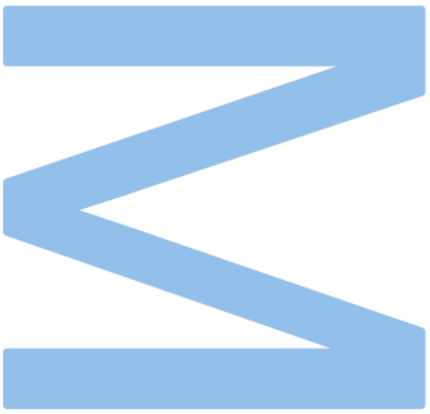
Microbe - Siderophores interactions for biocontrol of fish pathogen in aquaculture productions

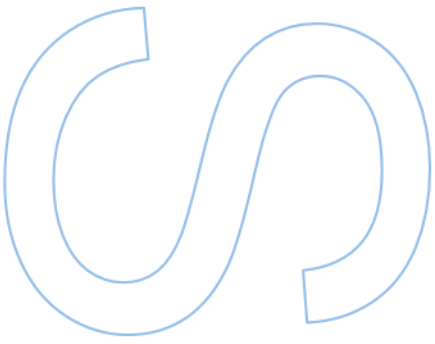
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Resumo

Como a população humana e a procura de alimentos aumentaram rapidamente nos últimos anos, a indústria da aquacultura tornou-se um dos sectores mais proeminentes para satisfazer as necessidades mundiais. No entanto, esta expansão exponencial é acompanhada por uma frequência crescente de surtos de doenças nos peixes, que ameaçam a saúde dos peixes e causam perdas financeiras consideráveis para o setor da aquacultura. Alguns dos agentes patogénicos, tais como *Tenacibaculum maritimum*, *Edwardsiella tarda* e *Vibrio anguillarum*, são predominantes em habitats marinhos e podem causar doenças em várias espécies de peixes. A presença de ferro é crucial para as bactérias patogénicas colonizarem o seu hospedeiro, mas também para a sua própria sobrevivência. Os sideróforos são agentes quelantes de ferro de baixo peso molecular segregados por organismos vivos, como bactérias, leveduras, fungos e plantas. Quando crescem em condições com baixo teor de ferro, as bactérias segregam estas moléculas quelantes de ferro para eliminar e solubilizar o ferro do ambiente extracelular, salvaguardando o fornecimento deste, que é essencial para o seu crescimento, replicação e metabolismo. Neste sentido, o principal objetivo deste trabalho foi avaliar as interações entre isolados bacterianos e sideróforos sintéticos para inibir três patogénicos de peixes, *T. maritimum*, *V. anguillarum* e *E. tarda*, responsáveis por elevadas taxas de mortalidade em sistemas de aquacultura. Para o efeito, foi testada uma biblioteca de 253 estirpes bacterianas, isoladas do biofiltro de um sistema RAS (Sistema de Recirculação de aquacultura) e 10 sideróforos em ensaios de suscetibilidade antimicrobiana para investigar a sua capacidade de suprimir os patogénicos mencionados. Em seguida, as melhores estirpes bacterianas e os melhores sideróforos foram testados, isolados ou combinados, numa experiência com água e matrizes do biofiltro de uma empresa de aquacultura.

Os resultados dos ensaios de difusão em disco e do riscado mostraram que nenhum dos isolados bacterianos apresentou uma inibição positiva contra *E. tarda*. Para *T. maritimum*, dos 253 isolados bacterianos, 30 foram capazes de inibir o crescimento de *T. maritimum* no teste de difusão em disco. Para além disso, nos ensaios do riscado, dos 253 isolados bacterianos, 62 mostraram capacidade de inibir o crescimento de *T. maritimum*. Quanto á *V. anguillarum*, apenas 57 dos 253 isolados bacterianos isolados bacterianos foram testados em ambos os ensaios, nos quais não foi observada qualquer inibição.

A identificação taxonómica foi realizada para os 74 isolados bacterianos que apresentaram potencial para inibir a *T. maritimum*, em que os géneros *Pseudomonas*, *Pseudoaltermonoas*, *Microbacterium* e *Cobetia* foram os mais abundantes. Foram testados 10 sideróforos contra os patógenos selecionados, em que nove dos quais mostraram atividade contra *T. maritimum*, dois mostraram atividade contra *E. tarda*, e nenhum contra *V. anguillarum.* Os resultados da experiência com água e matrizes do biofiltro, em que se testou a formulação de isolados bacterianos e o sideróforo, ainda estão a ser analisados. Estes resultados irão desvendar se os isolados e sideróforo escolhidos têm potencial para inibir a *T. maritimum* num meio mais complexo, e se a combinação de ambos pode ser mais eficaz e uma mais-valia para controlar o patogénico alvo.

Este trabalho mostrou que comunidades bacterianas do biofiltro RAS têm microrganismos com capacidade para inibir *T. maritimum*. Além disso, este trabalho evidenciou que os sideróforos são capazes de inibir diferentes patogénicos de peixes. Estas descobertas podem contribuir para o desenvolvimento de novas ferramentas biotecnológicas para melhorar a gestão de doenças no sector da aquacultura. No entanto, são necessários mais estudos sobre estes temas para compreender como a interação entre sideróforos e microrganismos pode ser utilizada para controlar e prevenir surtos de patogénicos em peixes e como afetam a comunidade microbiana do sistema RAS e o microbioma dos peixes.

Palavras-chave: Bactérias Probióticas, Bactérias Patogénicas de Peixes, Sistema de Recirculação Aquacultura (RAS), Sideróforos

Abstract

As the human population and food demand have rapidly increased in recent years, the aquaculture industry has become one of the most prominent industries to fulfill world needs. Nevertheless, this exponential expansion comes with an increasing frequency of fish disease outbreaks, threatening the fish's health and causing considerable financial losses to the aquaculture business. Some of the pathogens, such as *Tenacibaculum maritimum*, *Edwardsiella tarda* and *Vibrio anguillarum*, are prevalent in marine habitats and can cause diseases in several fish species. The presence of iron is crucial for pathogenic bacteria to colonize their host, but also for their own survival. Siderophores are low molecular weight iron chelating agents secreted by living organisms such as bacteria, yeasts, fungi, and plants. While growing under low-iron conditions, bacteria secrete these iron-chelating molecules to eliminate and solubilize iron from the extracellular environment, safeguarding the supply of this important metal that is essential for their growth, replication, and metabolism. In this vein, the main objective of this study was to assess the interactions between bacterial isolates and synthetic siderophores to inhibit three fish pathogens, *T. maritimum*, *V. anguillarum* and *E. tarda*, responsible for high mortality rates in aquaculture systems. To achieve that, a library of 253 bacterial strains, isolated from a RAS (Recirculating aquaculture system), and 10 siderophores were tested in antimicrobial susceptibility assays to investigate their ability to suppress the mentioned pathogens. Then, the best bacterial strains and siderophores were tested, alone or combined, in experiment using water and biofilter carriers from an aquaculture unit.

Results from disc diffusion and cross streak assays showed that none of the bacterial isolates displayed a positive inhibition against *E. tarda.* For *T. maritimum*, from the 253 bacterial isolates, 30 were able to inhibit *T. maritimum* growth in disc diffusion test. Moreover, in the cross-streak assays, from the 253 bacterial isolates, 62 displayed the ability to inhibit *T. maritimum. As* for *V. anguillarum*, only 57 out of 253 bacterial isolates were tested in both assays, in which no inhibition was observed.

Taxonomic identification was performed for 74 bacterial isolates that presented potential to inhibit the *T. maritimum,* in which *Pseudomonas, Pseudoaltermonoas, Microbacterium* and *Cobetia* were the most prevalent genera. Ten siderophores were tested against the selected pathogens, nine of which showed activity against *T. maritimum*, two showed activity against *E. tarda*, and none against *V. anguillarum*. The results of the experiment with water and biofilter carriers, in which the formulation of

bacterial isolates and the siderophore were tested, are still being analysed. These results will reveal whether the chosen isolates and siderophore have the potential to inhibit *T. maritimum* in a more complex environment and whether the combination of both could be more effective in controlling the target pathogen.

This work showed that the RAS biofilter's bacterial communities has microorganism with capacity to inhibit *T. maritimum.* In addition, this work highlighted that siderophores are capable to inhibit different fish pathogens. These finds can contribute for the development of new biotechnological tools to improve disease management in aquaculture sector. However, more studies focusing these topics are needed to understand how siderophore – microorganisms' interaction can be used as a toll to control and prevent fish pathogen outbreaks and how they affect the RAS natural microbial community and in the fish microbiome.

Keywords: Fish Pathogens, Probiotic Bacteria, Recirculating Aquaculture Systems, Siderophores

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List of Abbreviations

2,3–DHB	2,3-DIHYDROXYBENZOIC ACID
AMR	ANTIMICROBIAL RESISTANCE
AOB	AMMONIA OXIDIZING BACTERIA
BC	BIOFILTER CARRIERS
BMP	BEST MANAGEMENT PRACTICES
DFO	DESFERRIOXAMINE MESYLATE SALT
DMSO	DIMETHYLSULFOXIDE
EPS	EXOPOLYSACCHARIDES
FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
FFUP	FACULTY OF PHARMACY OF THE UNIVERSITY OF PORTO
LAB	LACTIC-ACID PRODUCING BACTERIA
LQOF	LABORATORY OF ORGANIC AND PHARMACEUTICAL CHEMISTRY
LPS	LIPOPOLYSACCHARIDES
MA	MARINE AGAR
MB	MARINE BROTH
MH	MUELLER-HINTON AGAR
NGS	NEXT GENERATION SEQUENCING
NOB	NITRITE OXIDIZING BACTERIA
OD	OPTICAL DENSITY
PCR	POLYMERASE CHAIN REACTION
PO	PRE-ONGROWING
QQ	QUORUM QUENCHING
QS	QUORUM SENSING
RAS	RECIRCULATING AQUACULTURE SYSTEM
R2A	REASONER'S 2A
SCN	STARCH – CASEIN – NITRATE AGAR
WE	WEANING
TDA	TROPODITHIETIC ACID
TE	TRIS – EDTA
TSB	TRYPTIC SOY BROTH
UP	UNIVERSITY OF PORTO

1. Introduction

1.1. Aquaculture Industry

The human population has grown exponentially over the last few decades, and with it, the need for food sources has grown proportionally. To address this demand, traditional food production methods have been changed, and new solutions such as aquaculture have been developed [1]. Aquaculture is a technology that uses freshwater or seawater for cultivation of organisms to increase the production of aquatic species like fish, crustaceans, mollusks, algae, and others. Being one of the most advanced and fastest growing food production industries in the world, aquaculture provides half of the fish and mollusks needed to feed the world's population, according to Food and Agriculture Organization (**Figure 1**) [2], [3]. Global aquaculture production reached 122.6 million tons in 2020, aquatic animals contributed for 87.5 million tonnes, whereas algae accounted for 35.1 million tonnes [3]. By the year of 2030 aquaculture production is projected to rise to 202 million tons [3]. From 2001 to 2018, aquaculture production grew at an annual average rate of 5.3% and aquaculture production has expanded by more than 600% since 1990 [4].

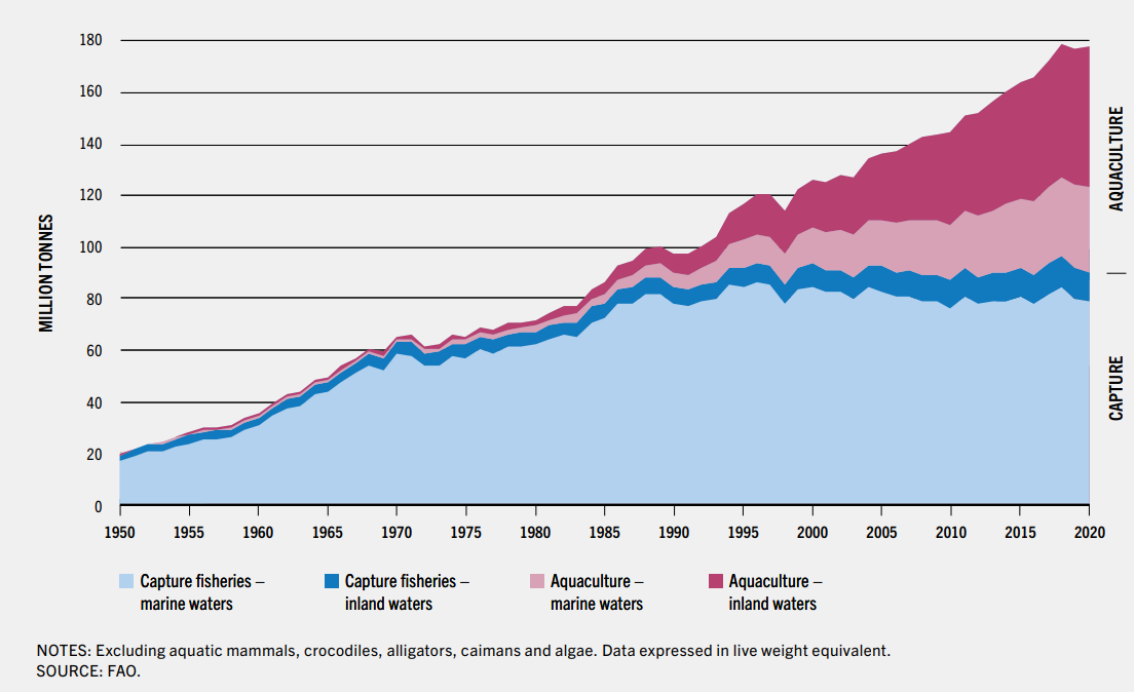


Figure 1 - World capture fisheries and aquaculture production until the year 2020. Source: FAO (2020) [3]

This growth can be due to improvements in water quality, adequate feed and the development of new functional diets, development of techniques to reduce losses

(selective breeding and hybridization), and the application of molecular genetic techniques (identification markers for stock discrimination, genetics of the pathogens organisms of commercial significant species, using techniques such as Polymerase Chain Reaction (PCR) for the detection of these pathogens, etc) [5]. Moreover, concerns about the impact on the environment, economy, and society have also evolved to assure a high-quality final product in a more sustainable approach. In fact, aquaculture has already been at fault for a wide range of serious environmental impacts, including habitat destruction (mangroves, rice fields, wetlands), water pollution and eutrophication, biotic depletion, disease and parasite transmission, and greenhouse gas emissions [6]–[9]. Recirculating Aquaculture Systems (RAS) are relatively new to the aquaculture sector in several countries, thus social acceptance is still a requirement for their widespread use, since it has some technological and economic obstacles, such as the need for a large initial investment and also the difficulty of adapting the system to the production of some species of fish [6]. Best management practices (BMP's) have been developed to ensure a final product with high-quality standards. These practices include maintaining an adequate water supply, application of pharmaceuticals, disinfectants, antibiotics, antivirals, and vaccinations to treat several diseases [4], [10], [11].

