Identification, phenotypic characterization and selection of gilthead seabream (*Sparus aurata*) associated bacteria for application as putative probiotics in fish larviculture

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Preface

The work presented in this thesis was performed at the Biological Sciences Research Group, Department of Bioengineering, Instituto Superior Técnico (Lisbon, Portugal), and at Estação Piloto de Piscicultura de Olhão - Instituto Português do Mar e da Atmosfera (EPPO-IPMA) during the period of September 2020 to September 2021, under the supervision of Prof. Dr. Rodrigo da Silva Costa and Dr. Nuno Miguel Formiga Borges. This thesis was supported by the Portuguese Foundation for Science and Technology (FCT), through the project PTDC/BIA-MIC/31996/2017 and through strategic funding provided to the Institute of Bioengineering and Biosciences (project UIDB/04565/2020).

This work contributed for a scientific poster presentation at the 1st Microbiome PT Summit by BioData.pt and ELIXIR-PT, Online event, February 4, 2021, entitled “Identification and characterization of bacteria with hydrolytic and antibacterial activities isolated from gilthead seabream (*Sparus aurata*) for application as probiotics in larviculture”, and a future scientific poster presentation at the Congress of Microbiology and Biotechnology by Nova University Lisbon, Online event, November 23-26, 2021, entitled “Identification, phenotypic characterization and selection of gilthead seabream (*Sparus aurata*) associated bacteria for application as putative probiotics in fish larviculture”. This work also resulted in the preparation of a scientific manuscript to be submitted for publication, named “Identification, phenotypic characterization and selection of gilthead seabream (*Sparus aurata*) associated bacteria for application as putative probiotics in fish larviculture”.

I confirm that the Portuguese ethical policies regarding the handling of animals for scientific studies were met. Direcção Geral de Alimentação e Veterinária (DGAV) accredited the personnel responsible for rearing and sampling the fish larvae used in this study. The methodology privileged animal well-being through minimally invasive handling and sampling procedures, following the guidelines of the European Directive 2010/63/EU and of the Portuguese national legislation (Decreto-Lei n.º 113/2013) on the protection of animals used for scientific purposes.

I declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly mentioned in the text and are included in the list of references.

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To all co-workers and friends from the MicroEcoEvo team my honest thank you, not only for making me feel at home and for all the funny game nights, which helped de-stress, but also for aiding and helping me with any and every information needed during my course.

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Abstract

Probiotics supplementation is a promising strategy to control pathogens in aquaculture, particularly during larviculture where the fish's immune system is underdeveloped. In this thesis, bacterial symbionts isolated from gilthead seabream (*Sparus aurata*) eggs, larvae, and juveniles were selected, based on taxonomical and physiological criteria, for their potential use as probiotics, and the best candidates assessed during a fish larvae rearing trial.

The studied symbionts (97 isolates) were classified into 32 genera in the *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla. Thirty-five non-pathogenic (based on current literature) and non-redundant isolates were characterized for hydrolytic enzyme (chitinases, proteases, amylases, lipases, and cellulases) production and antagonistic activity towards bacterial pathogens of fish. The isolates *Phaeobacter inhibens* L23 (best pathogen antagonist) and *Arthrobacter agilis* E13 (versatile producer of hydrolytic enzymes) were selected as co-inoculants of fish eggs and live feed (rotifers) and evaluated as probiotics in gilthead seabream larval rearing.

Using 16S rRNA gene amplicon sequencing, a decrease in abundance of opportunistic taxa (e.g., *Vibrionaceae*) and increase in abundance of putatively beneficial symbionts (e.g., *Rhodobacteraceae* species) was observed in probiotic-treated rotifers. While no differences in dry weight and length were observed between fish larvae fed probiotics-treated *versus* control rotifers, slightly higher larval survival rates were recorded under probiotic treatment by the end of the rearing trial. These results suggest that modulation of the rotifer-associated microbiome through co-inoculation of the here selected probiotics is feasible, yet further research is needed to improve fish larval wellbeing using rotifers as delivery systems of beneficial bacteria to fish.

Keywords: Antimicrobial activity; Aquaculture; Hydrolytic activity; Microbiome; Rotifers

Resumo

Suplementação probiótica é uma estratégia promissora para controlar patógenos na aquacultura, particularmente quando o sistema imunológico do peixe está subdesenvolvido (larvicultura). Nesta tese foram selecionados simbiontes bacterianos isolados de ovos, larvas e juvenis de dourada (*Sparus aurata*), baseado em critérios taxonómicos e fisiológicos, para o seu potencial uso como probióticos, e os melhores candidatos foram testados em larvicultura.

Os simbiontes estudados (97 isolados) foram classificados em 32 géneros dos filos *Proteobacteria*, *Firmicutes*, *Bacteroidetes* e *Actinobacteria*. Foram caracterizados 35 isolados não patogénicos (baseados na literatura atual) e não redundantes para a produção de enzimas hidrolíticas (quitinases, proteases, amílases, lípases e celulases) e atividade antagonista contra patógenos de peixes. Os isolados *Phaeobacter inhibens* L23 (melhor antagonista) e *Arthrobacter agilis* E13 (produtor enzimático versátil) foram selecionados para inocular ovos e alimento vivo (rotíferos) de dourada e testados como probióticos em larvicultura.

Através da sequenciação do gene 16S rRNA, observou-se uma diminuição da abundância de taxa oportunista (por exemplo, *Vibrionaceae*) e o aumento da abundância de simbiontes putativamente benéficos (por exemplo, espécies da *Rhodobacteraceae*) em rotíferos tratados com probióticos. Embora não tenham sido observadas diferenças no peso seco e no comprimento entre as larvas alimentadas com rotíferos tratados com probióticos *versus* controlo, taxas de sobrevivência larvais ligeiramente superiores foram registadas nas larvas tratadas. Estes resultados sugerem que a modulação do microbioma associado ao rotífero através da inoculação simultânea dos probióticos selecionados é viável, no entanto, são necessárias mais investigações para melhorar as larvas utilizando este sistema de entrega (rotíferos) de probióticos aos peixes.

Palavras-Chave: Atividade antimicrobiana; Aquacultura; Atividade hidrolítica; Microbioma; Rotíferos

List of Abbreviations

EPPO Estação Piloto de Piscicultura de Olhão

IPMA Instituto Português do Mar e da Atmosfera

FTS Flow-Through System

MMS Microbial Matured System

RAS Recirculating Aquaculture System

IMTA Integrated Multitrophic Aquaculture

FAO Food and Agriculture Organization of the United Nations

NGS Next Generation Sequencing

GI Gastrointestinal

VFs Virulence Factors

MRLs Maximum Residue Levels

EU European Commission

EMA European Medicines Agency

Codex FAO's Codex Alimentarius Commission

FFA Free Fatty Acids

ISAPP International Scientific Association for Probiotics and Prebiotics

min minute

h hour

s second

CC Colloidal Chitin

EAI Enzymatic Activity Index

OD Optical Density

TC-DNA Total Community DNA

DAH Days After Hatching

zOTU zero-radius Operational Taxonomic Unit

TL Total length

DW Dry Weight

PCoA Principal Coordinates Analysis

AAI Antagonistic Activity Index

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1. Introduction

1.1. Aquaculture

One of the greatest challenges that society has been facing over the past few years is achieving sustainable development goals that protect earth's ecosystems and at the same time allow humans to prosper. Several technologies have been developed or improved to fulfil the requirements for sustainable management of natural resources or food production. One example is the case of aquaculture (**Figure 1**) [1].

In the last few decades, aquaculture has grown into a global practice, playing a major role in global economy, food security and as a source of income, supporting around 200 million livelihoods [2]. Modern aquaculture is defined as the rearing of aquatic organisms, under controlled or semi-controlled conditions, where the organisms are provided with live and/or formulated feed, with the final intent of providing human food, promoting recreational fishing, preserving fish stocks of commercial value, among other applications [3].

Figure 1. Aquaculture station at Estação Piloto de Piscicultura de Olhão (EPPO), Algarve.

Facing climate change and a growing global population, as well as an increase in food demand, feeding future generations will most certainly emerge as a challenge, due to the depletion of marine natural resources (overexploitation) or pollution. Among several measures that need to be taken to confront this problem, while preventing depletion of earth's ecosystems, is the development and optimization of aquaculture techniques. Proper use of aquaculture practices may not only allow the recovery of endangered fish species, but also provide an efficient response to the increasing fish demand around the globe [1, 3, 4].

In aquaculture, the recent increase of species reared and the evolution of different forms of fish culturing demonstrate a clear trend and effort to provide a wide range of high-quality products to consumers, thus allowing for a vast consumer's choice and a sustainable environmental protection [5]. **Figure 2** presents some of the worlds most reared finfish [3] and the evolution of aquaculture supply contribution from 1950 to around 2005 [4].

Common name	Scientific name
African catfish	*Clarias gariepinus*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Bighead carp	*Aristichthys nobilis*
Bigmouth buffalo	*Ictiobus bubalus*
Black crappie	*Pomoxis nigromaculatus*
Blue catfish	*Ictalurus furcatus*
Blue tilapia	*Oreochromis aureus*
Bluegill	*Lepomis macrochirus*
Brook trout	*Salvelinus fontinalis*
Brown trout	*Salmo trutta*
Catla	*Catla catla*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Chum salmon	*Oncorhynchus keta*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Fathead minnow	*Pimephales promelus*
Gilthead sea bream	*Sparus aurata*
Goldfish	*Carassius auratus*
Grass carp	*Ctenopharyngodon idella*
Largemouth bass	*Micropterus salmoides*
Milkfish	*Chanos chanos*
Mossambique tilapia	*Oreochromis mossambicus*
Mrigal	*Cirrhinus mrigala*
Mud carp	*Cirrhina molitorella*
Muskellunge	*Esox masquinongy*
Nile tilapia	*Oreochromis niloticus*
Northern pike	*Esox lucius*
Pacu	*Colossoma metrei*
Pink salmon	*Oncorhynchus gorbuscha*
Plaice	*Pleuronectes platessa*
Rabbitfish	*Siganus* spp.
Rainbow trout	*Oncorhynchus mykiss*
Red drum	*Sciaenops ocellatus*
Rohu	*Labeo rohita*
Sea bass	*Dicentrarchus labrax*
Shiners	*Notropis* spp.

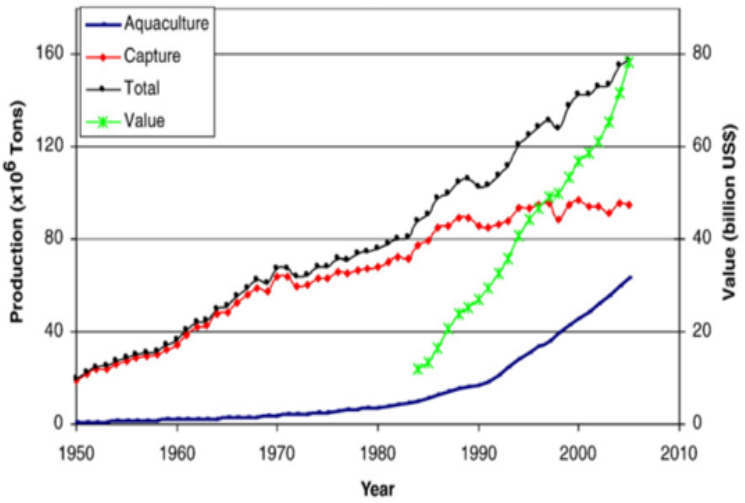


Figure 2. Common and scientific names of selected aquaculture species. Adapted from Stickney, (2000) (Left panel) [3]. Trend line showing aquaculture's contribution to the global supply of fish biomass for human consumption along the past decades. Image adapted from Sapkota *et al.*, (2008) (right panel) [4].

As visible, in this timeline (1950-2005) there is a clear finfish capture stabilization, mainly as from the 1990's, with an outstanding increase in the finfish value as well as the aquaculture production. Remarkably, the finfish market value rose from less than 20 billion US dollars to around 80 billion US dollars in this period. That shows an astonishing increase of 300% in value, in a little more than 50 years.

1.1.1. Aquaculture systems and operations

Aquaculture can be practiced in three different settings such as inland (mainly freshwater conditions) [6], coastal (mainly brackish conditions) [7] and open ocean (marine water) [8]. The features of an aquaculture system vary from fish to fish, since different fish require distinct salt concentrations, nutrients or water, and unique space conditions. The water salinity used in aquaculture for finfish rearing is dependent on these conditions and therefore may be of marine, brackish, or freshwater origin [5, 9].

However, there are many other characteristics that should be taken into consideration when planning aquaculture practices, that define the farm environment. For instance the temperature of the water (warm or cold), the physiographical zone (inland, coastal or marine) as well as the available area for the aquaculture practice and the water source (rain or tide fed farm, spring, seepage or ground water, sewage or diverted water) [5, 9].

Diverse rearing systems are used in aquaculture, and the design of the rearing system is to a large extent determined by the setting (inland, coastal, and open ocean) in which the aquaculture practice takes place. A few examples are: (i) Flow-Through System (FTS), with an inlet and outlet, maintaining a continuous water flow-through, hence preserving the required levels of water quality; (ii) Microbial Matured System (MMS), which is a modification of the FTS, through the implementation of a "hygienic barrier" usually obtained through disinfection using UV/Ozone or ultra-filtration of the intake water. In this type of system there is a bio filter (filter specially conceived for the growth of bacteria) with the intent of performing, for example, nitrification (biological oxidation of ammonia to nitrite followed by the oxidation of the nitrite to nitrate); (iii) Recirculating Aquaculture System (RAS), where there is a high percentage of water reusage (>90%). This system is a combination of mechanical processes (particle removal) and biological processes (*e.g.,* nitrification in a bio filter) [10]; (iv) Integrated Multitrophic Aquaculture (IMTA), where a strategy to combine several complementary organisms (*e.g.,* combination of filter-feeding organisms, such as shellfish, and seaweeds as organic and inorganic nutrient extractors, respectively) to optimize nutrient utilization and reduce solid waste is implemented thus recycling the fish alimentary residues [11]. One rearing example of commercially valuable fish species is the salmon or trout rearing, either through FTS or RAS [12, 13]. It begins with spawning, egg fertilization, incubation, development, and growth from egg to smolt (juvenile salmon), conducted in hatcheries located near to a source of high-quality freshwater. The hatchery production begins by combining and stripping eggs from brood stock. Then, the smolts are transferred to marine cultivation centers for supporting their growth (around 8-14 months, depending on species, water temperature and commercial targeted weight). After the fish reach the desired market weight, they are prepared for processing, selling, and finally, for consumption [13].

The fish feeding process is mainly composed by live feed (*e.g.,* rotifers, copepods, *Artemia* and microalgae) and formulated diets, which are the primary sources of bacteria, alongside with incoming water [10]. In fish hatcheries, where the conditions are considerably controlled, feeding accounts for 50-70% of production costs. Due to the eating behavior of the fish species, there is a considerable oral and feed waste manipulation that allows fish to have a more stable growth and longer survival, by removing contaminants in the water, which can be in the form of excessive food or the presence of organic matter, for instance fish feces. It also enables the producers to control the food waste, thus cutting unnecessary economical losses [14].

1.1.2. Aquaculture drawbacks

As previously mentioned, aquaculture has an environmentally positive effect on the worldwide stock of marine fish and presents an economic advantage when compared with traditional fishing. However, the rapid expansion of this industry also revealed to be problematic, eliciting side-effects of concern such as seasonal oversupply, disease, pollution, genetic deterioration, among others [2, 5, 15]. Many aquaculture pollution incidents, besides leading to environmental pollution, also lead to economic losses (*e.g.,* losses of batches due to poor storage or pathogenic infections) [5, 15]. There are four primary environmental effects of aquaculture:

(i) organic pollution and eutrophication (*e.g.,* excessive growth of marine plants, affecting the normal and desirable use of water) through the production of organic waste (*e.g.,* fish waste and uneaten feed), which is released into nature. In this category is also included the continuous discharge of untreated water, a source of nitrogen and phosphorus, which may lead to chronic elevation of the overall organic matter content in nature. Nonetheless, when compared with inputs derived from larger sources of nutrient pollution (*e.g.,* industrial sources of pollution), which can be substantial at a local scale, the untreated water inputs derived from aquaculture represent only a small portion. Still, this pollution could cause algal bloom, oxygen depletion, water quality reduction, death of corals and habitat destruction [5, 15].

(ii) chemical contamination, through the discharge of a variety of chemical waste used in aquaculture, including antibiotics, pesticides, water conditioners, disinfectants, among other contaminants. One hazard associated with these pollutants, mainly antibiotics, is the emergence of antibiotic resistant microbes. Many of the antibiotic resistant microbes that thrive in this adverse environment are pathogenic to fish, through various possible mechanisms such as toxin production and depletion of nutrients available to the host [5, 15].

(iii) use of low-value fish feed. The use of low-value fish as feed ingredients (*e.g.,* smaller fish captured in the wild) [16] to raise fish might not only affect indirectly aquatic ecosystems far away from the fish farm, due to the release of nitrogen and phosphorous into the environment, but can also directly influence these ecosystems by reinstating the same problem that aquaculture is trying to solve, which is the pressure on the stock of wild marine populations. To evade this problem, research on feed formulations with alternative protein sources (*e.g.,* insects) has been one of the key topics in aquaculture research and optimization [17];

(iv) biological contamination, which occurs when, for example, pathogenic bacteria are released into the environment. This contamination may disrupt the ecosystem through the fast multiplication and spread of these contaminants eventually causing disease outbreaks or harming other species [5, 15].

Another common drawback in aquaculture is the genetic deterioration of fish. The use of genetic engineering to produce genetically modified fish as a means of obtaining highly desired unnatural attributes (*e.g.,* specific fish genders, increased growth rate and greater tolerance towards higher temperatures and lower oxygen levels) [18, 19] may lead to a certain degree of genetic degradation (that is, loss of genetic diversity) within the reared species/population, although several generations under the same conditions are usually required for it to occur. This degradation could also happen due to an intense rearing environment, with monotonous conditions, independently of genetic engineering. This downside is mainly caused due to breeding mismanagement and might result in slow growth, poor quality of edible portions and low disease resistance of the reared fish. A related concern to this drawback is the unintentional release (escape) of these genetically modified fish into the wild, which may well spread unique diseases or parasites [5, 15, 20], and may even outcompete the native species [21]. In 2007, Krkošek *et al.*, reported that 99% of the native pink salmon (*Oncorhynchus gorbuscha*) population, in British Columbia, could collapse within eight years, due to the release or escape of farmed salmons infected with salmon lice (*Lepeophtheirus salmonis*) into the environment [22].

The creation of strict laws, regulations, and international oversight (*e.g.,* Food and Agriculture Organization of the United Nations, FAO) was established to avoid these situations and eventual ecological disasters (*e.g.,* native species population decline or specific inheritance of genetic disorders, for example deformations) [5, 15, 22, 23]. In economic terms, one of the main disadvantages of aquaculture is the high mortality rate in larval stages [24-26], mainly due to intensive rearing, infections or toxins produced by invasive pathogenic microbes. These pathogens take advantage of the fishes' most unfavorable stage, where the fish microbiome is developing and host animals are most susceptible to disease since their immune system is not completely developed [25, 27].

1.2. Symbiotic microbial communities

Symbiogenesis or endosymbiotic theory explains the similarity between some organelles and bacteria found in nature. This theory suggests that some organelles, such as mitochondria and chloroplasts, arose through (endo)symbiosis from an eukaryotic cell and aerobic bacteria [28]. Interactions between multicellular eukaryotes and prokaryotes started hundreds of millions of years ago and one of the most relevant scientific discoveries, in the last decades, was the revelation that a vast range of eukaryote-bacterial interactions, whether in shared ecosystems or intimate symbioses (*e.g.,* 90% of the bacterial species in termite guts are not found in other environments/hosts), dictate the ecophysiology and evolution of most multicellular organisms known to science. Since this discovery, several studies around the world were carried out to enable a deeper understanding of some of the deepest questions in current Biology: how bacteria facilitated the origin and evolution of animals; how animals and bacteria affect each other's genome; how ecological approaches can deepen our understanding of the multiple levels of animal-bacterial interaction [29, 30].

Symbiotic interactions are ubiquitous in nature, whether they are mutualistic or parasitic. In fact, these interactions can be classified into three different categories, based on whether the symbiont has beneficial, harmful, or no effect on the host: mutualism, where both the host and the symbiont are reciprocally benefited; commensalism, where the host is used by the symbiont (for the symbiont advantage) without being harmed or benefited; and parasitism, where the symbiont exploits the host as a resource, with consequent harm to the host [31].

1.2.1. Microbiomes

The aquaculture industry has become increasingly important in recent years, and high mortality rates during larviculture constitute a major bottleneck that is worthy being addressed to improve fish biomass production for human consumption. To accomplish this ambitious goal in a sustainable fashion, the scientific community is resorting to harmless and beneficial microbes to prevent fish disease, enhance fish health, and promote fish growth [2, 27]. These microbes can be isolated from specific tissues of the fish (*e.g.,* gut, skin, and gill) or from the surrounding environment (*e.g.,* water, surfaces, and food). Nevertheless, microbes from other origins (*e.g.,* terrestrial plants and animals) can also be used [24].

The microbial communities typically present in fish aquaculture systems can also be referred to as microbiomes, which may be defined as the total pool of microorganisms (including mutualist, commensal and pathogenic) present in a certain habitat, sample or host tissue, their genomes, interactions and the surrounding environmental conditions [32]. The realization that symbiotic microbial communities function as a fitness-enhancing factor to the benefit of their eukaryotic hosts was possible due to extensive studies and dedicated research of human-microbiome interactions [33, 34], plant-microbe interactions [35] and even of other animal hosts such as corals [36]. An intimate symbiotic relationship between distinct microbial consortia and humans, for instance, has by now been thoroughly described [37] (**Figure 3**).

There are many examples of co-regulation, by the host itself and its symbiotic microbes, of fundamental aspects of the host's physiology at the interface host/environment, such as the prevention of intestinal inflammatory disease [33], mediation of proneness towards obesity [38] and the prevention of alien organisms' invasion (such as bacterial pathogens) [39]. Additionally, individual variations in microbiome structure are reported to influence host health, which may be implicated in disease etiology and could also affect organismal physiology, including drug metabolism, toxicity, and nutrient absorption [30]. Therefore, it is reasonable to state that symbiotic microorganisms function as major promoters of their hosts' immune system, through diverse mechanisms. Recent studies demonstrated that human microbiome structure and composition might differ between healthy, lean and obese individuals, depending on the individual's origin and birth procedure (caesarean or natural birth), and on the origin of the sample (*e.g.,* different body sites), for example [34, 40, 41]. However, symbiosis is not restricted to humans. Due to vast ecological niches colonized by microorganisms, they present a massive variety of adaptive strategies and broad range of occurrence across several habitats and host organisms [42]. For example, microorganisms play an important role in association with plants and animals in both terrestrial and marine environments [30, 43-45].

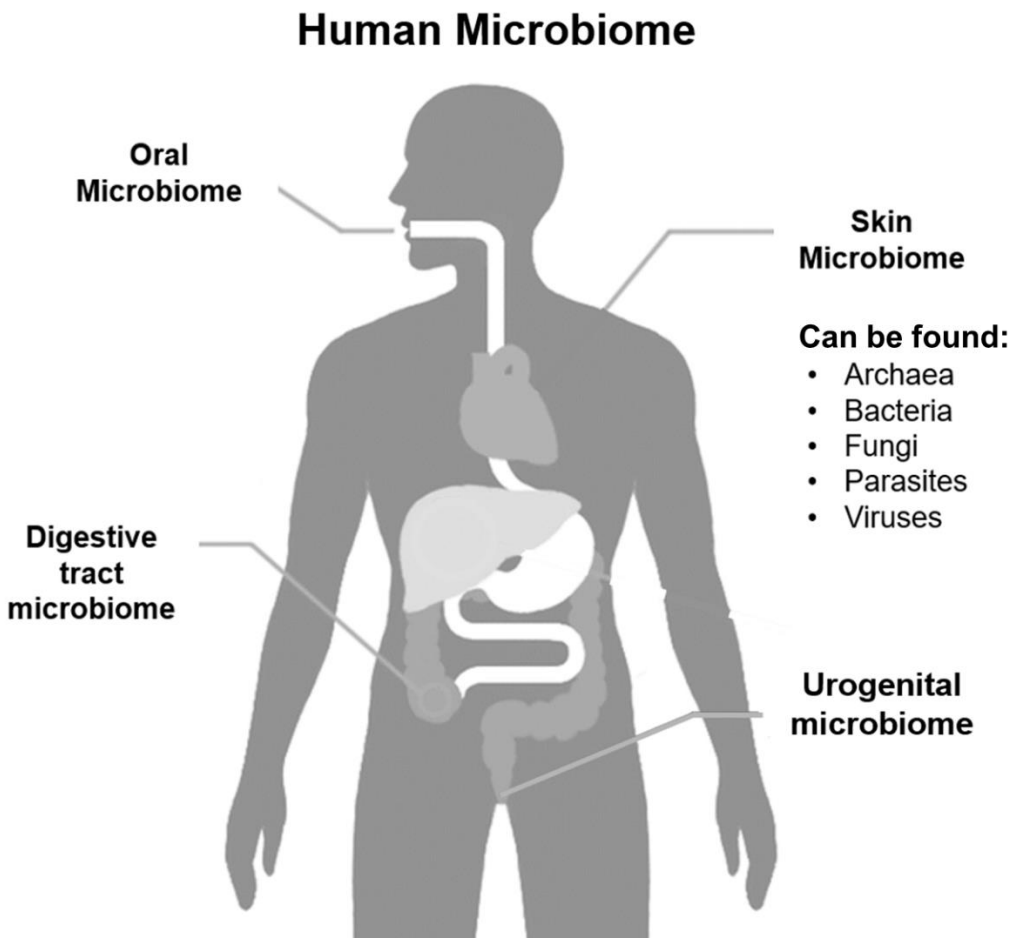


Figure 3. Representation of the human microbiome: oral microbiome, skin microbiome, urogenital microbiome, and digestive tract microbiome.

Symbiotic interactions between these hosts and their respective microbiome presumably allow ecosystems to maintain a dynamic equilibrium state, by enabling their hosts to survive, compete and evolve mainly in adverse conditions [46]. Nevertheless, despite the great developments around this theme, primarily due to new and better DNA sequencing techniques, much knowledge about microbe-microbe and/or host-microbe interactions is yet to be discovered. Due to the importance of microbiomes in the environment (*e.g.,* animals, fish, and soils) [47], better understanding of their diversity, inter-species interactions and response to changing environmental conditions is of utmost relevance [48]. To do so, the scientific community recurs to metagenomics, defined as "the study of the collective genomes recovered from environmental/host samples without prior cultivation" which allows the investigation of genome information on organisms that are not easily cultured in the laboratory. Therefore, it enables the systematic manipulation, investigation, and classification of all the genetic material obtained from environmental samples [49]. Besides the study of microbiomes in the natural environment (or in "environmental samples"), metagenomics-driven surveys of microbiomes can be conducted in animals and plants (host-associated microbiomes). Particularly, experimental studies are preferably performed using host models amenable to manipulation under controlled laboratory conditions, for instance mice as a canonical model for mammalian metabolism [48] and crop plants [50].

To conduct a microbiome study through culture-independent strategies, there are three main core workflows that one could follow: metagenomics for bio-exploration, with the goal of screening transformants for desired bioactivities [51]; 16S rRNA gene amplicon sequencing where the 16S rRNA gene is amplified for a biodiversity study; and full metagenome sequencing, where all genes are sequenced for a functional analysis [48, 52]. One example is the present study in which this Master thesis is inserted. In this thesis, 16S rRNA gene amplicon sequencing will be applied with the context of monitoring the prokaryotic communities of rotifers (*Sparus aurata* larvae live feed) inoculated with probiotics. Even so, culture-dependent techniques are extremely valuable since crucial approaches, such as probiotics or potential probiotics utilization (*e.g.,* the present study), require the ability of cultivating the microbe. Hence, expanding our ability to increasingly cultivate microorganisms from the environment is important.

1.2.2. Fish microbiome

Studies on microbial communities associated to fish started in the late 1920s. Back then, early studies of host-microbe and microbe-microbe interactions and their effects on fish were made, for example, by transplanting the skin mucosa of untreated fish, using a sterile loop, to fish whose skin mucus had been previously cleaned or “disinfected”, with, for instance, anti-microbial procedures such as absorbent cotton and sterilized water, thus removing all the slime present in the fish [53]. From then on, the study of microorganisms associated to fish has been deepened in all aspects. The recognition of the skin, gastrointestinal (GI) tract and gills as the major pathways for fish colonization by microorganisms (both pathogenic and beneficial) has emerged more than four decades ago [54]. Consequently, most of the pioneering studies of fish-associated microorganisms focused on these tissues, which in fact remain the center of attention of most of the modern, molecular-based studies of the fish microbiome to this date [55, 56].

