Universidade de Aveiro
2020

Joaquim Miguel Gonçalves de Sousa

Explorando o potencial de microrganismos na modulação do bacterioplâncton de aquacultura

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Newton Carlos Marcial Gomes, Investigador Principal do Centro de Estudos do Ambiente e do Mar e do Departamento de Biologia da Universidade de Aveiro e sob a coorientação do Doutor António Miguel de Oliveira Louvado, Investigador de Pós-Doutoramento do Centro de Estudos do Ambiente e do Mar e do Departamento de Biologia da Universidade de Aveiro.

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À Catarina, pela paciência.

o júri

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palavras-chave Aquacultura com sistema de recirculação, modulação de microbioma, fungos, *Pseudoalteromonas* inativada por calor

resumo

A comunidade microbiana de aquaculturas com sistema de recirculação (RAS) é essencial para a manutenção da qualidade da água e para a prevenção de doenças. A modulação do microbioma, através da promoção de uma elevada diversidade e de um aumento de microrganismos antagonistas, pode tornar um sistema de aquacultura mais resiliente contra o aparecimento de doenças. Tendo isto em conta, este trabalho procurou 1) isolar e caracterizar fungos da água e biofiltros de RAS para potencial aplicação como moduladores do microbioma em aquacultura e 2) avaliar o potencial de biomassa inativada por calor de *Pseudoalteromonas* spp. (HKP) na modulação da comunidade do bacterioplâncton de RAS.

No primeiro capítulo, os esforços de isolamento resultaram em 18 isolados de fungos pertencentes aos filos Ascomycota e Basidiomycota, sendo *Pseudotaeniolina globosa* o mais prevalente (11/18 isolados). Para além disso, três dos isolados foram identificados como *Vishniacozyma carnescens*. Outros isolados incluem a espécie de Ascomycota *Candida labiduridarum* e as espécies de Basidiomycota *Dioszegia hungarica, Tilletiopsis lilacina* e *Cystobasidium slooffiae*. Estas espécies podem produzir metabolitos secundários com potencial interesse biotecnológico, nomeadamente compostos antimicrobianos, e carotenoides, pelo que serão estudadas em trabalhos futuros, de modo a explorar a sua valorização no setor da aquacultura.

No segundo capítulo desta tese, a avaliação do efeito de HKP demonstrou que a estirpe HKP-SubTr2 tem o maior potencial para a modulação do bacterioplâncton de RAS. A adição de HKP-SubTr2 (potencial produtora do pigmento prodigiosina, com atividade antagonista) à água teve um claro efeito modulador nas comunidades do bacterioplâncton. Este tratamento enriqueceu significativamente a abundância relativa de Bacteriovoracales, Chitinophagales e Oceanospirillales em comparação com o controlo não tratado e com o tratamento com *Escherichia coli* DH5α, uma bactéria não pigmentada. Para além disso, o tratamento com HKP-SubTr2 diminuiu significativamente a abundância relativa de Rhodobacterales. Não houve quaisquer diferenças significativas nos parâmetros de qualidade testados entre os diferentes tratamentos e o controlo. Estes resultados indicam que o uso de biomassa inativada por calor poderá ser uma estratégia interessante para a modulação do bacterioplâncton de aquacultura. No entanto, são ainda necessários mais estudos, de modo a avaliar o seu potencial efeito na saúde dos peixes e na qualidade da água nos sistemas de aquacultura.

keywords Recirculating aquaculture systems, microbiome modulation, fungi, heat-killed *Pseudoalteromonas*

abstract The microbial community of recirculating aquaculture systems (RAS) is essential for the maintenance of water quality and disease management. Microbiome modulation, through the promotion of higher diversity and an increase in antagonistic microorganisms, can render an aquaculture system more resilient against disease. In line with this concept, this work aimed to 1) isolate and characterize fungi from the rearing water and biofilter of a fish RAS for potential application as microbiome modulators in the aquaculture sector and 2) assess the potential modulating effects of heat-killed biomass of *Pseudoalteromonas* spp. (HKP) on RAS bacterioplankton communities.

In the first chapter, our microbial isolation efforts resulted in 18 fungal isolates all belonging to the Ascomycota and Basidiomycota phyla, with *Pseudotaeniolina globosa* being the most prevalent (11/18 isolates). In addition, three of the isolates were identified as *Vishniacozyma carnescens*. Other organisms found include the Ascomycota species *Candida labiduridarum* and the Basidiomycota species *Dioszegia hungarica, Tilletiopsis lilacina,* and *Cystobasidium slooffiae*. These species may produce secondary metabolites with biotechnological potential, such as antimicrobial and antifungal compounds, and carotenoids, and further research is needed to explore their valorisation in the aquaculture sector.

In the second chapter of this thesis, the evaluation of the modulating effects of HKP showed that the strain HKP-SubTr2 had the greatest potential for RAS bacterioplankton modulation. The addition of HKP-SubTr2 (assumed to produce prodigiosin pigments, with possible antagonistic activity) to the rearing water was shown to have a clear modulating effect on aquaculture bacterioplankton communities. HKP-SubTr2 treatment significantly enriched the relative abundance of orders Bacteriovoracales, Chitinophagales, and Oceanospirillales in comparison with untreated control and treatment with unpigmented bacteria *Escherichia coli* HK-DH5α. In addition, the treatment significantly lowered the relative abundance of Rhodobacterales. No significant differences were found among the tested water quality parameters between the different treatments and the control. These findings indicated that the use of heat-killed microbial biomass may be an interesting strategy for aquaculture bacterioplankton modulation. However, further studies are necessary to investigate its potential effect on fish health and water quality during aquaculture production.

Table of contents

List of Figures

List of tables

List of abbreviations

2D – Two-dimensional
ASV – Amplicon sequence variants
ASW – Artificial seawater
BALO – *Bdellovibrio* and like organisms
BLAST – Basic local alignment search tool
BSA – Bovine serum albumin
DMSO– Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic acid
FAO – Food and Agriculture Organisation of the United States
HK – Heat-killed
HK-DH5α – Heat-killed *E. coli* DH5α
HKP – Heat-killed *Pseudoalteromonas* spp.
HKP-PtTr1 – Heat-killed *Pseudoalteromonas pisciscida* PtTr1
HKP-RH3 – Heat-killed *Pseudoalteromonas flavipulchra* RH3
HKP-SubTr2 – Heat-killed *Pseudoalteromonas rubra* SubTr2
ITS – Internal transcribed spacer
LAB – Lactic acid bacteria
MEGA– Molecular evolutionary genetics analysis
NCBI – National Center for Biotechnology Information
PCoA – Principal coordinates analysis
PCR – Polymerase chain reaction
PERMANOVA – Permutational analysis of variance
RAS – Recirculating aquaculture systems
RAPD– Randomly amplified polymorphic DNA
rRNA – Ribosomal ribonucleic acid
TAE – Tris-acetate-EDTA
TDA – Tropodithietic acid
TOC – Total organic carbon
UV – Ultraviolet
WHO – World health organization
YP – Yeast-peptone

General Introduction

General Introduction

1. Fish farming

Growing concerns regarding populational growth, climate change, and natural stock sustainability have led to the emergence of alternative sustainable approaches to secure aquatic food. In response, food production through aquaculture has experienced a rapid increase of 3.2% per year in the period from 1961 to 2018 (Food and Agriculture Organization of the United Nations, 2020). While aquaculture only provided about 7% of worldwide fish production in 1974, it currently accounts for 52% of all fish produced for human consumption (FAO, 2020), with finfish being by far the leading group of species produced by aquaculture, accounting for 85% of total aquaculture production in 2018 (FAO, 2020). Meanwhile, the capture of fish, crustaceans, molluscs, and other aquatic animals have persisted somewhat stable, with a mere 14% growth between 1990 and 2018, whilst aquaculture production has risen by 527% in the same period (FAO, 2020). Therefore, aquaculture currently represents an essential form of food production, with particular relevance in South-Eastern Asia, and more specifically, in China. Since 1990, China has produced more food by aquaculture than the rest of the world combined, accounting for 57.9% of the world's aquaculture production in 2018 (FAO, 2020). The increased aquatic food availability due to the explosion of aquaculture production has led to improved diets worldwide. Fish and other aquatic animals are a highly nutritious meal, containing high-quality protein, with essential amino acids, essential fats (such as long-chain omega-3 fatty acids), vitamins (B2 and D), and minerals (such as calcium, phosphorus, iodine, zinc, iron, magnesium and potassium) (Tørris et al., 2018).

The feed quality and cost and disease outbreaks are considered major limitations for the development of aquaculture techniques (FAO, 2020; White et al., 2018). High-quality feed that meets the farmed species' nutritional requirements and allows their growth is a major cost in fish aquaculture production ventures (White et al., 2018). Thus, it is relatively common that farming is performed without feed supplementation [e.g., the production of silver and bighead carp, bivalve molluscs, and other filter-feeding animals (FAO, 2020)]. Although non-fed aquaculture production leads to a lower yield, it may ensure food security. Likewise, the non-supplementation of feed reduces the environmental impact of aquaculture systems, as feed increases waste production and leads to a cascade of problems in water quality that eventually results in the proliferation of potentially harmful bacteria and fish disease or death (Dauda et al., 2019).

Disease outbreaks are a dire concern for producers as they can cause a rampant increase in mortality that can easily impact growth, yield and, in a worst-case scenario, the economic sustainability of the whole aquaculture production facility or sector (FAO, 2020). For example, Thailand, one of the largest producers and exporters globally, have had their global competitiveness challenged by significant losses in shrimp production, mostly due to disease outbreaks. Between 2010 and 2017, it is estimated that the Mahachai market, one of the principal seafood markets in Thailand, lost more than 7 billion US$ and that more than 4 billion US$ were lost in exports due to a decrease in production (Shinn et al., 2018). Globalisation, which facilitates the pathogen dissemination, and high stocking densities, which facilitate the spread of opportunistic pathogens by increasing the stress and decreasing the innate immune responses of fish, are two factors that are known to increase the impact of infectious diseases in the aquaculture sector (FAO, 2020; Reverter et al., 2020). Moreover, most countries have weak regulations regarding biosecurity measures, exacerbating this issue (FAO, 2020). Instead of adopting prevention measures, the biosecurity actions are often reactive, more costly, and less efficient in tackling the problem. Animals are routinely treated with antibiotics, leading to the emergence of antibiotic-resistant strains, which are difficult to treat and eradicate once settled in the aquaculture system (Santos & Ramos, 2018).

2. Recirculating aquaculture systems (RAS)

There are several types of aquaculture systems, which can be classified as extensive, semi-intensive, or intensive. The classification by intensity depends on the number of organisms present per water volume (*i.e.,* the stocking density) and how the feed is administered (Timmons, M. & Ebeling, J., 2010). Extensive aquaculture is a more traditional type of aquaculture, where ponds and lagoons (which can be natural or artificial) are maintained to facilitate the growth of the aquatic fauna, at a higher yield than what would be found in nature (Stickney, 2000). In semi-intensive aquaculture, the yield is increased by adding supplementary feed to the naturally available feed, allowing higher stocking densities and higher production rates. In intensive systems, all feed provided is delivered to fish in optimal feeding regimes, and abiotic parameters are maintained optimal for fish physiology. Intensive systems can be subdivided into two main system architectures: flow-through, in which water enters through a capture point, flows through all the tanks in the system and exits through a discharge point (Stickney, 2000), and recirculating systems.

This dissertation will focus on one type of intensive systems: recirculating aquaculture systems (RAS). In this type of aquaculture, water remains in a close

circuit and cycles back into the system, which allows easier control of all the physical-chemical parameters of the water (Timmons & Ebeling, 2010).

i. Advantages of RAS

RAS offers several advantages over other aquaculture systems. One obvious advantage is the use of 90-99% less water than flow-through systems, and less than 1% of the land area of extensive aquaculture systems (Timmons & Ebeling, 2010). Another advantage is a consistent yearly production, since there is absolute control over the environment, whereas flow-through systems may be influenced by the seasonal variance of abiotic parameters (Timmons & Ebeling, 2010). These systems also help mitigate the environmental dangers of other aquaculture types, since waste streams are controllable in sustainable ways (Midilli, Kucuk & Dincer, 2011).

One of the main advantages of RAS over other aquaculture systems, e.g.,flow-through systems, is the capacity for the establishment of a stable, autochthonous microbial community, which allows for better fish health and disease management (Attramadal et al., 2012). In comparison, flow-through systems are not isolated from the environment, thus allowing the exchange of pathogens, increasing the chance of disease outbreaks (Attramadal et al., 2012). It has been demonstrated that when RAS and flow-through systems were employed at the same feeding and rearing regimes, RAS had a more diverse and stable bacterial community (Attramadal et al., 2012). In contrast, the same study found flow-through systems had a larger fraction of opportunistic organisms, i.e., organisms capable of causing disease in immunosuppressed fish. In summary, RAS is environmentally sustainable, since it allows for greater conservation of water and heat, and allows for year-round production of safe and high-quality products (Attramadal et al., 2012; Midilli et al., 2011).

ii. Disadvantages of RAS

One major disadvantage of RAS over non-intensive aquaculture systems is its high initial investment, and maintenance and operating costs (Midilli et al., 2011). RAS implies the use of water treatment systems, which are expensive and need to be periodically maintained (Midilli et al., 2011). RAS operation also entails specialised labour with sufficient knowledge to operate the fish farm, which requires higher salaries (Lekang, 2013). Furthermore, the correct disposal of the high amounts of solid wastes (i.e., sludge) generated by the system is costly (Lekang, 2013). Therefore, research has looked at alternative means to reduce sludge production. These include, for instance, the development of higher quality feed, and improved feeding regimes that reduce unutilised feed in rearing water

(Dauda et al., 2019). Likewise, the valorisation of aquaculture waste (e.g., sludge and wastewater) for the production of feed products or additives, or in an integrated multi-trophic aquaculture system, can bring additional income to the venture and reduce waste (J. Lee et al., 2019; Milhazes-Cunha & Otero, 2017).

To break even, RAS facilities, as all types of intensive aquaculture systems, are required to rear fish at an optimal stocking density, higher than non-intensive systems. This intensification of aquaculture production can increase stress in rearing fish, which can be exacerbated by frequent handling (e.g., for vaccination, grading, and transport), and the low nutritional value of some feed (Madaro et al., 2015). The high stress to which fish are exposed severely reduces fish immunological response and can lead to disease outbreaks (Timmons & Ebeling, 2010).

3. Aquaculture microbiome in RAS

Microorganisms play a crucial role in the maintenance of a productive RAS. The overall aquaculture microbiome of a RAS is formed by microbial communities colonizing different biotopes (e.g., water, biofilter, and fish skin, mucus, gills, and gut). These communities will, for example, assist in nutrient recycling, pathogen suppression, host metabolism, and immune response (Bentzon-Tilia et al., 2016; Martins et al., 2018; Quigley, 2013). A stable and diverse microbiome can prevent pathogenic organisms from establishing and proliferating in the aquaculture system, by outcompeting pathogens for space and resources and producing antagonist compounds (Willey, Sherwood & Woolverton, 2011). For example, in animal hosts, beneficial colicinogenic *E. coli* strains (*i.e.,* capable of synthesizing a bacteriocin – colicin), have been found to inhibit enterohaemorrhagic pathogenic *E. coli* strains (Kamada et al., 2013).

Likewise, some bacterial strains belonging to the paraphyletic Roseobacter clade are known bacterial antagonists in aquaculture biotopes (Bentzon-Tilia et al., 2016; Hjelm et al., 2004). They produce several bioactive secondary metabolites, including broad-spectrum antibiotics and algaecides (Brinkhoff et al., 2004, Porsby et al., 2011). One such compound is the well-studied tropodithietic acid (TDA), capable of inhibiting several marine bacteria, and fish and human pathogens (Brinkhoff et al., 2004; D'Alvise et al., 2012; Hjelm et al., 2004; Porsby et al., 2011). Its mechanism of action is related to the disruption of the proton motive force, by depleting the transmembrane proton gradient through the exchange of extracellular protons for cytoplasmatic cations (Wilson et al., 2016). Disruption of the proton motive force prevents ATP production, resulting in cell death in a broad spectrum of bacteria. The culture broth of TDA-producing *Phaeobacter gallaeciensis* has been found to affect a wide range of bacteria,

especially Flavobacteria and Actinobacteria (Brinkhoff et al., 2004). D'Alvise et al. (2012) investigated the probiotic potential of this organism for fish larvae and their feed cultures and found that it significantly reduced the densities of *Vibrio anguillarum* in a culture of microalgae and rotifers while having no effect on these organisms. Moreover, *P. gallaeciensis* significantly increased the survival of cod (*Gadus morhua*) larvae challenged with *V. anguillarum* (D'Alvise et al., 2012).

Unfortunately, in intensive RAS, overcrowding conditions can severely suppress fish immune systems and increase the probability of the development of dysbiosis, which can ease the surge and spread of opportunistic bacteria in the system (Attramadal et al., 2012). While, initially, the immune system may be boosted by this stress, chronic stress can fragilize fish's primary barriers (e.g., fish skin, gills, and gastrointestinal tract), lead to reduced macrophage and lymphocyte counts and antibody activities, and increase levels of pro-inflammatory cytokines (Morey et al., 2015; Pasnik et al., 2009). Furthermore, evidence suggests that chronic stress can alter the composition and metabolic activity of gut microbiota, which can induce the activation of latent pathogenic viruses, and increase the host's susceptibility to opportunistic pathogenic bacteria (Karl et al., 2018; Morey et al., 2015; Pasnik et al., 2009). It has been recognised that immunosuppression can lead to a faster spread of infectious diseases in the system (Stevens et al., 2017).

As previously mentioned, biosafety is presently one of the main preoccupations of the aquaculture industry, as disease outbreaks can severely impact production, reduce revenue and affect the economic sustainability of a farm (FAO, 2020). Furthermore, this susceptibility to disease outbreaks is one of the principal disincentives for investment in the aquaculture sector (Brummett, 2013; FAO, 2020). The control of infectious diseases in aquaculture systems often relies on the therapeutic and prophylactic use of antibiotics, with severe consequences to fish, consumers, and the environment [e.g., spread of antibiotic resistance (Bentzon-Tilia et al., 2016; Santos & Ramos, 2018)]. Alternative strategies based on the modulation of aquaculture microbiomes are being sought out and can alter the current approach to disease management in aquaculture (Hai, 2015).

One way of doing this is through the application of probiotics (Pérez-Sánchez et al., 2018). These are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO & WHO, 2006). However, despite the several possible benefits of probiotics, there are various constraints to their application in aquaculture, such as the difficulty of scaling-up from an experimental to an industrial level (Castex et al., 2014), loss of viability after storage (Borges et al., 2021), and the possible environmental

issues of adding live microorganisms into the aquaculture environment (Lauzon et al., 2014). In the face of these issues, the use of prebiotics and postbiotics was suggested (de Almada et al., 2016; Gibson & Roberfroid, 1995). Instead of inserting new organisms into the environment, prebiotics stimulate the growth of the microbial populations already present (Gibson et al., 2017). On the other hand, evidence suggests some formulations of inanimate microorganisms (postbiotics) may also produce health benefits for the hosts (Aguilar-Toalá et al., 2018). Thus, inanimate biomass or metabolites produced by probiotic organisms can also be used to modulate microbial communities.

Several organisms can be used for this purpose. Lactic-acid bacteria (LAB) and bacteria belonging to the *Bacillus* genus are the most widely used probiotic organisms (N. -K. Lee et al., 2019; Szajewska et al., 2016). Since *Bacillus* spp. Are capable of endospore formation, their storage is easier, as they have an indefinite shelf-life without loss of viability. In addition, other Gram-positive bacteria with probiotic potential have been studied in aquaculture, including *Pseudomonas aeruginosa* and *Gordonia bronchialis,* for example (Giri et al., 2020; Shabanzadeh et al., 2016; Sheikhzadeh et al., 2017). Besides Gram-positive bacteria, other organisms, such as Gram-negative bacteria and yeasts can be used as probiotics.

Fungi may produce several secondary metabolites, including a variety of hydrolytic and oxidative enzymes (Amend et al., 2019; Bonugli-Santos et al., 2015), antimicrobial substances (Amend et al., 2019), pigments (Chreptowicz et al., 2019), and fatty acids (Vyas & Chhabra, 2017). Some of these metabolites could have interesting applications as microbiome modulators in aquaculture. For example, monounsaturated and poly-unsaturated fatty acids, produced by some yeast species (e.g., *Cystobasidium oligophagum*) can influence the function and fluidity of the intestinal membranes of fish, namely Atlantic charr (*Salvelinus alpinus*), leading to the establishment of different bacterial species in their microbiota (Ringø et al., 1998). Thus, it would be interesting to study the fraction of these organisms present in aquaculture systems, further aiming to assess the effect of their metabolic products on the modulation of aquaculture microbiomes.

Background, objectives, and strategy

There is a rising concern regarding food production and availability for the next generations, since traditional animal farming is environmentally unsustainable, requiring an abundance of natural resources (Palomo-Vélez, Tybur & van Vugt, 2018). Thus, given the increasing demand for fish in the past years, intensive RAS rose as one of the most interesting alternatives to traditional animal farming (Gómez et al., 2019). RAS is an environmentally sustainable form of aquaculture, requiring minimum water, land area, and energy to operate (Timmons & Ebeling, 2010). Proper management of RAS requires knowledge of the microbial communities present within the facility's biotopes, and control of their dynamics since microorganisms are essential for nutrient recycling and water quality maintenance. An imbalance in microbial community dynamics could lead to disease outbreaks, which are responsible for major economic impacts in aquaculture (Attramadal et al., 2012; FAO, 2020).

This dissertation will focus on two separate studies in the context of the AquaHeal project (MAR-02.01.01-FEAMP-0031). By modulating RAS rearing water microbiomes, this project aims to study the potential of microbial biomass to suppress the development of pathogens and improve natural fish barriers against them. Therefore, there are two primary aims of this dissertation, divided into two chapters:

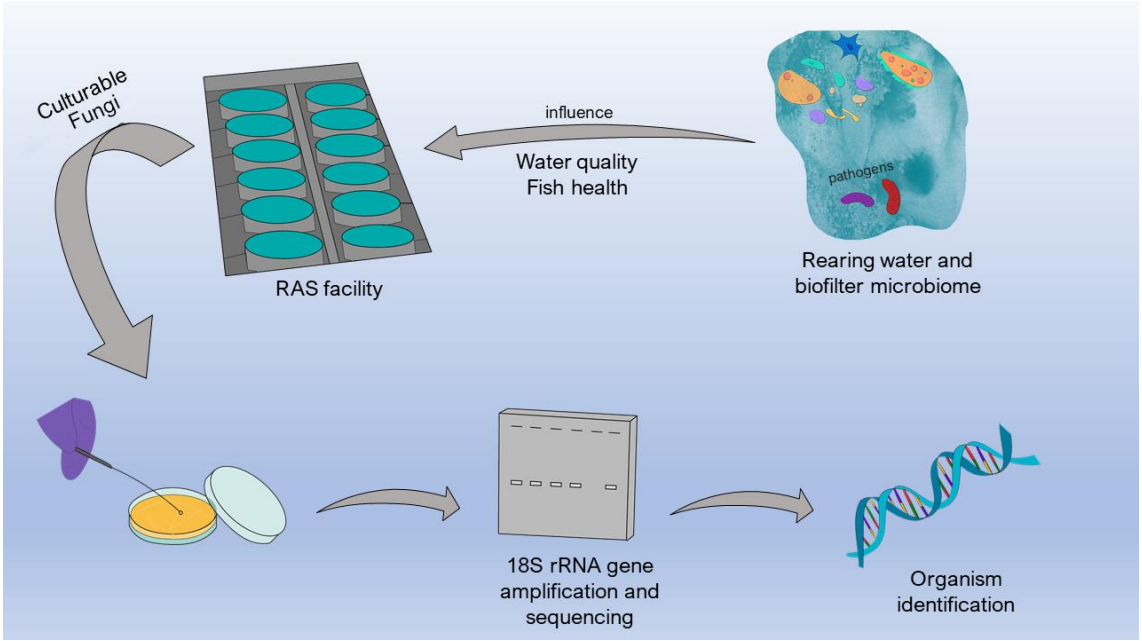
- The first chapter will focus on the isolation and molecular characterization of fungi from the rearing water and biofilter of European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) RAS. Moreover, we aim to review the current literature regarding their ability to produce biomolecules with potential applications for aquaculture microbiome modulation.
- The second chapter will assess the potential of heat-killed (HK) microbial biomass in modulating the bacterioplankton community composition of a European seabass and gilthead seabream RAS. For that purpose, the biomass of previously isolated marine strains of *Pseudoalteromonas* was produced, heat-killed, and added to Erlenmeyer flasks containing rearing water of an experimental RAS, and differences in bacterial community composition and water quality

parameters were evaluated.