However, one of the main challenges of intensive aquaculture production is controlling diseases outbreaks, which may spread quickly throughout fish communities [4], [11]. Some of the most common infections that affects fish communities in aquaculture systems are generally caused by bacteria (54.9%), viruses (22.6 %), parasites (19.4 %) and fungi (3.1 %) [12]. The uncontrolled use of antibiotics in aquaculture has contributed to the evolution of antimicrobial resistance (AMR), which can be a threat to both human and animal health in a global scale. Besides the appearance of antibiotic's resistant strains, antibiotic residues can accumulate in fish tissues [13] or can end up in aquatic habitats, which can have a vast impact on consumers health [14], [15]. For Instance, sedimentary analysis revealed that oxytetracycline persisted in marine sediment beneath cages of fish farm after administration, leading to a substantial increase in bacteria resistant to this antibiotic [16].

Transitioning to Recirculating Aquaculture Systems has become one of the most viable solutions to tackle the challenge of bacterial resistance, and the reason for this is that RAS allows a precise control of the environmental conditions, minimizing the environmental impact by, for example, recycling and reusing water efficiently [9]. In addition, since this system can be operated indoor, climate changes have a reduced impact on it and can function all year round without worrying with rainfall variation, global

warming, salinity fluctuation, ocean acidification, and conditions that traditional aquaculture productions have [6].

1.2. Recirculating Aquaculture Systems (RAS)

In the last two decades, RAS technology has undergone significant development [17]. Several European countries are transitioning to RAS systems, claiming environmental concerns as justification. However, these systems bring the possibility to diversify the production and market opportunities and ultimately increase the production efficiency, reducing operational risks and improving product quality [6]. RAS can be used in intensive fish and seafood production, being considered a more sustainable technology. They can be operated all year, in multiple locations, including near major seafood markets, and they are not impacted by seasonal or climate change factors [9]. These systems are designed to produce fish in places with poor biophysical conditions, limited water supply, poor water quality, and an unfavorable environment [18]. Besides, RAS allows for land-based farming in a controlled setting, minimizing direct interactions between production and the environment, which is a concern in aquaculture production in open sea [6], [19]. In RAS, fish are kept in tanks inside a controlled setting and water is purified by eliminating metabolic waste and uneaten food before being recirculated into the system. Mechanical and/or biological filtration, sterilization, and oxygenation are among the methods employed to purify the water in RAS (**Figure 2**) [19], [20].

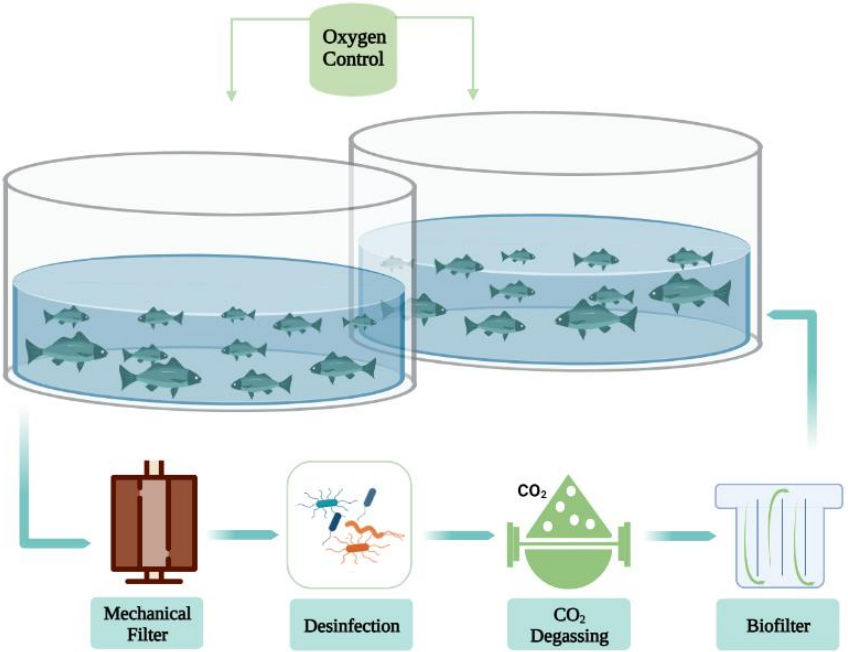


Figure 2 - General scheme of a recirculating aquaculture system

RAS provides multiple advantages for sustainable fish production, which includes reduction in water consumption (compared to traditional flow-through systems), small land footprint [9], [17], waste management (reducing the effluent waste [21]), nutrient recycling, biological pollution control, and biosecurity by minimizing the contact between fish populations and wild fish protecting them from disease transmission [6], [22]. All these factors can help to maintain an optimal production environment, animal safeguarding, and minimal environmental impact. Compared to other types of aquaculture systems, RAS has a more efficient use of energy and can control disease epidemics more effectively, resulting in less frequent outbreaks [23]–[25]. However, it is important to note that RAS requires a large initial investment, ongoing maintenance, and operation. It is a highly complex structure that requires complex equipment and technology [19].

1.2.1 Microbial Communities in RAS

Biofilters are a key component of the RAS, since they not only help producing high-quality water but also improve the overall quality and health of the fish. Biofilters are one of the most vital components in RAS, but also one of the most difficult to manage [26]. Biofilter has beneficial microbial populations, both autotrophic and heterotrophic bacteria, that are crucial for the reduction/removal of nutrient pollution, and for the water purification, enhancing welfare of the farmed organisms [27], [28]. Specifically, biofilters integrate aerobic and anaerobic microbial processes to eliminate waste products, such as nitrogen in the form of ammonia excreted by fish, and carbon and nitrogen accumulated from uneaten food and fecal matter produced by their metabolism [29]. The nitrification process in biofilters presents a challenge due to the accumulation of nitrite (NO_2^-) intermediate in the tank, which disrupts ion regulation and vital functions in fish [30], [31]. Besides, ammonia is also toxic for the fish [30]. To avoid this, a complete oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) is necessary, requiring specialized bacteria with the ability to perform this reaction. Ammonia oxidizing bacteria (AOB) are responsible for oxidizing ammonia to nitrite (e.g. *Nitrosomonas* and *Nitrosococcus* are common AOB found in RAS biofilters), nitrite oxidizing bacteria (NOB) are responsible for oxidizing nitrite to nitrate (*Nitrobacter* and *Nitrospira* are typical NOB in RAS biofilters) [32] and anammox bacteria (anaerobic ammonium oxidation), that are important in the conversion of ammonium and nitrite into dinitrogen gas, which is a harmless gas [33]-[34]. Heterotrophic bacteria have an important role in the mineralization of organic matter

resulting from the metabolism and uneaten food of the fish [27]. Rurangwa and Verdegem, (2015) reported that *Pseuomonas stutzeri, Ruegeria spp*., and *Roseobacter spp*. were the most common heterotrophic community species found in the RAS biofilter [27]. In addition, Almeida et al. (2021) studied the dynamics of the prokaryotic community in a Sole (*Solea senegalensis*) hatchery RAS unit and reported that the genera *Tenacibaculum*, *Sulfitobacter*, *Leucothrix*, *Novosphingobium*, *Marinicella*, *Pseudoalteromonas*, *Polaribacter*, *Schleiferia* and *Algibacter* were found in abundances higher than 3 % in the aquaculture unit. Moreover, they also reported the presence of the NOB *Nitrospira* and AOB *Nitrosomonas* in the WE (Weaning) and PO (Pre-Ongrowing) biofilter from a RAS [32], [35]. To ensure proper system functioning, a bacterial community that can efficiently manage water consumption, waste management, nutrient recycling and control fish pathogens in the aquaculture system would be ideal [36].

Table 1 - Major processes occurring in RAS system and the microorganisms that can be involved in the process [27].

Process	Microorganism
Nitrification	*Nitrosomas sp.* *Nitrosococcus sp.* *Nitrosopumilus sp.* *Ntrospira sp.* *Nitrobacter sp.*
Denitrification	*Pseudomonas sp.* *Thiomicrospira sp.* *Thiothrix sp.* *Paracoccus sp.*
Dissimilatory nitrate reduction to ammonia	Various Proteobacteria and Firmicutes phyla
Anaerobic ammonium oxidation	*Planctomycetes sp.* *Brocadia sp.*
Sulphate reduction	*Dethiosulfovibrio sp.* *Desulfovibrio sp.* *Bacteroides sp.*

Despite the numerous benefits of the bacterial community in aquaculture systems, they can face challenges in establishing itself. The biofilms found in the biofilters are composed by a broad spectrum of microbial populations which are competing for resources like oxygen, nutrients, and space. This competition affects mass transfer reactions and can significantly alter the performance and stability of the biofilm, depending on the culture conditions. Biofilms are characterized by three layers: an inner

layer composed of inert biomass near to the substrate, an intermediate layer dominated by the nitrifying population, and an outer layer dominated by heterotrophs [37]. This arrangement promotes the growth of heterotrophic bacteria, while the inner layers are restricted by the concentration of oxygen and nutrients [27]. Fdz-Polanco et al. (2000) discovered that the spatial distribution of the communities was non-uniform and concluded that the specific activities of ammonia oxidizers, nitrite oxidizers, and aerobic heterotrophs revealed a clear microbial segregation along the filter, depending on the oxygen entering the biofilter system [38]. Heterotrophic bacteria use a significant amount of oxygen which can hinder the nitrification process performed by the nitrifying community [29], [37], [38]. Therefore, successful biofilters operation is crucial for the operational efficiency of RAS.

Nevertheless, the biofilter bacterial community has the advantage of incorporating probiotic bacteria that can be crucial to help protect the fish against pathogenic bacteria [39]- [40]. Some beneficial bacterial genera can be found in RAS biofilters such as *Bacillus sp*. and *Pseudomonas sp*. [36], [41]. These bacteria can have different modes of action that protects the fish against pathogenic bacteria, by the production of antimicrobial substances, quorum-quenching abilities and maintaining the water quality, which is beneficial to the overall health of the fish [36]. The use of probiotic strains in the aquaculture sector has been studied and can be a viable alternative to the use of antibiotics and to maintain the health and well-being of different aquatic animals [36], [40], [42]–[44]. In aquaculture, the most studied probiotic candidates are lactic acid bacteria (LAB), such as *Lactococcus* and *Lactobacillus*, and *Bacillus* genus [43], [45]. Certain bacterial strains have the ability to enhance animal's immune system and offer resistance against pathogens [44]. These probiotic bacteria can be incorporated in feed to enhance fish immune system, improve growth performance, as well as to manage stress caused during transportation [42]. Moreover, these probiotic bacteria can also be used to change the microbiota composition of the fish intestine, contributing for their well-being.[42]

Aside from the benefits against bacterial pathogens and improvement of fish´s health, RAS microbial community can also create off-flavor-causing chemicals and they are a common problem in RAS [46]. The appearance of these off-flavors, which are caused by geosmin (trans-1,10-dimethyl-trans-9-decalol) and 2-metylisoborneol (1,2,7,7-tetramethyl-exo-bicyclo[2.2.1]-heptan-2-ol, MIB), are not yet understood in RAS but it is linked to organic-rich parts of the systems [27]. These two compounds are secondary metabolites produced by actinomycetes, myxobacteria and others [47]. The

primary concern with these off flavors' chemicals is that they can delay the harvest of the fish and render them unmarketable, making it crucial to take preventive measures and detect them early [27], [46], [47].

1.3 Pathogenic microbes in RAS

As mentioned above, the biofilter is a crucial component of RAS, responsible for maintaining water quality and fish welfare. However, pathogen accumulation within RAS biofilters can occur when certain conditions promote their proliferation [48]. For instance, pathogen outbreaks can occur when the tanks are overcrowding, causing stress and weaken to the immune system of the aquatic organisms. Moreover, poor water quality conditions (which leads to high ammonia and nitrite levels) and fluctuations in temperature and pH can also provide the optimal conditions for the proliferation of the pathogen [48]–[50]. These pathogens, which grow within microbial biofilms, are generally resistant antibiotics making them difficult to treat [39]. As a result, these pathogens will eventually detach from the biofilm, causing infectious diseases on the fish, upon contact [39], [50]. The relationship between pathogen, host, and environment determines the beginning and development of a fish disease [51].