Sequencing of the 16S rRNA gene is currently the gold standard for the fast identification of bacterial isolates, almost replacing early biochemical assay-based identification strategies in their entirety. Since this gene has a low mutation rate, and exists in every bacterium, it made it easy to characterize and develop phylogenetic studies [57]. When coupled to recently developed, Next Generation Sequencing (NGS) technologies, 16S rRNA gene sequencing can be employed to deliver deep taxonomic profiling of complex microbial communities without the need of prior cultivation [58, 59]. Presently, 16S rRNA gene-based taxonomic profiling of prokaryotic communities takes advantage of the sheer power of NGS and the existence of highly comprehensive, well curated databases to deliver accurate assessments of the composition of highly diverse microbiomes based on identifiable and traceable sequence heterogeneities within this target gene. The fish microbiome is now known to play several roles in fish physiology, such as epithelial differentiation and maturation, nutrition and aiding in the development of the innate immune system, which, altogether, may affect host fitness decisively. Considering the economic impact of diseases in fish aquaculture, the characterization of these microbiomes is imperative, since knowledge of their composition and how they can be managed can greatly contribute to future disease prevention [57].

There are a variety of factors that should be strictly controlled, since they can affect the microbial community associated to fish (**Table 1**) [15, 57]. Farming conditions, and human-caused (anthropic) stressors like the rise of the sea temperature and pollution, should also be rigorously regulated, since they can intensify bacterial diseases [57, 60].

In general, the fish microbiome is composed by four dominant phyla: *Proteobacteria, Bacteroidetes, Firmicutes* and *Actinomycetes*. For any given cultured fish species, the predominance of each phylum depends largely on the developmental stage, rearing system, feed regime and on the type of species itself [61].

Table 1. Overview of factors reported to influence the composition of the microbiomes of fish. Adapted from Merrifield *et al.*, (2015) **[57]**.

Factors		Details	Microbiome region
Dietary Factors	Dietary form	Pelleted *vs.* natural diet	GI, skin
	Trophic level	-	GI
	Food deprivation	Fed *vs*. unfed fish	GI, skin
	Dietary lipid	Lipid levels; lipid sources	GI
	Protein sources	Plant derived proteins; yeast protein concentrates; insect meals	GI
	Carbohydrates	Plant-derived carbohydrates; chitin	GI
	Feed additives	Probiotics; prebiotics; antibiotics/disinfectants; phytobiotics; immunostimulants	Eggs, GI, skin
Environmental factors	Water salinity	-	GI
	Seasonality	-	GI, gills
	Temperature	-	GI
	Wild vs. farmed fish	-	GI
	Water quality Toxicants	Metal nanoparticles; heavy metals	Eggs, GI, skin, gills
Developmental stage	-	Eggs *vs*. early-hatched larvae *vs*. fry vs. juveniles *vs*. adults	Egg surface, whole larval surface, specialized organs in adults (skin, GI, gills)
Other	Genetic	Different fish families within a species; transgenic individuals	GI, gills, skin
	Hierarchy/stress	Dominant vs. subordinate individuals	GI
	Stocking density	-	GI

The microbes belonging to these phyla promote several microbiome interactions such as the digestion of nutrients through the production of enzymes and the production of antagonistic compounds [57, 61]. Nevertheless, in the surrounding environment, fish pathogens such as *Staphylococcus epidermidis* [62], *Staphylococcus saprophyticus* [63], *Vibrio* spp. [64, 65], *Photobacterium* spp. [66], *Pseudoalteromonas piscicida* [57], among numerous others, may also be present and be part of a given microbiome [67-70]. These fish pathogens possess several defenses, nutrient acquisition, host colonization and virulence genomic traits and, in theory, present a difficult challenge for the management of aquaculture facilities and maintenance of fish health, either in early (larval) or in advanced (juvenile or adults) developmental stages [71].

The bacterial colonization cycle begins with the entry of the bacteria inside its host or its adhesion onto the host surface. To do so, bacteria possess several attributes like flagella (allows motility and adhesion), pili (allows adhesion) and chemotaxis proteins (allows a directed motility) (**Figure 4**). This first step is similar both in pathogenic and beneficial bacteria. The second step is bacterial establishment and multiplication in/on host tissue, often enabled through the secretion of extracellular polysaccharides followed by biofilm formation [71].

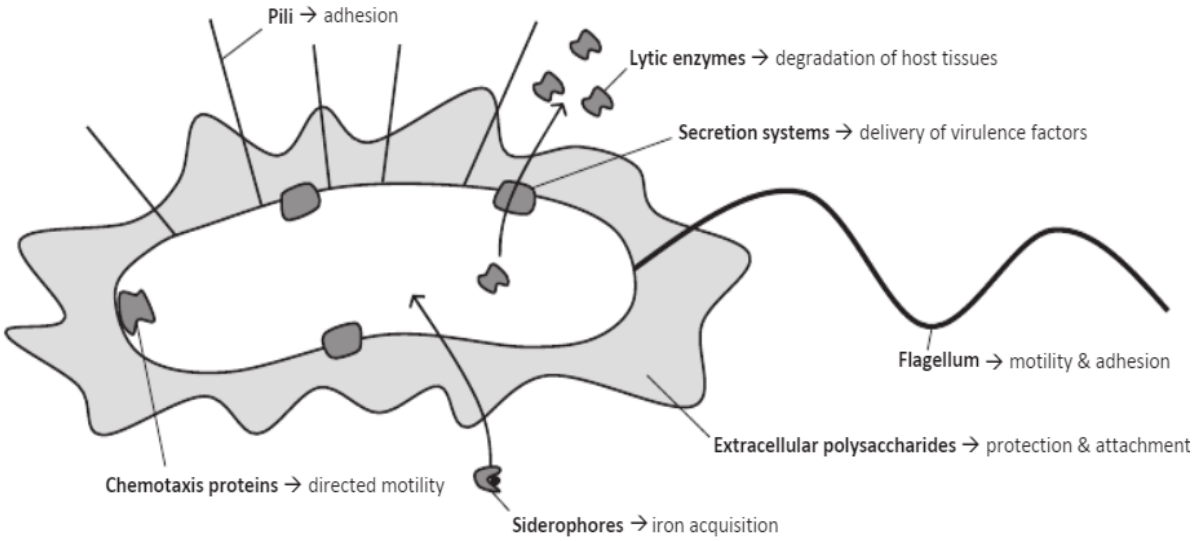


Figure 4. Schematic overview of virulence factors produced by pathogenic bacteria. Note that such "virulence factors" may as well help mutualistic, beneficial symbionts to establish positive interactions with their hosts. Adapted from Defoirdt, (2014) **[71].**

The formation of this biofilm protects the bacteria, allowing them to evade the fish defense system, in case of pathogenic microorganisms. Also, it allows bacteria to acquire and "share" nutrients across the biofilm, through small water channels, which help the continuous multiplication of pathogenic cells within the biofilm and consequent destruction of host cells and tissues, facilitating pathogen penetration. The damage caused to the host cells and tissues during penetration is due to the production of several virulence factors (VFs). The term virulence refers to the likelihood of a pathogen to cause infection [71]. However, VFs refer to the elements (*e.g.,* gene products) that enable a microorganism to colonize a certain niche where the organism thrives and causes tissue damage or systemic inflammation. VFs can include, for example, lytic enzymes (*e.g.,* proteases, chitinases and lipases), dispatched through a secretion system, protein toxins and cell-surface structures (*e.g.,* capsular polysaccharides and outer membrane proteins). It must be mentioned that lytic enzymes, pili, flagellum and the rest of the traits present in **Figure 4** (and many others not mentioned) may as well be attributes involved in environmental or host adaptation by several neutral or beneficial bacteria that might not necessarily be involved in pathogenesis [72]. The last step in the colonization cycle portrayed above is the release of the pathogen from the hosts' body into the environment [71].

As previously explained, pathogenic bacteria can affect the growth and development of the reared fish. To prevent fish infections and external lesions (in skin and gills), which eventually leads to fish death, the identification and characterization of potential fish pathogens, their interactions with the host and virulence factors, constitute a major goal, and could help conceiving more efficient disease prevention and/or treatment strategies [60].

1.2.2.1. *Sparus aurata* (gilthead seabream)

Sparus aurata (gilthead seabream), also known in Portugal as “dourada” (**Figure 5**), is one of the most important reared fishes worldwide, especially in Europe, representing up to almost 7% of the global aquaculture production [73, 74]. Over the last 20 years, its market value has rapidly increased along the Mediterranean basin, with 90% of the seabream production being concentrated in six countries: Turkey (37%), Greece (25%), Egypt (14%), Spain (9%), Tunisia (4%) and Italy (4%) [75, 76].

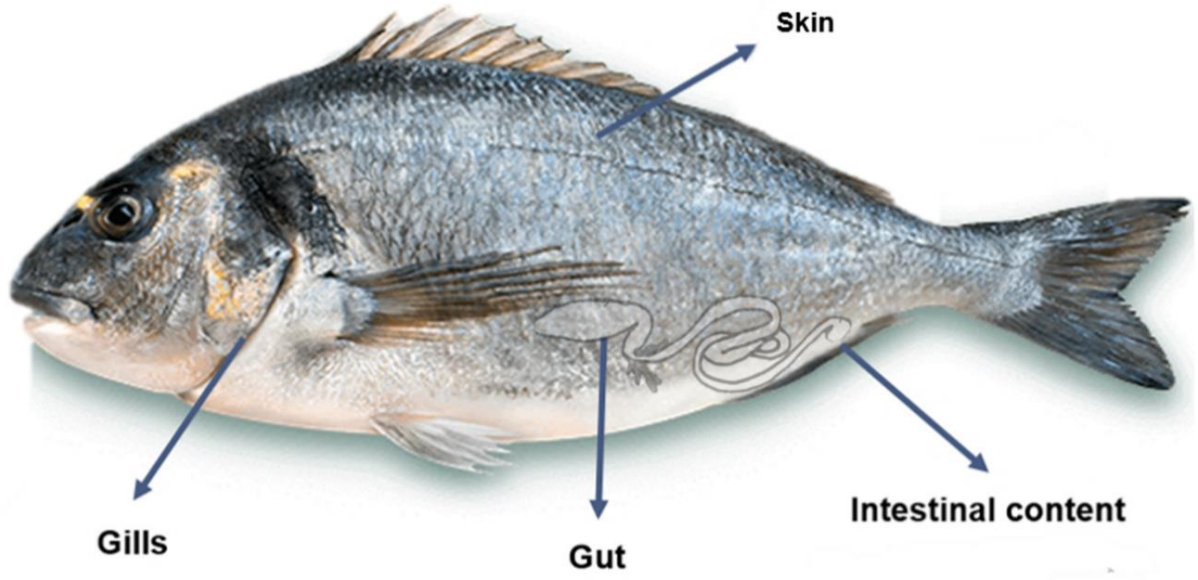


Figure 5. *Sparus aurata* (gilthead seabream) and fish body sites/organs (gills, skin, gut, and intestinal content) known to harbor microbiomes with distinct structure and taxonomic composition.

The continuous seabream rearing growth also generates new hurdles, from the quality of the farmed fish, under artificial conditions, compared with that of wild captures, to the high stocking density, which leads to difficulties related with poor hygiene conditions [73, 75]. However, one of the major issues in fish rearing, as previously stated, is the existence of pathogens that cause infectious diseases. These infections most often affect the productivity of the overall aquaculture, which in turn often presents a limiting factor for the development of this sector [77]. As an example, these infections can account for losses up to 10% at the end of the seabream on-growing production period [76]. Of all the fish life stages, the larval phases are the ones most affected by these pathogens since if these become infected the consequences might be a mortality up to 100% [77]. The adult fish microbiome is likely to present distinct structure and composition according to the body site or organ, such as the fish gut, gill and skin microbiome (**Figure 5**) [60, 78].

In *S. aurata* in particular, the gut microbiome is mainly composed by *Proteobacteria* (*e.g., Pseudoalteromonas* spp., *Vibrio* spp., *Glaciecola* spp., and *Photobacterium* spp.) and *Firmicutes* (*e.g., Bacillus* spp.) [78] while the gill and skin microbiomes are largely composed by *Proteobacteria* (*Rhodobacteraceae*, and *Vibrionaceae*) and *Bacteroidetes* (*Flavobacteriaceae*), mainly *Rubritalea* spp., *Vibrio* spp., and *Polaribacter* spp. [60].

1.2.3. Microbiome development

As previously shown in **Table 1**, numerous factors can influence the fish microbiome, not only during the juvenile and mature stages of the fish, but also during the microbiome formation, mainly in larval stages [42, 57, 79]. In comparison with skin, gill and even fish eggs microbial communities, the gut microbiome is thoroughly studied [57]. Nonetheless, the influence of host microbes on host physiology, health and survival is still poorly understood [57]. Upon hatching, most fish larvae possess a sterile and immature digestive system since they are fed by nutrient present in the yolk sac (their mouth is closed). Upon mouth opening and onset of heterotrophic feeding, larval exposure to early microbial colonizers is presumably limited, or shaped by the resources available. Consequently, the early gastrointestinal microbiome usually resembles the one present in microalgae, live feed, rearing water (as the larvae drink water for osmoregulatory purposes), or even the egg microbiome. The gut colonization process is characterized by continuous, successive replacement of microbial associates towards a complex assembly of gut connected microbes, and can vary according to the reared species, life stages, and rearing systems. Even between individuals belonging to the same species, the composition can vary immensely. Indeed, fish, as well as humans and other mammals, may display different microbial communities inside their GI tract, depending on the GI location (*e.g.,* foregut or hindgut). This shift in diversity is likely due to the difference in pH throughout the gut as well as due to the digestion process between the foregut and hindgut, which is collectively influenced by location, season, dietary input, and gut morphology [42, 57, 79, 80].

There are two main hypotheses for gut colonization: the first consists of a neutral or stochastic assembly that combines events of random dispersal of microorganisms, that lead the microbes to the intestine, being these the sole responsible for the intestinal community final profile. The second hypothesis consists of a non-neutral or deterministic model, where the microbial GI community is formed due to host selective pressures, host-microbe and microbe-microbe interactions as well as active dispersal by the host. Although microbial community assembly processes in fish larvae are much less studied, it is likely that the combined effects of deterministic and stochastic events simultaneously play a role in shaping the structure of microbiomes in early developmental stages of fish [42].

1.3. Fish disease control: old *versus* new

1.3.1. Classic methods: antibiotics, metals, and other agrochemicals

One of the major concerns of the aquaculture industry worldwide is the prevention of pathogen colonization. However, the majority of preventive measures are extremely strict and difficult procedures that often are not applied or, when applied, there is no certainty that they are efficacious and a mistake may lead to severe economic losses [81, 82]. In aquaculture, many of the infections caused by pathogens such as *Staphylococcus* spp. and *Vibrio* spp. tend to lead to higher fish mortality rates or to several post consumption infections. Nevertheless, fish mortality is not the only criterion to evaluate the damage effects. Many times, morbidity, which leads to poor growth and weight loss, and eventually to a decrease in fish survival, also contributes substantially to farming losses [83]. With the purpose of mitigating these losses and maintaining a sustainable aquaculture, both economically and environmentally, pathogen control is a major factor that needs to be addressed [84]. The basic strategy, applied globally, is the use of chemotherapeutics and medicinal herbs as pathogen control, or through vaccines and immunostimulants to improve host control of microbial diseases [83]. Due to the existence and accessibility of antimicrobial compounds (like sulfamerazine), disease prevention in aquaculture using vaccination as a routine methodology was limited. However, from the mid to the late 70's, due to an increased interest in fish farming, vaccination was getting more attention as a means of fish disease prevention. This turn of events had several reasons such as the high costs of chemical intervention, the increasing appearance of antibiotic resistant fish pathogens, and environmental concerns [84-86]. The effective prevention of several bacterial infections in fish by vaccination is by now well documented, for instance against vibriosis, through immersion or intraperitoneal injection of the vaccine [84].

A vaccine is a substance used for the stimulation of the immune system with the intent of antibodies production and consequently a certain degree of immunity against one or more diseases [84, 87]. On the other hand, antibiotics are defined as a group of synthetic or natural compounds which either kill bacteria or inhibit their growth. Due to an increase of the aquaculture practice in the last few decades, there was a decrease in the sanitary conditions and an increase in the stressful conditions regarding overall fish rearing, including high fish and farm densities and lack of appropriate barriers between farms. The risk of bacterial infections raised so much that the implementation of antibiotics was a need and not a means of precaution [4].

An example of this was the implementation of antibiotics in Norway in 1987 to prevent the total crash of the fish farming industry (**Figure 6**) [88]. Studies previously performed in the 1970s, in the USA, demonstrated a clear effectiveness of a fish immersion vaccine against vibriosis, which led to a decline of the use of antibiotics in Norway, as visible in **Figure 6** [88]. However, a new disease, furunculosis caused by *Aeromonas salmonicida* emerged and, as immersion vaccines proved ineffective against this pathogen, injectable vaccines containing adjuvants were developed in the early 1990s. In present days, the usage of antibiotics in aquaculture has been restricted (*e.g.,* the use of chloramphenicol, has been banned in China and in the European union [89]. Other examples include the restriction of spectinomycin and rifampin in the USA [90]).

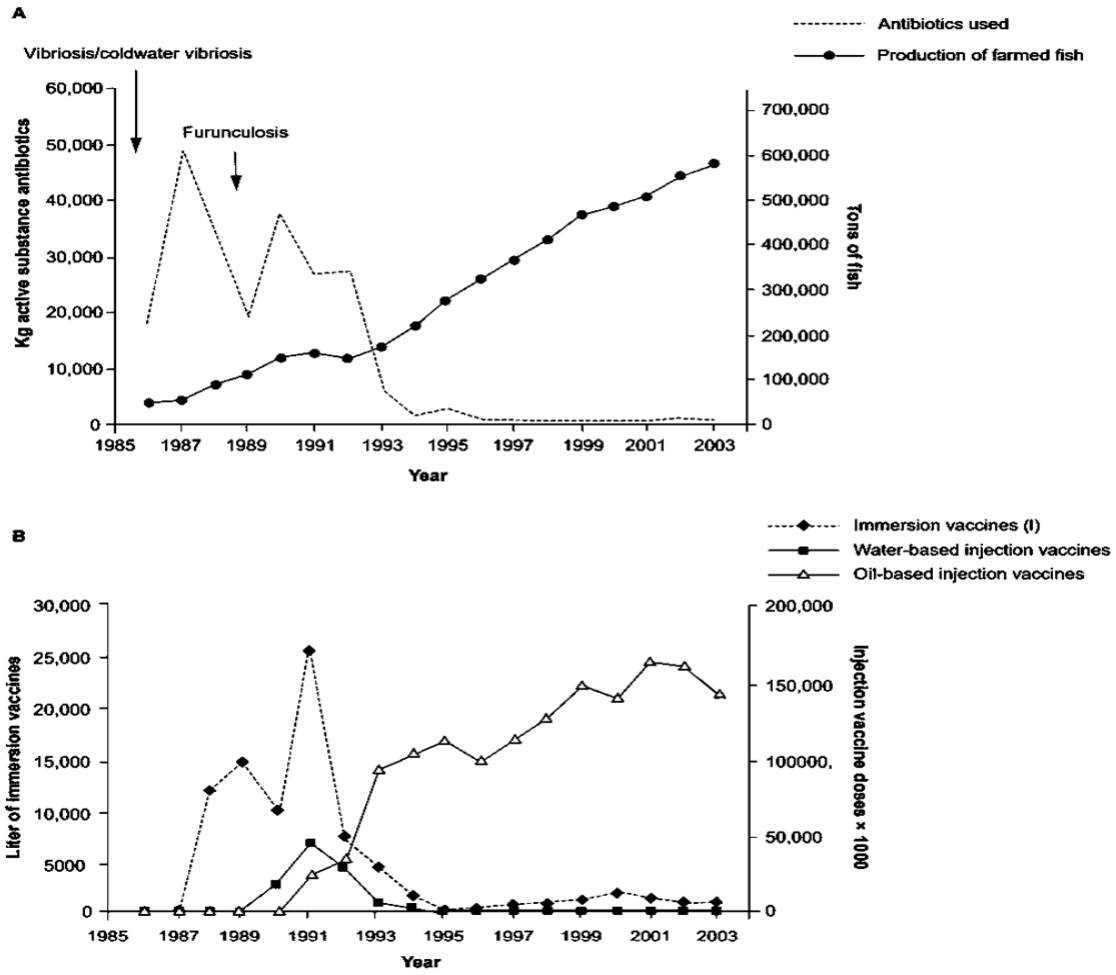


Figure 6. The use of antibiotics (A) and different types of vaccines (B), during the growth of Norwegian aquaculture industry from 1986 to 2003**.** Adapted from Sommerset *et al.*, (2005) **[88].**

Maximum Residue Levels (MRLs) were established by the European Legislation which might differ depending on the geographical area and on the agencies that estipulate the required levels. For example, if a certain antibiotic has not yet been restricted in one area, it may not have a MRL in that geographical region or it might have different MRLs (if restricted) depending on the acting agency in that region. In most of the developing countries, there are no established MRLs for antibiotics and, when considering the major aquaculture producing countries and organizations, there are still many authorized antibiotics (**Table 2**). The major agencies operating in Europe are the European Commission (EU), European Medicines Agency (EMA), Norwegian Veterinary Institute, the Norwegian Food Safety Authority, FAO's Codex Alimentarius Commission (Codex), and government ministries [91, 92].

Despite the prevalent use of vaccines in present days, the excess use of antibiotics in the past, mainly in the 1980-1990 decades, and in low-income countries, has raised two major concerns: the impact of drugs and their constituent compounds on the environment and the development of antibiotic-resistant bacteria. For instance, until 2009, in Korea, there had been the application of 27 different antibacterial agents, of which 23 were antibiotics [83].

Table 2. List of authorized antibiotics which have been used among the major aquaculture-producing countries and organizations. Adapted from Chen *et al.*, (2020) [91].

Country	Nº of authorized antibiotics	Antibiotics
China	13 (33 antibiotics used from 2008 to 2018)	doxycycline, enrofloxacin, florfenicol, flumequine, neomycin, norfloxacin, oxolinic acid, sulphadiazine, sulphamethazine, sulphamethoxazole, sulphamonomethoxine, thiamphenicol, and trimethoprim
Vietnam	30 (39 antibiotics used from 2008 to 2018)	amoxicillin, benzylpencillin, ciprofloxacin, cloxacillin, colistin, chlortetracycline, cypermethrim, danofloxacin, dicloxacillin, difloxacin, emamecyin, erythromycin, flumequine, neomycin, oxolinic acid, ormetoprinm, oxytetracycline, oxacillin, paromomycin, sarafloxacin, sulfadimethoxine, sulfadiazine, sulfamonomethoxine, sulfamethoxazole, sulfamethazine, spectinomycin, tetracycline, tilmicosin, trimethoprim, and tylosin
U.K.	5	oxytetracycline, oxolinic acid, amoxicillin, sarafloxacin, and cotrimazine
U.S.A	4	oxytetracycline, florfenicol, sulfadiazine/trimethoprim, and sulfadimethoxine/ormetoprim
Italy	6	tetracycline, oxytetracycline, amoxicillin, flumequine, and sulfadiazine/trimethoprim
Brazil	2	florfenicol and oxytetracycline
Thailand	14 antibiotics used from 2008 to 2018	amoxicillin, enrofloxacin, norfloxacin, oxytetracycline, ormetoprim, penicillin, sulfadiazine, sulfadimethoxine, sulphamonomethoxine, sulfadimethoxine, sulphaguanidine, trimethoprim, tribrissen, and tetracycline
South Korea	17 antibiotics used from 2008 to 2018	amoxicillin, ciprofloxacin, chlortetracycline, enrofloxacin, erythromycin, florfenicol, nalidixic acid, ormetoprim, oxolinic acid, oxytetracycline, sulfadiazine, sulphachloropyridazine, sulphamethoxazole, sulfadimethoxine, sulphamethazine, trimethoprim, and tetracycline
Chile	19	amoxicillin, chloramphenicol, doxycycline, enrofloxacin, erythromycin, florfenicol, flumequine, furazolidin, gentamyin, neomycin, norfloxacin, oxolinic acid, oxytetracycline, sulphadiazine, sulphamethazine, sulphamethoxazole, sulphamonomethoxine, thiamphenicol, and trimethoprim
Bangladesh	12	amoxicillin, chlortetracycline, doxycycline, erythromycin, oxytetracycline, penicillin G, sulfadiazine, sulfamethazine, sulfamethizole, sulfamethoxazole, trimethoprim, and tylosin
Japan	11	amoxicillin, carbolic acid, doxycycline, erythromycin, fosfomycin, oxolinic acid, lincosamide, oxytetracycline, sulphamonomethoxine, sodium alkane sulfonate, and thiamphenicol
U.S. FDA	4	florfenicol, oxytetracycline, and sulfadimethoxine/ormetoprim
FAO 2005	4 + SA (sulfonamides) antibiotics authorized	florfenicol, oxytetracycline, sarafloxacin, eythromycin, and sulfonamides

Since many of the drugs used across numerous industries (agriculture, therapeutic purposes, and animal husbandry) are recalcitrant and hardly degradable - such as quinolones, which are absorbed deeply into sewage sludge, soils, sediments, and aquatic environments - they may become toxic to humans and animals, due to life-threatening contaminations causing high death tolls (*e.g.,* chloramphenicol has been associated with serious toxic effects such as fatal aplastic anemia. In the case of quinolones, they can be directly toxic or be the source of human resistant pathogens) [79, 92-94].

Antibiotics generally eliminate most bacteria in a colony. Even so, the existence of mutants, most likely due to pharmaceutical compounds in the environment or due to the excessive use of drugs, can lead to antibiotic resistance by microbial strains, which will gain a selective advantage, due to the acquisition of drug resistant genes via horizontal gene transfer events (*e.g.,* genes present in plasmids that are laterally transferred from one cell to the other), thus increasing proliferation in the environment impacted by the drug(s) in question [90]. Although the use of antibiotics was the most important explored option for disease control, several other approaches have been attempted, throughout the years, to control microbial diseases in aquaculture settings. One approach was the use of metals, like copper-based antifouling metals, that were applied to slow down the process of biofouling (accumulation of microbes that are generally ubiquitous, and which may lead to future complications [95]), and the use of altered fish feeds (amended with, or composed of, various metals to fulfil certain mineral requirements) [4]. Besides not being an efficient option for disease control, metal-based solutions could also lead to human exposure, due to the metal excess in farmed fish. The application of other chemicals for prevention and treatment, also called agrochemicals such as pesticides, antifungals, disinfectants, fertilizers, and other compounds used in water treatments have also been largely attempted [15, 96]. However, in resemblance with the metals method, this could lead to substantial environmental and human health threats [4].

In summary, the use of vaccines, antibiotics, metals, agrochemicals, among other approaches (*e.g.,* genetically modified fish) has raised substantial concerns regarding environmental and human health friendly practices in aquaculture. The input of formulated feeds and the application of agrochemicals, antibiotics, and other inputs, led to an extreme chemical contamination not only in aquaculture facilities, but also in the environment, destabilizing surrounding ecosystems through the increase of for example antibiotic resistant-bacteria, reef coral mortality events and habitat destruction, to name a few effects [4, 5, 15]. So, in the last few decades, the aquaculture industry has been the target of many regulations and investigations to prevent its major bottleneck: anthropic pollution. One of the foremost explored methods, as previously stated, is the application of harmless and helpful microbes, also known as probiotics, as substitutes of antibiotics and chemicals [2, 27].

1.3.2. Probiotics: an alternative to classic methods

The term “probiotic” was firstly coined in 1965 by Lilly and Stillwell to describe substances secreted by one microbe which stimulated the growth of another [97]. However, in this thesis the adopted definition is the one reviewed and published by ISAPP (International Scientific Association for Probiotics and Prebiotics) in 2013, which states that a probiotic is a “live microorganism that, when administered in adequate amounts, confers a health benefit on the host” [98]. Since 1974, probiotics were explored, studied, and even applied daily to improve human health, for example, through the consumption of fermented milks (*e.g., Lactobacillus* spp.). These applications are still used to this date and have been successfully applied in other production sectors and animals [99]. A broad range of microalgae (*e.g., Tetraselmis* spp.), yeasts (*e.g., Debaryomyces* spp. and *Saccharomyces* spp.) and Gram-positive (*e.g., Bacillus* spp. and *Lactococcus* spp.) as well as Gram-negative bacteria (*e.g., Aeromonas* spp. and *Alteromonas* spp.) have been evaluated and used as probiotics both for humans and animals [99-101]. In recent years, probiotics have also been used as a means to control diseases in aquaculture systems [100], supplementing, or, in some cases, replacing the use of antimicrobial compounds, since many pathogens are emerging as resistant to current antibiotics (see **Table 2** for a more precise perspective of the antibiotics still used these days) [24, 100].

Given that marine animals are susceptible to countless pathogens from water, food, and even other animals, there is an urgent need for the protection of these marine animals [102]. As mentioned above, probiotics can be used as an alternative to traditional methods, such as antibiotics. But why?

Pharmaceuticals stimulate a physiological response in bacteria, animals, humans and in other organisms, to protect against any invasive microorganisms [79, 93]. However, besides killing most of the pathogenic bacteria, they also kill most of the beneficial ones [24]. The use of probiotics, as an alternative to traditional methods, may aid in the protection of the hosts against microbial diseases through natural mechanisms or the action of (probiotic-derived) substances, such as the competition for biological surfaces, antagonistic capabilities, and competition for the natural resources. One example is the production of siderophores, which are organic, iron-complexing chemicals, that act in iron uptake [24, 102], allowing the probiotic to capture iron from the environment, thus “stealing” the iron from other potential pathogens, inhibiting their growth (**Figure 7**) [24, 103].