These chapters reflect the experimental work done and are independent of each other. Each chapter will include a small, more directed, introduction and review of current literature, a description of the materials and methods used, a presentation and discussion of results, and a conclusion.

Chapter One: Isolation of fungi from a Recirculating Aquaculture System

Graphical abstract



I. INTRODUCTION

As mentioned earlier, knowledge of the microbial community inhabiting the different RAS biotopes is crucial for its management, since these organisms can recycle nutrients and maintain water quality, improving fish health and avoiding disease outbreaks (Attramadal et al., 2012; Ruan et al., 2015). The optimisation of high-throughput sequencing technologies has enabled a more profound knowledge of microbial composition and diversity of environmental habitats (Kennedy et al., 2020). Regardless, the culture of these organisms is still an essential process for their study and potential application, since there are still several taxa without cultured representatives, remaining severely unexplored (Bollmann et al., 2010). Thus, the field would benefit from more studies regarding the culturable microbial diversity, and from the development of novel culture techniques, including innovative culture media, incubation, and inoculation parameters, allowing for a better understanding of the functional roles of these unexplored taxa in environmental microbiomes (Böllman & Martienssen, 2020; Zhu Chen et al., 2013).

Our understanding of aquaculture microbiomes has increased in recent years (Duarte et al., 2019; Martins et al., 2018). However, most research has focused more on bacterial diversity than on other domains. Nonetheless, a recent study by Boaventura et al. (2018) reported on the micro-eukaryotic communities of a turbot (*Scophtalmus maximus)* and sole (*Solea senegalensis*) RAS, and found that these were mostly composed of organisms affiliated to the Stramenopiles (e.g., *Labyrinthulea*); Alveolata (e.g., *Ciliophora* and *Apicomplexa*), Opisthokonta (*Choanozoa*) and Fungi taxonomic groups. Several members of these taxa (e.g., *Labyrinthulea, Ciliophora,* and *Choanozoa*) are characteristically known to be bacterial grazers (i.e., they feed on bacteria), thus having an essential role in bacterial dynamics in the system, which may in turn influence water quality and fish health.

Regarding fungi, it is estimated that there are more than 10.000 species of marine fungi, ubiquitously distributed through marine environments, whose diversity and environmental roles are not fully elucidated (Amend et al., 2019). Given that fungi produce a variety of hydrolytic and oxidative enzymes, they may have significant environmental roles, such as the cycling of carbon and other nutrients (Amend et al., 2019; Bonugli-Santos et al., 2015) and production of bioactive compounds (e.g., antimicrobial substances), which may influence the composition and dynamics of other microbes (Amend et al., 2019). Some efforts have been made to study the culture-dependent and independent fraction of fungi in aquaculture settings, but mostly focusing on harmful pathogenic or mycotoxic

fungi. For example, Silva et al. (2011) isolated and identified the culturable fungal fraction present in nursing water of juvenile and adult *Litopenaeus vinnamei. Aspergillus, Penicillium, Fusarium,* and *Cladosporium* were found to be the most common genera, with the first two being more ubiquitous. These are opportunistic fungi, capable of producing mycotoxins, which may decrease aquaculture productivity, and lead to severe economic impacts (Greco, Pardo & Pose, 2015). These results are in line with other studies. Viegas et al. (2019) found a presence of *Cladosporium* in biofilter samples and *Penicillium* in waters of RAS, in addition to *Aureobasidium;* and Zhang et al. (2012) found that *Aspergillus* and *Penicillium* were the most prevalent genera of culturable fungi associated with gorgonians from the South China Sea, having also found the presence of *Fusarium* and *Cladosporium* species.

Given the wide variety of secondary metabolites produced by fungi, which could have interesting applications as microbiome modulators in the aquaculture sector, this chapter aims to isolate and characterize fungi from the rearing water and biofilter of a European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) RAS. The microbial isolates were characterised by comparing their 18S rRNA gene sequences with those available in public databases, and phylogenetic analysis. After this was accomplished, a literature review was performed, aiming to investigate whether these isolates had potential for future application as microbiome modulators.

II. MATERIALS AND METHODS

Samples of rearing water and biofilter were obtained from an experimental fish farm (RiaSearch Lda, Murtosa, Portugal) rearing gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), with both species of fish contained within the same recirculating system. This fish farm employs a recirculating aquaculture system (RAS) composed of 250 L or 350 L rearing tanks, a mechanical filter for solids (50 µm), a protein skimmer pump, an ozone generator, active trickling biofilter made up of polypropylene "bio-barrels", and UV sterilisation. Water samples were collected directly from rearing tanks in 500 ml sterile glass bottles, and biofilter samples were randomly picked and placed in sterile 50 ml Falcon tubes. Samples were immediately transported back to the laboratory, in ice, and used within 24 h of the sample collection.

1. Culture media

Artificial seawater (ASW) was prepared using a commercially available Red Sea Coral Pro Salt mixture (Red Sea Fish Pharm, Herzliya, Israel) at a final salinity of 18 ppt. pH was adjusted at 7.2-7.6. Salinity and pH conditions were chosen in accordance with RAS rearing water conditions.

Yeast-peptone (YP) medium was prepared in sterile ASW and contained 1 $g.l^{-1}$ yeast extract (Merck Millipore, Burlington, MA, USA), 1 $g.l^{-1}$ peptone (Merck Millipore, Burlington, MA, USA), 500 $mg.l^{-1}$ streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 300 $mg.l^{-1}$ penicillin (Sigma-Aldrich, St. Louis, MO, USA), based on the methods of Gupta et al. (2013). YP-agar medium was prepared by adding 15 $g.l^{-1}$ of agar (VWR, Radnor, PA, USA) to the YP medium. Antibiotic stock solutions were filter-sterilised and then added to the autoclaved medium after cooling.

2. Fungi isolation

The isolation procedure was adapted from the direct plating technique previously reported by Gupta et al. (2013). Microbial communities of rearing water were recovered by filtering water samples (100 ml) through a 0.22 µm polycarbonate membrane on a filtration manifold system (Merck Millipore, Burlington, MA, USA) by vacuum pumping. Afterwards, the membrane was placed in a sterile 50 ml Falcon tube, along with 2.85 – 3.45 mm sterile glass beads and 1 ml of sterile ASW (18 ppt), and vortexed for 1 min to detach microorganisms (10^2 microbial cell density in relation to RAS water). Ten-fold serial dilutions [10^1, 10^0, and 10^{-1} (in relation to rearing water)] were spread (100 µl) with a Drigalski spatula onto YP-agar medium plates before incubation.

Microbial cells of biofilter were obtained by, first, cutting biofilter samples

into small pieces with heat-sterilised pliers, which were placed inside a sterile 50 ml Falcon tube, along with 2.85 – 3.45 mm sterile glass beads and 1 ml of sterile ASW (18 ppt). The samples were vortexed for 1 min, and the aqueous fraction (10^0) and a ten-fold dilution (10^{-1}) of dislodged cell suspensions were spread (100 µl) onto YP-agar medium plates.

YP-agar medium plates were incubated at 21 ºC for a maximum of 3 weeks and inspected daily for the growth of any colonies. Distinct colonies were selected and reinoculated on fresh YP-agar plates with a sterile loop by the streak plate method. In order to obtain pure colonies, the culture purification process was repeated at least three times.

3. Sample storage

After purification, microbial isolates were inoculated in sterile 250 ml Erlenmeyer flasks, containing 50 ml of YP broth, and incubated at room temperature with agitation (120 rpm) until sufficient growth was apparent. Aliquots of the well-grown microbial cultures were prepared for storage of microbial isolates [with the addition of glycerol at a final concentration of 30% (v/v)] and DNA extraction. Glycerol aliquots were stored at -80°C in our laboratory's microbial collection. Microbial cultures were also reinoculated in YP-agar plates to confirm purity and YP-agar slants to be used as working stocks.

4. DNA extraction

Microbial biomass was produced in YP broth as described above and harvested by centrifugation at 3.000 g for 15 min. The microbial pellet was resuspended in 1 ml of absolute ethanol (ITW Reagents, PanReac AppliChem GmbH, Darmstadt, Germany) and stored at -20 ºC until DNA extraction.

The DNA extraction protocol was adapted from Ghosh et al. (2013). The cell suspensions in ethanol were centrifuged at 16.000 rpm for 1 min. The supernatant was discarded, and the pellet was resuspended in 500 µl of DNA extraction buffer (200 mM Tris-HCl, 200 mM NaCl, 25 mM EDTA, pH = 8) pre-heated at 65 ºC. The mixture was transferred to sterile screw-capped microtubes, to which 0.2 g of 0.25 – 0.5 mm and one 2.85 – 3.45 mm sterile glass beads were previously added. Samples were first incubated at 65 ºC for 10 min, and then cells were lysed by shear force using a FastPrep-24™ homogeniser (MP Biomedicals, Santa Ana, CA, USA), processed twice at 6.0 ms^{-1} for 30 s separated by a 30 s pause. Afterwards, samples were incubated for 10 min at 65ºC, and insoluble cellular components were pelleted by centrifugation at 15.000 rpm for 10 min. The supernatant was collected and mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). This mixture was homogenised,

incubated at room temperature for 5 min, and centrifuged at 15.000 rpm for 10 min. The ethanol precipitation method was used to concentrate and purify the DNA samples. DNA pellets were resuspended in 60 µl of nuclease-free water (MP biomedicals, USA).

DNA quantity and quality were assessed by measuring absorbance at 260, 230, and 280 nm using NanoDrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were then stored at -20 ºC. The commercial kit FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) was used, following the manufacturer's protocol, for DNA extraction of two isolates (E and M), which were more refractory to lysis with phenol-chloroform extraction.

5. Polymerase chain reaction (PCR) amplification

PCR was used to amplify 18S rRNA gene fragments for subsequent sequencing and phylogenetic analysis (Field et al., 1988). The primers used for the amplification of this gene were the universal primers Euk_18Sa (5'–AAC CTG GTT GAT CCT GCC AGT–3') and Euk_18Sb (5'–GAT CCT TCT GCA GGT TCA CCT AC–3'). The final volume of the PCR reaction mix was 25 µl: containing 1 µl (approximately 20 ng) of template DNA, 12.5 µl of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µg of bovine serum albumin (BSA), 2% (v/v) of dimethyl sulfoxide (DMSO), and 0.1 µM of each primer, and the rest of the volume of nuclease-free water (MP Biomedicals, Santa Ana, CA, USA). PCR protocol was based on P. Chen et al. (2017), with modifications. After optimisation, the PCR conditions were as follows: initial denaturation at 94 ºC for 2 min, followed by 38 cycles of denaturation at 94 ºC for 45 s, annealing at 55 ºC for 1 min and extension 72 ºC for 2 min, ending with a final extension step at 72 ºC for 10 min.

Amplification of all PCRs was confirmed by electrophoresis at 100 V for 30 min in 1% (w/v) agarose gel with added nucleic acid staining solution (RedSafe™, iNtRON, Sangdaewon-dong, South Korea) in 1x TAE buffer. PCR products ran alongside a molecular-weight size marker (GenRuler 1 kb Plus DNA Ladder, Thermo Fisher Scientific, Waltham, MA, USA). The gel was visualised under UV light, and the image was captured by ChemiDoc™ XRS+ molecular imager (BioRad, Hercules, CA, USA). Amplicons were sequenced externally (Eurofins, Ebersberg, Germany).

6. Phylogenetic analysis

The DNA sequences obtained in this study were compared to the ones existing in GenBank [National Center for Biotechnology Information (NCBI,

Bethesda, MD, USA)] database through the Basic Local Alignment Search Tool (BLAST) [NCBI, Bethesda, MD, USA, https://blast.ncbi.nlm.nih.gov/Blast.cgi, (accessed 10/04/2020)], in order to obtain the phylogenetically closest neighbours.

18S rRNA gene sequence top matching hits were used to construct a phylogenetic tree using the MEGA7 (Molecular Evolutionary Genetics Analysis Version 7.0.26, Pennsylvania State University, PA, USA) programme package. Neighbour-joining trees were constructed using the Timura 3-pattern model (T92), with gamma-distributed rates among sites (G), after a model selection test was performed to find the best model. A total of 1000 rounds of bootstrap were conducted. *Nephridiophaga blaberi* (Fungi *incertae sedis*) was used as an outgroup.

III. RESULTS AND DISCUSSION

In this study, 18 fungal strains were isolated from water and biofilter biotopes from a fish RAS. The colony morphology of the isolates is shown in **Figure 1** and **Table 1.**

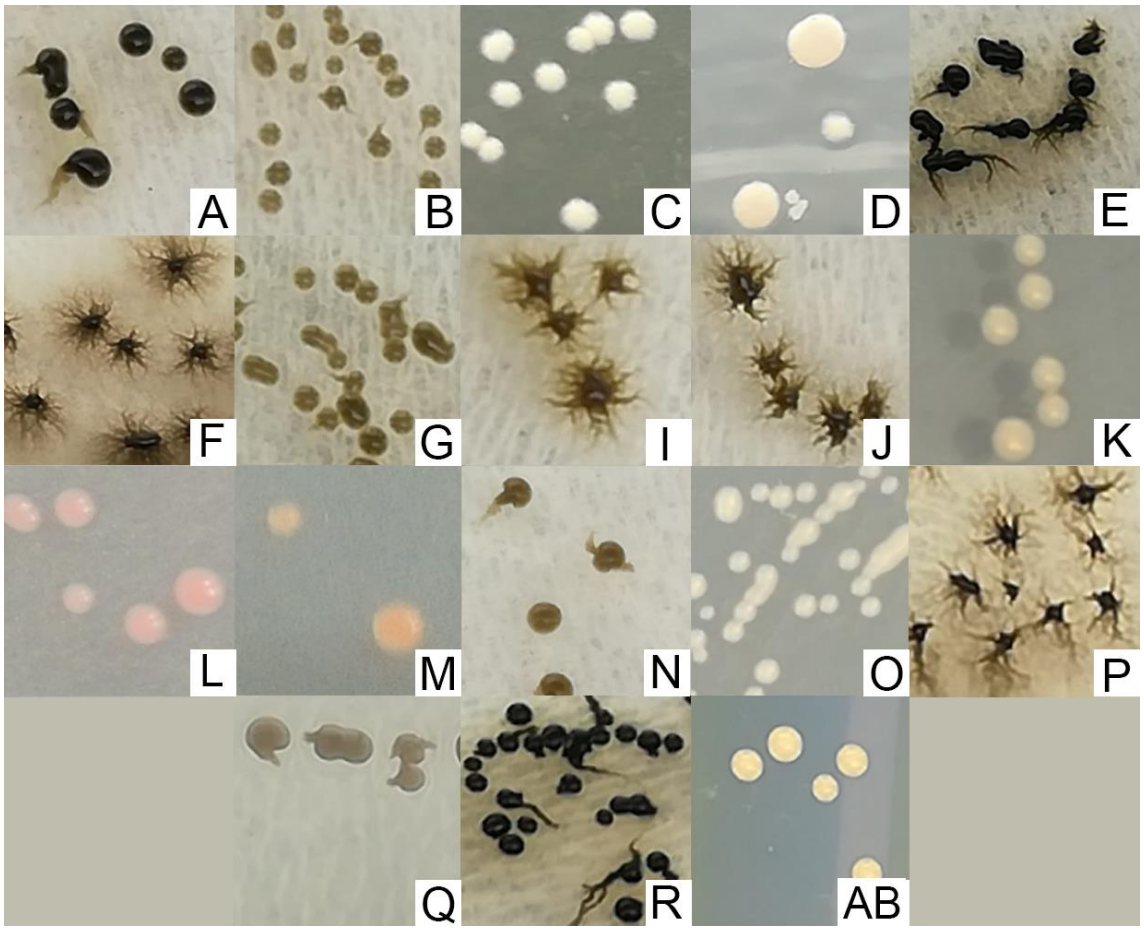


Figure 1 - Photograph of each of the isolates obtained in this study. The white box identifies each of the isolates.

Table 1 - Colony morphology of the fungal isolates.

Sample type	ID	Macroscopic appearance	Colour
Water	A	Round colonies, filamentous	Black
	B	Round colonies, filamentous	Greenish brown
	C	Roundish colonies with irregular outlines, which spread across the plate. No filaments.	Beige
	D	Roundish colonies with irregular outlines, which spread across the plate. No filaments.	Beige
	E	Round colonies, filamentous	Black
	F	Irregular starry shape, filamentous	Dark brown
	G	Round colonies, filamentous	Greenish brown
	I	Irregular starry shape, filamentous	Greenish brown
	J	Irregular starry shape, filamentous	Dark brown
	K	Round colonies, no filaments	Beige
	M	Round colonies, no filaments	Salmon
	N	Round colonies, filamentous	Greenish brown
	O	Round colonies, no filaments	Beige
	P	Irregular starry shape, filamentous	Black
	Q	Round colonies, filamentous	Brown
	AB	Round colonies, no filaments	Pale yellow
Biofilter	L	Round colonies, no filaments	Pink
	R	Round colonies, filamentous	Black

Here, the BLAST (NCBI, Bethesda, MD, USA) was used to compare the obtained sequences with the ones present in the GenBank database (NCBI, Bethesda, MD, USA) (**Table 2)**. The sequences of their closest relatives were aligned in MEGA7, and the obtained phylogenetic tree is shown in **Figure 2.** The BLAST search showed the all the isolates obtained in this study were closely related (≥99% of sequence similarity) to known fungi belonging to the Ascomycota or Basidiomycota phyla.

Of the species isolated, *Pseudotaeniolina globosa* (A, B, E, F, G, I, J, P, Q, and R), a meristematic fungus, characterised by its slow growth, thick cell walls, and presence of melanin (de Leo et al., 2003), was the most prevalent species,

representing 11/18 isolates (water and biofilter samples) (**Table 2**). These filamentous fungi present a black or dark green/brown colouration (**Figure 1**). *P. globosa* has been isolated from extreme environments, and the melanisation might help it endure extreme conditions, such as low and high temperatures, solar irradiation, and low water activities (de Leo et al., 2003, Pangallo et al., 2015). Such characteristics may act as a defence mechanism against the process of water purification in RAS, which uses ozone and UV radiation (Teitge et al., 2020). This species belongs to the Capnodiales order, the second largest of the Dothidiomycetes class, which contains several plant and human pathogens, endophytes, saprobes, and epiphytes (Crous et al., 2009). In aquaculture, an *Aureobasidium* spp., belonging to this order, has previously been detected in water samples of an experimental RAS rearing sea urchin (*Paracentrotus lividus*) and sea cucumber (*Holothuria tubulosa*) (Viegas et al., 2019). However, *P. globosa* tends to be oligotrophic, being commonly found adhering to inert surfaces, such as rock or painted wood (Pangallo et al., 2015). Despite having been found in the aorta of a deceased human patient after aortic aneurysm surgery, they displayed no sign of on-going infection or inflammatory process, and the isolated fungi were unable to grow at 37ºC, thus being likely that *P. globosa* was contaminating the medical devices used (Kurzai et al., 2008). There is no published evidence that this species may act as human or fish pathogens.

Table 2 - BLAST search of 18S rRNA gene sequences of the isolates obtained in this study.

ID	Kingdom	Phylum	Class	Order	Family	Genus	Species	Accession no.	% Identity	Reference
A	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
B	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
C	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Bullerabasidiaceae	*Vishniacozyma*	*V. carnescens*	NG 060988.1	99.91	Takashima et al., 2003
D	Fungi	Basidiomycota	Exobasidiomycetes	Entylomatales	*incertae sedis*	*Tilletiopsis*	*T. lilacina*	NG 062410.1	99.90	Wang et al, 2015
E	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
F	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
G	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
I	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
J	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
K	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Bullerabasidiaceae	*Vishniacozyma*	*V. carnescens*	NG 060988.1	99.91	Takashima et al., 2003
L	Fungi	Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*	*C. slooffiae*	AB126653.1	99.91	Nagahama et al., 2006
M	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Bullerabasidiaceae	*Dioszegia*	*D. hungarica*	NG 060983.1	98.77	Takashima et al., 1999
N	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
O	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Bullerabasidiaceae	*Vishniacozyma*	*V. carnescens*	NG 060988.1	99.91	Takashima et al., 2003
P	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
Q	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
R	Fungi	Ascomycota	Dothideomycetes	Capnodiales	*incertae sedis*	*Pseudotaeniolina*	*P. globosa*	NG 062782.1	99.61	Crous et al. 2009
AB	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Candida*	*C. labiduridarum*	NG 063271.1	99.84	Suh, Nguyen & Blackwell, 2007

Isolate AB, obtained from water samples, was identified as *Candida labiduridarum*, a member of Debaryomycetaceae (Ascomycota). This isolate had high similarity (99.84%) with a strain previously isolated from plant-associated insects (Suh et al., 2008). Strains affiliated to the *Candida* genus are ubiquitously found in marine and other aquatic environments (Navarrete & Tovar-Ramírez, 2014) and have been found to colonise the gastrointestinal tract of several fish [e.g.,mullet (*Mugil* spp.), tambatinga (*Colossoma macropomum* ♀ x *Piaractus brachypomus* ♂), croaker (*Cilus gilberti*) and rainbow trout (*Onchorhynchus mykiss*) (Caruffo et al., 2015; Navarrete & Tovar-Ramírez, 2014; Pinheiro et al., 2018) and humans (Kim & Sudbery, 2011). Nonetheless, many species of this genus are opportunistic human pathogens, including *C. albicans* and *C. auris* (Alfouzan et al., 2019; Kim & Sudbery, 2011). Interestingly, several species of this genus have been studied as potential fish probiotics. In a recent study, Hansen et al. (2019) demonstrated that dietary supplementation of *C. utilis* increased the growth performance of Atlantic salmon (*Salmo salar*) parr. Furthermore, it has been recently reported that *C. labiduridarum* might produce antifungal metabolites with antagonistic activities against *Sclerotinia sclerotiorum*, a plant pathogenic fungus (Cavalcanti et al., 2020).

Several isolates (4) were found to be affiliated to the Tremellales microbial group. Isolates C, K, and O, obtained from water samples, were identified as *Vishniacozyma carnescens* (= *Cryptococcus carnescens*), saprophyte fungi, which can be opportunistic pathogens in humans and animals, causing disease in immunocompromised hosts (Danesi et al., 2014). Isolate M, also isolated from water samples, on the other hand, was identified as *Dioszegia hungarica,* previously referenced as *Cryptococcus hungaricus* and *Bullera armeniaca*. (Takashima et al., 2011). *D. hungarica* is a yeast, able to produce carotenoid pigments, characterized by yellow to deep-orange colouration (Takashima et al., 2011). In fact, the salmon-orange colouration of this yeast is evident in **Figure 1**. These pigments are commonly used as functional ingredients and feed supplements in aquaculture. Besides rendering the fish visually more attractive, especially salmon, which can influence the customer in favour of purchasing it, carotenoids are semi-essential nutrients, promoting fish health and growth (Nakano & Wiegertjes, 2020). Among the biological functions of carotenoids in fish, promotion of immune responses, a decrease of cellular damage and oxidative stress, and effects on digestion and reproduction can be highlighted. Besides, previous studies have shown a microbiome modulation effect of carotenoids in fish, namely a study in juvenile pacú (*Piaractus mesopotamicus)* by Rossi et al. (2020), which revealed a modification of its gut microbial community by dietary supplementation of 225 $g.kg^{-1}$ of β-carotene. Given that *D.*

hungarica has been shown to be able to colonize RAS water by the present study, this yeast is an attractive candidate for future studies aiming at evaluating its potential to produce dietary bioactive carotenoids in RAS wastewater.

