided about the intestinal fish microbiota is based on culture-dependent approaches (Clements *et al.* 2014), which usually only allow a limited information on the bacterial diversity and composition, since many bacteria are uncultivable (Navarrete *et al.* 2009). The recent introduction of molecular techniques, such as PCR-DGGE (Polymerase Chain Reaction - Denaturating Gradient Gel Electrophoresis), for the detection and quantification of microorganisms allowed a greater understanding of the gut composition and diversity (Gomez and Balcazar 2008, Clements *et al.* 2014). PCR-DGGE is based on an amplification of PCR products with the same size and different sequences, from a hypervariable region (V3) of the 16S rRNA gene. The products are separated by electrophoresis on a denaturation gradient gel with increasing concentration of the denaturating agent that induces double strand-DNA separation, and thus migration through the electrophoresis gel, dependent on base composition (Ercolini 2004). DNA bands can be extracted and sequenced, allowing species identification.

1.4.2 Probiotics

The conventional approach to avoid the occurrence of bacterial outbreaks was, until a few years ago, the massive use of in-feed antibiotics as preventive and therapeutic measure. This practice exerted a selective pressure on the commensal and environmental bacteria, and exacerbated the potential for antimicrobial resistance development among pathogenic bacteria, while also deteriorating the environment (Balcázar *et al.* 2006, Hai 2015). In fact, antibiotic resistance genes among pathogenic aquatic bacteria (Rhodes *et al.* 2000), and their potential transference to human pathogens, have been reported (Aitken *et al.* 2016).

Nowadays, several alternative methods have been developed and established due to the concern of aquaculture sustainability. One of the most effective preventive measures suggested to control bacterial diseases in the sector is the use of probiotics (Verschuere *et al.* 2000, Kesarcodi-Watson *et al.* 2008, Newaj-Fyzul *et al.* 2014, Hai 2015). Probiotics are “live organisms which when administrated in adequate amounts confer a health benefit on the host”. They are believed to (1) enhance the immune response of the host, (2) compete for adhesion sites and nutrients/energy, (3) produce

natural antimicrobial compounds (NACs), (4) promote growth and survival rates, (5) improve water quality and (6) contribute to enzymatic digestion (Gatesoupe 1999, Verschuere *et al.* 2000, Balcázar *et al.* 2006, Kesarcodi-Watson *et al.* 2008, Hai 2015). With all these modes of action, probiotics, when administrated in proper ways, reset the beneficial microbiota enhancing the immune system and therefore leading to a reduction of antibiotics and their accumulation on water (Verschuere *et al.* 2000, Gomez and Balcazar 2008).

The ability of probiotics to adhere and colonize the mucosal epithelium of the gastrointestinal tract is essential for establishing these bacteria as competitive indigenous microbiota, and therefore reduce the incidence of opportunistic bacteria. However, the colonization of the gut surface not necessarily implies the competition for adhesion sites has the only protective action (Verschuere *et al.* 2000, Balcázar *et al.* 2006, Hai 2015), and the production of antimicrobial compounds by some probiotics is also a valuable characteristic to control the proliferation of pathogens, enhancing the host resistance to infections (viruses, bacteria, fungi and parasites) (Hai 2015).

The selection of probiotics is a rigorous and detailed process that evaluates some important characteristics, such as the absence of virulent resistance genes, their acceptance and persistence in the host (Verschuere *et al.* 2000, Balcázar *et al.* 2006, Gomez and Balcazar 2008, Kesarcodi-Watson *et al.* 2008, Hai 2015). Probiotics administration is diversified, being possible to, for example, add to live food (artemia, rotifers and copepods), include in diets, bath in bacterial suspensions and add directly into the water (Balcázar *et al.* 2006, Hai 2015).

The most common bacterial species used as probiotics in the biological control are the lactic acid bacteria (e.g. *Lactobacillus* sp.) and members of the genus *Vibrio*, *Pseudomonas* or *Bacillus* (Verschuere *et al.* 2000, Hong *et al.* 2005). Although *Bacillus* species have been commonly used as probiotics in humans and animal practices for more than 50 years, the scientific interest in the immunostimulatory properties of these species has only occurred in the past 15 years. *Bacillus* species, are Gram-positive, aerobic and endosporeforming bacteria, common in soil, water, dust and air, especially because of their ability to disperse (Hong *et al.* 2005, Cutting *et al.* 2009, Cutting 2011).

The *Bacillus* spore is an extreme resistant form that can survive to extreme physical and chemical insults, and therefore is produced when the environmental conditions become too hostile for the vegetative cell survival, such as decline in nutrients or water (Duc *et al.* 2003, Barbosa *et al.* 2005, Cutting *et al.* 2009, Cutting 2011). This dehydrated form can persist indefinitely in this state, but when exposed to water, nutrients and favourable environmental conditions will germinate leading to a new vegetative cell (Casula and Cutting 2002, Duc *et al.* 2003, Cutting *et al.* 2009).

Bacillus spores are being used as in-feed additives (Hong *et al.* 2005, Nayak 2010, Newaj-Fyzul *et al.* 2014) because of their advantages over other non-sporeforming bacteria, such as their remarkably robust structure allowing an unlimited shelf-storage; their easy production in large scale and possibility to be desiccated, facilitating their incorporation in feed/food during processing without losing characteristics, while possessing a low cost production (Barbosa *et al.* 2005, Hong *et al.* 2005, Cutting *et al.* 2009, Cutting 2011).

Another important characteristic of *Bacillus* spores is their ability to survive the gastric barrier with low pH. Some *Bacillus* spores, administrated orally by association with food and water (Nayak 2010), are able to survive the transit across the gastric barrier and germinate in the small intestine since this part of the intestine possess a microenvironment enough for the bacterial growth and proliferation, being usually found and isolated from the gut of animals, insects, humans (Hong *et al.* 2005, Tam *et al.* 2006, Cutting *et al.* 2009). Thus, spores that survive across the gut and proliferate in small intestine perform their probiotic actions by preventing the colonization of the pathogenic bacteria (Casula and Cutting 2002, Tam *et al.* 2006).

In the aquaculture sector, *Bacillus* species have been reported for their ability to enhance the immune system and growth of sea bream, white shrimp, and other fish species (Salinas *et al.* 2008, Avella *et al.* 2010, Sun *et al.* 2010, Das *et al.* 2013, Ramesh *et al.* 2015), improve water quality by remediation (Xie *et al.* 2013, Hai 2015), decrease the pathogenic strains inside the intestine of white shrimp by competitive exclusion (Li *et al.* 2007), increase the survival rates and disease resistance of black tiger shrimp, turbot, sea bass larvae and others (Rengpipat *et al.* 2003, Vaseeharan and Ramasamy 2003, Ziaei-Nejad *et al.* 2006, Balcazar and Rojas-Luna 2007, Touraki *et al.* 2012, Das *et al.* 2013, Chen *et al.* 2016). *Bacillus* have also been reported as producers of important Natural Antimicrobial Compounds (next section).

1.4.3 Natural Antimicrobial Compounds

Bacillus species produce Natural Antimicrobial Compounds or NACs, which can be metabolites, peptides or proteins. For example, Bacteriocin-like substances are small ribosomal antimicrobial peptides produced by the major lineages of bacteria including *Bacillus* (Abriouel *et al.* 2011, Dobson *et al.* 2012, Allen *et al.* 2014, Egan *et al.* 2016) and own a huge and broader antimicrobial activity, against important human and animal infectious pathogens (Abriouel *et al.* 2011, Sahoo *et al.* 2016).

The continuous incidence of bacterial infections in farmed fish led to a research effort in the past few years towards NACs based therapies to overcome the undesirable

effects of antibiotics (Sahoo *et al.* 2016). NACs-producing *Bacillus*, active against fish pathogens, have been found in numerous habitats (Table 2) and, since the gut microbiota has been reported as an unlimited source of pharmacological molecules, recent studies have been performed to find new NACs producers in the fish gut (Sahoo *et al.* 2016). When NACs are produced in the gut of animals, they might help their producers to constitute a barrier against the proliferation of a broad range of opportunistic microorganisms (Verschuere *et al.* 2000, Austin 2005, Hong *et al.* 2005, Kesarcodi-Watson *et al.* 2008, Abriouel *et al.* 2011, Dobson *et al.* 2012, Egan *et al.* 2016), by directly kill the pathogenic strain or by colonizing certain niche facilitating the dominance of the producer strains or also by function as signalling peptides (Dobson *et al.* 2012). However, NACs capacity to treat and control pathogens biofilm formation is less-studied. Biofilms are structured associations of microorganisms, generally involving a strong colonization of liquids or solids surface, in which the bacterial cells are protected against external insults, such as antibiotics, contributing to the increase of virulence. (Flemming *et al.* 2016) Marine microorganisms, in particular, *Bacillus* species, have been recently documented as secretors of important compounds to regulate or inhibit pathogens biofilm formation (Sayem *et al.* 2011, Dusane *et al.* 2013, Pletzer and Hancock 2016) and therefore control bacterial infections. Importantly, and contrary to antibiotics that impose selective pressure resulting in the emergence of antibiotic-resistance, NACs are directed to non-essential functions like biofilms, and thus are unlikely to induce resistance (Sumi *et al.* 2014).

The production of NACs and the sporulation capacity offer a broad spectrum of industrial applications of *Bacillus* species like animal growth and immune system promoters (Sumi *et al.* 2014, Sahoo *et al.* 2016). An efficient research for NACs will allow new therapeutic measures to control fish bacterial diseases in aquaculture, allowing the reduction of chemicals and consequently combat multi-drug resistance among pathogenic bacteria, thus contributing to public health.

Table 2. *Bacillus* species with antimicrobial activity against important bacterial fish pathogens

Bacillus species	Origin	Bactericidal spectrum	Reference
B. subtilis	Shrimp pounds	*V. anguillarum*	Vaseeharan and Ramasamy (2003)
		V. harveyi	
		P. damselae	
		V. harveyi	Balcazar and Rojas-Luna (2007)
	Fish gut	*Aeromonas* spp.	Newaj-Fyzul *et al.* (2007)
		A. salmonicida	Banerjee *et al.* (2016)
		Aeromonas spp.	
	NCBMI[#] (Type strain)	*V. anguillarum*	Touraki *et al.* (2012)
			Zeigler *et al.* (2008)
	Marine sponges	*A. hydrophila*	Phelan *et al.* (2013)
		V. anguillarum	
		S. aureus	
B. subtilis; B. aerophilus	Fish gut	*Aeromonas* spp.	Thankappan *et al.* (2015)
B. amyloliquefaciens	Mangrove	*Vibrio* species	Xu *et al.* (2014)
B. amyloliquefaciens	Fish gut	*V. anguillarum*	Chen *et al.* (2016)
		Vibrio spp.	
B. pumilus; B. mojavensis	Coastal sediments	*V. harveyi*	Liu *et al.* (2015)
		S. aureus	
B. licheniformis; B. pumilus	Fish gut	*Aeromonas* spp.	Ramesh *et al.* (2015)
Bacillus sp.	Fish gut	*A. salmonicida*	Nandi *et al.* (2016)
		A. hydrophila	

[#] NCBMI: National Collection of Industrial Food and Marine Bacteria, Aberdeen, Scotland, UK

1.5 Objectives

Knowing the important influence of a balanced gut microbiota on fish health and the factors affecting such relationship, such as environmental conditions, feeding habits, feed itself or fish genetics, one objective of this work was to evaluate if, when subjecting two fish-species with different feeding habits (omnivorous vs carnivorous) to the same diet, the gut microbiota remains species-specific or if it is modulated by diet towards a similar gut microbiota composition. This was accomplished by a culture-independent method involving the PCR amplification of the bacterial DNA present on the intestinal samples of both fish species at the beginning and at the end of the feeding trial. The 16S rRNA gene polymorphisms were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) and different microbial diversity indices calculated.