As previously mentioned, the same metabolic features (*e.g.,* siderophore production) that may help a pathogenic bacterium can also help a beneficial/probiotic bacterium. The interpretation of the bacterium behavior (pathogenic or beneficial) based only on physiological/genomic features is one of the crucial challenges in this field of research. Besides providing protection against foreign bacteria (*e.g.,* pathogens), probiotics can also positively influence the host’s feed consumption and weight gain, through the production and secretion of hydrolytic enzymes (*e.g.,* cellulases, amylases, proteases, and lipases) which can improve the hosts’ appetite by degrading indigestible components while providing more palatable, lower molecular weight compound to the host, increasing the production of vitamins and the detoxifying effect [104].

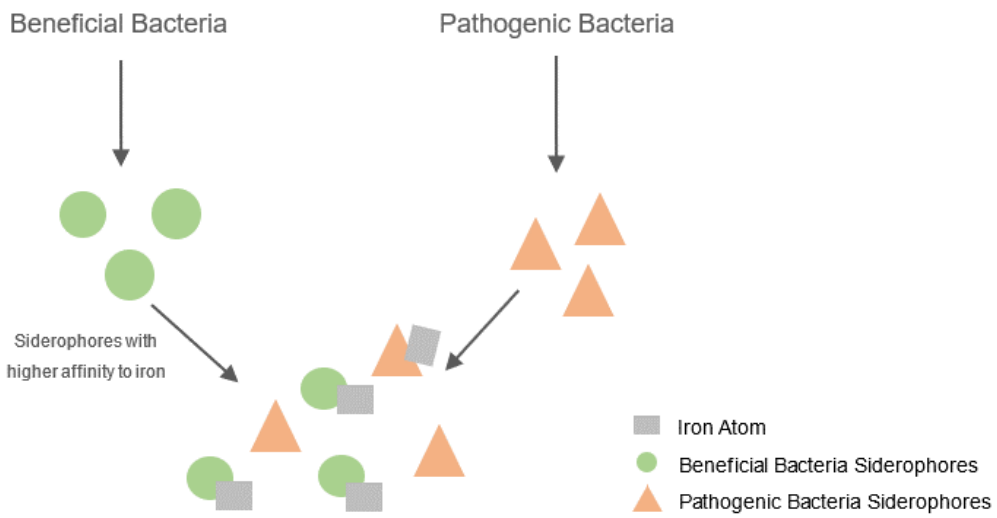


Figure 7. Iron uptake by beneficial and pathogenic bacteria through siderophore production. Beneficial bacteria could produce siderophore with higher affinity to iron, thus having a higher iron acquisition. Therefore, the pathogenic bacteria do not obtain as much iron as needed for their growth, which could lead to suppression of their pathogenic activity.

Examples of these enzymes include (i) lipases either alone or conjugated with esterases, whose biological function is to catalyze the hydrolysis of triacylglycerol into diacylglycerol, monoacylglycerol, free fatty acids (FFA) and glycerol [105]; (ii) amylases which are enzymes that catalyze the hydrolysis of starch into glucose (starch is a polymeric carbohydrate consisting of different glucose polymers joined by glycosidic bonds) [106, 107]; (iii) proteases which the main biological function is to degrade proteins (*e.g.,* elastin, collagen, and proteoglycans) aiding several times in host defense *in vivo* [108, 109]; (iv) cellulases which are enzymes produced by several microorganisms such as bacteria, fungi and protozoa, by termites, and in the digestive tract of ruminants with the main purpose of catalyzing the cellulose hydrolysis [110] and, (v) finally, chitinases that degrade one of the most abundant polysaccharides on the planet, which is chitin. This biopolymer is found in the exoskeleton of insects, fungi, yeast, and algae, and in the internal structures of other vertebrates [111].

There is a vast range of probiotic properties among microorganisms and to solve ecological, human, and economic concerns pervasive to several sectors of activity, scientists are resorting to probiotics. Their natural antimicrobial and organic matter degradation activity makes them a perfect target for innovative biocontrol and bioremediation practices, respectively, posing as a promising approach to mitigate a range of problems faced by aquatic species worldwide [24, 112, 113]. In aquaculture, two main types of probiotics exist: gut probiotics and water probiotics. The gut probiotics can blend with feed and be administrated orally, to enhance the activity of the beneficial gut microbiota. The water probiotics can grow in water medium and inhibit the pathogenic bacteria by consuming all available nutrients, eliminating them through starvation [24]. Among all routes of probiotic administration, the supplementation of the rearing water is the only method which can be applied at any fish life stage [114]. To improve the growth, maintenance and activity of the probiotics, on numerous occasions prebiotics are used, which may be defined as probiotic feed, like glucan, oligosaccharides, fructo-oligosaccharides, inulin, among others [115]. However, according to the ISAPP definition followed by this thesis, prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [116].

1.3.2.1. Selection and application of probiotics

In various studies, probiotics are applied during the early stages of fish development, the larval stage. During this phase, it might be feasible to manipulate the microbiome of the larval digestive system through the addition of probiotics, either in the culture water or via the live feed, as a viable option to, for example, vaccines, since these might be difficult to apply to smaller fishes and early life stages, when many infections occur (*e.g.,* vaccination by injection is labor intensive and require the fish to be over a certain amount in size, thus being difficult to administer such vaccines in fry or smaller fish stages) [88]. This way, due to the ease of application of probiotics during early fish developmental stages, it may be feasible to increase the survival rate of host species in aquaculture [79]. As mentioned, in the first life stages, most marine fish larvae possess a sterile and immature digestive system, which is colonized by the egg microbiota at the time of hatching or through water-borne bacteria [42, 57, 79, 80].

The initial fish colonizers can be probiotic or pathogenic bacteria and, since fish larvae rely on their weak innate immune system (non-specific), if the primary colonizers are pathogens, this can lead to high mortality rates. Since probiotics can out-compete pathogenic strains, it seems plausible to argue that the addition of these live, health-enhancing microorganisms, could not only increase host survival but also growth rates and weight gain, among other desirable characteristics [26, 79, 112, 113]. Further development of probiotics for commercial use in aquaculture requires deep research, extensive and detailed trials, and economic evaluation of its use. The inappropriate selection of a microorganism to function as a probiotic may lead to experimental failure and economical losses. There are several selection steps (**Figure 8**) that should be followed for the proper choice of a probiotic strain. However, they should be fine-tuned depending on the host species and the surrounding environment [24, 112].

The first step consists of the collection of background information about fish rearing practices and design, to determine whether a probiotic application is feasible. Then, the acquisition of a large pool of potential probiotics is a major step worth considering. This pool may well be acquired by studying the microbiomes present in the host or in the surrounding environment. However, there might be some good candidates outside these two possibilities. After acquiring a pool of putative probiotics, they need to be screened for potential probiotic activities, by performing, for example, antagonistic and enzymatic tests. The best candidates should be selected based on their ability to produce inhibitory compounds (*e.g.,* bacteriocins) or on their ability to compete for nutrients (*e.g.,* siderophore production) [24, 112, 117-119]. These tests can be performed through the use of the adequate medium and incubation conditions for one particular “probiotic feature” (*e.g.,* media with milk, to assess the presence of proteases or media with starch, to evaluate the presence of amylases). Proper conduction of bioactivity screenings for the determination of probiotic features among bacteria is fundamental to foster the choice of correct probiotic candidates for *in vivo* experiments. The next step consists of the evaluation of whether the best candidates can survive under stress and non-stress conditions. Characteristics like the ability to adhere efficiently to intestinal epithelial cells and survival from the transition through the gastrointestinal tract of the host (*e.g.,* resistance to low pH, proteases, and bile salts) should, ideally, be evaluated.

Finally, the two last steps should be an *in vivo* evaluation (*e.g.,* introduction of probiotics into hosts, followed by the assessment of their performance at the laboratory scale) and an application of the probiotic candidates with the greatest potential under rearing conditions, to assess the practical application, using dry or liquid inoculant forms [24, 112, 117-119].

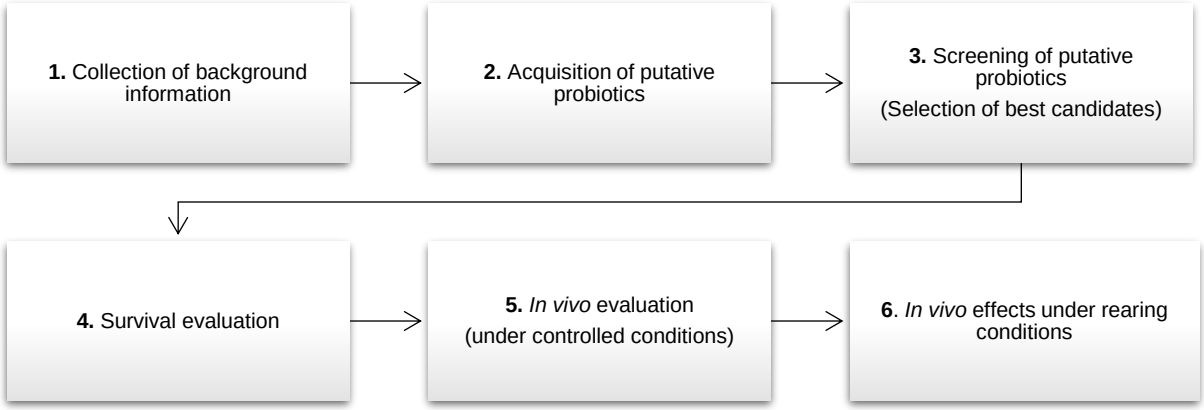


Figure 8. Steps for the selection and application of probiotics in aquaculture.

The probiotic can be provided to fish in dry form, alongside with feed or applied in the water, or in liquid form. In liquid forms, the probiotics are usually applied directly in the hatcheries or blended with farm feed. In general, liquid forms give more positive results in lesser time than dry forms, since in liquid forms they are metabolically active, ready to act and can be applied anytime and, in dry forms, there is a need for brewing and of meeting very specific conditions before and during application [24].

1.3.2.2. Benefits and limitation of probiotics

Although the exact mode of action of many probiotics may be unknown, it is likely that they play a crucial role in the host's immune responses and in the interaction between these responses and intestinal bacterial communities [120]. Therefore, the use of probiotics can lead to several benefits (**Figure 9**) since they can function as:

- Growth promoters, by working as a source of nutrients, vitamins, and digestive enzymes, or by simply enhancing the hosts digestive enzymes, which helps the host's nutrient absorption and growth performance [117, 120].
- Sources of inhibitory compounds, by releasing several chemicals such as siderophores, proteases, bacteriocins, among others, that inhibit both Gram-positive and Gram-negative bacteria [24, 117].
- Competitors against pathogenic microbes for adhesion sites and nutrients, thus incapacitating pathogens to grow and proliferate [24, 117, 120].
- Functional immunostimulants, through non-specific immune system stimulation [24]. The interaction between the host's intestinal epithelial cells and bacterial cells results in a physical and immunological barrier enabling enhanced disease control [120]. Immune system stimulation against infectious diseases occurs through the production of signaling molecules and can be controlled through the activation of lysozymes and peroxidases, and through phagocytic activity, among other mechanisms [24, 120].

- Promoters of water quality, due to the ability of probiotics to degrade organic matter and inhibit antibiotic-resistant bacteria [24, 120].

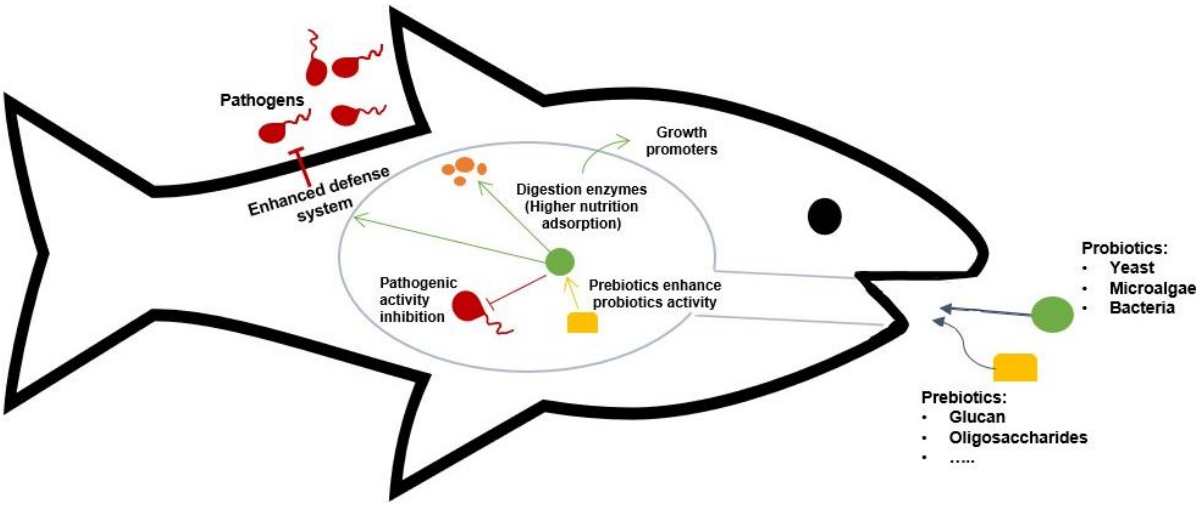


Figure 9. Examples of probiotics and prebiotics activities in fish. Probiotics are directly or indirectly absorbed by the host, as well as prebiotics (in this example, as probiotics enhancers). In the hosts' gut, probiotics will improve, for example, both the gut digestive system (*e.g.,* by the production of digestive enzymes) and the host defense system (*e.g.,* by inhibiting the activity of pathogens in the hosts' body).

Moreover, probiotics may also be used to promote antiviral and antifungal activities, and to mitigate stressful conditions (*e.g.,* high temperature, crowding and water temperature) [24, 117, 120, 121]. Probiotics have also been proposed as new anti-infective strategy in aquaculture, through the disruption of the quorum sensing system of pathogens [122].

A vast range of putative probiotics has been evaluated and applied in aquaculture. A detailed summary of different probiotics, their sources and the beneficial effects on the fish host is given in **Table 3** [117]. Despite the many benefits, probiotics also hold some limitations. First, they should not be administered with other chemicals or drugs during treatment of diseases since they are easily destroyed. Further, the use of probiotics is rather preventive than therapeutic, since they are mostly used to prevent diseases caused by pathogenic bacteria and not to treat the disease, and their effectiveness might be better when administered in sterile water, before any contamination [24].

Table 3. Examples of probiotic species used in aquaculture, their source and beneficial effects on the host species. Adapted from Chauhan and Singh, 2018 [117].

Probiotic species	Source of probionts	Beneficial effects
Lactobacillus spp. (*e.g., L. plantarum, L. fermentum and L. acidophilus*)	Seawater, sediment or host (*e.g.,* Rainbow trout and *Paralichthys olivaceus*)	Stimulate growth performance, feed efficiency, antimicrobial compounds against pathogens (*e.g., S. aureus*, *Streptococcus* spp. and *E. coli*) and best immune responses, among others.
Lactococcus spp. (*e.g., Lactococcus lactis*)	Host (*e.g., Marsupenaeus japonicas*)	Stimulate growth performance, feed efficiency, antimicrobial compounds against pathogens (*e.g., S. aureus*, *V. parahaemolyticus* and *E. coli*), reduce the adhesion of pathogens, improved phagocytic activity, among others.
Bacillus spp. (*e.g., B. cereus, B. subtilis* and *B. coagulans*)	Seawater, sediments, host, and commercial product (*e.g., Puntius conchonius* and *Cyprinus carpio*)	Enhance the non-specific immune parameters, improve resistance against pathogenic *Vibrio* spp., Inhibit the growth of *Aeromonas hydrophila*. Growth enhancers and cellular components which exhibit bactericidal activity against the fish pathogens, among others.
Nitrosomonas spp. *Nitrobacter* spp.	Commercial product	Improve water quality and lower the pathogenic (*Pseudomonas* spp.) bacterial load in fish ponds.
Kocuria spp. *Rhodococcus* spp.	Host (*e.g., Oncorhynchus mykiss* (Rainbow trout))	Produce extracellular enzymes (secondary metabolites) which are inhibitory to *V. anguillarum, V. ordalii, E. coli, P. aeruginosa* and *S. aureus*.
Others: *Streptococcus faecium*; *Paenibacillus polymyxa*; *Enterobacter* spp.; *Pediococcus acidilactici* spp.; *Enterococcus faecium*; *Saccharomyces cerevisiae*; *Vibrio* spp.; *Solibacillus silvestris*	Seawater and sediments, commercial product and host	Best growth performance and feed efficiency, antagonistic activities, immunity enhancers, among others.

1.4. Objectives and hypothesis

The major aim of this thesis was to select, based on taxonomical and physiological criteria, bacterial symbionts of fish from early developmental stages (eggs, larvae, and juveniles) and assess the best candidates for their potential use as probiotics during a fish larval rearing trial. During this phase, it might be feasible to manipulate the microbiome of larval digestive system through the addition of probiotics, either in the culture water or via the live feed thus, hopefully, increasing the survival rate of host species in aquaculture [79]. However, this hypothesis still needs to be thoroughly verified across multiple larviculture systems. To this end, the following specific objectives were delineated in this thesis:

1. Identify a collection of 97 bacterial isolates previously retrieved from gilthead seabream eggs, larvae and juveniles using molecular taxonomy methodology (*e.g.*,16S rRNA gene sequencing).

2. Couple taxonomic identity, determined in objective 1, to *in vitro* bioactivity screenings (*e.g.*, hydrolytic enzymes activity and antagonistic activity against fish pathogens) of non-pathogenic isolates, and choose two potential probiotic candidates with complementary physiological attributes.

3. Determine whether inoculation of rotifers with the chosen test probiotics alter the composition of the rotifer-associated bacterial communities, highlighting shifts in abundance of potentially pathogenic and beneficial bacterial groups, if existent.

4. Assess the probiotic capacities of the selected candidates in a gilthead seabream larval rearing trial, using inoculation of fish live feed (*e.g.*, rotifers) as probiotic delivery mode to fish. To achieve this objective, parameters such as larval body weight, length and survival in the presence and absence of the test strains were determined during the rearing trial.

2. Materials and Methods

2.1. Cultivation of heterotrophic bacteria from gilthead seabream

The starting material of this Master thesis consisted of a pool of 97 bacterial isolates (32 isolates from eggs, 31 from larvae, and 34 from juveniles) previously cultivated from gilthead seabream by Borges *et al*. (unpublished data), before the beginning of this thesis. The preparation of the fish samples for cultivation of aerobic, heterotrophic bacteria, as well as the methods used for the cultivation are explained in **Annex I**. The isolates previously obtained in this study were coded as follows: the first uppercase letter refers to the fish development stage (E, egg; L, larva; J, juvenile used at batch 1; and J2, juvenile used at batch 2); the second uppercase letter indicates the isolation medium used or treatment (No second letter, Reasoner's 2A agar diluted (dR2A); T, Trypticase Soy Agar (TSA); M, Man, Rugosa and Sharpe medium (MRS); B, heat treatment at 80°C for 10 min and grown on R2A dilution medium); the isolates were then numbered serially per cultivation medium (variants "A" or "B" for the same isolate code describe different colonies obtained during the colony purification process). All isolates were subjected to molecular DNA extraction and 16S rRNA gene PCR before the beginning of the Master thesis.

2.2. Taxonomic identification of the isolates

A summary of the methods used for molecular DNA extraction and 16S rRNA gene PCR, completed before the start of my Master thesis, is given here. Briefly, microorganisms were isolated from *Sparus aurata* eggs, larvae, and juveniles by serial dilution plating technique on various media (half-strength R2A medium agar (dR2A): R2B (1.8 g/L), agar (15 g/L), and Artificial Sea Water (ASW; 1 L): NaCl (23.38 g/L), $MgSO_4 \cdot 7H_2O$ (2.41 g/L), $MgCl_2 \cdot 6H_2O$ (1.90 g/L), $CaCl_2 \cdot 2H_2O$ (1.11 g/L), KCl (0.75 g/L) and $NaHCO_3$ (0.17 g/L); TSA with 1.5% NaCl (media composition in **Annex II**); and MRS with ASW (**Annex II**)). After incubation, colonies with distinct morphological appearances were selected and re-streaked until pure colonies were obtained. For the growth of isolates in liquid medium prior to the preparation of glycerol stocks for storage and to DNA extractions for 16S rRNA gene sequencing, Marine Broth (MB; 40 g/L) was used. Genomic DNA of the isolates grown in MB medium (24-48 h, 25°C, 200 rpm) was extracted with the Wizard Genomic DNA purification kit (Promega), following the instructions from the manufacturer. PCR amplification of the 16S rRNA gene fragments was performed using 27f/1492r primers and PCR products were sequenced with the Sanger method at StabVida (Portugal) using the 27f (forward) primer. As previously stated, a collection of 97 bacterial 16S rRNA gene sequences and respective microorganisms, were available as starting material for this thesis. The raw 16S rRNA sequence data were analyzed using CodonCode Aligner software and bad quality 5'- and 3'-regions were removed. As a first step of this thesis, the 16S rRNA gene-based taxonomic identification of the isolates was conducted in august 2020, using the Classifier and Sequence Match tools of the Ribosomal Database Project (RDP). The parameters used were: Strain: Type; Source: Isolates; Size: ≥1200; and Quality: Good.

In addition, sequences were matched on the NCBI database using the BLASTn algorithm (default parameters with limit to sequences from type material), which allows us to identify the closest type-strains to our queries. All isolate sequences were submitted to the “DECIPHER's Find Chimeras” web tool [123] to search for putative chimeras, using default parameters.

2.2.1. Phylogenetic analysis of cultivated bacterial symbionts of fish

Phylogenetic trees based on all isolates 16S rRNA gene sequences (non-redundant included) were constructed using MEGA X version 10.1 [124] after multiple alignments were performed using the Muscle algorithm with default parameters. The closest type-strains were also included in the phylogenetic analysis. Evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model (Tamura Nei model was used for the *Actinobacteria* tree) [125]. All trees were drawn to scale with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion). To assess the robustness of tree branches, a 500 replicates bootstrap analysis was performed [124].

2.3. Bioactivity screening of cultivated bacterial symbionts of fish

As a result of the taxonomic and phylogenetic analysis performed above, 45 out of 97 isolates in total, representative of non-redundant 16S rRNA gene sequences, were subjected to *in vitro* screenings for potentially probiotic properties (detailed below). Only potential probiotic or isolates with no documentation of pathogenic behavior were evaluated for their properties, while well-known pathogenic taxa were not subjected to phenotypic screenings.

2.3.1. Extracellular hydrolytic enzyme production

The selected isolated bacteria were screened to produce lipase, amylase, cellulase, protease, and chitinase enzymes in agar plate assays. For all the following assays, each isolate was pre-cultivated in MB medium at 25°C for three days (ODs above 2), and all the extracellular hydrolytic enzymes screening assays were performed at least in biological triplicates.

For the determination of lipolytic activity, the bacterial cultures (2 µL) were spotted on Marine Agar medium (MA) supplemented with 1% (v/v) tween 80 (oleic acid monoester of polyoxyethylene sorbitan) and incubated at 25°C. After seven days of incubation, the appearance of a white precipitation around the colony indicated the presence of lipolytic activity [126]. The lipase hydrolyzes the subtract tween 80 into glycerol and free fatty acids (*e.g.,* esters of oleic acid). The latter binds to the calcium present in the medium, forming insoluble, white crystals around the colony [127]. For the detection of amylolytic and cellulolytic activities, the bacterial cultures (2 µL) were spotted on MA supplemented with 1% (w/v) starch and 1% (w/v) cellulose, respectively, and incubated for seven days at 25°C. For visualization, a 1% (w/v) Lugol's iodine reagent was poured onto the plate for 30 s. Then, the reagent was removed and after 20 min the test results were recorded. The presence of a clear halo around the colony was indicative of amylolytic and cellulolytic activities. This method is based on the reaction of iodine with the sugar polymer, originating a blue-black color.

A clear area around the colony indicates the absence of polymer due to its hydrolysis by the extracellular amylases or cellulases produced by the isolate [106, 107, 110]. The Lugol's iodine reagent was prepared as following: 5 g of KI was dissolved in 15 mL of H_2O. Then, while heating and stirring, 2.5 g of I_2 were added to the previous solution. Finally, the volume was adjusted to 50 mL. For the detection of proteolytic activity, the isolate cultures (2 µL) were spotted on MA plates supplemented with 1% (w/v) skimmed milk powder (autoclaved at 100°C for 1 h and added to the autoclaved MA medium). The plates were incubated for seven days at 25°C. The milk-derived casein proteins present in the medium were degraded by the present proteases, which generate a transparent halo around the colony [108, 109]. For chitinolytic activity, a freshly-grown culture of each isolate (2 µL) was spotted on MA containing 1% (w/v) of colloidal chitin (CC). The plates were incubated for seven days at 25ºC. For visualization, Lugol's iodine reagent was used as previously described to facilitate chitin degradation visualization. A clear halo around the colony was recorded as chitin hydrolysis, indicating the presence of chitinase enzymes [111, 128]. The CC was prepared from β-chitin powder following the procedures of Hsu and Lockwood, (1975) [129] and Kuddus and Ahmad, (2013) [130], with slight modifications. Briefly, 5 g of chitin powder were slowly added to 150 mL of HCl 37% and kept for 75 min at 30ºC, with rigorous stirring. The solution was then transferred slowly to 500 mL of ice-cold (roughly 4ºC) distilled water for CC precipitation. The CC was filtered through filter paper and washed by re-suspending in 1 L of distilled water at 4ºC (this last step was performed two times). Then, using a vacuum pump, the CC was once again filtered and then resuspended in 200 mL of distilled water at 4ºC (this step was performed two times). The pH of this solution was then adjusted to 3.5 with 10 M NaOH.

2.3.1.1. Enzymatic activity index estimates

The extent/strength of bioactivity displayed by the isolates in each of the agar diffusion tests described above was expressed as Enzymatic Activity Index (EAI) [131] using **Equation 1**.

$$EAI = \frac{(\text{colony diameter} + \text{halo diameter}(D_{c+h}))}{\text{colony Diameter } (D_c)} \qquad \textbf{Equation 1}$$

An example of the extracellular hydrolytic enzyme assay for lipase and the respective EAI measurement (blue lines) is present in **Figure 10.**

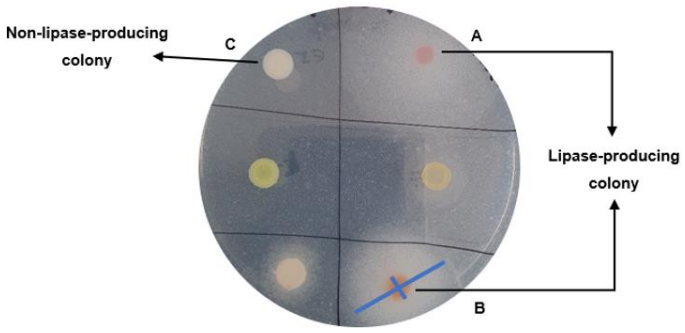


Figure 10. Lipase screening method, using tween 80 as a substrate in the culture medium. Colonies A and B produce a white halo around the colony, thus indicating the formation of a calcium complex and, consequently, lipase production. Conversely, colony C does not produce a white halo, thus indicating an absence of the calcium complex, hence no production of lipases. The blue lines illustrate how the enzymatic activity index was determined using **Equation 1**.

2.3.2. Antagonistic activity of potential probiotics against fish pathogens

To assess the antagonistic activity of the potential probiotics, two assays (the cross-streak and the soft-agar overlay assays) were employed against indicator pathogenic strains (*Vibrio parahaemolyticus*, *Photobacterium damselae* subsp. *piscicida*, and *Streptococcus iniae*). Before the antagonistic assays, *V. parahaemolyticus* and *P. damselae* strains were cultivated in MB medium for three days at 25°C, and *S. iniae* was grown in Tryptic Soy Broth with Yeast Extract (TSB-YE) medium at 37°C for three days.

In the cross-streak assay, each isolate (25 µL) was seeded by a single streak line (2 cm) in the middle of the MA plate. After incubation at 25°C for seven days, the plates were seeded with the pathogenic strains by a single streak (5 µL) perpendicular to the central streak, without touching the probiotic candidate to avoid any contamination. Then, the plates were incubated for three days at 25°C for *V. parahaemolyticus* and *P. damselae*, or 37ºC for *S. iniae*. The antimicrobial activity was analyzed by the growth inhibition of the pathogenic strain [132]. A control plate consisting of the pathogenic lines was also prepared.

In the soft-agar overlay assay, bacterial isolates (2 µL) were spotted on MA plates and incubated for four days at 25°C. After the spot development, falcon tubes with 3 mL of MB soft-agar (for *V. parahaemolyticus* and *P. damselae*) or TSB-YE soft-agar (for *S. iniae*) were maintained in a water bath at 45ºC. The soft-agar of MA medium was prepared with MB (40 g/L) and 0.75% (w/v) of agar. To each correspondent falcon tube, each pathogen was added (final optical density at 600 nm (OD_{600}) of 0.049 for *V. parahaemolyticus*; 0.035 for *P. damselae*; and 0.142 for *S. iniae*). The falcon tubes containing the media plus pathogens were stirred using vortexing for 3 s, and then poured onto the MA plates (with the isolates spots). After gelification of the soft-agar (30 min), the plates were incubated for three days at 25ºC for *V. parahaemolyticus* and *P. damselae*, and at 37ºC for *S. iniae.* As a control, the same protocol was conducted with plates without the potentially probiotic isolates.

