Interaction between fish probiotic roseobacters and the natural microbiota in aquaculture settings

Dittmann, Karen Kiesbye

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Interaction between fish probiotic roseobacters and the natural microbiota in aquaculture settings

PhD thesis

by

Karen Kiesbye Dittmann

May 2019

Technical University of Denmark

Department of Biotechnology and Biomedicine

Section for Microbial and Chemical Ecology

Bacterial Ecophysiology and Biotechnology group

Preface

This thesis describes the work outcome of my PhD study at the Technical University of Denmark (DTU). It marks the finale of a project which began on June 1st, 2016 and ended on May 31st, 2019. The project was funded by a PhD stipend from DTU.

The work was mainly carried out at the Department of Biotechnology and Biomedicine, DTU, under the supervision of Professor Lone K. Gram and Assistant Professor Mikkel Bentzon-Tilia. It also included at 4 ½ months stay supervised by Associate Professor Suhelen Egan at the at the Centre for Marine Bio-Innovation, School of Biological, Earth and Environmental Sciences, The University of New South Wales in Sydney, Australia.

Karen Kiesbye Dittmann

Kgs. Lyngby, May 2019

This thesis is based on the following papers:

Paper 1:

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

Paper 2:

Dittmann, K.K., Sonnenschein, E.C., Egan, S., Gram, L. & Bentzon-Tilia, M. (2019). Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities. *Environ Microbiol Rep*., **11**(3), 401–413. https://doi.org/10.1111/1758-2229.12698

Paper 3:

Dittmann, K.K., Porsby, C.H., Goncalves, P., Mateiu, R.V., Sonnenschein, E.C., Bentzon-Tilia, M., Egan, S. & Gram, L. (2019). Tropodithietic acid induces oxidative stress response, cell envelope biogenesis and iron uptake in *Vibrio vulnificus*. *Environ Microbiol Rep*. **Accepted**. https://doi.org/10.1111/1758-2229.12771

Paper 4:

Dittmann, K.K., Rasmussen, B.B., Melchiorsen, J., Sonnenschein, E.C., Gram, L. & Bentzon-Tilia, M. (2019). *Roseobacter* probiotics affect lower-trophic level microbiomes in marine aquaculture. Manuscript in preparation.

Furthermore, I have contributed to the following article during my PhD study (not included in this thesis):

Rasmussen, B.B., **Dittmann, K.K.**, Gram, L. & Bentzon-Tilia, M. (2019). Upscaling probiotic *Phaeobacter* spp. in *Tetraselmis suecica* algae cultures. Manuscript in preparation

Conference contributions:

Dittmann, K.K., Sonnenschein, E.C., Gram, L. & Bentzon-Tilia, M. (2016). Impact of tropodithietic acid-producing *Phaeobacter inhibens* on eukaryote-associated microbial communities. Poster presentation at the Annual Congress of the Danish Microbiological Society, 14th November 2016. Copenhagen, Denmark.

Dittmann, K.K., Goncalves, P., Porsby, C.H., Sonnenschein, E.C., Bentzon-Tilia, M., Gram, L. & Egan, S. (2018). The effect of sub-lethal levels of the *Roseobacter* secondary metabolite, tropodithietic acid, on gene expression of *Vibrio vulnificus*. Poster presentation at the 17th International Symposium on Microbial Ecology (ISME17), 12th – 17th August 2018. Leipzig, Germany

Summary

Aquaculture is the fastest growing protein producing sector in the world and this growth is required to feed the growing world population. Microbial diseases are a major bottle-neck in aquaculture, which must be controlled to avoid great, economic losses. Adult fish can be vaccinated against the most common bacterial diseases. However, the vaccines cannot be used on fish larvae because they have underdeveloped immune systems. Antibiotics are commonly used for acute treatment of infection, however, this increases the risk of antibiotic resistance dissemination. Therefore, more sustainable, preventive measures are sought and probiotics has been proposed as one of the solutions. Probiotics are "*live organisms which when administered in adequate amounts confer a health benefit on the host*" (FAO and WHO, 2001). Tropodithietic acid (TDA) producing members of the *Roseobacter* group, such as *Ruegeria* spp. and *Phaeobacter* spp., have potential as probiotics in aquaculture. They have repeatedly been isolated from aquaculture environments and they can reduce mortality of fish larvae challenged with pathogens. However, it is uncertain how the probiotic treatment affects the commensal microbiome of the larvae.

The **purpose** of the present PhD project was to determine how probiotic *Phaeobacter inhibens* affect the natural microbiota in marine eukaryote systems related to aquaculture. Given that roseobacters are commonly found in complex communities of marine eukaryotes in nature and that they are indigenous to the aquaculture environment, the main **hypothesis** of this work is that *P. inhibens* can establish itself in microbiomes associated with aquaculture-related eukaryotes and protect the host with minor impact on the commensal bacteria.

In this study, 16S rRNA amplicon taxonomics was used to characterize the microbiota of different trophic levels – *Tetraselmis suecica* (microalga), *Acartia tonsa* (copepod), and *Scophthalmus maximus* (turbot) larvae – and determine the changes in diversity induced by treatment with probiotic *P. inhibens*. Interestingly, the structure of the microbial community associated with the lower trophic levels were shifted in the presence of *P. inhibens*, though not for the fish larval community. The effect was specific and targeted taxa closely related to the probiotic bacterium. Despite previous studies suspecting the live-feed to be vectors of infection, these microbiotas had low abundance of *Vibrio* spp. commonly causing disease in fish larvae. In contrast, the turbot egg microbiome were dominated by vibrios, however, these were suppressed after 24 hours incubation and kept stable - most likely due to inherent roseobacters or the added probiotic.

In nature, members of the *Roseobacter* group are often found in association with marine eukaryotes such as algae and molluscs. Secondary metabolite production is believed to be involved in these interactions, though it is uncertain how they shape the microbiome. In microalgal blooms, roseobacters increase in abundance, which suggests that they play a role in the course of the bloom and they likely impact the microbiome. In this study, two model systems – *Emiliania huxleyi* (microalga) and *Ostrea edulis* (European flat oysters) – were used to study how the secondary metabolite producer *P. inhibens* affects the diversity and composition of the associated microbiomes. Roseobacters were indigenous to both communities and addition of *P. inhibens* caused substantial changes in the structure of the low-complexity microbiome of *E. huxleyi*, though not to the more complex oyster microbiomes. The impact was specific to vibrios and pseudoalteromonads, which were decreased in abundance.

The role of TDA in host-bacteria, bacteria-bacteria interactions is unknown. A mode of action has been proposed for TDA, but it is based on studies of *Escherichia coli* rather than marine, non-TDA-producing bacteria which are more likely to encounter TDA in their surroundings. In this study, a transcriptomics approach was used to study how a sub-lethal concentration of TDA affected the fish and human pathogen, *Vibrio vulnificus*. Exposure to TDA triggered a defense response to reactive oxygen species and iron depletion in *V. vulnificus*. Furthermore, there were indications of switch to a biofilm phenotype, which could explain why inherent resistance and tolerance is rarely observed.

This thesis concludes that TDA-producing *P. inhibens* causes minor impact on the microbiomes of various marine eukaryotes. The changes are highly specific to the commensal microbiome; in part decreasing related taxa, in part decreasing the abundance of putative pathogens such as vibrios. The molecular mechanism of TDA and role is still uncertain, but these data indicate that TDA induces a phenotypic switch in the target organism to protect the cells. Given the ease of introduction, the targeted effect, and the lack of resistance development, the application of *P. inhibens* as probiotic in aquaculture is highly promising.

Resumé (in Danish)

Akvakultur er den hurtigst voksende, protein-producerende sektor i verden og den vækst er nødvendig for at brødføde den voksende verdensbefolkning. Mikrobielle sygdomme er en markant flaskehals i akvakultur og de skal holdes under kontrol for at undgå store, økonomiske tab. Voksne fisk kan vaccineres mod de mest almindelige bakterielle sygdomme. Disse vacciner virker dog ikke på fiskelarver, idet de har underudviklede immunforsvar. Antibiotika bliver almindeligvis brugt mod infektioner i udbrud, men dette øger risikoen for spredning af antibiotikaresistens. Mere bæredygtige, forebyggende metoder er derfor efterspurgt og probiotika er en af de foreslåede løsninger. Probiotika er "levende organismer, som, når de administreres i passende mængder, giver en sundhedsfordel til værten" (FAO and WHO, 2001). Tropodithietic acid (TDA) producerende medlemmer af *Roseobacter* gruppen, såsom *Ruegeria* spp. og *Phaeobacter* spp., har potentiale som probiotika i akvakultur. De er gentagne gange blevet isoleret fra akvakulturmiljøer og de kan reducere dødeligheden blandt fiskelarver inficeret med patogener. Det er dog uvist, hvordan den probiotiske behandling påvirker larvernes kommensale mikrobiomer.

Formålet med dette PhD studium var at klarlægge, hvordan probiotiske *Phaeobacter inhibens* påvirker den naturlige mikrobiota i marine eukaryote systemer relateret til akvakultur. Givet at roseobactere almindeligvis findes i komplekse mikrobielle samfund i og på marine eukaryoter i naturen, og at de er naturligt forekommende i akvakultursystemer, er den primære **hypotese** for dette arbejde, at *P. inhibens* kan etablere sig i mikrobiomer tilhørende akvakulturrelaterede eukaryoter og beskytte værten uden betydelig påvirkning af de kommensale bakterier.

I dette studium blev 16S rRNA amplicon taxonomics brugt til at karakterisere mikrobiotaerne relateret til de forskellige trofiske niveauer – *Tetraselmis suecica* (mikroalger), *Acartia tonsa* (vandlopper) og *Scophthalmus maximus* (pighvarlarver) – samt til at klarlægge diversitetsændringer forårsaget af behandling med probiotiske *P. inhibens*. Strukturen af mikrobielle samfund relateret til lavere trofiske niveauer ændrede sig ved tilstedeværelsen af *P. inhibens*, men dette skete ikke for fiskelarvemikrobiomet. Effekten var specifik og målrettet taksonomiske grupper, der er nært beslægtede med den probiotiske bakterie. Til trods for, at tidligere studier har mistænkt foderorganismer for at være smittebærere, var mængden af *Vibrio* spp., der ofte forårsager sygdom i fiskelarver, lav i disse systemer. Derimod var pighvaræg-mikrobiomet domineret af vibrioer. Disse blev dog undertrykt i løbet

af 24 timers inkubation og holdt på et stabilt niveau – sandsynligvis grundet tilstedeværelsen af naturlige roseobactere eller den tilsatte probiotiske bakterie.

I naturen er medlemmer af *Roseobacter* gruppen ofte observeret i association med marine eukaryoter såsom alger og bløddyr. Man mener, at produktion af sekundære metabolitter er involveret i disse interaktioner, men det er uvist, hvordan de påvirker mikrobiomet. I mikroalgeopblomstringer øges tilstedeværelsen af roseobactere, hvilket indikerer, at de spiller en rolle i opblomstringens forløb, og at de sandsynligvis påvirker mikrobiomet. I dette studium blev to modelsystemer – *Emiliania huxleyi* (mikroalgen) og *Ostrea edulis* (Europæiske fladøsters) – brugt til at undersøge, hvordan den sekundære metabolit-producerende *P. inhibens* påvirker diversiteten og sammensætningen af mikrobiomerne tilknyttet modelorganismerne. Roseobactere tilhørte de kommensale mikrobiomer. Tilføjelsen af *P. inhibens* forårsagede betydelige ændringer i strukturen af det mindre komplekse *E. huxleyi* mikrobiom, men ikke i det mere komplekse østers mikrobiom. Indvirkningen var specifik mod vibrioer og pseudoalteromonader, hvis tilstedeværelse blev mindsket.

TDAs rolle i interaktioner mellem vært og bakterier, samt mellem bakterier og andre bakterier er ukendt. En virkningsmekanisme for TDA er blevet foreslået, men den er baseret på studier af *Escherichia coli* fremfor marine, ikke-TDA-producerende bakterier, som med større sandsynlighed vil støde på TDA i deres omgivelser. I dette studium blev transkriptomundersøgelser anvendt til at undersøge, hvordan en ikke-dræbende koncentration af TDA påvirker den fiske- og humanpatogene bakterie, *Vibrio vulnificus*. Eksponering for TDA udløste et forsvarsrespons imod oxidanter (”reactive oxygen species”) og jernmangel i *V. vulnificus*. Derudover var der indikationer på et skift til en biofilm fænotype, hvilket kan forklare, hvorfor nedarvet resistens og tolerance sjældent er set.

Denne afhandling konkluderer, at TDA-producerende *P. inhibens* har minimal indvirkning på mikrobiomer relateret til forskellige marine eukaryoter. Ændringerne er yderst specifikke i det kommensale mikrobiom; til dels mindskes mængden af nærtbeslægtede taksonomiske grupper, til dels mindskes mængden af potentielle patogener såsom vibrioer. TDAs molekylære virkningsmekanisme og dets rolle er endnu uvis, men disse data indikerer at TDA inducerer et fænotypisk skift for at beskytte cellerne. Letheden af introduktion, den målrettede effekt og manglen på resistensudvikling er lovende for anvendelsen af *P. inhibens* som probiotika i akvakultur.