Antibiotics were initially used to quickly control or limit the spread of diseases caused by different pathogens. However, this approach led to the emergence of antibiotic-resistant strains over time, since antibiotics were extensively used as a preventive measure rather than a therapeutic [11], [52], [53]. As a result, alternative treatments were explored to combat the spread outbreaks such as administration of antimicrobial peptides [54], bacterial probiotics [40], immunostimulants and quorum sensing (QS) inhibitors [42], [55]. Two of the most effective treatments that have been utilized are UV and ozone, either used independently or in combination. Additionally, chemotherapy, vaccines, and other strategies such as salinity and temperature manipulation have been employed to reduce the spread of the disease [39], [56].

To develop new therapeutics for prevention and control of major diseases in aquaculture fish production, multidisciplinary studies are needed to understand and characterize the potential fish pathogens, the biology of the fish hosts, and how the environmental factors can affect the interaction between the pathogen and the host [51], [57].

Bacterial pathogens such as *Aeromonas salmonicida*, *Vibrio (Listonella) anguillarum*, *Vibrio (Aliivibrio) salmonicida, Yersinia ruckeri*, *Tenacibaculum maritimum*

and *Edwardsiella tarda* (**Table 2**) are among the most known pathogens responsible for the fish infections in the aquaculture industry [58]–[60].

Table 2 – List of some pathogen's genus found in aquaculture systems

Pathogen Bacteria	References
Acinetobacter spp.	[27]
Aeromonas hydrophila	[61]
Aeromonas salmonicida.	[62]
Enterobacter cloacae	[63]
Yersinia ruckeri	[64]
Photobacterium damsela	[65]
Vibrio cholerae	[66]
Tenacibaculum maritimum	[67]
Plesiomonas shigelloides	[68]
Edwardsiella tarda	[69]
Vibrio anguillarum	[70]

This work will focus on targeting three pathogens, *T*. *maritimum*, *E. tarda* and *V. anguillarum* since these three pathogens are known to cause a variety of diseases in different fish species, not only in RAS but also in more conventional aquaculture systems.

1.3.1 *Tenacibaculum maritimum*

T. maritimum, formerly known as *Flexibacter maritimus*, first described in 2001 by Suzuki [71], is a Gram-negative, filamentous bacteria that is mostly found in marine or estuarine environments [67]. This specie belongs to the phylum Bacteroidota, and it is known for causing an ulcerative disease known as tenacibaculosis in fish aquaculture farms around the world [56]. This disease causes gross lesions on the surface of the fish body, such as ulcers, necrosis, mouth rot (also known as yellow mouth), frayed fin and tail, resulting, most of the times, in the death of the animal (**Figure 3**) [72]. Previous studies suggests that seawater plays an important role in the horizontal transmission of *T. maritimum* [56]. Still, a study conducted by Saldarriaga-Córdoba and Avendaño-

Herrera et al. (2005) [73] have identified several strategies in the evolution and pathogenicity mechanisms of *Tenacibaculum* species, including iron acquisition mechanisms [74], copper homeostasis, resistance to tetracycline and fluoroquinolones, pathogenic genomic islands and phages [73].

This infection generates serious economic losses in the aquaculture industry due to the high treatment costs, product losses, along with the increasing mortality rate caused by this disease [75].



Figure 3 - Clinical signs of tenacibaculosis in Atlantic salmon. *Salmo salar L.* were exposed to different species of *Tenacibaculum* (*T. maritimum* NLF-15 (E-H) and *T. dicentrarchi* (A-D)) and photos were taken with different times of exposure. (A, B) were taken on day-1 post-exposure, (C, D) were taken on d 2 post-exposure. (E, F) were taken on d 9–10 post-exposure, (G, H) were taken on d 17–18 post-exposure. Ulcerations and hemorrhages are present, predominately around the jaws (B, C, F, G), but also on the flanks and fins of fish (A, D, E, H). (C, D, G, H) display an exaggerated form of mouth rot; and ulcerations on the flank went beyond the epidermis into the musculature (D, H). (Adapted from [76])

Up till now, treatment of *T. maritimum* is mainly limited to the use of antibiotics and certain disinfectants [77]. A study conducted by Avendaño-Herrera et al. (2005) showed that *T. maritimum* strains are susceptible to amoxicillin, nitrofurantoin, florfenicol, oxytetracycline, trimethoprim-sulfamethoxazole, enrofloxacin and flumequine [77]. However, to reduce the appearance of multi-drug resistance strain, probiotics can be a new alternative to the use of antibiotics. For instance, a study conducted by Tesdorpf et al. (2022) reported that the probiotic bacteria (*Phaeobacter piscinae* S26), that are tropodithietic acid (TDA)-producing bacteria, were capable of killing pathogenic *Tenacibaculum* species such as *T. maritimum*, through in vitro assays [78].

1.3.2 *Vibrio anguillarum*

V. anguillarum is a fish pathogen that represents a serious threat to the aquaculture sector. *V. anguillarum* is a rod-shaped, gram-negative bacterium, member of the Pseudomonadota phylum (**Figure 4**). This specie is polarly flagellated, does not produce spores, is facultatively anaerobic, halophilic and it is mostly found in marine environments [79].

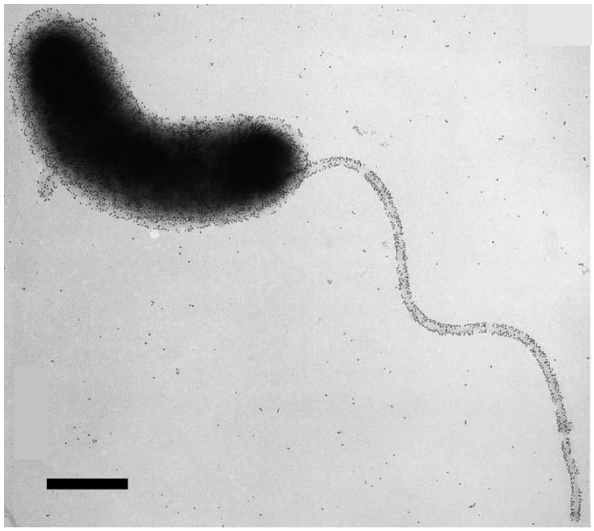


Figure 4 - Immunogold electron microscopy of whole cell of *V.anguillarum*. (Adapted from [79])

This pathogen can cause vibriosis, an infection that can led to flesh rot, necrosis, circulatory haemorrhage, erythema, and ultimately to death **(Figure 5**) [80]. Typically, exposure to this pathogen leads to death within days [80], [81].



Figure 5 - Clinical signs of Vibriosis in various part of the European seabass body. (Adapted from [82])

Several studies have been conducted seeking to understand the complete mechanisms of infection of this pathogen [80], [83]. Current knowledge suggests that adhesion mechanisms to the organism, along with the adhesion site (skin or gut) is crucial for this pathogen to infect the host [80], [84]. Moreover, another important infection mechanism is the iron sequestration system, which can either be dependent or independent of siderophores [74], [80]. Additionally, the presence of a polysaccharide chain in pathogen, lipopolysaccharides (LPS) structure, does not activate the target organism's immune system response, leading to the infection of the host [85].

1.3.3 *Edwardsiella tarda*

E. tarda is a gram-negative, rod-shaped, facultative anaerobic bacteria, peritrichously flagellated, that belongs to the Pseudomonadota phylum [60]. This pathogen has been reported to cause major economic losses in important fish species since 1962 [60]. This pathogen is an intracellular pathogen found in marine and freshwater environments and can infect different types of cells in the target organism [69]. *E. tarda* can infect a wide variety of species, including fish, reptiles, birds and humans [69].

E. tarda causes a disease known as Edwardsiellosis. This disease leads to the infection that can cause ascites, hernia, exophthalmia and severe lesions of internal organs (**Figure 6**) [86].

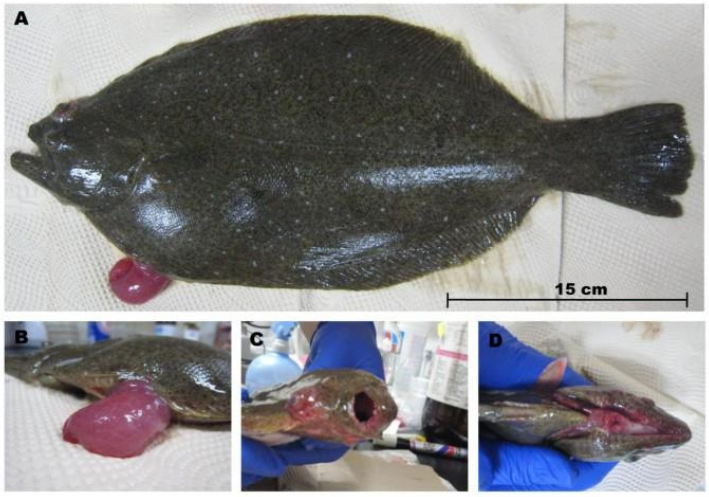


Figure 6 - Clinical signs of Edwardsillosis in olive flounder (A: external lesions, B: Abdominal distension, C: Exophthalmia and opacity of the eye. D: Peripheral hyperemia in mandible lesion). (Adapted from: [86])

Various mechanisms have been uncovered regarding *E. tarda* virulence and pathogenicity. In fact, this pathogen is able to withstand and adapt to changes in temperature, pH, and salinity [86]. Moreover, *E. tarda* uses a variety of substances and

mechanisms to infect and survive in the host [86], which include the type III and type VI secretion systems, iron uptake regulators, hemolysins [87], siderophores production, and QS [86], [88], [89].

Antibiotics and vaccines are the most common therapeutics that is used to treat Edwardsiellosis [69]. There are several types of vaccines developed for this purpose, including whole cell bacterins, recombinant vaccines such as recombinant FimA, DnaJ, FlgD, [90], DNA vaccines, and live attenuated or wild type avirulent *E. tarda* vaccines [86], [91]. Still, there are other alternative treatments such as use of immunostimulants, phytobiotics, and bacterial probiotics [91]–[93].

1.4 Siderophores as biocontrol of pathogens

Siderophores are very small, highly-affinity iron-chelating substances that are released by microorganisms like bacteria and fungus. Their name means "iron carrier" in Greek and are among the most potent (highest affinity) Fe^{3+} binding agents that are currently known [94]. While their roles are still being recognized, a growing number of studies have revealed their importance in the medical and environmental research [95]–[98]. In fact, siderophores possess a wide range of chemical structures, specialized features, and the capacity to bind to different metals aside from iron, making them significant in environmental applications. They can be used in biosensors [99], as chelating agents, in biocontrol of plant [100] in biocontrol of fish diseases, and in the bioremediation of environmental pollutants such as metals [98], [101], [102]. According to the chemical groups involved in iron binding, bacteria's siderophores can be classified into three main families: catecholates, hydroxamates and carboxylates (**Figure 7**) [103].

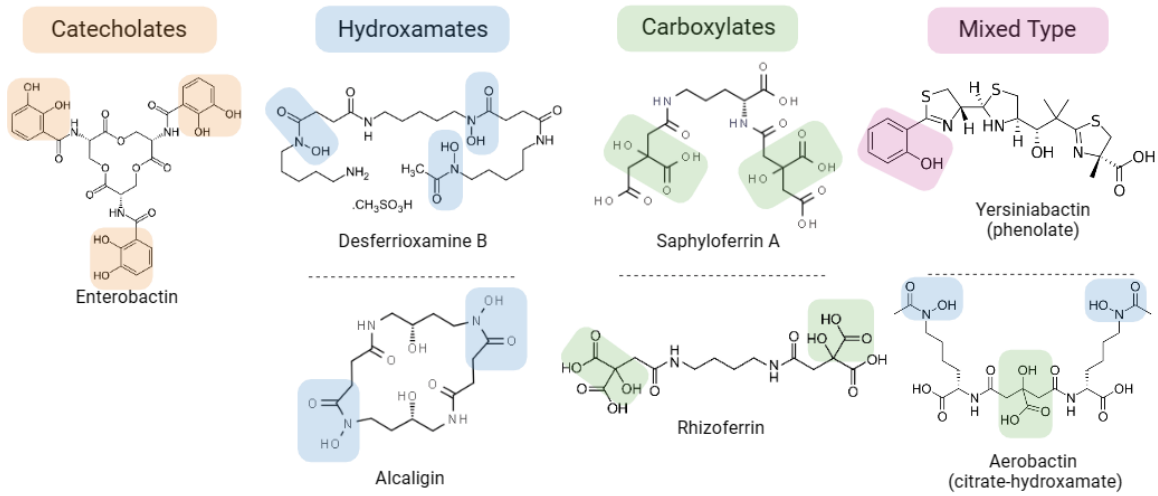


Figure 7 - Three main families of siderophores based on the chemical groups involved in iron binding produced by gram-positive and gram-negative bacteria. Binding groups of the mixed siderophores: catechol (orange), hydroxamate (blue), carboxylate (green) and phenolate (purple).

Practically all kinds of life require iron as a vital component. Iron is necessary for several biological functions, including oxygen transport, DNA replication, energy production and protection from oxidative stress [94]. Over 100 enzymes involved in both primary and specialized metabolism possess iron-containing cofactors such as heme groups or iron-sulfur clusters [104]. Bacterial pathogens also require iron to thrive and spread disease within their vertebrate hosts [105]. However, the free iron present in human and animal body fluids is insufficient for bacterial survival [106]. Typically, iron is normally kept in iron storage protein ferritin or complexed within the heme porphyrin ring as a cofactor of hemoglobin or myoglobin [95]. This results in an insufficient amount of iron present in the host to support the pathogen's survival. Therefore, the production of siderophores (**Figure 8**) is one of the most common approaches for accumulation of iron.