Isolate D was identified as *Tilletiopsis lilacina*. This species is of *incertae familiae*, given that the Exobasidiomycetes are not a monophyletic group (Q.-M. Wang et al., 2015). This genus has been previously found in marine water samples collected in Australia (Andreakis et al., 2015), associated with jellyfish (*Nemopilema nomurai*) (Yue et al., 2015) and South China Sea gorgonians (Zhang et al., 2012), and in deep-sea sediments (P. Singh et al., 2010). Yue et al. (2015) revealed that a *Tilletiopsis* spp. had antibacterial activity against human pathogens, namely *Staphylococcus aureus* and *Salmonella enterica*. Besides, co-culture with *Aspergillus versicolor* revealed the presence of multiple metabolites that were not present in the pure culture, and also anti-fungal activity against *Rhizoctonia solani* and *Botyris cinerea*. Likewise, a previous study had found that *T. albescens* had anti-fungal activity, namely against *A. versicolor* and *Aspergillus sydowii* (Zhang et al., 2012). Given the unknown metabolic potential of *T. lilacina*, further studies aiming to explore its antibacterial and antifungal potential in aquaculture would be of great interest, since these compounds can be useful for microbiome modulation.

The strain L, isolated from biofilter samples, was identified as *Cystobasidium slooffiae.* This organism was previously classified as *Rhodotorula slooffiae* and is an oleaginous yeast (Capus et al., 2016), i.e.,capable of accumulating more than 20% of their biomass as intracellular lipids (Vasconcelos, 2019). Given the light pink colouration of this isolate, and its close phylogenetic relationship to *C. slooffiae,* it is likely that this organism produces carotenoid pigments (Naghavi et al., 2013). This yeast species has also been reported to produce indole-3-acetic acid, an auxin that is of crucial importance for plant growth and development (Ramos-Garza et al., 2016). Therefore, given the diversity of metabolic products produced by *C. slooffiae*, it would be interesting to study isolate L further, aiming to assess the effect of its metabolites on the modulation of fish gut microbiomes.

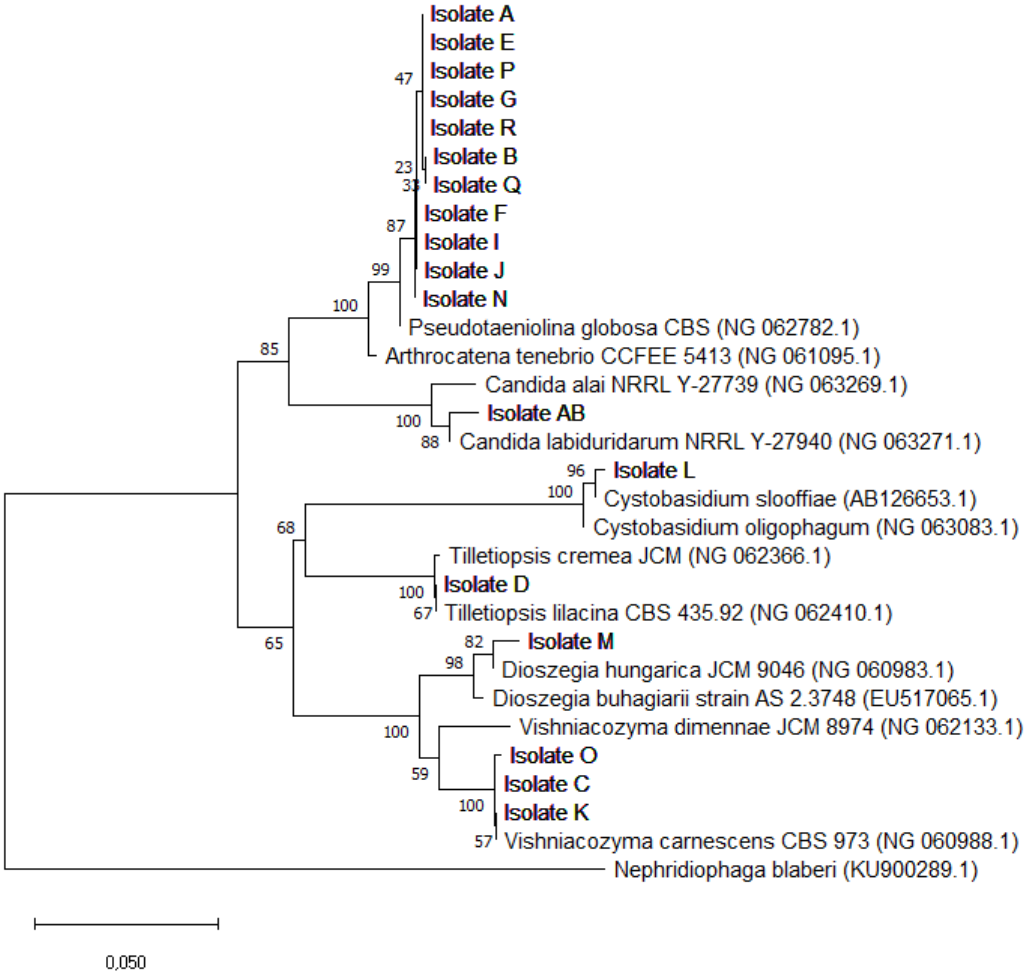


Figure 2 - Phylogenetic tree of the 18S rRNA gene from all the isolates (highlighted in bold) and their closest phylogenetic neighbours found by BLAST (accession numbers in parenthesis), constructed by the neighbour-joining method, using the Timura 3-parameter substitution model. Bootstrap values are indicated in percentage at the nodes and were obtained from 1000 bootstrap replicates. The phylogeny was inferred from an alignment with a length of 1187 base-pairs. The scale bar corresponds to 0.050 change per nucleotide. *Nephridiophaga blaberi* was used as an outgroup.

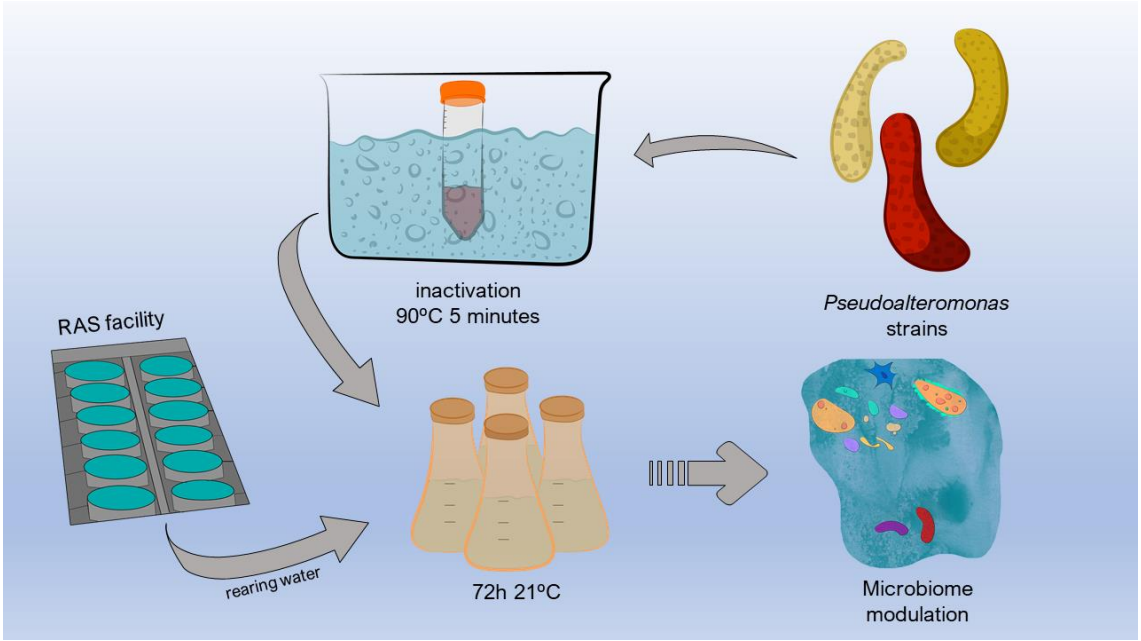
IV. CONCLUSION

In this chapter, we aimed to isolate and identify fungi from the rearing water and biofilter samples of a fish RAS with potential for microbiome modulation in the aquaculture sector. The results showed that all the isolated organisms belong to the Ascomycota and Basidiomycota taxonomic groups. 11/18 of the identified fungi were phylogenetically related to *Pseudotaeniolina globosa,* a fungal species with dark hyphae commonly found as a plant endo- or epiphyte. Other isolated organisms include the Ascomycota species *Candida labiduridarum,* and members of the Basidiomycota phylum: *Cystobasidium slooffiae, Tilletiopsis lilacina, Vishniacozyma carnescens,* and *Dioszegia hungarica*.

While no culture-independent surveys were performed, it is likely that the culture-based methods employed in the present study do not reflect the vast fungi diversity in RAS rearing waters and biofilters. However, this approach was successful in isolating fungal strains with potential biotechnological application in the aquaculture sector. For instance, isolate D and AB were closely related to *T. lilacina* and *C. labiduridarum,* respectively, which have previously been reported to produce antibacterial and antifungal compounds. Similarly, isolates L and M have shown close phylogenetic relation to *C. slooffiae* and *D. hungarica,* respectively, which may produce metabolic products with biotechnological potential, namely indole-3-acetic acid and carotenoid pigments, respectively. Thus, future studies will be conducted, aiming to assess the effect of these interesting fungi in the modulation of aquaculture microbiomes.

Chapter Two: Modulation of RAS microbiome using heat-killed microbial biomass

Graphical abstract



I. INTRODUCTION

Current disease control strategies in aquaculture mostly rely on the prophylactic and therapeutic use of antimicrobial treatments, and vaccination. However, both efforts are known to have several disadvantages in preventing disease outbreaks. By indiscriminately affecting the whole microbial community, antimicrobial strategies may result in a drastic reduction in microbial load, diversity, and richness, affecting pathogens and beneficial antagonistic bacteria alike. Following a disturbance in the system, fast-growing r-strategists (potentially opportunistic pathogens) can quickly colonise available niches, due to the high concentration of substrates (nitrogen and carbon) and low competition, leading to the exponential growth of a few species and a decrease in diversity (Hess-Erga, 2010). Thus, these approaches may lead to a higher abundance of opportunistic pathogens and lower microbial diversity, fragilizing the system and increasing the chance of disease outbreak (Attramadal et al., 2012). Likewise, the indiscriminate use of antibiotics in aquaculture can lead to the rise of antibiotic resistance (Santos & Ramos, 2018). Meanwhile, although effective, vaccination has limited applicability regarding certain fish species, developmental stages, and pathogens. Furthermore, fish immune systems are not yet fully understood, thus rendering it difficult to develop vaccines (Adams, 2019).

Therefore, alternative disease control and prevention approaches must be developed to improve aquaculture resilience to diseases during fish production. These approaches could rely on modulating the aquaculture microbiome by promoting a higher diversity and enrichment of beneficial microbes. These strategies are currently mainly focused on the microbial modulation of the fish gut microbiome using probiotic and prebiotic feed supplements and ignore the effect the rearing water microbiome has on fish health and disease prevention. However, recent research suggests that the environmental conditions and antagonistic microorganisms present in rearing water may have a greater influence on fish health than was previously thought (Dittmann et al., 2017). A stable and healthy aquaculture microbiome can decrease the relative abundance of pathogens, avoiding the development and spread of diseases (Martins et al., 2018). Hence, studying these dynamics could hold great potential to prevent disease outbreaks in aquaculture settings.

Pseudoalteromonas is a genus of Gram-negative, aerobic, marine bacteria, which are known to inhibit the growth of various putative pathogenic bacteria [e.g., *Vibrio harveyi, Vibro nigripulchritudo,* and *Vibrio parahaemolyticus* (Offret

et al., 2019; Richards et al., 2017; Sorieul et al., 2018)] and eukaryotes, including algae, fungi and *Hydractinia* larvae (Alker et al., 2020). Furthermore, *Pseudoalteromonas* strains, isolated from aquaculture systems, have been shown to have antagonistic activity against *Vibrio splendidus* DMC-1 and *Pseudoalteromonas* HQ, two relevant marine pathogens (Zhao Chen et al., 2019; Hjelm et al., 2004). Due to their antagonist properties, a great interest has emerged in using *Pseudoalteromonas* species for pathogen suppression in aquaculture systems (Offret et al., 2019, Richards et al., 2017) and its use as a probiotic supplement has been hypothesized in previous studies (Zhao Chen et al., 2019; Hjelm et al., 2004). Several studies have been performed, evaluating the *in vitro* and *in vivo* effect of live *Pseudoalteromonas* spp. in a diverse range of marine organisms, including abalone (*Haliotis tuberculate*) (Offret et al., 2019), turbot (*Scophtalmus maximus*) (Hjelm et al., 2004), and prawns (*Litopenaeus stylirostris*) (Sorieul et al., 2018). A recent study showed that a *Pseudoalteromonas* hCg-6 suspension in seawater (10^6 CFU.mL^{-1}) significantly increased abalone survival (16%-40%) in an infection-inducing challenge experiment with *V. harveyi* ORM4 (Offret et al., 2019). Likewise, the addition of *Pseudoalteromonas* NC201 to the rearing water (10^5 CFU.mL^{-1}) led to an increase in the survival of prawns challenged with *V. nigripulchritudo* (Sorieul et al., 2018). In addition, the probiotic treatment led to an increase in survival following osmotic stress.

Despite the benefits of using live probiotics in aquaculture, previous studies have suggested that the health-promoting effects of some probiotics are independent of their viability (Aguilar-Toalá et al., 2018). Thus, new concepts, such as the alternative use of inanimate microorganisms (postbiotics) have been developed to investigate their potential health benefits. The use of inanimate microorganisms was suggested to avoid the environmental concerns and storage constraints of using live bacteria (Borges et al., 2021). Methods to inactivate bacteria include ionizing or UV radiation, high pressure, sonication, high voltage, and heat-inactivation (Aguilar-Toalá et al., 2018; Borges et al., 2021; Choudhury & Kamilya, 2019). Heat inactivation is the most common method due to its low cost and relatively easy implementation (Choudhury & Kamilya, 2019). This process for the production of heat-killed (HK) biomass leads to membrane disruption, loss of nutrients and ions, ribosome aggregation, DNA and protein denaturing, and inactivation of enzyme activity (Choudhury & Kamilya, 2019; Wegh et al., 2019). However, this method can also lead to increased cell coarseness and roughness, which affects the adhesion of the cell to the host, possibly decreasing the advantageous potential of the inanimate biomass when applied to gut microbiomes (de Almada et al., 2016).

The ability of inanimate microorganisms to promote biological responses similar to those of probiotics has been demonstrated *in vivo*. For example, inanimate lactic acid bacteria (such as HK *Lactobacillus plantarum*), have been reported to improve the immune parameters of animal hosts, and to increase their survival when exposed to common aquaculture pathogens, such as *Aeromonas hydrophila* and *Streptococcus agalactiae* (Dash et al., 2015; Dawood et al., 2015a, 2015b, 2015c, 2016; Tung et al., 2009; Van Nguyen et al., 2019; Yang et al., 2015; H. Zhou et al., 2019). Likewise, positive effects on growth and feed efficiency were reported in red seabream [*Pagrus major*, (Dawood et al., 2015b, 2015c, 2016)], kuruma shrimp [*Marsupenaeus japonicus*, (Tung et al., 2009)], Nile tilapia [*Oreochromis niloticus*, (Van Nguyen et al., 2019)], amberjack [*Seriola dumerili*, (Dawood et al., 2015a)], sea-cucumber [*Apostichopus japonicus*, (Yang et al., 2015)] and in giant freshwater prawn [*Macrobrachium rosenbergi,* (Dash et al., 2014, 2015)] fed HK *Lactobacillus*. Likewise, the supplementation of inanimate *Bacillus* strains (e.g., *Bacillus amyloliquefaciens* and *Bacillus clausii)* in feed has been shown to improve the innate immune responses in catla [*Catla catla,* (Kamilya et al., 2015; S. T. Singh et al., 2017)] and orange-spotted grouper [*Epinephelus coioides*, (J. Wang et al., 2017)]. Meanwhile, HK *Pseudomonas aeruginosa* improved the immune and physiological parameters of carp (*Cyprinus carpio*) and improved fish resistance against *A. hydrophila* (Giri et al., 2020). HK biomass of *Gordonia bronchialis* improved growth performance and enhanced immune parameters of rainbow trout (*Oncorhynchus mykiss*) (Shabanzadeh et al., 2016; Sheikhzadeh et al., 2017), and HK *Pediococcus pentosaceus* improved the growth performance and health of red seabream juveniles (Dawood et al., 2016).

Unlike probiotics, postbiotics are incapable of colonizing fish gut microbiomes. Nevertheless, evidence suggests that they are capable of modulating the structure and function of the gut microbiome, leading to improved immunity and growth performance (Borges et al., 2021). However, the effect of inanimate bacterial feed supplements, in comparison to their live counterparts, can be ambiguous. While some studies reported increased innate immunity-related parameters when using postbiotics (J. Wang et al., 2017), others, in contrast, show a more prominent effect of live probiotic supplements (Lazado et al., 2010). Nonetheless, there is currently a consensus that postbiotics may be an effective strategy to improve animal health.

In line with the concept of postbiotics, in this chapter, we hypothesized that HK biomass of *Pseudoalteromonas* spp. (HKP) can modulate the bacterioplankton diversity and composition of RAS. Thus, in the first part of this chapter, we evaluated the effects of three HKP strains on RAS bacterioplankton

communities, in a preliminary study using DGGE. In the second part, we performed an in-depth analysis of the effects of a selected HKP (*Pseudoalteromonas rubra* SubTr2) biomass on RAS bacterioplankton diversity, composition, and water quality.

II. MATERIALS AND METHODS

1. Bacterial strains and growth conditions

The bacterial strains (RH3, PtTr1, and SubTr2) used in this study were previously isolated from marine sponges during the Ecotech-Sponge project (PTDC/BIA-MIC/6473/2014–POCI-01-0145-FEDER-016531). The BLAST similarity search of 16S rRNA gene sequences indicated that these strains are closely related to *Pseudoalteromonas flavipulchra* NCIMB 2033 (99.73% identity), *Pseudoalteromonas piscicida* IAM 12932 (99.89% identity), and *Pseudoalteromonas rubra* ATCC 29570 (99.55% identity), respectively (unpublished data). *Escherichia coli* DH5α (Thermo Fisher Scientific, Waltham, MA, USA), a derivative of *E. coli* K12 strain commonly used for cloning and genetic studies, *was* also included in the experiment as a reference strain. *E. coli* DH5α is unpigmented and thought to not produce antagonist substances or induce antibiosis *in vitro* (T. Zhou et al., 2012).

Bacteria were grown in Difco™ Marine Broth 2216 (MB) (Thermo-Fisher Scientific, Waltham, MA, USA), apart from *E. coli* DH5α, which was grown in LB medium (Thermo Fisher Scientific, Waltham, MA, USA). Marine agar (MA) plates were obtained by adding agar powder (VWR, Radnor, PA, USA) to MB at 15 $g.l^{-1}$. ASW was prepared as described in the previous chapter.

Bacterial stock cultures from the EcoTech-Sponge culture collection (http://www.cesam.ua.pt/ecotech/index2.html) were reactivated by their inoculation in MB medium, and incubation at 25ºC at 150 rpm until growth was visible. The bacterial cultures were then streaked on MA plates, to confirm purity and provide working aliquots for subsequent steps. Before each assay, a stock culture of each bacterium was inoculated in 50 ml of MB and grown overnight at 25ºC and 150 rpm agitation. In the case of *E. coli* DH5α, the medium used was LB, and incubation was at 37ºC. An aliquot (500 µl) of the pre-inoculum was transferred to 50 ml of fresh medium and grown for 72 h at the same conditions described above.

2. Preparation of HK microbial biomass

After incubation, bacterial cultures were centrifuged at 2.500 g and 4°C for 1 h, the supernatant was discarded, and the biomass pellet was resuspended in 2 ml ASW. The pellet was centrifuged and resuspended again in ASW two more times to wash the biomass and avoid residual nutrient carryover. For inactivation, the pellet obtained for each strain was incubated in a water bath at 90°C for 5 min (Yan et al., 2015). An aliquot of the pellet of each strain was streaked onto agar

plates of their respective culture media before and after the inactivation step, to confirm the microbial biomass inactivation. Plates were incubated at their respective temperature and inspected for growth every 24 h, for a total of 5 days. Confirmed heat-killed bacterial biomasses were lyophilized and stored until use.

3. Evaluation of the effects of HK microbial biomass on RAS bacterioplankton community

Rearing water was collected in a sterile container at the outlet of fish production tanks of an experimental RAS rearing European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) (RiaSearch Lda, Murtosa, Portugal). Water was immediately transported back to the laboratory in ice and used in the experiments upon arrival. Two independent experimental assays were performed.

i. Screening of potential modulators

In the first experiment, three treatments were established with HKP-RH3, HKP-PtTr1, and HKP-SubTr2, to evaluate the effects of these treatments on the bacterioplankton. The experiment (4 replicates per treatment) was performed in 250 ml Erlenmeyer flasks, containing 50 ml of rearing water with and without HK biomass (20 $mg.l^{-1}$). The flasks were incubated at 21 ºC for 72h, and 20% (v/v) of the rearing water from each flask was replaced (at 24h intervals) with 0.2 µm filter-sterilised rearing water containing 20 $mg.l^{-1}$ of their respective modulator.

After the incubation period, the bacterioplankton was retrieved by filtration through a 0.2 µm polycarbonate membrane (Merck Millipore, Burlington, MA, USA). Filter membranes were immersed in absolute ethanol and stored at -20 ºC until DNA extraction.

ii. In-depth evaluation of the HKP-SubTr2 treatment

HK-SubTr2 was chosen for a subsequent experiment, aiming for an in-depth evaluation of its effects on the bacterioplankton and water quality parameters. In addition, HK biomass of *E. coli* DH5α (prepared as previously described for HKP) was used as a reference, given it is unpigmented and thought not to produce antagonistic substances *in vitro* (T. Zhou et al., 2012). Negative control of aquaculture water without HK microbial biomass was also prepared, to evaluate the changes of the bacterioplankton communities after incubation. The experiments were performed as described above, except that 50 ml samples (4 replicates) of RAS rearing water were also analysed, to characterise the initial communities, prior to the experiment.

At the beginning and end of the experiment, water samples were collected from

each flask for flow cytometry and physical-chemical analysis (described in the following sections a. and b.). pH was measured daily.