Table of Contents

Preface i

Summary iii

Resumé (in Danish) v

1. Introduction & outline 1

2. Microbiomes in aquaculture 5

2.1. Microbiomes associated with reared fish 6

2.2. Methods to study microbiomes 9

2.2.1. 16S rRNA amplicon taxonomics 10

2.3. Conclusions 11

3. Microbiome management in aquaculture 12

3.1. Water conditioning and bioremediation 12

3.2. Probiotics in aquaculture 14

3.2.1. Roseobacters as probiotics in aquaculture 19

3.3. Commercial application of microbiome management in aquaculture 24

3.4. Conclusions 26

4. Roseobacters & TDA 28

4.1. Colonization of surfaces and interactions with eukaryotes 29

4.2. Tropodithietic acid 34

4.2.1. Activity spectrum of TDA 35

4.2.2. The mode of action for TDA 35

4.2.3. Impact of TDA at sub-inhibitory concentrations and potential function 36

4.2.4. Resistance to TDA 39

4.3. Conclusions 40

5. Concluding remarks and future perspectives 42

6. Acknowledgements 45

7. References 46

Paper 1

Paper 2

Paper 3

Paper 4

1. Introduction & outline

The world population is growing and is expected to reach 9.8 billion individuals by 2050 (United Nations, Department of Economic and Social Affairs, 2017). This increases the demand for food production, especially high-quality protein such as fish. Wild fish and shellfish reservoirs are depleting; in 2015, 93 % of the fish stocks were either maximally, sustainably fished (59.9 %) or over-exploited (33.1 %) (FAO, 2018). Farmed fish is an alternative solution to meet the demand. The aquaculture industry is rapidly growing and the amount of farmed fish produced for human consumption surpassed the wild catches a few years ago (Figure 1). By 2030, the aquaculture sector is projected to reach 109 million tonnes of product output (FAO, 2018). The increasing demand in combination with an increased focus on sustainability and ethics from the general public put pressure on the aquaculture industry to deliver high quantities of quality outputs through environmentally desirable production.

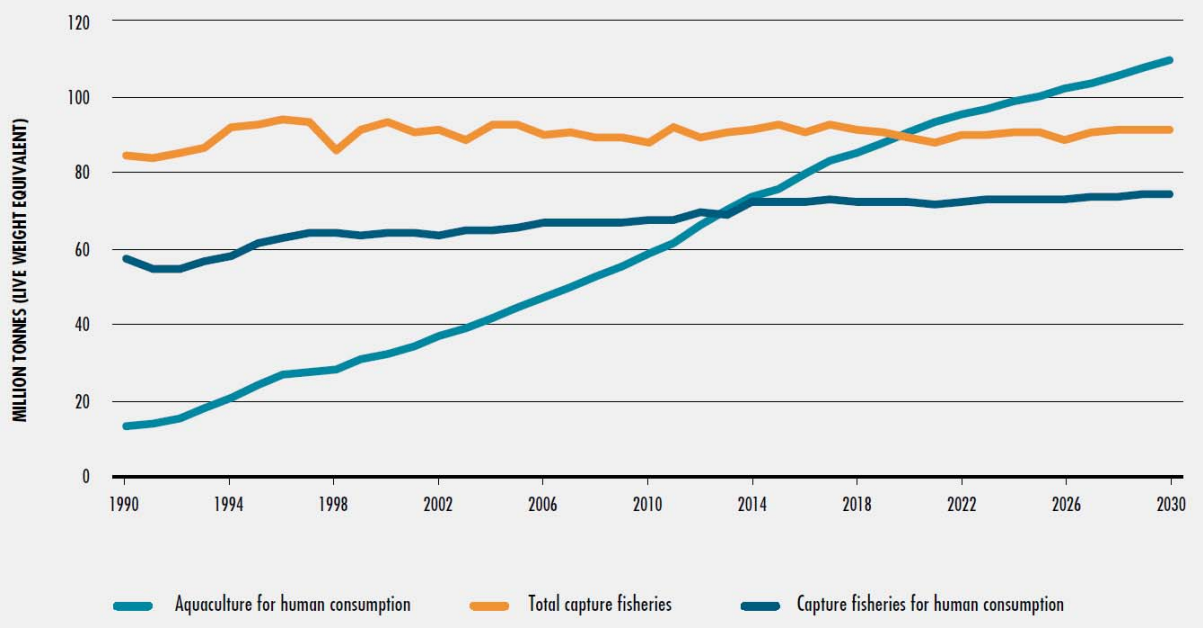


Figure 1: Global aquaculture production and capture fisheries for the period 1990 to 2030. The blue graphs reflect the aquaculture (light shade) and capture fisheries (dark shade) for human consumption. The orange graph reflects the total capture fisheries. Modified from FAO (2018).

From hatching of the eggs to full-grown adults, farmed fish are reared in different tanks or nets with many individuals in a confined space. The intense farming increases the environmental and social stressors, which, as a consequence, makes the fish more vulnerable

to infections and spread of disease. Bacteria are the most common causes (about 55 %) of disease, though viral, fungal, and parasitic infections are also observed in these systems (Kibenge et al., 2012). Many bacteria can cause disease in fish, however, members of the genus *Vibrio* are common fish pathogens in aquaculture (Thompson et al., 2004; Toranzo et al., 2005) and also the target pathogen of the work in this thesis.

Antibiotics are deployed in the event of acute infection. In some countries, the use of antibiotics as prophylactic treatment is still permitted and applied (Cabello, 2006; Cabello et al., 2013; Miranda et al., 2018). Stricter regulation landscapes do exist, such as in the European Union (EU), where the EU Veterinary Medicinal Products Directive has banned the non-therapeutic prophylactic use of antibiotics in 2001 (Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products). However, the use of antibiotics increases the selective pressure for and the risk of spreading of antibiotic resistance among the commensal microbiota members (Cabello et al., 2013; Higuera-Llantén et al., 2018; Miranda et al., 2018). This should be avoided given the antibiotic crisis we are facing (Cooper & Shlaes, 2011); an increasing number of observed multi-drug resistant pathogens combined with a lack of new antibiotics being developed.

Alternatives are sought to circumvent the use of antibiotics to minimize economical losses and bacterial antibiotic resistance occurrence. Vaccines have been developed and are working against the most common pathogens in adult fish (Ringø et al., 2014; Sommerset et al., 2005). The deployment of vaccines in combination with stricter regulatory oversight of antimicrobial use and aquaculture management (i.e. hygiene and biosecurity) has decreased the antimicrobial use in the Norwegian aquaculture by 99 % from 1987 to 2013 despite a major increase in the production output (300,000 tonnes in 1996; 1.2 mio. tonnes in 2013) (Norwegian Ministry of Health and Care Services, 2015; The review on antimicrobial resistance, 2015). However, these vaccines are not working on fish larvae given their underdeveloped immune systems. Thus, other preventive measures are needed. Bacteriophage therapy (Rørbo et al., 2018; Silva et al., 2014; Tan et al., 2014), Quorum Sensing (QS) disruption (Zhao et al., 2015; Zhao et al., 2018), enrichments (Crab et al., 2010; Crab et al., 2012; Hari et al., 2004; Xu et al., 2013), and probiotics (D'Alvise et al., 2013; Grotkjær, Bentzon-Tilia, D'Alvise, Dierckens, et al., 2016) are some of the proposed solutions. Probiotics – the use of beneficial bacteria that when applied have a beneficial effect on the host (FAO and WHO, 2001) – have been studied for decades and their effects have

been tested in many, different kinds of aquaculture-related systems. Most studies have focused on improving the gut microbiome of the farmed fish by deployment of Firmicutes, though their origin is not necessarily marine.

Bioactive members of the Gram-negative *Roseobacter* group have been proposed as probiotics in marine systems. Particularly, the tropodithietic acid (TDA) producing genus *Phaeobacter* has repeatedly shown promising efficiency in warding off pathogenic *Vibrio* spp. while imposing minimal effect on the live-feed for the fish larvae and the fish larvae (D'Alvise et al., 2012, 2013; Hjelm et al., 2004). Resistance to TDA is difficult to induce (Porsby et al., 2011; Rasmussen et al., 2016), though tolerance has been observed (Dittmann, Sonnenschein et al., 2019; Harrington et al., 2014). The mechanism of action of TDA on marine bacteria, as well as the impact of TDA-producers on the inherent microbiota found in aquacultures, remain to be understood. The microbiota of, for instance, algae used as live-feed in aquaculture is central to the growth and well-being of the algae. It is therefore of great importance to understand how the addition of a probiotic organism (over extended periods of time) affects the commensal microbiota and not just the target pathogen. In this particular study, the activity of the probiotic bacteria is assumed to be caused, predominantly, by one molecule, TDA. Understanding the mechanism of action (on other bacteria) of this molecule is also a way in which potential short- and long-term effects on the commensal microbiota can be assessed.

The **purpose** of this PhD project was to determine how probiotic *Phaeobacter inhibens* affect the natural microbiota in marine eukaryote systems related to aquaculture. Given that roseobacters are commonly found in complex communities of marine eukaryotes in nature, and that they are indigenous to the aquaculture environment, though in low abundance, the main **hypothesis** of this work is that *P. inhibens* can establish itself in microbiomes associated with aquaculture-related eukaryotes and protect the host with minor impact on the commensal bacteria. The main part of the work has focused on microbiome characterization (paper 2 and 4). Another part relates to the influence and mechanism of action of TDA on pathogenic *Vibrio vulnificus* by a transcriptomic approach (paper 3).

This thesis consists of an overview section and four papers/manuscripts. The overview section introduces microbiomes, microbiome management, probiotics, and the probiotic species investigated in this project. Chapter 2 defines microbiomes in an aquaculture context including state-of-the-art technologies available to study these complex systems. Based on

this knowledge, chapter 3 is focused on management of microbiomes and the exploitation of beneficial bacteria (probiotics) towards favorable conditions in aquaculture settings. Chapter 4 describes the *Roseobacter* group, particularly focused on the members producing TDA and how they interact with other bacteria as well as eukaryotes. The experimental work and results obtained during this project are summarized in paper 2, 3, and 4, while highlights of the results are also included in this thesis.

The overall **goals** of this research is to 1) provide more knowledge on the microbiotas related to aquacultures, 2) understand how the addition of TDA-producing *P. inhibens* changes the bacterial microbiome diversity and determine the target-spectrum of the probiotic effect, and 3) elucidate the mechanism of action of TDA in relation to marine, non-TDA-producers. This knowledge is essential for the risk assessment of *P. inhibens* with regards to future applications in aquaculture.

2. Microbiomes in aquaculture

Farming of fish and shellfish in aquacultures creates a unique microbial environment. Every batch of reared animals comes into a "new" environment - cleaned and disinfected ponds or tanks – where levels of dissolved organic matter quickly rise. At certain life stages, the fish and shellfish are moved to new tanks and the cycle is restarted. While this discontinuous culture cycle is likely stressful to the reared animals, it also affects the microbial community associated with the fish. The high nutrient levels and repeated disinfection between batches promote proliferation of fast-growing opportunistic bacteria rather than a stable microbial community (Skjermo & Vadstein, 1999; Verschuere et al., 2000).

About 10 % to 15 % of fish larvae survive and grow into juveniles in the aquaculture industry (Vadstein, Attramadal, Bakke, & Olsen, 2018). Several studies have indicated that the major losses of larvae are due to detrimental interactions and dysbiosis in the microbiota of the fish larvae (Kanther & Rawls, 2010; Vadstein, Attramadal, Bakke, Forberg, et al., 2018; Vadstein et al., 2013; Vestrum, Luef, et al., 2018). Antibiotics can be used to avoid these fish larvae population crashes, though the understanding of why these crashes suddenly occur is still uncertain. To some extent, this problem originates in the lack of understanding the microbiome and the interactions occurring at that scale.

A microbiota is "*the assemblage of microorganisms present in a defined environment*" while a microbiome "*refers to the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eurkaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions*" (Marchesi & Ravel, 2015). While these two concepts are linked, they are also often used indiscriminately. Microbiomes and microbiotas can be defined at various levels from an entire animal to parts of the animal such as the gut or skin microbiome. In aquacultures, multiple microbiomes impact the production including the rearing water, biofilters, the rearing tanks, microalga, live-feed (rotifers, *Artemia*, copepods), and the fish. All of these microbiotas are interlinked and interact through feeding and exchange of metabolites (Figure 2). This chapter will focus on the microbiomes associated with fish, live-feed, and feed for live-feed.