In marine environments, *Bacillus* species have been reported as producers of new diseases-preventive molecules. Therefore the ultimate goal of the present work was to isolate, identify and characterize sporulating microorganisms, in particular *Bacillus* species capable to produce Natural Antimicrobial Compounds (NACs) active against important fish pathogens. To this aim, sporeformers were isolated from the gastrointestinal tract of three important fish species, assuming that probiotics originated from the gut of the target animal and from the ecological niche of the target pathogen, would be potentially more effective and ethically more acceptable to use. The isolates were identified, based on the partial sequencing of the 16S rRNA gene, and subject to antimicrobial tests to evaluate their bactericidal capacity, which included the production of extracellular NACs capable of suppressing pathogens growth and biofilm formation. Isolates were also screened for the presence of antibiotic resistance.

2. Materials and Methods

2.1 Experimental Trial

The trial was performed at the experimental facilities of the Marine Zoological Station, Faculty of Sciences, Porto University, and fish handling and procedures were based on the recommendations of the EEC Committee (2010/63/EU) for care and use of laboratory animals. White sea bream juveniles were obtained from IPMA, Olhão, Portugal, while gilthead sea bream juveniles from Atlantik Fish, Algarve, Portugal. Following 15 days of quarantine period, fish were acclimatized for additional 15 days in a thermo-regulated water recirculation system, continuously supplied with filtered seawater and equipped with 6 tanks (100 L capacity) (Figure 5).

Figure 5. Thermo-regulated recirculation water system

Triplicate groups with 10 fish of gilthead sea bream (body weight of 62 g) and white sea bream (body weight of 40 g) were distributed to each tank. Fish were fed by hand twice daily, 6 days a week, until apparent visual satisfaction with the same commercial diet (Skretting, Stavanger, Norway) containing 16% of lipids and 47% of protein. The trial lasted 6 weeks and during this time water temperature was maintained at 22±1 ºC, salinity averaged 35 g/L and dissolved oxygen was kept near saturation (7 mg/L). Fish were kept in natural photoperiod.

2.2 Sampling

Sampling procedures occurred in 2 different occasions: the first one, before the beginning of the experimental trial, was defined as initial time (TI), while the second one

happened at end of the experimental period and was defined as final time (TF). In both samplings, 9 fish of each species (3 per tank) were randomly sampled and killed by a lethal dose of anaesthesia (ethylene glycol monophenyl ether), 4 hours after the morning meal to ensure that fish intestines were full. Fish were weighted and the intestines were carefully removed and separated from adipose tissue (Figure 6). With sterile tools, faeces were squeezed out of the intestines and collected into properly labelled tubes. Then, intestines were opened in their length to expose the intestinal mucosa which was scrapped and collected into another labelled tube. Samples (150 mg) were used for isolation of sporeforming species (next section) and the rest frozen in liquid nitrogen and then stored at - 80 ºC for posterior DNA extraction and identification of gut bacterial community. To obtain a higher diversity of sporeforming bacteria, it was also sampled intestine contents of European sea bass present in the experimental facilities and fed with the same diet.

Figure 6. Sampling of gilthead sea bream (left), European sea bass (middle) and white sea bream (right) intestines for collection of faeces and intestinal mucosa.

2.3 Isolation, selection and characterization of sporeforming bacteria

To select the aerobic bacterial sporeforming isolates, around 150 mg of each faecal sample, previously collected, were diluted in buffered peptone water before freezing with liquid nitrogen and homogenized by vigorous vortexing. Then, serial dilutions (10^{-0}, 10^{-1}, 10^{-2}) were prepared in Bott & Wilson (B&W) salts (Annex 1) and 100 µL were plated in Luria Bertani (LB) agar plates (Becton, Dickinson and Company, USA), after a 20 min heat treatment at 65 ºC (Nicholson and Setlow 1990, Barbosa *et al.* 2005).

Following an incubation at 37 ºC for 48 h, colonies obtained from each fish-species were counted and randomly selected by their different morphologies. All selected isolates were purified by re-streaking on LB agar plates, numbered and stored at -80 ºC in 30% glycerol. To confirm spore production, isolates were grown overnight at 37 ºC in

solid Difco sporulation medium or DSM (Becto Dickinson and Company, US), and then observed by phase-contrast microscopy (Nicholson and Setlow 1990, Barbosa *et al.* 2005). To determine isolates catalase activity, a small amount of each fresh-LB colony was ressuspended into 5 µL of 3% Hydrogen Peroxide (H_2O_2) solution in a microscope slide; the production of air bubbles was considered positive (Barbosa *et al.* 2005).

2.4 DNA extraction from pure bacterial cultures

The bacterial genomic DNA of sporeformers was extracted from overnight liquid cultures, based on the method of Pitcher *et al.* (1989) with few modifications. Each bacterial-cells pellet was gently homogenized with 250 µL of TE buffer solution containing 50 mg/mL lysozyme. Then, 5 µL of RNAse (from a 10 mg/mL solution) were added and pellets were incubated at 37 ºC for 1 hour, followed by the addition of 50 µL of 10% SDS and 3 µL of proteinase K (from a 20 mg/mL solution) with 30 minutes of incubation at 55 ºC, promoting cell walls, proteins and RNA degradation. 500 µL of GES solution (Annex 1) and 250 µL of ammonium acetate 7.5 M were added to precipitate all remaining proteins and then tubes were cooled on ice. The extraction of nucleic acids started with 500 µL of phenol:chloroform:isoamyl-alcohol (25:24:1), and after the collection of the aqueous phase, additional 500 µL of chloroform:isoamyl-alcohol (24:1) allowed a re-extraction of the aqueous phase. The DNA was precipitated with 0.6 volumes of isopropanol followed by incubation on ice and centrifugation. The pellet was washed with 500 µL of ice-cold 70% ethanol and dried at 37 ºC for 10 minutes. DNA was finally dissolved in 100 µL ultrapure water and stored at 4 ºC. To ensure that the procedure was effective, 5 µL of DNA were resolved in 1% agarose gel electrophoresis for 30 minutes at 120 V containing GelRed (Biotium). The gel was then visualized on a Gel Doc EZ System (Bio-Rad, EU) using the Image Lab software v4.0.1 (Bio-Rad) to check the presence of DNA in the samples.

2.5 DNA extraction from fish intestinal samples

For extraction of bacterial DNA from fish faeces and mucosas, around 300 mg of sample (each sample was a pool of three fish of the same tank to reduce variability) were weighted to a 2 mL bead-beater (Sigma-Aldrich, Buchs, Switzerland) tube previously prepared with 500 µL STE buffer (0.1 M NaCl, 10 mM Tris, 1 mmM EDTA, pH 8) and 0.5 g of glass beads (Sigma-Aldrich G8772). Samples were then homogenized twice for 30 seconds in the bead-beater at 2500 speed with an interval of at least 30 seconds on

ice. Following a 15 minutes incubation at 75 ºC, with gentle agitation every 5 minutes, tubes were centrifuged for one minute at 13000 g and 500 µL of supernatant was transferred to new sterile 2 mL Eppendorf tubes. The tubes, after the addition of 50 µL of lysozyme (from a 10 mg/mL solution) and 5 µL of RNAse (from a 10 mg/mL solution) were incubated at 37 ºC for 1 hour. From this point the protocol used for bacterial DNA extraction from pure cultures (previous section) was strictly followed.

2.6 16S rRNA gene amplification and sequencing

For identification of sporeforming fish isolates, a DNA fragment containing almost the complete 16S rRNA gene (~1465 bp) was amplified by polymerase chain reaction (PCR) using primers 16S-27Fand 16S-1492R (Table 3). Each 50 µL reaction contained 31.70 µL of water (Sigma-Aldrich, Buchs, Switzerland), 5 µL of 10 x DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 5 µL of 2 mM of each dNTP (Thermo Scientific, Vilnius, Lithuania), 2.5 µL of 10 µM of each primer (STAB Vida, Lisboa, Portugal), 0.3 µL of Dreamtaq DNA polymerase enzyme (Thermo Scientific, Vilnius, Lithuania), and 3 µL of DNA template. The program consisted in an initial denaturation step (95 ºC for 5 minutes) followed by 35 cycles of denaturation (95ºC, 30 seconds), annealing (55 ºC, 30 seconds) and extension (72ºC 1.30 minutes) and a final extension step (72 ºC, 10 minutes).

Table 3. Oligonucleotide primers used in this study

Primer name	Primer sequence (5'-3')	Reference
16S-27F	AGAGTTTGATCMTGGCTCAG	Lane (1991)
16S-1492R	GGYTTACCTTGTTAYGACTT	"
16S-358F	CCTACGGGAGGCAGCAG	Muyzer *et al.* (1993)
CG-16S-358F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG	"
16S-517R	ATTACCGCGGCTGCTGG	"

To assess the faecal and mucosal intestinal microbiota composition, polymorphism analyses of 16S rRNA gene was done by Denaturing Gradient Gel Electrophoresis (DGGE). Bacterial 16S rRNA gene internal fragments were amplified by a touchdown PCR using primers 16S-358F (which has a GC clamp at the 5´end) and

16S-517R (Table 3), yielding a 233 bp DNA fragment. To perform this reaction, a mixture of 24.77 µL of water (Sigma), 10 µL of GoTaq Buffer 5x (PROMEGA), 5 µL of each dNTPs (2mM, PROMEGA), 2.5 µL of each primer (10 µM 16S-358F with a CG clamp at the 5' end and 10 µM 16S-517R; Table 3) and 0.25 µL of GoTaq polymerase (PROMEGA) were added to 5 µL of DNA template. The touchdown PCR consisted of a 94 ºC incubation for 5 min followed by 10 cycles of 64 ºC, 1 min, 65 ºC, 1 min and 72 ºC, 3 min. The annealing temperature was decreased 1 ºC at every cycle, until reaching 55 ºC. Thus, final 20 cycles of 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 3 min. Final extension was at 72 ºC for 10 min.