2.4. Determination of growth parameters for the two selected potential probiotics

After evaluation of the bioactivity screenings performed above, two strains were selected as potential probiotics to be used in an *in vivo* fish larval rearing experiment. Growth curves for the two selected strains were established to determine the growth parameters (specific growth rate and doubling time). Pre-cultures of both candidates (*Arthrobacter agilis* E13 and *Phaeobacter inhibens* L23) were prepared by inoculating 3 mL of MB medium with a 10 µL inoculation loop from a fresh culture and incubated at 25°C with agitation (150 rpm). After three days, cell density was estimated spectrophotometrically by measuring the optical density at 600 nm. These pre-cultures were used to inoculate 150 mL fresh MB medium to a final OD_{600} = 0.1 and grown at 25ºC in an orbital shaker (150 rpm). Cell densities of the cultures were recorded during 50 h. Growth curves of the two potential probiotics were done in biological triplicates (**Figure 11**). The isolate's *Arthrobacter agilis* E13 growth rate (µ) and the duplication time (Dt) obtained were 0.10 h^{-1} (SD = 0.02) and 9.66 h, respectively, as determined through the slope of the isolate growth curve (**Figure 11,** left panel). After 24 h, the *Arthrobacter agilis* E13 culture exited the lag phase and started growing exponentially.

Regarding the isolate *Phaeobacter inhibens* L23, the μ and the Dt obtained were 0.44 h^{-1} (SD = 0.05) and 2.28 h, respectively, also determined through the slope of the isolate growth curve (**Figure 11,** right panel). After two hours, the *Phaeobacter inhibens* L23 culture exited the lag phase and started growing exponentially.

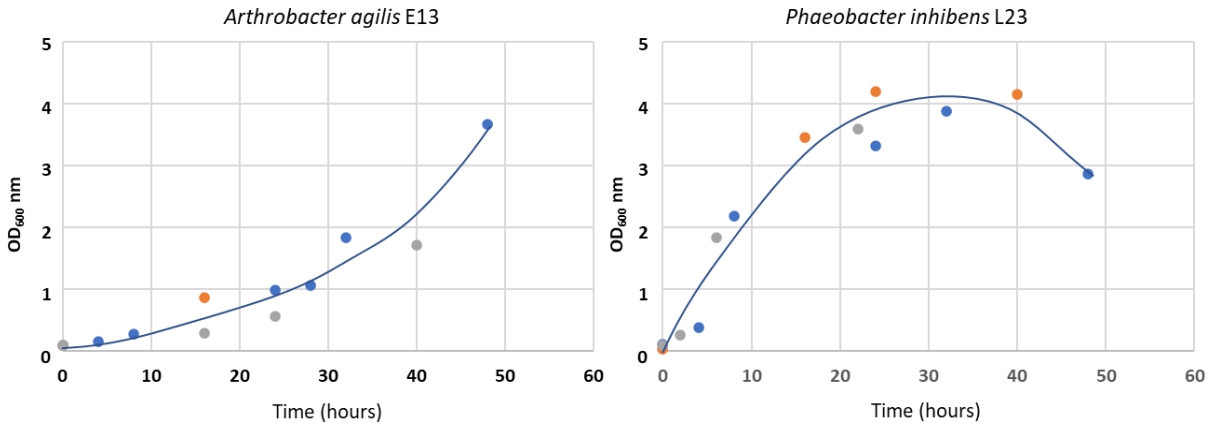


Figure 11. Partial growth curve of *Arthrobacter agilis* E13 (left plot) and growth curve of *Phaeobacter inhibens* L23 (right plot) using triplicates values (blue, orange, and grey dots) grown for two days (48 h).

To determine the number of Colony Forming Units (cfu) per mL along the growth curve, cultures with OD_{600} = 1 were 10-fold diluted in MB. Then, 100 μL of each dilution was plated on MA and incubated for four days. After incubation, the colonies were counted to determine the number of cfu/mL for each organism. The cfu/mL obtained for an OD_{600} = 1 was 9.8 x 10^7 for *Arthrobacter agilis* E13 and 6.1 x 10^8 for *Phaeobacter inhibens* L23. To prepare the biomass of both potential probiotics for the larval trial, the isolates were cultivated as previously described. The cells were grown until reaching a final OD_{600} around 1. Then, the cells were harvested by centrifugation (4000 rpm) for 10 min at 25°C. The supernatant was discarded, and the pellets were resuspended in 5 mL of sterile ASW (**Figure 12**), and subsequently the OD_{600} was estimated.

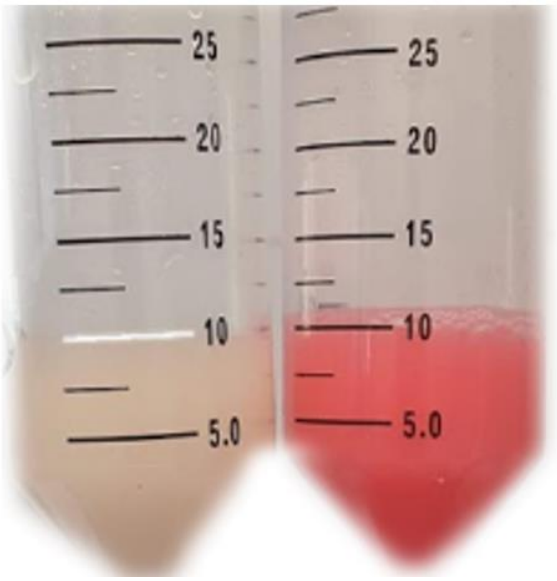


Figure 12. Culture of the selected isolates *P. inhibens* L23 (left) and *A. agilis* E13 (right), grown in MB medium, directly used for rotifers and *S. aurata* eggs treatment at EPPO-IPMA (Olhão, Portugal).

2.5. Production of microalgae, rotifers, and *Artemia*

The production of live feed for the gilthead seabream (*Sparus aurata*) larvae trial was carried out at Estação Piloto de Piscicultura de Olhão (EPPO), Instituto Português do Mar e da Atmosfera (IPMA) (Olhão, Portugal), designated hereafter as "EPPO-IPMA" [133], according to in-house protocols. Briefly, microalgae (*Nannochloropsis oculata* and *Isochrysis galbana*) were added to help maintain the water quality and the live feed nutritional profile. Rotifers (*Brachionus* spp.) were produced in a batch culture system enriched with the commercial product RedPepper® (Bernaqua NV, Belgium) and algae following the supplier's indications. For the preparation of rotifers with probiotics, a mixture of probiotics was added to the enriching batch culture for 12 h at 28-30°C. Probiotics were added together in equal proportions (1:1) to make a final concentration of 1 x 10^6 cfu/mL for each potential probiotic in rotifer suspensions provided to fish larvae. No changes in mobility were observed among probiotic-enriched rotifers when compared with control rotifers using a 10x magnification stereoscopic microscope. To evaluate the microbiota of whole-body rotifers in the absence and presence of a probiotic mixture, samples (n = 3 of the same tank per condition, wet weight = 0.2 g) were taken after 12 h of growth, washed with sterile ASW, frozen in liquid nitrogen, and stored at -80°C. *Artemia nauplii* and *Artemia metanauplii* were obtained from Viet Nam Brine Shrimp (VNBS from Golden Lotus Trading LLC, USA) and from Salt Lake Aquafeed (Catvis BV, Netherlands), respectively. All *Artemia* cysts were decapsulated as described by Pousão-Ferreira, (2006) [134]. Subsequently, *Artemia nauplii* were harvested at hatching to be used, while *Artemia metanauplii* were harvested at hatching to be enriched with the commercial product RedPepper® according with supplier's indications.

2.5.1. Total DNA extraction, quantification, and PCR amplification of 16S rRNA genes from control and probiotic-treated rotifers

The DNeasy® Power Soil® Kit (QIAGEN®, Germany) was used to extract the total community DNA (TC-DNA) from rotifers and its associated microbiome, with and without probiotics (200 mg of rotifers each sample, washed three times with ASW), according to the manufacturer's protocol with slight modifications. First, the samples were homogenized in 500 µL of ASW for 2 min using a mortar/pestle prior to DNA extraction. Second, the initial step of the protocol was modified: instead of adding 0.25 µL of sample, the entire homogenate prepared above (whole biomass) was added to the PowerBead tube provided. Two additional steps were added after the first supernatant transfer to a new collection tube: one step where 100 µL of a 10 mg/mL lysozyme solution was added to the collection tube containing the sample (for a final concentration of 1 mg/mL), followed by incubation for 1 h at 37°C, and a second step where 100 µL of a 20 mg/mL proteinase K solution was added to the collection tube containing the sample (for a final concentration of 2 mg/mL), followed by incubation for 1 h at 55°C [58]. The DNA samples were then quantified using the Qubit Fluorometric Quantification device (Invitrogen), according to the manufacturer's protocol, and the NanoDrop device (ThermoScientific).

Briefly, the Qubit device allows us to quantify the DNA or RNA present in a sample, quickly and specifically, through fluorescence. When a fluorescent dye bounds to the target molecules (DNA or RNA) it emits fluorescence, which is captured by the device, and estimates of nucleic acid concentrations based on the intensity of the fluorescent signal of two standard solutions. For DNA quantification, the Qubit™ double strand Broad range (dsDNA BR) and High Sensitivity (dsDNA HS) assay kits were used. The NanoDrop spectrophotometer is a full-spectrum, UV-Vis spectrophotometer that allows us to quantify and assess the purity of DNA, RNA, proteins and more, from sample volumes as small as 0.5 µL (in this work the volume used was 2 µL).

2.5.1.1. 16S rRNA gene amplicon sequencing

Probiotic enriched rotifers (n = 3) and standard rotifers (n = 3) were examined for bacterial community diversity and composition using Illumina MiSeq (2 x 300 PE; 20.000 reads per sample) of 16S rRNA gene reads amplified from "total community" DNA (TC-DNA) samples. For the Illumina sequencing, each DNA template was used in a PCR amplification with 515F* (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R* (5'-GGACTACNVGGGTWTCTAAT-3') primer set, which targets the V4 hypervariable region of bacterial 16S rRNA genes. A PCR was then conducted using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 95ºC for 5 min, followed by 35 cycles of 95°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 10 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Samples were then multiplexed using unique dual indices and pooled together in equal proportions based on their molecular weight and DNA concentrations.

Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR products were used to prepare an Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq apparatus following the manufacturer's guidelines.

2.5.1.2. Analysis of 16S rRNA gene sequencing profiles

The sequence data derived from the sequencing process was processed using the MR DNA ribosomal and functional gene analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Sequences were depleted of primers, short sequences (<150 bp) and sequences with ambiguous base calls were removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised. The unique sequences identified with sequencing or PCR point errors were removed, followed by chimera removal, thereby providing a denoised sequence or zOTU (zero-radius Operational Taxonomic Unit) or ASVs (Amplicon Single Variants) thus allowing a correct biological identification, distinguishing sequences with even a single difference (level of specificity not achievable when using a 97% identity threshold as with traditional OTUs), providing greater resolution of all biological sequences.

Final zOTUs were taxonomically classified using BLASTn against a curated database derived from NCBI and compiled into each taxonomic level (e.g., genera, family, class and phylum) into both “reads” and “percentage” files. Reads files contained the actual number of sequences while the percent files contained the relative (proportion) percentage of sequences within each sample that map to the designated taxonomic classification (*e.g.,* if 100 sequences of a total of 1000 sequences in a sample are classified as *Staphylococcus* then the relative percentage of this genus in the sample is 10%). The taxonomic composition of each sample using bar charts for the taxonomic level genera, family, class and phylum is shown as well as an ordination analysis of zOTU profiles (see details below).

2.6. Rearing conditions of *Sparus aurata*

Sparus aurata eggs were obtained naturally from brood stock adapted to captivity at EPPO-IPMA. Eggs were incubated at 18.0 ± 0.5°C in 0.2 m^3 cylindric-conical fiberglass tanks at a density of 0.5 g/L for a period of 48 h. Two tanks were used for eggs incubation: one for control eggs and one for eggs incubated with the isolates *Phaeobacter inhibens* L23 and *Arthrobacter agilis* E13, for 1 h. One day after hatching (DAH), fish larvae were distributed across eight tanks (0.2 m^3) at a density of 100 larvae/L (four control tanks and four probiotic tanks). The water in the tanks was static during the first two days. From 3 to 18 DAH, fish larvae were kept in a flow-through system with water recirculation (25% per hour) by draining through a 80 µm mesh size filter during the day and 150 µm during the night. The water exchange rate increased with the age of the larvae, with 30% per hour from day 19 to day 20, 35% from 21 to 23 DAH and 40% until the end of the trial (35 DAH). Likewise, the mesh size of the filters was changed over time (150 µm during the day and 350 µm during the night from 19 to 21 DAH and 500 µm from 22 to 35 DAH).

Water temperature was maintained at 18.0 ± 1.0°C, salinity at 36 ± 1 psu, dissolved oxygen (DO) at 7.0 ± 1.05 mg/L and light intensity at approximately 800 lux. Photoperiod was of 14 h light, starting at 9 am, and 10 dark until the end of the experiment (35 DAH). The green-water technique (addition of microalgae to the rearing tanks to help maintain the water quality and the live feed nutritional profile) was applied using a mixture of *Nannochloropsis oculata* and *Isochrysis galbana* (1:1) since mouth opening (3 DAH) until the end of the trial. Gilthead seabream larvae were fed rotifers enriched with algae and enrichment media (Red Pepper, Bern Aqua NV, Belgium) since mouth opening until 22 DAH, *Artemia nauplii* were introduced at 15 DAH until 19 DAH and *Artemia metanauplii* were given since 20 DAH until the end of the larval rearing trial (35 DAH) (**Figure 13**). Live preys were provided *ad libitum* considering a minimum concentration of 5 rotifers per mL due to the deficient larvae mobility during the early life stages, and 130.000 to 900.000 *Artemia* per day (0.65 and 4.5 *Artemia* per mL respectively) in each tank.

2.7. Larval rearing trial set-up and sample collection

The *Sparus aurata* larvae trial comprised two treatments (Control and Probiotic treatments) with four replicate tanks each treatment. The overall larval trial scheme is presented in **Figure 13**. As previously stated, the eggs were incubated at 18 ± 0.5°C in 0.2 m^3 cylindric-conical fiberglass tanks at a density of 0.5 g/L for two days and the eggs surface was not disinfected. Half of the eggs were incubated with a potential probiotic mixture (1 x 10^6 cfu/mL both for *Arthrobacter agilis* E13 and *Phaeobacter inhibens* L23) in a volume of 1 L for 1 h. After incubation, the volume was increased until 15 L. After hatching (1 DAH), fish larvae were distributed across eight tanks (0.2 m^3) at a density of 100 larvae/L (*e.g.,* four tanks for the control treatment and four tanks for the probiotic treatment). The feeding protocol of the four tanks corresponding to the probiotic treatment included the provision of rotifers enriched with probiotics whilst the control treatment tanks included the provision of non-enriched rotifers. Every tank was equally fed. Live preys were distributed four times a day according to amount given per meal predicted in the protocol.

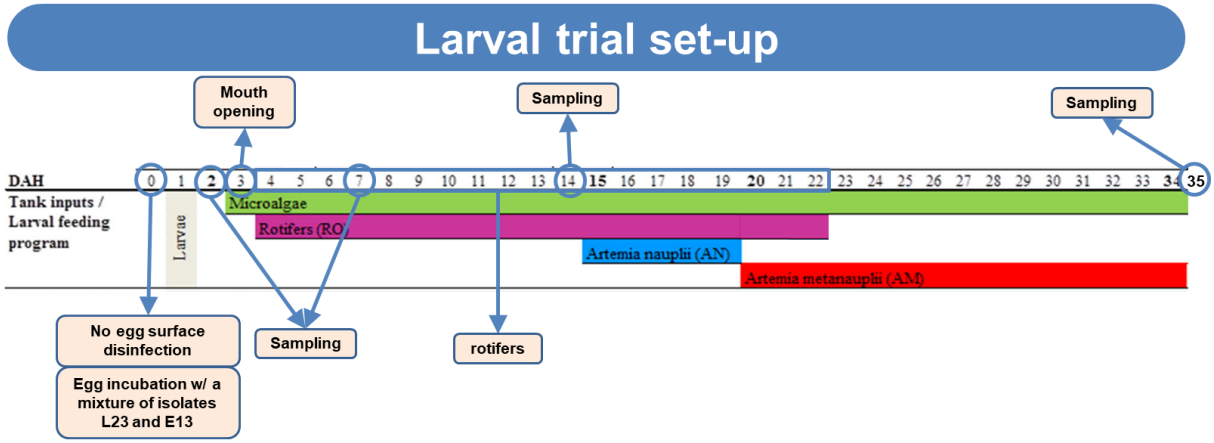


Figure 13. Experimental design and sampling methodology. No egg surface disinfected occurred in this trial. Probiotic-treated tanks derived from eggs incubated with the selected isolates (*P. inhibens* L23 and *A. agilis* E13), while control tanks derived from eggs without treatment. At 3 DAH the larvae opened their mouth and were fed with microalgae until the end of the experiment. During this period, besides microalgae, larvae were fed with rotifers (probiotic-enriched rotifers for probiotic-treated tanks and control rotifers for control tanks) and *Artemia* (*Artemia nauplii* and *Artemia metanauplii*). Larvae samples were taken at 2, 7, 14 and 35 days after hatching, for biometry analysis and for bacterial community profiling. Adapted from Califano *et al.*, (2017) [58].

To evaluate the effect of rotifers enriched with a mixture of probiotics on larval performance, 10 larvae samples per tank were collected at 2, 7, 14, and 35 DAH for biometry (total length and dry weight) analysis. The remainder larvae at 35 DAH were used for survival analysis. Finally, the samples were frozen in liquid nitrogen and stored at -80°C until further use. Each single, individual larval sample length was measured in a Nikon SMZ1000 Zoom Stereo Microscope with a zoom ratio of 10:1, making use of a Nikon Ds-Fi2 camera in the Nikon DS-L3 program. A pool of larvae sample (10 larvae per sample) was used to determine the dry weight (DW). For that purpose, the larvae were washed up in distilled water and stored at -20ºC until they were freeze-dried and properly weighed. The growth of *S. aurata* larvae was assessed in terms of weight and length gain percentage at different developmental stages. The weight gain percentage was calculated based on the standard equation (**Equation 2**), and the length gain was evaluated based on the standard equation (**Equation 3**).

$$\text{Weight gain (\%)} = \frac{\text{(final weight - initial weight)}}{\text{initial weight}} * 100 \qquad \textbf{Equation 2}$$

$$\text{Length gain (\%)} = \frac{\text{(final length - initial length)}}{\text{initial length}} * 100 \qquad \textbf{Equation 3}$$

The survival percentage was determined by counting the leftover larvae in the tanks at the end of the trial (**Equation 4**). The mortality was counted every day, although these results were not considered since several larvae were degraded to be considered a reliable count.

$$\text{Survival (\%)} = \frac{\text{number of larvae in each tank}_{35\ \text{DAH}}}{\text{initial larvae in each tank}} * 100 \qquad \textbf{Equation 4}$$

2.8. Statistical Analysis

All data regarding larval length, dry weight and survival were subjected to statistical analysis using "IBM SPSS Statistics v21.0" software for Windows (Armonk, NY: IBM Corp.). All results were expressed as "mean ± standard deviation". The data referring to the growth of different treatments were submitted to an unidirectional variance analysis (one-way ANOVA), to evaluate for the existence of significant differences between the two different treatments. The assumptions for performing this type of analysis were evaluated through the Shapiro-Wilk test, to assess the normality of the data, and through the Leven's test to assess their homogeneity. When the results showed significance difference ($P < 0.05$), the means between treatments were compared using Tukey's *post hoc* test. A Student's *t*-test was applied to results of survival at the end of the trial. Statistical significance was evaluated with 95% confidence. Moreover, a statistical analysis for the 16S rRNA gene amplicon sequencing data were performed using the statistical analysis tool "PAST v4.05" [135]. Principal Coordinates Analysis (PCoA) was performed on a Bray-Curtis similarity matrix obtained from zOTU relative abundance. Permutational analysis of variance (PERMANOVA) was then conducted to determine whether the structure of prokaryotic communities, at the zOTU level, in control *versus* treated rotifers was significantly different. Additionally, the bioinformatics software "STAMP v2.1.3" [136] was applied, using the two groups Welch's *t*-test, with a p-value filter of 0.05 (meaning it is only showed the results below this value) to determine whether the relative abundance of specific taxa, at several taxonomic levels (genus, family, class and phylum), significantly shifted in control *versus* probiotic-treated rotifers.

3. Results and Discussion

High mortality during fish larviculture is one of the main reasons for economic losses in the aquaculture industry [26]. Several products, such as antibiotics, vaccines, and probiotics, have been introduced in aquaculture management to improve larval survival rates [5, 15, 88]. In general, antibiotic administration has been reduced or substituted by novel antibiotics to prevent the emergence of antibiotic-resistant bacteria [94]. In contrast, probiotic application to control pathogenic bacteria and to promote fish growth has increased in the last decades, since it has been considered a sustainable strategy [100]. However, most of the commercially available probiotics in aquaculture were isolated from non-fish sources and, therefore, colonization, survival, and efficacy of these probiotics in the fish gut are questionable. Hence, the identification of novel probiotics from the fish host is a timely demand in this field of research.

3.1. Taxonomic identification and phylogenetic analysis of isolates

In the present study, a total of 97 isolates were recovered from three different fish developmental stages (eggs, larvae, and juvenile guts) of *S. aurata* using a culture-dependent approach. Partial 16S rRNA gene sequences were obtained for all the 97 isolates. Decipher v11.5 [123] was used to detect chimeric sequences (*e.g.,* artificial sequences generated from two or more phylogenetically different DNA templates during PCR amplification). From all sequences analyzed, only six sequences (E5, E10, E15, E21, L4, and L13) were assigned as potential chimeras. Nonetheless, these sequences (around 6% of all isolates) were revalidated as non-chimeric, since the sequence chromatograms did not reveal any background interference in those specific regions, and the NCBI/RDP databases gave good overall results.

The closest type-strain for each isolate was assigned using the Sequence Match tool from the Ribosomal Database Project, which provides quality-controlled Bacterial and Archaeal 16S rRNA gene sequences [137]. In addition, the best type-strain match was also retrieved from the NCBI database. The taxonomic assignment of each isolate based on these two databases is shown in **Table 4**. Through these databases, the isolates were assigned to six classes encompassing 32 genera of bacteria: *Alphaproteobacteria* class (*Phaeobacter, Roseobacter, Shimia, Sulfitobacter, Lentilitoribacter, Ruegeria, Sagittula*, and *Tateyamaria*); *Gammaproteobacteria* class (*Photobacterium, Alteromonas, Enterobacter, Pseudoalteromonas, Psychrobacter, Alcanivorax, Simiduia*, and *Vibrio*); *Cytophagia* class (*Pontibacter*); *Bacilli* class (*Staphylococcus, Bacillus* and *Lactococcus*); *Actinobacteria* class (*Kocuria, Streptomyces, Microbacterium, Arthrobacter, Knoellia, Micrococcus, Dermacoccus*, and *Brachybacterium*); and *Flavobacteria* class (*Aquimarina, Tenacibaculum, Psychroserpens*, and *Polaribacter*).

Table 4. Complete list of all isolates cultured from *Sparus aurata* and their respective taxonomic affiliation based on 16S rRNA gene sequencing.

RDP		NCBI	
Type-strain (Accession number) *	**Isolate [a] (S_ab score)**	**Type-strain (Accession number) ****	**Isolate [a] (Identity; %)**
Shimia haliotis WM35 (KC196071)	E3 (0.984); E4 (0.981)	*Shimia sagamensis* JAMH 011 (NR_137204.1)	E3 (98.48); E4 (98.26)
Sulfitobacter porphyrae SCM-1 (AB758574)	E5 (0.993)	*Sulfitobacter porphyrae* SCM-1 (NR_125460.1)	E5 (99.30)
Lentilitoribacter donghaensis BH-4 (JX139717)	E6 (0.988)	*Lentilitoribacter donghaensis* BH-4 (NR_132291.1)	E6 (98.80)
Ruegeria scottomollicae LMG 24367 (AM905330)	E7 (1)	*Epibacterium scottomollicae* LMG 24367 (NR_042675.1)	E7 (100)
Sagittula marina F028-2 (HQ336489)	E8 (0.999)	*Sagittula marina* F028-2 (NR_109096.1)	E8 (99.88)
Tateyamaria pelophila SAM4 (AJ968651) ***	E12 (0.973); E22 (0.983)	*Roseovarius scapharcae* MA4-5 (NR_145897.1);	E12 (97.79); E22 (98.25)
Roseobacter denitrificans OCh 114 (L01784) ***	E1 (0.981)	*Roseobacter denitrificans* OCh 114 (NR_102909.1)	E1 (98.52)
Phaeobacter inhibens T5 (AY177712)	L23 (0.995)	*Phaeobacter piscinae* 27-4 (NR_159171.1)	L23 (99.77)
Vibrio diabolicus HE800 (X99762)	E24 (0.986)	*Vibrio antiquarius* EX25 (CP001805.1)	E24 (99.22)
Alcanivorax borkumensis Sk2 (Y12579)	E10 (0.995); E15 (0.991); E21 (0.991)	*Alcanivorax borkumensis* SK2 (MN186605.1)	E10 (99.69); E15 (99.50); E21 (99.50)
Simiduia agarivorans SA1 (EF617350)	E11 (0.696)	*Halioxenophilus aromaticivorans* (AB809162.1)	E11 (98.70)
Vibrio owensii R-40496 (GU078672)	L10 (0.987)	*Vibrio alginolyticus* NBRC (NR_121709.1)	L10 (99.14)
Pseudoalteromonas spongiae UST010723-006 (AY769918) ***	L14 (0.991); L3 (0.989)	*Pseudoalteromonas spongiae* UST010723-006 (CP011039.1) ***	L14 (99.12); L3 (99.54)
Enterobacter xiangfangensis 10-17 (HF679035)	L13 (0.997)	*Enterobacter hormaechei* subsp. *Xiangfangensis* 10-17 (MK603176.1)	L13 (99.67)
Psychrobacter nivimaris 88/2-7 (AJ313425)	LT1 (0.995)	*Psychrobacter nivimaris* 88/2-7 (KY471041.1)	LT1 (100)
Vibrio sinaloensis CAIM 797 (DQ451211)	L18 (0.982)	*Vibrio sinaloensis* CAIM 797 (NR_043858.1)	L18 (98.22)
Vibrio anguillarum NCMB 6 (AM235737)	L15 (1)	*Vibrio anguillarum* DSM 21597 (CP010084.1	L15 (100)
		Vibrio qinghaiensis Q67 (CP022741.1)	L15 (100)
Vibrio cyclitrophicus LMG 21359 (AM162656)	L16 (0.996)	*Vibrio cyclitrophicus* LMG 21359 (NR_115806.1)	L16 (99.78)
Alteromonas gracilis 9A2 (AB920393)	L21 (0.997)	*Alteromonas gracilis* (AB920393.1)	L21 (99.66)
Alteromonas simiduii BCRC 17572 (DQ836766)	L4 (0.995)	*Alteromonas macleodii* ATCC 27126 (CP003841.1)	L4 (99.61)
Vibrio ichthyoenteri DSM 14397T (AJ421445)	J2-T1 (0.995)	*Vibrio sinensis* BEI233 (MG797701.1)	J2-T1 (98.79)
Photobacterium damselae ATCC 33539 (AB032015)	J2-3 (0.999); J2-2 (0.998); J2-T3 (0.998); J2-5 (0.998); J2-1A (0.997); J6 (0.998); J10 (0.996); J11 (0.998); J12 (0.998)	*Photobacterium damselae* ATCC 33539 (NR_113783.1) (FJ971859.1)	J2-3 (99.90); J2-2 (99.77); J2-T3 (99.78); J2-5 (99.77); J2-1A (99.71); J6 (99.78); J10 (99.61); J11 (99.78); J12 (99.87)
Photobacterium damselae NCIMB 2058 (X78105) ***	J2-2 (0.998); J2-5 (0.998); J2-1A (0.997)	*Photobacterium damselae* NCIMB 2058 (X78105) ***	J2-2 (99.77); J2-5 (99.77); J2-1A (99.71)
Vibrio jasicida TCFB 0772 (AB562589)	J2-8 (0.997)	*Vibrio jasicida* TCFB 0772 (NR_113182.1)	J2-8 (99.69)