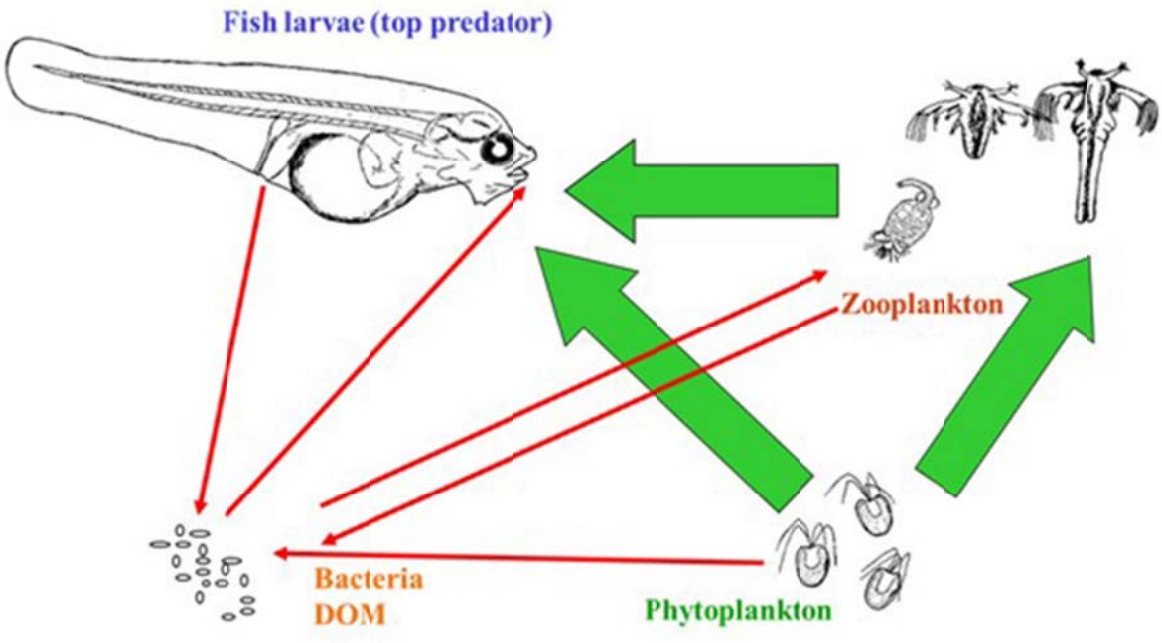


Figure 2: The complex food-web of first-feeding fish larvae in aquacultures. The arrows indicate the interactions between the different trophic levels (Vadstein, Attramadal, Bakke, Forberg, et al., 2018).

2.1. Microbiomes associated with reared fish

The microbiotas associated with reared fish is dependent on the fish species (Li et al., 2012). The microbiome develops throughout the life cycle of the animal (Bledsoe et al., 2016; Wilkes Walburn et al., 2019). Early bacterial colonization of the gastrointestinal tract of fish larvae is highly influenced by the uptake of bacteria from the rearing water and the feed (Blanch et al., 1997; Ingerslev et al., 2014; Wilkes Walburn et al., 2019). Some of the earlier, culture-dependent studies of reared fish, turbot and halibut, larvae reported a succession from oxidative Gram-negative rods dominating the early stages to *Vibrio* spp. dominating the later stages that were feeding on live-feed such as rotifers, *Artemia*, and copepods (Blanch et al., 1997; Munro et al., 1994; Verner-Jeffreys et al., 2003). In this study, we observed that turbot egg microbiomes were dominated by members of the Vibrionales and Alteromonadales orders, while the larval microbiome within the first few days of hatching changed to be dominated by the Alteromonadales with presence of other orders such as Flavobacteriales (Bacteroidetes), Oceanospirillales (Proteobacteria), Pseudomonadales (Proteobacteria), Rhodobacterales (Proteobacteria), and Vibrionales (Proteobacteria) in lower abundances (Dittmann, Rasmussen, Melchiorsen, et al., 2019). Other studies have also started to characterize and map the microbiomes related to larvae from other, aquaculture-relevant

species such as rainbow trout (Ingerslev et al., 2014), tilapia (Giatsis 2015), and cod (Bakke et al., 2015). These studies have revealed a much broader diversity and thereby bridging some of the knowledge gap.

The impact of feed type and feed nutrient composition also has a major impact on the gut microbiome composition. A culture-independent study on reared Yellowtail Kingfish (*Seriola lalandi*) showed that the microbiota shifted from being dominated by Proteobacteria to being dominated by Firmicutes, when the larval feed changed from live-feed to formulated pellets (Wilkes Walburn et al., 2019). Another study on adult Yellowtail Kingfish observed that the gut microbiota of fish reared in aquaculture was dominated by Firmicutes, while the gut microbiota of the wild fish was dominated Proteobacteria (Ramírez & Romero, 2017). Along the same line, artificial feeding decreased the bacterial species diversity of wild Atlantic cod held in captivity for 6 weeks (Dhanasiri et al., 2011). Ringø *et al.* (2006) observed that the digestive tract of adult cod fed with fish meal were dominated by *Brochothrix* spp. and *Carnobacterium* spp. (Gram-positive genera) while the digestive tract of cod fed with soy bean meal were dominated by *Chryseobacterium* spp. (Gram-negative), *Psychrobacter glacincola* (Gram-negative), and *Carnobacterium* spp.. Plant-based feed shifted the microbiome of rainbow trout larvae to Firmicutes, while marine diet (i.e. fish meal and fish oil) shifted the microbiome to dominance of Proteobacteria (Ingerslev et al., 2014). Altogether, these studies illustrate not only that the gut microbiomes depend on the feed (plant-based vs. fish-based), but also on the feed preference of the fish species.

Live-feed have often been suspected as infection vectors in aquaculture larval rearing (Hansen & Olafsen, 1999; Olafsen, 2001). However, similarly to the fish larval microbiome, the knowledge on the live-feed microbiotas is scarce. *Vibrio* spp. are naturally associated with zooplankton (Colwell et al., 2003; Kaneko & Colwell, 1973; Sochard et al., 1979; Vezzulli et al., 2015) and culture-dependent studies have observed that *Vibrio* spp. were dominating the microbiome (Montanari et al., 1999; Sochard et al., 1979). A culture-independent study on copepods from the North Atlantic Ocean observed that the microbiome was dominated by Gammaproteobacteria, particularly *Pseudoalteromonas* spp., and Rhodobacteraceae were associated with the transient, food microbiome (Moisander et al., 2015). Similarly, Gammaproteobacteria of the Alteromonadales and Oceanospirillales orders dominated the microbiome of *Acartia tonsa* nauplii in this study, but the abundance of Vibrionales was less than 2 % of the microbiome (Dittmann, Rasmussen, Melchiorsen, et al., 2019). Bakke *et al.* (2015) observed that the copepod microbiome was dominated by

Alphaproteobacteria (mainly Rhodobacteraceae) and Flavobacteria, but the Vibrionaceae were less than 1 % of the microbiome. Hence, the presumed dominance of vibrios observed in culture-dependent studies does not necessarily reflect the whole bacterial community of copepods.

Microbiotas of *Artemia* and rotifers were also assessed in the study by Bakke *et al.* (2015). While the rotifer culture was dominated by Actinobacteria and Alphaproteobacteria (mainly Rhodobacteracea), the *Artemia* cultures were solely dominated by Alphaproteobacteria (mainly Rhodobacteracea). Furthermore, Vibrionaceae were only observed in the *Artemia* cultures. Høj *et al.* (2009) reported that the microbiota of newly hatched nauplii was dominated by Gammaproteobacteria and Planctomycetales. Furthermore, isolates of the genera *Vibrio*, *Pseudomonas*, *Micrococcus*, *Brevundimonas*, *Sphingomonas*, and *Rhizobium* could be retrieved from *Artemia* surface-treated with antibiotics (Høj et al., 2009). Califano *et al.* (2017) observed that the rotifers from a gilthead seabream hatchery were dominated by a single operational taxonomic unit classified as a *Loktanella* sp., while the *Artemia* nauplii were dominated by Flavobacteriaceae, Rhodobacteraceae, and *Paracoccus* sp.. Hence, the microbiota of the live-feed is dependent on the cultivation environment rather than the host.

Microalgae are used as feed for the live-feed and fish larvae are in some aquacultures reared in "green water" with high loads of microalgae. Some of the favored microalgal genera for larval feeds include *Chaeotoceros*, *Thalassiosira*, *Tetraselmis*, *Isochrysis*, and *Nannochloropsis* (Duerr, 1998). Despite their extensive use, their microbiomes, particularly in relation to aquaculture settings, are scarcely studied. Biondi *et al.* (2017) observed that the microbiome of *Tetraselmis suecica* was dominated by Proteobacteria – mainly members of the *Roseobacter* group and the Rhizobiales order – and Bacteroidetes from the Flavobacteriales order. A similar community composition was observed in the *T. suecica* cultures used in this study, although Planctomycetes (Phycisphaerales) were also prominent members of the microbiota (Dittmann, Rasmussen, Melchiorsen, et al., 2019). Feeding *Tetraselmis* spp. and *Chlorella minutissima* to *Artemia* decreased the load of total bacterial and presumptive *Vibrio* spp. in the *Artemia*, most likely due to the presence of bioactive Gram-positive bacteria in the microbiomes of the algae (Makridis et al., 2006; Olsen et al., 2000). Furthermore, feeding Atlantic salmon with *T. suecica* reduced mortalities caused by *Aeromonas salmonicida*, *Serratia liquefaciens*, *Vibrio anguillarum*, *Vibrio salmonicida*, and *Yersinia ruckeri* type I (Austin et al., 1992). Hence, the microbiotas of the different trophic layers in an aquaculture are intimately linked and they have high influence on each other.

However, it is still uncertain what bacterial species are indicators of a "healthy" microbiome. This also emphasizes the need for understanding the diversity of the individual microbiotas as well as their function in relation to the other microbiotas.

2.2. Methods to study microbiomes

The earliest studies of aquaculture bacterial communities were based on classical, microbiological methods; cultivation and isolation of bacteria, as well as phenotypic and genotypic characterization (e.g. (Blanch et al., 1997; Munro et al., 1994)). Fingerprinting techniques, such as polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and terminal restriction fragment length polymorphism (T-RFLP), enabled broader analysis of the microbial community as a whole and the option to compare the microbiomes of different niches in a culture-independent way (Fjellheim et al., 2012; Hovda et al., 2007; Pond et al., 2006). Fjellheim *et al.* (2012) showed that there was no correlation between richness and diversity results obtained from T-RFLP on 16S rRNA amplicons and culture-dependent phenotyping methods. This is most likely due to culturability; 90 % to 99 % of marine bacteria cannot be cultured at standard laboratory conditions (Glöckner & Joint, 2010), and thus, the culture-dependent studies only reflect the 1 % to 10 % of bacteria that could grow on agar plates and/or in liquid medium.

The fingerprinting techniques do not provide taxonomic classification to the bacteria without the use of sequencing. Sequencing of the bands has resulted in a certain level of taxonomy in some studies (Fjellheim et al., 2012; Hovda et al., 2007). However, this does not provide information about individual members at genus and species levels. With the rise and dissemination of Next Generation Sequencing and omics technologies as well as development of open-source, easy-to-use data handling pipelines, it is now possible to study diversity, community composition, taxonomy of the community members, and function of the microbiomes. Combinations of methods such as amplicon sequencing (taxonomy), metagenomics (taxonomy and genetic potential), metatranscriptomics (gene expression), proteomics (protein expression), and metabolomics (metabolites) can be used to understand interactions in complex microbiomes. In this study, 16S rRNA amplicon sequencing was used to assess diversity and taxonomic distribution of marine eukaryote-associated microbiotas.

2.2.1. 16S rRNA amplicon taxonomics

Currently, one of the most widely used method is 16S rRNA amplicon sequencing (taxonomics). The method is based on PCR amplification of conserved regions of the 16S rRNA genes on genomic DNA from the environment. In this study, the 16S rRNA V4 region was amplified (Dittmann, Rasmussen, Melchiorsen, et al., 2019; Dittmann, Sonnenschein, et al., 2019), however, other regions and combinations of multiple regions have also been used. The choice of region determines the taxonomic resolution. Several analysis pipelines - e.g. DADA2 (Callahan et al., 2016), mothur (Schloss et al., 2009), QIIME (Caporaso et al., 2010), QIIME 2 (Bolyen et al., 2018), USEARCH (Edgar, 2010), and VSEARCH (Rognes et al., 2016) – have been developed to process 16S rRNA amplicon sequencing data. The choice of pipeline is dependent on available computer power, programming language preference, size of data set, and to some extend also personal preference. In this study, we used mothur (Dittmann, Sonnenschein, et al., 2019) and QIIME 2 (Dittmann, Rasmussen, Melchiorsen, et al., 2019). Mothur is relatively easy to approach in the sense that it can run in Windows on a regular laptop and the pipeline is standardized to take the data from raw reads to Operational Taxonomic Units (OTUs), as well as calculate measures of alpha- and beta diversity, including statistics. The data sets in the second taxonomics study were too large and diverse for our available computer power to handle, which was why we transferred to QIIME 2. This pipeline is more flexible and plugins from DADA2 (denoising, chimera removal, generation of Amplicon Sequence Variant, ASV, table) and VSEARCH (classification) can be used, though it is dependent on running in a UNIX environment. QIIME 2 can be used for calculating the measures of alpha- and beta-diversity, statistical analysis, and visualizations, but the R packages Phyloseq and Vegan were used for that purpose in this study (Dittmann, Rasmussen, Melchiorsen, et al., 2019).