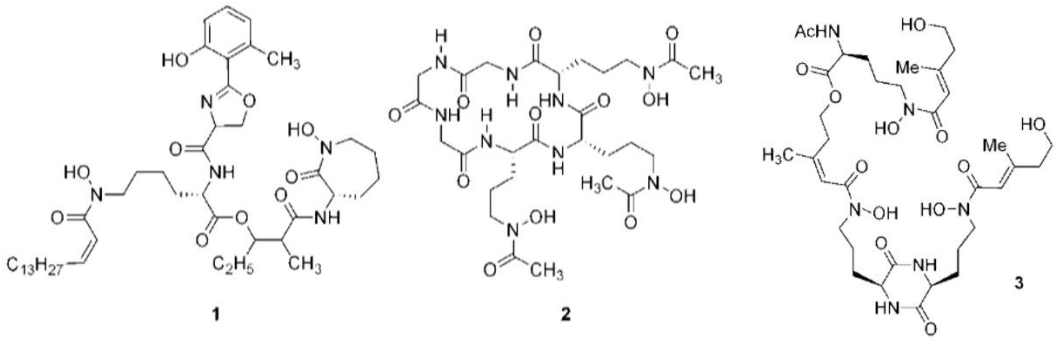


Figure 8 – Three different siderophores isolated from bacteria between 1949-1952. 1) mycobactin; 2) ferrichrome; 3) coprogen

Several bacteria, either gram-positive or gram-negative, can synthesize siderophores. In the **Table 3** are described some siderophore-producing bacteria and the respective siderophore.

Pathogens can develop several acquiring iron mechanisms, one of which is through the production of siderophores. In such cases, the competition between the two siderophores-producing bacteria can become more complex. Thus, the affinity constants between the siderophores or other modes of action will dictate the success or not of the infection [96], [107], [108]. For example, from a perspective of competition between pathogenic and probiotic bacteria, the rivalry between the siderophores produced by them is important. For instance, there are pathogenic bacteria and probiotic bacteria that can either produce or not siderophores. There are a lot of interactions that can happen

between bacteria (pathogenic or benefic) that produces siderophores and these interactions can occur in different ways. If both bacteria are producers, if the siderophore produced by the pathogenic quelates strongly the iron, the pathogen will have advantage against the probiotic bacteria.

Moreover, if the pathogenic bacteria is the only siderophore producer, either the beneficial bacterial is affected by the lack of iron or it can become a “cheater”, which means that it can absorb the siderophore produced by the pathogenic bacteria. This interaction can occur when these bacteria have specific receptors to uptake the siderophore, leaving the pathogen in disadvantage. The same can happen if the probiotic bacteria is a siderophore producer and the pathogenic bacteria is not [109].

Table 3 - Example of siderophore producing bacteria and respective siderophore

Type of Siderophores	Microbial Siderophores	Microorganism	References
Catecholate	Enterobactin	*Escherichia coli*	[110]
		Salmonella typhimurium	[111]
	Bacillibactin	*Bacillus anthracis*	[110]
		Bacillus subtilis	[112]
	Vibriobactin	*Vibrio cholerae*	[110]
Hydroxymate	Ferrichrome	*Ustilago sphaerogena*	[110], [113]
	Desferrioxamine B	*Pseudomonas fluorescens*	[114]
	Desferrioxamine E	*Pseudomonas fluorescens*	[114]
	Desferrioxamine G	*Pseudomonas fluorescens*	[114]
	Aerobactin	*Pseudomonas fluorescens*	[98]
	Ferribactin	*Pseudomonas fluorescens*	[98]
	Gonobactin	*Neisseria gonorrhea*	[98]
	Nocobactin	*Neisseria meningiditis*	[98]
Carboxylate	Rhizobactin	*Rhizobium spp.*	[115]
	Staphyloferrin A	*Staphylococcus hyicus*	[115]
Mixed type	Aerobactin	*Escherichia coli*	[116]
	Yersiniabactin	*Yersinia pestis*	[117]
	Parabactin	*Paracoccus denitrificans*	[118]
	Carboxymycobactin	*Mycobacterium tuberculosis*	[119]

In the management of fish diseases, the competition between the siderophore produced by bacteria, such as probiotic or pathogenic bacteria, and the mechanism of storage used by the host is crucial [108]. The siderophore has a stronger chelating

potential and stability than the transferrins, and therefore, will limit virulence and bacterial interactions by pathogenic bacteria [108], [120]. *Bacillus cereus* is a well-known siderophore-producing bacteria that can inhibit the growth of the fish pathogen *Aeromonas* hydrophila [121].In addition, different studies reported the potential of siderophore-producing bacteria, such as *B. cereus* (NRRL 100132), *Pseudomonas fluorescens* (F19/3 and AH2) and *Aeromonas media* (A 199) to inhibit the growth of several pathogens such *A. salmonicida* and several species of the *Vibrio* genus [96], [122], [123].

1.5 Objectives

The main objective of this study was to assess the interactions between RAS bacterial isolates and synthetic siderophores to inhibit three fish pathogens, *T. maritimum*, *V. anguillarum* and *E. tarda*, responsible for high mortality rates in aquaculture systems. To achieve that, a library of 253 bacterial strains, isolated from a RAS system, and 10 siderophores synthesized in a laboratory were tested in antimicrobial susceptibility assays to investigate their ability to suppress the mentioned pathogens. Then, the best bacterial strains and siderophores were tested against *T. maritimum*, alone or combined, in an microcosms experiment using water and biofilter carriers from an aquaculture unit, to mimic some of the conditions found in a RAS biofilter. To achieve these objectives, this dissertation is divided into 4 chapters. In the first chapter, it is presented an introduction about aquaculture production, RAS and its microbial communities and different pathogens that concern aquaculture, and the use of siderophores as biocontrol for fish pathogens. In the chapter 2, it is described all the methodology, that was divided in 3 main experiments 1) Potential of RAS bacterial strains to inhibit different fish pathogens; 2) Siderophores inhibition tests against fish pathogens and 3) Microorganism-siderophore interaction for inhibition of *T. maritimum*. In the chapter 3, it is presented the results and discussion of the 3 main experiments and in the chapter 4 it is described the main conclusions of this work.

2. Material and methods

2.1 Potential of RAS bacterial strains to inhibit different fish pathogens

2.1.1 Isolation of RAS bacterial strains

In a previous study conducted by Almeida (2022) [124], correlation and network analysis (using prokaryotic NGS data collected from water and biofilter carriers from a RAS system) were conducted to evaluate the potential negative interaction between RAS procaryotic community and the fish pathogen *T. maritimum*. Afterwards several bacterial genera with negative interactions with *T. maritimum* were identified, and specific cultivation approaches were developed seeking their isolation. Then, water and several biofilter carries were collected from the same biofilter from the RAS system used in the previous study (Almeida 2022). The biofilter carriers were placed in 15mL tubes with 0.85 % of NaCl saline solution to release the biofilm within the carriers. Both water and the biofilm solution were spread ten-fold dilutions (up to 10^{-6}) in the selected media **(Figure 9)** in duplicates: commercial media Marine Agar (MA; Conda S.A), Reasoner's 2A (R2A; VWR Chemicals), unformulated media Starch–Casein–Nitrate agar (SCN) **(Table 4)** and M1 agar (**Table 4).** Half of the plates were incubated at room temperature and the other half were incubated at 28 ºC. Bacterial colonies with different morphological features were visually identified (**Figure 10**), purified in the respective agar media and preserved in glycerol (25 %) and saline solution (0.85 %) at -80 ºC. In total 253 bacterial isolated were preserved [124]. In addition, biomass of each bacterial strain was collected into a tube containing 100 µL of Tris-EDTA buffer (pH 8) and kept at -20 ºC for further DNA extraction.

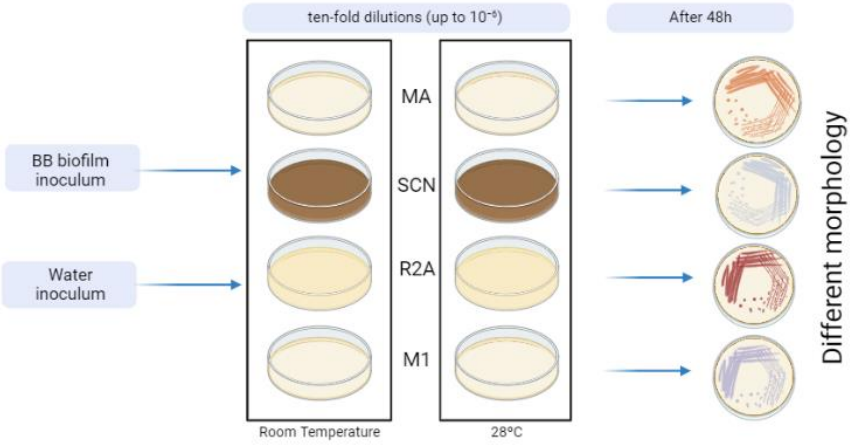


Figure 9 - Schematic representation of the isolation process of the culturable bacterial communities in water and biofilter carriers from a RAS biofilter.

Table 4 - Composition of SCN and M1 solid media used in the isolation of RAS bacterial strains

Media	Composition	Amount (g/L)
SCN*	Starch	10
	Casein sodium salt from bovine milk	0.3
	K_24PO_4	2
	KNO_3	2
	NaCl	2
	$MgSO_4 \cdot 7H_2O$	0.05
	$CaCO_3$	0.02
	$FeSO_4 \cdot 7H_2O$	0.01
	Agar	17
M1**	Starch	10
	Yeast extract	4
	Peptone from milk solids	2
	Agar	17

* 1L of distiled water
**1L of seawater

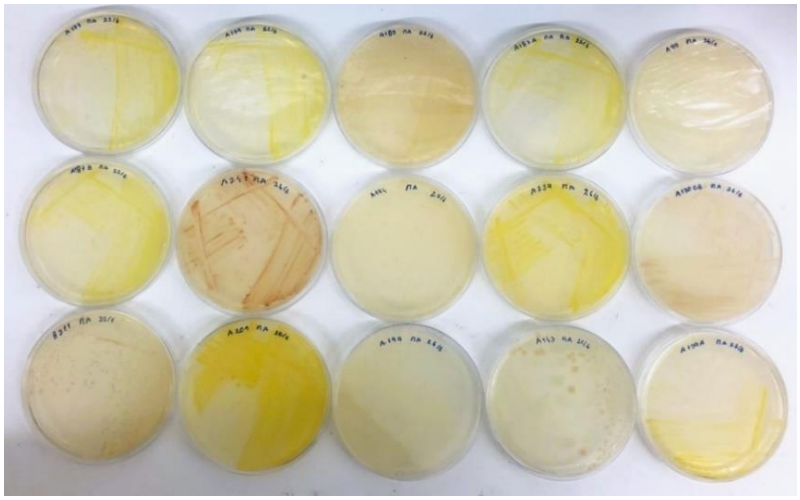


Figure 10 - Different bacterial strains isolated from water and biofilter carriers.

In this study, the potential of these 253 bacterial isolates was explored for the inhibition against three fish pathogens, *T. maritimum*, *V. anguillarum* and *E. tarda.*

2.1.2 Assays preparation

To conduct the assays, *T. maritimum*, *V. anguillarum* and *E.tarda* were cultivated in MA, M1 and TSA agar plates, respectively, and incubated for 48 hours at 28 ºC.

All the bacterial isolates were then cultivated on the media on which they were isolated (MA, M1, R2A and SCN). Since the tests were carried out on the pathogens' optimum growth media (MA, M1 and TSA), all bacterial isolates were grown on the medium on which the test was carried out. For *T. maritimum*, which is a pathogen that grows only on high salinity media, all the tested bacterial isolates were grown in MA, and the ones that did not grown on MA media were not tested against this pathogen. The same procedure was applied for *E. tarda*, and *V. anguillarum*, before the inhibition assays. The potential of each bacterial isolate to inhibit the selected pathogens was assessed using two different assays: disc diffusion and cross streak assays.

2.1.3 Disc diffusion method

For this experiment, for the 253 bacterial isolates, the inoculum of each was prepared in different liquid media (depending on the pathogen) and incubated in an orbital shaker (brand) at 28 °C for 48 hours (**Figure 11**). After that, all bacterial cultures were centrifuge at 3.5 rpm for 1 minute.

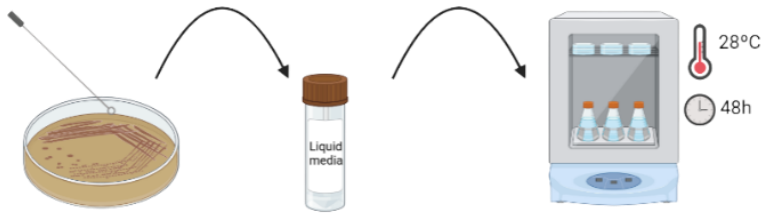


Figure 11 - Schematic of the preparation of the isolate's inoculum for the disc diffusion susceptibility test

In the meantime, the inoculum of each pathogen was prepared in the respective culture media (Tryptic Soy Broth (TSB; Liofilchem® S.r.l), Marine Broth (MB; Conda S.A) or M1 Broth) with an optical density (OD) between 0.8-1.2, at 625 nm. The pathogen

inoculum was spread evenly all over the plate using a swab as described in the **Figure 12.**

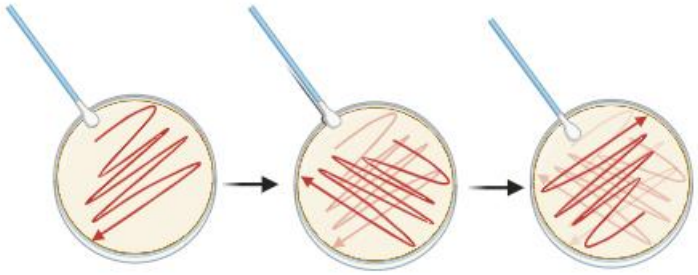


Figure 12 - Spreading of the pathogen inoculum. Spread evenly all over the plate using a swab, turned 90º and spread again, then rotated another 90º and spread a third time.