All PCR reactions occurred on a T100™ Thermal Cycler (Bio-Rad, EU). PCR products (5 µL) were resolved by electrophoresis in a 1% agarose gel for 45 minutes at 120 V containing GelRed (Biotium, Fremont, California, USA) in 1XTris-Acetate EDTA (TAE) buffer and visualized on a Gel Doc EZ System (Bio-Rad, EU) using the Image Lab software v4.0.1 (Bio-Rad, EU) to check for product size. The amplified products were sent to STABVIDA (Caparica, Portugal) for sequencing with the primers 16S-27F for the identification of the sporeformers isolates, and 16S-358F for the identification of DGGE bands.

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

2.7 Polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE)

10 µL of each PCR product were loaded on an 8% acrylamide gel composed of a denaturing gradient of 30 to 70% 7 M urea/40%formamide. Electrophoresis occurred in a DCode™ Universal Mutation Detection System (Bio-Rad, EU) at 60ºC, 65V during 16,5h in 1xTAE buffer. Gel was then stained with SYBR Gold Nucleic Acid Gel Stein during 1h, imaged on a Gel Doc EZ System (Bio-Rad, EU) with the Image Lab software v4.0.1 (Bio-Rad, EU) and the DGGE banding patterns transformed into presence/absence matrices using the Quantity One 1-D Analysis Software v4.6.9 to measure each band intensity. Relative similarities between species and replicates where calculated with Primer software v7.0.5.

Bands of interest were marked in the gel photograph and were then excised using a scalpel blade, cleaned with EtOH between each band. The excised gel bands were then placed into previously prepared Eppendorf tubes with 20 µL sterile ddH_2O and kept at 4 ºC overnight to allow resuspension of the DNA. Tubes were then vortexed for 15 seconds, centrifuged for 1 minute at 13,000 g and 5 µL used as DNA template to perform a PCR amplification similar to the one described for the 16S rRNA gene fragments amplification of faecal and mucosa samples, but using a forward primer lacking the GC-clamp (Table 3).

2.8 Bacterial strains and culture conditions

The sporeforming bacterial strains isolated from fish guts were denominated as "producer strains", and used to evaluate their capacity to produce Natural Antimicrobial Compounds (NACs). All isolates were routinely grown in LB medium at 37ºC with agitation (120 rpm). The laboratory strain *Bacillus subtilis* 168 (Zeigler *et al.* 2008) was used as control. *Vibrio harveyi*, *Vibrio anguillarum*, *Aeromonas salmonicida*, *Aeromonas bivalvium*, *Aeromonas veronii*, *Staphylococcus aureus* and *Photobacterium damselae* were selected as "indicator strains" based on their pathogenicity on fish. All the indicator strains were grown aerobically in BHI medium at room temperature, with the exception of *S. aureus* that grew at 37ºC. All strains after purification were stored at -80ºC in 25% glycerol.

2.9 Screening for antimicrobial activity

i) Colony overlay assay

All producer strains grown aerobically overnight at 37ºC were inoculated as a 5 µL spot on LB agar plates (Barbosa *et al.* 2005). After 24h growth at 37ºC, cells were killed by exposure to chloroform vapours for 25 minutes, followed by replacement of plate covers and aeration for 25 min. Colonies were then overlayed with 8 mL of Brain Heart Infusion (BHI) soft agar (containing 0.7% agar) that had been inoculated with 100 µL of the indicator strains grown overnight in BHI, at OD_{600} ~ 0.1. Plates were inverted for 1 hour allowing the agar solidification prior the incubation at 25ºC (for *A. salmonicida, A. veronii, A. bivalvium, V. anguillarum, V. harveyi* and *P. damselae*) and 37ºC (for *S. aureus*). Zones of growth inhibition around the colonies after 24h were considered positives and radius was measured. All plates were photographed in a Gel-Doc™ XR+ System, using the Image Lab™ Software (Bio-Rad, EU).

ii) Microplate growth inhibition test

Six producer strains with the best profile from the previous task, were tested with the microplate growth inhibition assay (Papa *et al.* 2015). 100 µL of the indicator bacterial cultures (OD_{600} ~ 0.1) were added to 96-well flat bottomed polystyrene plates, and then each well was filled with 100 µL cell-free supernatant. Supernatants were obtained after centrifugation (13.000 rpm, 4ºC, and 15 minutes) and posterior sterilization with 0.22 µm cellulose acetate filter (VWR, Europe). 200 µL of BHI medium alone (without bacteria) were used as negative control and 200 µL of each bacterial culture (in medium, without supernatant) were used as positive control. The 96-well microplate was incubated aerobically at room temperature, and the optical density (OD_{600}) was measured at 0, 1, 2, 3, 4, 5, 22, 23, 24 hours with a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc.) to establish the bacterial growth pattern in the presence or absence of producer strains supernatant.

iii) Well diffusion assay

In the well diffusion assay, 2 mL of each producer strain grown aerobically overnight at 37ºC was centrifuged at 13.000 rpm, 4ºC for 15 minutes. Supernatants were sterilized by passage through a 0.22 µM cellulose acetate filter (VWR, Europe), and preserved on ice until use. The dilutions (OD_{600} ~ 0.1) of the indicator strains were spread on BHI agar plates with a cotton swab (in 3 different directions). Then 9 mm diameter wells were punched and 100 µL of each cell-free supernatant were added. Plates were incubated 24h at 37ºC or 25ºC (depending on the bacterial species) inverted. Zones of growth inhibition were considered positives and radius was measured. All plates were photographed in Gel-Doc™ XR+ system using the Image Lab™ Software (Bio-Rad, EU).

2.10 Inhibition of biofilm formation

The ability of sporeformers to inhibit the biofilm formation of fish pathogenic strains was tested by a modification of Papa *et al.* (2015) method. In brief, 100 µL of "indicator" pathogenic bacterial cultures (OD_{600} ~ 0.1) were added to 96-well flat bottomed polystyrene plates. Each well was filled with 100 µL of cell-free supernatant of each sporeforming "producer" strain. BHI medium alone (200 µL, without bacteria) was used as negative control and 200 µL of each bacterial culture (in medium alone, without supernatant) were used as positive control. After 24h of aerobic incubation at room temperature, the remaining cells were removed and the wells were washed three times with 250 µL of Phosphate-Buffered Saline (PBS) and allowed to dry in an inverted

position. Each well was stained with 250 µL of 0.1% crystal violet for 15 minutes at room temperature and rinsed twice with 250 µL of double distilled water. After all wells dry (in an inverted position), the dye bound to adherent cells was solubilized in 250 µL of 20% glacial acetic acid and 80% ethanol for 30 minutes at room temperature. The final quantification of biomass was accessed by measuring the optical density at 590nm in a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc.). The data is composed by three independent experiments.

2.11 Antibiotic susceptibility test

The antibiotic susceptibility was determined by Kirby-Bauer method (Biemer 1973) using antimicrobial susceptibility discs (Oxoid Limited, Thermo Fisher Scientific Inc.). Summarizing, the bacterial inoculums (obtained from ressuspending an isolated colony in 1% of NaCl), with the optical density adjusted to 0.5 McFarland standard units, were spread (in three different directions to guarantee full coverage) with a cotton swab in 20 mL MH (Muller-Hinton) agar plates, and the antibiotics were distributed on the plates with a disk dispenser. The antibiotic disks used were Teicoplanin (TEC_{30}), Vancomycin (VA_{30}), Chloramphenicol (C_{30}), Tetracycline (TE_{30}), Erythromycin (E_{15}), Gentamycin (CN_{10}), Kanamycin (K_{30}) and Streptomycin (S_{10}) following the recommendations of the European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed (EFSA-FEEDAP 2012). After 24 hours of incubation at 37 ºC organisms were classified as Sensitive (S), Intermediate (I) and Resistant (R) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Table S2, Supplementary tables).

2.12 Statistical Analysis

The statistical analyses were done using the SPSS 23.0 software package for Windows. Before any test, data were subject to a Levene's test, to ensure the homogeneity of variances that complies the requirements of ANOVA.

The DGGE banding patterns were used to calculate the relative similarities between species and replicates with Primer software v7.0.5. Species Richness was established with the use of Margalef's diversity index while Shannon-Weaver index was used to establish Species Diversity and Bray-Curtis method to represent the similarity percentages (SIMPER) between studied groups. A two-way ANOVA, was then ran with the obtained parameters using species and time as fixed factors and a one-way ANOVA

was used in case of a significant interaction. When *p*-values were significant ($p<0.05$), means were compared with Tukey´s test.

Differences in the number of isolates obtained from the intestinal contents between fish species were analysed by one-way ANOVA, and significant differences ($p<0.05$) among means were determined by the Tukey's test. A repeated measures ANOVA and an one-way ANOVA were performed to evaluate the 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.

3. Results

During the experimental trial, specimens of white sea bream (WSB), gilthead sea bream (GSB) were fed with the same commercial diet, and submitted to the same husbandry conditions to guarantee that differences in their gut microbiota were due to their different gastrointestinal tracts and feed behaviours and not due to their origin (aquaculture farm).

The zootechnical parameters and growth performance, such as *initial* and *final weight* of each species, *feed intake*, *feed efficiency* (that shows the relation between the feed intake and actual growth), and the *specific growth rate* (that presents a percentage of weight gain in relation to the length of the experiment), are presented in Table 4. Besides no mortalities registered, white sea bream and gilthead sea bream juveniles experienced normal growth rates and feed intake, according to each species specifications.

Table 4. Growth performance and feed utilization efficiency of white sea bream (WSB) and gilthead sea bream (GSB) through the experimental process

Species	WSB	GSB
Initial Weight (g)	40.8±0.12	61.7±0.09
Final Weight (g)	48.9±0.36	113.1±5.22
Weight Gain (g)[1]	80.3±2.72	513.5±52.02
Feed Intake (g kg ABW -1day-1) [2]	10±0.24	20.7±1.83
Feed Efficiency [3]	0.50±0.03	0.79±0.06
Specific Growth Rate [4]	0.50±0.01	1.68±0.13

Mean values and standard error of the mean (±SE) are presented for each parameter (n=3)

[1]Weight Gain: Final body weight –Initial body weight

[2]Feed Intake:((feed intake (g dry matter / fish) x 1000) / (ABW x nb days)

[3]Feed efficiency: wet weight gain/dry feed intake

[4]Specific growth rate (SGR): 100 × ((LN(Final body weight)-LN(Initial body weight))/(time in days)

3.1 Gut Microbiota Diversity Analysis

The microbial community profiling of ESB and GSB intestines (faeces and mucosas) was studied by polymorphism analyses of the variable V3 region of the 16S rRNA gene using DGGE, at the beginning (TI) and at the end (TF) of the experimental trial. Following DGGE analysis of each PCR product, a Bray-Curtis dendogram showed that two out of three replicates for each species presented similar

banding patterns with one always failing to cluster (Figure 7). The Figure further shows that both in the digesta and mucosa samples, the similarity between replicates increase from the beginning to the end of the experiment, with digesta samples being more similar (>70%) than mucosa ones (<60%).