While 16S rRNA taxonomics is becoming relatively affordable, it still has some pitfalls. Extraction of representative (if not all) genomic DNA, degradation of DNA, amplification biases in the PCR, and chimeric amplification products are some of the common errors, which can be introduced prior to sequencing. Furthermore, amplifying a short fraction of a highly conserved gene, such as the 16S rRNA gene, limits the taxonomic resolution window and only the bacterial community is assessed. This can be mediated by metagenomics, where all of the genomic DNA is sequenced. However, this is still an expensive method to apply, the required computer capacity is beyond the regular benchtop computers, and the DNA extraction biases are still an issue with this method.

2.3. Conclusions

The microbiomes of aquacultures are highly dynamic and the fish microbiota is influenced by the rearing water, the feed, and environmental factors. To date, there are only few studies on the aquaculture related microbiomes and more work is needed to determine 1) what a healthy fish larval microbiome is, 2) what the differences are between larval microbiomes related to different fish species, and 3) which factors cause dysbiosis leading to crashes in fish populations.

The technologies for studying microbiome diversity and function are rapidly developing. One of the key strengths of the Next Generation Sequencing technologies is that a lot of data are obtained. Combinations of the –omics technologies have the potential to answer the more complex questions on interactions and functionality of the microbiomes, which could lead to more rational microbial management and microbiome engineering in aquacultures.

In the following chapter, this thesis will focus on strategies for how aquaculture-related microbiomes can be managed and engineered to increase the welfare and yield of farmed fish.

3. Microbiome management in aquaculture

Several alternative solutions to microbial management strategies replacing antibiotic deployment have been proposed for aquacultures; this includes water control, enrichment of favorable functions, phage-therapy, and probiotics. All of the technologies will alter the existing microbiome to a presumed "healthier" version or will use beneficial bacteria to control unwanted pathogens. Some of these principles will briefly be described below with the main focus being on fish larval probiotics.

3.1. Water conditioning and bioremediation

Improving and stabilizing the water quality is of great importance to ensure balance in aquaculture systems. Temperature, salinity, pH, and oxygen levels are the strongest environmental drivers of aquatic microbial communities (Campbell & Kirchman, 2013; Herlemann et al., 2011; Liu et al., 2015; Lozupone & Knight, 2007; Meron et al., 2011; Sunagawa et al., 2015; Wright et al., 2012). The chemical properties of the input rearing water such as temperature, oxygen, salinity and pH are controlled in aquacultures to avoid environmental stressors from fluctuations (Bentzon-Tilia et al., 2016). Introduction of pathogenic microorganisms through the inlet water has also been a major concern. Therefore, the water can be sterilized through UV irradiation or ozonation (Summerfelt, 2003). If the system is closed, re-circulating the water is an option to keep costs low and avoiding exchange with the environment (Attramadal et al., 2012). Fish tank water contains high loads of dissolved organic matter and the system is self-polluting with accumulation of nitrogen and phosphorus (Schneider et al., 2005). Especially ammonium and nitrite are problematic in intensive fish rearing (Avnimelech, 1999) because these compounds are toxic to the animals. Therefore, they should be removed or converted to other, non-toxic compounds prior to outlet of the water to the environment or re-introduction of the water into the fish tanks. This can be done by application of recirculating aquaculture systems (RAS). The idea is to condition the water using microbial communities. First, the water can be filtrated mechanically to remove accumulating particles of organic matter (Bentzon-Tilia et al., 2016). The water is then passed through biofilters, which are abiotic structures with biofilms coating the relatively large surface areas. The biofilms are composed of autotrophic, nitrifying bacteria – ammonium oxidizing *Nitromonas* spp. and nitrite oxidizing *Nitrospira* spp. – which convert ammonium to nitrate (Foesel et al., 2008). Marine RAS biofilters can also contain members oxidizing sulfide (Cytryn et al., 2005). However, the community composition of the biofilters is unique to each RAS and it is highly influenced by factors such as the fish feed,

management routines, the fish-associated microbiota, water chemical properties, and microbial selection pressure in the filter community (Attramadal et al., 2012; Blancheton et al., 2013; Schreier et al., 2010). Given the variability in these factors, it can be difficult to establish and maintain a working biofilter which is consistently efficient and safe in terms invasion of pathogens.

Besides improving water quality, RAS can also be utilized for a slightly different purpose. Sterilization of the water for the fish larvae is a necessity, but it also diminishes competition between bacteria, and thus, it gives room for domination of fast-growing, opportunistic pathogens already in the rearing water. Microbial maturation – re-colonization of the water by non-opportunistic bacteria – using biofilters could be a solution. Skjermo *et al.* (1997) showed reduced proliferation of pathogens after hatching of turbot (*Scophthalmus maximus*) eggs and increased survival of Atlantic halibut (*Hippoglossus hippoglossus*) yolk sac larvae, when the rearing water was matured after sterile-filtration. Attramadal *et al.* (2012) observed a more stable and diverse microbial community composition with a lower fraction of opportunists in comparison to conventional flow-through systems. This strategy can also lower the mortality of Atlantic cod larvae (Attramadal et al., 2012, 2014) by selecting for slow growing, competition-specialized bacteria with affinity for resources (K-selection) (Attramadal et al., 2014; Vestrum, Attramadal, et al., 2018).

Improving the water quality and directing the aquaculture community composition can also be done in a relatively simple, low-technology way through bioflocs. The technology is based on the balance of carbon and nitrogen; if the concentrations are well-balanced in the water, nitrogenous waste such as ammonium will be converted to bacterial biomass (Schneider et al., 2005). Adding extra carbon to a system with high loads of ammonium and carbon-limitation stimulates heterotrophic bacterial growth, which in turn increases the nitrogen-uptake (Avnimelech, 1999) and improves the water quality. This also creates accumulation of bacteria in flocs, which the reared animals eat, and thus, improve growth (Crab et al., 2010, 2012; Hari et al., 2004; Xu et al., 2013).

Improving the aquaculture rearing environment by shifting the microbiota to utilize favorable functions already found in the microbial community is an elegant concept. While it has been proven that RAS biofilters and bioflocs can be used for manipulating the water microbiome – both chemically and microbially – the currently established methods are often more coincidental rather than rationally designed. Seeding the systems with synthetic communities

of bacteria with known, beneficial functions, including probiotics, could be one route to streamline and minimize variability in the production (Bentzon-Tilia et al., 2016; Dittmann, Rasmussen, Castex, et al., 2017). One way, proposed by Bentzon-Tilia *et al.* (2016), would be to select and combine nitrifying and denitrifying bacteria with probiotics, which would enable the biofilter to mediate the conversion of the nitrate, nitrite, and ammonium to nitrogen gas, as well as release probiotics to the rearing water (Figure 3).

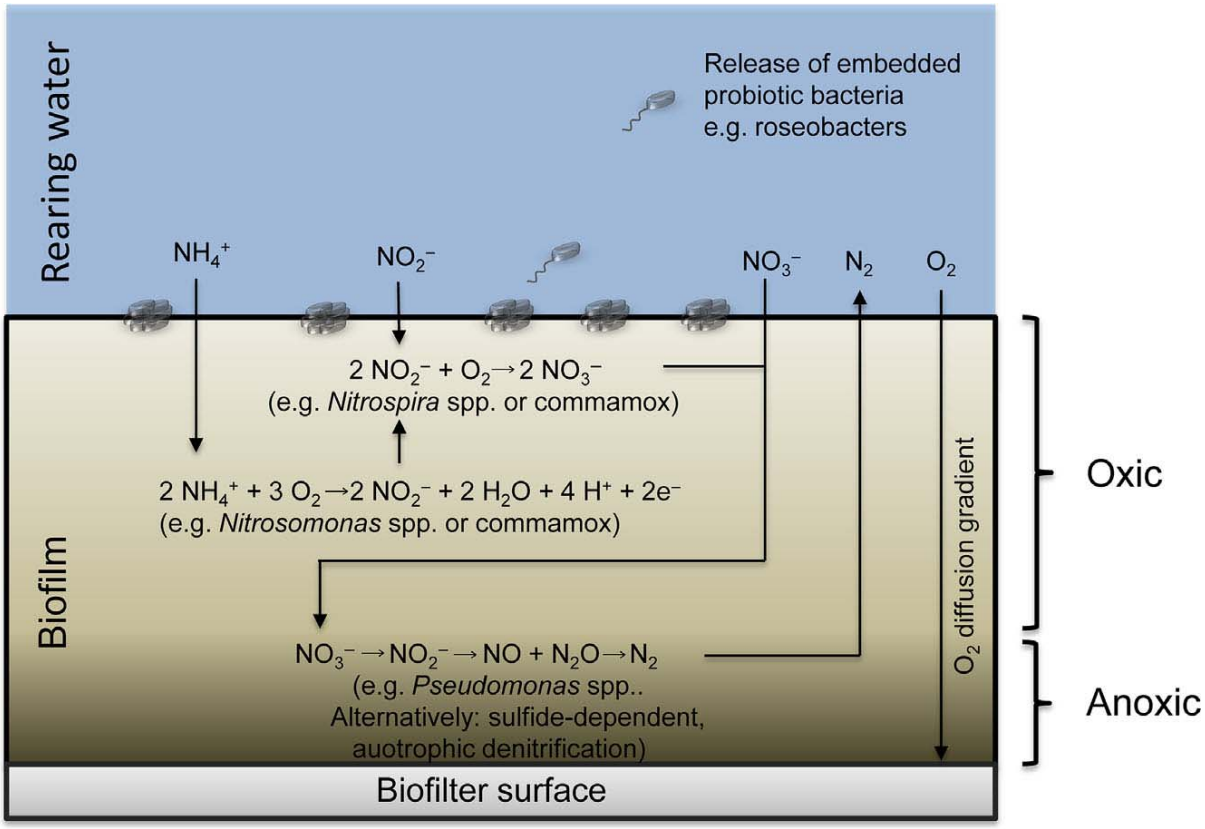


Figure 3: Engineering strategy proposed by Bentzon-Tilia *et al.* (2016) for seeding biofilters using a synthetic community of microorganisms with needed functions in a biological aerated filter (BAF) for use in microbial reconditioning of rearing water. The anoxic layer could be dominated by heterotrophs or autotrophs carrying out denitrifying processes, whereas the oxic layer could be dominated by nitrifying bacteria. Probiotics could also be embedded in the top layer to be released to the rearing water (Bentzon-Tilia et al., 2016).

3.2. Probiotics in aquaculture

Probiotics are "*live organisms which when administered in adequate amounts confer a health benefit on the host*" (FAO and WHO, 2001). In literature, there are many variations in the definition of probiotics and most of them are specific to a health benefit conferred in the gut of the host (e.g. Fuller, 1989; Gatesoupe, 1999). Historically, many of these definitions are associated with the Gram-positive, lactic acid bacteria (LAB) deployed in humans and

terrestrial animals. The increasing interest in aquaculture farmed fish and shellfish has also lead to investigations of probiotics for this industry. However, there are some pronounced differences between terrestrial and aquatic animals, which need to be considered when designing probiotics for aquaculture. Farmed fish and shellfish are highly influenced by the microbiome of the surrounding water (Defoirdt, Sorgeloos, & Bossier, 2011; Verschuere et al., 2000); they are in constant contact with the water and continuously ingest it. The aquaculture ecosystem does not only support the life of the eukaryote and the commensal bacteria, but also of (opportunistic) pathogens, which can reach high densities in this favorable environment (Moriarty, 1998). Opportunistic pathogens such as *Vibrio* spp. do not only invade the host through the gut, but they can also invade fish through the gills and the skin (Spanggaard et al., 2000; Weber et al., 2010). While the wording of the probiotics definition is debated, the FAO and WHO definition from 2001 is broad enough to include probiotics acting on the gut system as well as in/on other organs of the fish including indirect actions in the water. Hence, this definition will be used in this PhD thesis.

For the past decades, the beneficial effects of probiotics have been extensively studied *in vitro* (typically as inhibition of pathogens) and to a lesser extend *in vivo* (Table 1). However, the mechanisms behind the effects are still largely uncertain, if even uncovered, and only partial explanations are provided, given the methodological and ethical limitations concerning animal trials (Ringø et al., 2014; Tinh et al., 2008; Verschuere et al., 2000). Some of the suggested mechanisms include (i) competitive exclusion through production of inhibitory compounds, (ii) competition for nutrients, chemicals, or energy, (iii) adhesion site competition, (iv) contribution to digestion, (v) contribution to macro- and micronutrients, (vi) enhancement of immune response, and (vii) reduction of virulence through QS manipulation. These will be covered below in the descriptions of the probiotic candidates.

Table 1: Probiotic bacterial candidates.