After that, several blank discs were placed in the plate and were inoculated with 15 µL of each bacterial isolate supernatant, as shown in **Figure 13**. Liquid media was used as negative control, while enrofloxacin (1 mg/mL) was used as positive control for *T. maritimum* and *E. tarda,* and oxytetracycline (1 mg/mL) was used as positive control for *V. anguillarum*. The plate was incubated at 28 °C for 48 hours. After incubation, inhibition halos were measured.

The disc diffusion tests were performed in three separate times, spaced within 48 hours of each other. Each test was conducted at 48, 96 and 144 hours after the initial incubation of the bacterial isolate inoculum. These times were chosen to align with the 3 different phases of bacterial growth (lag, exponential and stationary phase).

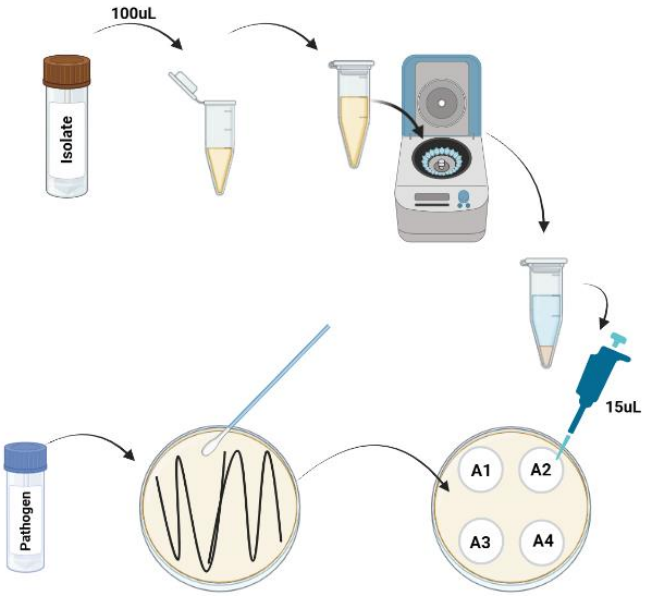


Figure 13 - Schematic of the disc diffusion assay.

2.1.4 Cross Streak method

To perform the cross-streak assay, both pathogen and the bacterial strain were streaked on a single plate. For that, the pathogen was evenly spread at the centre of the plate, and the bacterial isolate was streaked on each side, as demonstrated **Figure 14.** The plates were incubated at 28 ºC for 48 hours. The pathogen inhibition was measured by seeing an inhibition zone in the centre where the pathogen was spread.

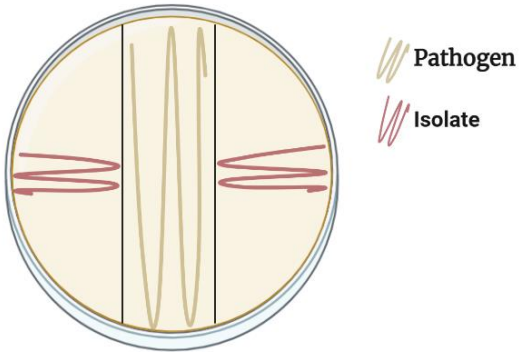


Figure 14 – Schematic representation of cross streak assay

2.1.3 Taxonomic identification of the bacterial isolates

The bacterial isolates with potential to inhibit the selected fish pathogens in the previous methods were identified through 16S rRNA gene sequencing. For that, DNA extraction of each bacterial isolate was performed using E.Z.N.A® Bacterial DNA Kit (Omega Bio-tek, Inc., GA, USA) following the manufacturer's protocol. Afterwards, DNA was quantified using DeNovix DS – 11 FX Series Spectrophotometer/Fluorometer (DeNovix Inc, USA). Then, extracted DNA was amplified by Polymerase Chain Reaction (PCR) using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5’–CGGTTACCTTGTTACGACTT-3’). For that, PCR was carried out using 1-3 µL of DNA template, 1 µL of each primer and 5 µL of MyTaq Mix 2x (Bioline, Meridian Bioscience). Sterile water was added to the samples containing 1-2 µL of DNA template to complete the 10 µL reaction volume. The PCR reaction program is described in **Table 5**.

Table 5 - PCR reaction program used for the amplification of 16S rRNA gene

Steps	Temperature	cycles	Time
Denaturation	95°C	1	2 min
Aneling	94°C	30	30 seg
	48°C		90 seg
	72°C		2 min
Extension	72°C	1	10 min
	15°C	1	∞

PCR products were visualized in a 1.5 % electrophoresis agarose gel. Amplicons were purified and sequenced by GenCore, I3S (Instituto de Investigação e Inovação em Saúde, Porto, Portugal). The raw sequences provided by the company were analyzed utilizing Geneious Prime software (2021.2.1) and the final consensus sequences were submitted GenBank from NCBI, for taxonomic identification. For the blast, NCBI nucleotide collection and 16S ribosomal RNA sequences (Bacteria and Archea) databases were used (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The database EzBioCloud [125] was also used to compare the results provided among the different databases.

2.2. Siderophores inhibition tests against fish pathogens

2.2.1. Siderophores

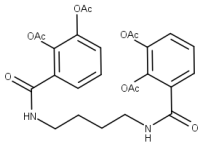
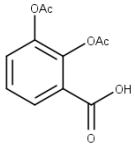
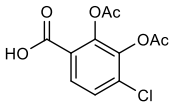
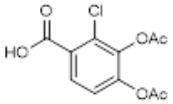
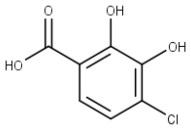
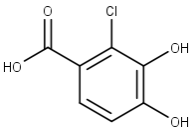
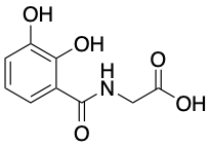
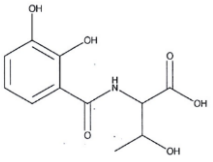
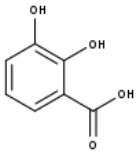
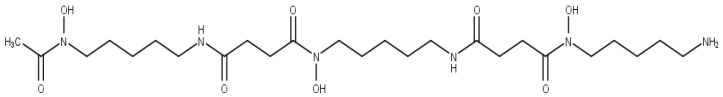
Siderophore AG2 (Itoic acid), siderophore mimetics (MA72, MA59, MA70, MA69, MA67, MA68, AG4, and 2,4-dihydroxybenzoic acid (2,3-DHB) were previously synthesized by other MSc and PhD students at the Laboratory of Organic and Pharmaceutical Chemistry (LQOF) of the Faculty of Pharmacy of the University of Porto (FFUP). Deferoxamine mesylate salt (DFO) was purchased to Sigma-Aldrich. All siderophores are derived from the siderophore 2,3-DHB, a common siderophore moiety. Modifications were performed to build a structure-activity relationship by increasing the number of carbon atoms in the carbon chain.

2.2.2. Siderophores inhibition assays

To conduct the inhibition assays, siderophores solutions with a final concentration of 1 mg/mL were prepared by dissolving each siderophore in dimethylsulfoxide (DMSO). In the **Table 6** is provided the list of synthetic siderophores used in this study, most of which are from the catecholate family and hydroxamate family (DFO). For these assays, *T. maritimum*, V. *anguillarum* and *E.tarda* were cultivated in Mueller-Hinton Agar (MH), a standardized medium usually used in antimicrobial assays [126]. After 48h, the

inoculum for each pathogen was prepared with an OD between 0.08 – 0.10 (at λ = 625 nm) and then, the pathogen was spread evenly throughout the entire plate (**Figure 12**). Afterwards, several blank discs for antimicrobial susceptibility assays (Liofilchem® S.r.l) were placed on the plate and inoculated with 15 µL of each siderophore solution (1 mg/mL). Moreover, Mueller-Hinton Broth (MHB) was used as negative control, enrofloxacin (1 mg/mL) was used as positive control for *T. maritimum* and *E. tarda,* and oxytetracycline (1 mg/mL) was used as positive control for *V. anguillarum*. In addition, black discs with DMSO (99 %) were also tested against the three pathogens, to assess if this solvent can be toxic to the tested strains. The plates were incubated at 28 ºC **(Figure 15**). Results were observed after 24 hours and 48 hours.

Table 6 - Synthetic siderophores tested against *T. maritimum*, *V. anguillarum* and *E. tarda*

Sample	Structure	Molar mass (g/mol)
MA72		528.51
MA59		238.2
MA70		272.64
MA69		272.64
MA67		188.56
MA68		188.56
AG2		211.17
AG4		255.23
2,3-DHB		154.12
DFO		560.69

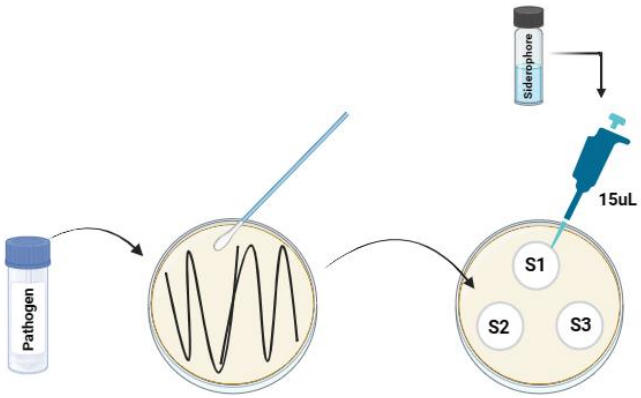


Figure 15 – Schematic representation of the siderophore disc diffusion assay.

2.3. Microorganism-siderophore interaction for inhibition of *T. maritimum*

2.3.1 Microcosms experiments

In the previous experiments, several bacterial isolates and several siderophores presented potential to inhibit the pathogen *T. maritimum*, in the screening assays. However, to fully understand their potential for pathogen biocontrol in a more realistic scenario, experiments using water and biofilter carriers from an aquaculture industry were assembled. For that, 5 bacterial isolates from 3 different genera, that tested positive against *T. maritimum*, *Tritonibacter sp.*, *Pseudoalteromonas sp.* and *Cobetia sp.*, were selected to be a part of a probiotic bacterial formulation. Regarding the siderophores, DFO, (one of the siderophores with the capacity to inhibit this pathogen), was selected for this experiment and since the synthesis of other siderophores were not possible within the given timeframe.

Microcosms were assembled containing 6 biofilter carriers and 20 mL of water from a biofilter, from an aquaculture RAS system. In this experiment, 8 distinct conditions were tested, in triplicates, as described in **Figure 16**.

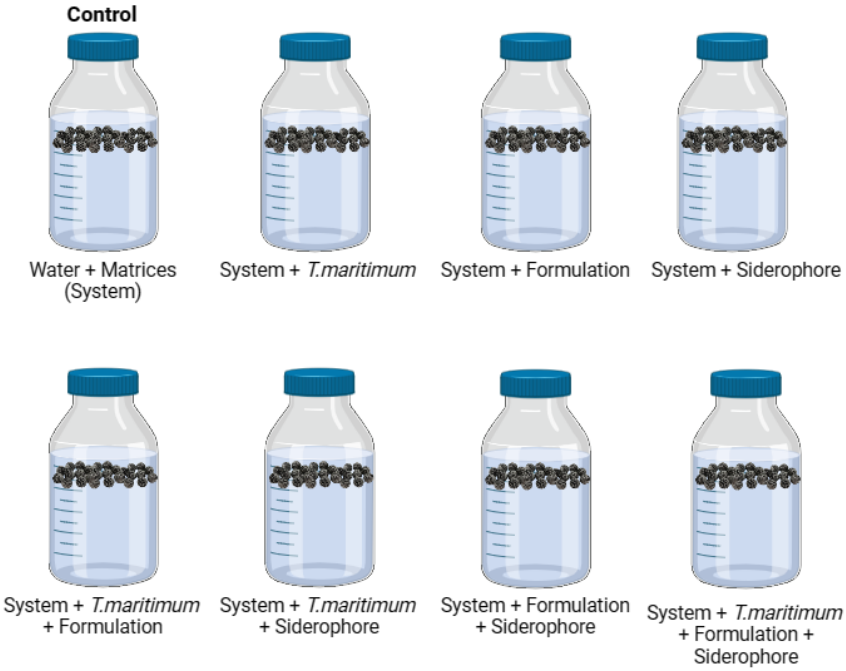


Figure 16 – Experimental design of the experiments using water and biofilter carriers collected from a RAS system. Siderophore corresponds to DFO and Formulations corresponds to the consortium of 5 bacterial isolates belonging to 3 different genera (*Tritonibacter sp., Pseudoalteromonas sp.* And *Cobetia sp.*).