Psychrobacter submarinus KMM 225 (AJ309940)	J2-1B (0.985)	*Psychrobacter submarinus* KMM 225 (NR_025457.1)	J2-1B (98.50)
Bacillus hwajinpoensis SW-72 (AF541966)	L12 (0.951); L19 (0.980); LB1 (0.971); LB3 (0.963); LB4 (0.997)	*Alkalihalobacillus hwajinpoensis* SW-72 (MW227498.1)	L12 (99.14); L19 (99.66); LB1 (99.57); LB3 (99.46); LB4 (99.66)
Bacillus plakortidis P203 (AJ880003)	JB3 (0.996)	*Bacillus plakortidis* P203 (NR_042383.1)	JB3 (99.63)
Bacillus oceanisediminis H2 (GQ292772)	J2-10 (0.993); JB2 (0.993); EB4 (0.995); EB2 (0.993); EB3 (0.995)	*Bacillus oceanisediminis* H2 (NR_118440.1)	J2-10 (99.87); JB2 (99.87); EB4 (99.45); EB2 (100); EB3 (99.45)
Bacillus halmapalus DSM 8723 (X76447)	E9 (0.987)	*Bacillus halmapalus* DSM 8723 (NR_026144.1)	E9 (98.66)
Bacillus cereus ATCC 14579 (AE016877)	J2-B1 (0.999)	*Bacillus paramycoides* MCCC 1A04098 (MT508531.1)	J2-B1 (100)
Staphylococcus epidermidis ATCC 14990 (D83363)	J2-9 (0.998); J7 (0.999)	*Staphylococcus epidermidis* ATCC 14990 (KT989845.1)	J2-9 (99.00); J7 (99.00)
Staphylococcus saprophyticus ATCC 15305 (AP008934)	J1 (0.995) J2 (0.997)	*Staphylococcus saprophyticus* ATCC 15305 (CP035294.1)	J1 (99.65)
		Staphylococcus edaphicus P5085 (MW111172.1)	J2 (100)
Bacillus safensis FO-036b (AF234854) / *B. pumilus* ATCC 7061 (AY876289)	J2-4 (1) / J2-4 (1)	*Bacillus safensis* NBRC 100820 (MG645269.1) / *B. pumilus* ATCC 7061 (NR_043242.1)	J2-4 (100) / J2-4 (100)
Bacillus idriensis SMC 4352-2 (AY904033)	JB1 (0.999)	*Bacillus idriensis* SMC 4352-2 (NR_043268.1)	JB1 (99.86)
Lactococcus lactis NCDO 604T (AB100803)	LT3 (0.998); L6 (0.997)	*Lactococcus lactis* FDAARGOS_865 (CP065737.1)	LT3 (99.82); L6 (99.68)
Micrococcus aloeverae AE6 (KF524364)	J17 (1); E25 (0.997); J3 (0.997)	*Micrococcus luteus* NCTC 2665 (MN075406.1)	J17 (100); E25 (99.74); J3 (99.65)
Micrococcus yunnanensis YIM 65004 (FJ214355)	J8 (0.999)	*Micrococcus yunnanensis* YIM 65004 (NR_116578.1)	J8 (99.89)
Kocuria polaris CMS76or (AJ278868)	J2-6 (0.988)	*Kocuria himachalensis* JCM 13326 (LC113906.1)	J2-6 (98.00)
Arthrobacter agilis DSM 20550 (X80748)	E13 (0.998); J16 (0.997)	*Arthrobacter bussei* (MN080869.1)	E13 (100); J16 (99.89)
Dermacoccus nishinomiyaensis (X87757)	E26 (0.995)	*Dermacoccus nishinomiyaensis* FDAARGOS_1119 (CP068484.1)	E26 (99.81)
Knoellia locipacati DMZ1 (HQ171909)	E18 (0.984); E19 (0.989); E28 (0.989)	*Knoellia locipacati* DMZ1 (NR_109064.1)	E18 (98.38); E19 (98.85); E28 (98.85)
Microbacterium maritypicum DSM 12512 (AJ853910)	LT4 (0.986); L20 (0.992); L9 (0.997); L17 (0.999); L24 (0.997); L26 (0.996)	*Microbacterium maritypicum* DSM 12512 (MK424289.1)	LT4 (98.63); L20 (99.22)
		Microbacterium algeriense G1 (MK480726.1)	L9 (99.78); L17 (99.89); L24 (99.66); L26 (99.63)
Brachybacterium paraconglomeratum LMG 19861 (AJ415377) / *B. conglomeratum* (AB537169)	EB1 (0.999) / (0.999)	*Brachybacterium paraconglomeratum* LMG 19861 (NR_025502.1) / *B. conglomeratum* J1015 (NR_104689.1)	EB1 (99.89) / (99.89)
Brachybacterium rhamnosum LMG 19848 (AJ415376)	J9 (0.999)	*Brachybacterium rhamnosum* H-6S (NR_042109.1)	J9 (99.88)
Streptomyces tendae (D63873) / *S. rubrogriseus* (AB184681)	J13 (0.991) J15 (0.991) J14 (0.996)	*Streptomyces mutabilis* DSM 40169 (KC954557.1)	J13 (99.27)
		Streptomyces flavoviridis JCM 4372 (MT760523.1)	J15 (99.22)
		Streptomyces tendae JCM 4610 (MT760583.1)	J14 (99.66)
Tenacibaculum mesophilum MBIC1140 (AB032501)	L25 (0.986); L8 (0.980); L11A (0.974); L5Amarelo (0.986); J2-B2 (0.984)	*Tenacibaculum mesophilum* DSM 13764 (CP045192.1)	L25 (98.64); L8 (97.98); L11A (98.75); L5Amarelo (98.64); J2-B2 (98.37)
Tenacibaculum discolor LL0411.1.1 (AM411030)	L2Verde (0.999); L2Amarelo (0.998)	*Tenacibaculum discolor* LL04 11.1.1 (NR_042576.1)	L2Verde (99.89); L2Amarelo (99.82)

Polaribacter marinivivus GYSW-15 (KM017972)	E27 (0.973); E20 (0.969); E16 (0.968); E17 (0.966); E14 (0.966)	*Polaribacter marinivivus* GYSW-15 (NR_134790.1)	E27 (97.29); E20 (96.90); E16 (96.76); E17 (96.60); E14 (96.61)
Polaribacter porphyrae LNM-20 (AB695286)	E23 (0.961)	*Polaribacter lacunae* HMF2268 (NR_156151.1)	E23 (96.64)
Psychroserpens mesophilus KOPRI 13649 (DQ001321)	E2 (0.977)	*Psychroserpens mesophilus* KOPRI 13649 (NR_043453.1)	E2 (97.67)
Aquimarina muelleri KMM 6020 (AY608406)	L1 (0.988)	*Aquimarina muelleri* KMM 6020 (NR_025823.1)	L1 (98.79)
Pontibacter korlensis X14-1 (DQ888330	J5 (0.966)	*Pontibacter brevis* XAAS-2 *(NR_159274.1)*	J5 (97.46)
Pontibacter saemangeumensis GCM0142 (JN607163)	J4 (0.976)	*Pontibacter saemangeumensis* GCM0142 (NR_109499.1)	J4 (97.61)

[a] Isolate code (ID): first uppercase letter - fish development stage (E, egg; L, larva; J, juvenile); second uppercase letter - medium used for the isolation (no 2nd letter, R2A diluted medium; T, TSA medium; M, MRS medium; B, heat treatment); one number – number of the colony (A, B, colonies obtained during the purification process); *e.g.,* J2, isolate obtained from a second batch of juveniles.
* Type-strains retrieved from RDP sequence match on August 04, 2020.
** Type-strains retrieved from NCBI on May 20, 2021.
*** The nucleotide sequence contains "N", which could represent any of the four nucleotides (A, T, G, C).

Around 81% of the species identified in this study were already isolated from aquatic environments (*Alcanivorax borkumensis* [138], *Aquimarina muelleri* [139], *Alteromonas gracilis* [140], *Arthrobacter agilis* [141], *Bacillus oceanisediminis* [142], *Simiduia agarivorans* [143], *Sagittula marina* [144], *Lentilitoribacter donghaensis* [145], *Microbacterium maritypicum* [146], *Roseobacter denitrificans* [147], *Ruegeria scottomollicae* [148], *Tateyamaria pelophila* [149], *Pontibacter saemangeumensis* [150], *Polaribacter marinivivus* [151], *Psychrobacter submarinus* [152] *Psychroserpens mesophilus* [153], and *Pontibacter korlensis* [154]) and aquatic species such as algae (*Polaribacter porphyrae* [155] and *Sulfitobacter porphyrae* [155]), sponges (*Bacillus hwajinpoensis* [156], *Bacillus plakortidis* [157], *Pseudoalteromonas spongiae* [158], *Psychrobacter nivimaris [159],* and *Micrococcus yunnanensis* [160]), fishes (*Bacillus safensis*/*Bacillus Pumilus* [161], *Lactococcus lactis* [162], *Phaeobacter inhibens* [163], *Bacillus cereus* [164], *Enterobacter xiangfangensis* [165], *Pho. damselae* [166], *Staphylococcus epidermidis* [62], *Staphylococcus saprophyticus* [63], *Tenacibaculum discolor* [167], *Tenacibaculum mesophilum* [168], *Vibrio anguillarum* [163], *Vibrio diabolicus* [73], *Vibrio ichthyoenteri* [169], *Vibrio jasicida* [170] and *Brachybacterium conglomeratum/B. Paraconglomeratum* [171, 172]), shrimps (*Vibrio owensii* [173], and *Vibrio sinaloensis* [174]), mussels (*Vibrio cyclitrophicus* [175]), and abalone (*Shimia haliotis* [176]). To our knowledge, only *B. safensis*/*B. pumilus* [161], *Pho. damselae* [78], *S. epidermidis* [62], *V. anguillarum* [177], *V. diabolicus* [73], and *V. owensii* [73] have been previously isolated from *Sparus aurata.* Some of these species have already been used as a potential probiotic in aquaculture. For example, *L. lactis* was used as probiotic candidate in *Sparus aurata*, but no significant differences were found between groups for the feed conversion ratio or specific growth rates. However, the final body weight of fish fed with this bacterium was significantly higher than that of the control group [178]. Another example is the potential probiotic activity of *B. pumilus*, which was applied in Indian major carp where the treated fish showed higher total erythrocyte count, hemoglobin concentration and hematocrit concentrations when compared with other bacterial treatments (*Bacillus licheniformis* and *Bacillus cereus*) [179]. Furthermore, Grotkjær *et al.*, (2016) [180] demonstrated that *P. inhibens* in *Artemia* and algae cultures originated a significant reduction of pathogen growth [180].

According to the current literature, the remaining species (around 19%) such as *Streptomyces rubrogriseus/S. tendae* [181, 182], *Bacillus idriensis* [183], *Dermacoccus nishinomiyaensis* [184], *Kocuria polaris* [185], *Knoellia locipacati* [186], *Micrococcus aloeverae* [187], *Bacillus halmapalus* [188], and *Brachybacterium rhamnosum* [150] were found to be usually retrieved from soil, humans, terrestrial animals or plants. Likely, the documentation of these species, which are not usually retrieved from aquatic environments, relies on the fact that that aquaculture facilities are environments built in land-water transition areas with manifold allochthonous/artificial inputs of organic carbon. As a result, it might be normal that these systems present a quite large variety of bacterial species from different environments.

In this thesis, a threshold of 97% 16S rRNA gene homology with type-strains of known species was used as indicator of possible new species, as reviewed and discussed by Zhang *et al.*, (2013) [189]. However, it should be taken in consideration that this threshold is just an indicator and not an assurance that a new species has been found. Based on NCBI, the isolates E14, E16, E17, and E20 closely related with *Polaribacter marinivivus* (96.61; 96.76; 96.60; 96.90), and E23, closely related with *Polaribacter lacunae* (96.64), showed percent identities in 16S rRNA gene sequences below this threshold with their closest type-strains, strongly suggesting that the above-mentioned isolates might represent novel bacterial species. Moreover, representative genomes of the closest species to the isolates E14, E16, E17, and E20 are currently not available, impeding us to perform genome-wide assessments to more appropriately infer whether our strains truly represent novel bacterial species. Since the results presented in this thesis (*e.g.,* 16S rRNA gene sequences) alone do not suffice to underscore the status of these isolates as novel species, their genomic DNA will be, in the near future, sent for sequencing in the scope of the ongoing collaborative project GEBA (Genomic Encyclopedia of Bacteria and Archaea) headed by the Joint Genome Institute (JGI), USA. Subsequently, comparative genomics and a deeper biomarker composition profiling analysis should also be conducted (*e.g.,* lipids and carbohydrates profile) to support the classification of these isolates as novel species.

The use of two databases such as NCBI and RDP allowed us to achieve a better data analysis and interpretation, as well as a good foundation for isolate comparisons. The NCBI database was used in a first instance to quickly determine the closest type-strain to our isolates as well as obtain their percent identity, which is retrieved through local alignments between the query and database sequences using specific scoring matrices. Then, RDP was used to obtain results with higher resolution, since this database is more curated than NCBI, presenting mechanisms that identify possible chimeras after submission, possessing also a kmer oriented approach to search for closest 16S rRNA gene relatives which are supposedly more refined than percent homologies between sequences alone [123]. Briefly, this database presents a score (S_ab score) which corresponds to the fraction that results from the number of (unique) 7-base oligomers shared between our query sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences. However, RDP has not been updated since 2016 (information displayed on the database website). Therefore, this database might not include the more recently proposed and/or validated names at the time of this writing.

The two databases offered identical results at the phylum, genus, and species taxonomic levels. Therefore, based on the taxonomic assignments retrieved from the RDP database, the relative abundance of each cultivated phyla (**Figure 14**) and genera (**Figure 15**) in different *S. aurata* developmental stages (eggs, larvae, and juvenile guts) was assessed. Nonetheless, it is worth noting that, at the genus and species levels, the results retrieved from both databases were not 100% identical. For instance, isolates E11, E12 and E22 displayed different results at the genus level, while isolates J2-T1, J2-B1, J17, E25, J3, J2-6, E13, J16, E23, J5, L23, E24, L10, and L4 presented different results at the species level (**Table 4**).

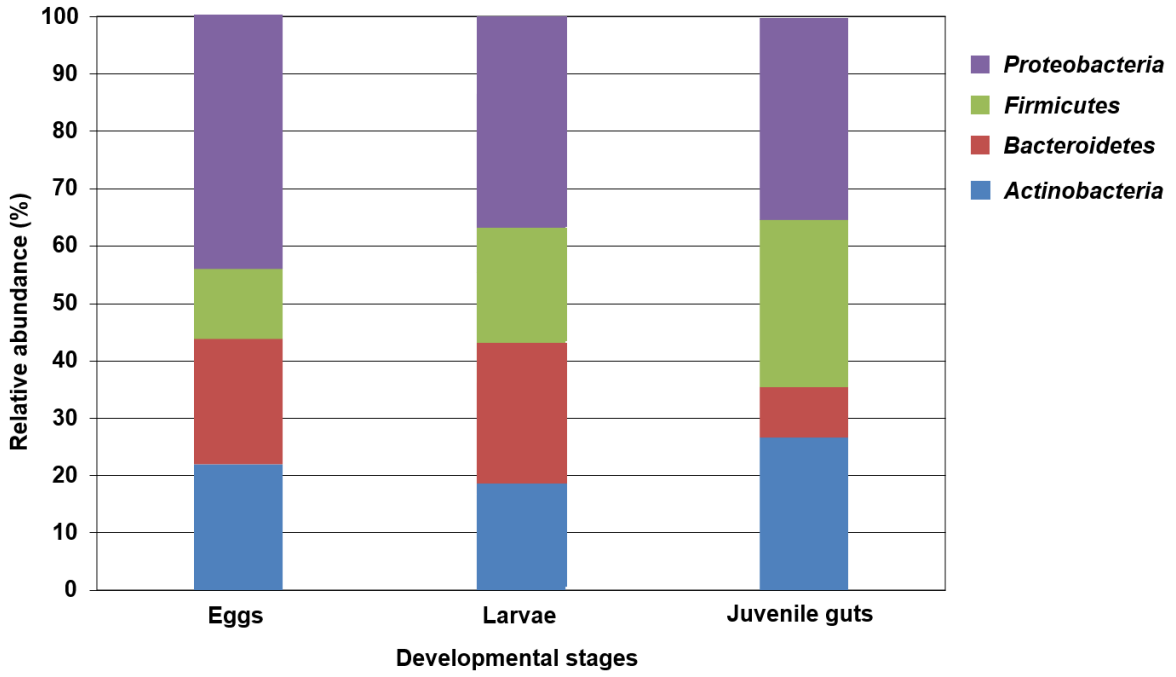


Figure 14. Relative abundance of cultivated bacterial phyla in the three fish developmental stages (*Sparus aurata* eggs, larvae, and juvenile guts).

Isolated bacteria were mainly distributed among four bacterial phyla. From the fertilized eggs through larval and juvenile stages, *Proteobacteria* was the dominant phylum. The phylum *Firmicutes,* which in eggs constituted less than 15% of the cultivated bacterial community, increased in abundance in both larvae and juvenile stages (for around 20% and 30%, respectively). In contrast, *Bacteroidetes* decreased in abundance in the juvenile stage. At last, *Actinobacteria* decreased from the egg to the larval stage. However, from the latter to the juvenile stage this phylum increased considerably in relative abundance.

The most represented bacterial genera in the egg stage (**Figure 15**) were *Roseobacter* (16%), *Ruegeria* (9%), *Vibrio* (9%), and *Polaribacter* (9%), while in the larval stage (**Figure 15**) the genera *Microbacterium* (19%), *Tenacibaculum* (19%), *Bacillus* (16%), and *Vibrio* (13%), were most dominant. Finally, highest proportions of *Photobacterium* (25%), *Bacillus* (17%), and *Streptomyces* (14%) were found in juvenile guts (**Figure 15**). We observed that genera belonging to the *Gammaproteobacteria* class (within the *Proteobacteria* phylum) increased in abundance as the fish host developed, that is, at the larval and juvenile (gut) developmental stages.

Indeed, most of the isolates considered as pathogens in this study belonged to the *Gammaproteobacteria* class, with the genus *Vibrio* representing 13% of the 38% pathogenic isolates of the total larval culturable community (which is a 4% increase from the previous developmental stage (eggs)), and the genus *Photobacterium* representing 25% of the 64% pathogenic isolates of the total culturable community from the juvenile guts (representing a 100% increase from the two previous developmental stages (eggs and larvae)). This may rank as one of the main factors underpinning for high mortality rates in larviculture, since most of these isolated pathogens are known for their ability to cause lethal infections in several fish at different developmental stages (*e.g., Pho. damselae* [166] and *Vibrio anguillarum* [190]). The *Firmicutes* and *Actinobacteria* increase could be associated, to a large extent, with the intake of water and feed (*e.g.,* rotifers, *Artemia*, formulated feed, and algae) by the larvae/juvenile, since many of the species documented here belonging to these phyla are common to marine environments and hosts. Indeed, increases in *Firmicutes* abundance along fish developmental stages have been often observed among gilthead seabream trials, being usually associated with dietary changes as discussed by Borges *et al.*, (2021) [61] and Moroni *et al.*, (2021) [178], both of which concluded that the increase of *Firmicutes* was directly related with the intake of feed [61, 178]. Moreover, Moroni *et al.*, (2021), also observed an increase of *Actinobacteria* in experimental diets [178].

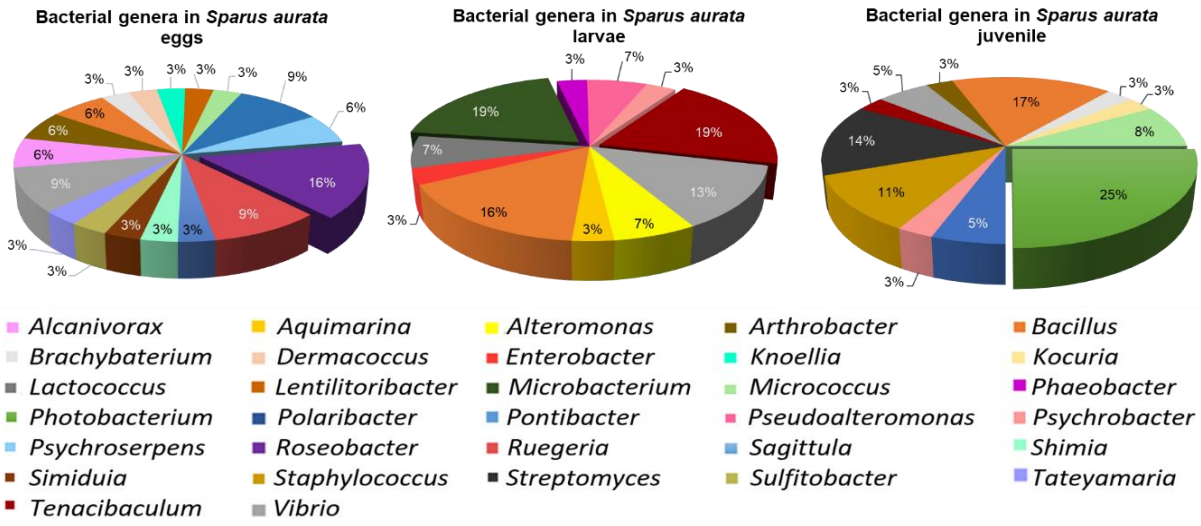


Figure 15. Genus-level composition and 16S rRNA gene-based richness of bacteria isolated from *Sparus aurata* eggs, larvae and juveniles.

However, it should be taken into consideration that in this study a culture-dependent strategy was used to capture potential probiotic candidates for fish larviculture, which is a less comprehensive technique when it comes to cataloguing bacterial diversity than the culture-independent approach. Indeed, cultivable bacteria may represent only a small portion of the microbial community present in a rich environment such as the gut of a fish sample [161]. As far as we know, this is the first time that a culture-dependent strategy to the study of the *S. aurata* microbiome through different life stages is employed, before their application as putative probiotics. After isolation, insights into the role of such microorganisms in the environment can be gained for example through media manipulation (*e.g.,* iron reduction). Moreover, cultured microorganisms can be categorized taxonomically with confidence, aiding in the depth of knowledge on microbial diversity [191].

Employing a culture-independent approach, Califano *et al.*, (2017) [58] and Nikouli *et al.*, (2019) [192] reported, in general, similar results at the phylum level to the ones obtained in this study via cultivation: the microbiome of *S. aurata* was dominated by the phylum *Proteobacteria* in the early developmental stages. The relative abundance of this phylum in the late larval developmental stage decreases, while there is an increase of the phylum *Firmicutes*. Interestingly, in the abovementioned studies, the phylum *Actinobacteria* was observed in vestigial abundance [58, 192]. In contrast, around 23% of all isolates retrieved across the three developmental stages in the present study belonged to the phylum *Actinobacteria*. Nonetheless, through the application of molecular techniques, Califano *et al*., (2017) and Nikouli *et al*., (2019) found 34 and 19 bacterial phyla, respectively, which is nine times and almost five times more, respectively, than the ones found in the present study (four phyla) [58, 192]. Likewise, when comparing genera across the two studies (Califano *et al.*, (2017) and the present study), significant changes between both methods were found. For example, Califano *et al*., (2017) found genera such as *Loktanella, Actinobacillus, Paracoccus,* and other uncultured bacteria in higher abundance than in this study [58]. In contrast, genera such as *Vibrio, Photobacterium, Roseobacter, Ruegeria, Bacillus, Tenacibaculum, Streptomyces,* and *Microbacterium* were found at a much higher proportions when using a culture-dependent method rather than molecular methods. In fact, most of these genera were virtually not found by molecular techniques, mainly the pathogenic genera such as *Vibrio, Tenacibaculum* and *Photobacterium*. These differences are not unexpected, in fact, they are quite ordinary as discussed by Hardoim *et al.*, (2014) [193]. In this study, Hardoim *et al.*, (2014) demonstrated that 15 to 18 bacterial phyla were found in *Marcottages spinosulus* and *Ircinia variabilis* sponges using cultivation-independent methods. However, when comparing the results obtained with those retrieved with a cultivation-dependent method, the *Proteobacteria* phylum (mainly *Alphaproteobacteria* and *Gammaproteobacteria* classes) dominated the cultured bacterial community. In turn, cultivation-independent methods revealed between 200 and 220 OTUs in *S. spinosulus* and *I. variabilis*, respectively, while via culturing only 33 and 39 OTUs were found in these species. Even so, despite the significantly large OTUs community found with the cultivation-independent method, around 50% of all cultured OTUs escaped detection by this method [193]. Overall, these studies demonstrate that differences between these techniques are quite common, and that both techniques may be used in a complementary fashion to enable a comprehensive description of bacterial diversity in the natural environment.

To infer about the identity between isolates from this study and their closest type-strains, phylogenetic trees based on the 16S rRNA gene sequences of the bacterial isolates and respective closest type-strains for each class of bacteria were constructed and are presented in **Figure 16**. The 16S rRNA gene has a slow mutation rate, meaning it can be used as a reliable molecular clock enabling us to understand the phylogenetic relationships among prokaryotes without fully elucidating the evolutionary changes that this molecule undergoes [194]. However, due to its slow evolutionary rate, often times it is difficult to distinguish between closely related species or strains based solely on 16S rRNA gene sequences.

In the present study, members of the *Gammaproteobacteria* class clearly dominated the bacterial communities in larvae and juvenile samples, as previously discussed and evidenced by the trees shown in **Figure 16**, whilst the genera belonging to the *Alphaproteobacteria* class were most dominant in eggs. No genus was particularly dominant among the *Alphaproteobacteria*. Contrarily, within the *Gammaproteobacteria* the well-known fish pathogen *Photobacterium* was the genus with the highest number of isolates (n = 9). All these nine isolates were placed into the same branch in the *Gammaproteobacteria* phylogenetic tree sharing virtually 100% 16S rRNA gene identity with the species *Photobacterium damselae*. In this phylogenetic tree is to also notice the high diversity of *Vibrio* species. The *Bacteroidetes* phylum also presented a dominant genus (*Polaribacter,* n = 6), with the species *Polaribacter marinivivus* dominating this phylum. Regarding the *Actinobacteria* phylum, *Microbacterium maritypicum* was clearly dominant (n = 6). Finally, the phylum *Firmicutes* was mainly represented by the genus *Bacillus* with 15 isolates, comprising seven different species (*B. hwajinpoensis, B. plakortidis, B. oceanisediminis, B. halmapalus, B. cereus, B. safensis/pumilus,* and *B. idriensis*) with *B. hwajinpoensis* and *B. oceanisediminis* being the species sharing the same, highest number of isolates (n = 5). Also, to note was the presence of two *Staphylococcus* spp., which are also notable and recognized pathogens. The patterns seen across the phylogenetic trees corroborate the taxonomic classification obtained and proposed in **Table 4.** To highlight is the fact that isolates L10 and E24, belonging to the *Vibrio* genus (*Gammaproteobacteria*), are far away from their own closest type-strains *V. owensii* and *V. diabolicus*, respectively in the *Gammaproteobacteria* tree. Despite having a gene similarity between their closest type-strain above the established threshold (almost 100%) in the NCBI database, the score given by RDP was around 0.98. Other examples include isolate E11 (*Gammaproteobacteria*), presumably belonging to the *Simiduia* genus, which presents a 98.70% gene similarity with its closest type-strain *S. agarivorans* accordingly to NCBI and an extremely low score in RDP (0.696). Furthermore, isolates E25 and J3 (*Actinobacteria*) belonging to the genus *Micrococcus*, despite having higher than 99% and 0.99 scores in NCBI and RDP, respectively, with the type-strain of the species *M. aloeverae,* both present a high divergence from their type-strain in the *Actinobacteria* tree. The same happens for the isolates L25, L8, L11A, L5Amarelo, and J2-B2 (gene similarity and S_ab score between 97-98% and 0.97-0.98, respectively) belonging to the genus *Tenacibaculum* (*Bacteroidetes*), closely related to the type-strain *T. mesophilum*, and isolate E23 (*Bacteroidetes*) belonging to the *Polaribacter* genus, closely related to the type-strain *P. porphyrae* which has a gene similarity below 97% and a S_ab score of 0.961.

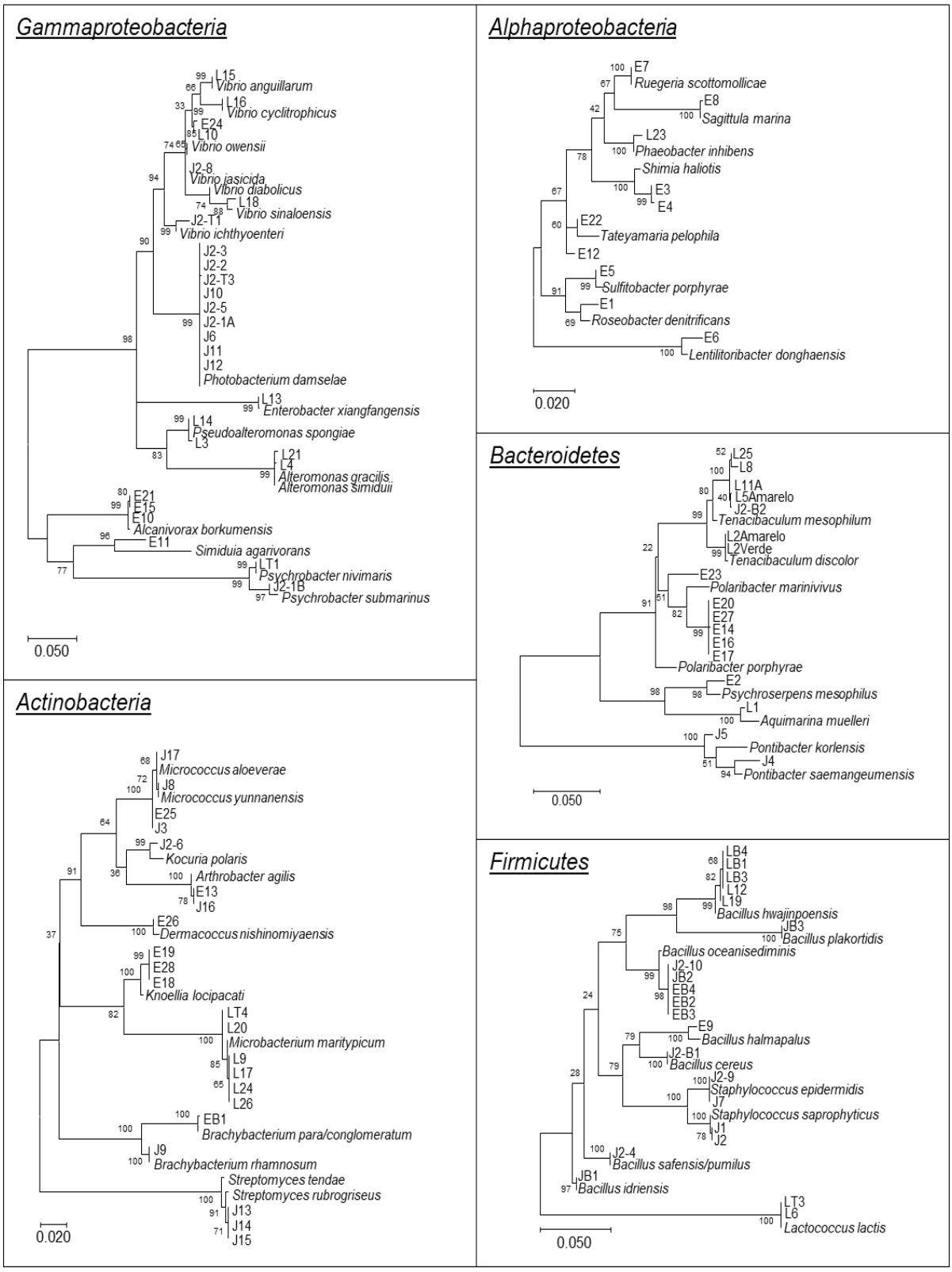


Figure 16. Phylogenetic trees of partial 16S rRNA gene sequences showing the relationship between bacteria isolated from *Sparus aurata,* and their respective closest type-strains. The tree was constructed with the Maximum Likelihood statistical method using the Tamura-Nei substitution model (Kimura's two-parameter model for *Actinobacteria* tree) with complete deletion of the gaps/missing data. The numbers at the branch nodes indicate the bootstrap support based on 500 replicates (*e.g.,* 100 on a branch means that in the 500 replicates, the branch and the isolates always have that same position). The accession numbers of sequences from type-strains are in **Table 4**. On the bottom left of each tree is the branch scale for the respective tree.