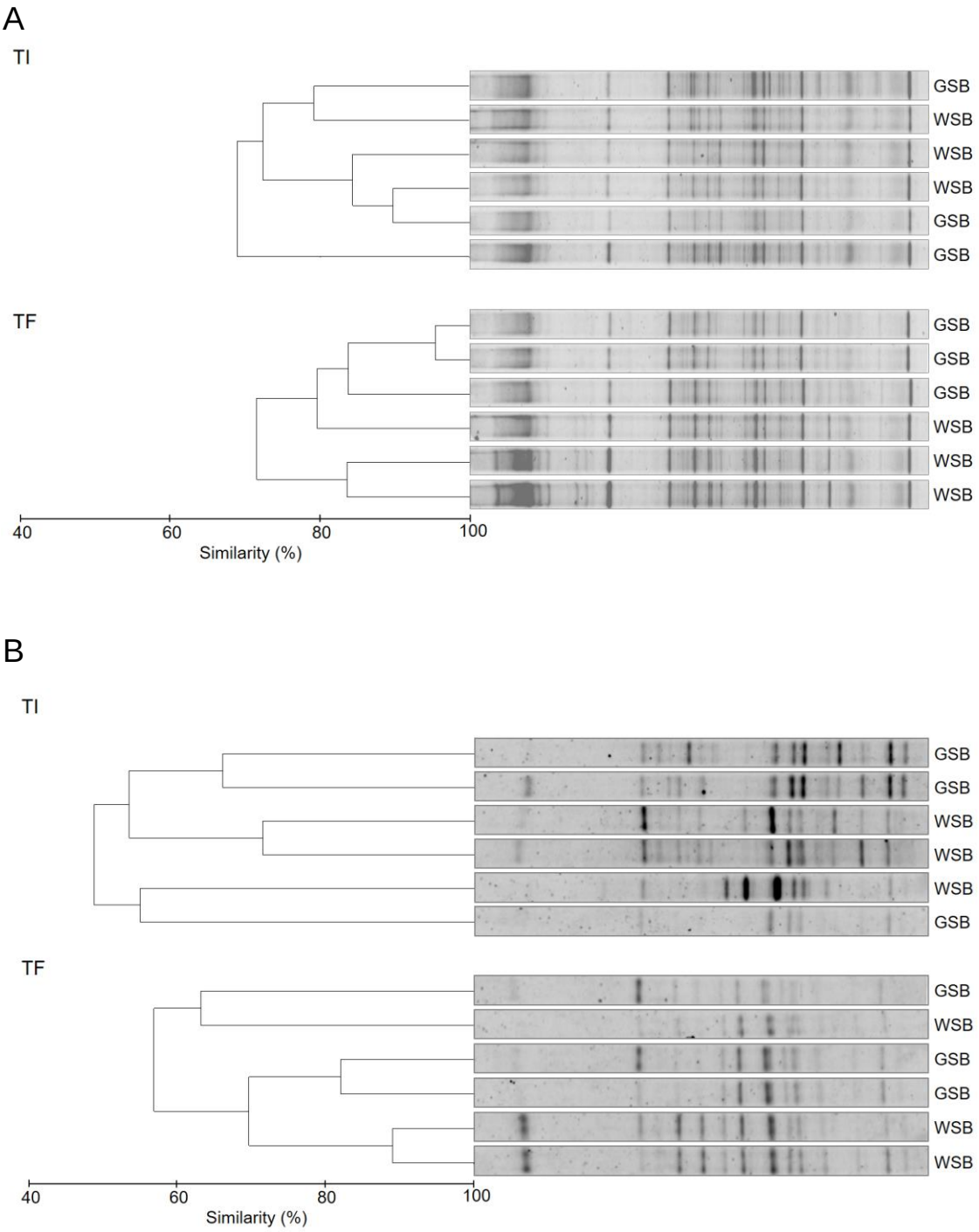

Figure 7. Dendograms and PCR-DGGE fingerprints of the digesta **(A)** and mucosa **(B)** microbiota recovered from white sea bream (WSB) and gilthead sea bream (GSB) at initial time (TI) and final time (TF) of the experimental trial.

In the digesta samples the two-way ANOVA analysis (Table 5) showed that although not statistically significant, the number of OTU's, species diversity and richness tend to increase from TI to TF in WSB and maintaining their numbers in GSB. The percentage of similarity however, increases on both WSB and GSB with the two-way ANOVA showing significant differences (p<0.05) in the time factor. Interaction between both factors, species and time of sampling, showed a significant difference in number of in similarity percentage (p<0.05), with a one-way ANOVA showing that in GSB the time factor had a significant influence on the similarity of the replicates. Results also showed that in the mucosa samples (Table 6) the number of OTU's, species diversity, richness and percentage of similarity between replicates suffer a reduction when compared to the digesta samples. Also, and besides no significant differences in every parameter analysed, the OTUs, richness and diversity slightly reduce their numbers and the similarity percentage of the replicates tend to increase with time of sampling in both species.

Table 5. Ecological parameters obtained from PCR-DGGE fingerprints of the digesta microbiota recovered from white sea bream (WSB) and gilthead sea bream (GSB) at initial time (TI) and final time (TF) of the experimental trial.

Time	TI		TF	
Species	WSB	GSB	WSB	GSB
OTUs[1]	21.7±2.1	25.3±7.4	28.7±3.5	25.7±1,5
Richness[2]	1.2±0.1	1.4±0.4	1.6±0.2	1.4±0,1
Diversity[3]	3.3±0.1	3.4±0.2	3.5±0.2	3.4±0
SIMPER similarity (%)[4]	80.2±8.5	67,4±5.6	80.0±5.2	87.3±7.0

Two-way ANOVA

Variation source	Time	Species	Interaction
OTUs[1]	ns	ns	ns
Richness[2]	ns	ns	ns
Diversity[3]	ns	ns	ns
SIMPER Similarity (%)[4]	*	ns	*

One-way ANOVA

Variation source	Time
WSB	ns
GSB	*

Values presented as means ± standard deviation (±SD) (n = 3 per treatment pooled from 9 fish)

[1]OTUs: Average number of operational taxonomic units

[2]Margalef species richness: d=(S-1)/log(N)

[3]Shannons diversity index: H'=-∑(pi(lnpi))

[4]SIMPER, similarity percentage within group replicates

ns, non-significant (p>0.05); *p<0.05

Table 6. Ecological parameters obtained from PCR-DGGE fingerprints of the mucosa microbiota recovered from white sea bream (WSB) and gilthead sea bream (GSB) at initial time (TI) and final time (TF) of the experimental trial.

Time	TI		TF	
Species	WSB	GSB	WSB	GSB
OTUs[1]	10.6±4.1	12.3±3.1	9.7±2.3	8.3±0.6
Richness[2]	1.1±0.4	1.2±0.3	1.0±0.2	0.9±0,6
Diversity[3]	2.3±0.4	2.5±0.3	2.2±0.3	2.1±0.7
SIMPER similarity (%)[4]	54.2±15.2	57.2±7.4	70.0±8.2	66.7±19.8

Two-way ANOVA

Variation source	Time	Species	Interaction
OTUs[1]	ns	ns	ns
Richness[2]	ns	ns	ns
Diversity[3]	ns	ns	ns
SIMPER Similarity (%)[4]	ns	ns	ns

Values presented as means ± standard deviation (±SD) (n = 3 per treatment pooled from 9 fish)

[1]OTUs: Average number of operational taxonomic units

[2]Margalef species richness: d=(S-1)/log(N)

[3]Shannons diversity index: H'=-∑(pi(lnpi))

[4]SIMPER, similarity percentage within group replicates

ns, non-significant (p>0.05)

The identification of selected DGGE bands from the digesta profile is shown in table 7. Sequence numbers match the ones on Figure 8 with only the matches with high and reliable parameters being added to the table. Almost every match was either from the Proteobacteria or Firmicutes phylum with *Propionibacterium acnes* being the only representant of the Actinobacteria phylum and *Calothrix desertica* the only Cyanobacteria. Among the Proteobacteria phylum it was detected one band of *Acinetobacter*, one of *Pseudomonas*, one of *Vibrio*, one *of Luteimonas* and also one band belonging to the *Lysobacter* genera. Regarding the Firmicutes phylum, bacteria belonging to *Lactobacillus*, *Enterococcus*, and also *Bacillus* genera were present in the selected bands (one band each).

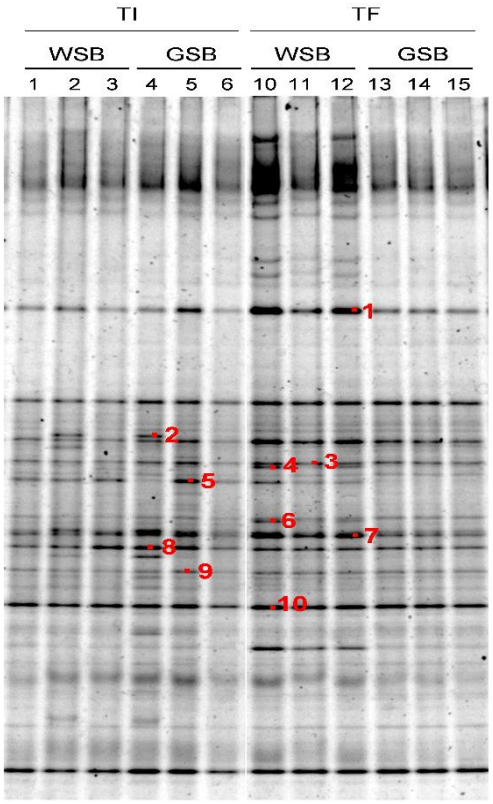

Figure 8. PCR-DGGE fingerprints of the microbiota found in the intestine of white sea bream (WSB), gilthead sea bream (GSB) at the initial and final time. The black numbers correspond to each sample analysed composed by two pools of faeces, and the red numbers inside the figure represent the bands excised for sequencing.