After the incubation period, the bacterioplankton was retrieved by filtration through a 0.2 µm polycarbonate membrane and stored as previously described.

iii. Flow Cytometry

Flow cytometry analysis was performed in the water samples using the commercial kit LIVE/DEAD BacLight Bacterial Viability and Counting Kit (ThermoFisher Scientific, Waltham, MA, USA). However, the viability of cells was not assessed. Thus, the manufacturer's protocols were followed with modifications, as follows: an aliquot of 1 ml was retrieved from each Erlenmeyer flask for flow cytometry analysis. 1.5 µl of 3.34 mM SYTO 9 Nucleic acid stain was added to an aliquot of 988 µl of each sample and incubated for 15 min, in the dark. 10 µl of the microsphere standard were added to the samples, after being resuspended in the vortex, and samples were analysed by flow cytometry, using an Attune® Acoustic Focusing Cytometer (Applied Biosystems, Foster City, CA, USA).

iv. Water quality parameters

Ammonia/ammonium, nitrite, and nitrate were quantified from the 0.2 µm-filtered rearing water by visible light spectrometry, using colorimetric methods appropriate for seawater samples (Spectroquant-Merck, Kenilworth, NJ, USA). Concentrations were calculated from measured absorbances based on linear regression of calibration curves [NO_3^- (R^2=0.9979); NO_2^- (R^2=0.9953); NH_3/NH_4^+ (R^2=0.9958)]. Total organic carbon (TOC) was quantified to evaluate the effect of modulator addition on the organic carbon content of the system. This analysis was performed externally at A3lab (Ílhavo, Portugal) using infrared detection [SM 5310]. pH was measured using a calibrated Consort C932 electrochemical analyser (Consort BVBA, Turnhout, Belgium).

4. DNA Extraction

Filter membranes were thoroughly cut into small pieces using a sterilised pair of scissors. DNA was extracted from all filter membranes using the commercial kit FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's instructions.

5. 16S rRNA gene DGGE analysis

DNA samples were analysed by denaturing gradient gel electrophoresis (DGGE). In the first experiment, this approach was used as a fast and cost-effective approach to screen the effect of the different HK biomasses on the

bacterioplankton communities. In the second experiment, DGGE was used as a preliminary analysis of the bacterial communities and for quality control, allowing the evaluation of the quality of the DNA extracted and the reproducibility of the experimental results. For these purposes, the 16S rRNA gene was amplified, using a nested-PCR approach (i.e., using two sets of primers in two successive PCRs), following published methods by Gomes et al. (2008).

In the first PCR, the universal bacterial primers – F-27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and R-1492 (5'-GGT TAC CTT GTT ACG ACT T-3') – were used to amplify this gene (Weisburg et al., 1991). The PCR mix (25 µl) contained 1 µl (approximately 20 ng) of template DNA, 12.5 µl of DreamTaq PCR Master Mix (2X) (ThermoFisher Scientific, Waltham, MA, USA), 2% (v/v) DMSO, 2.5 µg of BSA and 0.1 µM of each primer. Following 5 min of denaturation at 94ºC and 25 thermal cycles of 1 min at 94 ºC, 1 min at 56ºC, and 2 min at 72ºC, the reaction ended with a final extension step at 72ºC for 10 min. The ca. 1450 bp amplicons obtained in the first PCR were then used as the template for a second PCR, using bacterial DGGE primers – F984-GC (5'-[GC-clamp] AAC GCG AAG AAC CTT AC-3') and R1378 (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') –, following a published protocol (Heuer et al., 2001), but only 25 cycles were used. The primers used cover the sequence variation located in the hypervariable regions V6-V9.

The ca. 473 bp amplicons obtained in the second PCR were analysed by DGGE, using the DCode™ Universal Mutation Detection System (BioRad, Hercules, CA, USA). These amplicons were applied to a 40-60% denaturing gradient polyacrylamide gel. Electrophoresis was run in 1x TAE buffer at 60 ºC and a constant voltage of 80 V for 960 min (16h). The gels were silver-stained following published guidelines (Heuer et al., 2001), and digitalised using an EPSON Pro scanner (Epson, Nagano, Japan).

6. 16S rRNA gene amplicon high-throughput sequencing

In the second experiment, for in-depth analysis of bacterioplankton community composition, 16S rRNA gene amplicon high-throughput sequencing was performed. First, the hypervariable region V4 of this gene was PCR-amplified using the primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3'), containing a barcode, and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'), and using HotStarTaq® Plus Master Mix Kit (Qiagen, Hilden, Germany). PCR conditions were as follows: 94 ºC for 3 min, 28 cycles of 94 ºC for 30 s, 53 ºC for 40 s and 72 ºC for 60 s, and a final extension step at 72 ºC for 5 min. The amplicons were observed in 2% agarose gel and purified, using calibrated AMPure XP Beads (Beckman Coulter, Brea, CA, USA).

Library preparation and sequencing were performed in a MiSeq sequencing platform at Molecular Research LP (www.mrdnalab.com; Shallowater, TX, USA), following standard Illumina procedures (Illumina, San Diego, CA, USA).

i. Sequencing data analysis

QIIME2 (version 2020.8) was used to analyse the 16S rRNA gene amplicon libraries (Bolyen et al., 2019). The DADA2 plugin (Callahan et al., 2016) was used to trim sequences (final length 205 bp), which were demultiplexed and used to generate the amplicon sequence variants (ASV) abundance table. Taxonomy was assigned to ASV using a sci-kit-learn Naïve Bayes classifier (Bokulich et al., 2018), based on the SILVA database for the amplified region (V4) (version 138.1, released August 27, 2020) at 99% similarity. The classifier is available at https://docs.qiime2.org/2020.8/plugins/; accessed 17/11/2020.

7. Statistical analysis

i. DGGE profiles

Bacterial community DGGE profiles were analysed using the BioNumerics software (version 7.6.3, Applied Maths, Sint-Martens-Latem, Belgium), as described by Smalla et al. (2001). The background was removed, using the rolling disk method with an intensity of 8 (relative units), and lanes were normalised. The data obtained in this software were exported as a band intensity matrix.

This matrix was imported into the RStudio software (version 4.0.2, R Project for Statistical Computing, Vienna, Austria), and differences between treatments were evaluated by principal coordinates analysis (PCoA). For that purpose, data were log(x+1) transformed, and the vegdist() function of the vegan package (Oksanen et al., 2019) was used to produce a distance matrix, using the Bray-Curtis index as a dissimilarity measure (Bray & Curtis, 1957). Then, the cmdscale() function of the R stats package (R Core Team, 2020) was used to generate the PCoA.

To test the significance of the differences between treatments, permutational analysis of variance (PERMANOVA) was performed, using the adonis() function of the vegan package (Oksanen et al., 2019). The Bray-Curtis distance matrix was used as the response variable, and the different treatments as a factor, with 999 permutations.

ii. High-throughput sequencing data analysis

The ASV abundance table obtained in QIIME2 software (version 2020.8, Bolyen et al., 2019) was imported to R software (version 4.0.2, R Project for Statistical Computing, Vienna, Austria) for removal of singleton chloroplast,

mitochondrial, and other non-bacterial ASV. ASV with >99% similarity to *P. rubra* SubTr2 (MK533559) and *E. coli* DH5α (CP026085.1:465734-467287) were searched and removed from the library. The rarefy() function of the vegan package (Oksanen et al., 2019) was used to estimate the total rarefied ASV richness of the ASV abundance table, and the rrarefy() function was used to subtract a rarefied ASV table from the dataset. This table was used for statistical computing and plot generation, using the vegan, stats, and ggplot2 packages (Oksanen et al., 2019; R Core Team, 2020; Wickham, 2016).

The diversity() function of the vegan package (Oksanen et al., 2019) was used to infer the Shannon diversity index, which was divided by the log(x+1) number of ASVs to calculate Pielou's evenness. The log(x+1) transformed ASV abundance table was used to generate a distance matrix, using the vegdist() function of the vegan package (Oksanen et al., 2019) and the Bray-Curtis index as dissimilarity measure (Bray & Curtis, 1957). The cmdscale() function of the R stats package was used to generate the PCoA (R Core Team, 2020).

PERMANOVA, using the adonis() function of the vegan package (Oksanen et al., 2019), was used to assess the significance of differences between treatments, using the Bray-Curtis distance matrix as the response variable, the treatments as factors, and 999 permutations. The mean relative abundance of the ten most abundant phylum and order level descriptions in each treatment was plotted, using the barplot2() function of the ggplot2 package (Wickham, 2016). Taxa with significant differences between treatments were only represented graphically when their relative abundance was superior to 2.5% in either treatment.

iii. Water quality parameters

The significance of differences in each parameter between different treatments was assessed by PERMANOVA, using the adonis() function of the vegan package (Oksanen et al., 2019), in the RStudio software (version 4.0.2, R Project for Statistical Computing, Vienna, Austria), using the treatment as factor and 999 permutations.

III. RESULTS

Part ONE – Preliminary study of potential heat-killed biomass

DNA extraction and PCR-DGGE analysis were successful for all samples (**Figure 3**). Since this first experiment was used as a preliminary study of the potential of HK biomass, DGGE was used as a cost-effective and fast way of assessing the effect of the different treatments.

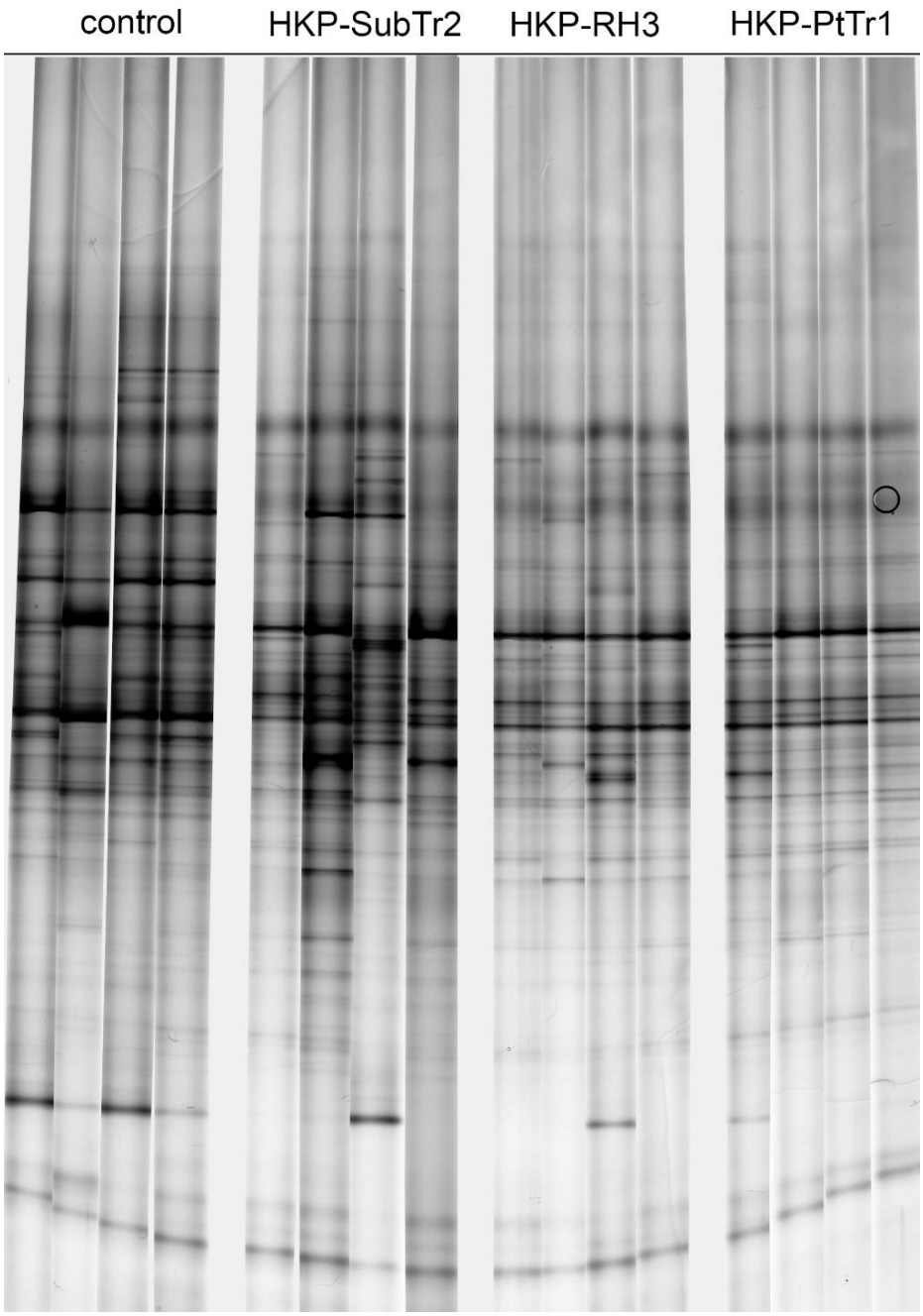


Figure 3 - Comparison of DGGE patterns of 16S rRNA gene fragments amplified from: control (untreated RAS water), HKP-SubTr2-treated samples, HKP-RH3-treated samples, and HKP-PtTr1-treated samples.

The PCoA revealed a clear separation between the control and the different

treatments, along the horizontal axis, which is the axis spanning the most variation (28.93%) (**Figure 4**). There is also a clear separation along the vertical axis, spanning the second most variation (17.52%), of the samples treated with HKP-SubTr2 compared to the other two treatments.

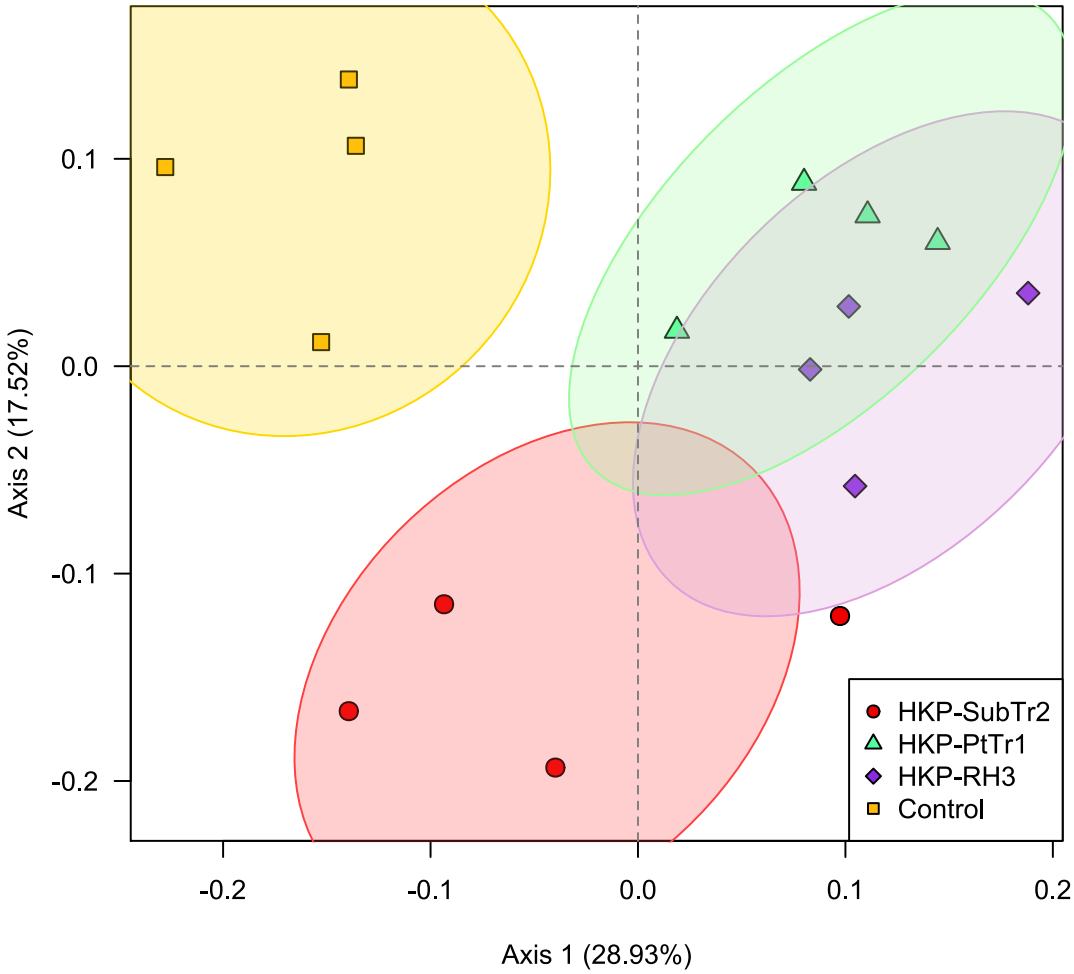


Figure 4 - Principal Coordinates Analysis (PCoA) of the 16S rRNA gene DGGE profiles of the treated samples. Yellow represents the untreated control; red represents the samples treated with HKP-SubTr2; green represents samples treated with HKP-PtTr1, and purple represents samples treated with HKP-RH3. The ellipses represent the 95% confidence ellipse of each treatment.

Overall, significant differences were observed between the control and water treated with HKP (PERMANOVA: *p*-value < 0.05; **Table 3**). However, no significant variation was observed between the HKP-RH3 and HKP-PtTr1 treatments (*p*-value > 0.05).

Table 3 - PERMANOVA between 16S rRNA gene DGGE profiles of different treatments in the first experiment. When differences are significant (p-value < 0.05), results are marked with *.

Analysis	R^2	$F_{1,6}$	*p*
Control x HKP-SubTr2	0.24656	3.4122	0.035*
Control x HKP-RH3	0.20613	5.1708	0.030*
Control x HKP-PtTr1	0.24765	3.4799	0.038*
HKP-SubTr2 x HKP-RH3	0.19080	3.7161	0.039*
HKP-SubTr2 x HKP-PtTr1	0.23220	3.2886	0.031*
HKP-RH3 x HKP-PtTr1	0.19553	1.7510	0.086

Despite all HK treatments having had a significant effect on bacterial composition, HKP-SubTr2 was chosen for a subsequent experiment. This bacterium was chosen, given its ability to produce red pigments, assumed to be prodigiosin or derivates. Thus, the second part of this chapter aims to study the effects of HKP-SubTr2 on aquaculture water microbiota, including an unpigmented bacterial strain, which is thought not to produce antagonist compounds, *E. coli* DH5α (T. Zhou et al., 2012), as reference.

Part TWO - Evaluation of heat-killed biomass of *Pseudoalteromonas rubra* SubTr2 on the modulation of bacterioplankton communities

i. DGGE profiles

Similar to the previous experimental results, DNA extraction and PCR-DGGE analysis were successful for all samples (**Figure 5**). These results revealed that the extracted DNA possessed a good quality for subsequent high-throughput sequencing analysis. Likewise, it was possible to observe prominent differences between the DGGE profiles of the initial RAS water samples and incubated samples and marked differences between the DGGE profile of HKP-SubTr2-treated samples in relation to the control or HK-DH5α treatment.

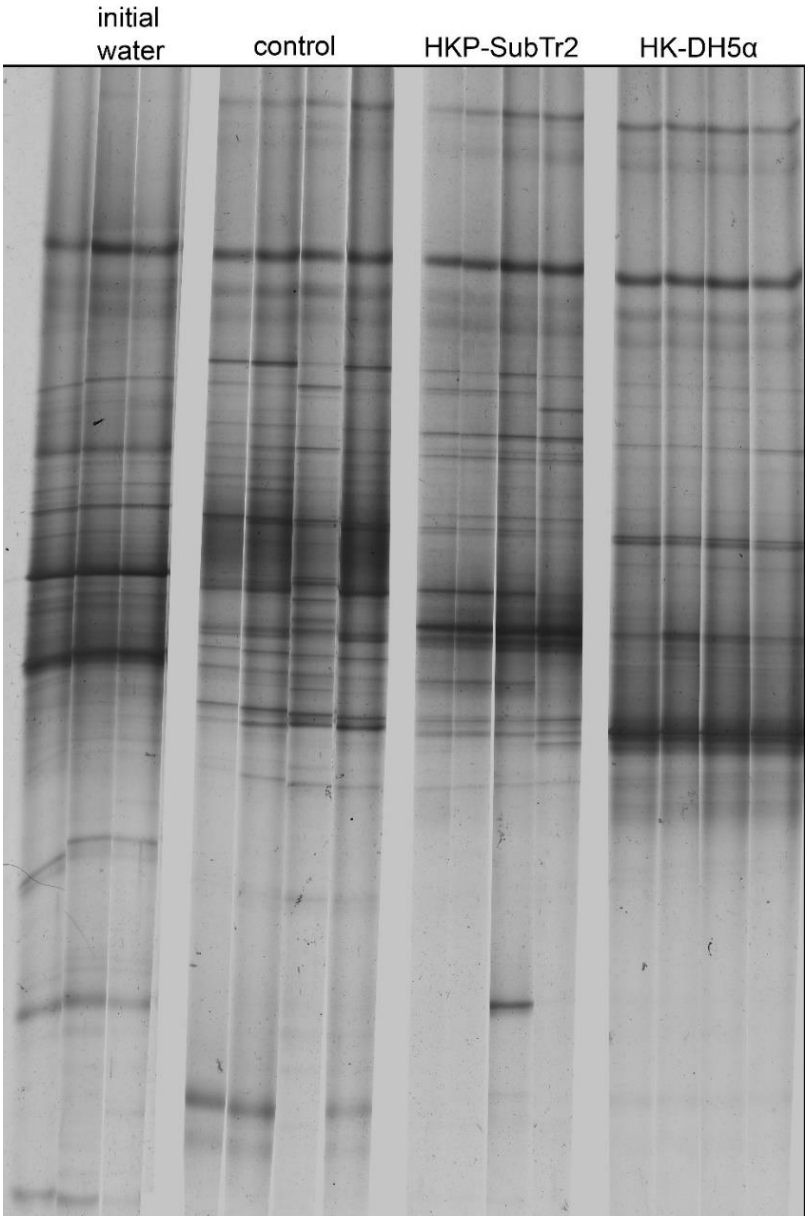


Figure 5 - Comparison of DGGE patterns of 16S rRNA gene fragments amplified from: the initial RAS rearing water samples, control (untreated RAS water), HKP-SubTr2-treated samples, and HK-DH5α-treated samples.

These results were further confirmed by PCoA, represented in **Figure 6**, which revealed clear separation in the DGGE profiles of the initial RAS water compared to all the post-incubation samples, along the horizontal axis, spanning the most variation (31.39%). PERMANOVA confirmed this (**Table 4**), revealing significant (*p*-value < 0.05) variation between the initial water sample and untreated control, as well as the RAS water treated with HKP-SubTr2 and HK-DH5α.

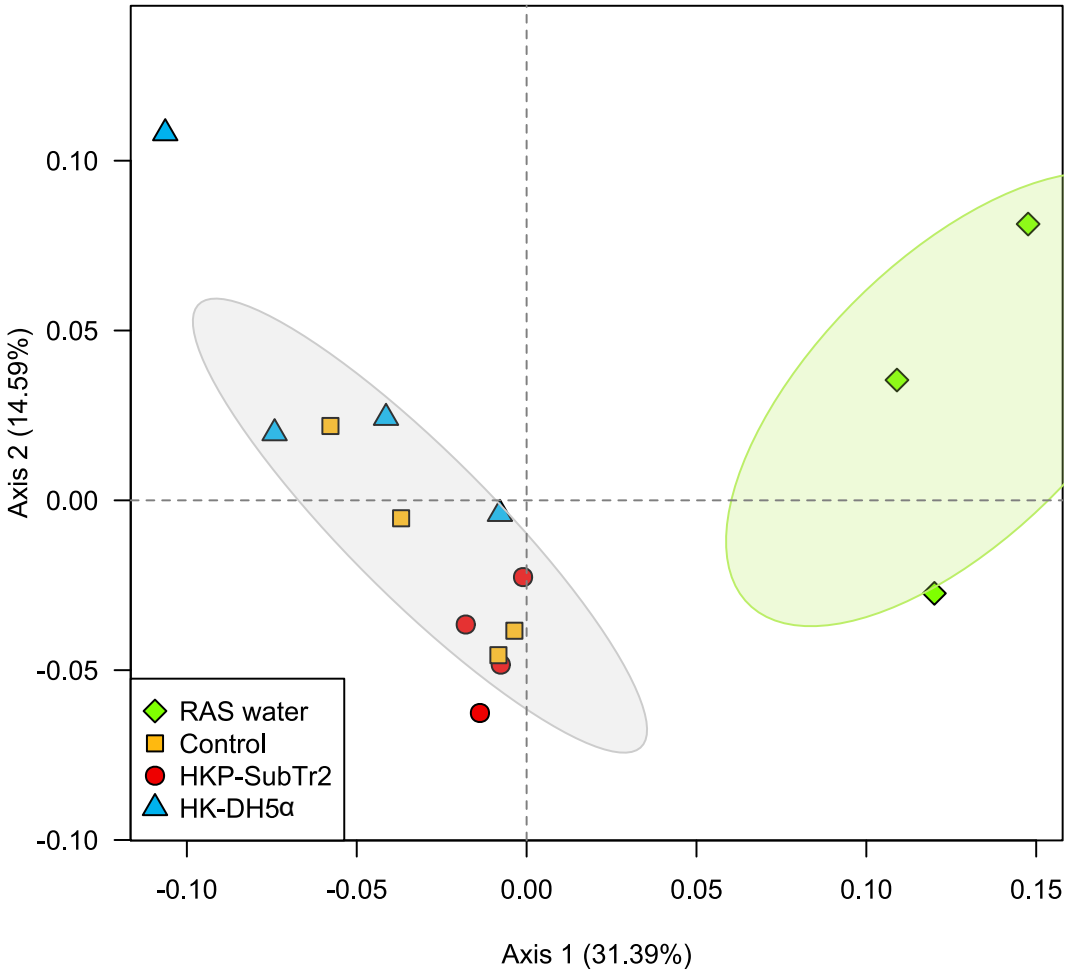


Figure 6 - Principal Coordinates Analysis (PCoA) of the 16S rRNA gene DGGE profiles of the treated samples. Green represents the initial RAS water samples, yellow represents the untreated control, red represents the samples treated with HKP-SubTr2, and blue represents the samples treated with HK-DH5α. The green ellipse represents the 95% confidence ellipse of the distribution of unincubated samples, while the grey ellipse represents the incubated samples, showing that these two groups are clustered independently.