3.2. Selection of isolates with potential probiotic properties

The main goal of the characterization performed via bioactivity screenings of the isolates in this thesis was to select the two best complementary isolates (one isolate with the best enzymatic activity and another isolate with the best antagonistic activity). In **Table 5,** a summary of antagonistic and enzymatic activity features, retrieved from available literature, of the species phylogenetically closest to our isolates is shown, along with their classifications as "probiotics", "pathogens" and "unknowns" based on these features.

Based on literature reports, several bacterial phylotypes isolated in this study such as *Bacillus safensis* [195-197]/*pumilus* [197-199]; *Bacillus hwajinpoensis* [156]; *Lactococcus lactis* [200]; *Streptomyces rubrogriseus* [182, 201]/*tendae* [181, 202] and *Phaeobacter inhibens* [203], have previously been identified as probiotics. Moreover, no pathogenicity towards animals and plants has been described for the abovementioned microorganisms. Therefore, the isolates described here which were closest related with these bacterial species were classified as potential probiotic strains (**Table 5**). In contrast, other species such as *Bacillus halmapalus* [188]; *Bacillus oceanisediminis* [142, 204]; *Brachybacterium conglomeratum* [172]/*paraconglomeratum* [171]; *Brachybacterium rhamnosum* [150]; *Microbacterium maritypicum* [205]; *Roseobacter denitrificans* [147, 206]; *Micrococcus yunnanensis* [207]; *Arthrobacter agilis* [208, 209]; *Pseudoalteromonas spongiae* [210]; *Alcanivorax borkumensis* [211]; *Pontibacter korlensis* [154] and *Psychrobacter nivimaris* [159] had never been used as probiotics, but possessed several putative probiotic characteristics, such as siderophore and hydrolytic enzymes production, antimicrobial activities, and biosynthesis of plant growth promoting molecules. Consequently, isolates closely related with these species were classified as "unknown" strains from the perspective of their validated use as probiotics (**Table 5**). The remaining isolates, all closely related with species that showed pathogenicity or may function as opportunistic pathogens towards animals or plants were classified as potential pathogenic strains (**Table 5**).

Only those isolates classified as "potential probiotic" and "unknown" strains were selected for further characterization (a total of 45 isolates). However, *Lactococcus* sp. (n = 2), *Alcanivorax* sp. (n = 2), *Lentilitoribacter* sp. (n = 1), *Pontibacter* sp. (n = 1), *Pseudoalteromonas* sp. (n = 2), *Simiduia* sp. (n = 1) and *Tateyamaria* sp. (n = 1) grew poorly in MB medium and could not be fully characterized. Therefore, a total of 35 isolates were characterized regarding hydrolytic enzymatic and antagonistic activities.

Table 5. Classification and characteristics of the closest bacterial species (from RDP) to the isolates retrieved in this study as potential probiotics, pathogenic and "unknown" (from the standpoint of probiotic *versus* pathogenic behavior) taxa based on literature reports. The existence of literature reports stating the isolation from finfish is also exhibited.

Category	Organisms (RDP)	Characteristics	Isolated in finfish	Refs.
Potential Probiotic	*Bacillus hwajinpoensis*	Symbionts of marine sponges with potential biotechnological and biopharmaceutical applications	No	[156]
	Bacillus safensis; B. Pumilus	Several positive probiotic tests in *Sparus aurata* and Tilapia fish larviculture, among other applications	No; Yes	[195-197]; [197-199]
	Lactococcus lactis	Pathogen inhibitor, used in fermentation of food, growth promoters, among others	Yes	[162, 200]
	Phaeobacter inhibens	Pathogen inhibition	Yes	[163, 203]
	Streptomyces rubrogriseus; S. Tendae	Enzymatic activities, antibacterial and antifungal properties	No	[182, 201]; [181, 202]
Pathogen	*Bacillus cereus*	Human pathogen	Yes	[117, 164]
	Bacillus idriensis	Possible Human pathogen	No	[183]
	Dermacoccus nishinomiyaensis	Human pathogen	No	[184]
	Enterobacter xiangfangensis	Dog pathogen	Yes	[165, 212, 213]
	Kocuria polaris	Opportunistic Pathogen in humans	No	[185]
	Micrococcus aloeverae	Human and plant pathogen	No	[187, 214]
	Photobacterium damselae	Fish pathogen	Yes	[166]
	Psychrobacter submarinus	Possible Human pathogen	No	[215]
	Staphylococcus epidermidis	Human and fish pathogen	Yes	[62]
	Staphylococcus saprophyticus	Human pathogen	Yes	[63, 216, 217]
	Tenacibaculum discolor	Fish pathogen	Yes	[167]
	Tenacibaculum mesophilum	Possible fish pathogen	Yes	[167]
	Vibrio anguillarum	Fish pathogen	Yes	[190]
	Vibrio cyclitrophicus	Mussel pathogen	No	[175]
	Vibrio diabolicus	Human pathogen	Yes	[73, 218]
	Vibrio ichthyoenteri	Fish pathogen	Yes	[169]
	Vibrio jasicida	Lobster larvae pathogen	Yes	[170, 219]
	Vibrio owensii	Shrimp pathogen	No	[173]
	Vibrio sinaloensis	Shrimp pathogen	No	[174]
Unknow *	*Alcanivorax borkumensis*	Siderophore production	No	[211]
	Arthrobacter agilis	Plant growth promoter, antifungal compound and iron acquisition inducer in plants	No	[208, 209]
	Bacillus halmapalus	Amylase producer	No	[188]
	Bacillus oceanisediminis	Possible industrial applications (*e.g.,* reservoir of heavy-metal resistance genes)	No	[142, 204]
	Brachybacterium conglomeratum; B. Paraconglomeratum	Proteolytic activity, biosurfactant producers and antimicrobial agents	Yes	[172]; [171]
	Brachybacterium rhamnosum	Chlorophenol-degrading activity which can be used as antimicrobial agents	No	[150]
	Microbacterium maritypicum	Soybean pathogen suppresser	No	[205]
	Micrococcus yunnanensis	Plant growth promoter and antimicrobial metabolites	No	[160, 207]
	Pontibacter korlensis	Lipopeptide biosurfactant producer	No	[154]
	Pseudoalteromonas spongiae	*Hydroides elegans* larval growth and survival enhancer	No	[158, 210]
	Psychrobacter nivimaris	Possible siderophore production	No	[159]
	Roseobacter denitrificans	Possible pathogen inhibitor	No	[147, 206]

* To our knowledge the following microorganisms were classified as unknown strains since no information about pathogenicity and probiotic activity was found in the literature: *Aquimarina muelleri, Bacillus plakortidis, Simiduia agarivorans, Alteromonas simiduii; Alteromonas gracilis; Pontibacter saemangeumensis; Polaribacter marinivivus; Polaribacter porphyrae; Psychroserpens mesophilus; Lentilitoribacter donghaensis; Sulfitobacter porphyrae; Knoellia locipacati; Ruegeria scottomollicae; Sagittula marina; Shimia haliotis*; and *Tateyamaria pelophila.*

3.2.1. Hydrolytic activities

To evaluate whether the isolates were able to produce hydrolytic enzymes, enzymatic activities were performed for the detection of lipases, cellulases, proteases, chitinases and amylases, in each selected isolate (**Table 6**). Most of the isolates (71%) exhibited at least one hydrolytic activity. However, only eight isolates (*Bacillus halmapalus* E9*, Arthrobacter agilis* E13*, Aquimarina muelleri* L1*, Alteromonas simiduii* L4*, Alteromonas gracilis* L21*, Bacillus hwajinpoensis* LB3*, Bacillus oceanisediminis* J2-10 and JB2) possessed the five hydrolytic activities assessed. In contrast, 10 strains (*Phaeobacter inhibens* L23, *Shimia haliotis* E3, *Shimia haliotis* E4, *Sulfitobacter porphyrae* E5, *Ruegeria scottomollicae* E7, *Sagittula marina* E8, *Polaribacter marinivivus* E16, *Tateyamaria pelophila* E22, *Polaribacter porphyrae* E23, and *Polaribacter marinivivus* E27) did not show any hydrolytic activity.

Table 6. Production of extracellular hydrolytic enzymes (enzymatic activity index). The average for each enzymatic assay (at least biological triplicates) is present in the table**.**

Closest RDP type-strain	ID	Hydrolytic activities (Average ± SD*)				
		Lipases	Cellulases	Proteases	Amylases	Chitinases
Alteromonas gracilis	L21	3.02 ± 0.32	4.34 ± 0.06	1.56 ± 0.12	1.90 ± 0.24	3.00 ± 0.49
Alteromonas simiduii	L4	3.44 ± 0.31	3.60 ± 0.15	2.48 ± 0.23	1.46 ± 0.24	5.06 ± 0.30
Aquimarina muelleri	L1	4.11 ± 0.59	5.59 ± 0.73	3.70 ± 0.30	3.76 ± 0.36	4.91 ± 0.80
Arthrobacter agilis	J16	4.09 ± 0.09	3.48 ± 0.28	2.07 ± 0.07	nd	3.20 ± 0.00
Arthrobacter agilis	E13	3.20 ± 0.53	4.43 ± 1.09	2.96 ± 0.50	3.00 ± 0.20	4.25 ± 0.25
Bacillus halmapalus	E9	2.43 ± 0.43	2.07 ± 0.07	3.57 ± 0.14	1.83 ± 0.17	2.61 ± 0.48
Bacillus hwajinpoensis	LB3	2.33 ± 0.11	1.72 ± 0.06	2.87 ± 0.07	1.24 ± 0.01	2.05 ± 0.11
Bacillus hwajinpoensis	L19	1.96 ± 0.31	1.56 ± 0.06	3.53 ± 0.22	nd	1.93 ± 0.07
Bacillus oceanisediminis	J2-10	1.93 ± 0.07	2.69 ± 0.06	2.35 ± 0.46	1.68 ± 0.14	3.23 ± 0.02
Bacillus oceanisediminis	JB2	2.00 ± 0.00	2.80 ± 0.05	1.97 ± 0.22	1.58 ± 0.22	3.35 ± 0.21
Bacillus plakortidis	JB3	nd	nd	3.79 ± 0.04	nd	nd
Bacillus safensis; B. pumilus	J2-4	2.62 ± 0.24	nd	4.04 ± 0.54	nd	nd
Brachybacterium para/conglomeratum	EB1	2.09 ± 0.09	nd	nd	nd	3.38 ± 0.63
Brachybacterium rhamnosum	J9	nd	4.00 ± 0.00	1.69 ± 0.19	2.32 ± 0.18	4.67 ± 0.83
Knoellia locipacati	E19	nd	2.79 ± 0.54	nd	nd	nd
Knoellia locipacati	E28	3.16 ± 0.13	4.58 ± 0.89	nd	nd	2.63 ± 0.13
Microbacterium maritypicum	L9	nd	1.96 ± 0.19	nd	nd	2.60 ± 0.40
Microbacterium maritypicum	L24	nd	1.92 ± 0.22	nd	nd	3.00 ± 0.67
Micrococcus yunnanensis	J8	2.01 ± 0.09	nd	nd	nd	2.85 ± 0.35
Phaeobacter inhibens	L23	nd	nd	nd	nd	nd
Polaribacter marinivivus	E16	nd	nd	nd	nd	nd
Polaribacter marinivivus	E27	nd	nd	nd	nd	nd
Polaribacter porphyrae	E23	nd	nd	nd	nd	nd
Pontibacter saemangeumensis	J4	-	2.50 ± 0.50	2.55 ± 0.52	nd	2.73 ± 0.48
Psychrobacter nivimaris	LT1	2.79 ± 0.34	nd	nd	nd	nd
Psychroserpens mesophilus	E2	2.39 ± 0.11	nd	1.66 ± 0.22	nd	nd
Roseobacter denitrificans	E1	4.94 ± 0.96	nd	nd	nd	nd
Ruegeria scottomollicae	E7	nd	nd	nd	nd	nd
Sagittula marina	E8	nd	nd	nd	nd	nd
Shimia haliotis	E3	nd	nd	nd	nd	nd
Shimia haliotis	E4	nd	nd	nd	nd	nd
Streptomyces rubrogriseus; S. tendae	J14	2.07 ± 0.38	3.97 ± 0.15	nd	2.58 ± 0.25	3.75 ± 0.25
Streptomyces rubrogriseus; S. tendae	J13	2.67 ± 0.10	4.11 ± 0.74	nd	2.28 ± 0.36	3.29 ± 0.46
Sulfitobacter porphyrae	E5	nd	nd	nd	nd	nd
Tateyamaria pelophila	E22	nd	nd	nd	nd	nd

"nd", no activity detected; "-", no growth observed; *SD, Standard Deviation.

The isolate with the highest lipase activity was *Roseobacter denitrificans* E1 (EAI = 4.94), **Figure 17-A.** This was an expected result since isolates belonging to this species had already been reported to exhibit lipase activity [220]. In the present study, other isolates such as *Aquimarina muelleri* L1 (EAI = 4.11), and *Arthrobacter agilis* J16 (EAI = 4.09) also displayed considerable lipase activity and studies of lipase production for these species have also been reported [221, 222]. Regarding cellulose and starch degradation, *Aquimarina muelleri* L1 presented the highest cellulose and amylase activity (enzymatic activity index of 5.59 and 3.76, respectively) (**Table 6** and **Figure 17-B** and **E**). To our knowledge, cellulose activity was never reported for *Aquimarina muelleri*. In contrast, amylase activity has been described for *A. muelleri* LMG 22569^T. Strain LMG 22569 was isolated from seawater, and was observed to hydrolyze starch, casein, chitin and tween 20, 40, and 80 [222]. These results were also verified in the present study, since isolate L1 showed activity in all hydrolytic tests. Several isolates, namely *Bacillus safensis/pumilus* J2-4, *Bacillus plakortidis* JB3, *Aquimarina muelleri* L1, *Bacillus hwajinpoensis* L19 and *Bacillus halmapalus* E9 showed high enzymatic activity index values for protease activity (between 3.50 and 4.00). *Bacillus safensis/pumilus* J2-4 presented the highest protease activity with an EI of 4.04 (**Table 6** and **Figure 17-C**). To our knowledge, protease activity was detected for the first time for the species *Bacillus plakortidis* (strains JB3). Berrada *et al.*, (2012) [223] demonstrated the production of proteases in the strain *Bacillus safensis* B582 [223], obtained from two hypersaline environments, located in Larache [223]. Similarly, the production of proteases has been described for species closely related with isolates L1 (*A. muelleri*), E9 (*B. halmapalus*), and L19 (*B. hwajinpoensis*) [156, 222, 224]. The highest chitin-degradation activity (EAI = 5.06) was observed for *Alteromonas simiduii* L4 (**Table 6** and **Figure 17-D**). According to Chiu *et al.*, (2007) [225], the strain *Alteromonas simiduii* AS1^T, isolated from water samples collected from the Er-Jen River estuary, Tainan, Taiwan, was tested for the production of chitinases through the growth of the strain on a modified PY plate medium containing colloidal chitin. The isolate presented growth within 15 and 20 days of incubation, which indicates the production of chitinases and consequent hydrolysis of chitin, since this was the only source of carbon, which is needed for the bacterium to grow [225].

The methods used in this study for the retrieval and measurement of enzymatic activities, revealed to be adequate for our objectives since they allowed both an excellent quantitative and a qualitative measurement of the isolate enzymatic activity. Most of the results presented a great yield and quality ratio between the colony size and the enzymatic halo, which permitted a clear identification and selection amongst enzymatic producers and non-producers for the tested enzymes. It is well documented that probiotics could alter the digestive enzymatic profile of their larval host, throughout the evolution of the larval digestive system, which develops and changes as the larva grows [226, 227]. Tovar-Ramirez *et al.*, (2004) [228] documented that seabass (*Dicentrarchus labrax*) larvae fed with live yeast (*Debaryomyces hansenii* CBS 8339), incorporated in their diet, showed improved activity and concentrations of lipase [228]. As a result of the present study, the bacteria isolated from the digestive system of *S. aurata* have been shown to export, *in vitro*, hydrolytic enzymes which can be directly related with the isolation location (*e.g.,* fish gut), since this is the location where unattractive complex molecules (*e.g.,* chitin), eaten by the fish, will be digested.

Therefore, it is plausible that bacteria thriving in the fish gut (and in the overall microbiomes present in the fish such as skin and gills) present some sort of ability to obtain resources for their survival and proliferation. The use of the host-microbiome system as a source of probiotics may elicit tremendous positive side effect since these bacteria are naturally established within the host defense system. It has been suggested that one of the beneficial features of producing hydrolytic enzymes is the increase of the food digestibility, thus releasing more nutrients into the host diet and enhancing feed utilization, modulating the overall growth of the fish [104].

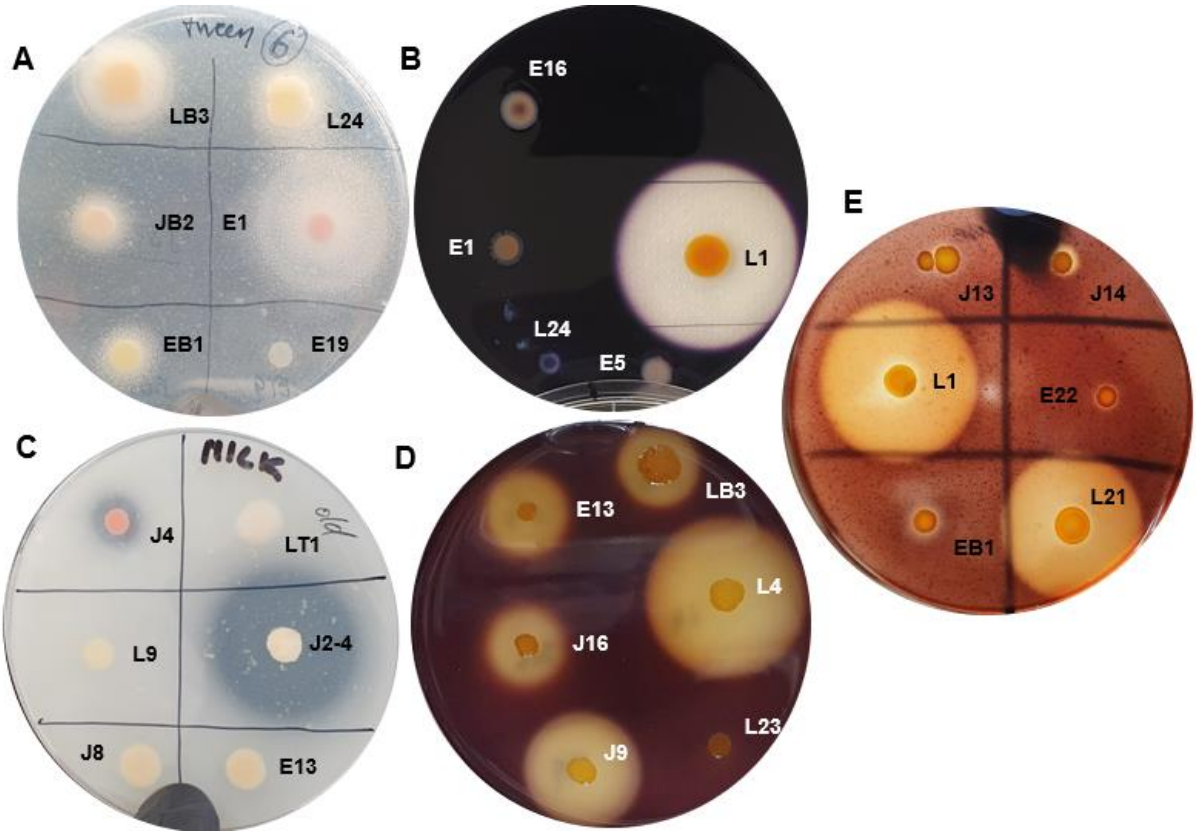


Figure 17. Lipase screening results for *Roseobacter denitrificans* (E1). Lipase screening method, using tween 80 as a substrate in the culture medium. The colonies produce a white halo around the colony, thus indicating the formation of a calcium complex. Therefore, there is an indication of lipase production, Plate A; Amylase and cellulase screening results for *Aquimarina muelleri* (L1), Plate B and E. Amylase screening method, using starch as a substrate in the culture medium. A halo around the colony is formed due to the absence of starch in that region, visualized by Lugol's iodine reagent, which means that the starch once present in that area was hydrolyzed (Plate B). Cellulase screening method, using cellulose as a substrate in the culture medium. A halo around the colony formed due to the degradation of cellulose, stained with Lugol's iodine reagent (Plate E); Protease screening results for *Bacillus safensis/pumilus* (J2-4). Protease screening method, using milk as a substrate in the culture medium. A halo around the colony is formed due to the degradation of milk-derived casein, Plate C; Chitinase screening results for *Alteromonas simiduii* (L4). Chitinase screening method, using CC from β-chitin powder as a substrate in the culture medium, stained with Lugol's iodine reagent. A halo around the colony is formed due to the degradation of chitin, Plate D.

Additionally, the production of these enzymes can lead to an increase of the reared fish overall health, since they might inhibit the colonization of the intestine by pathogens or inhibit their activity [104]. In general, this modulation, and fish intestinal microbiome manipulation, can have a positive impact on aquaculture, allowing fish to have a better growth and heating-up the circular economy using natural resources, such as the naturally occurring symbiotic bacteria that thrive in association with fish, for more efficient fish production.

3.2.2. Antagonistic activities

In this study, potential probiotic bacteria were selected based on their antimicrobial activity against pathogens. The antimicrobial activity of the selected isolates (35 strains) was preliminary done by cross-streak assay (section 3.2.2.1.). Based on the results of this assay, the isolates that showed inhibitory effect on at least one fish pathogenic strain were selected for the further assessment using the soft-agar overlay assay (section 3.2.2.2.).

3.2.2.1. Cross-streak assay

The cross-streak assay was used as a fast-screening assay to qualitatively determine the antibacterial activity of the 35 selected isolates against three pathogenic indicator strains (*Pho. damselae*, *S. iniae*, and *V. parahaemolyticus*). This assay consisted of striking the desired isolate on the middle of the plate, after which the pathogen was streaked, seven days afterwards the desired isolate incubation, in a perpendicular direction to the isolate (**Figure 18**).

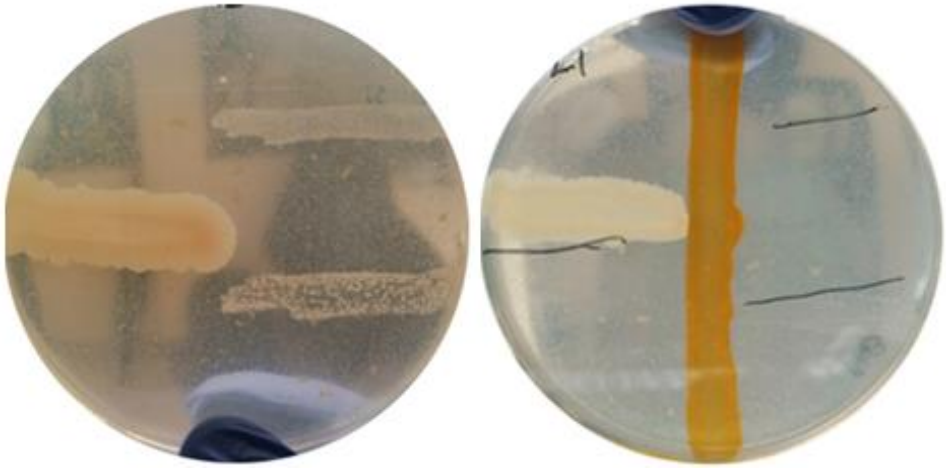


Figure 18. The plate on the left exemplifies a negative control test where no potential antagonist is streaked on the center of the plate, while fish pathogens *Vibrio parahaemolyticus* (left side of the plate, middle strike), *Photobacterium damselae* subsp. *piscicida* (right side of the plate, upper strike) and *Streptococcus iniae* (right side of the plate, lower strike) have been streaked for visualization of growth in the absence of potential antagonists. The plate on the right shows the antagonistic results obtained for *Aquimarina muelleri* strain L1. On the right side of the plate there is no, or too little growth of the pathogens *Streptococcus iniae* (lower strike) and *Photobacterium damselae* (upper strike), due to the inhibitory activity by strain L1. On the left side of the plate, there was no growth inhibition of the pathogen *Vibrio parahaemolyticus*.

Out of the 35 isolates, only seven strains (*Aquimarina muelleri, Phaeobacter inhibens, Bacillus safensis* or *B. pumilus, Alteromonas simiduii, Alteromonas gracilis, Bacillus oceanisediminis* and *Microbacterium maritypicum*; 20% of the tested isolates) showed consistent inhibitory activity against at least one fish pathogen. However, the results showed poor reproducibility since most of the isolates did not demonstrate a consistent inhibition throughout the streak assays (biological triplicates). The poor reproducibility could be related to the irregular production of the antibacterial compounds by the isolates, the irregular growth of the pathogenic strains, or due to the technique itself. Since this assay did not allow us to determine antagonistic activity in a robust manner and did not deliver a quantitative measurement of the inhibition, the isolates that showed inhibitory activity against at least one fish pathogen (22 out of 35 isolates) were then subjected to another assay, namely the soft-agar overlay assay.

3.2.2.2. Soft-agar overlay assay

The soft-agar overlay assay is a quantitative assay used in this study to improve reproducibility and allow a better measurement of the inhibitory activity of the tested isolates. This assay consists of growing a spot of the isolate on a plate, and then covering the plate with soft agar previously seeded with one pathogen, thus creating a layer on top of the medium where the test isolates were grown (**Figure 19**). This assay was likewise used in this thesis to determine the ability of the tested isolates to inhibit the growth of the same three pathogens: *Pho. damselae*, *S. iniae*, and *V. parahaemolyticus.*

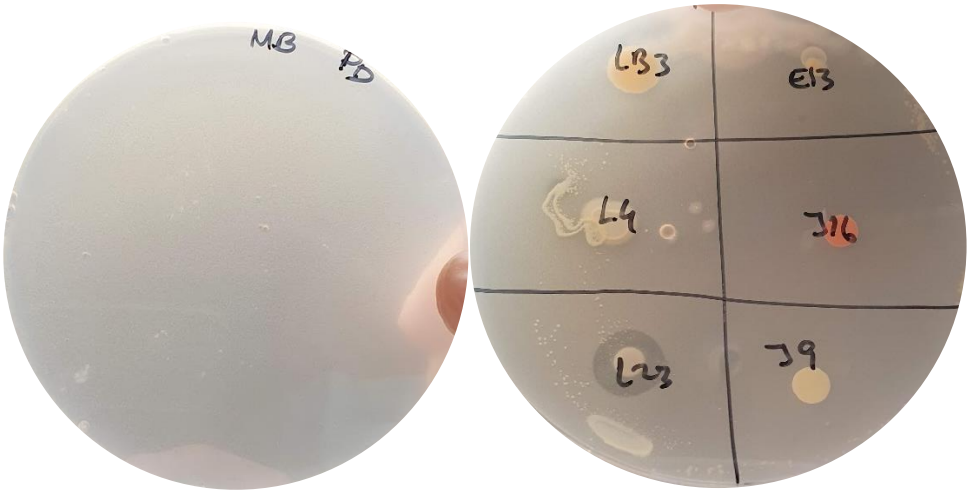


Figure 19. Example of an overlay assay result, where soft agar seeded with the pathogen (in this case, *Photobacterium damselae* subsp. *piscicida*) is layered over the culture medium spotted with the potential probiotic. The plate on the left shows a negative control result, where *Photobacterium damselae* subsp. *piscicida* was laid on top culture medium without any test strain. The halo observed on the right plate (bottom left colony) represents antibacterial activity by strain *Phaeobacter inhibens* L23 isolate against *Photobacterium damselae* subsp. *piscicida*.