Table 7. Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of the intestinal communities of white sea bream (WSB) and gilthead sea bream (GSB)

Band	Closest known species (BLAST)	ID (%)	Accession nr.
1	*Lactobacillus aviarius*	99	NR_044703.2
2	*Acinetobacter* sp.	95	NR_117621.1
3	*Pseudomonas* sp.	93	NR_117822.1
4	*Enterococcus* sp.	87	NR_114785.2
5	*Vibrio sp.*	93	NR_122060.1
6	*Luteimonas aquatica*	87	NR_044323.1
7	*Bacillus subtilis; Virgibacillus halodenitrificans*	91	NR_102783.
8	*Lysobacter dokdonensis*	83	NR_115948.1
9	*Calothrix desertica*	80	NR_114995.1
10	*Propionibacterium acnes*	100	NR_040847.1

3.2 Gut sporeformers selection and characterization

Heat-treated intestinal contents of white sea bream (WSB), gilthead sea bream (GSB) and European sea bass (ESB) were used to obtain sporeforming gut bacteria capable of producing NACs active against important fish pathogens. The isolates obtained aerobically in LB agar plates were firstly counted, and calculate the number of CFU (**colony-forming unit**) in the original samples of each species (white sea bream contained around $9.0x10^2$ CFU, in gilthead sea bream $4.6x10^3$ CFUs and in European sea bass $3.5x10^3$ CFU). The One-way ANOVA analysis revealed the absence of significant differences in the spore numbers between the species analysed (Data not shown).

Following selection and purification, 176 isolates representing different colony morphologies and samples, were chosen for analysis (61 from GSB, 51 from WSB and 64 from ESB). The morphological diversity of representative fish isolates FI314, FI326, FI330, FI347, FI353, FI354, FI359, FI368, FI376, FI424, FI429, FI436, FI442, FI480 compared to the reference strain *B. subtilis* 168 is illustrated in Figure 9.

Spore production of each isolate was confirmed by phase-contrast microscopy, revealing that 98% of the isolates produce endospores of different sizes and shapes (Table 8 and data not shown). Also 98% of isolates shown to be catalase positive, indicating that these are probably *Bacillus* species and not aerotolerant strains of *Clostridium* spp. (catalase negative) (Table 8).

Identification of the 41 most promising isolates (active against the pathogens tested, see next section), by partial sequencing the 16S rRNA gene (~700 kb) revealed a clear abundance of *B. subtilis* in the guts of GSB, WSB and ESB (Table 8). According to BLAST of the GenBank nonredundant (nr) nucleotide database, besides the predominant species *B. subtilis* (54%), *B. licheniformis* and *B. methylotrophicus* represented 9% and *B. amyloliquefaciens* corresponded to 7% of the identified strains, while others species are present in small numbers. There are also some strains that identification at a species level was not possible (10%), although all the isolates exhibited 97% or higher rRNA gene sequence identity to this database. A comparison to the Sequence Match package of the Ribosomal Database Project 11, revealed that *B. subtilis* represented 71%, in a lower extent *B. licheniformis* and *B. amyloliquefaciens* (~10% each), while other organisms such as *B. safensis* or *B. pumilus* (~2% each) were equivalent distributed (data not shown).

Table 8. Identification and characterization of the 41 sporeformers isolated from the intestinal contents of white sea bream (WSB), gilthead sea bream (GSB) and European sea bass (ESB).

Isolate	Source	Tank	16S rRNA gene analysis		Sporulation test	Catalase test
			Closest known species	% ID [a]		
FI300	GSB	1	*Bacillus sp.*	98	+	+
FI302	GSB	1	*B. subtilis*	98	+	+
FI304	GSB	1	*B. subtilis*	99	+	+
FI307	GSB	2	*B. licheniformis*	98	+	+
FI314	GSB	2	*B. subtilis*	97	++	+
FI321	GSB	2	*Bacillus sp.*	99	++	+
FI324	GSB	2	*B. cereus*	99	++	+
FI326	GSB	2	*B. subtilis; B. cereus*	99	++	+
FI330	GSB	2	*B. subtilis*	98	++	+
FI333	GSB	3	*B. methylotrophicus*	97	+	+
FI335	GSB	3	*B. methylotrophicus*	98	++	+
FI338	GSB	3	*B. subtilis*	98	++	+
FI342	GSB	3	*B. licheniformis*	98	++	+
FI347	GSB	3	*Bacillus sp.*	98	+	+
FI348	GSB	3	*B. licheniformis*	99	+	+
FI353	WSB	1	*B. subtilis*	96	+	+
FI354	WSB	1	*B. subtilis*	98	+	+
FI355	WSB	1	*B. subtilis*	98	+	+
FI359	WSB	1	*B. subtilis*	98	++	+
FI361	WSB	1	*B. licheniformis*	99	++	+
FI367	WSB	2	*B. amyloliquefaciens; B. methylotrophicus*	99	+/-	-
FI368	WSB	2	*B. subtilis*	98	+	+
FI373	WSB	2	*B. subtilis*	97	+	+
FI375	WSB	3	*B. pumilus*	97	+	+
FI376	WSB	3	*B. subtilis*	97	++	+
FI377	WSB	3	*B. subtilis*	98	++	+
FI378	WSB	3	*B. subtilis*	99	+	+/-
FI387	WSB	3	*B. subtilis*	98	++	+
FI390	WSB	3	*Bacillus sp.*	99	+	+
FI401	ESB	1	*B. subtilis*	97	+	+
FI414	ESB	1	*B. methylotrophicus*	99	+	+
FI423	ESB	2	*B. amyloliquefaciens*	99	++	+
FI424	ESB	2	*Bacillus sp.*	99	+	+
FI429	ESB	2	*B. amyloliquefaciens*	100	+	+
FI436	ESB	3	*B. subtilis*	98	++	+
FI442	ESB	3	*B. subtilis*	98	++	+
FI455	ESB	3	*B. subtilis*	97	+	+
FI456	ESB	3	*B. subtilis*	99	+	+
FI464	ESB	3	*B. safensis*	99	+	+
FI469	ESB	3	*B. subtilis*	98	+	+
FI480	ESB	3	*B. subtilis*	99	+	+

[a]Closest organism using BLAST based on partial sequences of 16S rRNA gene (700kb)

++ Great positive reaction or formation of spores;+ Positive reaction or formation of spores; +/- Slow reaction or limited formation of spores; - Negative reaction or no formation of spores.

3.3 Screening gut sporeformers for NACs

The entire collection of 172 isolates capable of producing endospores, was screened for the presence of natural antimicrobial compounds (NACs) able to antagonize different fish pathogens, using a colony overlay assay (Barbosa *et al.* 2005). Around 52% of gut sporeformers produced NACs active against at least one of the pathogenic strains tested. The method allowed the selection of 14 isolates with the most promising antimicrobial activities, namely FI314, FI326, FI330, FI347, FI353, FI354, FI359, FI368, FI376, FI424, FI429, FI436, FI442, and FI480. These 14 sporeformers were tested in the same conditions and the representative results are illustrated in Figure 10. Strains FI314, FI330 and FI442 were successful in inhibiting all pathogenic strains tested with exception of *A. salmonicida*. FI347 was only active against *A. veronii*. FI354 shown to be effective against *S. aureus*. FI359, FI368, FI376 and FI436 inhibited *S. aureus, V. harveyi*, *A. veronii* and in a small extent *P. damselae*. FI424 was capable of inhibiting *S. aureus* and *P. damselae*, while FI429 was only active against *P. damselae*. FI480 had successful results against *S. aureus*, *V. harveyi* and *A. veronii*. Isolates FI314, FI330 and FI442 shown impressive antagonistic capacity against the growth of *P. damselae* as demonstrated in Figure 11.

For the six producer strains with the best antimicrobial profile in the previous experiments, a microplate growth inhibition test was realized in which the growth inhibition for each indicator pathogen was evaluated when cultured in BHI medium alone (control) or in the presence of cell-free supernatant of the producer *Bacillus* strains FI314, FI330, FI359, FI376, FI442 and FI480 (Figure 12A). Growth of *A. veronii*, *A. bivalvium*, *V. harveyi*, *V. anguillarum*, *P. damselae* and *S. aureus* was significantly inhibited by the cell-free supernatant of strains FI314, FI330, which suggests that these isolates might produce promising extracellular compounds with NAC activity. On a small-scale FI442 was capable to significantly reduce the growth of *V. anguillarum* and *P. damselae* and, also FI376 shown activity against *V. anguillarum* and *S. aureus*. (Table 9). *A. salmonicida* was the only pathogenic strain that none of the *Bacillus* isolates tested were capable of inhibiting.

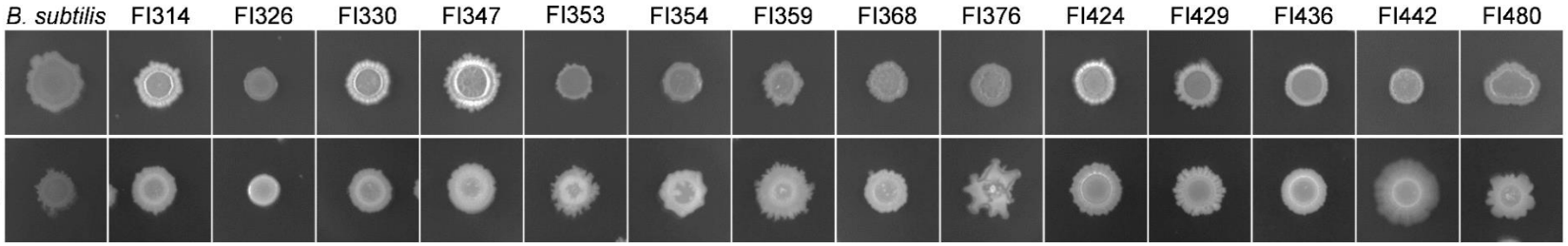

Figure 9. Morphological diversity of representative sporeforming fish isolates (FInumbers on top) obtained from intestinal contents. Photographs of colonies grown 24h in LB (Luria-Bertani) and DSM (Difco Sporulation Medium) agar medium, are at the same scale. The laboratory strain *B. subtilis* 168 was used as a control.