Probiotic strain	Isolation source	Target pathogen	Probiotic effect	Host	Reference
Bacillus sp. LT3	Whiteleg shrimp (*Penaeus vannamei*)	*V. campbellii*	Degradation of AHLs, decreasing activity of pathogen, enhancing innate immune response of *Artemia* larvae	Brine shrimp larvae (*Artemia franciscana*)	Defoirdt *et al.* (2011), Niu *et al.* (2014)
Bacillus sp. QSI-1	*Carassius auratus gibelio*, intestine gut	*A. hydrophila*	Quorum quenching reducing pathogenicity	Zebrafish (*Danio rerio*)	Chu *et al.* (2015)
Bacillus spp.	NA	Luminous *Vibrio* spp.	NA	Prawns	Moriarty (1998)
Lactobacillus fermentum CLFP 242	Rainbow trout (*Oncorynchus mykiss*), intestine	*A. hydrophila*, *A. salmonicida*, *Y. ruckeri*	Inhibition of pathogen adhesion	Rainbow trout (*Oncorynchus mykiss*) - *in vitro*	Balcázar *et al.*(2008)
Lactobacillus plantarum CLFP 238	Rainbow trout (*Oncorynchus mykiss*), intestine	*A. hydrophila*, *A. salmonicida*	Inhibition of pathogen adhesion	Rainbow trout (*Oncorynchus mykiss*) - *in vitro*	Balcázar *et al.*(2008)
Lactococcus lactis CLFP 101	Rainbow trout (*Oncorynchus mykiss*), intestine	*A. hydrophila*, *A. salmonicida*, *Y. ruckeri, V. anguillarum*	Inhibition of pathogen adhesion, antibacterial effect	Rainbow trout (*Oncorynchus mykiss*) - *in vitro*	Balcázar *et al.*(2008)
Pediococcus acidilactici	Rainbow trout (*Oncorynchus mykiss*) larvae, gut and feed	*P. damnosus*, *L. monocytogenes*, *L. innocu, L. garvieae*	Bacteriocin production (antagonism)	NA	Araújo *et al.*(2015, 2016)
Phaeobacter gallaeciensis BS107 (DSM 17395)	Seawater in scallop (*Pecten maximus*) cultures	*V. anguillarum*	Antagonism	Cod larvae (*Gadus morhua*), copepods (*Acartia tonsa*), rotifers (*Brachionus plicatilis*), *Artemia*	D'Alvise *et al.* (2010, 2012), Neu *et al.* (2014), Rasmussen *et al.* (2018)
Phaeobacter sp. S26	Mediterranean aquacultures	*V. anguillarum*	Antagonism	*Tetraselmis suecica*, *Artemia*	Grotkjær *et al.* (2016)
Phaeobacter sp. S60	Mediterranean aquacultures	*V. anguillarum*	Antagonism	*Tetraselmis suecica*, *Artemia*	Grotkjær *et al.* (2016)
Pseudomonas fluorescens AH2	Iced freshwater fish (*Lates niloticus*)	*A. salmonicida, V. anguillarum*	Iron competition (siderophores)	Rainbow trout (*Oncorynchus mykiss* Walbaum) - only vibriosis	Gram *et al.* (1999, 2001)
Roseobacter 27-4	Turbot Larvae (*Scophthalmus maximus*) Rearing Units	*V. anguillarum*	Antagonism	Turbot larvae (*Scophthalmus maximus* L.)	Hjelm *et al.,* (2004), Planas *et al.* (2006),
Shewanella putrefaciens Pdp11	Gilthead seabream, *Sparus aurata* (L.), skin	*V. harveyi, P. damselae* subsp piscicida	Colonization of mucus and adhesion reduction of the pathogens, improve growth of fish juveniles, modulate the intestinal microbiota, modulate expression of immune-related genes	Gilthead seabream (*Sparus aurata* (L.)), Senegalese sole (*Solea senegalensis* (Kaup))	Chabrillón *et al.* (2005), Cordero *et al.* (2016), Sáenz de Rodrigáñez *et al.* (2009), Tapia-Paniagua *et al.* (2014), Varela *et al.* (2010).
Vibrio alginolyticus	Sea water	*V.parahaemolyticus*	Antagonism	Whiteleg shrimp (*Litopenaeus vannamei*)	Garriques & Arevalo (1995)
Vibrio alginolyticus	Shrimp aquaculture	*A. salmonicida, V. anguillarum, V. ordalii*	Antagonism	Atlantic salmon (*Salmo salar* L.)	Austin *et al.* (1995)

Some of the most studied probiotic candidates belong to the Firmicutes phylum, namely LAB and bacilli (Araújo et al., 2016; Balcázar et al., 2008; Carnevali et al., 2004; Gatesoupe, 1991, 1994; Moriarty, 1998; Venkat et al., 2004). These probiotics have been successful in humans and livestock, and the bacilli as biocontrol in horticulture, though they are not adapted to nor common in the marine environment. LAB can tolerate acidic pH and bile salts, which enable them to survive in gut systems (Balcázar et al., 2008; Bentzon-Tilia et al., 2016; Merrifield et al., 2010). These bacteria can colonize the intestinal mucus, where they are believed to act as an infection barrier and assist in the processing and uptake of feed, which in turn can promote growth of the fish (Ringø et al., 2010; Vieco-Saiz et al., 2019). *Pediococcus acidilactici* was isolated from the gut of rainbow trout larvae (*Oncorhynchus mykiss*) as well as their feed (Araújo et al., 2016; Araújo et al., 2015). The strains were bioactive against common fish pathogens, in part due to bacteriocin production, and they performed well in safety assessment as they did not display antibiotic resistance, produce hemolysins, or degrade gastric mucin (Araújo et al., 2016). Other LAB such as *Carnobacterium maltaromaticum*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactococcus lactis*, and *Leuconostoc mesenteroides* have also been isolated from the intestines of salmonids (Balcázar et al., 2007). Some of these strains – *L. lactis* CLFP 101, *L. plantarum* CLFP 238, and *Lactobacillus fermentum* CLFP 242 – were tested for their antibacterial effect and their ability to inhibit adhesion of the fish pathogens *Aeromonas hydrophila*, *A. salmonicida*, *Y. ruckeri,* and *V. anguillarum* to intestinal mucus from rainbow trout (*in vitro*) (Balcázar et al., 2008). Only *L. lactis* CLFP 101 reduced adhesion of all the tested pathogens in the mucus assay, and supernatant from the LAB strain inhibited growth of all pathogens, too. *L. fermentum* CLFP 242 reduced adhesion of all pathogens except *V. anguillarum*, but its supernatant did not show antibacterial activity, indicating that its probiotic potential is most likely not due to production and secretion of antimicrobial agents. This was also the case for *L. plantarum* CLFP 238 and its ability to inhibit adhesion was restricted to the tested *Aeromonas* spp.. Hence, their probiotic mode of action is specific at species level, if not strain level. Thus, all probiotic candidates would have to be tested *in vivo* to determine their exact activity spectrum and potential for application.

Bacilli have also been observed to improve survival of reared shrimp and controlling luminous *Vibrio* spp. (Moriarty, 1998). *Bacillus* sp. can also protect the live-feed (*Artemia*) and increase survival by decreasing the activity of *Vibrio campbellii* and enhancing the innate immune response of the *Artemia* larvae (Niu et al., 2014). Defoirdt *et al.* (2011) isolated

Bacillus spp. from whiteleg shrimp and European sea bass, which could degrade N-acyl-homoserine lactones (AHL). Degrading the AHLs can disrupt the QS modulated phenotypes such as virulence. Quorum quenching *Bacillus* sp. QSI-1 reduced pathogenicity of *A. hydrophila* in zebrafish (*Danio rerio*) and thereby improved the survival rate (Chu et al., 2015). Hence, probiotic effect does not have to be due to competition, but it can also be due to modulation of behavior in the microbiota. It will not necessarily decrease the pathogen load and an imbalance might still let the opportunists gain dominance.

While LAB strains seem somewhat promising as probiotics in aquaculture, it is important to assess both strengths and weaknesses. If they are to be used as probiotics in larvicultures, they may not serve their full purpose in the early fish life stages, because the gastrointestinal tract is not fully developed and the microbiome inside the larvae is transient – being an extension of the microbiota in the tank (Bentzon-Tilia et al., 2016) – which is not (yet) dominated by Firmicutes. Hence, other species, that are adapted to and act in the marine environment are likely more suitable at this stage. Proteobacteria such as *Pseudomonas* spp., *Shewanella* spp., *Vibrio* spp., and members of the *Roseobacter* group have been proposed as non-LAB probiotics (Chabrillón, Rico, Arijo, et al., 2005; Chabrillón, Rico, Balebona, & Morinigo, 2005; Dittmann et al., 2017; Garriques & Arevalo, 1995; Gram et al., 2001; Gram et al., 1999; Porsby & Gram, 2016; Prado et al., 2009; Tapia-Paniagua et al., 2014), especially due to their antagonism against pathogens. Gram *et al.* investigated the probiotic potential of *Pseudomonas fluorescens* strain AH2 against *V. anguillarum* and *A. salmonicida* (Gram et al., 2001, 1999). The growth of both pathogens was inhibited *in vitro* by *P. fluorescens* AH2 and the effect was increased during iron-limited growth conditions. This indicated that part of the probiotic effect could be due to iron competition (siderophores), though the experimental conditions did not allow for an exact determination of this (Gram et al., 1999). While the probiotic *Pseudomonas* could protect rainbow trout against vibriosis (Gram et al., 1999), furunculosis caused by *A. salmonicida* in Atlantic salmon (*Salmo salar* L.) was unaffected by the probiotic treatment (Gram et al., 2001). Hence, it is not possible to predict a "good" probiotic *in situ* based on *in vitro* experimental results; *in vivo* trials of probiotic candidates against different target pathogens in different fish systems are necessary to determine their spectrum of activity.

Despite their pathogenicity to some fish and shellfish (Ben Kahla-Nakbi et al., 2009; Cao et al., 2018; Gómez-León et al., 2005), addition of *Vibrio alginolyticus* to the culture water, could reduce the occurrence of *Vibrio parahaemolyticus* and increase the survival of whiteleg

shrimp, *Litopenaeus vannamei* (Garriques & Arevalo, 1995). Similarly, bathing Atlantic salmon in culture of a *V. alginolyticus* strain - used as disease control in shrimp aquaculture in Ecuador - reduced mortality of the fish challenged with *A. salmonicida* and to a lesser extent salmon challenged with *V. anguillarum* and *Vibrio ordalii* (Austin et al., 1995). Both studies suggested that the probiotic properties came from antagonism towards the target pathogens. *Shewanella putrefaciens* Pdp11 isolated from the skin of healthy gilthead seabream, *Sparus aurata* (L.) (Chabrillón, Rico, Balebona, et al., 2005), was able to colonize the mucus and reduce adhesion of the pathogens *Vibrio harveyi* and *Photobacterium damselae* subsp piscicida, both in gilthead seabream and in Senegalese sole, *Solea senegalensis* (Kaup) (Chabrillón, Rico, Arijo, et al., 2005; Chabrillón, Rico, Balebona, et al., 2005). Further studies have revealed that *S. putrefaciens* Pdp11 is able to improve growth when added to the feed of juveniles of both fish species (Sáenz de Rodrigáñez et al., 2009; Varela et al., 2010). The strain can also modulate the intestinal microbiota and expression of immune-related genes (Tapia-Paniagua et al., 2014; Varela et al., 2010) during high-stocking induced stress (Cordero et al., 2016; Tapia-Paniagua et al., 2014; Varela et al., 2010). Altogether, this indicates that probiotics, exemplified by *S. putrefaciens* Pdp11, can have multiple mechanisms, which act together to protect and improve health of aquaculture related animals.

3.2.1. Roseobacters as probiotics in aquaculture

Members of the *Roseobacter* group, mainly *Phaeobacter* spp., have shown great potential as probiotics in aquaculture. They have been isolated in multiple aquaculture units (Grotkjær, Bentzon-Tilia, D'Alvise, Dourala, et al., 2016; Porsby et al., 2008; Ruiz-Ponte et al., 1998), which indicates that they might play a more or less important role in the microbiome in some farms. *Phaeobacter gallaeciensis* BS107 can antagonize *V. anguillarum in vitro* and protect cod (*Gadus morhua*) larvae from vibriosis (D'Alvise et al., 2012). The antagonistic effect was likely due to production of the secondary metabolite TDA, given that a TDA-negative mutant did not protect the larvae to the same extend (Figure 4). Similarly, Grotkjær *et al.* (2016) observed that TDA-producing *Phaeobacter* sp. S26 and *Phaeobacter* sp. S60 isolated from Mediterranean aquacultures could reduce growth of *V. anguillarum* in non-axenic microalgae, *T. suecica*, and *Artemia* systems (used as live-feed in aquacultures). Altogether, this would argue that TDA-producing *Phaeobacter* spp. used as probiotics could not only protect the larvae from infection but also prevent proliferation and introduction of pathogens through the live-feed.