Based on our results, from the previous technique, *Phaeobacter inhibens* L23 inhibited *Pho. damselae*, *S. iniae*, and *V. parahaemolyticus*, while *Bacillus oceanisediminis* J2-10 inhibited *Pho. damselae* (**Table 7**). In this case, since we are not only evaluating the production of enzymes, but the antagonistic activity, EAI from **Equation 1** was denominated AAI (Antagonistic Activity Index).

Table 7. Antibacterial activity of the selected isolates following the soft-agar overlay method. The halos were measured according to **Equation 1.** The average (at least biological duplicates) and the standard deviation (SD) for each antagonistic test is present in the table.

RDP type-strain (Isolates)	**Isolate ID**	**AAI (Average ± SD)**		
		Vibrio parahaemolyticus	***Photobacterium Damselae* subsp. *piscicida***	***Streptococcus iniae***
Phaeobacter inhibens	L23	1.43 ± 0.09	2.40 ± 0.10	1.58 ± 0.09
Bacillus oceanisediminis	J2-10	nd	3.00 ± 0.30	nd

"nd", no activity detected.

The isolate *Phaeobacter inhibens* L23 displayed a wide range of inhibition since it could inhibit the growth of the three pathogens (two Gram-negative bacteria namely *V. parahaemolyticus* (AAI = 1.43) and *Pho. damselae* (AAI = 2.40), and one Gram-positive bacterium namely *S. iniae* (AAI = 1.58)). In contrast, the isolate *Bacillus oceanisediminis* J2-10, only displayed antibacterial activity against *Pho. damselae* (AAI = 3.00). The remaining isolates *B. oceanisediminis* (JB2), *P. saemangeumensis* (J4), *M. yunnanensis* (J8), *B. rhamnosum* (J9), *S. rubrogriseus*/*S. tendae* (J13 and J14), *A. agilis* (J16 and E13), *B. safensis/B. pumilus* (J2-4), *A. muelleri* (L1), *A. simiduii* (L4), *M. maritypicum* (L9 and L24), *A. gracilis* (L21), *B. hwajinpoensis* (LB3 and L19), *S. haliotis* (E4), *B. halmapalus* (E9), *K. locipacati* (E28), and *B. para/conglomeratum* (EB1) did not show any or consistent results under our experimental conditions and were not considered for further antagonistic assays.

The implementation of these two assays (cross-streak assay and soft-agar overlay assay) in combination enabled the conduction of a more dedicated screening methodology, by discarding the isolates without any antagonistic activity through a previous fast-screening assay, and at the same time advancing to the selection and testing of the isolates with the best antagonistic activity. Although the cross-streak assay was helpful as a fast-screening assay it is felt that the conditions of this assay should be extensively adapted, according to the organism being studied, as a means to improve the measured activities. In contrast, the soft-agar assay generated reproducible results among replicates. The usual lack of antagonistic activity observed among the test strains (only two out of 22 strains showed positive and consistent results) were likely due to inability of the test isolate to grow in such adverse conditions ("environment" with a heavy pathogen load). However, this assay also presented its difficulties, mainly in the overgrowth of some pathogens, which could lead to an exceedingly inhibitory growth conditions for the isolates, and the colony displacement and consequent wild growth of the isolates evaluated throughout the medium. The genus *Phaeobacter* (more specifically, the species *P. inhibens*) has been demonstrated to inhibit the growth of pathogenic bacteria belonging to genera such as *Vibrio*, *Photobacterium,* and *Streptococcus* [229]. The substances (antagonistic compounds) produced by bacteria may be synthesized as either primary or secondary metabolites and therefore have different modes of inhibitory action. One of the usual secondary metabolites excreted by bacteria are antibiotics that, although produced in small quantities, inhibit or kill other microorganisms [4]. *P. inhibens* is one example of antibiotic producing bacterium by synthesizing Tropodithietic Acid (TDA). Due to the production of this natural antibiotic (among other reasons), *P. inhibens* has also been identified as a probiotic, as previously shown in **Table 6.** Since isolate L23 is closely related with *P. inhibens*, it might hold the potential to synthesize this antibiotic due to the fact that it also produces a brownish dye, dispersed around the colony (indicative of TDA production by *P. inhibens*) [163]. Nonetheless, the production of this particular antibiotic was not evaluated in this thesis. Therefore, the remarkable antagonism exhibited by this isolate may possibly occur due to the production of this antibiotic which has been proven to have antibacterial activity against pathogens in, for example, *Artemia* cultures [163]. In contrast, *B. oceanisediminis*, to which the isolate J2-10 is closely related, had no reports regarding antagonistic activity against *Photobacterium* or any other pathogenic bacterial taxon.

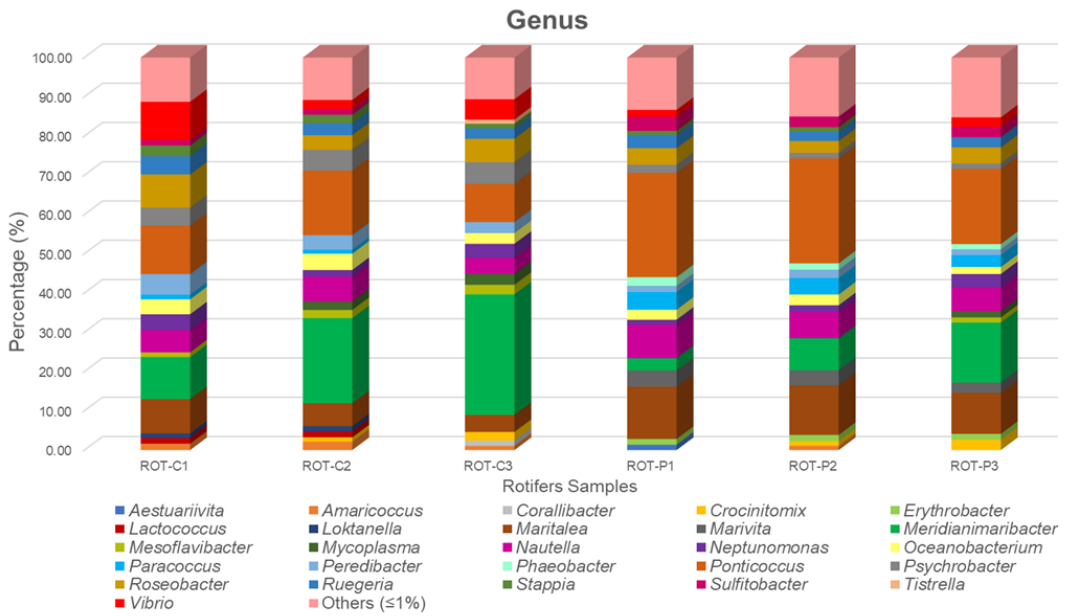
This is an interesting result, since this species had already been identified to be a conceivable choice for industrial application due to the presence of a heavy-metal resistance genes reservoir, and for nematode and fungal control, in plants, due to nematicidal and fungal activity, respectively [142, 223, 230]. This bacterium was also found to produce a biosurfactant (surfactin) which can be used as a powerful antibiotic [231].

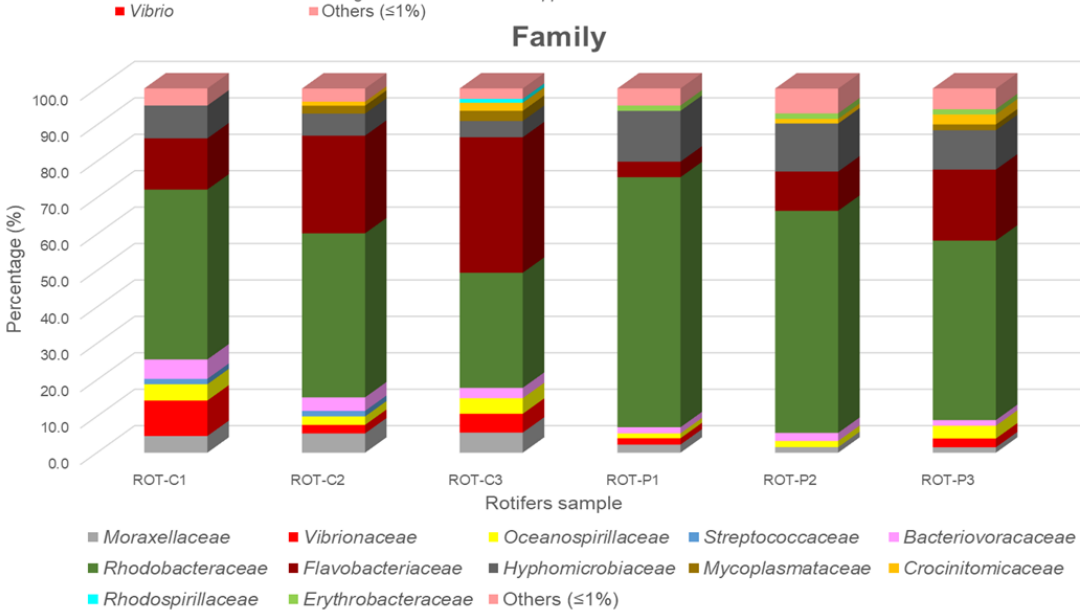
3.3. Selection of probiotics for the larval trial

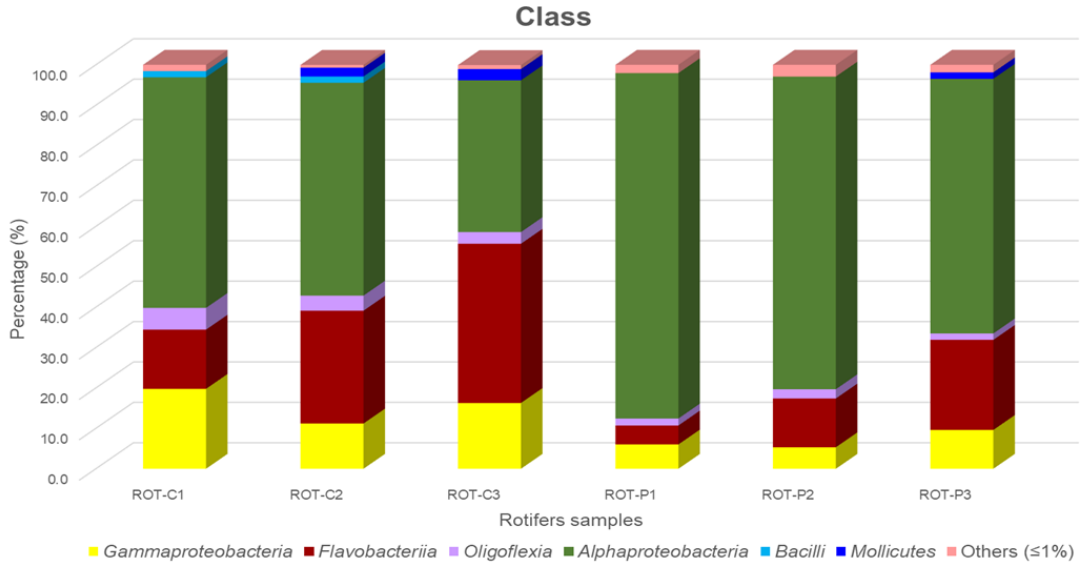
Based on the previous data and results, the two best isolates were chosen to be evaluated as potential probiotics in a larval rearing trial. As previously discussed, one of this study's objective, besides the identification and characterization of non-pathogenic bacteria with hydrolytic and antibacterial activities, was to select two isolates with the best activities of each assay (hydrolytic and antagonistic) to attempt a complementary and balanced action of the putative probiotics. The isolates with better hydrolytic activity in general (high activity in all enzymatic tests), based on the data obtained during this study, were *A. muelleri* L1 and *A. agilis* E13. Despite exhibiting better results, *A. muelleri* L1 was not selected since some species of the *Aquimarina* genus were found to be opportunistic pathogens in lobsters, for example by degrading the chitin shell of these marine animals, through chitinolytic activity [232]. In contrast, *A. agilis* E13 has been suggested as plant growth promoter and phytopathogenic fungi inhibitor [208, 209], and therefore was the isolate selected for the larval trial. The isolate with the best antagonistic activity (antibacterial activity against all three indicator strains) was *P. inhibens* L23. Therefore, this isolate was also chosen for the larval trial. A literature review for these two isolates regarding colonization, biofilm formation and stress resistance (conditions valued for potential probiotics) was conducted. From this analysis it was concluded that *P. inhibens* is an excellent colonizer and a biofilm former in glass and in marine environments, including hosts (*e.g.,* finfish skin and mollusks shells) [233]. This bacterium is also resistant to acidic stress since the production of TDA is known to induce acidic environments [234]. Regarding *A. agilis*, a study suggests that this bacterium is not able to produce any biofilm in ceramic roof tiles [235]. Nevertheless, no other studies were found for the ability to produce biofilms by this bacterium, thus not excluding the possibility of forming biofilms in other systems, since this strain was obtained from the gut of *Sparus aurata*. Regarding stress resistance, this bacterium was found to resist and adapt to thermal and salt stress conditions [141]. A brief description of *A. agilis* and *P. inhibens* can be found in **Annex III.**

3.4. Effects of probiotics supplementation on rotifer-associated bacterial communities

To assess the probiotic capacity of both isolates on growth and survival of *S. aurata* larvae, rotifers enriched simultaneously with *Arthrobacter agilis* E13 and *Phaeobacter inhibens* L23 (cell densities of 1 x 10^6 cfu/mL) were produced (ROT-P). As a control, rotifers were grown in the same conditions without probiotic supplementation (ROT-C). Total community DNA samples were obtained from rotifers with and without probiotics. Probiotic enriched rotifers and control rotifers were examined for bacterial community diversity and composition, through 16S rRNA gene amplicon sequencing, using Illumina technology (**Figure 20**).


Genus
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Percentage (%)
ROT-C1
ROT-C2
ROT-C3
ROT-P1
ROT-P2
ROT-P3
Rotifers Samples
Aestuariivita
Amaricoccus
Corallibacter
Crocinitomix
Erythrobacter
Lactococcus
Loktanella
Maritalea
Marivita
Meridianimaribacter
Mesoflavibacter
Mycoplasma
Nautella
Neptunomonas
Oceanobacterium
Paracoccus
Peredibacter
Phaeobacter
Ponticoccus
Psychrobacter
Roseobacter
Ruegeria
Stappia
Sulfitobacter
Tistrella
Vibrio
Others (≤1%)


Family
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percentage (%)
ROT-C1
ROT-C2
ROT-C3
ROT-P1
ROT-P2
ROT-P3
Rotifers sample
Moraxellaceae
Vibrionaceae
Oceanospirillaceae
Streptococcaceae
Bacteriovoracaceae
Rhodobacteraceae
Flavobacteriaceae
Hyphomicrobiaceae
Mycoplasmataceae
Crocinitomicaceae
Rhodospirillaceae
Erythrobacteraceae
Others (≤1%)


Class
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percentage (%)
ROT-C1
ROT-C2
ROT-C3
ROT-P1
ROT-P2
ROT-P3
Rotifers samples
Gammaproteobacteria
Flavobacteriia
Oligoflexia
Alphaproteobacteria
Bacilli
Mollicutes
Others (≤1%)

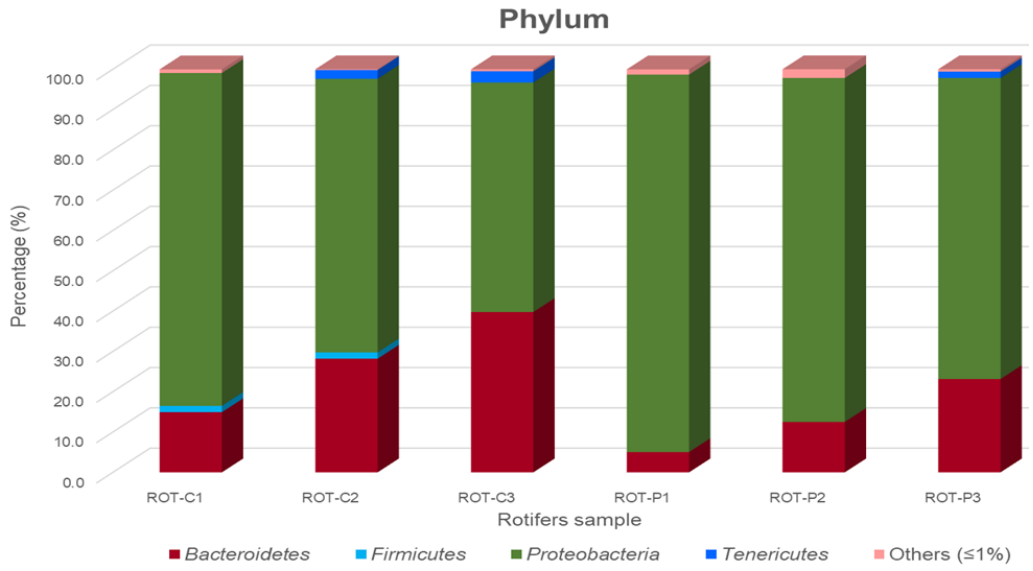


Figure 20. Probiotic enriched rotifers and control rotifers bacterial community composition representation (zOTUs), obtained through 16S rRNA gene amplicon sequencing, using Illumina technology. The taxonomic levels such as genus, family, class and phylum, and the taxa with the highest presence (higher than 1%) on the rotifers control samples (ROT-C1, ROT-C2, and ROT-C3) and treated rotifer samples (ROT-P1, ROT-P2, and ROT-P3) are shown. The remaining taxa with a presence lower or equal to 1% were grouped in the category "Others".

Despite a small increase in the relative abundance of *A. agilis* and *P. inhibens* in probiotic-enriched rotifers (from an average of 0.01% to 0.04%, and 0.52% to 1.74%, respectively), the addition of these inoculants, although not making them dominant in the system, caused a considerable change in the structure of the microbiomes of the rotifers. For instance, there was an increase in relative abundance of the *Rhodobacteraceae* (with an average increase of almost 20% across the probiotic-treated rotifers, thus dominating the microbiome, **Figure 20-Genus and Family chart**) which are acknowledged to contain species/strains with probiotic and/or beneficial properties towards marine animals [58, 61, 180, 233]. Examples of genera belonging to this family include *Paracoccus, Ponticoccus*, and *Phaeobacter*, which demonstrated significant increases, confirmed through a Welch's *t*-test (**Figure 23**). As an example, *Phaeobacter* in the control rotifers samples, has a presence lower than 1% or even zero, and in the treated rotifers samples the presence of this specie is higher than 1%, which may be related with the great ability of this species to form biofilms (**Figure 20-Genus chart**) [233]. Contrarily, in the presence of inoculants when compared with the microbiome of the control rotifers, pathogens belonging to the *Vibrio* genus fell in abundance, with an average decrease of almost 5% (for example the *Vibrio* genus has an relative abundance average of 6% in the ROT-C samples, whilst in the ROT-P samples this average fell to 2%), and genera belonging to the *Flavobacteriaceae* family (*e.g., Meridianimaribacter* [236]), which is known for harboring opportunistic species [237], also displayed an average decrease of 15% from the ROT-C to the ROT-P samples. Interestingly, no *Photobacterium* zOTUs were found in any sample. Overall, the *Proteobacteria* phylum, more specifically the *Alphaproteobacteria* class, clearly dominated all the rotifer-associated bacterial communities, especially among the treated rotifers. In general, it appears that the inoculants are moving the rotifers' microbiome to a less detrimental state compared to the control ones, decreasing the pathogenic species thus allowing more space for *Rhodobacteraceae* species to increase in abundance.

To gain better insights into this hypothesis (existence of a shift from a microbiome composed of several different genera to a more concise microbiome), the zOTU data was subjected to a multivariate, ordination analysis using Principal Coordinates Analysis (PCoA) to determine whether the communities in control *versus* treated rotifers were significantly different (**Figure 21**).

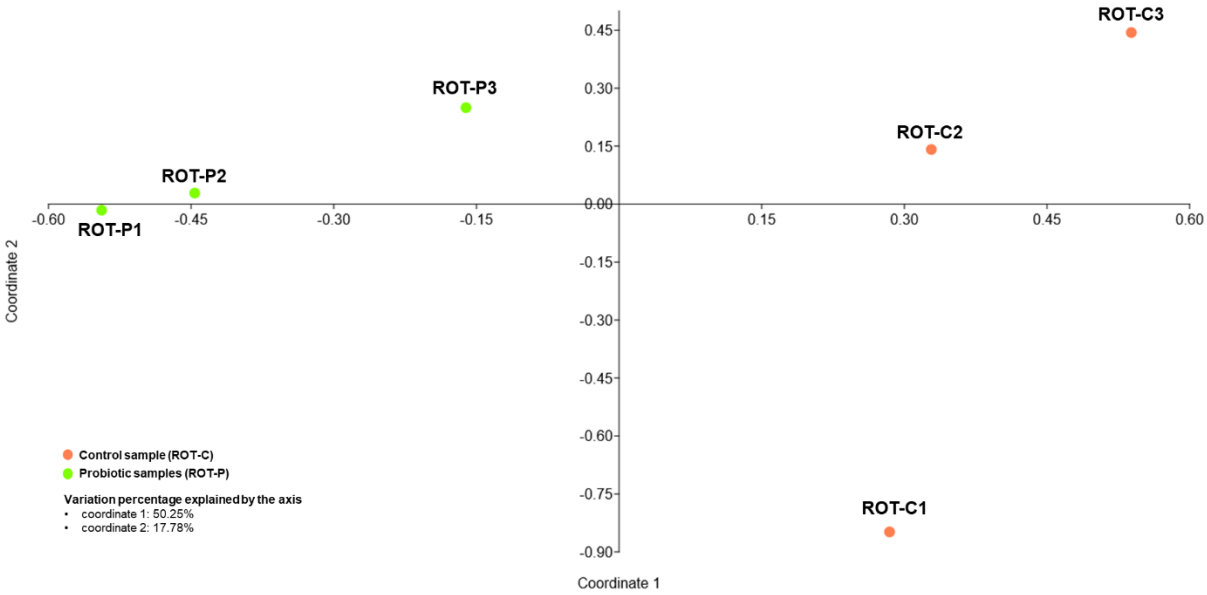


Figure 21. Principal Coordinate Analysis (PCoA) performed on a Bray-Curtis similarity matrix obtained from zOTUs relative abundance data in control (ROT-C) vs treated rotifer samples (ROT-P)) microbiome zOTUs. Coordinate 1 and coordinate 2 explain 50.25% and 17.78%, respectively (68.03% explained when combining the two coordinates), of the total dataset variation.

The obtained PCoA diagram showed that two groups were clearly formed and separated (three samples each group): the control "group" (with significant variation within the control samples) and the treated rotifers group (less variance within the treated samples). Coordinate 1 displays the highest percent explanation of the total community variation (50.25%), while coordinate 2 explained 17.78% of the total community variation. An interesting result is the lower variation in bacterial community structure among the rotifer-treated samples, which therefore had a higher resemblance and proximity between each other thus giving strength to the proposed hypothesis. In contrast, the three samples of the control group were farther away from each other, thus suggesting higher beta diversity (variation in community composition) obtained through 16S rRNA gene amplicon sequencing. In spite of a clear separation of control and probiotic treated rotifers samples along coordinate 1 of the ordination diagram, the structure of the rotifer-associated prokaryotic communities was considered not to differ significantly among control and treatment sample groups as determined by PERMANOVA (P = 0.11). Therefore, another deep statistical analysis was performed using the "STAMP v2.1.3" bioinformatic tool, by applying the two groups Welch's *t*-test, with a p-value filter higher than 0.05 (below or equal to this value, the results show significant difference and are the ones showed). This program allows us to understand if the relative abundance of each individual genus, family, class, and phylum had a significant shift as well as the direction of that shift (increasing percentage in control and decreasing in the treated rotifers, and *vice-versa*). These results can be observed in **Figure 22.**

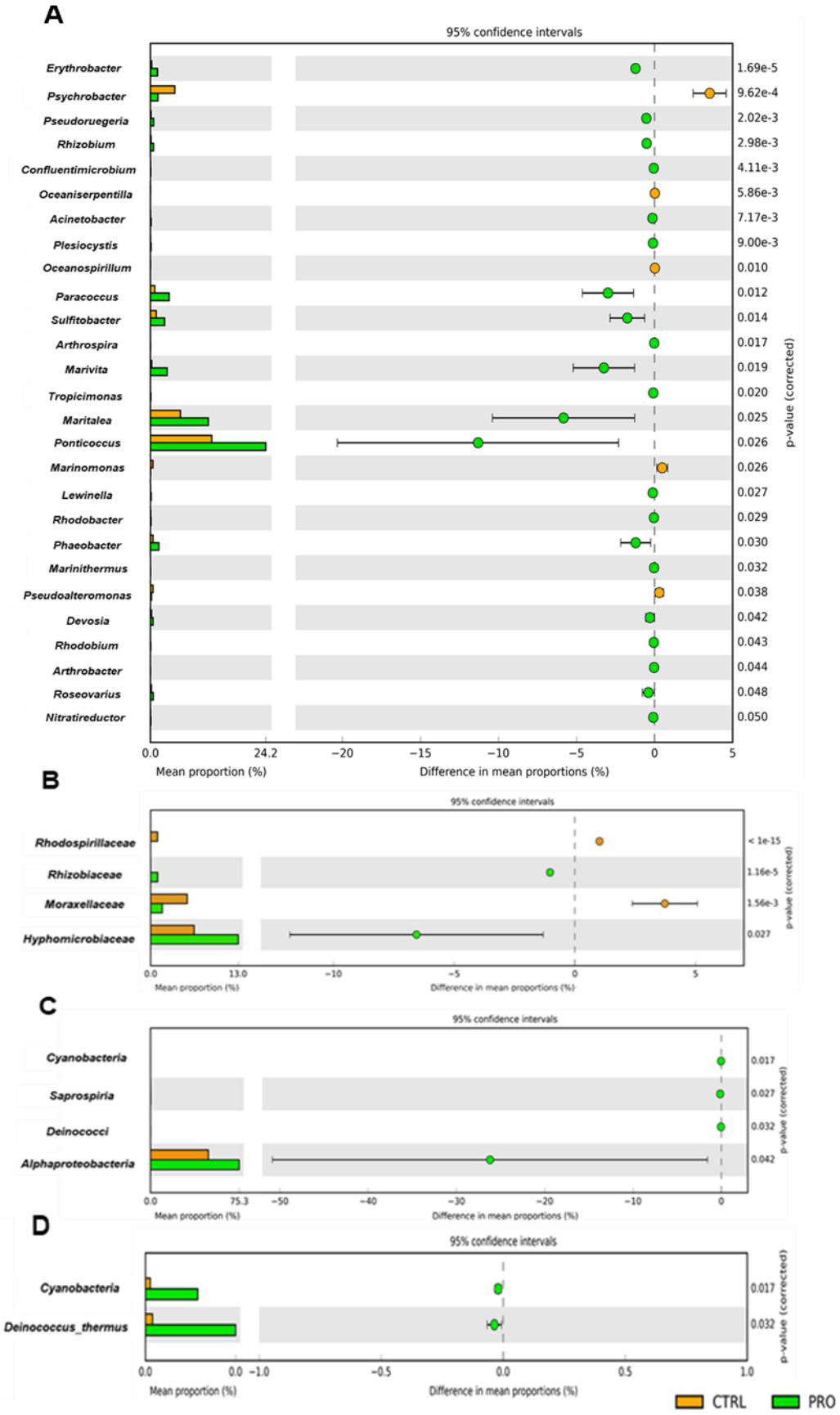


Figure 22. Extended error bar chart using the two groups Welch's *t*-test, showing the genera (A), families (B), classes (C), and phyla (D) with significant statistical difference (P < 0.05) for each sample (control (CTRL) vs treated rotifers (PRO)). The mean proportion (%), as well as the difference in the mean proportion (%) is shown. This test has 95% interval confidence, and the p-values (corrected) are sorted from the lowest to the highest.

From all the zOTUs analyzed, for each taxonomic level, 27 genera (out of 110), four families (out of 48), four classes (out of 17), and two phyla (out of 10) presented a significant shift in their relative abundance. From these, the genera *Erythrobacter*, *Paracoccus*, *Sulfitobacter*, *Marivita*, *Maritalea*, *Ponticoccus*, and *Phaeobacter* (**Figure 22-A**), the families *Hyphomicrobiaceae* and *Rhizobiaceae* (**Figure 22-B**), the class *Alphaproteobacteria*, **(Figure 22-C)** and the phylum *Deinococcus_thermus* (**Figure 22-D**) had the highest variation increases (in the probiotic (PRO) direction) due to an increase in abundance from the control to the treated rotifers (**Figure 20**). The *Deinococcus_thermus* phylum had a significant shift in the relative abundance even though it belonged to the phyla with less or equal 1% presence in the overall bacterial community profile, before and after the treatment (**Figure 20**). In contrast, the genus *Psychrobacter*, and the families *Rhodospirillaceae* and *Moraxellaceae* had the highest increase in variation (in the control (CTRL) direction) due to a significant decrease of the relative abundance from the control to the treated rotifers (**Figure 20**). Curiously, the *Vibrio* genus and the *Flavobacteriaceae* family did not display a significant shift in their relative abundance in control *versus* treated rotifers. This is a surprising result since both displayed an obvious decrease in their percentage in the latter samples (**Figure 20**). However, after performing a Welch's *t*-test we verified that despite this clear decrease in the treated rotifers the difference between abundance means of control and treated groups was not significant for both taxa. Another surprising result was the absence of a significant shift of the *Rhodobacteraceae* family and phyla *Proteobacteria* and *Bacteroidetes*, despite their increase in treated rotifers as revealed in **Figure 20**. The presence of a significant shift with a low variance in the mean proportion difference of both isolates administered to the rotifers was also an interesting outcome since it might indicate that the probiotic inoculation led to the survival and increase of both isolates in the larvae. Altogether, the results shown in **Figures 20** and **22** suggest that the treatment with inoculants moved the rotifers microbiome to an apparent less detrimental state compared to the control ones. At the beginning of this Master thesis, the hypothesis/question if it was feasible to manipulate the live feed microbiome through the use of probiotics was raised. The findings described above suggest this hypothesis to be true. Moreover, the shift present in the rotifers communities might also answer the hypothesis that a shift in the larvae microbiome might have occurred, that is, a change in the larvae microbiome from a detrimental state, with the presence of several pathogenic bacteria, to a more beneficial community composition with for example *Rhodobacteraceae* standing out and controlling the microbiome, while having a positive effect on larvae overall biometrics and survival. The larvae analysis is ongoing, and this hypothesis will be addressed in the future.