The different conditions were designed to evaluate: a) if the bacterial formulation and the selected siderophore, alone or combined, are able to inhibit *T. maritimum;* b) evaluate the effects of both siderophores and bacterial formulation in the natural microbial community from a RAS system. For the treatments containing the pathogen, *T. maritimum* inoculum was prepared with an OD of 0.2 (λ = 600 nm) in 0.85 % of NaCl saline solution and was inoculated in the respective flaks. As for the bacterial formulation, inoculum of the 5 bacterial isolates were separately prepared by mixing 1 loop of biomass in 1 mL of saline solution (0.85 %) and then, were mixed together in equal proportions. Afterwards, the flaks from the treatments containing formulation were inoculated, starting with optical density of 0.2 (λ = 600 nm). Moreover, the flasks from the treatments containing siderophore were doped with 1 mg/mL of DFO. All flasks were incubated at 25 ºC. This experiment was conducted over a period of one month, during which specific days were allocated for sampling and water change as described in **Table 7** and **Figure 17**.

Table 7 - Experimental schedule describing the sampling days and water changes during the experiment.

Days	Maintenance	
0	Sampling	Water change
3	Sampling	
7	Sampling	
10		
14	Sampling	
17		
21	Sampling	
24		
28		
31	Sampling	

At each 3-4 days pattern (**Figure 17**), the water from the microcosms were changed to ensure the presence of nutrients and carbon sources. For that, 10 mL of water and the biofilter carriers were transfer to new sterile flasks containing 10 mL of water from the RAS biofilter, previously filtered with 0.22 µm MCE membrane filters (MF-MiliporeTM). All flasks from treatments containing siderophores were doped with 0.5 mg/mL of DFO. In the sampling days (day 0, 3, 7, 14, 21, 31), one biofilter carrier was collected from each flask for DNA extraction. Moreover, 10 mL of water was collected for HPLC and nutrient analysis. All the samples were stored at -20 ºC for further analysis.

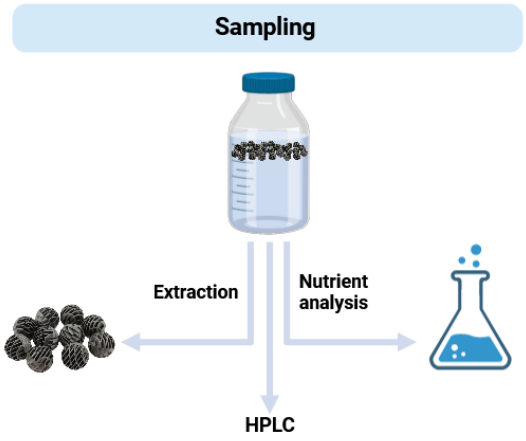


Figure 17- Schematic representation of the sampling procedure.

2.3.2. DNA extraction for prokaryotic community characterization by Next-generation sequencing

DNA from biofilter carriers was extracted using DNeasy Power Soil Kit (QIAGEN, Merck KGaA, Darmstadt, Germany) following the manufacture's protocol. Before starting the protocol, biofilter carriers were placed in 5 mL tubes containing 800 µL of CD1 solution from the DNeasy Power Soil Kit and were centrifuged at 4350 *g* for 15 minutes. Then, the tube containing the biofilter carriers and CD1 solution were submitted to a quick vortex (20-30s), followed by another centrifugation for 5 minutes at same speed. The supernatant was then added to the PowerBead Pro tube and the instructions provided by the manufacture were followed. Extracted DNA was quantified using Qubit™ dsDNA assay kit using Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific) in accordance with manufacturer instructions. DNA samples were sent to Genoinseq (Cantanhede, Portugal) for high-throughput sequencing of the V4-V5 hypervariable region (≈412 bp) of the16S rRNA gene by Illumina MiSeq platform.

2.3.3. Nutrient analysis

Nutrient content in each treatment was assessed by spectrophotometry (VWR V-1200 spectrophotometer). For that, 10 mL of water sample was collected from each flask and filtered through a 25 mm 0.45 µm MCE syringe filter (VWR™ International, North America). The analysis focused on four key nutrients: phosphates, ammonia, nitrites and nitrates. A standardized protocol was followed for the analysis of each nutrient. Ammonia Concentration was assessed using the Grasshoff & Johannsen [127], method, an adaptation of Koroleff (1970) [128].

Nitrite and phosphate concentrations were measured following the protocol provided by Grasshoff et al. (2009) [129]. Nitrate concentration was quantified using the adaptation of the spongy cadmium reduction technique reported by Jones (1984) [130].

3. Results and discussion

3.1 Potential of RAS bacterial strains to inhibit different fish pathogens

3.1.1. Disc diffusion assay and cross streak assay

A library of 253 bacterial isolates were tested against *E. tarda* and *T. maritimum.* Results from disc diffusion and cross streak assays showed that none of the bacterial isolates displayed a positive inhibition against *E. tarda.* For *T. maritimum,* only 217 bacterial isolates were tested, since 36 did not have the capacity to grow on MA medium. From the 217 bacterial isolates, 30 were able to inhibit *T. maritimum* growth in disc diffusion test (**Figure 18A**). It is worth mentioning that 11 bacterial isolates only displayed positive inhibition after 48 or 96 or 144 hours (only in one of the assays). In these cases, the result was considered negative, and it is necessary to repeat the assay to confirm these results. Moreover, in the cross-streak assays, from the 217 bacterial isolates, 62 displayed the ability to inhibit *T. maritimum.* In addition, 9 bacterial isolates were actually inhibited by the pathogen (**Figure 18B and 18C**). Some bacterial isolates were considered partially positive when the bacterial isolate only displayed a small inhibition against the pathogen (**Figure 18D**).

For *V. anguillarum*, only 57 out of 253 bacterial isolates were tested in both assays, in which no inhibition was observed. The remaining screening assays against this pathogen (for the 196 bacterial isolates) will be conducted in the future.

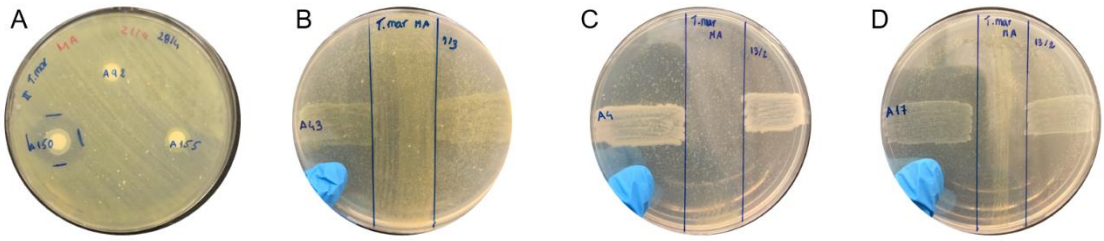


Figure 18 – Inhibition of *T. maritimum* in disc diffusion assays (A) and cross streak assays (B, C). A: positive inhibition by the isolate A150 and negative inhibition by the isolates A92 and A155. B: Isolate A43 cannot inhibit *T. maritimum*. C: Isolate A4 has positive inhibition against *T. maritimum.* D: Isolate A17 demonstrated a partially positive against *T. maritimum.*

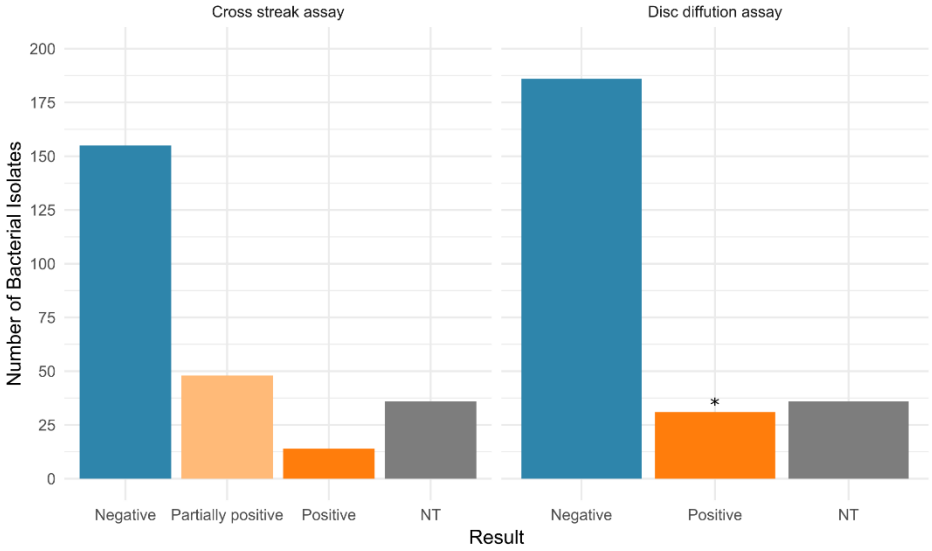


Figure 19 - Number of bacterial isolates that displayed inhibition/or no inhibition against *T. maritimum* in the disk diffusion and cross-streak assays. NT: not tested. Barplot produced with ggplot2 package in R Studio. * Representation of total positive inhibitions after 48, 96 and 144 hours of bacterial isolate inoculum growth.

In the disc diffusion assays, it was observed a higher number of bacterial isolates with positive inhibition against *T. maritimum* (**Figure 19**). This method is usually used to determine the susceptibility of pathogens against different compounds. In the assay conducted in this work, it was used the supernatant of the bacterial isolate and, in it, are present not only the bacterial isolate but also possible metabolites produced by them, which can be responsible for the inhibition. While the cross-streak method is used to screen the antagonism between different microorganisms. So, both methods can lead to different results, as observed in this work. These results shows that the RAS bacterial community has in their structure, bacteria capable of inhibiting the pathogen *T. maritimum*. Probiotic bacteria can fight and supress different pathogens through different modes of action such as competitive exclusion through the production of inhibitory compounds, competition for nutrients, chemicals, or energy, adhesion site competition, enhancement of immune response, and reduction of virulence through disruption of QS, as a new anti-infective strategy [131]. The use of probiotic bacteria has been increasingly studied as an alternative to the use of antibiotics [123]. Probiotics can improve the immune system of the target organisms, can present antibacterial properties, and they can interact with or antagonize other enteric bacteria in the organism by resisting colonization or directly suppressing and lowering the occurrence of opportunistic infections [44], [132].

A study conducted by Dalmin et al. (2001) showed that the regular addition of probiotic *Bacillus spp.*to a shrimp culture pond, increased the population of heterotrophic

bacteria and *Bacillus spp.* and decreased *Vibrio* population (species not specified) after each application. Additionally, the pounds with the *Bacillus spp.* displayed optimum transparency, low organic matter content and low counts of pathogenic *Vibrios* [133]. A more recent study conducted in 2021 by Santos and co-authors studied the quorum quenching (QQ) ability of 200 *Bacillus* spp. isolated from the guts of different farmed fish species to inhibit fish pathogen*, Aeromonas spp. (*LMG 3780 and LMG 2844 strains and fish isolates), *Vibrio spp*. (DSM 21597, LMG 2850 and LMG 13545 strain and fish isolate), *Photobacterium damselae* (LMG 7892)*, Edwardsiela tarda* (LMG 2793)*,* and *Shigella sonnei* (LMG 10473). They found that bacterial isolates identified as *B. subtilis, B. vezelensis*, and *B. pumilus* all substantially decreased *E. tarda* pathogenicity in zebrafish larvae, improving survival by 50% [93]. The genus *Bacillus* spp. has been described as producer of QS molecules. QS is a cell-to-cell communication method through which bacteria coordinate their population size and activity. QS molecules have been suggested as a possible approach to control bacterial infections in aquaculture by disrupting pathogen communication QS.

3.1.2. Taxonomic identification

Taxonomic identification was performed for 74 bacterial isolates that tested positive against *T. maritimum*, in both or either of the inhibition assays (**Figure 20**). From these 74 bacterial isolates, 35 were isolated from biofilter carriers (BC) and 40 from the water (W). In total, 29 different genera were identified among the 74 bacterial isolates, in which the genera *Sulfitobacter, Roseivivax, Rheinheimera, Pseudoalteromonas, Morganella, Microbacterium, Halomonas, Cobetia* and *Acinetobacter* were isolated in both matrices (**Figure 20**). Moreover, the bacterial genera *Tritonibacter, Strenotrophomonas, Staphylococcus, Roseibium, Polaribacter, Pseudomonas, Maritalea* and *Amaricoccus* were only isolated from the water sample (W). Furthermore, the bacterial genera *Ruegeria, Psychrobacter, Pseudovibrio, Pseudosulfitobacter, Marinobacter, Lelliottia, Janibacter, Enterococcus, Cytobacillus, Castellaniella* and *Albirhodobacter* were only isolated from the biofilter carriers (BC).

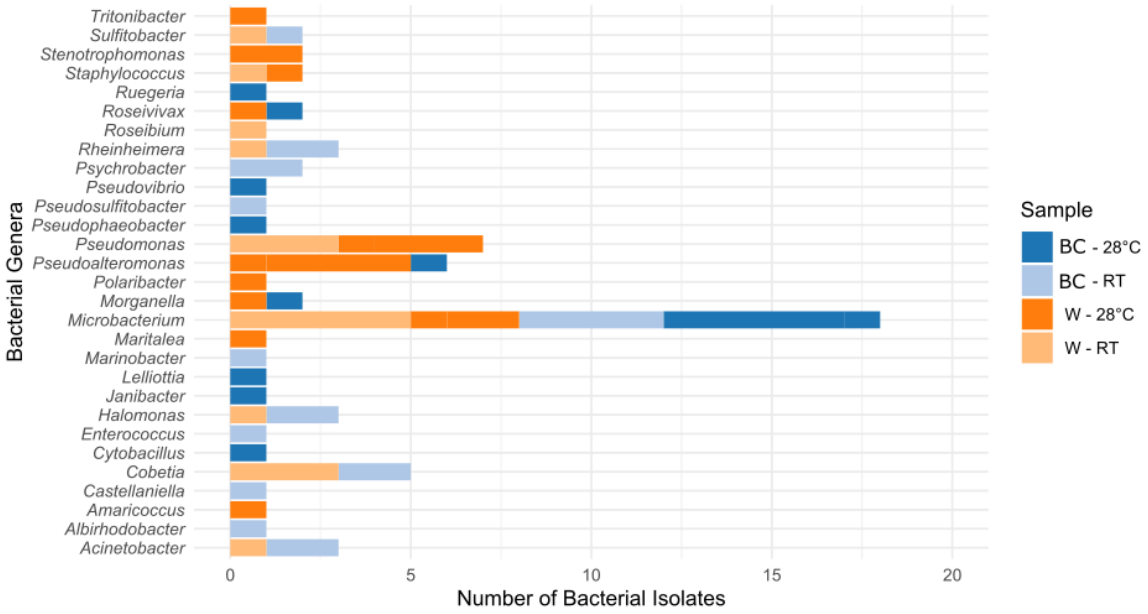


Figure 20 - Taxonomic identification of bacterial isolates that tested positive against *T. maritimum* BC: biofilter carriers W: water. RT: incubated at room temperature; 28ºC - incubated at 28ºC in an incubator.