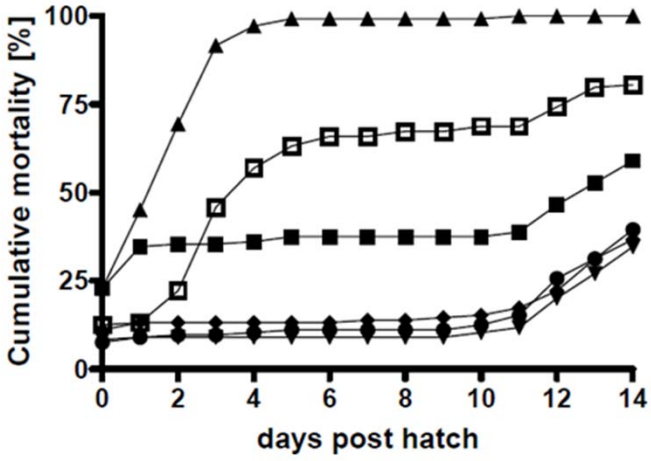


Figure 4: Mortality of cod larvae challenged with *Vibrio anguillarum*. Single larvae cultures were inoculated with TDA-producing *Phaeobacter gallaeciensis* (wild type) and *V. anguillarum* (•) or a TDA-negative mutant of *P. gallaeciensis* and *V. anguillarum* (□). The following controls were included: untreated control (▪), only *V. anguillarum* (▲), only TDA-producing *P. gallaeciensis* (▼), and only TDA-negative *P. gallaeciensis* (♦) (D'Alvise et al., 2012).

Given the interest in the biotechnological application of TDA-producers as probiotics in the aquaculture industry, it is of utmost importance that neither TDA nor the TDA-producer are harmful to the production animals and their live-feed. So far, no study has reported negative effects on the fish larvae (D'Alvise et al., 2012; D'Alvise et al., 2010; Hjelm et al., 2004; Planas et al., 2006) and the live-feed organisms *Artemia* (Grotkjær, Bentzon-Tilia, D'Alvise, Dourala, et al., 2016; Neu et al., 2014), rotifers (D'Alvise et al., 2012), and copepods (Rasmussen et al., 2018). TDA has so far not been associated with harm to the algae, however, some TDA-producers – i.e. *P. inhibens, P. gallaeciensis*, and *Phaeobacter piscinae* – also produce algicidal compounds called roseobacticides (Sonnenschein et al., 2018). Roseobacticides share some common features, like the tropone ring and at least one sulphur atom, with TDA, as well as part of the biosynthetic pathway (Seyedsayamdost, Carr, et al., 2011; Seyedsayamdost, Case, et al., 2011). Some microalgal species - *Rhodomonas salina*, *Thalassiosira pseudonana*, and *Emiliania huxleyi* – are negatively affected by the exposure to these algicidal compounds (Sonnenschein et al., 2018). Interestingly, *T. suecica* was not affected by rosebacticides (Sonnenschein et al., 2018), which is positive if TDA-roseobacticide-producers should be applied as probiotics in aquaculture units where *T. suecica* is used as feed for the live-feed.

Besides potentially causing harm to the reared animals and the live-feed, the addition of a high load of a probiotic bacterium with pronounced bioactivity could potentially cause imbalance in the microbiota. Geng *et al.* (2016) observed that TDA caused shifts in the

microbiome structure of the microalga, *Nannochloropsis salina*. In the present study, the impact of adding a probiotic TDA-producing *P. inhibens* to the microbiotas related to three different trophic layers – i.e. microalgae (*T. suecica*), copepod nauplii (*A. tonsa*), and turbot (*Scophthalmus maximus*) eggs/larvae - from aquaculture systems was assessed (Dittmann, Rasmussen, Melchiorsen, et al., 2019). Overall, the probiotic treatment had minor impact on the richness and diversity of the microbiomes. The structure of the communities associated with the lower trophic levels was significantly shifted. Interestingly, this was not obvious when assessing the community composition. The microalgal community composition was stable and no pronounced shifts were observed. However, a decrease of Rhodobacterales – particularly members closely related to *P. inhibens* – was observed in the copepod microbiota (Figure 5) and the turbot microbiota (Dittmann, Rasmussen, Melchiorsen, et al., 2019). This has previously been observed in the microbiomes of the microalgae, *E. huxleyi* (Dittmann, Sonnenschein, et al., 2019) and *Thalassiosira rotula* (Majzoub et al., 2019). This would indicate that roseobacters compete for the same niches and modulate their abundance according to which related taxa that are present. From an applied point of view, it is promising that the added probiotic strain establishes itself in the microbiota.

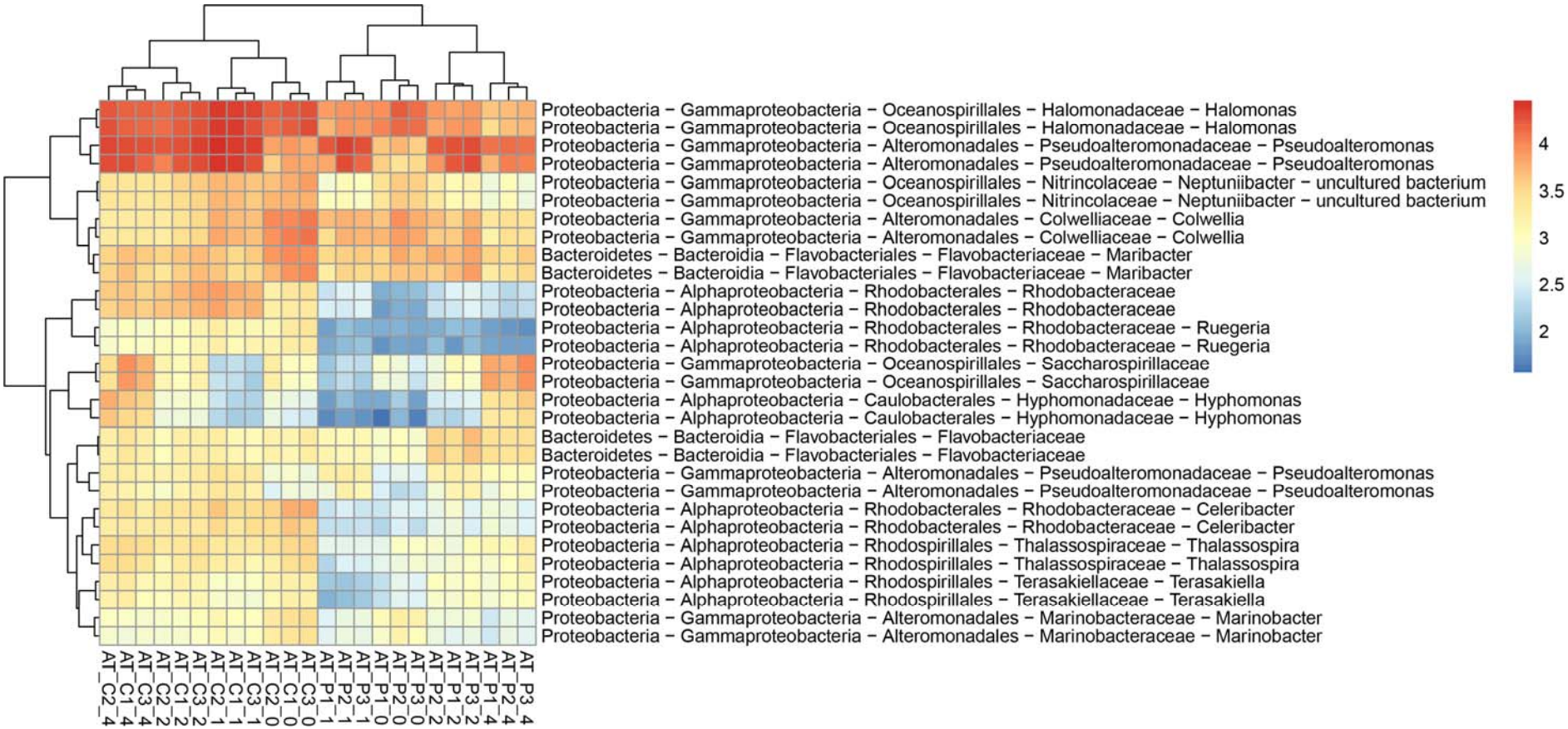


Figure 5: Relative abundances of the 30 most abundant Amplicon Sequence Variants (ASVs) observed in the *Acartia tonsa* (AT) microbiome. The populations were either untreated (controls, C) or exposed to probiotic *Phaeobacter inhibens* DSM 17395 (P). Each population was sampled at day 0, 1, 2, and 4. The relative abundances have been $\log_{10}(x+1)$ transformed. Each row represent a unique ASV and the assigned taxonomy is listed next to the plotted relative abundances (Dittmann, Rasmussen, Melchiorsen, et al., 2019).

As mentioned previously, entry of *Vibrio* spp. has been linked to live-feed. In this study, we only observed vibrios in the turbot larval microbiota and their relative abundances were stable regardless of treatment (Figure 6) (Dittmann, Rasmussen, Melchiorsen, et al., 2019). TDA-production is necessary for the probiotic effect against vibriosis (D'Alvise et al., 2012). Majzoub *et al.* (2019) used both a TDA-producing *P. inhibens* 2.10 (WT) and a variant (NCV12a1) with reduced antagonistic effect; the microbiotas developed in the same way regardless of the bioactivity. In both this study and the study by Majzoub *et al.* (2019), *Phaeobacter*-like species were already present in the microbiomes. If TDA-producers are already present, and the added probiotic bacterium replaces the inherent genera, then the net amount might be indifferent to the vibrios in the system. However, taxonomics data is not sufficient to understand the mechanisms behind the probiotic effect. To understand the interactions in artificial microbiomes, we need to comprehend the ecology of roseobacters and the mechanism of action for TDA, which will be elaborated on in chapter 4 of this thesis.

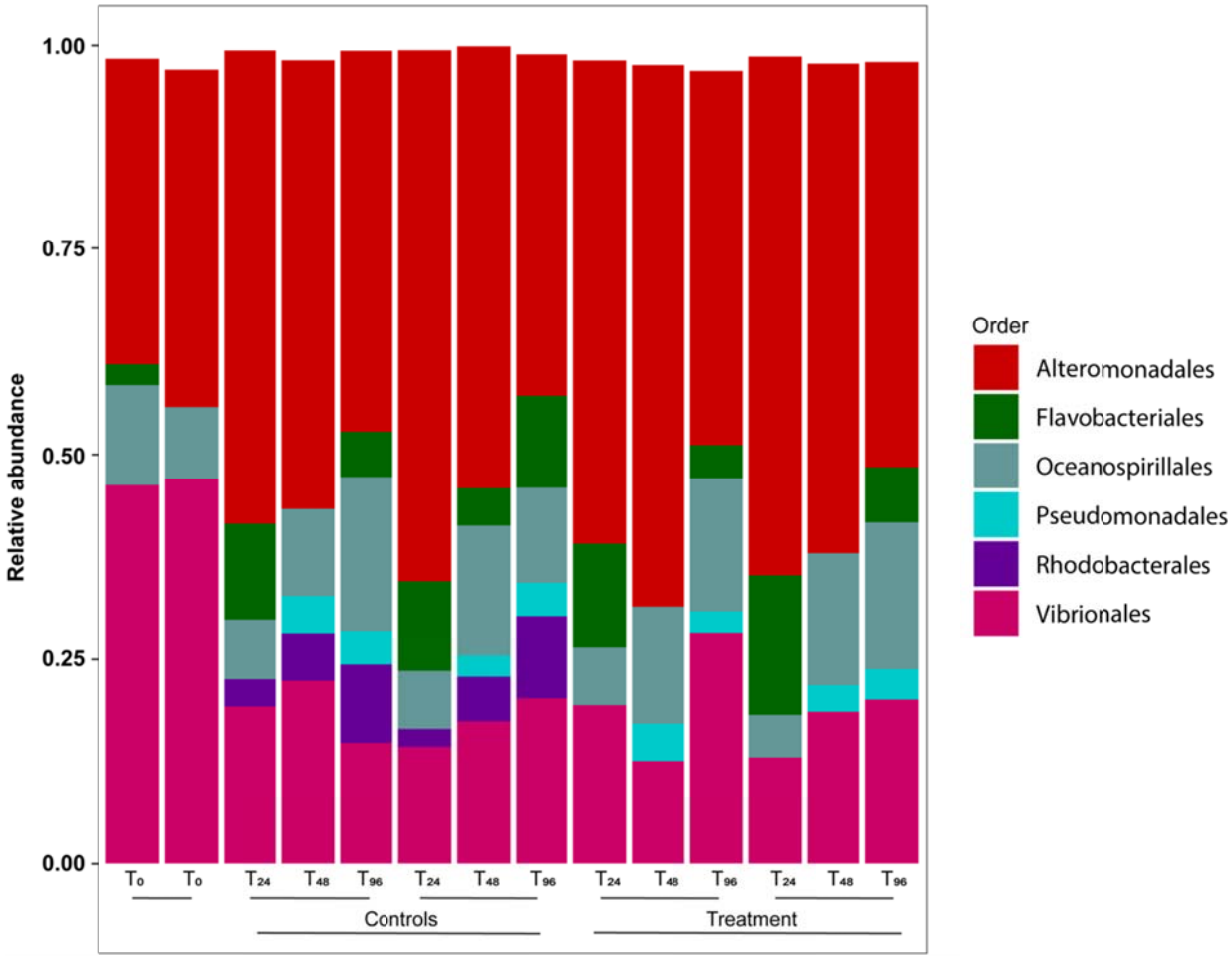


Figure 6: Community composition of the microbiota of turbot eggs and larvae. The populations were either untreated (Controls) or exposed to probiotic *Phaeobacter inhibens* DSM 17395 (Treatment). The community was sampled at 0, 24, 48, and 96 hours post-exposure in duplicates. The bars illustrate the

relative abundances of all the bacterial orders observed on the eggs / larvae. Only orders with relative abundance above 2 % were included and Amplicon Sequence Variants (ASVs) containing the added *P. inhibens* was removed from the dataset prior to plotting. T_0: untreated time zero control (Dittmann, Rasmussen, Melchiorsen, et al., 2019).