A second PCoA, represented in **Figure 7** was performed without the data regarding the initial water samples for a clearer evaluation of the variation between treatments. In this graph, it is possible to see three different clusters, one for each treatment. The most variance occurs between the RAS water treated with HKP-SubTr2 and HK-DH5α, along the horizontal axis, spanning 31.49% of

the variation.

PERMANOVA revealed significant (*p*-value < 0.05, **Table 4**) variation between the untreated control and each of the treatments. As revealed by the PCoA, there were significant differences between the treatment with HKP-SubTr2 and HK-DH5α.

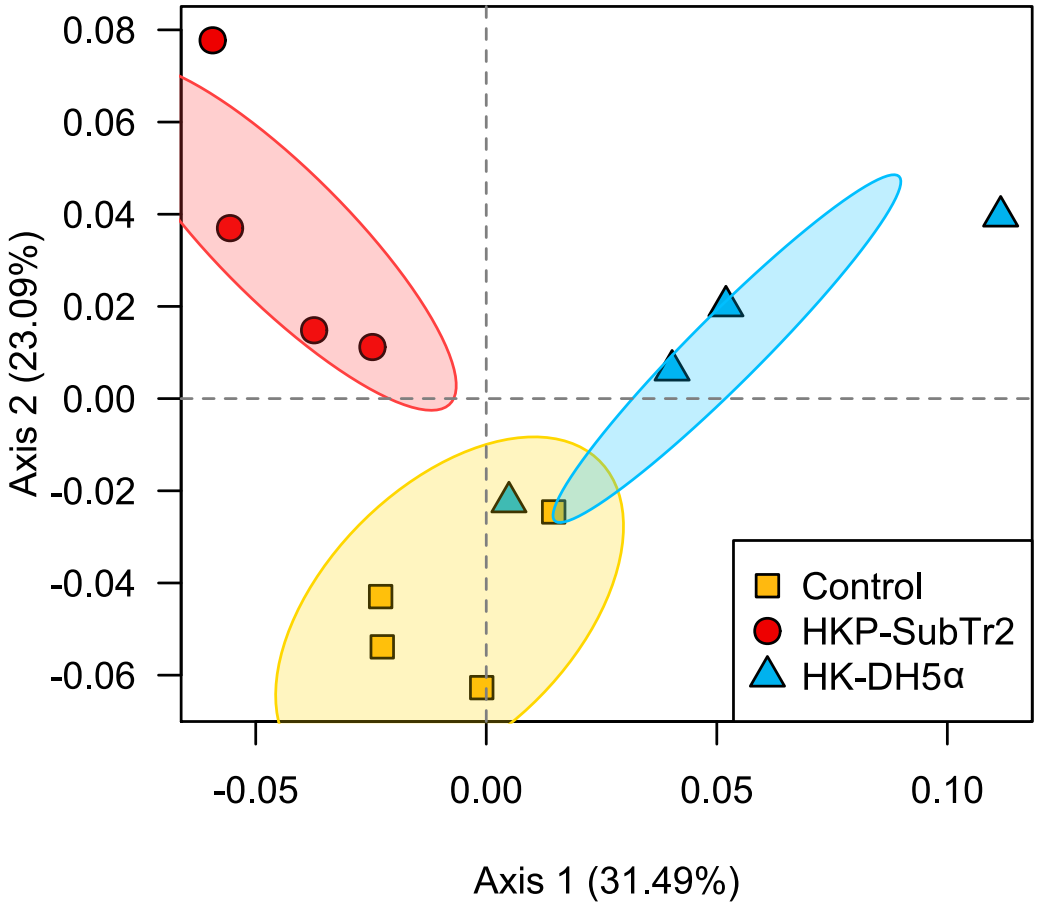


Figure 7 - Principal Coordinates Analysis (PCoA) of the 16S rRNA gene DGGE profiles of the treated samples. Yellow represents the untreated control, red represents the samples treated with HK-SubTr2, and blue represents samples treated with HK-DH5α. The ellipses represent the 95% confidence ellipse of each treatment.

These results reveal significant differences in the 16S rRNA gene fingerprinting patterns of the RAS water treated with HKP-SubTr2 when compared to the untreated control and the unpigmented HK-DH5α. Thus, it can be inferred that HKP-SubTr2 treatment has had significant effects on the microbiome of RAS rearing water.

Table 4 - Pairwise comparison between 16S rRNA gene DGGE profiles of different treatments in the second experiment. When differences are significant (p-value < 0.05), results are marked with a *.

Analysis	R^2	$F_{1,6}$	*P*
Initial water x Control	2.1700	5.6087	0.025*
Initial water x HKP-SubTr2	1.4343	6.4647	0.035*
Initial water x HK-DH5α	3.7163	6.2948	0.022*
Control x HKP-SubTr2	2.4091	3.6771	0.031*
Control x HK-DH5α	2.7894	2.8235	0.033*
HKP-SubTr2 x HK-DH5α	5.1505	3.8944	0.029*

ii. Water quality parameters

Information regarding the nitrate (NO_3^-), nitrite (NO_2^-), and ammonia/ammonium (NH_3/NH_4^+) concentration in each of the samples, as well as total organic carbon (TOC), is present in **Table 5**.

Table 5 - Mean concentration values and standard deviation of ammonia/ammonium, nitrites, nitrates, and total organic carbon (TOC) in the different samples before and after incubation.

Sample	NO_3^- (mg/L)	NO_2^- (mg/L)	NH_3/NH_4^+ (mg/L)	TOC (mg/L)
Initial water	144.4	0.226	0.536	8.02
Control	178.9 ± 1.7	0.291 ± 0.095	2.437 ± 1.482	10.1 ± 2.6
HKP-SubTr2	176.5 ± 2.4	0.419 ± 0.046	4.072 ± 0.831	10.1 ± 0.7
HK-DH5α	173.5 ± 10.0	0.397 ± 0.021	6.206 ± 1.597	9.0 ± 1.1

The concentration of all parameters was higher under experimental conditions in comparison to the initial water. However, no significant variation was detected between treatments and the control for neither of the parameters (**Table 6**).

Table 6 - Pairwise comparison of NO_3^-, NO_2^-, NH_4^+/NH_3 and TOC concentration between the different samples.

	Analysis	R^2	$F_{1,6}$	*p*-value
NO_3^-	Control x HKP-SubTr2	3.8454	0.0471	0.867
	Control x HK-DH5α	44.328	0.4405	0.770
	HKP-SubTr2 x HK-DH5α	45.719	0.3488	0.758
NO_2^-	Control x HKP-SubTr2	0.0335	5.8687	0.068
	Control x HK-DH5α	0.0285	4.7352	0.087
	HKP-SubTr2 x HK-DH5α	0.0076	0.7522	0.538
NH_3/NH_4^+	Control x HKP-SubTr2	1.6203	5.6029	0.086
	Control x HK-DH5α	2.3697	14.755	0.053
	HKP-SubTr2 x HK-DH5α	1.4353	5.8759	0.087
TOC	Control x HKP-SubTr2	2.4091	0.0001	0.966
	Control x HK-DH5α	2.7894	0.5389	0.586
	HKP-SubTr2 x HK-DH5α	5.1505	2.4958	0.156

pH was measured every day of incubation. These results are present in **Table 7**.

Table 7 - Mean values and standard deviation of pH in different samples during the 3 days of incubation.

	Initial	24h	48h	72h
Control	7.685	7.990 ± 0.028	8.007 ± 0.027	7.745 ± 0.397
HKP-SubTr2	7.685	7.915 ± 0.015	7.926 ± 0.023	7.917 ± 0.078
HK-DH5α	7.685	7.952 ± 0.008	7.905 ± 0.029	7.744 ± 0.517

No significant changes (*p*-value > 0.05) were found between the final pH of either of the treatments (**Table 8).**

Table 8 - Pairwise comparison of pH between the different samples.

Analysis	R^2	$F_{1,6}$	*p*
Control x HKP-SubTr2	1.9879	0.9634	0.514
Control x HK-DH5α	0.6715	0.2084	0.813
HKP-SubTr2 x HK-DH5α	2.0496	1.0003	0.482

iii. Flow cytometry analysis

Flow cytometry analysis revealed a decrease in cell densities between the initial water sample and the HK biomass treated samples (HK-DH5α and HKP-SubTr2), represented in **Figure 8.**

Table 9 - Pairwise comparison of cell density (cells/ml) in different samples.

Analysis	R^2	$F_{1,6}$	*p*
Control x HKP-SubTr2	$3.75x10^{14}$	0.8586	0.479
Control x HK-DH5α	$5.58x10^{14}$	0.4190	0.583
HKP-SubTr2 x HK-DH5α	$5.58x10^{14}$	0.4190	0.598

Meanwhile, no significant changes have been detected between the control and the HK-biomass treatments (**Table 9**).

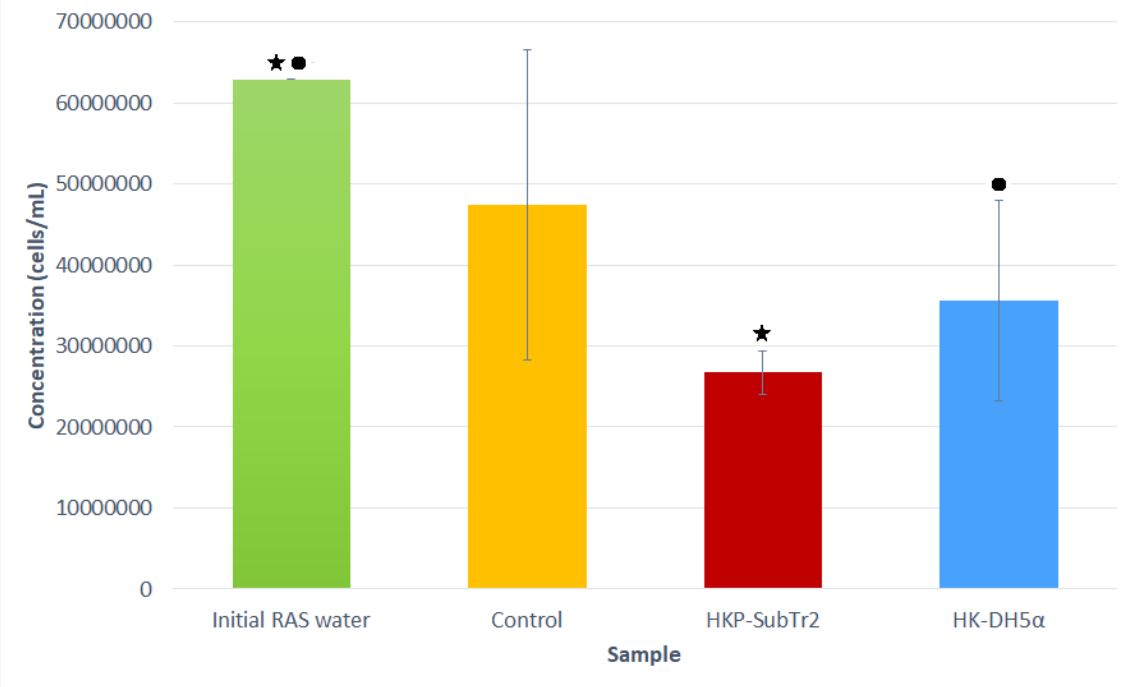


Figure 8 – Bar-plot of bacterial load revealed by flow cytometry. The bars represent the mean value of cells/ml of: green – initial RAS water, yellow – control (untreated RAS water), red – RAS water treated with HKP-SubTr2, blue – RAS water treated with HK-DH5α. The error bars represent the standard deviation. Similar symbols indicate significant differences between treatments.

iv. Structural diversity analysis of the bacterioplankton communities

Overall, the results (**Table 10**) show no difference in evenness, rarefied richness, and Shannon's diversity of samples exposed to either HK biomass in comparison to control (*p*-value > 0.05, **Table 11**).

Table 10 - Alpha diversity analysis of the ASV composition for each of the samples.

	Evenness	Rarefied richness	Shannon's diversity
Control	0.55 ± 0.03	1.93 ± 0.01	3.55 ± 0.20
HK-DH5α	0.57 ± 0.03	1.94 ± 0.02	3.61 ± 0.23
HKP-SubTr2	0.54 ± 0.02	1.91 ± 0.02	3.42 ± 0.16

Interestingly, in line with the DGGE community profiles, the PCoA analysis of bacterioplankton ASV composition (**Figure 9**) revealed a clear separation between the control and the HK treatments (HK-DH5α and HKP-SubTr2), along the horizontal axis, which is the first and foremost axis (represents 28.21% of total variation). Simultaneously, it is also possible to observe clear segregation between the two treatments (HK-DH5α and HKP-SubTr2) along the vertical axis, spanning the second most variation (20.58%).

Table 11 - Pairwise comparison of evenness, RR, and Shannon's diversity between samples.

		R^2	$F_{1,6}$	*p*-value
Control x HK-DH5α	**Evenness**	0.00699	0.0468	0.8359
	RR	0.00207	0.8496	0.3922
	Shannon's diversity	0.37853	0.0089	0.9280
Control x HKP-SubTr2	**Evenness**	0.00556	0.8162	0.4011
	RR	0.00231	1.4402	0.2753
	Shannon's diversity	0.29131	0.899	0.3797
HK-DH5α x HK-SubTr2	**Evenness**	0.00496	1.4676	0.2713
	RR	0.00234	4.2410	0.0851
	Shannon's diversity	0.24115	1.3457	0.2901

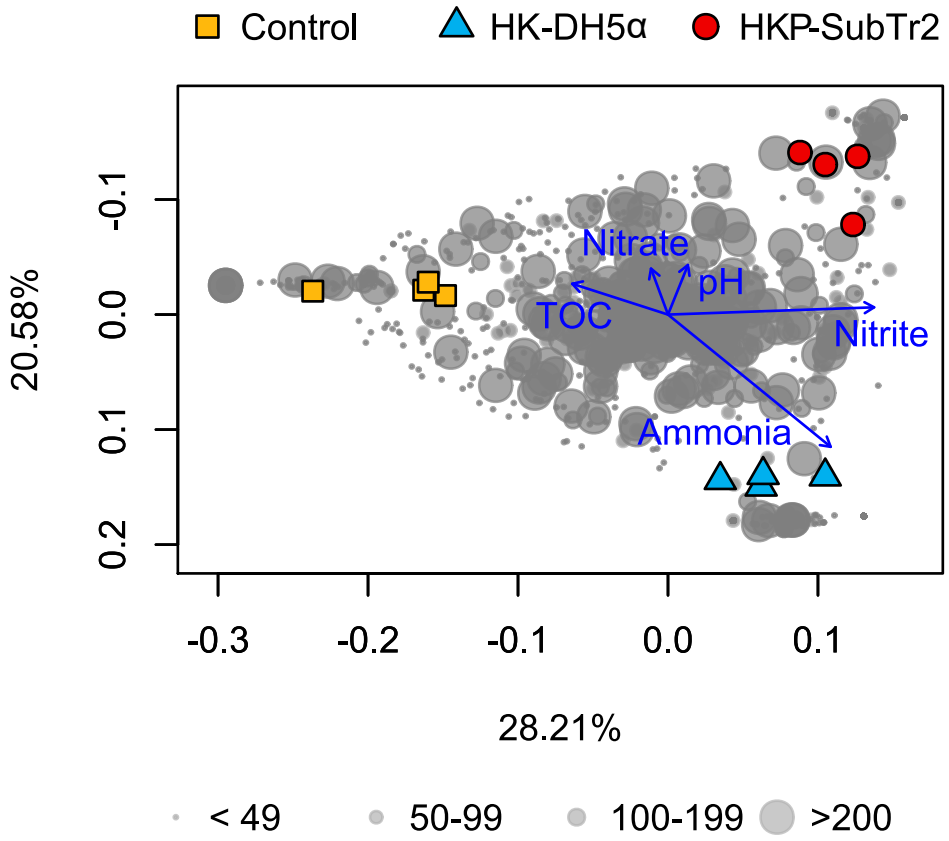


Figure 9 - PCoA of the bacterioplankton ASV composition. The grey symbol represents each ASV's score, while its size represents their relative abundance. The envfit() function of the vegan package (Oksanen et al., 2019) was used to fit the variance in environmental parameters onto the PCoA ordination.

The differences observed in the PCoA were further confirmed by PERMANOVA, which confirmed significant differences between the bacterioplankton composition of all treatments (**Table 12)**.

Table 12 - Pairwise comparison of ASV differences between samples.

	R^2	$F_{1,6}$	*p*-value
Control x HK-DH5α	0.39278	3.8811	0.036*
Control x HKP-SubTr2	0.41114	4.1892	0.022*
HK-DH5α x HKP-SubTr2	0.40254	4.0425	0.029*

The bacterioplankton communities were associated with differences in water quality parameters. Nitrite, which was higher in the HK treatments (HK-DH5α and HKP-SubTr2) than in control, was a significant predictor of the bacterial composition of these samples (*p*-value for horizontal axis = 0.010). Meanwhile, ammonia was significant predictor for the HK-DH5α bacterioplankton community (*p*-value for horizontal axis = 0.012; and vertical axis = 0.010). No significant associations were found between changes in the other water quality parameters

and the bacterioplankton community composition.

v. Composition analysis of dominant ASVs

Table 13 - Dominant phyla in the bacterioplankton of samples.

Relative abundance (%)	Control	HK-DH5α	HKP-SubTr2
Proteobacteria	62.44 ± 10.17	40.90 ± 0.77	44.75 ± 6.55
Bacteroidota	33.16 ± 11.63	44.91 ± 4.05	39.91 ± 4.59
Verrucomicrobiota	1.44 ± 1.25	1.23 ± 0.37	1.08 ± 0.26
Bdellovibrionota	1.25 ± 0.34	3.04 ± 0.68	4.39 ± 0.85
Patescibacteria	0.50 ± 0.22	8.60 ± 4.99	9.33 ± 6.23

At the phylum level (**Table 13, Figure 10**), Proteobacteria and Bacteroidota were the main bacteria present in all treatments. There was a significant (p-value < 0.05) increase in the relative abundance of Bdellovibrionota (HK-DH5α: p-value = 0.002; HKP-SubTr2: p-value < 0.001), Patescibacteria (HK-DH5α: p-value = 0.003; HKP-SubTr2: p-value = 0.005) and Proteobacteria (HK-DH5α: p-value = 0.003; HKP-SubTr2: p-value = 0.024) in the samples treated with HK biomass in comparison to control. Moreover, the HKP-SubTr2 treatment had a significant and higher relative abundance of ASVs related to the Bdellovibrionota (p-value = 0.047) than samples exposed to HK-DH5α.

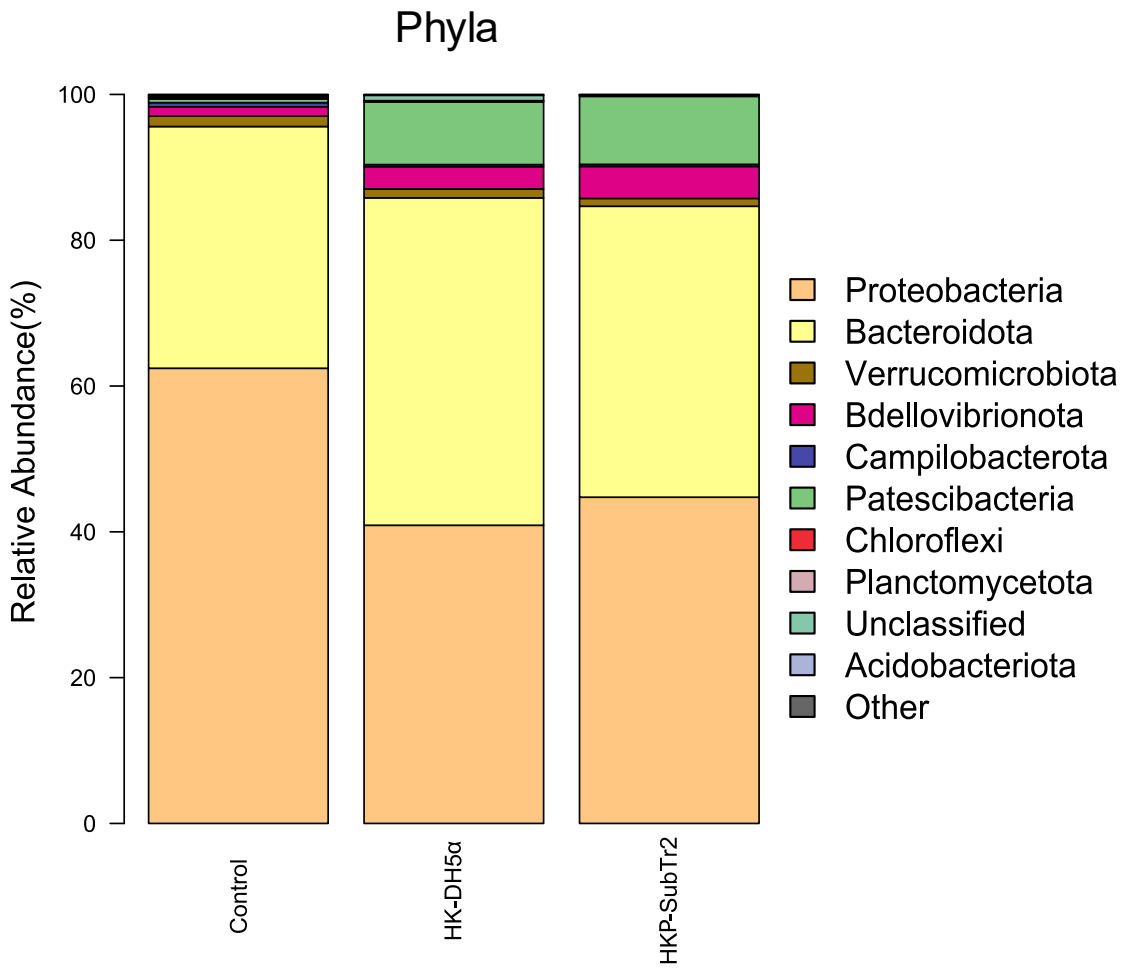


Figure 10 - Relative abundance (%) of the top phyla of bacterioplankton in control, HK-DH5α, and HKP-SubTr2 samples. Only the top 10 phyla are included in the bar-plot.