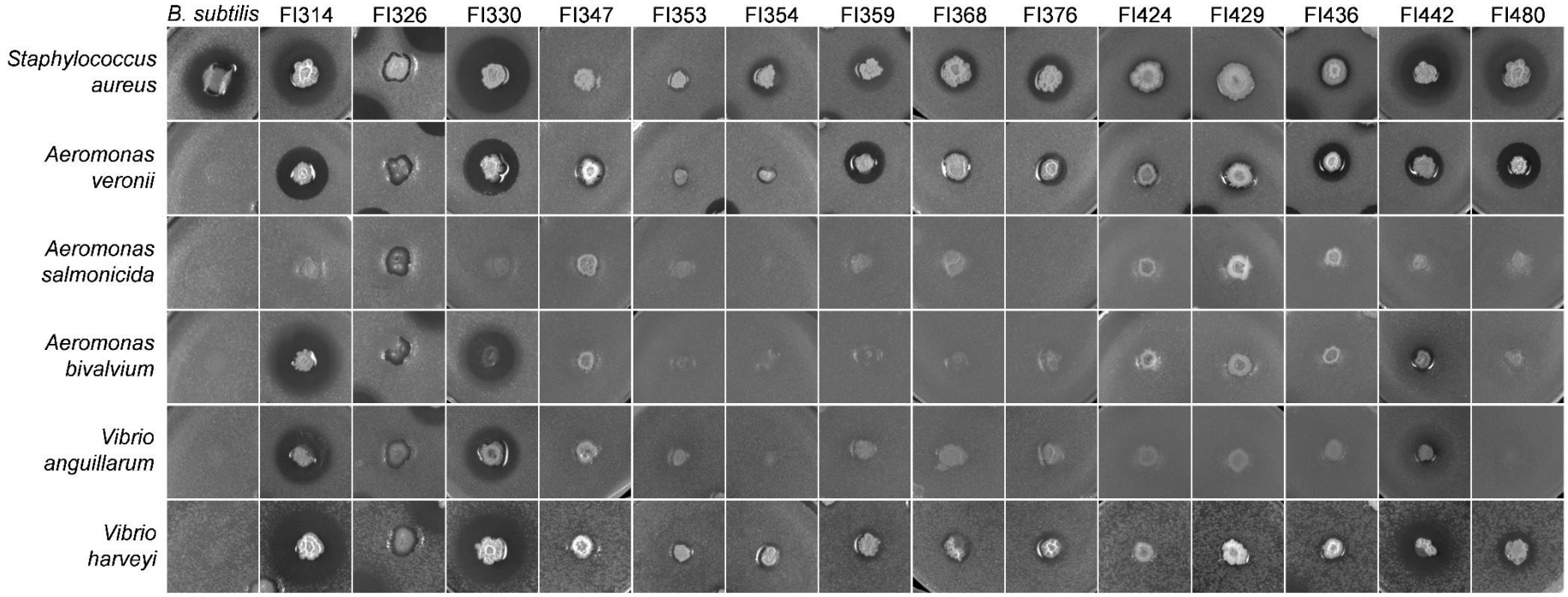

Figure 10. Formation of growth inhibition zones for the indicator pathogenic strains *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi* and *S. aureus* around colonies of producer sporeforming fish isolates (FInumbers on top). The laboratory strain *B. subtilis* 168 was used as a control. All photos are at the same scale.

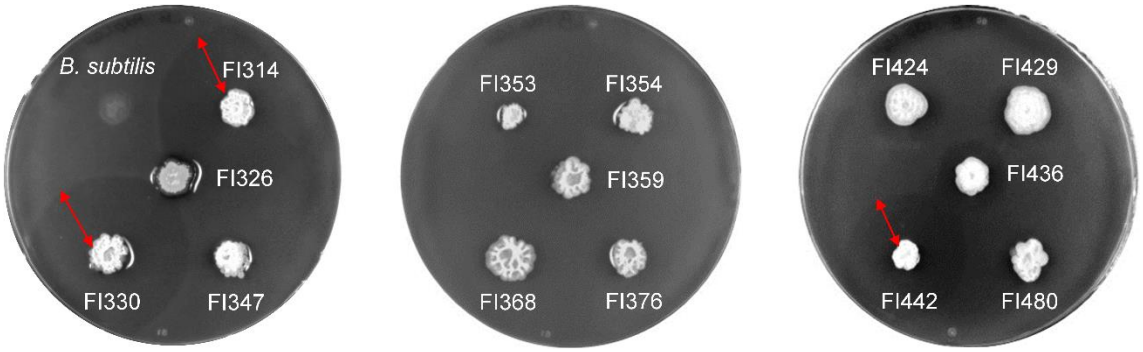

Figure 11. Formation of growth inhibition zones (red arrows) for the indicator pathogenic strain *Photobacterium damselae* around colonies of producer sporeforming fish isolates FI314, FI326, FI330, FI347, FI353, FI354, FI359, FI368, FI376, FI424, FI429, FI436, FI442 and FI480. The laboratory strain *B. subtilis* 168 was used as a control.

Table 9. Statistical analysis of the ability of sporeforming isolates (Bsub and FInumbers) to inhibit the bacterial growth of different fish pathogenic strains.

Strain	*A. salmonicida*	*A. veronii*	*A. bivalvium*	*V. anguillarum*	*V. harveyi*	*P. damselae*	*S. aureus*
Bsub[#]	ns	ns	ns	ns	ns	ns	ns
FI314	ns	*	***	***	**	***	**
FI330	ns	*	***	***	**	***	**
FI359	ns	ns	ns	*	ns	ns	ns
FI376	ns	ns	ns	*	ns	ns	**
FI442	ns	ns	ns	*	ns	*	ns
FI480	ns	ns	ns	*	ns	ns	ns

ns, non-significant (p>0.05); *p<0.05; **p<0.01; ***p<0.001
[#] Bsub stands for the laboratory strain *B. subtilis* 168

The 3 *Bacillus* isolates with the most promising NAC activities were further tested with a standard agar-well diffusion assay (Figure 12B). The cell-free supernatants of strains FI314 and FI330 were able to antagonize different fish pathogens, such as *S. aureus*, *A. bivalvium*, *V. harveyi* and *P. damselae*. FI442 demonstrated a discrete inhibition of *Vibrio anguillarum* growth.

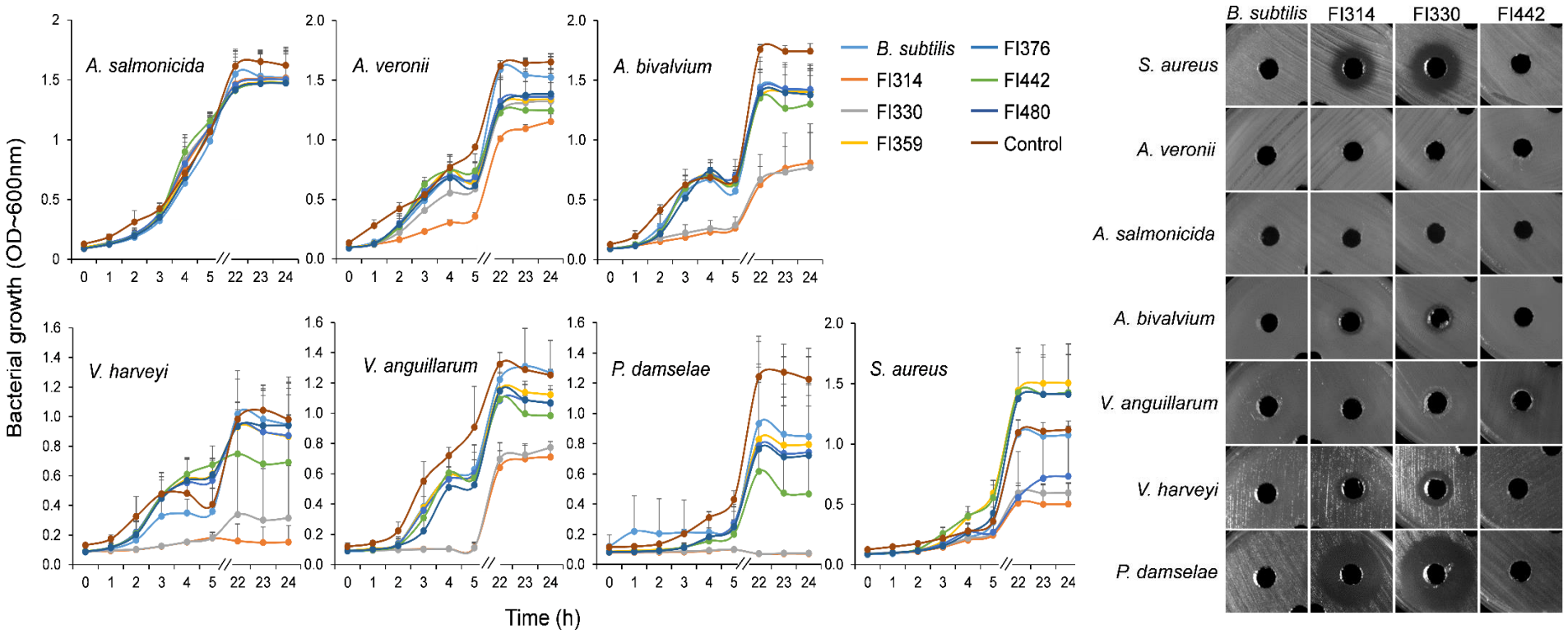

Figure 12. (A) Microplate growth inhibition assays of the indicator pathogenic strains *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *S. aureus* and *P. damselae* when cultured in BHI medium alone (control) or supplemented with cell-free supernatant of the producer sporeforming isolates FI314, FI330, FI359, FI368, FI376, FI436, FI442 and FI480. Optical density was measure at an absorbance of 600nm. **(B)** Formation of growth inhibition zones for the indicator pathogenic strains *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *S. aureus and P. damselae* around the wells with cell-free supernatant of the strains FI314, FI330 and FI442. All photos are at the same scale. The laboratory strain *B. subtilis* 168 was used as a control in both experiments.

3.4 Inhibition of biofilm formation

The three *Bacillus* strains with the best antimicrobial profile (FI314, FI330 and FI442) were analysed for their ability to interfere with one of the virulence mechanisms associated to pathogenic bacteria, namely biofilm formation. The results regarding the anti-biofilm activity of cell-free supernatants of all three *Bacillus* strains are represented in Figure 13. A one-way ANOVA analysis revealed that the biofilm production of *A. veronii* and *P. damselae* was significantly decreased in the presence of FI314, FI330 and FI442 cell-free supernatant. For *A. salmonicida* all three *Bacillus* strains were able to reduce the attachment of biofilm, although a significant difference was only observed with FI442. *V. anguillarum* was the pathogen with the highest value of biofilm formation and besides the FI314 interference with the surface attachment of this, no additional significant differences were detected. Although no statistically significant differences could be found in the biofilm formation capacity of *V. harveyi* and *S. aureus,* a tendency to a weaker attachment was observed when the cell-free supernatants of FI314 and FI330 were used (Figure 13).