Among the 74 bacterial isolates, the genus *Microbacterium*, from the Actinomycetota phylum, and the genera *Pseudomonas* and *Pseudoalteromonas,* from the Pseudomonadota phylum were the most genera found that inhibited *T.maritimum*. As described in the **Table 8,** all bacterial isolates identified as *Microbacterium sp.* displayed positive inhibition almost exclusively in cross- streak assay. Regarding the bacterial isolates belonging to the Pseudomonadota phylum, there is no assay that stands out the most. Both disc diffusion assay and cross-streak assay demonstrated positive inhibition against *T. maritimum*.

Table 8 - Identification of bacterial isolates that displayed inhibition (cross-streak, disc diffusion or both) against T. maritimum. √: Partially positive; √: Positive; **x**: Negative; * Not tested.

Isolate ID	Bacterial Genera	Cross streak	Disc difussion		
			48h	96h	144h
A 1	*Pseudoalteromonas sp.*	√	√	√	√
A 2	*Pseudoalteromonas sp.*	√	√	√	√
A 3	*Pseudoalteromonas sp.*	√	√	√	√
A 4	*Pseudoalteromonas sp.*	√	√	√	√
A 5	*Polaribacter sp.*	√	x	x	x
A 8	*Microbacterium sp.*	√	x	x	x
A 13	*Morganella sp.*	√	x	x	x
A 16	*Pseudomonas sp.*	√	x	x	x
A 17	*Morganella sp.*	√	x	x	x
A 21	*Stenotrophomonas sp.*	x	√	√	*

A 25	*Microbacterium sp.*	√	x	x	x
A 26	*Acinetobacter sp.*	√	x	x	x
A 27	*Psychrobacter sp.*	√	x	x	x
A 28	*Rheinheimera sp.*	x	√	√	√
A 31	*Pseudomonas sp.*	√	x	x	x
A 32	*Microbacterium sp.*	√	x	x	x
A 33	*Rheinheimera sp.*	√	x	x	x
A 36	*Microbacterium sp.*	√	x	x	x
A 40	*Sulfitobacter sp.*	√	x	x	x
A 46	**NOT IDENTIFIED**	x	√	x	x
A 48	**NOT IDENTIFIED**	√	x	x	x
A 53	*Cobetia sp.*	√	√	√	√
A 55	*Psychrobacter sp.*	√	x	x	*
A 56	*Castellaniella sp.*	√	x	x	x
A 58	*Acinetobacter sp.*	√	√	x	x
A 66	*Enterococcus sp.*	√	x	x	*
A 71	**NOT IDENTIFIED**	x	x	x	√
A 73	*Cobetia sp.*	√	√	√	√
A 79A	**NOT IDENTIFIED**	x	√	x	x
A 82	*Rheinheimera sp.*	x	√	√	√
A 83	*Halomonas sp.*	√	x	x	x
A 84	*Albirhodobacter sp.*	√	x	x	√
A 85	*Cobetia sp.*	√	√	√	√
A 89	*Pseudophaeobacter sp.*	√	x	x	x
A 100	*Ruegeria sp.*	√	x	√	√
A 101	**NOT IDENTIFIED**	x	x	√	x
A 104	**NOT IDENTIFIED**	x	√	√	√
A 105	*Tritonibacter sp.*	√	√	√	√
A 105B	*Tritonibacter sp.*	x	√	√	√
A 106	*Maritalea sp.*	x	√	√	√
A 108	**NOT IDENTIFIED**	x	√	√	x
A 109	*Microbacterium sp.*	√	x	√	x
A 111	**NOT IDENTIFIED**	x	√	x	x
A 112	*Pseudomonas sp.*	√	√	√	√
A116	*Amaricoccus sp.*	√	x	x	x
A 121	**NOT IDENTIFIED**	x	x	x	√
A 122	*Microbacterium sp.*	√	x	x	*
A 124	*Stenotrophomonas sp.*	x	x	√	√
A 125	*Pseudomonas sp.*	√	x	x	*
A 126	*Staphylococcus sp.*	√	x	x	√
A 127	*Halomonas sp.*	x	√	√	√
A 128	*Cobetia sp.*	√	√	√	√
A 129	*Cobetia sp.*	√	√	√	√
A 130 C	*Roseibium sp.*	x	√	√	√
A 131A	**NOT IDENTIFIED**	x	√	x	x
A 131C	*Microbacterium sp.*	√	x	x	x
A 132A	*Microbacterium sp.*	√	x	x	x

A 133	*Marinobacter sp.*	x	√	√	√
A 134	*Pseudosulfitobacter sp.*	x	x	√	√
A 135	*Halomonas sp.*	√	x	x	x
A 137	*Sulfitobacter sp.*	x	x	√	√
A 138	**NOT IDENTIFIED**	x	x	x	√
A 141A	*Microbacterium sp.*	√	x	x	x
A 150	*Acinetobacter sp.*	√	x	√	*
A 151	*Pseudomonas sp.*	x	√	√	√
A 152	*Pseudomonas sp.*	√	x	x	x
A 153A	*Staphylococcus sp.*	√	x	x	x
A 154A	*Microbacterium sp.*	√	x	x	x
A 154B	*Microbacterium sp.*	√	x	x	x
A 155A	*Microbacterium sp.*	√	x	x	x
A 173	*Microbacterium sp.*	√	x	x	x
A 175	*Microbacterium sp.*	√	x	x	x
A 180	*Janibacter sp.*	√	x	x	x
A 181	*Microbacterium sp.*	√	x	x	x
A 182B	*Cytobacillus sp.*	√	x	x	x
A 183A	*Lelliottia sp.*	x	√	√	√
A 185	**NOT IDENTIFIED**	x	√	x	x
A 188	*Microbacterium sp.*	√	x	x	x
A 191	*Roseivivax sp.*	√	x	x	x
A 192	*Roseivivax sp.*	√	x	x	x
A 198	*Pseudoalteromonas sp.*	√	√	√	√
A 202	Pseudovibrio sp.	√	x	x	x
A 205	**NOT IDENTIFIED**	√	x	x	x
A 208	**NOT IDENTIFIED**	√	x	x	x
A 208.1B	*Microbacterium sp.*	√	x	x	x
A 209	*Pseudomonas sp.*	√	x	x	x
A 211A	*Pseudoalteromonas sp.*	x	√	√	√
A 221B	*Microbacterium sp.*	x	√	√	√

Some studies have reported the probiotic potential of *Microbacterium* sp. In fact, *Microbacterium sp.* was used previously as probiotic in a multi-strain probiotic mixture with the bacterial strains *Pseudoalteromonas, Ruegeria* and *Vibrio* [134]. Skjermo et al. (2015) administered a mix of 4 candidate probiotic strains, *Microbacterium sp.* (ID3-10), *Ruegeria* sp. (RA4-1), *Pseudoalteromonas* sp. (RA7-14) and *Vibrio sp.* (RD5-30) through the feed nutrition and water treatment of cod larvae, throughout 24 hours, to evaluate the probiotic bacteria colonization. They reported that the only candidate that was found in the larval microbiota was the *Microbacterium* ID3-10 [135]. In another study, conducted by Fjellheim and co-authores in 2010, which had the goal of characterizing a pool of 500 probiotic bacteria candidates from the dominant intestinal microflora of cod larvae, that produce antimicrobial compounds against the pathogenic bacterium *V.*

anguillarum. They have described these bacteria phenotypically (uniqueness, dominance, and fermentative ability), their antagonism, adhesion to mucus, growth in mucus, production of extracellular enzymes, fish bile resistance and haemolytic properties. In the end, 5 bacterial isolates improved the survival of cod larvae, including bacterial isolates from the genus *Microbacterium* [136].

From the phylum Pseudomonadota, there are some studies that reported the use of *Pseudomonas sp.* strains (specifically *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) as a potential probiotic in fish farming. In fact, in a study conducted by Giri et al. (2012), *P. aeruginosa* showed potential to improve the immunity and disease resistance of tropical freshwater fish *Labeo rohita against the pathogen Aeromonas hydrophila,* through dietary supplementation [137]*.* Another study conducted by Gram and co-authors (1999) reported that in iron-limited conditions*, P. fluorescens* was capable of inhibiting the growth of *V. anguillarum*, whereas in iron-rich condition the same did not applied [138]. Moreover, Wuertz et al. (2023) showed that two different species of *Psychrobacter* genus (*Psychrobacter nivimaris* and *Psychrobacter faecalis*) were capable to reduce the mortality rates associated to *T. maritimum* (0 % and 8 % of mortality was observed after treatment comparing with the control that had 20 % mortality) [139]. As for the genus *Pseudoalteromonas,* their capacity to produce bioactive molecules, including antimicrobial compounds, has already been described [140]. In a work developed in 2016 by Wiebke Wesseling and co-authors, two different systems, infected with *V. anguillarum,* were inoculated with *Pseudoalteromonas sp*. strain MLms gA3 (immobilized in ceramics or in alginate microencapsulation). The authors concluded that both systems exhibited promising anti-*Vibrio* activities since, after two weeks, the viable cell count of the pathogen decreased [141]. In another study, Fuente and co-authors (2015) conducted a screening using 80 Gram negative bacterial strains, aiming to inhibit three salmon pathogens, *V. anguillarum*, *Aeromonas hydrophila* and *Flavobacterium psychrophilum*. They reported that 10 of those bacterial strains were identified as belonging to *Pseudomonas* genus and nine of them were siderophore producers, which suggested that these strains can be an important for the biocontrol of fish pathogen. Despite this findings, one of the 10 strains did not produce siderophore, but had the capacity to inhibit the pathogen [142]. Pathogenic bacteria have developed advanced mechanisms to obtain iron from their hosts or from the surrounding environment. One of these mechanisms is the synthesis of siderophores to obtain the iron needed for their survival and pathogenicity [98]. The use of probiotic bacteria that produce siderophores has shown to be a promising solution in minimizing the virulence of bacterial infections in fish [123]. By synthesizing siderophores, the probiotic bacteria

can compete with the siderophores produced by pathogens, thereby blocking its access to iron (or other metals like Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, etc), and reducing their virulence. Therefore, if two competing strains produce their own exclusive siderophore, the competition between them can be influence by many factors, such as, the number of produced siderophores, iron-binding properties and the kinetics of siderophore formation and bacteria's siderophore receptors [109].

Regarding the other bacterial genera identified in this study, some of them have been described as having probiotic effects. In fact, the bacterial genera *Tritonibacter, Pseudovibrio, Ruegeria* and *Sulfitobacter* were associated to the production of tropodithietic acid (TDA). This compound induces the disruption of target microorganisms' proton motive force and, presumably, its iron-chelating capabilities [143]–[145]. Additionally, the bacterial genera *Stenotrophomonas, Acinetobacter*, *Cobetia, Enterococcus* and *Halomonas* have been reported as probiotic strains against different fish pathogens, such as *Aeromonas hydrophila, Vibrio harveyi* and *E. tarda* [146]–[150]. *Marinobacter sp.*and *Polaribacter sp* were reported as producers of beneficial metabolites, such as antioxidant compounds and exopolysaccharides (EPSs), with probiotic and prebiotic properties, respectively [151], [152]. Regarding the remaining bacterial genera, namely *Roseivivax, Roseibium, Rheinheimera, Pseudosulfitobacter, Pseudophaeobacter, Morganlla, Maritaela, Albirhodobacter, Amaricocus, Castellaniella, Cytobacillus, Janibacter* and *Leliotia,* no data was found reporting their probiotic activity against different pathogens. Therefore, to our best knowledge, this is the first study reporting their potential to inhibit fish pathogen. Still, more studies are needed to investigate their potential as probiotic bacteria in a more complex scenario.

In the present work, the tested bacterial isolates were only capable to inhibit *T. maritimum*. In fact, the methodology was designed to isolate bacteria with negative correlations with *T. maritimum*, a goal that was achieved, since several isolates tested positive against *T.maritimum*. Nevertheless, since aquaculture industry faces many challenges related with pathogen outbreaks, other fish pathogens were tested in this study. None of the bacterial isolates were capable to inhibit *E. tarda.* In addition, for *V. anguillarum*, although the screening assays were not conducted for all the bacterial isolates (only 57 were tested), the ones that were tested did not inhibit this pathogen. To fulfil this gap, for both pathogens, network and correlation analysis should be conducted in the future to identify the RAS procaryotic community that can have a negative correlation with *V. anguillarum* and *E. tarda.* With this analysis, it is possible to design

specific cultivation methods to isolate the key bacteria that can have potential to inhibit both pathogens.