At the order level, it is possible to observe more differences between treatments (**Table 14, Figure 11**). The dominant orders in the control samples were Alteromonadales (33.63 ± 5.13%), followed by Flavobacteriales (29.81 ± 10.87%) and Rhodobacterales (13.48 ± 5.64%). In both HK biomass treatments, there was a significant increase in the relative abundance of Bacteriovoracales (HK-DH5α: *p*-value = 0.002, HKP-SubTr2: *p*-value < 0.001), Chitinophagales (HK-DH5α: *p*-value = 0.024, HKP-SubTr2: *p*-value< 0.001) and JGI_0000069-P22 taxonomic group of the candidate phylum Gracilibacteria (HK-DH5α: *p*-value = 0.003, HKP-SubTr2: *p*-value = 0.005). In addition, treatment with HKP-SubTr2 led to an enrichment in the relative abundance of Rhodospirillales (*p*-value = 0.020), Oceanospirillales (*p*-value < 0.001) and Kordiimonadales (*p*-value < 0.001).

Table 14 - Dominant orders in the bacterioplankton of samples.

Relative abundance (%)	Control	HK-DH5α	HKP-SubTr2
Alteromonadales	33.63 ± 5.13	9.47 ± 4.19	3.32 ± 0.95
Flavobacteriales	29.81 ± 10.87	35.42 ± 1.87	26.87 ± 4.44
Rhodobacterales	13.48 ± 5.64	10.75 ± 2.46	2.99 ± 0.90
Oceanospirillales	5.78 ± 2.02	11.74 ± 5.16	26.45 ± 6.30
Cytophagales	2.30 ± 1.06	5.40 ± 1.51	3.38 ± 0.94
Verrucomicrobiales	1.23 ± 1.15	1.10 ± 0.34	0.87 ± 0.24
Bacteriovoracales	1.19 ± 0.35	3.01 ± 0.70	4.34 ± 0.85
Rhodospirillales	1.23 ± 1.15	1.93 ± 1.05	3.21 ± 0.30
JGI_0000069-P22	0.38 ± 0.26	8.32 ± 4.89	9.25 ± 6.23
Chitinophagales	0.77 ± 0.22	3.90 ± 1.17	9.37 ± 1.50
Vibrionales	1.08 ± 1.08	2.57 ± 0.50	0.62 ± 0.27
Cellvibrionales	0.68 ± 0.35	0.20 ± 0.12	1.89 ± 1.26
Pseudomonadales	1.03 ± 0.82	0.27 ± 0.27	1.27 ± 2.35
Kordiimonadales	0.23 ± 0.05	0.35 ± 0.17	1.08 ± 0.17

Curiously, both HK treatments led to a significant decrease (*p*-value < 0.05) in the relative abundance of ASV affiliated to the Alteromonadales order (HK-DH5α: *p*-value < 0.001, HKP-SubTr2: *p*-value < 0.001). In addition, treatment with HKP-SubTr2 significantly decreased the relative abundance of ASVs related to the Rhodobacterales order (*p*-value = 0.003).

The most abundant genera within Alteromonadales were *Glaciecola* (27.49 ± 5.17%), *Colwellia* (3.28 ± 1.43%) and Alteromonas (2.08 ± 2.23) (**Table 15**). Meanwhile, the relative abundance of *Glaciecola* was highly decreased in both treatments (HK-DH5α: *p*-value < 0.001; HKP-SubTr2: *p*-value < 0.001), as well as *Colwellia* (HK-DH5α: *p*-value = 0.003; HKP-SubTr2: *p*-value = 0.006).

Within the Rhodobacterales order, the genera *Yoonia-Loktanella* (6.68 ± 2.03%) and *Celeribacter* (2.10 ± 1.69%) were the most prominent in the control samples and highly decreased in HKP-SubTr2 samples (*p*-value < 0.001 and = 0.044, respectively).

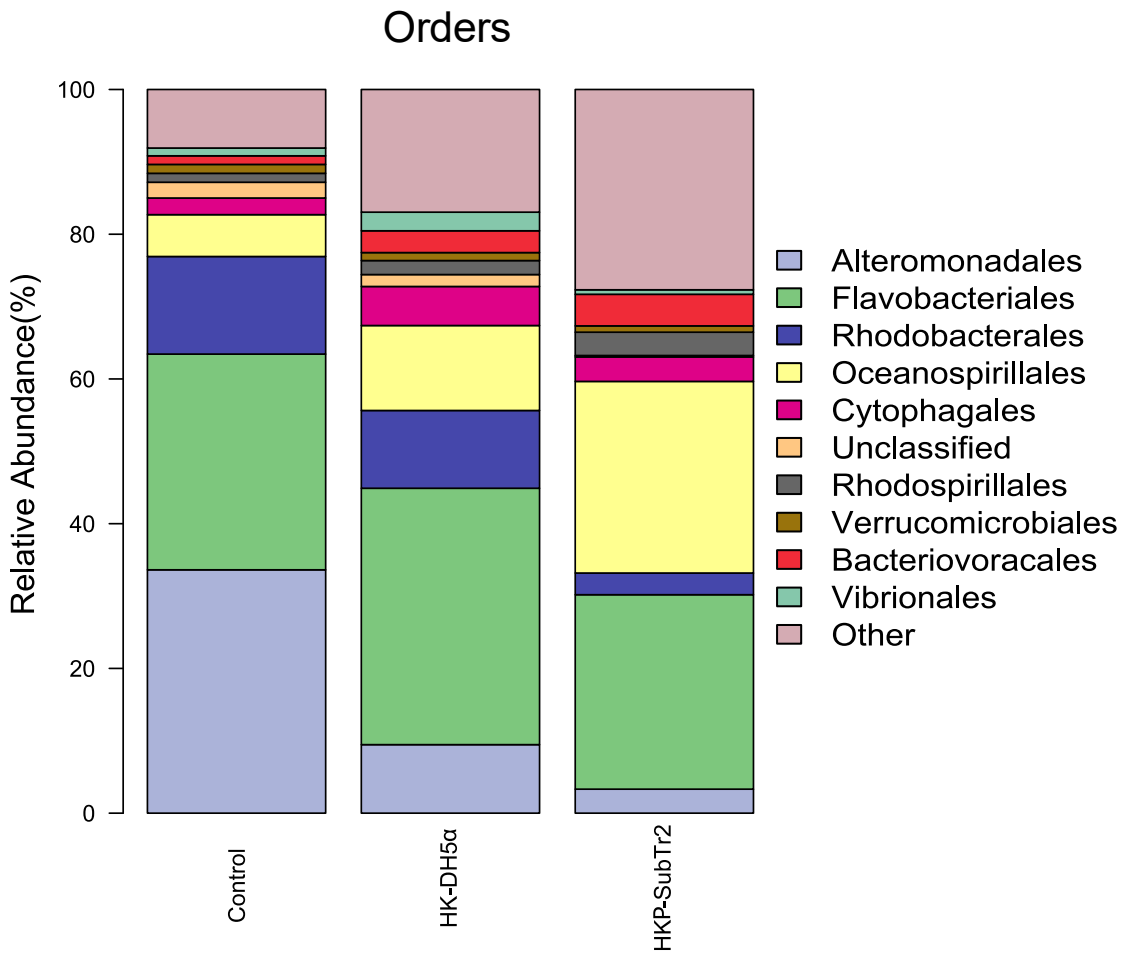


Figure 11 - Relative abundance (%) of top orders of bacterioplankton in control, HK-DH5α, and HKP-SubTr2 samples. The significant increase in "Other" orders in HK-DH5α and HKP-SubTr2 treated samples are mostly due to a significant increase in the orders Chitinophagales and JGI_0000069-P22. Only the top 10 orders were considered in the bar-plot.

Interestingly, HK biomasses significantly increased the relative abundance of bacterial populations belonging to the *Peredibacter* genus (HK-DH5α: *p*-value = 0.002; HKP-SubTr2: *p*-value < 0.001), belonging to the Bdellovibrionota phylum. The treatment with HK biomass also led to an increase in the abundance of Chitinophagales. Such a trend was mostly due to an increase in the relative abundance of *Edaphobaculum* (HK-DH5α: *p*-value < 0.01; HKP-SubTr2: *p*-value < 0.01).

It might be interesting to note that the treatment with HK-DH5α led to a significant increase in the genus *Tenacibaculum* (*p*-value < 0.001), which was not verified in the treatment with HKP-SubTr2.

ASVs with close similarity to *P. rubra* SubTr2 and *E. coli* DH5α were searched and removed from the library, as the results could be influenced by their presence. The inanimate biomass was added to the respective treated samples

and ASVs related to both species were thus present in high relative abundances in these samples. ASV associated with *P. rubra* SubTr2 had a relative abundance of 16.76 ± 0.17 % in the samples treated with HKP-SubTr2, whereas ASV associated with *E. coli* DH5α had a relative abundance of 26.31 ± 3.16 % in the samples treated with HK-DH5α, prior to removal. The removal of these ASV led to a relative abundance of 0.40 ± 0.17% of *Pseudoalteromonas* in the HKP-SubTr2-treated samples, closer to control (0.25 ± 0.17%), while the genus *Escherichia* had negligible abundances in all samples (< 0.01%).

Table 15 - Dominant genera in the bacterioplankton of samples.

Relative abundance (%)	Control	HK-DH5α	HKP-SubTr2
Glaciecola	27.49 ± 5.17	6.68 ± 3.73	1.16 ± 0.73
Yoonia-Loktanella	6.68 ± 2.03	7.17 ± 0.46	1.06 ± 0.46
NS3a marine group	4.42 ± 0.70	0.01 ± 0.02	0.01 ± 0.02
Colwellia	3.28 ± 1.43	0.60 ± 0.17	0.93 ± 0.12
Tenacibaculum	2.66 ± 1.78	18.43 ± 2.11	2.75 ± 1.71
Celeribacter	2.10 ± 1.69	1.01 ± 0.14	0.44 ± 0.21
Alteromonas	2.08 ± 2.23	0.29 ± 0.09	0.16 ± 0.05
Luteibaculum	1.87 ± 1.16	0.95 ± 0.26	0.07 ± 0.04
Fabibacter	1.84 ± 0.94	4.72 ± 1.05	2.59 ± 0.75
Pseudohongiella	1.65 ± 0.76	0.36 ± 0.10	0.45 ± 0.14
Oceaniserpentilla	1.40 ± 0.35	0.95 ± 0.65	1.27 ± 0.51
Peredibacter	1.17 ± 0.34	2.99 ± 0.70	4.11 ± 0.83
Oceanospirillum	0.64 ± 1.20	9.22 ± 5.46	22.43 ± 6.92
JGI_0000069-P22	0.39 ± 0.27	8.44 ± 4.92	9.25 ± 6.23
Edaphobaculum	0.43 ± 0.24	3.70 ± 1.15	8.93 ± 1.65
Vibrio	1.08 ± 1.08	2.57 ± 0.50	0.60 ± 0.28
Vicingus	0.20 ± 0.13	2.57 ± 1.53	0.60 ± 0.28
Thalassotalea	0.13 ± 0.08	1.50 ± 0.50	0.54 ± 0.11
Aquimarina	0.67 ± 1.17	1.49 ± 0.98	0.12 ± 0.06
Owenweeksia	0.43 ± 0.19	0.65 ± 0.42	1.34 ± 0.62

IV. DISCUSSION

As mentioned in the previous section, PCoA concerning the preliminary study of HKP revealed significant variation between the control and treatments with HKP biomasses. These results suggested that all HKP biomasses had a significant modulating effect on the microbiota of RAS rearing water. HKP-SubTr2 was chosen for the second experiment, in which it was analysed alongside an HK reference strain (HK-DH5α). Results obtained were similar to the first experiment, with PCoA showing a clear separation between the HKP-SubTr2 treatment and both the control and HK-DH5α treatment.

Curiously, the SubTr2 strain is a close phylogenetic relative of *P. rubra* ATCC 29570 (99.55% identity). Like other *Pseudoalteromonas* spp., *P. rubra* is known to produce pigments that may have interesting biotechnological potential (Offret et al., 2016, Richards et al., 2017). The primary pigments produced by *P. rubra* strains include cycloprodigiosin, prodigiosin, 2-methyl-3-hexyl-prodiginine, 2-methyl-3-butyl-prodiginine, and 2-methyl-3-heptyl-prodiginine (Setiyono et al., 2020). Interestingly, *P. rubra* SubTr2 presents a red colouration and was assumed to produce these same pigments or analogous ones. Prodigiosin was first isolated from soil bacteria *Serratia marcescens*, but several marine bacteria are capable of producing this and derivate pigments, including the genera *Beneckea, Pseudovibrio, Vibrio, Streptomyces, Pseudomonas, Pseudoalteromonas, Hahella,* and *Zooshikella* (Setiyono et al., 2020). It seems to have several biological functions, including antimicrobial, larvicidal, and anti-nematoid, immunomodulation, and antitumoral activities (Lin et al., 2020). Its mechanism of antimicrobial activity seems to be through the induction of ROS and inhibition of microcystin, causing the leakage of the cell membrane, though how oxidative stress and prevention of ROS scavenging are triggered is still unknown. In a study published earlier this year, Setiyono et al. (2020) tested the pigments produced by two *P. rubra* strains against several pathogenic organisms, reporting that cycloprodigiosin at a concentration of 20 µg/mL had a great inhibitory effect against *Staphylococcus aureus.* Likewise, other pigments present in different *Pseudoalteromonas* species have been associated with antifouling and inhibitory activities against algal spores, invertebrate larvae, bacteria, and fungi (Pachaiyappan et al., 2020).

The treatment with both HK biomasses led to a non-significant (p-value > 0.05) increase in the concentration of nitrite, in relation to the control samples. Similarly, treatment with HKP-DH5α led to a non-significant increase of ammonium/ammonia concentration, in relation to control. Nitrite and ammonia

are highly toxic for fish, even at low concentrations. Nitrite oxidises the iron in the haemoglobin molecule, converting it to methaemoglobin (this leads to brown blood disease, named after the colouration of this compound), which affects the capacity of oxygen transport (Timmons & Ebeling, 2010). Likewise, ammonia is toxic to all vertebrates, being capable of permeating the cell membranes through aquaporins, due to structural similarity to H_2O. Its toxicity mechanism is likely the displacement of K^+ and depolarisation of neurons, which leads to the activation of an NMDA-type glutamate receptor, causing an influx of excess Ca^{2+}, culminating in cell death (Randall & Tsui, 2002; Timmons & Ebeling, 2010). Therefore, the increase in this parameter, though non-significant, is not ideal and should be evaluated further in later studies, especially when the effects of HK biomass treatment on fish health are assessed.

Flow cytometry analysis revealed that, despite the decrease in cell densities in both HK biomass treatments in comparison to initial RAS water, there were no significant differences between treatments. These results are relevant and indicate that the HK microbial biomasses tested in this study did not promote bacterial proliferation. Such an effect is desirable in real situations, as it is important to maintain the microbial load of the rearing water near or at the maximum microbial carrying capacity (Attramadal et al., 2016). Diversity parameters are key in modulating aquacultures' microbiomes as systems at a maximized diversity are known to be less susceptible to disease (Kühsel & Blüthgen, 2015). In a system close to its maximum carrying capacity, selective pressure reduces the chances of the proliferation of opportunistic bacteria, since harmless bacteria are already occupying these environmental niches. Thus, these communities tend to be more stable, and capable of sustaining most perturbations, having a higher diversity and lower density of opportunistic organisms, which lowers the chances of disease outbreaks (Vestrum et al., 2018). However, treatment with HK biomass (HKP-SubTr2 and HK-DH5α) had no significant effect on the RAS bacterioplankton community α-diversity parameters analysed.

Furthermore, taxonomic analysis of the bacterioplankton revealed that Alteromonadales, Flavobacteriales, Rhodobacterales, and Oceanospirillales were the dominant orders in the control sample. This is in accordance with previous studies which found these orders to be the main constituents of the RAS bacterioplankton community (Duarte et al., 2019; Martins et al., 2013). Within the Alteromonadales order, the most abundant genera were *Glaciecola, Colwellia,* and *Alteromonas*. All members of *Glaciecola* have been isolated from marine

environments (Xiao et al., 2019) and this genus has been previously found dominating the microbiome of macroalgae cultivated in integrated multi-trophic aquaculture facilities (Califano et al., 2020). Similarly, all members of *Colwellia* have been isolated from marine environments, including polar and subtropical marine waters, and coastal waters (Harrison et al., 2014). A recent study reported the presence of *Colwellia* in the water and biofilms of a fish farm harbouring European seabass (Roquigny et al., 2021). These bacterial groups all have similar ecological roles (S. Cao et al., 2019), being important for carbon and nutrient recycling in marine environments, including hydrocarbon contaminants (Harrison et al., 2014).

Interestingly, the HKP-SubTr2 treatment had a significant and higher abundance of Bacteriovoracales in comparison to control, which was mostly represented by genus *Peredibacter*. This genus belongs to the *Bdellovibrio*-and-like-organisms (BALO) taxonomic group (Davidov & Jurkevitch, 2004, Müller et al., 2011), which are obligate predatory prokaryotes known to prey exclusively on Gram-negative bacteria (Williams & Piñeiro, 2006). BALO isolated from fish ponds have been reported to contribute to fish health and growth performances, reducing the incidence of diseases caused by *Aeromonas hydrophila* and *Vibrio alginolyticus* (Kandel et al., 2014). In fact, evidence suggests that *Vibrio* species are amongst the most susceptible to predation by BALO (Williams & Piñeiro, 2006). In line with this, a decrease in the relative abundance of *Vibrio* was verified for the treatment with HKP-SubTr2, though this variation was not significant for either of the treatments.

HKP-SubTr2 treatment also led to an increase in the order Chitinophagales, mostly represented by the genus *Edaphobaculum*. This order belongs to the phylum Bacteroidetes, recognised as an important constituent of marine bacterioplankton, especially in pelagic zones (Thomas et al., 2011). These organisms are known for catabolising complex polysaccharides, such as starch, cellulose, xylans, and pectins. Furthermore, they play an important role in protein metabolism, due to the production of proteases (Rajillić-Stojanović & de Vos, 2014). The order Chitinophagales is no different and, as the name indicates, some species within this order are capable of chitin degradation (e.g., *Chitinophaga pinensis*), and some are known cellulose degraders (e.g., *Chitinophaga oryziterrae*) (Rosenberg, 2014). The management and removal of solids is a crucial step for any recirculating aquaculture system, as they are extremely detrimental to fish, accumulating in gills and impairing respiration (Timmons & Ebeling, 2010). Therefore, the increase in the relative abundance of these organisms could be beneficial, by reducing the amount of suspended organic matter. Furthermore, some members of the Chitinophagales order have

been found to produce several secondary metabolites, including antimicrobial and antifungal compounds, which could have interesting antagonistic activity (Beckmann et al., 2017, Loudon et al., 2014). Nevertheless, *Edaphobaculum* is a recently described genus, and there is, thus, little published information on its metabolic capacities (M. Cao et al., 2017).

In addition, treatment with HKP-SubTr2 led to an enrichment in the relative abundance of Oceanospirillales. These are Gram-negative bacteria, halophilic or halotolerant, and widespread in marine environments, as well as RAS bacterioplankton (Duarte et al., 2019; Liao et al., 2020). Being underpinning members of the bacterioplankton, they play important environmental roles, namely the degradation of complex organic compounds, including hydrocarbonates, through excretion of hydrolytic enzymes and emulsifying agents (Jensen et al., 2010). Furthermore, they are involved in symbiotic relationships with several marine invertebrates, such as sea urchins, corals, mussels, and bone-eating worms (Y. Cao et al., 2014).

The treatment with HKP-SubTr2 also led to a significant decrease in the relative abundance of Rhodobacterales. As mentioned in the introduction section, some members of the Rhodobacterales order (e.g., Roseobacter clade) are known antagonists in aquaculture, producing several bioactive secondary compounds, capable of inhibiting several fish pathogens (Bentzon-Tillia et al., 2016; Brinkhoff et al., 2004; Hjelm et al., 2004; Porsby et al., 2011). Therefore, the decrease in the relative abundance of this order is not an ideal result since a high abundance of Rhodobacterales could be beneficial in aquaculture systems.

Curiously, the treatment with HK-DH5α increased the relative abundance of *Tenacibaculum,* a genus associated with fish disease in aquaculture (Småge et al., 2016), an effect which was not verified in the treatment with HKP-SubTr2.

Overall, these results show that the utilization of HK biomasses can modulate the RAS bacterioplankton composition. HKP-SubTr2, without incrementing bacterial abundance, clearly altered the composition of the bacterioplankton of a RAS. This effect was distinctive from the HK-DH5α treatment, indicating that it was species-specific. HKP-SubTr2 increased the abundance of beneficial taxonomic groups associated with grazing and polymer degradation (e.g., Bacteriovoracales and Chitinophagales). However, this treatment also led to a decrease in the beneficial order Rhodobacterales. Therefore, further studies are required to confirm its potential as a modulator of aquaculture microbiomes and a promoter of fish well-being.

V. CONCLUSION

This chapter aimed to evaluate the effect of heat-killed *Pseudoalteromonas* spp. on the RAS bacterioplankton communities in controlled experiments. DGGE-PCR analysis from a preliminary study involving three HK *Pseudoalteromonas* spp. led to the selection of HKP-SubTr2 to be used in the second experiment.

The results from the second experiment showed that treatment with HKP-SubTr2 influenced the bacterioplankton composition, without augmenting bacterial abundance. This strain led to an increase in beneficial taxonomic groups, such as Bacteriovoracales and Chitinophagales, which are involved in bacterial grazing and organic matter degradation. However, this treatment also led to a decrease in Rhodobacterales, which are a beneficial group of organisms in aquaculture microbiomes. The effects observed were distinct from the HK-DH5α treatment, indicating species-specificity. Overall, the impacts of HKP-SubTr2 treatment on the bacterioplankton communities did not affect the water quality parameters analysed in this study.

Our findings indicated, for the first time, that the use of heat-killed microbial biomass may be an interesting strategy for the modulation of aquaculture bacterioplankton. However, further studies are necessary to investigate their potential effect on fish health and water quality during aquaculture production.

Concluding remarks and future prospects

CONCLUDING REMARKS AND FUTURE PROSPECTS

Aquaculture production is one of the most interesting alternatives to traditional animal farming. Nonetheless, one of the most critical issues faced by aquaculture facilities is the control of infectious diseases, which is typically reliant on the therapeutic and prophylactic use of antibiotics. These strategies have severe consequences for fish health and the environment. Thus, current research is preoccupied with developing alternative strategies to control the microbial communities in aquaculture. In line with this trend, this dissertation aimed to study the potential of microorganisms to suppress the development of pathogens in aquaculture and improve natural fish barriers against them.

Thus, in the first chapter, we aimed to isolate and characterize the culturable fungal fraction of the rearing water and biofilters of a European seabass and gilthead seabream experimental RAS. The results revealed the presence of fungal strains associated with *Tilletiopsis lilacina* and *Candida labiduridarum*, which may produce antibacterial and antifungal compounds, and *Cystobasidium slooffiae* and *Dioszegia hungarica,* which may produce indole-3-acetic acid and carotenoid pigments, respectively. Therefore, this study led to the isolation of fungal strains with potential biotechnological applications in the aquaculture sector. Future studies will consequently aim to study the effect of these strains on the modulation of aquaculture microbiomes.

The second chapter addressed the potential of heat-killed *Pseudoalteromonas* spp. to modulate the bacterioplankton composition of a European seabass and gilthead seabream experimental RAS. The findings highlight that HKP-SubTr2, closely related to *Pseudoalteromonas rubra* ATCC 29570, and thus assumed to produce prodigiosin or analogous pigments, influenced the RAS bacterioplankton composition. This strain led to an increase in the abundance of beneficial taxonomic groups, including bacterial grazers and organic matter degraders, such as Bacteriovoracales and Chitinophagales, without increasing the bacterial load. These effects did not alter the water quality parameters tested in this study and seemed to be species-specific, as they were distinct from the effects of HK-DH5α treatment.