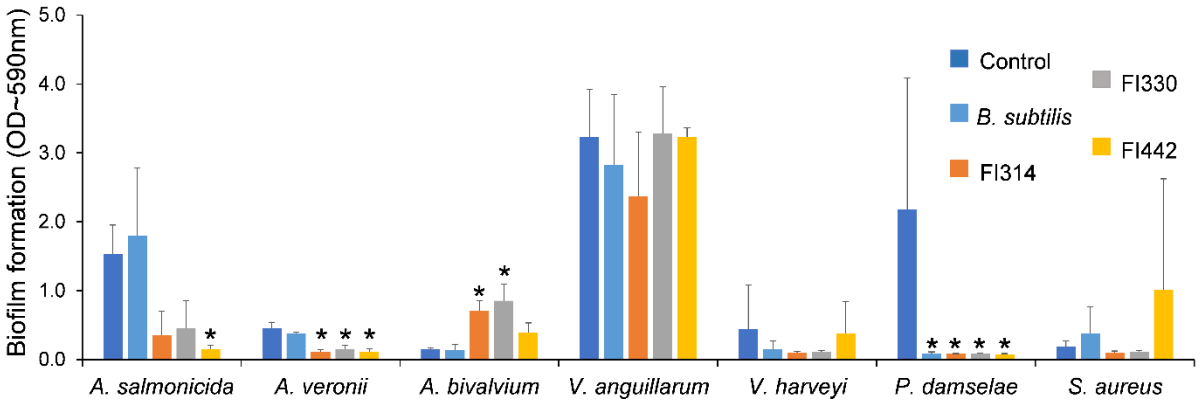

Figure 13. Biofilm formation of the indicator pathogenic strains *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *P. damselae* and *S. aureus* when cultured in BHI medium alone (control) or supplemented with cell-free supernatant of the sporeforming isolates FI314, FI330 and FI442. Biofilm developed during 24 h was stained with 0.1% crystal violet and the optical density was measure at an absorbance of 590 nm. The laboratory strain *B. subtilis* 168 was used as a control for bacterial growth. Significant differences ($p < 0.05$) in relation to control are represented by an asterisk (*).

3.5 Antibiotic susceptibility

In general, all the strains obtained from fish guts were sensitive to the antibiotics tested, as shown in Table 10. The exceptions were the isolates FI314 and FI480 that presented an intermediate susceptibility to Streptomycin (S_{10}) and FI436 with the same pattern to Tetracycline (TE_{30}).

Table 10. Sensibility of sporeformers to the antibiotics Teicoplanin (TEC30), Vancomycin (VAN30), Chloramphenicol (C30), Tetracycline (TE30), Erythromycin (E15), Gentamycin (CN10), Kanamycin (K30), Streptomycin (S10). The laboratory strain *B. subtilis* 168 was used as a control.

STRAIN	Antibiotic Susceptibility							
	TEC30	VA30	C30	TE30	E15	CN10	K30	S10
B. sub 168	S	S	S	S	S	S	S	S
FI314	S	S	S	S	S	S	S	I
FI326	S	S	S	S	S	S	S	S
FI330	S	S	S	S	S	S	S	S
FI347	S	S	S	S	S	S	S	S
FI353	S	S	S	S	S	S	S	S
FI354	S	S	S	S	S	S	S	S
FI357	S	S	S	S	S	S	S	S
FI368	S	S	S	S	S	S	S	S
FI376	S	S	S	S	S	S	S	S
FI424	S	S	S	S	S	S	S	S
FI429	S	S	S	S	S	S	S	S
FI436	S	S	S	I	S	S	S	S
FI442	S	S	S	S	S	S	S	S
FI480	S	S	S	S	S	S	S	I

S- Sensitive; I- Intermediate

4. Discussion

It is currently assumed that the commensal gut microbiota can be modulated by nutritional, genetic and environmental factors (Gomez and Balcazar 2008, Perez *et al.* 2010, Maslowski and Mackay 2011), but the exact mechanisms behind these influences are yet to be fully understood. For example, from all the literature found, this is the first report evaluating the effect of the same diet on the gastrointestinal microbial community of two fish species with different feeding behaviours (gilthead sea bream- carnivorous vs white sea bream- omnivorous). Both sea bream species reared for six weeks with the same commercial diet and under optimal rearing conditions, experienced growth rates similar to the ones described by others, in which gilthead sea bream juveniles (Venou *et al.* 2003, Enes *et al.* 2008) showed higher growth rates than white sea bream juveniles (Ozorio *et al.* 2006, Sa *et al.* 2008c), despite of these ones having a larger growth rate than the first ones if comparing their larval stage (Abellan and Garcia-Alcazar 1995).

Some studies in terrestrial organisms have reported diet as a controlling factor of gut microbial diversity (Ley *et al.* 2008a, Yun *et al.* 2014, Graf *et al.* 2015), and this observation also applies to fish, where diets modulate the gut microbiota (Kormas *et al.* 2014, Larsen *et al.* 2014, Li *et al.* 2014, Perez-Cobas *et al.* 2015, Zarkasi *et al.* 2016). The DGGE analysis of the gastrointestinal microbial community of both fish species under study, revealed that white sea bream maintained the similarity values between replicates, while gilthead sea bream raised the similarity percentage from the beginning to the end of the trial, when subjected to a carnivorous commercial diet. In agreement with our results, Cerezuela *et al.* (2013) has subject gilthead sea bream to a commercial diet and reported a higher similarity between replicates (~75%) after 4 weeks of trial. Additionally, it has been demonstrated that in mammals (Ley *et al.* 2008a) and also in fish (Ward *et al.* 2009, Larsen *et al.* 2014) the microbial diversity in the gastrointestinal tract increases from carnivorous to omnivorous to herbivorous when animals are subject to their own diet. In accordance with this, we observed that the omnivorous species (white sea bream), although being fed with a carnivorous diet, showed higher values in species richness, diversity and OTUs (both in digesta and mucosa samples) than the carnivorous, gilthead sea bream. Opposing the raw values of every parameter of the intestinal digesta against the ones from the intestinal mucosa, reveals lower richness and diversity indices in the mucosa associated microbiota, as already shown in other studies with different fish species (Kim *et al.* 2007, Wu *et al.* 2012, Gajardo *et al.* 2016), indicating the poor fraction of bacteria present in the intestinal digesta with power to colonize the intestinal mucosa layer (Kim *et al.* 2007, Gajardo *et al.* 2016).

Studies performed in gut samples of terrestrial mammalians display a dominance of the Firmicutes and Bacteroidetes phylum (Ley *et al.* 2008b, Qin *et al.* 2010), but in our study, although a dominance of Firmicutes is maintained, Bacteroidetes were replaced by Proteobacteria, as also shown in other studies performed on fish like gilthead sea bream (Kormas *et al.* 2014, Estruch *et al.* 2015), rainbow trout (Kim *et al.* 2007) and grass carps (Han *et al.* 2010, Wu *et al.* 2012, Larsen *et al.* 2014). Among the phyla previously described, the microorganisms found in the gastrointestinal tract of the fish species analysed in this study were closely related to bacteria belonging to *Lactococcus*, *Vibrio*, *Enterococcus*, *Pseudomonas*, *Acinetobacter*, *Luteimonas*, *Lysobacter* and *Bacillus* genera.

Lactic acid bacteria, such as *Lactococcus* spp., are commonly present in the gut of healthy fish and have received special attention due to their beneficial effects as probiotics, by preventing the proliferation of opportunistic bacteria, which is important in the health-maintenance of industrial animal farms (Kesarcodi-Watson *et al.* 2008, Perez *et al.* 2010). *Vibrio* spp. are usually found in aquatic environments, being often isolated from the intestine of marine species (Perez *et al.* 2010), and even though some *Vibrio* species are pathogenic to fish, others, such as *V. algynoliticus* are known for their beneficial characteristics, like the competitive exclusion of opportunistic pathogens (Gatesoupe 1999, Thompson *et al.* 2010, Hai 2015). *Enterococcus* and *Pseudomonas*, also previously found in fish intestinal contents (Perez *et al.* 2010), are usually associated with important fish and human infections (Frans *et al.* 2011, Austin and Austin 2012), although some strains have already been reported as potential probiotics (Hai *et al.* 2007, Hai 2015). The presence of soil and water bacteria (*Acinetobacter* sp., *Luteimonas aquatica*, *Lysobacter dokdonensis* and also the cyanobacteria *Calothrix desertica*) could be attributed to the ingestion of the surrounding water, which came directly from the sea and probably carrying these organisms. Finally, *Bacillus species* and in particular *Bacillus subtilis,* are known as beneficial due their antimicrobial activities against a broad-range of pathogenic species and are also frequently detected in the gastrointestinal tract of different animals, including humans and fish. Since *Bacillus* species are capable of entering the gastrointestinal tract associated with food or in water, germinate and grow in the gut (Casula and Cutting 2002, Barbosa *et al.* 2005, Tam *et al.* 2006, Hong *et al.* 2009), the presence of these organisms in gut samples was somehow expected.

Although the DGGE method was proven as an effective method of microbiota diversity analysis, it is not able to quantify the exact amount of each taxon present in each sample and should therefore be used as an indication and not an absolute proof of the real diversity degree of microbial communities (Ercolini 2004, Kim *et al.* 2007). In the present study, patterns of the same species and same sampling time showed differences

in proportions, like observed in previous studies (Zhu *et al.* 2002, Kim *et al.* 2007), proving how this method can severely underestimate bacterial diversity. A powerful approach to overcome the limitations of DGGE method is the use of metagenomics (Simon and Daniel 2009), which, by high-throughput sequencing, allows a greater understanding of the real gut microbial diversity.

The intestinal microbiota is considered a pool of potential probiotics with important biological functions in the animal industry sector (Perez *et al.* 2010, Roeselers *et al.* 2011, Larsen *et al.* 2014). *Lactobacillus* spp., *Bacillus* spp., *Vibrio* spp. and also *Pseudomonas* spp. have been investigated for their promising antibacterial actions against pathogens and their application as probiotics in the field (Gatesoupe 1999, Verschuere *et al.* 2000, Gomez and Balcazar 2008, Salinas *et al.* 2008). One important criteria used in strain selection is the capacity to minimize pathogens growth by competitive exclusion or production of antimicrobial molecules (Verschuere *et al.* 2000, Cutting 2011, Dobson *et al.* 2012, Hai 2015). In this study, we successfully isolated from the gut of white sea bream, gilthead sea bass and European sea bass, sporeforming *Bacillus* species to be tested for their potential as producers of natural antimicrobials or NACs.

Although *Bacillus* spp. were traditionally considered soil organisms, their continuous isolation from water environments and most importantly, from the gastrointestinal tract of several animals including fish, let to the current believe that *Bacillus* spores comprise their natural life-cycle inside the animal gut (Casula and Cutting 2002, Barbosa *et al.* 2005, Tam *et al.* 2006, Hong *et al.* 2009, Zhou *et al.* 2014). Thus, it was not surprising that a great variety of spores (based on morphological differences) could be isolated from the faecal samples examined, further suggesting that these organisms may play an important role in the microbial balance of the gastrointestinal tract in aquatic animals.

By sequencing the 16S rRNA gene, all identified isolates were assigned to the *Bacillus* genus, being *Bacillus subtilis* the most prevalent species (>50%). Identification to the species level was, as expected, not possible in some isolates, since the use of a single molecular marker (such as 16S rRNA) limits the taxonomic analysis in close relative species groups (Maughan and Van der Auwera 2011, Tu and Lin 2016). This was the case of isolates FI333, FI335, FI367 and FI429 belonging to the *B. subtilis* clade (*B. subtilis, B. vallismortis, B. mojavensis, B. atrophaeus, B. amyloliquefaciens, B. methylotrophicus, B. licheniformis, B. sonorensis* and *B. tequilensis*), and isolates FI324 and FI326 that can belong to the *B. subtilis or B. cereus* clade (*B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis and B. cytotoxicus*) (Connor *et al.* 2010, Bhandari *et al.* 2013).