Nevertheless, this work unveiled the potential of several bacterial strains to inhibit *T. maritimum*. These results can contribute to the development of new probiotic formulations to prevent or control *T. maritimum* outbreaks in RAS aquaculture production. However, more studies are needed to fully characterize the probiotic potential of this bacterial strains and their efficiency in real RAS aquaculture systems.

3.2. Siderophores inhibition tests against fish pathogens

Disc diffusion assays were conducted against *T. maritimum, V. anguillarum* and *E. tarda*. For that, a total of 10 siderophores were tested (**Table 6**). The results showed that most of the selected siderophores were able to inhibit *T. maritimum* after 24h of incubation, being the only exception the siderophore AG4. Still, the siderophores DFO, MA59, MA67, MA68, MA69, MA70, MA72, AG2 and 2,3-DHB were the ones that displayed the highest inhibition halos (Figure 21). For the pathogen *E. tarda*, only MA72 and DFO were able to inhibit the pathogen after 24 hours (**Figure 21**). None of the selected siderophores were able to inhibit the growth of *V. anguillarum*. (**Table 9**)

All siderophores were dissolved in DMSO, thus, to ensure that the observed inhibition was due to the presence of siderophore and not from the DMSO, the solvent was tested against the 3 pathogens. The results showed that the DMSO did not inhibit the 3 pathogens.

Table 9 - Siderophores inhibition potential against T. maritimum, V. anguillarum and E. tarda after 48 hours. √: Positive; X Negative

Sideroforos	*Tenacibaculum maritimum*	*Vibrio anguillarium*	*Edwardsiella tarda*
MA59	√	x	x
MA67	√	x	x
MA68	√	x	x
MA69	√	x	x
MA70	√	x	x
MA72	√	x	√
DFO	√	x	√
AG2	√	x	x
2,3-DHB	√	x	x
AG4	x	-	x
DMSO	x	x	x

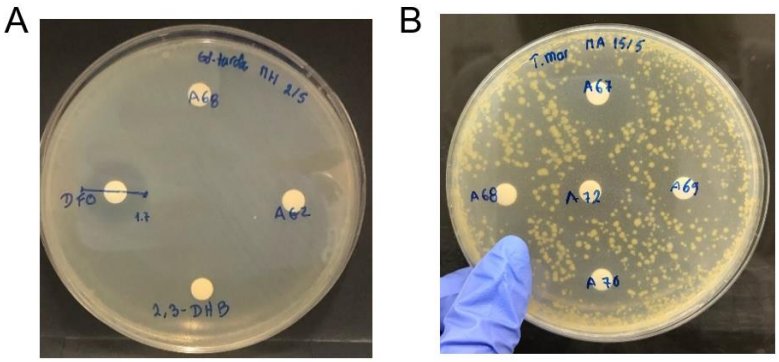


Figure 21 – *Siderophores* inhibition assays for *E. tarda* (A) and *T. maritimum* (B).

All pathogens, *T. maritimum*, *V. anguillarum* and *E. tarda* are siderophore-producing bacteria [74], [153], [154]. Between the three, only *V. anguillarum* and *E. tarda* have their siderophore described. *V. anguillarum* have been associated to the production of 3 siderophores: anguibactin [155], vanchrobactin [156] and piscibactin [157], and depending on the serotypes (O1, O2 and O3), *V. anguillarum* can be highly virulent [158]. *E. tarda* can produce 1 siderophore, vibrioferrin [159]. *T. maritimum* can synthesize a hydroxamate siderophore however, this siderophore hasn't been fully characterized [74].

According to Avendaño-Herrera and co-authors (2005), *T. maritimum* has at least two types of mechanisms to acquire iron: the synthesis of siderophores and the use of heme groups as iron sources by direct binding [74]. In this study, the synthetic siderophores seemed to have overpowered the iron acquisition mechanisms used by *T. maritimum*. There is a lack of literature about the inhibition of *T. maritimum* by

siderophores. Therefore, further studies must be conducted to unveil the interaction between synthetic siderophores and *T. maritimum*.

Regarding *V. anguillarum*, none of the synthetic siderophores were capable to inhibit the pathogen. Some studies have been described that this pathogen is virulent since it can produce a different variety of siderophores. Additionally, the siderophores that *V. anguillarum* synthesize are from the catechol family, which is known to have the highest iron (III) affinity among any binding group class [103], [158]. Most of the synthetic siderophores tested in this work belong to the catechol family, but natural siderophores are more complexed than the ones that were tested in this work, which may explain why no inhibition was observed. As mentioned before, siderophore-producing bacteria are capable of inhibit this pathogen and have been used as probiotic before [109]. A lot is known about biosynthesis process, iron acquisition systems, regulation, and transport of *V. anguillarum* [160]. However, to our knowledge there is no data available about the use of siderophores as a strategy to control *V. anguillarum* infection.

At last, *E. tarda* was only inhibit by two synthetic siderophores, MA72 (catechol family) and DFO (hydroxamate family). The siderophore produced by this pathogen, vibrioferrin, describe in literature, is a member of carboxylate family, and its unique feature is its relatively weak iron binding properties compared to other siderophore classes [161], which may explain why both synthetic siderophore inhibit the pathogen. Similar to the previous pathogens, there are also a lack of studies performed about the use of synthetic siderophores to prevent the infection caused by *E.tarda*.

A study conducted by Erin K McCreary and co-authors in 2021, showed promising results of using Cefiderocol, a synthetic conjugate composed by cephalosporin moiety and a catechol-type siderophore, for the treatment of carbapenem-resistant infections. In their work, infections caused by *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacterales* Gram-negative bacilli, *Stenotrophomonas maltophilia*, *Burkholderia sp* and *Achromobacter* species were studied and undergoing clinical trials. For *Enterobacterales,* Cefiderocol seems to be a reasonable alternative when other b-lactam agents show resistance. However, for the other infections there is still a lot to uncover, and a singularity of this antibiotic candidate is its cell entry, which facilitates bacterial cell entry using active iron transport [162].

Furthermore, the commercially known siderophore DFO is a medication used for iron toxicity and aluminium toxicity, sold as Desferal. DFO is used to remove iron excess from the body in anemia or thalassemia patients who have many blood transfusions. DFO is also used with other medicines to treat acute iron poisoning,

especially in small children [163]. In this work, DFO was used to understand its capabilities to inhibit the *T. maritimum* in experiments using water and biofilter carriers from an aquaculture unit.

To fully understand the pathogenic strategies employed by these pathogens and the role of siderophores in their pathogenicity, more studies are needed, including studying these pathogens under iron-supplemented and iron depleted media.

3.3. Microorganism-siderophore interaction for inhibition of *T. maritimum*

The results from this experiment are still being analysed. The samples were sent for Next Generation Sequencing (NGS) however, the results are not available yet. Regarding the nutrient analysis, phosphate and nitrite analysis were concluded but, to evaluate the nutrients concentration in each treatment, it is necessary to conclude the ammonia and nitrate analysis.

This experiment aimed to assess the microorganism-siderophore interaction for inhibition of *T. maritimum,* in a more real scenario. For that, experiments were conducted using water and biofilter carriers collected from a RAS aquaculture system. With this experiment, it is possible to investigate how siderophores and bacterial probiotic formulation, combined or alone, can inhibit or control the spread of *T. maritimum*. This experiment was designed to respond to several hypotheses: 1) Does the probiotic formulation and/or the siderophore have the potential to inhibit *T. maritimum*? 2) Can the formulation compete with the natural procaryotic community from the biofilter? 3) Does the probiotic formulation and/or the siderophore have any effect on the structure and dynamics of the procaryotic community? 4) Can the microorganism-siderophore interaction be the best approach to develop a biocontrol technology to control and prevent *T. maritimum* outbreaks? In this study, a bacterial probiotic formulation composed of 5 different isolates from 3 different genera (*Tritonibacter sp., Pseudoalteromonas sp.* and *Cobetia sp.*) was tested instead of using a single probiotic strain. Previous studies have investigated the efficacy of single versus multi-strain probiotics, but there is still insufficient data to conclude which is the best option [164], [165]. Procaryotic community analysis will unveil if natural procaryotic communities from the biofilter are affected by the addition of probiotic bacterial formulation to inhibit *T. maritimum*. If no significant changes in the natural community are observed and *T. maritimum* relative abundance decreases or disappears, it indicates that the use of the

formulation may be a valuable way to manage *T. maritimum* infection in aquaculture. The use of multi-strain probiotics in aquaculture units has been advocated [166], [167]. In fact, multi-strain probiotics composed by *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, and *Pediococcus pentosaceus* enhanced the growth, immune response, and survival of *Labeo rohita* fish after a challenge with *Aeromonas hydrophila* [168]. However, no studies were found using the probiotic bacteria selected in this study or the use of multi-strain probiotics against *T. maritimum*. The efficiency of probiotic bacterial formulation will be assessed. The results from the disc diffusion and cross-streak assay were promising however, the probiotic bacterial formulation has to adapt and to compete for nutrients and carbon sources with the natural community from the biofilter and the success of the bacterial formulation can be compromised. Additionally, this experiment seeks to understand how siderophore DFO behaves in a natural environment. Siderophores are known to chelate iron (Fe^{3+}), but it is unclear how they will behave when they are in contact with the procaryotic community from RAS. In fact, it is unclear if the DFO will target specifically the pathogen or will target specific bacteria from the biofilter microbiome or even the probiotic bacterial formulation. The application of DFO as pathogen bioagent has already been demonstrated for *Piscirickettsia salmonis* [169], where DFO siderophore displayed a negative impact on the pathogen bacterial cells [169].

Furthermore, NGS analysis will disclose if the combined use of the probiotic formulation and DFO enhances the *T. maritimum* inhibition. Studies combining DFO with antibiotic has been reported [170]. In that study, methicillin-resistant *Staphylococcus aureus* and metallo-β-lactamase producers – *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were inhibit by the synergetic activity of siderophore and antibiotics (deferoxamine-B+ampicillin) [170]. However, in a study conducted by Gatesoupe (1997), the activity of DFO and the probiotic bacteria did not present further improvement compared to the activity of both separately, when they were used to inhibit *Vibrio splendidus* [171]. Even though synergetic effects of combined use of DFO with other compounds has been previously demonstrated, there is a lack of knowledge regarding how DFO will behave combined with bacterial probiotics.

At last, nutrient analysis will be essential to determine if the water from the biofilter had enough nutrients to stimulate the natural microbial community and the probiotic bacterial formulation. The water from each treatment was changed at each 3 days however, it is impossible to predict if the nutrients were enough to stimulate the community during this period of time.

The results from this experiment will contribute for the development of technologies to prevent or control *T. maritimum* outbreaks, in RAS aquaculture units.

4. Conclusion

RAS has been increasingly used to improve the aquaculture industry and make it more environmentally friendly. RAS allows a huge production of a great diversity of fish and marine animals and provides the ability to have more control over several aspects, such as water quality, waste reduction and disease control. However, pathogen outbreaks are still a major concern in the aquaculture sector. In order to prevent and control fish pathogen outbreaks, new biocontrol technologies are needed. Making the transition to the use of probiotics and other greener alternatives is the best option for moving towards a more sustainable aquaculture production. The use of probiotics has several benefits, such as improvement on fish production, prevent and control various diseases and improve the immune functions of captive animals. In addition, the use of probiotics can be presented as an alternative to the use of antibiotic.

The main goal of this work was to evaluate the interactions between RAS bacterial isolates and synthetic siderophores to inhibit three fish pathogens, *T. maritimum*, *V. anguillarum* and *E. tarda*, responsible for high mortality and financial losses in aquaculture systems.

This work revealed that there are bacteria in the RAS biofilter capable of inhibiting *T. maritimum*. Additionally, the genera *Pseudoalteromonas, Pseudomonas* and *Microbacterium* were the most abundant among the bacterial isolates that tested positive against *T. maritimum*. To our best knowledge, none of these genera had been described as to having probiotic activity against *T. maritimum*. However, some of these bacterial isolates were associated to the inhibition of other fish pathogens, such as *V. anguillarum*. Curiously, none of the tested isolates so far were capable to inhibit this pathogen. Moreover, results obtained for susceptibility test with synthetic siderophores showed that siderophores from the catechol and hydroxamate family were able to inhibit the growth of *T. maritimum* and *E. tarda*. The siderophores MA72 and DFO turned out to be the best to achieve that goal. There is a lack of studies regarding the use of siderophores to control the fish pathogens. The results from this work contributed to understand how important and efficient siderophores can be to supress fish pathogens.

To try to understand the interaction between probiotic bacteria and siderophores, an experiment was conducted using water and biofilter carriers from a RAS aquaculture unit. In the end, this was designed to answer to several hypothesis: 1) Does the probiotic formulation and/or the siderophore have the potential to inhibit *T. maritimum*? 2) Can the formulation compete with the natural procaryotic community from the biofilter? 3) Does

the probiotic formulation and/or the siderophore have any effect on the structure and dynamics of the procaryotic community? 4) Can the microorganism-siderophore interaction be the best approach to develop a biocontrol technology to control and prevent *T. maritimum* outbreaks? The results from this experiment will be crucial to answer these hypotheses and to understand how this technology can be applied in a real aquaculture unit.

The results from this work provided additional insights into the development of new tools to combat and fish prevent outbreaks. However, more studies focusing these topics are needed to understand how siderophore – microorganisms' interaction can be used as a toll to control and prevent fish pathogen outbreaks and how they affect the RAS natural microbial community and in the fish microbiome.

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