This study describes an original approach for modulating aquaculture microbiomes, indicating, for the first time, the potential effects of HK biomass on the bacterioplankton communities of RAS. Nevertheless, more research is necessary to determine the potential effects of HK biomass on fish health and water quality during aquaculture production.

References

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish & Shellfish Immunology*, *90*, 210–214. https://doi.org/10.1016/j.fsi.2019.04.066

Aguilar-Toalá, J. E., Garcia-Varela, R., Garcia, H. S., Mata-Haro, V., González-Córdova, A. F., Vallejo-Cordoba, B., & Hernández-Mendoza, A. (2018). Postbiotics: An evolving term within the functional foods field. *Trends in Food Science & Technology*, *75*, 105–114. https://doi.org/10.1016/j.tifs.2018.03.009

Alfouzan, W., Dhar, R., Albarrag, A., Al-Abdely, H. (2019). The emerging pathogen *Candida auris*: a focus on the Middle-Eastern countries. *Journal of Infection and Public Health. 12*, 451-459. https://doi.org/10.1016/j.jiph.2019.03.009

Alker, A. T., Delherbe, N., Purdy, T. N., Moore, B. S., Shikuma, N. J. (2020). Genetic examination of the marine bacterium *Pseudoalteromonas luteoviolacea* and effects of its metamorphosis-inducing factors. *Environmental Microbiology. 22*(11), 4689-4701. https://doi.org/10.1111/1462-2920.15211

AlMatar, M., & Makky, E. A. (2016). *Cladosporium cladosporioides* from the perspectives of medical and biotechnological approaches. *3 Biotech*, *6*, 4. https://doi.org/10.1007/s13205-015-0323-4

Amend, A., Burgaud, G., Cunliffe, M., Edgcomb, V. P., Ettinger, C. L., Gutiérrez, M. H., Heitman, J., Hom, E. F. Y., Ianiri, G., Jones, A. C., Kagami, M., Picard, K. T., Quandt, C. A., Raghukumar, S., Riquelme, M., Stajich, J., Vargas-Muñiz, J., Walker, A. K., Yarden, O., & Gladfelter, A. S. (2019). Fungi in the Marine Environment: Open Questions and Unsolved Problems. *MBio*, *10*:e01189-18. https://doi.org/10.1128/mBio.01189-18

Andreakis, N., Høj, L., Kearns, P., Hall, M. R., Ericson, G., Cobb, R. E., Gordon, B. R., & Evans-Illidge, E. (2015). Diversity of Marine-Derived Fungal Cultures Exposed by DNA Barcodes: The Algorithm Matters. *PLOS ONE*, *10*(8), e0136130. https://doi.org/10.1371/journal.pone.0136130

Attramadal, K. J. K., Minniti, G., Øie, G., Kjørsvik, E., Østensen, M.-A., Bakke, I., & Vadstein, O. (2016). Microbial maturation of intake water at different carrying capacities affects microbial control in rearing tanks for marine fish larvae. *Aquaculture*, *457*, 68–72. https://doi.org/10.1016/j.aquaculture.2016.02.015

Attramadal, K. J. K., Salvesen, I., Xue, R., Øie, G., Størseth, T. R., Vadstein, O., & Olsen, Y. (2012). Recirculation as a possible microbial control strategy in the production of marine larvae. *Aquacultural Engineering*, *46*, 27–39. https://doi.org/10.1016/j.aquaeng.2011.10.003

Beckmann, A., Hüttel, S., Schmitt, V., Müller, R., & Stadler, M. (2017). Optimization of the biotechnological production of a novel class of anti-MRSA antibiotics from *Chitinophaga sancti*. *Microbial Cell Factories*, *16*, 143. https://doi.org/10.1186/s12934-017-0756-z

Bentzon-Tilia, M., Sonnenschein, E. C., & Gram, L. (2016). Monitoring and managing microbes in aquaculture—Towards a sustainable industry. *Microbial Biotechnology*, *9*(5), 576–584. https://doi.org/10.1111/1751-7915.12392

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*, 90. https://doi.org/10.1186/s40168-018-0470-z

Bollmann, A., Palumbo, A. V., Lewis, K., & Epstein, S. S. (2010). Isolation and Physiology of Bacteria from Contaminated Subsurface Sediments. *Applied and Environmental Microbiology*, *76*(22), 7413–7419. https://doi.org/10.1128/AEM.00376-10

Böllman, J. & Martienssen, M. (2020). Comparison of different media for the detection of denitrifying and nitrate reducing bacteria in mesotrophic aquatic environments by the most probable number method. *Journal of Microbiological Methods*. *168*. 105808. https://doi.org/10.1016/j.mimet.2019.105808

Bolyen, E., Rideout, J. R., Dillon, M. R., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*, 852–857. https://doi.org/10.1038/s41587-019-0209-9

Bonugli-Santos, R. C., dos Santos Vasconcelos, M. R., Passarini, M. R. Z., Vieira, G. A. L., Lopes, V. C. P., Mainardi, P. H., dos Santos, J. A., de Azevedo Duarte, L., Otero, I. V. R., da Silva Yoshida, A. M., Feitosa, V. A., Pessoa, A., & Sette, L. D. (2015). Marine-derived fungi: Diversity of enzymes and biotechnological applications. *Frontiers in Microbiology*, *6,* 269. https://doi.org/10.3389/fmicb.2015.00269

Borges, N., Keller-Costa, T., Sanches-Fernandes, G. M. M., Louvado, A., Gomes, N. C. M., & Costa, R. (2021). Bacteriome Structure, Function, and Probiotics in Fish Larviculture: The Good, the Bad, and the Gaps. *Annual Review of Animal Biosciences*, *9*(1). https://doi.org/10.1146/annurev-animal-062920-113114

Bray, J. R., & Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349. https://doi.org/10.2307/1942268

Brinkhoff, T., Bach, G., Heidorn, T., Liang, L., Schlingloff, A., & Simon, M. (2004). Antibiotic Production by a Roseobacter Clade-Affiliated Species from the German Wadden Sea and Its Antagonistic Effects on Indigenous Isolates. *Applied and Environmental Microbiology*, *70*(4), 2560–2565. https://doi.org/10.1128/AEM.70.4.2560-2565.2003

Brummett, R. (2013). Growing Aquaculture in Sustainable Ecosystems. *Agricultural and Environmental Service Department Notes. 5.* 4 p.

Califano, G., Kwantes, M., Abreu, M. H., Costa, R., & Wichard, T. (2020). Cultivating the Macroalgal Holobiont: Effects of Integrated Multi-Trophic Aquaculture on the Microbiome of *Ulva rigida* (Chlorophyta). *Frontiers in Marine Science*, *7*, 52. https://doi.org/10.3389/fmars.2020.00052

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*, 581–583. https://doi.org/10.1038/nmeth.3869

Caruffo, M., Navarrete, N., Salgado, O., Díaz, A., López, P., García, K., Feijóo, C. G. & Navarrete, P. (2015). Potential probiotic yeasts isolated from the fish gut protect zebrafish (*Danio rerio*) from a *Vibrio anguillarum* challenge. *Frontiers in Microbiology*. *6*. 1093. https://doi.org/10.3389/fmicb.2015.01093

Cao, M., Huang, J., Li, J., Qiao, Z., & Wang, G. (2017). *Edaphobaculum flavum* gen. nov., sp. nov., a member of family *Chitinophagaceae*, isolated from grassland soil. *International Journal of Systematic and Evolutionary Microbiology*, *67*(11), 4475–4481. https://doi.org/10.1099/ijsem.0.002316

Cao, S., He, J., Zhang, F., Lin, L., Gao, Y., & Zhou, Q. (2019). Diversity and community structure of bacterioplankton in surface waters off the northern tip of the Antarctic Peninsula. *Polar Research*. *38.* https://doi.org/10.33265/polar.v38.3491

Cao, Y., Chastain, R. A., Eloe, E. A., Nogi, Y., Kato, C., & Bartlett, D. H. (2014). Novel psychropiezophilic Oceanospirillales species Profundimonas piezophila gen. nov., sp. nov., isolated from the deep-sea environment of the Puerto Rico trench. Applied and environmental microbiology, 80(1), 54–60. https://doi.org/10.1128/AEM.02288-13.

Capus, A., Monnerat, M., Ribeiro, L. C., de Souza, W., Martins, J. L., & Sant'Anna, C. (2016). Application of high-content image analysis for quantitatively estimating lipid accumulation in oleaginous yeasts with potential for use in biodiesel production. *Bioresource Technology*, *203*, 309–317. https://doi.org/10.1016/j.biortech.2015.12.067

Castex, M., Durand, H., & Okeke, B. (2014). Issues with Industrial Probiotic Scale-up. In: D. Merrifield & E. Ringø (Eds.), *Aquaculture Nutrition* (pp. 347–359). https://doi.org/10.1002/9781118897263.ch13

Cavalcanti, V. P., Araújo, N. A. F., Machado, N. B., Costa Júnior, P. S. P., Pasqual, M., Alves, E., Schwan-Estrada, K. R. F., & Dória, J. (2020). Yeasts and *Bacillus* spp. as potential biocontrol agents of *Sclerotinia sclerotiorum* in garlic. *Scientia Horticulturae*, *261*, 108931. https://doi.org/10.1016/j.scienta.2019.108931

Chen, P.-C., Chiang, K.-P., & Tsai, S.-F. (2017). *Pelagostrobilidium liui* n. sp. (Ciliophora, Choreotrichida) from the Coastal Waters of Northeastern Taiwan and an Improved Description of *Pelagostrobilidium minutum* Liu et al., 2012. *Journal of Eukaryotic Microbiology*, *64*(5), 579–587. https://doi.org/10.1111/jeu.12392

Chen, Zhao, Chang, Z., Zhang, L., Jiang, Y., Ge, H., Song, X., Chen, S., Zhao, F., Li, J. (2019). Effects of water recirculation rate on the microbial community and water quality in relation to the growth and survival of white shrimp (*Litopenaeus vannamei*). *BMC Microbiology. 19,* 192. https://doi.org/10.1186/s12866-019-1564-x

Chen, Zhu, Liu, Y., Liu, L. Z., Wang, X. J. & Liu, Z. P. (2013). Heterotrophic Bacterial Community Structure of Multistage Biofilters in a Commercial Pufferfish *Takifugu rubripes* RAS. *Advanced Materials Research. 726-731.* 1621-1627. https://doi.org/10.4028/www.scientific.net/AMR.726-721.1621

Choudhury, T. G., & Kamilya, D. (2019). Paraprobiotics: An aquaculture perspective. *Reviews in Aquaculture*, *11*(4), 1258–1270. https://doi.org/10.1111/raq.12290

Chreptowicz, K., Mierzejewska, J., Tkáčová, J., Młynek, M., & Čertik, M. (2019). Carotenoid-Producing Yeasts: Identification and Characteristics of Environmental Isolates with a Valuable Extracellular Enzymatic Activity. *Microorganisms*, *7*(12), 653. https://doi.org/10.3390/microorganisms7120653

Crous, P. W., Schoch, C. L., Hyde, K. D., Wood, A. R., Gueidan, C., de Hoog, G. S., & Groenewald, J. Z. (2009). Phylogenetic lineages in the *Capnodiales*. *Studies in Mycology*, *64*, 17–47. https://doi.org/10.3114/sim.2009.64.02

D'Alvise, P. W., Lillebø, S., Prol-Garcia, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, Ø., & Gram, L. (2012). *Phaeobacter gallaeciensis* Reduces *Vibrio anguillarum* in Cultures of Microalgae and Rotifers, and Prevents Vibriosis in Cod Larvae. *PLOS ONE*, *7*(8):e43996. https://doi.org/10.1371/journal.pone.0043996

Danesi, P., Firacative, C., Cogliati, M., Otranto, D., Capelli, G., & Meyer, W. (2014). Multilocus sequence typing (MLST) and M13 PCR fingerprinting revealed heterogeneity amongst *Cryptococcus* species obtained from Italian veterinary isolates. *FEMS Yeast Research*, *14*(6), 897–909. https://doi.org/10.1111/1567-1364.12178

Dash, G., Raman, R. P., Pani Prasad, K., Makesh, M., Pradeep, M. A., & Sen, S. (2014). Evaluation of *Lactobacillus plantarum* as feed supplement on host associated microflora, growth, feed efficiency, carcass biochemical composition and immune response of giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). *Aquaculture*, *432*, 225–236. https://doi.org/10.1016/j.aquaculture.2014.05.011

Dash, G., Raman, R. P., Pani Prasad, K., Makesh, M., Pradeep, M. A., & Sen, S. (2015). Evaluation of paraprobiotic applicability of *Lactobacillus plantarum* in improving the immune response and disease protection in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). *Fish & Shellfish Immunology*, *43*(1), 167–174. https://doi.org/10.1016/j.fsi.2014.12.007

Dauda, A. B., Ajadi, A., Tola-Fabunmi, A. S., & Akinwole, A. O. (2019). Waste production in aquaculture: Sources, components and managements in different culture systems. *Aquaculture and Fisheries*, *4*(3), 81–88. https://doi.org/10.1016/j.aaf.2018.10.002

Davidov, Y., & Jurkevitch, E. (2004). Diversity and evolution of *Bdellovibrio*-and-like organisms (BALOs), reclassification of *Bacteriovorax starrii* as *Peredibacter starrii* gen. nov., comb. nov., and description of the *Bacteriovorax–Peredibacter* clade as *Bacteriovoracaceae* fam. nov. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1439–1452. https://doi.org/10.1099/ijs.0.02978-0

Dawood, M. A. O., Koshio, S., Ishikawa, M., & Yokoyama, S. (2015a). Effects of Partial Substitution of Fish Meal by Soybean Meal with or without Heat-Killed *Lactobacillus plantarum* (LP20) on Growth Performance, Digestibility, and Immune Response of Amberjack, *Seriola dumerili* Juveniles. *BioMed Research International*, *2015*, Article ID 514196. 11 p. https://doi.org/10.1155/2015/514196

Dawood, M. A. O., Koshio, S., Ishikawa, M., & Yokoyama, S. (2015b). Effects of heat killed *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance and immune response of red sea bream, *Pagrus major*. *Aquaculture*, *442*, 29–36. https://doi.org/10.1016/j.aquaculture.2015.02.005

Dawood, M. A. O., Koshio, S., Ishikawa, M., & Yokoyama, S. (2015c). Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*. *Fish & Shellfish Immunology*, *45*(1), 33–42. https://doi.org/10.1016/j.fsi.2015.01.033

Dawood, M. A. O., Koshio, S., Ishikawa, M., & Yokoyama, S. (2016). Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C. *Fish & Shellfish Immunology*, *54*, 266–275. https://doi.org/10.1016/j.fsi.2016.04.017

de Almada, C. N., Almada, C. N., Martinez, R. C. R., & Sant'Ana, A. S. (2016). Paraprobiotics: Evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. *Trends in Food Science & Technology*, *58*, 96–114. https://doi.org/10.1016/j.tifs.2016.09.011

de Leo, F., Urzı, C., & de Hoog, G. S. (2003). A new meristematic fungus, P*seudotaeniolina globosa. Antonie van Leeuwenhoek*, *83*, 351–360. https://doi.org/10.1023/A:1023331502345

Dittmann, K. K., Rasmussen, B. B., Castex, M., Gram, L., & Bentzon-Tilia, M. (2017). The aquaculture microbiome at the centre of business creation. *Microbial Biotechnology*, *10*(6), 1279–1282. https://doi.org/10.1111/1751-7915.12877

Duarte, L. N., Coelho, F. J. R. C., Oliveira, V., Cleary, D. F. R., Martins, P., & Gomes, N. C. M. (2019). Characterization of bacterioplankton communities from a hatchery recirculating aquaculture system (RAS) for juvenile sole (*Solea senegalensis*) production. *PLOS ONE*, *14*(1), e0211209. https://doi.org/10.1371/journal.pone.0211209

Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R., & Raff, R. A. (1988). Molecular Phylogeny of the Animal Kingdom. *Science*, *239*(4841), 748–753. https://doi.org/10.1126/science.3277277

FAO (2020). The State of World Fisheries and Aquaculture 2020. *Sustainability in action.* Rome. https://doi.org/10.4060/ca9229en

FAO & WHO. (2006). Probiotics in food: Health and nutritional properties and guidelines for evaluation - Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Rome.

Ghosh, P., Chattopadhyay, S. K., Adhikari, S., Saha, S., Mondal, S., Kader, A., Dey, S. T., Bandyopadhyay, T. K., & Sengupta, C. (2013). A high throughput DNA extraction method from chemotypically heterogeneous plant species. *Protocol Exchange*. https://doi.org/10.1038/protex.2013.018

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology*, *14*, 491–502. https://doi.org/10.1038/nrgastro.2017.75

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary Modulation of the Human Colonie Microbiota: Introducing the Concept of Prebiotics. *The Journal of Nutrition*, *125*(6), 1401–1412. https://doi.org/10.1093/jn/125.6.1401

Gomes, N. C. M., Borges, L. R., Paranhos, R., Pinto, F. N., Mendonça-Hagler, L. C. S. & Smalla, K. (2008). Exploring the diversity of bacterial communities in sediments of urban mangrove forests. *FEMS Microbiology Ecology,* *66*(1), 96-109. https://doi.org/10.1111/j.1574-6941.2008.00519.x

Gómez, B., Munekata, P. E. S., Zhu, Z., Barba, F. J., Toldrá, F., Putnik, P., Bursać Kovačević, D., & Lorenzo, J. M. (2019). Challenges and opportunities regarding the use of alternative protein sources: Aquaculture and insects. In: F. Toldrá (ed.). *Advances in Food and*

Nutrition Research (Vol. 89, Chapter 7, pp. 259–295). https://doi.org/10.1016/bs.afnr.2019.03.003

Greco, M., Pardo, A. & Pose, G. (2015), Mycotoxigenic Fungi and Natural Co-Ocurrence of Mycotoxins in Rainbow Trout (*Oncorhynchus mykiss*) Feeds. *Toxins, 7,* 4595-4609. https://doi.org/10.3390/toxins7114595

Gupta, A., Wilkens, S., Adcock, J. L., Puri, M., & Barrow, C. J. (2013). Pollen baiting facilitates the isolation of marine thraustochytrids with potential in omega-3 and biodiesel production. *Journal of Industrial Microbiology & Biotechnology*, *40*(11), 1231–1240. https://doi.org/10.1007/s10295-013-1324-0

Hai, N. V. (2015). The use of probiotics in aquaculture. *Journal of Applied Microbiology*, *119*(4), 917–935. https://doi.org/10.1111/jam.12886

Hansen, J. Ø, Hofossæter, M., Sahlmann, C., Ånestad, R., Reveco-Urzua, F. E., Press, C. M., Mydland, L. T. & Øverland, M. (2019). Effect of *Candida utilis* on growth and intestinal health of Atlantic salmon (*Salmo salar*) parr. *Aquaculture*. *511*. 734239. https://doi.org/10.1016/j.aquaculture.2019.734239

Harrison, J. P., Schratzberger, M., Sapp, M. & Osborn, A. M. (2014). Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms. *BMC Microbiology, 14:*232. https://doi.org/10.1186/s12866-014-0232-4

Hess-Erga, O.-K. (2010). Recolonization by heterotrophic bacteria after UV irradiation or ozonation of seawater; a simulation of ballast water treatment. *Water Research*, *44*(18), 5439–5449. https://doi.org/10.1016/j.watres.2010.06.059

Heuer, H., Wieland, G., Schönfeld, J., Schönwälder, A., Gomes, N. C. M., & Smalla, K. (2001). Bacterial community profiling using DGGE or TGGE analysis. In: P. A. Rochelle (Ed.), *Environmental molecular microbiology: Protocols and applications* (Ch. 13, pp. 177–190).

Hjelm, M., Bergh, Ø., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birkbeck, H., & Gram, L. (2004). Selection and Identification of Autochthonous Potential Probiotic Bacteria from Turbot Larvae (*Scophthalmus maximus*) Rearing Units. *Systematic and Applied Microbiology*, *27*(3), 360–371. https://doi.org/10.1078/0723-2020-00256

Ina-Salwany, M. Y., Al-saari, N., Mohamad, A., Mursidi, F., Mohd-Aris, A., Amal, M. N. A., Kasai, H., Mino, S., Sawabe, T., & Zamri-Saad, M. (2019). Vibriosis in Fish: A Review on Disease Development and Prevention. *Journal of Aquatic Animal Health*, *31*(1), 3–22. https://doi.org/10.1002/aah.10045

Jensen, S., Duperron, S., Birkeland, N.-K., & Hovland, M. (2010). Intracellular *Oceanospirillales* bacteria inhabit gills of *Acesta* bivalves. *FEMS Microbiology Ecology*, *74*(3), 523–533. https://doi.org/10.1111/j.1574-6941.2010.00981.x

Kamada, N., Chen, G. Y., Inohara, N., & Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology*, *14*, 685–690. https://doi.org/10.1038/ni.2608

Kamilya, D., Baruah, A., Sangma, T., Chowdhury, S., & Pal, P. (2015). Inactivated Probiotic Bacteria Stimulate Cellular Immune Responses of Catla, *Catla catla* (Hamilton) In Vitro. *Probiotics and Antimicrobial Proteins*, *7*, 101–106. https://doi.org/10.1007/s12602-015-9191-9

Kandel, P. P., Pasternak, Z., van Rijn, J., Nahum, O., & Jurkevitch, E. (2014). Abundance, diversity and seasonal dynamics of predatory bacteria in aquaculture zero discharge systems. *FEMS Microbiology Ecology*, *89*(1), 149–161. https://doi.org/10.1111/1574-6941.12342

Karl, J. P., Hatch, A. M., Arcidiacono, S. M., Pearce, S. C., Pantoja-Feliciano, I. G., Doherty, L. A., & Soares, J. W. (2018). Effects of Psychological, Environmental and Physical Stressors on the Gut Microbiota. *Frontiers in Microbiology*, *9*, 2013. https://doi.org/10.3389/fmicb.2018.02013

Kennedy, S. R., Prost, S., Overcast, I., Rominger, A. J., Gillespie, R. G. & Krehenwinkel H. (2020). High-throughput sequencing for community analysis: the promise of DNA barcoding to uncover diversity, relatedness, abundances and interactions in spider communities. *Development Genes and Evolution, 230,* 185-201. https://doi.org/10.1007/s00427-020-00652-x

Kim, J., Sudbery, P. (2011). *Candida albicans,* a Major Human Fungal Pathogen. *The Journal of Microbiology. 49*(2), 171-177. https://doi.org/10.1007/s12275-011-1064-7

Kühsel, S. & Blüthgen, N. (2015). High diversity stabilizes the thermal resilience of polinator communities in intensively managed grasslands. *Nature Communications, 6,* 7989. https://doi.org/10.1038/ncomms8989

Kurzai, O., Keith, P., Hoog, S. de, Abele-Horn, M., & Frosch, M. (2008). Postmortem isolation of *Pseudotaeniolina globosa* from a patient with aortic aneurysm. *Mycoses*, *46*(3–4), 141–144. https://doi.org/10.1046/j.1439-0507.2003.00866.x

Lauzon, H. L., Pérez-Sánchez, T., Merrifield, D. L., Ringø, E., & Balcázar, J. L. (2014). Probiotic Applications in Cold Water Fish Species. In: D. Merrifield & E. Ringø (Eds.), *Aquaculture Nutrition* (Ch. 9, pp. 223–252). https://doi.org/10.1002/9781118897263.ch9

Lazado, C. C., Caipang, C. M. A., Gallage, S., Brinchmann, M. F., & Kiron, V. (2010). Expression profiles of genes associated with immune response and oxidative stress in Atlantic cod, *Gadus morhua* head kidney leukocytes modulated by live and heat-inactivated intestinal bacteria. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *155*(3), 249–255. https://doi.org/10.1016/j.cbpb.2009.11.006

Lee, N. K., Kim, W. S. & Paik, H. D. (2019). *Bacillus* strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. *Food Science Biotechnology, 28*, 1297-1305. https://doi.org/10.1007/s10068-019-00691-9.