Many *Bacillus* species, including the ones currently used as human and animal probiotics, are known to produce Natural Antimicrobial Compounds (NACs) capable of minimizing or inhibiting the pathogens growth and proliferation (Duc *et al.* 2004, Hong *et al.* 2005, Abriouel *et al.* 2011, Cutting 2011). In accordance with our approach, many strains with potent and broad range inhibitory activity have been isolated from the gut of the target animals such as humans, broiler chickens or pigs (Barbosa *et al.* 2005, Guo *et al.* 2006, Fakhry *et al.* 2008, Ahire *et al.* 2011, Gu *et al.* 2015). In fact, six earlier studies have reported the isolation of *Bacillus* spp. from the gastrointestinal tract of fish with inhibitory capacity against important fish pathogens, with special focus on *Aeromonas* species (Newaj-Fyzul *et al.* 2007, Ramesh *et al.* 2015, Thankappan *et al.* 2015, Banerjee *et al.* 2016, Chen *et al.* 2016, Nandi *et al.* 2016). In this study, we went further in our investigation and observed that more than 50% of the *Bacillus* strains that were found associated with the gut of marine-fish species are active against at least one of the tested pathogenic strains, suggesting that the fish *Bacillus* community may have an important role in protecting its hosts against opportunistic bacteria. Based on consecutive screenings we were able to select 14 promising isolates, exhibiting inhibitory actions against gram positive and gram negative fish pathogens. Only the growth of *A. salmonicida* was not affected by any of the isolates, suggesting that this species may be resistant to the antimicrobial compounds produced by all *Bacillus* spp. tested, which emphasises the high-level of resistance that this bacteria displays over antibiotics and other compounds, and its persistence inside the host (Dallaire-Dufresne *et al.* 2014, Menanteau-Ledouble *et al.* 2016).

Besides showing potent and broad antimicrobial capacity, the 14 *Bacillus* isolates were susceptible to a series of antibiotic classes, including the ones demanded by EFSA as mandatory to comply with minimal safety requirements (EFSA-FEEDAP 2012,Cabello *et al.* 2016). This is particularly important because the extensive use of antibiotics as prophylactic and therapeutic agents in animal husbandry in general, and in aquaculture in particular, has contributed to the emergence of antibiotic resistance genes among bacteria, leading to environmental, animal and human health problems (Cabello *et al.* 2016). And although the increasing concern about this thematic along the years had resulted in the development and use of probiotics as a prophylactic approach, some studies indicate a mislabelling of the bacterial strains included in probiotic products, and in some cases, the inclusion of strains harbouring multidrug resistances (Hoa *et al.* 2000, Duc *et al.* 2004). For example, analysis to a probiotic used in Vietnamese shrimp farms revealed the presence of antimicrobial resistance genes against important antibiotics used in humans and animals (Noor Uddin *et al.* 2015). The fact that the sporeformers

selected in our study do not possess any antimicrobial resistance, strongly suggests that these are putatively safe to incorporate a future probiotic product.

It is known that many of the NACs produced by *Bacillus* and other bacterial species are extracellular molecules released to the surrounding environment (Abriouel *et al.* 2011, Egan *et al.* 2016). Some recent studies have reported *Bacillus* spp. isolated from a diversity of ecosystems (fish gut, marine sponges, sediments and water) as producers of extracellular compounds capable of inhibiting important fish pathogens such as *Aeromonas salmonicida*, and other *Aeromonas* species (Newaj-Fyzul *et al.* 2007, Phelan *et al.* 2013, Thankappan *et al.* 2015, Banerjee *et al.* 2016, Nandi *et al.* 2016), *V. anguillarum*, *V. harveyi* and other *Vibrio* species (Vaseeharan and Ramasamy 2003, Touraki *et al.* 2012, Phelan *et al.* 2013, Liu *et al.* 2015, Chen *et al.* 2016), *P. damselae* (Vaseeharan and Ramasamy 2003, Touraki *et al.* 2012), and also *S. aureus* (Touraki *et al.* 2012, Liu *et al.* 2015). In agreement with these studies, our results highlight the antimicrobial activity in the cell-free supernatant of FI314, FI330 and in a small extent FI442 against the different pathogenic strains tested, suggesting that the inhibitory molecule(s) possess an extracellular nature. By using two independent cell-free supernatant tests, a microplate growth inhibition assay and a well-diffusion assay, it became evident the power of FI314 and FI330 NACs against *S. aureus*, *V. harveyi*, *P. damselae*, and *A. bivalvium*. It was also possible to observe some influence on the bacterial growth of *A. veronii* and *V. anguillaru*m, although not sufficient for a complete inhibition. The lack of activity against *A. salmonicida* was in agreement with the results observed in the initial screenings.

Although we cannot rule out that the other isolates tested might be producing unstable extracellular molecules, from our observations we can assume that the 3 sporeforming isolates mentioned FI314, FI330 and FI442 are both exporting their antimicrobial molecules to the surrounding environment and producing a more resilient molecule that does not degrade or loses function when the centrifugation/filtration procedures are applied.

Adding to pathogens growth and proliferation, other important bacterial characteristics are known to promote resilience to antimicrobial treatments and capacity to cause disease. That is the case of biofilms, which are bacterial aggregates characterized by their high tolerance to stress situations, such as a higher resistance to conventional antibiotics due to horizontal gene transfer, being therefore associated to chronic and re-emerging diseases with particular significance in the medical and industrial fields (Dusane *et al.* 2013, Nastro *et al.* 2013, Flemming *et al.* 2016). *Bacillus* species have been reported as effective in controlling biofilm formation of a broad range of pathogens, like *Aeromonas* and *Vibrio* species, *E. coli*, *S. aureus* and other

opportunistic bacteria (Nithya *et al.* 2011, Sayem *et al.* 2011, Dusane *et al.* 2013, Nastro *et al.* 2013, Wu *et al.* 2013, Shanthi *et al.* 2016). In the present study, the extracellular bioactive compounds of FI314, FI330 and FI442, were able to significantly reduce the biofilm formation of *A. salmonicida*, *A. veronii* and *P. damselae.* The antimicrobial compounds possess a variety of modes of action (Dobson *et al.* 2012) that may not directly inhibit the bacterial growth, but reduce the pathogen defence mechanisms, such as biofilms, and therefore increase the chances of controlling their proliferation. This is the particular case of *A. salmonicida*, in which the tested NACs were not capable to control its bacterial growth, but significantly reduced its biofilm formation, opening the possibility to develop a new prophylactic and/or therapeutic approach to deal with this problematic fish pathogen and zoonotic agent.

6. Conclusions and Future Perspectives

The work presented in this dissertation is a relevant contribution to the understanding of the gut microbial diversity found in marine aquaculture fish species with different feeding habits. When comparing the omnivorous white sea bream with the carnivorous gilthead sea bream, we observed that the intestinal microbiota similarity between replicates significantly increased in gilthead sea bream along time, suggesting that the carnivorous fish gut microbiota became more homogenous than the omnivorous fish gut microbiota. Additionally, we also observed that, in accordance with previous studies, there is a trend for a richer and more diverse gut microbiota of omnivorous species (white sea bream), although being fed with a carnivorous diet, than the carnivorous gilthead sea bream. Since some studies have reported that besides feeding habits, the host genetic background might have a great influence on the gut microbiota, it would be interesting in a future study to analyse if two genetically apart species converge their gut microbiota when subject to the same diet.

The results here presented also revealed that when applying a culture-dependent and selective analysis of the gut microbiota, a great diversity of sporeformers, in particular *Bacillus* spp., can be found in association with the gastrointestinal tract of fish species with different feeding habitats. Moreover, more than 50% of this endosporeforming community shows a capacity to produce natural antimicrobial compounds (NACs) active against different important fish pathogens, known to cause severe diseases and economic losses to the aquaculture sector. The present study allowed the selection of three fish gut isolates, FI314, FI330 and FI442 with important characteristics including the production of antimicrobial and anti-biofilm extracellular compounds, and the absence of antimicrobial resistances. From the preliminary identification based on the 16S rRNA gene sequence, all the strains are *B. subtilis*, a species generally regarded as safe (GRAS status; EFSA-FEEDAP (2012)), highlighting their probiotic potential.

Based on these *in vitro* tests, the three strains seem to be potentially good candidates to be used as probiotics or as source of bioactive molecules able to antagonize important bacterial fish pathogens. To achieve such a goal, a series of further tests need to be performed. For instance, the purification of the extracellular compounds responsible for the antimicrobial and biofilm activities observed, will allow to identify and fully characterize the NACs and respective properties, further elucidating the potential to be used as disease-preventive molecules in the aquaculture field. Taking in consideration that bacteriocin-like substances are also capable of suppressing pathogens quorum-sensing (cell-cell communication), another objective of this work is to

test the entire collection of gut sporeformers for the production of anti-quorum-sensing (or quorum-quenching) molecules, by using biosensors (Ng and Bassler 2009). Once the full characterization of such important compounds is achieved, *in vivo* tests will allow to evaluate their safety and effectiveness in preventing the occurrence of bacterial diseases in important aquaculture fish species, using challenging experiments.

The publication of a manuscript in a peer-reviewed journal, gathering all the work and results presented in this dissertation, is currently under preparation.

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7. Annexes

7.1 Solutions composition

B&W salts:

- KH_2PO_4 pH 7.2 : 7.6 g
- K_2HPO_4 pH 7.2: 12.4 g
- Sodium citrate: 1 g
- $(NH_4)_2SO_4$: 6 g
- ddH_2O to 1000 mL

GES solution:

- guanidine thiocyanate: 60 g
- EDTA 0.5M, pH 8.0: 20 mL
- 10% N-lauroysarcosine solution: 5mL
- ddH_2O to 100mL

7.2. Supplementary tables

Table S1. Zone diameter breakpoints for Gram-positive bacteria using the standardized disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute).

Antibiotic name	Disc code	Disc content (µg)	Zone diameter (mm)		
			Resistant (R)	Intermediate (I)	Sensitive (S)
Teicoplanin	TEC	30	<= 10	11-13	>=14
Vancomycin	VA	30	<=11	11-14	>=15
Chloramphenicol	C	30	<=12	13-17	>=18
Tetracycline	TE	30	<=14	15-18	>=19
Erythromycin	E	15	<=13	14-22	>=23
Gentamycin	CN	10	<=12	13-14	>=15
Kanamycin	K	30	<=13	14-17	>=18
Streptomycin	S	10	<=10	11-14	>=15