The effects of probiotic *P. inhibens* on aquaculture-related microbiomes is notable, though it is not possible to predict whether the impact is positive, negative, or even indifferent to the microbiota balance. Studies over extended periods of time, assessing the impact of addition route (e.g. microalgal, live-feed, rearing water) and the dose is necessary to optimize the probiotic effect with minimal harm to the microbiome prior to commercial application.

3.3. Commercial application of microbiome management in aquaculture

Protection of the animals and faster growth is of great interest to commercial aquaculture, in order to live up to regulations and sustainability-focused governmental stakeholders and consumers. While the ideas for improving the aquaculture industry in a sustainable way is getting increasing attention, the implementation and routine application of products targeting the microbiome in this sector is still in its infancy (Dittmann et al., 2017). An editorial describing the status quo of commercialized microbiome-focused products for the aquaculture industry, and looking into the "crystal ball" of the future aquaculture industry (Dittmann et al., 2017), was included in this thesis. Some products are already on the market and they fall into two categories: 1) targeting the water and pond environment, and 2) targeting the gut microbiome (feed and feed additives) (Table 2). The products available for targeting the water and environment, are generally focused on improving the water quality and decreasing the self-polluting dissolved organic matter as well as toxic compounds (ammonium, nitrite, and hydrogen sulphide). The discontinuous culture cycles of aquacultures leaves little to no room for establishment of a mature, healthy microbiome. The water and environmental products could be applied to seed biofilters and prime the rearing environment, thus, potentially excluding the opportunity for pathogens to get a "head start" and establish themselves.

Table 2: Commercially available microbial solutions for improvement of aquaculture microbiomes. References on the products can be found in the published article. Modified from Dittmann *et al.* (2017).

Target environment	Company	Product	Purpose	Composition
Water and pond	AquaInTech	PRO4000X, AquaPro B, AquaPro EZ	Degrade organic matter, reduce ammonia, *Vibrio* reduction	2 strains of Bacillus - *Bacillus subtilis, Bacillus licheniformis*
	Biomin	Aquastar	Stabilize water quality, improves pond bottom quality and support the gut health of fish and shrimp	Formula not publicly available
	Keeton Industries	Waste & Sludge Reducer	Improve water and bottom quality, pathogen control	*Bacillus cereus* RRRL B-30535
	Keeton Probiotics	ShrimpShield, PondToss	Degrade organic sludge, improve feed efficiency	Formula not publicly available
	Lallemand	Lalsea Biorem	Degrade organic matter, reduce ammonia, pathogen control, stabilize pH	7 specific bacterial strains
	NovoZymes	Pond Plus	Pathogen control, Decomposition of organic substances	Spore forming bacteria
	NovoZymes	Pond Dtox	Hydrogen sulfide control	*Paracoccus pantotrophus*
	NovoZymes	Pond Protect	Ammonia and nitrite reduction	*Nitrosomonas eutropha*, *Nitrobacter winogradskyi*
Gut microbiome (feed, feed additive)	AquaInTech	Aquapro F	Organic matter degradation, improved digestion of feed	Five strains of bacillus combined
	Evonik	EcoBiol	Improve gut health	*Bacillus amyloliquefaciens* CECT 5940
	Keeton Probiotics	FeedTreat	Degrade organic sludge and Improve feed efficiency	Combination of Lymnozyme and Waste & Sludge Reducer Preblend
	Lallemand	Bactocell®	Reduce deformities across fish species, improve gut health across a range of fish and shrimp species	*Pediococcus acidilactici* (MA18/5M)
	Rubinum	TOYOCERIN®	Promote growth, increase specimen homogeneity, improve intestinal mucosa	*Bacillus cereus* var. *toyoi*

The commercial feed and feed additives are intended as growth promoters through aid for feed digestion (Dittmann et al., 2017), which could help controlling the colonization of the fish gut. These are mostly Gram-positive bacteria, namely bacilli, and only one of the products – Bactocell® by Lallemand - was approved by the European Food Safety Authority (EFSA) for use in aquaculture feed (Commission Regulation [EC] No. 911/2009 and Commission Implementing Regulation [EU] No. 95/2013) by the time of writing. Indeed, they are somewhat "easier" to get on the market given their status as "Qualified Presumption of Safety (QPS)" or "Generally Regarded As Safe" (GRAS)(Dittmann et al., 2017). Furthermore, *P. acidilactici* has been isolated from the gut of rainbow trout larvae (Carlos Araújo et al., 2015). However, marine fish and shellfish microbiomes are in many cases dominated by Gram-negative bacteria (Egerton et al., 2018; Gatesoupe, 1999). Thus, an

avenue of new possibilities would be commercialization of probiotics of marine origin (Dittmann et al., 2017), which requires in-depth knowledge of marine fish microbiomes and the microbial drivers of a healthy microbiota.

One of the key issues has been – and still is – the lack of knowledge on the microbiome constituents present in the aquacultures and how that is impacted by manipulations (Dittmann et al., 2017; Dittmann, Rasmussen, Melchiorsen, et al., 2019). The dissemination and "normalization" of Next-Generation Sequencing technologies as well as accessible, understandable bioinformatics tools for data processing has significantly increased the amount of studies trying to facilitate the understanding of fish and shellfish microbiome diversity and functionality (Egerton et al., 2018). It is becoming evident that the microbiomes of fish are versatile – from species to species, one environment to the other, wild vs. captured – and they develop throughout the life-cycle of the fish, in part due to changing feed (Egerton et al., 2018; Wilkes Walburn et al., 2019). Having knowledge about the different microbiomes and how they change due to stressors – e.g. environmental and social stress as found in aquacultures – would pave the path to rational microbiome engineering. Thus, the future probiotics could be single-cultures or mixtures of bacteria, which would be more suited to 1) survive in the environment, 2) exert the desired effect, and 3) cause minimal damage to the existing microbiota, given a rational development towards aquatic/marine microbiomes.

3.4. Conclusions

Managing microbiomes can be done in several ways – from giving preferential treatment to certain microbial members through targeted feeding to introducing new microbial members with desirable traits and functions. However, all actions will impose a change and thereby an imbalance of the microbiome. Stabilizing in a new microbial balance should ideally benefit the system to improved health, but prediction of the effect is challenging, and it could also range from no or minor effect to diminished or harmed health of the system. Therefore, it is of crucial importance to 1) know the microbiome of the system you are intending to manage, and 2) assess the potential consequences of imposing changes to the system e.g. by pilot or full-scale studies using Next Generation Sequencing technologies to monitor the changes.

Probiotics have shown great potential to reduce the load of pathogens or modify their behavior based on numerous *in vitro* and *in vivo* studies. However, the exact "mode of action" is still uncertain for many candidates and very few studies have assessed their impact on the microbiomes found in aquaculture. This study is focused on the probiotic candidate, *P.*

inhibens. Despite it being a prominent secondary metabolite producer, minor effects on the microbiomes exposed to probiotic concentrations are observed and they are highly specific to other roseobacters and vibrios. This suggests that addition of TDA producers to aquaculture systems is beneficial, however, the interactions at the molecular level is still uncertain, and it would need to be tested in real aquaculture systems where fish and live-feed interact with each other.

In order to understand their behavior in artificial systems, it is important to take their behavior in nature into consideration. The following chapter is focused on the ecology of the TDA-producing roseobacters and the molecular mechanisms underlying TDA activity.

4. Roseobacters & TDA

The *Roseobacter* group (previously "*Roseobacter* clade") are Gram-negative α-Proteobacteria. Roseobacters are almost exclusively isolated from hypersaline and marine environments, either tolerating or requiring salt for living (Buchan et al., 2005). Members of this group are distributed across the world oceans, from the polar to the temperate oceans (Selje et al., 2004), accounting for 2 % to 8 % of the surface water bacterioplankton (Sunagawa et al., 2015; Wietz et al., 2010). In coastal waters, roseobacters are highly abundant and constitute up to 20 % to 40 % of the microbiome (Buchan et al., 2005; Moran et al., 2003; Prabagaran et al., 2007). Especially the bacterial communities of phytoplankton blooms are dominated by roseobacters (González et al., 2000), but these bacteria are also observed in the microbiotas of other eukaryotes such as macroalgae (Rao et al., 2005) and molluscs (Prado et al., 2009; Ruiz-Ponte et al., 1998; Wegner et al., 2013).

The highly diverse ecological niches, where members of the *Roseobacter* group can be found, point to a metabolic versatility and adaptability. The *Roseobacter* group members are "ecological generalists" (Moran et al., 2007; Newton et al., 2010). Both phototrophs – e.g. *Roseobacter litoralis* and *Roseobacter denitrificans* – and heterotrophs – e.g. *Phaeobacter* spp. - can be found among the members of the group (Buchan et al., 2005; González & Moran, 1997; Newton et al., 2010). Roseobacters also play a major role in the oceanic sulfur cycling (González et al., 2000; Malmstrom et al., 2004) – both inorganic and organic forms of sulfur. Particularly, some members are able to assimilate and metabolize the algal osmolyte, dimethylsulfoniopropionate (DMSP) to the climate influencing gas dimethyl sulfide (DMS) (Miller & Belas, 2004; Moran et al., 2003). Other ways of gaining energy is through oxidation of inorganic sulfur and carbon monoxide (Buchan et al., 2005; Moran et al., 2004; Sorokin, 1994, 1995).

From a genomic point of view, they have variable genomes – averaging at 4.4 Mb (Buchan et al., 2005) – and they often harbor plasmids (Buchan et al., 2005; Moran et al., 2004). These plasmids often carry metabolically important features such as photosynthetic gene clusters (Petersen et al., 2012), secondary metabolite biosynthesis genes (Berger et al., 2012; Brinkhoff et al., 2004), and type IV secretion systems (Petersen et al., 2013), which adds to the physiological diversity of this prominent bacterial group.

4.1. Colonization of surfaces and interactions with eukaryotes

Some members of the *Roseobacter* group are excellent biofilm formers and they colonize both abiotic and biotic surfaces. Dang and Lovell (2000) observed that these bacteria were some of the earliest and most prominent colonizers of polymer surfaces. For example, *Phaeobacter* spp. colonize the walls of the fish larval rearing tanks (Hjelm et al., 2004) in aquaculture units. *P. inhibens* strain 2.10 was able to colonize the surface of a diatom within 2 days of incubation (Majzoub et al., 2019), further emphasizing their versatile ability to rapidly colonize these surfaces.

Roseobacters are known for interacting with eukaryotes, both symbiotically and pathogenically. These interactions are believed to be mediated by bacterial motility and chemotaxis, QS, and antimicrobial biosynthesis. Some of the best described interactions involve phytoplankton. Coccolithophorid microalgae and dinoflagellates produce DMSP (González et al., 2000; Zubkov et al., 2001, 2002), which can be metabolized by roseobacters (Miller & Belas, 2004), but the compound can also act as a chemoattractant (Seymour et al., 2010). Hence, the algae attract and feed the bacteria. However, symbiosis is not a one-way bargain; in return for continuous nutrition, bacteria feed the microalgae with supplements such as vitamins (Cooper et al., 2015; Croft et al., 2005) and/or protection against predation (Seyedsayamdost, Case, et al., 2011). Such a relationship has been proposed between *P. inhibens* and the coccolithophorid microalga, *E. huxleyi* (Figure 7) (Seyedsayamdost, Case, et al., 2011). At symbiotic conditions, the algae produces DMSP and provides the *P. inhibens* with a biofilm surface in return for growth-promoting compounds – phenylacetic acid and indole-3-acetic acid – and protection (Segev et al., 2016; Seyedsayamdost, Case, et al., 2011). The protective effect comes from the production of the antibiotic TDA and its tautomer thiotropocin. When the algae age, they release *p*-coumaric acid, which along with indole-3-acetic acid triggers a production of algicidal compounds, roseobacticides, by *P. inhibens* (Segev et al., 2016; Seyedsayamdost, Carr, et al., 2011; Seyedsayamdost, Case, et al., 2011). Thus, the mutualistic relationship and the *Phaeobacter* becomes pathogenic to the individual algae – potentially, it also escalates the termination of algal blooms in nature.