Lee, J., Kim, I.-S., Emmanuel, A., & Koh, S.-C. (2019). Microbial valorization of solid wastes from a recirculating aquaculture system and the relevant microbial functions. *Aquacultural Engineering*, *87*, 102016. https://doi.org/10.1016/j.aquaeng.2019.102016

Lekang, O.-I. (2013). Traditional Recirculation and Water Re-use Systems. In: *Aquaculture Engineering* (2nd ed., Ch. 14, pp. 190–200). https://doi.org/10.1002/9781118496077.ch14

Liao, H., Lin, X., Li, Y., Qu, M., & Tian, Y. (2020). Reclassification of the Taxonomic Framework of Orders *Cellvibrionales*, *Oceanospirillales*, *Pseudomonadales*, and *Alteromonadales* in Class *Gammaproteobacteria* through Phylogenomic Tree Analysis. *mSystems*, *5*(5), e00543-20. https://doi.org/10.1128/mSystems.00543-20

Lin, S.-R., Chen, Y.-H., Tseng, F-J., Weng, C.-F. (2020). The production and bioactivity of prodigiosin: *quo vadis?*. *Drug Discovery Today, 25*(5), 828-836. https://doi.org/10.1016/j.drudis.2020.03.017.

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P. C. & Harris, R. N. (2014). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Frontiers in Microbiology. 5.* 441. https://doi.org/10.3389/fmicb.2014.00441

Madaro, A., Olsen, R. E., Kristiansen, T. S., Ebbesson, L. O. E., Nilsen, T. O., Flik, G., & Gorissen, M. (2015). Stress in Atlantic salmon: Response to unpredictable chronic stress. *Journal of Experimental Biology*, *218*, 2538–2550. https://doi.org/10.1242/jeb.120535

Martins, P., Cleary, D. F. R., Pires, A. C. C., Rodrigues, A. M., Quintino, V., Calado, R., & Gomes, N. C. M. (2013). Molecular Analysis of Bacterial Communities and Detection of Potential Pathogens in a Recirculating Aquaculture System for *Scophthalmus maximus* and *Solea senegalensis*. *PLoS ONE*, *8*(11), e80847. https://doi.org/10.1371/journal.pone.0080847

Martins, P., Coelho, F. J. R. C., Cleary, D. F. R., Pires, A. C. C., Marques, B., Rodrigues, A. M., Quintino, V., & Gomes, N. C. M. (2018). Seasonal patterns of bacterioplankton composition in a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system. *Aquaculture*, *490*, 240–250. https://doi.org/10.1016/j.aquaculture.2018.02.038

Midilli, A., Kucuk, H., Dincer, I. (2011). Environmental and sustainability aspects of a recirculating aquaculture system. *Environmental Progress & Sustainable Energy, 31*(4), 604-611. https://doi.org/10.1002/ep.10580

Milhazes-Cunha, H., & Otero, A. (2017). Valorisation of aquaculture effluents with microalgae: The Integrated Multi-Trophic Aquaculture concept. *Algal Research*, *24, Part B*, 416–424. https://doi.org/10.1016/j.algal.2016.12.011

Morey, J. N., Boggero, I. A., Scott, A. B., & Segerstrom, S. C. (2015). Current directions in stress and human immune function. *Current Opinion in Psychology*, *5*, 13–17. https://doi.org/10.1016/j.copsyc.2015.03.007

Müller, F. D., Beck, S., Strauch, E. & Linscheid, M. W. (2011). Bacterial Predators Possess Unique Membrane Lipid Structures. *Lipids. 46*. 1129-1140. https://doi.org/10.1007/s11745-011-3614-5

Nagahama, T., Hamamoto, M., Nakase, T., Shimamura, S., & Horikoshi, K. (2006). Phylogenetic relationship within the Erythrobasidium clade: Molecular phylogenies, secondary structure, and intron positions inferred from partial sequences of ribosomas RNA and elongation factor-1a genes. *Journal of General and Applied Microbiology*, *52*(1), 37–45. https://doi.org/10.2323/jgam.52.37

Nakano, T., & Wiegertjes, G. (2020). Properties of Carotenoids in Fish Fitness: A Review. *Marine Drugs*, *18*(11), 568. https://doi.org/10.3390/md18110568

Navarrete, P., Vergara & Tovar-Ramírez, D. (2014). Use of yeasts as probiotics in fish aquaculture. In: M. P. Hernandez-Vergara & C. I. Perez-Rostro (eds.) *Agricultural and biological sciences-sustainable aquaculture techniques*. (pp. 135-172). https://doi.org/10.5772/57196

Offret, C., Desriac, F., Le Chevalier, P., Mounier, J., Jégou, C., & Fleury, Y. (2016). Spotlight on Antimicrobial Metabolites from the Marine Bacteria *Pseudoalteromonas*: Chemodiversity and Ecological Significance. *Marine Drugs*, *14*(7), 129. https://doi.org/10.3390/md14070129

Offret, C., Rochard, V., Laguerre, H., Mounier, J., Huchette, S., Brillet, B., Le Chevalier, P., & Fleury, Y. (2019). Protective Efficacy of a *Pseudoalteromonas* Strain in European Abalone, *Haliotis tuberculata*, Infected with *Vibrio harveyi* ORM4. *Probiotics and Antimicrobial Proteins*, *11*(1), 239–247. https://doi.org/10.1007/s12602-018-9389-8

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2019). vegan: Community Ecology Package. *R package version 2.5-6* https://CRAN.R-project.org/package=vegan

Pachaiyappan, A., Sadhasivam, G., Kumar, M. & Muthuvel, A. (2020). Biomedical Potential of Asthaxanthin from Novel Endophytic Pigment Producing Bacteria *Pontibacter korlensis* AG6. *Waste and Biomass Valorization*. https://doi.org/10.1007/s12649-020-01169-0

Palomo-Vélez, G., Tybur, J. M., van Vugt, M. (2018). Unsustainable, unhealthy, or disgusting? Comparing different persuasive messages against meat consumption. *Journal of Environmental Psychology, 58, 63-71*. https://doi.org/10.1016/j.jenvp.2018.08.002

Pangallo, D., Bučková, M., Kraková, L., Puškárová, A., Šaková, N., Grivalský, T., Chovanová, K. and Zemánková, M. (2015), Microbial survey of epoxy resin sculpture. *Environmental Microbiology*, 17: 462-479. https://doi.org/10.1111/1462-2920.12523

Pasnik, D. J., Evans, J. J., & Klesius, P. H. (2009). Stress in Fish. In: H. E. Roberts (Ed.), *Fundamentals of Ornamental Fish Health* (pp. 33–38).

Pérez-Sánchez, T., Mora-Sánchez, B., & Balcázar, J. L. (2018). Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends in Microbiology*, *26*(11), 896–903. https://doi.org/10.1016/j.tim.2018.05.002

Pinheiro, R., Rodrigues, A., Santos, J., Costa, J., Pereyra, C., Torres, A., Rosa, C., Santos, A. & Muratori, M.. (2018). Occurrence and diversity of yeast species isolated from fish feed and tambatinga gut. *Latin American Journal of Aquatic Research*. *46*(4), 837-842. https://doi.org/10.3856/vol46-issue4-fulltext-22

Porsby, C. H., Webber, M. A., Nielsen, K. F., Piddock, L. J. V., & Gram, L. (2011). Resistance and Tolerance to Tropodithietic Acid, an Antimicrobial in Aquaculture, Is Hard To Select. *Antimicrobial Agents and Chemotherapy*, *55*(4), 1332-7. https://doi.org/10.1128/AAC.01222-10

Quigley, E. M. M. (2013). Gut Bacteria in Health and Disease. *Gastroenterology & Hepatology*, *9*(9), 560-569.

R Core Team. (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. Vienna, Austria. https://www.R-project.org/

Rajilić-Stojanović, M. and de Vos, W.M. (2014), The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev, 38: 996-1047. https://doi.org/10.1111/1574-6976.12075.

Ramos-Garza, J., Bustamante-Brito, R., Ángeles de Paz, G., Medina-Canales, Ma. G., Vásquez-Murrieta, M. S., Wang, E. T., & Rodríguez-Tovar, A. V. (2016). Isolation and characterization of yeasts associated with plants growing in heavy-metal- and arsenic-contaminated soils. *Canadian Journal of Microbiology*, *62*(4), 307–319. https://doi.org/10.1139/cjm-2015-0226

Randall, D. J. & Tsui, T. K. N. (2002). Ammonia toxicity in fish. *Marine pollutions bulletin, 45*(1-12), 17-23. https://doi.org/10.1016/S0025-326X(02)00227-8.

Reverter, M., Tapissier-Bontemps, N., Sarter, S., Sasal, P., Caruso, D. (2020). Moving towards more sustainable aquaculture practices: a meta-analysis on the potential of plant-enriched diets to improve fish growth, immunity and disease resistance. *Reviews in Aquaculture*, *13*(1), 537-555. https://doi.org/10.1111/raq.12485

Rosenberg E. (2014) The Family Chitinophagaceae. In: Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F. (eds) The Prokaryotes. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-38954-2_137.

Richards, G. P., Watson, M. A., Needleman, D. S., Uknalis, J., Boyd, E. F., Fay, J. P. (2017). Mechanisms for *Pseudoalteromonas piscicida*-induced killing of vibrios and other bacterial pathogens. *Applied Environmental Microbiology*. 83:e00175-17. https://doi.org/10.1128/AEM.00175-17

Ringø E, Bendiksen HR, Gausen SJ, Sundsfjord A & Olsen RE. (1998). The effect of dietary fatty acids on lactic acid bacteria associated with the epithelial mucosa and from faecalia of Arctic charr, Salvelinus alpinus (L.). *Journal of Applied Microbiology, 85*(5):855-64. https://doi.org/10.1046/j.1365-2672.1998.00595.x.

Roquigny, R., Mougin, J., Le Bris, C., Bonnin-Jusserand, M., Doyen, P., & Grard, T. (2021). Characterization of the marine aquaculture microbiome_ A seasonal survey in a seabass farm. *Aquaculture*, *531*, 735987. https://doi.org/10.1016/j.aquaculture.2020.735987

Rossi, L. T., Sharpen, A. R., Zimmermann, J. A., Olivero, C. R., Zbrun, M. V., Frizzo, L. S., Signorini, M. L., Bacchetta, C., Cian, R. E., Cazenave, J., Soto, L. P., & Drago, S. R. (2020). Intestinal microbiota modulation in juvenile Pacú (*Piaractus mesopotamicus*) by supplementation with *Pyropia columbina* and β-carotene. *Aquaculture International*, *28*, 1001–1016. https://doi.org/10.1007/s10499-020-00508-1

Ruan, Y.-J., Guo, X.-S., Ye, Z.-Y., Liu, Y., & Zhu, S.-M. (2015). Bacterial Community Analysis of Different Sections of a Biofilter in a Full-Scale Marine Recirculating Aquaculture System. *North American Journal of Aquaculture*, *77*, 318–326. https://doi.org/10.1080/15222055.2015.1017128

Sadat Naghavi, F., Hanachi, P., Soudi, M. R., Saboora, A., & Ghorbani, A. (2013). Evaluation of the Relationship between the Incubation Time and Carotenoid Production in *Rhodotorula Slooffiae* and *R. Mucilaginosa* Isolated from Leather Tanning Wastewater. *Iranian journal of basic medical sciences, 16*(10), 1114–1118.

Santos, L., & Ramos, F. (2018). Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. *International Journal of Antimicrobial Agents*, *52*(2), 135–143. https://doi.org/10.1016/j.ijantimicag.2018.03.010

Setiyono, E., Adhiwibawa, M. A. S., Indrawati, R., Prihastyanti, M. N. U., Shioi, Y., & Brotosudarmo, T. H. P. (2020). An Indonesian Marine Bacterium, *Pseudoalteromonas*

rubra , Produces Antimicrobial Prodiginine Pigments. *ACS Omega*, *5*(9), 4626–4635. https://doi.org/10.1021/acsomega.9b04322

Shinn, A. P., Pratoomyot, J., Griffiths, D., Trong, T. Q., Vu, N. T., Jiravanichpaisal, P., & Briggs, M. (2018). Asian Shrimp Production and the Economic Costs of Disease. *Asian Fisheries Science*, *31S*, 29–58. https://doi.org/10.33997/j.afs.2018.31.S1.003

Singh, P., Raghukumar, C., Verma, P., & Shouche, Y. (2010). Phylogenetic diversity of culturable fungi from the deep-sea sediments of the Central Indian Basin and their growth characteristics. *Fungal Diversity*, 40, 89-102. https://doi.org/10.1007/s13225-009-0009-5

Singh, S. T., Kamilya, D., Kheti, B., Bordoloi, B., & Parhi, J. (2017). Paraprobiotic preparation from *Bacillus amyloliquefaciens* FPTB16 modulates immune response and immune relevant gene expression in *Catla catla* (Hamilton, 1822). *Fish & Shellfish Immunology*, *66*, 35–42. https://doi.org/10.1016/j.fsi.2017.05.005

Szajewska H., Konarska, Z. & Kolodziej, M., (2016). Probiotic Bacterial and Fungal Strains: Claims with Evidence. *Digestive Diseases, 34,* 251-259. https://doi.org/10.1159/000443359.

Småge, S. B., Frisch, K., Brevik, Ø. J., Watanabe, K., & Nylund, A. (2016). First isolation, identification and characterisation of *Tenacibaculum maritimum* in Norway, isolated from diseased farmed sea lice cleaner fish *Cyclopterus lumpus* L. *Aquaculture. 464,* 178-184. https://doi.org/10.1016/j.aquaculture.2016.06.030

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., & Berg, G. (2001). Bulk and Rhizosphere Soil Bacterial Communities Studied by Denaturing Gradient Gel Electrophoresis: Plant-Dependent Enrichment and Seasonal Shifts Revealed. *Applied and Environmental Microbiology*, *67*(10), 4742–4751. https://doi.org/10.1128/AEM.67.10.4742-4751.2001

Sorieul, L., Wabete, N., Ansquer, D., Mailliez, J.-R., Pallud, M., Zhang, C., Lindivat, M., Boulo, V., & Pham, D. (2018). Survival improvement conferred by the *Pseudoalteromonas* sp. NC201 probiotic in *Litopenaeus stylirostris* exposed to *Vibrio nigripulchritudo* infection and salinity stress. *Aquaculture*, *495*, 888–898. https://doi.org/10.1016/j.aquaculture.2018.06.058

Stickney, R. R. (2000). *Encyclopedia of Aquaculture*. John Wiley & Sons, Inc.

Suh, S.-O., Nguyen, N. H., & Blackwell, M. (2008). Yeasts isolated from plant-associated beetles and other insects: Seven novel *Candida* species near *Candida albicans*. *FEMS Yeast Research*, *8*(1), 88–102. https://doi.org/10.1111/j.1567-1364.2007.00320.x

Takashima, M., Deak, T., & Nakase, T. (2001). Emendation of *Dioszegia* with redescription of *Dioszegia hungarica* and two new combinations, *Dioszegia aurantiaca* and *Dioszegia crocea*. *The Journal of General and Applied Microbiology*, *47*(2), 75–84. https://doi.org/10.2323/jgam.47.75

Takashima, M., Van, B. H., An, K.-D. & Ohkuma, M. (2011). *Dioszegia rishiriensis* sp. nov., a novel yeast species from soil collected on Rishiri Island, Hokkaido, Japan. *International Journal of Systematic and Evolutionary Microbiology. 61*(7). 1736-1739. https://doi.org/10.1099.ijs.0.025254-0

Teitge, F., Peppler, C., Steinhagen, D., & Jung-Schroers, V. (2020). Water disinfection by ozonation has advantages over UV irradiation in a brackish water recirculation aquaculture system for Pacific white shrimp (*Litopenaeus vannamei*). *Journal of Fish Diseases*, *43*(10), 1259–1285. https://doi.org/10.1111/jfd.13238

Thomas, F., Hehemann, J.-H., Etienne, R., Mirjam, C., Gurvan, M. (2011). Environmental and Gut Bacteroidetes: The Food Connection. *Frontiers in Microbiology, 2,* 93. https://doi.org/10.3389/fmicb.2011.00093

Timmons, Michael B. & Ebeling, James L. (2010). Introduction to Recirculating Aquaculture Technology. In: *Recirculating Aquaculture* (2nd ed., pp. 1–38).

Tørris, C., Småstuen, M. C., & Molin, M. (2018). Nutrients in Fish and Possible Associations with Cardiovascular Disease Risk Factors in Metabolic Syndrome. *Nutrients*, *10*(7), 952. https://doi.org/10.3390/nu10070952

Tung, H. T., Koshio, S., Teshima, S., Ishikawa, M., Yokoyama, S., Ren, T., Hirose, Y. & Phuong, N. D. T. (2009). Effects of Heat-Killed *Lactobacillus plantarum* Supplemental Diets on Growth Performance, Stress Resistance and Immune Response of Juvenile Kuruma Shrimp *Marsupenaeus japonicus* Bate. *Aquaculture Science*, *57*(2), 175–184. https://doi.org/10.11233/aquaculturesci.57.175

Van Nguyen, N., Onoda, S., Van Khanh, T., Hai, P. D., Trung, N. T., Hoang, L., & Koshio, S. (2019). Evaluation of dietary Heat-killed *Lactobacillus plantarum* strain L-137 supplementation on growth performance, immunity and stress resistance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, *498*, 371–379. https://doi.org/10.1016/j.aquaculture.2018.08.081

Vasconcelos, B., Teixeira, J. C., Dragone, G. & Teixeira, J. A. (2019). Oleaginous yeasts for sustainable lipid production—From biodiesel to surf boards, a wide range of “green” applications. *Applied Microbiology and Biotechnology, 103,* 3651-3667. https://doi.org/10.1007/s00253-019-09742-x

Vestrum, R. I., Attramadal, K. J. K., Winge, P., Li, K., Olsen, Y., Bones, A. M., Vadstein, O., & Bakke, I. (2018). Rearing Water Treatment Induces Microbial Selection Influencing the Microbiota and Pathogen Associated Transcripts of Cod (*Gadus morhua*) Larvae. *Frontiers in Microbiology*, *9*, 851. https://doi.org/10.3389/fmicb.2018.00851

Viegas, C., Esteves, L., Faria, T., Pombo, A., Caetano, L. A., Quintal-Gomes, A., Twarużek, M., Kosicki, R., Grajewski, J., & Viegas, S. (2019). Fungal diversity and mycotoxin distribution in echinoderm aquaculture. *Mycotoxin Research*, *35*, 253–260. https://doi.org/10.1007/s12550-019-00352-0

Vyas S. & Chhabra, M. (2017). Isolation, identification and characterization of *Cystobasidium oligophagum* JRC1: A cellulase and lipase producing oleaginous yeast. *Bioresource Technology, 223*, 250-258. https://doi.org/10.1016/j.biortech.2016.10.03.

Wang, J., Yang, H.-L., Xia, H.-Q., Ye, J. -d., Lu, K.-L., Hu, X., Feng, Y., Ruan, L., & Sun, Y.-Z. (2017). Supplementation of heat-inactivated *Bacillus clausii* DE5 in diets for grouper, *Epinephelus coioides,* improves feed utilization, intestinal and systemic immune responses and not growth performance. *Aquaculture Nutrition*, *24*(2), 821–831. https://doi.org/10.1111/anu.12611

Wang, Q.-M., Begerow, D., Groenewald, M., Liu, X., Theelen, B., Bai, F.-Y., & Boekhout, T. (2015). Multigene phylogeny and taxonomic revision of yeasts and related fungi in the Ustilaginomycotina. *Studies in Mycology*, *81*, 55–83. https://doi.org/10.1016/j.simyco.2015.10.004

Wegh, C. A. M., Geerlings, S. Y., Knol, J., Roeselers, G. & Beizer, C. (2019). Postbiotics and Their Potential Applications in Early Life Nutrition and Beyond. *International Journal of Molecular Sciences. 20*(19):4673. https://doi.org/10.3390/ijms20194673

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697–703. https://doi.org/10.1128/JB.173.2.697-703.1991

White, P. G., Shipton, T. A., Bueno, P. B., & Hasan, M. R. (2018). Better management practices for feed production and management of Nile tilapia and milkfish in the Philippines*. FAO Fisheries and Aquaculture Technical Paper No. 614*. Rome, FAO. 98 pp.

Willey, J., Sherwood, L., & Woolverton, C. J. (2011). Normal Microbiota of the Human Body. In: *Prescott's Microbiology* (6th ed., pp. 735–742).

Williams, H. N., & Piñeiro, S. (2006). Ecology of the Predatory *Bdellovibrio* and Like Organisms. In: E. Jurkevitch (ed.), *Predatory Prokaryotes.* Microbiology Monographs (Vol. 4, pp. 213–248). Springer Berlin Heidelberg. https://doi.org/10.1007/7171_2006_058

Wilson, M. Z., Wang, R., Gitai, Z., & Seyedsayamdost, M. R. (2016). Antibiotic and anticancer activities of TDA. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(6), 1630–1635. https://doi.org/10.1073/pnas.1518034113

Xiao, Y.-K., Yan, Z.-F., Kim, Y., Lee, H.-M., Trinh, H., Yang, J.-E., Won, K.-H., Yi, T.-H., & Kook, M. (2019). *Glaciecola amylolytica* sp. nov., an amylase-producing bacterium isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*, *69*(4), 957–963. https://doi.org/10.1099/ijsem.0.003222

Yan, Y.-Y., Xia, H.-Q., Yang, H.-L., Hoseinifar, S. H., & Sun, Y.-Z. (2015). Effects of dietary live or heat-inactivated autochthonous *Bacillus pumilus* SE5 on growth performance, immune responses and immune gene expression in grouper *Epinephelus coioides*. *Aquaculture Nutrition*, *22*(3), 698–707. https://doi.org/10.1111/anu.12297

Yang, H., Han, Y., Ren, T., Jiang, Z., Wang, F., & Zhang, Y. (2015). Effects of dietary heat-killed *Lactobacillus plantarum* L-137 (HK L-137) on the growth performance, digestive enzymes and selected non-specific immune responses in sea cucumber, *Apostichopus japonicus* Selenka. *Aquaculture Research*, *47*(9), 2814–2824. https://doi.org/10.1111/are.12731

Yue, Y., Yu, H., Li, R., Xing, R., Liu, S., & Li, P. (2015). Exploring the Antibacterial and Antifungal Potential of Jellyfish-Associated Marine Fungi by Cultivation-Dependent Approaches. *PLOS ONE*, *10*(12), e0144394. https://doi.org/10.1371/journal.pone.0144394

Zhang, X.-Y., Bao, J., Wang, G.-H., He, F., Xu, X.-Y., & Qi, S.-H. (2012). Diversity and Antimicrobial Activity of Culturable Fungi Isolated from Six Species of the South China Sea Gorgonians. *Microbial Ecology*, *64*, 617–627. https://doi.org/10.1007/s00248-012-0050-x

Zhou, H., Gai, C., Ye, G., An, J., Liu, K., Xu, L., & Cao, H. (2019). *Aeromonas hydrophila*, an Emerging Causative Agent of Freshwater-Farmed Whiteleg shrimp *Litopenaeus vannamei*. *Microorganisms*, *7*(10), 450. https://doi.org/10.3390/microorganisms7100450

Zhou, T., Chen, D., Li, C., Sun, Q., Li, L., Liu, F., Shen, Q., & Shen, B. (2012). Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. *Microbiological Research*, *167(7)*, 388–394. https://doi.org/10.1016/j.micres.2012.01.003