Overall, these were significant results since for most cultured marine fish species the most suitable prey at first feeding are rotifers (usually *Brachionus* spp.) [238], which are crucial for the fish/larvae health not only by potentially acting as vehicle of bacteria (beneficial or pathogenic), but also as a means for growth and durable life. Manipulation of the microbiomes associated with the live feed provided to the larvae has potential application in the delivery of probiotics onto reared species. The density of bacterial communities in rotifers is approximately 5×10^3 cells per individual and attempts to feed rotifers with a considerably higher bacterial load to fish larvae has proven unsuccessful. Moreover, the growth of the probiotic on the live feed depends on several factors such as the type of probiotic, duration of exposure and initial quantity, and the state (dead or alive) of the feeding organism [79].

As the live feed's bacterial load increases, it may well reach levels, and/or microbial communities might develop, that may negatively affect the health of the host larvae [79]. For example, Munro *et al.*, (1999) [239] found that rotifers with an overload of bacteria led to poorer larval growth when fed to turbot larvae. In contrast, when UV-treated rotifers with significantly decreased bacterial load were provided, the turbot larvae displayed a higher survival rate [239]. Because rotifers are highly relevant as feed for fish larval rearing but may simultaneously represent a vector of pathogenic bacteria to fish larvae, applying probiotics to rotifers may constitute an effective approach to prevent proliferation of bacterial diseases among the feeding larvae. Moreover, the fish may also be too sensitive to foreign inoculants. Thus, it is likely that the delivery of probiotics through the inoculation of live feed renders a more cost-effective and efficient method than simply applying probiotics to the rearing tank-water (*e.g.,* in cases where there is water renewal in the larval rearing system, attachment of probionts to live feed may be preferable since it results in lower inoculant volumes) [79]. Zink *et al.*, (2013) [240] demonstrated that not only is possible to apply probiotics in live feed, but it is also possible to use probiotics as an approach to improve rotifers. In the mentioned study, the authors assessed a commercially available *Bacillus* spp. probiotic blend on population growth dynamics of the rotifer *Brachionus plicatilis*. This addition improved rotifer culture population growth rate, suggesting potential production benefits from its exploitation [240]. In the present study, the above-mentioned factors were not fully studied due to lack of time and resources. Hence, the study focused directly on the application of the two test isolates (L23 and E13) to the live feed (rotifers) and its effect on the growth and survival of the feeding larvae.

3.5. Effects on growth and survival of *S. aurata* larvae treated with probiotic-enriched rotifers

To evaluate the probiotic capacity of both isolates, *Arthrobacter agilis* E13 and *Phaeobacter inhibens* L23 were added simultaneously (cell densities of 1 x 10^6 cfu/mL) into one independent tank containing fish eggs (probiotic tank). As a control, the same fish egg amount was added to another tank (control tank). After egg hatching, the larvae were then distributed into eight tanks (four control tanks from the initial egg control tank and four probiotic tanks from the initial egg tank incubated with the isolates). Then, from 4 DAH to 22 DAH, probiotic-enriched rotifers were given as feed to the probiotic fish larvae independent tanks whilst larvae in the four independent rearing control tanks were fed with standard rotifers (*i.e.*, without potential probiotics). The total larval length (TL), dry weight (DW), and survival were evaluated and compared for the two treatments (**Figure 23**). TL and DW were similar for both treatments (control *versus* probiotic) not showing statistically significant differences ($P > 0.05$), since the beginning until the end of the trial, as shown in **Figure 23**. The survival data analysis also did not show any statistically significant difference between the two treatments ($P > 0.05$), even when calculated from hatching, presenting a survival of 4.55% for control treatment and 5.35% for probiotic treatment, nor after discarding larvae that died before mouth opening, with a survival of 5.55% for control treatment and 6.53% for probiotic treatment (**Figure 23**).

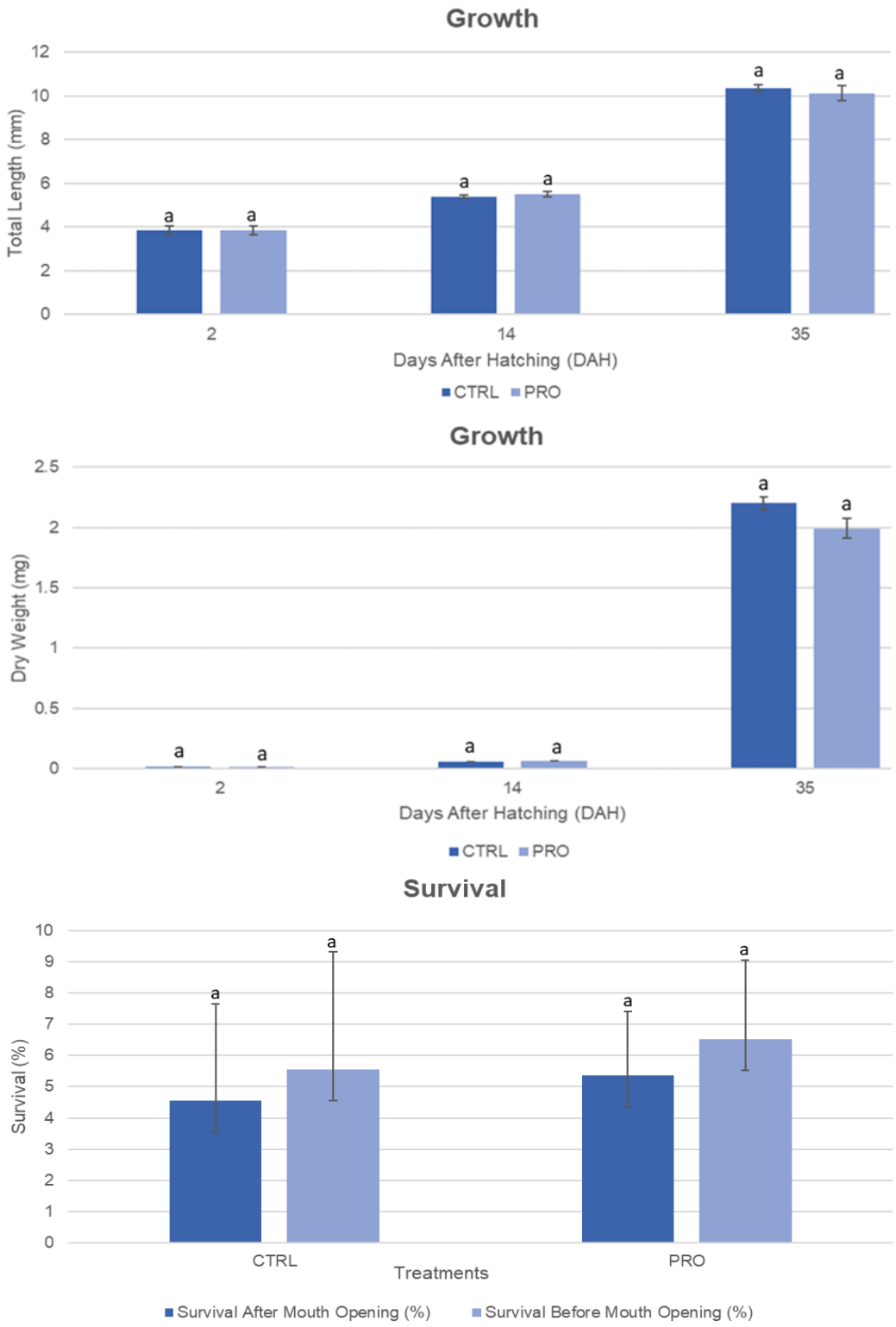


Figure 23. Larval length (mm) and dry weight (mg) biometric data comparison between the control batch (larvae with no potential probiotics; dark blue) and larvae fed with probiotic-treated rotifers (light blue) for samples taken at 2, 14, and 35 DAH is shown. The larval survival from the 1 DHA (before mouth opening; bottom left panel) is shown as well as the larval survival from the 3 DHA (after mouth opening; bottom right panel). The standard deviation is presented for all data. All statistical analysis were performed using "IBM SPSS Statistics 21.0 software". The data referring to the growth of different treatments were submitted to a unidirectional variance analysis (one-way ANOVA), to assess the existence of significant differences between the two different treatments. The Shapiro-Wilk test was used to evaluate the normality of the data, and the Leven's test to evaluate their homogeneity. When the results showed significance, the means between treatments were compared using Tukey's *post hoc* test. A Student's *t*-test was applied to results of survival at the end of the trial. Statistical significance was assessed with a confidence level of 95%. No statistical difference between the treatment and control tanks was observed, indicated in the figure by the letter "a" on top of each bar.

However, despite no statistical difference was observed for the biometric and survival data between the two experiments (control *versus* probiotic), the latter showed a slight survival increase of 0.98% when calculated from hatching and an increase of 0.80% when evaluated after mouth opening, which might suggest that probiotic treatment optimization (*e.g.,* dose and duration) could lead to positive results. In a study carried on *Sparus aurata*, Carnevali *et al.*, (2004) [241] applied two bacterial strains *Lactobacillus fructivorans* AS17B and *Lactobacillus plantarum* 906, previously isolated from *S. aurata* and human feces, respectively. This study demonstrated that the administration of these two bacterial strains significantly decreased larval mortality after 39 days when compared with the control group. In the same study, the authors observed a probiotic influence on gut colonization, with *L. plantarum* colonization being induced by the treatment, and the microbiome control by *L. fructivorans* after 66 days post-hatching [241]. Regarding the two isolates used in the present study (*P. inhibens* and *A. agilis*), no studies covering the probiotic action of the isolates in *Sparus aurata* fish larval cultures was found (as far as we know). Therefore, this is the first study to use any of the isolates as potential probiotics in this particular fish larval trials. Nevertheless, *P. inhibens* has already been used as a putative probiotic in turbot larvae (*Scophthalmus maximus*) culture. In this study, the supplementation effect of the isolate on turbot larvae microbiome was evaluated. Although the addition of *P. inhibens* as part of a probiotic regime did not appear to cause major imbalances on the community structure associated with turbot larvae, the relative abundance of closely related taxa from the *Roseobacter* group were reduced [242]. Moreover, several studies have reported the antagonistic activity of *P. inhibens* against many pathogens such as *V. anguillarum*, *V. harveyi* and *V. vulnificus* [163, 243].

In the present work, the low survival of the treated larvae group might be related with several factors, which raises numerous questions and hypothesis: was there any intake of the potential probiotics by the fish (selective ingestion); the putative probiotics were eaten but did not survive in the digestive tract; did the isolates do not display any probiotic activity studied *in vitro;* was the dosage, the dosage frequency, and duration enough for bacterial colonization and consequent growth. Even so, the decrease of the relative abundance of pathogens in the live feed under probiotic treatment might suggest that pathogen control may be possible using both strains and/or that the larvae may be more resistant to a potential disease outbreak. To evaluate this hypothesis, one future approach might go through another *in vivo* test, through the addition of a pathogen at the end of the larval trial or during the trial itself. It has been suggested by Verschuere *et al.*, (2000) [244] that putative probiotics should be tested *in vivo,* by challenging them through the addition of a representative pathogen when biological control is the objective, so that their effect on growth and/or survival parameters could be assessed. In fact Gildberg *et al.*, (1998) [245], observed that twelve days after infection of Atlantic cod fry with a pathogenic *V. anguillarum* strain, cumulative mortality was reduced in fish given feed supplemented with the diatom *Chaetoceros divergens*. However, at the end of the study the mortality levels were equal throughout the tanks [244, 245]. Other factor that should be taken into consideration is the dosage and dosing frequency. If the goal is for probiotics establishment in the hosts' microbiome and contribution with their probiotic activities, their introduction should be regular and studies on the effect of different probiotic concentrations on the live feed, or larvae, should be conducted [104].

Optimum inoculation densities for host-associated probiotics must be carefully determined to avoid overdosing, which could result in lower efficacy while increasing costs. Moreover, the fish, or the fish microbiome, may be too sensitive to the potential probiotics used [104]. In this thesis, to prevent such susceptibility issues, all isolates were obtained from the fish itself. Nevertheless, issues occurred during the course of the larval rearing trial and the bacterial densities had to be adjusted, confirming the importance of dosage and dosage frequency. A study conducted by Grotkjær *et al.*, (2016) [180] determined that a concentration of 10^6 CFU/mL was sufficient for *P. inhibens* to maintain its cell densities throughout *Artemia* culture trials, independently of the background microbiome [180]. Hence, the bacterial density used in this study (10^6) was very close of the optimal and suggested bacterial density and it is likely that, isolates L23 and E13 will be detectable in the larval samples taken in this study at 2, 7, 14 and 35 DAH using future 16S rRNA gene sequencing.

Despite the biometric data and survival analysis did not show any significant variations between the control and the treated larvae, a 16S rRNA gene amplicon sequencing (similarly to the rotifers 16S rRNA gene amplicon sequencing) to assess the larvae samples community profiles and infer about the isolates proliferation in the fish microbiome is ongoing. These results might help us understand if the rotifers with altered community and the isolates themselves could have created a positive shift in the larvae microbiome, thus giving the larvae a substantial lead to tackle pathogenic genera. The main factor that should be deliberately and thoroughly decided before the beginning of any project is the probiotic source. Despite several studies considering and using probiotics from a distinct source than the host in the study itself, many of the aquaculture probiotic studies across the scientific community seem to be species-specific, meaning the probiotics used were isolated from the same host or are directed to the host on where they are assessed. Therefore, the probiotic could have a positive result in a desirable fish species (the same species they were initially isolated from), but not on other species. Despite the various developments in this area, additional studies are needed where both host-associated probiotics (*e.g.,* from fish microbiomes) and probiotics obtained from other sources (*e.g.,* from marine sediments or water) are used in the same study [104]. In this thesis, if any effect was or tends to be positive in *S. aurata*, it is not guaranteed that it will work on other marine or terrestrial species.

4. Conclusions and Future Work

At the beginning of the Master thesis, through the use of a culture-dependent approach, 97 isolates were obtained from three different *Sparus aurata* life stages: eggs, larvae, and juveniles. From this pool, 35 isolates were characterized for their bioactivities, and it was found that the dominant phylum across all three life stages was *Proteobacteria* which included *Photobacterium* spp. (the most dominant genus in the juvenile stage). In the larvae stage one of the most dominant genera was *Vibrio*. These results could help explain the high mortality rates found in larviculture since species belonging to these genera are known for their bacterial infections in fish and consequent fish mortality. The work conducted here allowed the identification of several novel fish-associated bacterial strains possessing bioactivities presumably related to fish growth promotion and biocontrol. From these results, it was determined that the two isolates most appropriate for the desirable outcome (to increase and enhance larval survival and biometrics, respectively) were *Phaeobacter inhibens* L23 and *Arthrobacter agilis* E13. Together, these isolates were expected to complement each other in the larval trial, through the application of their bioactivities (antagonism and hydrolytic enzymes production by *P. inhibens* and *A. agilis*, respectively) on the live feed (rotifers), larvae and eggs. Using independent triplicates to assess rotifers' bacterial communities, it was clear that the treated rotifers presented an increase of *Rhodobacteraceae* [61], and a decrease of known pathogenic (and opportunistic) species, likely due to the combining bioactivities of the two added isolates. Likewise, when compared with the control rotifers, in the treated ones, these species (*P. inhibens* and *A. agilis*) were found in higher amounts (in the *P. inhibens* case surpassing 1% of the overall bacterial community). This gives strength to the hypothesis that the positive shift created in the treated rotifers microbiome was caused due to the application of the isolates, and that it might be possible to observe the same positive shift in the larvae-associated microbiome (analysis being conducted at the moment). However, when considering the biometrics and survival results from the larval samples, no significant differences between the control and the treated larvae was found. Nevertheless, a slight survival increase in the treated larvae can be seen (with a significant lower standard deviation than the control larvae samples) which might also be the result of the isolates bioactivities.

In this thesis, time and resources availability, and networking logistics were severely affected by the Covid-19 pandemic, especially considering the extended effort needed to select the two best candidates for larviculture application. Therefore, some steps concerning the characterization of the two best candidates were overlooked such as the production of different beneficial compounds (*e.g.,* secondary metabolites such as vitamins) and the isolates *in vitro*/*in vivo* ability to attach to the larval intestinal mucus and consequent growth characteristics in the latter (*e.g*., short lag period and doubling time). Instead, these were substituted by literature review of the two selected isolates, when possible (*e.g*., resistance to stress and biofilm formation). Regarding the assays employed, both antagonistic and enzymatic assays showed to be appropriate for the selection of the two best candidates. Nevertheless, in the enzymatic assays different selective and non-selective media should or must be used in the future to attempt to grow and evaluate a higher range of isolates.

Concerning the antagonistic assays, only one isolate per plate in the soft-agar assay should idealistically be used. However, due to the lack of time several isolates were used per plate. This identification and characterization is relevant, mainly on *S. aurata* fish-associated bacterial strains, since it is the first report (as far as we know) to isolate and characterize cultivable bacteria from this commercially valuable fish employing a culture-dependent approach. It is also the first known study so far to apply either of the isolates (*P. inhibens* and *A. agilis*) in *Sparus aurata* fish larviculture.

As previously discussed, rotifers are considered one of the most suitable prey for a first feeding and one of the most important vectors of bacteria to the larvae, whether beneficial or harmful [79, 238]. As a result, in a future research project or in a future continuity of this project the study should, firstly, evaluate the best vehicle (*e.g.,* live feed or water) to transport the isolate to the eggs/larvae. In this study, it was demonstrated that the bacterial strains used as putative probiotics altered the microbiome structure of the rotifers, despite not being dominant in the microbiome. Therefore, adding the knowledge discussed throughout this thesis, to the results obtained, it is clear that further studies must focus on the isolates ability to colonize and thrive on the live feed microbiome, for example, through the use of different isolate concentrations, and consequent effects assessment of the isolates on the feed growth, mobility, survival, and ability to modify or reduce the pathogenic load (or increase the load of beneficial bacteria) of the microbiome. Only then, idealistically, the research should go through an *in vivo* larviculture trial (from the eggs to the larvae) to assess the larvae biometric and survival data, and the gut bacterial content. The decrease of the relative abundance of pathogens in the live feed under probiotic treatment might also suggest that pathogen control may be possible using both strains and/or that the larvae may be more resistant to a potential disease outbreak. To evaluate this hypothesis, in the future, the putative probiotics could be evaluated *in vivo,* by challenging them through the addition of a representative pathogen, to better understand the future effects of the isolates on the larvae growth and/or survival parameters.

A further extension of the project could lead to a range of positive consequences both in the management of coastal ecosystems and in the sustainable development of the aquaculture sector as well as a positive effect on a fundamental public health issue: the spread of infectious diseases in intensive aquaculture, with potential consequences for human health. To do so, several hurdles need to be tackled and solved, such as regulations which could prevent the production and distribution of probiotics across aquaculture facilities and markets, mainly in the European industry.

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Appendix

Annex I – Microorganism isolation and cultivation

Eggs, larvae, and juveniles from gilthead seabream (*Sparus aurata*) were collected from the EPPO-IPMA (Olhão, Portugal). Samples of around 200 eggs were washed and suspended in 1.0 mL of sterile 0.9% NaCl. The larvae suspension (around 1 mL) was immersed in benzalkonium chloride (0.1%, w/v) for approximately 15 s. Then, the larvae were washed several times with sterile 1.5% NaCl (final washed volume = 250 mL) and resuspended in 1 mL of sterile 1.5% NaCl. The juvenile hindguts (around 1 cm; average weight of 0.108 g) were cut in small pieces and suspended in 1.0 mL of sterile 0.9% NaCl and 1.5% NaCl. Each suspension was homogenized in a mortar/pestle (2 min), transferred into a 15 mL falcon and the volume was adjusted to 10 mL with corresponding NaCl solution. After homogenization, glass beads were added to the homogenates and vortex for 1 min. Finally, ten-fold serial dilutions of homogenates were prepared and plated (100 µL of each dilution) on: (i) half-strength R2A (in ASW) agar medium; (ii) TSA (in 1.5% NaCl) agar medium; and (iii) MRS (in ASW) agar medium plates. To selectively isolate *Bacillus* species, 500 µL of dilution 10^{-1} of all samples were heated at 80°C for 10 min. Heated samples were spread (100 µL) on half-strength R2A medium agar plates. All plates were incubated for 2 to 15 days at 18°C under aerobic conditions. Biological triplicates were performed for all fish development stage, except for larvae, which was done a single time.

Annex II – Media composition

Media composition: Artificial Sea Water (ASW): NaCl (23.38 g/L), $MgSO_4 \cdot 7H_2O$ (2.41 g/L), $MgCl_2 \cdot 6H_2O$ (1.90 g/L), $CaCl_2 \cdot 2H_2O$ (1.11 g/L), KCl (0.75 g/L) and $NaHCO_3$ (0.17 g/L); half-strength R2A medium agar: R2B (1.8 g/L), agar (15 g/L), and 1 liter ASW; TSA (Tryptone Yeast Extract agar) in 1.5% NaCl: tryptone (10 g), Yeast extract (5 g), NaCl (15 g/L), and agar (15 g/L); MRS (De Man, Rogosa and Sharpe) with ASW: MRS (61.2 g/L), 1 mL/L Tween 80, and 1 L ASW. Colonies with different morphological characteristics (color, size, and form) from each sample were selected, sub-cultured in liquid marine broth (MB; 40 g/L) (10 mL) in T-flasks and stored in sterile glycerol (15% v/v) at -80°C.

Annex III - Brief description of *Arthrobacter agilis* and *Phaeobacter inhibens*

A. agilis is a psychrotrophic (capable of growth and reproduction in low temperatures, ranging from -20°C to +10°C), Gram-positive bacterium, firstly isolated from the Pangong Lake, a subglacial lake in northwestern Himalayas, India [246]. It belongs to the *Actinobacteria* phylum, *Actinobacteria* Class, *Micrococcales* Order, and *Micrococcaceae* Family. Its cells have 0.8 to 1.2 µm diameter sphere shape, that occurs in pairs and tetrads. On MB agar, colonies are circular, entire, slightly convex, smooth, and matte with a usually beige to orange color (**Figure 24**). This is a non-sporulated bacterium, and motile due to the presence of, usually, three flagella. However non-motile strains may occur [247].

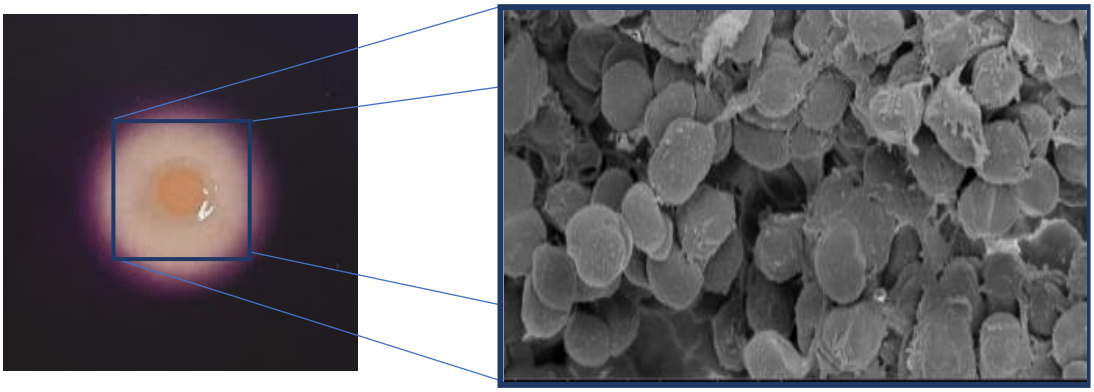

Figure 24. On the left: Colony of isolate E13, identified in this study as *A. agilis* on MA medium, supplemented with starch, stained with 1% Lugol's iodine reagent. On the right side: Scanning Electronic Microscope (SEM) picture of a pure *A. agilis* culture in TSB medium. Picture adapted from Tescari *et al.*, (2018) [248].

This chemoorganotrophic (requires an organic source of carbon and metabolic energy) and aerobic bacterium is known for being a plant growth promoter [246]. *A. agilis* hydrolyzes starch, tween 80, (as corroborated in this study), gelatin and esculin. *A. agilis* strain E13 obtained in this study presented, as discussed, chitinase, lipase, protease, amylase, and cellulose enzymatic activity. *A. agilis* is a catalase and oxidase positive species and grows well at temperatures between 20 and 30°C, while no or poor growth occurs at 37ºC. This bacterium is susceptible to penicillin, streptomycin, chloramphenicol, tetracycline, erythromycin, novobiocin, ampicillin, carbenicillin, and gentamicin. However, it is resistant to lysozyme. Its natural habitats are water, soil, and human skin. The GC content of the DNA is 67.0 to 69.0% and the type-strain is ATCC 966 (= DSM 20550 = CCM 2390) [247].

Phaeobacter inhibens cells are rod-shaped with 1.4-1.9 µm x 0.6-0.8 µm. Colonies on agar are smooth and convex with regular edges that become brownish after 24 h of incubation at 20ºC, and dark brown after 48 h of incubation, with diameter up to 0.80 mm. In MB, it has the tendency to form aggregates (**Figure 25**) [249].

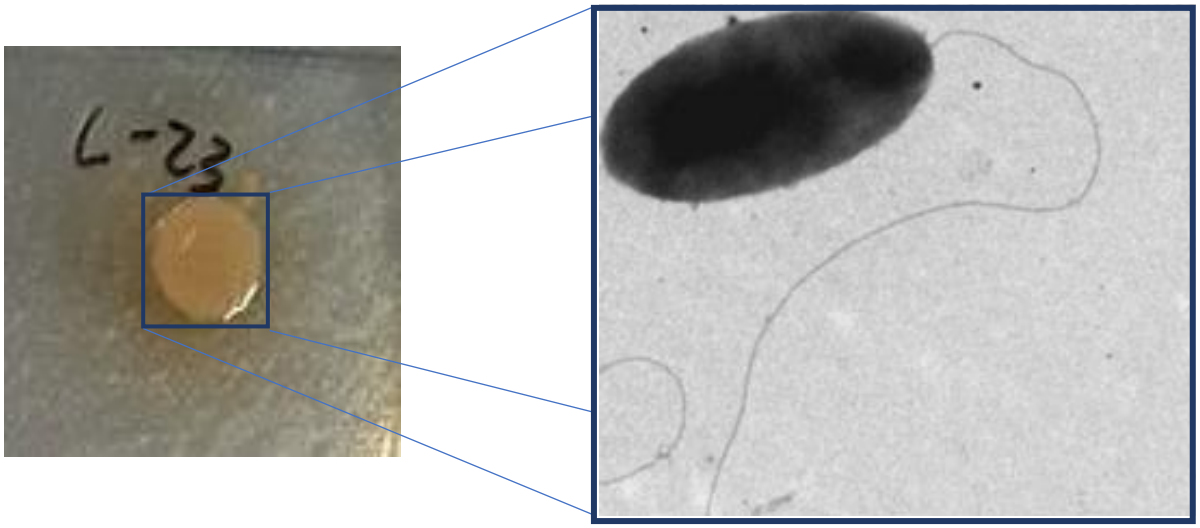

Figure 25. On the left: Colony of isolate L23, identified in this study as *P. inhibens* on MA medium, supplemented with tween 80. On the right side: Transmission Electron Micrograph picture of a negatively stained *Phaeobacter inhibens* cell in MB medium. Picture adapted Martens *et al.*, (2006) [249].

Cells grow at temperatures ranging from 4ºC to 36ºC, with an optimum temperature between 27ºC and 29ºC, and pH ranging from 6.0 to 9.5 (optimum pH 7.5). They also grow in the presence of Na^+ concentrations of 0.01 M to 1.5 M, optimal salinity is between 0.51 and 0.68 M, and no growth is observed in the absence of Na^+. This bacterium is oxidase negative and catalase positive. Moreover, it is amylase, tween, and gelatinase negative, as corroborated by the assays performed in this study. Cells produce an antibiotic (TDA) during the exponential growth phase. This compound creates a brownish halo around the colony, as visible in **Figure 25** (left image; isolate L23, from the present study). The cells are susceptible to penicillin G, streptomycin sulphate and chloramphenicol. The DNA GC content is 55.7%. The type-strain is $T5^T$ (=DSM 16374^T =LMG 22475^T) [249].