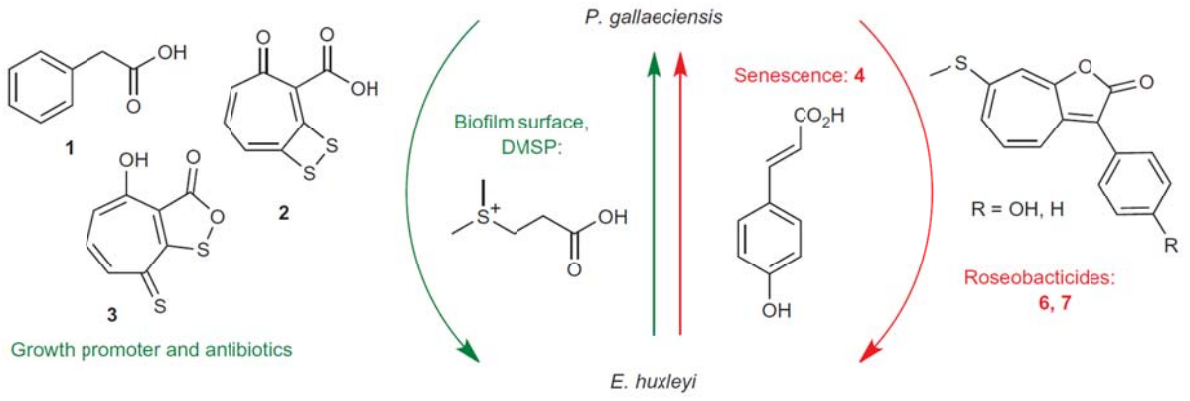


Figure 7: Proposed model for interactions between the microalga, *Emiliania huxleyi*, and *Phaeobacter inhibens* (prev. *Phaeobacter gallaeciensis*). At symbiotic conditions (green arrows), *P. inhibens* provide *E. huxleyi* with growth promoters and protection (antibiotics) in return for nutrients and a surface to form biofilm on. When the algae age (senesces) and release *p*-coumaric acid, *P. inhibens* starts to produce roseobacticides (algicidal compounds) and turns pathogenic (red arrows) (Seyedsayamdost, Case, et al., 2011).

Symbiotic relationships have also been proposed for members of the *Roseobacter* group and several squid species from diverse marine environments (Barbieri et al., 2001; Collins et al., 2012; Grigioni et al., 2000; Gromek et al., 2016; Kaufman et al., 1998; Pichon et al., 2005). In these interactions, the roseobacters colonize and dominate the bacterial community of the reproductive organs (accessory nidamental glands) (Barbieri et al., 2001; Collins et al., 2012; Grigioni et al., 2000; Kaufman et al., 1998; Pichon et al., 2005) and in some cases they also colonize the egg cases (Collins et al., 2012; Pichon et al., 2005). It is believed that *Phaeobacter* spp. are passed on to the eggs of the Hawaiian bobtail squid, *Euprymna scolopes*, by incorporation in the jelly coat (Collins et al., 2012). Gromek *et al.* (2016) characterized *Leisingera* sp. JC1, isolated from the jelly coat of *E. scolopes* eggs, and observed that it was highly bioactive against pathogenic *Vibrio* spp., partly due to the production of the secondary metabolite indigoidine. They suggested that the presence of roseobacters – in this case *Leisingera* sp. JC1 – could have a protective role in egg defense and/or influence the microbial community composition due to production of siderophores, AHLs, and antimicrobials.

Pathogenic interactions between roseobacters and eukaryotes have also been observed. *Nautella* sp. R11 (previously *Ruegeria* sp. R11) and *P. gallaeciensis* LSS9 can cause bleaching of the red alga *Delisea pulchra* (Case et al., 2011; Fernandes et al., 2011; Zozaya-Valdes et al., 2015), though bleaching in nature is likely caused by multiple, opportunistic pathogens (Zozaya-Valdes et al., 2015). Genomic comparisons of the pathogenic, R11 and

LSS9 isolates, and non-pathogenic isolates revealed putative virulence factors in all genomes, though one QS-dependent regulator was unique to the pathogenic isolates (Zozaya-Valdes et al., 2015). While the exact molecular mechanisms behind the pathogenicity are uncertain, it has been speculated that members of the indigenous community can switch from a symbiotic to pathogenic lifestyle, when environmental conditions compromise the host's chemical defenses (Case et al., 2011; Fernandes et al., 2011). Another *Roseobacter* group member, *Roseovarius crassostreae*, can cause juvenile oyster disease and massive mortalities among hatchery-raised American oysters, *Crassostrea virginica* (Boardman et al., 2008; Boettcher et al., 2000; Boettcher et al., 2005). However, the mechanisms of pathogenicity remain uncertain.

Despite numerous studies observing roseobacters in microbiomes of eukaryotes and testing their *in vitro* phenotypes – such as production of secondary metabolites and bioactivity, QS-abilities, and biofilm formation – the knowledge on how they behave and impact microbial communities *in vivo* and *in situ* is scarce. In the present study, two marine eukaryotes – *E. huxleyi* and *Ostrea edulis* (European flat oyster) – model systems were constructed to investigate the impact of TDA-producing *P. inhibens* on the associated bacterial community (Dittmann, Sonnenschein, et al., 2019). Interestingly, the richness was significantly increased in the complex oyster microbiota (Figure 8), which is an indicator of healthy oysters (King et al., 2012; Wegner et al., 2013), though this change was not in the microalgal microbiota.

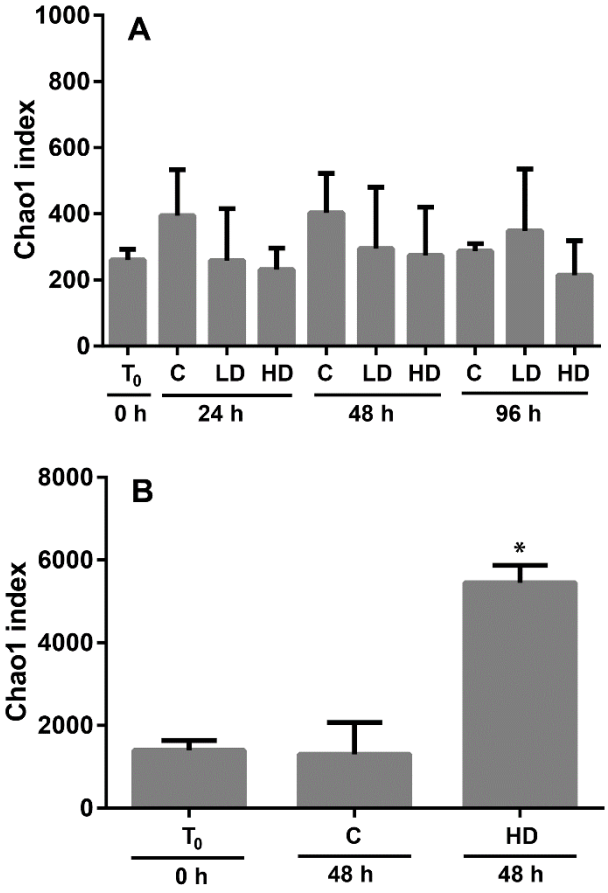


Figure 8: Bacterial richness in microbiotas of (A) coccolithophorid microalga, *Emiliania huxleyi*, and (b) European flat oysters, *Ostrea edulis*. The bars represent the average Chao1 richness estimate for co-cultures/animals exposed to *Phaeobacter inhibens* DSM 17395 at a low dose (LD) or high dose (HD), as well as untreated controls (T_0, C) sampled at time point 0 h, 24 h, 48 h, and 96 h. The error bars represent the standard deviation. Statistical significance ($p \leq 0.05$) is indicated by an asterisk. Please note the difference in the y-axis (Dittmann, Sonnenschein, et al., 2019).

The effect of *P. inhibens* was highly targeted; *Vibrio* spp. and *Pseudoalteromonas* spp. were reduced, while other species such as *Colwellia* spp., *Winogradskyella* sp., *Marinicella* sp., and *Neptuniibacter* sp. were either unaffected or increase in abundance (Figure 9) (Dittmann, Sonnenschein, et al., 2019). This would indicate that both antagonistic and synergetic interactions are occurring. The minor, highly specific effects of *P. inhibens*, that we observed at high abundances of the *Phaeobacter* in the microbiota, could potentially be explained by indigenous presence of TDA-producers and adaptation of the existing microbiota to these secondary metabolite producers prior to our experiments. TDA tolerant bacteria have previously been isolated from marine sponge microbiotas containing TDA-producing *Pseudovibrio* spp. (Harrington et al., 2014). Our observations of OTUs being unaffected or proliferating in the presence of TDA-producing *P. inhibens* could indicate, that tolerance mechanisms could have evolved in our model systems, or that TDA might have a different

function than being an antimicrobial. To elucidate these areas, the mode of action of TDA needs to be considered. This will be addressed below.

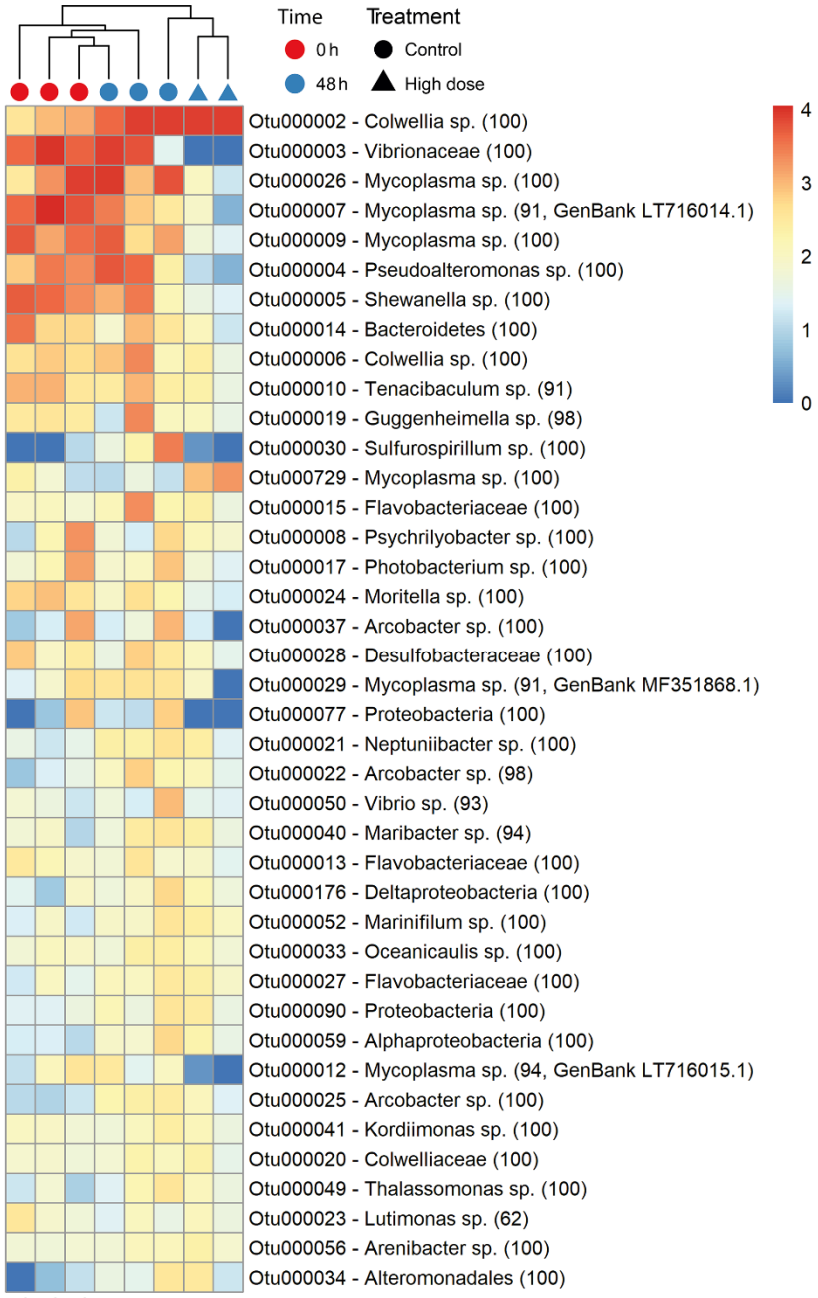


Figure 9: Relative abundances of the 40 most abundant OTUs observed in the *Ostrea edulis* microbiome exposed to *Phaeobacter inhibens* DSM 17395 over time (0 h to 48 h). The relative abundances have been $\log_{10}(x+1)$ transformed. The individual samples (columns) are arranged according to their Bray-Curtis distances between them (tree not drawn to scale). The assigned taxonomy is listed next to the OTU identifier together with their identity scores (%) (Dittmann, Sonnenschein, et al., 2019).

4.2. Tropodithietic acid

TDA is a tropone with a disulfide bridge and a carboxylic acid group (Figure 10). It exists in a tautomeric equilibrium with thiotropocin (Greer et al., 2008). The first description of TDA was in 2003 and it came from *P. inhibens* T5 (orig. *Ruegeria* sp.) isolated in the German Wadden Sea (Brinkhoff et al., 2004; Liang, 2003). However, thiotropocin was discovered a couple of decades before in a *Pseudomonas* sp. collected from soil (Kintaka et al., 1984; Tsubotani et al., 1984). Since then, TDA has repeatedly been observed in various *Pseudovibrio* spp. (Harrington et al., 2014; Penesyan et al., 2011), *Ruegeria* spp. (prev. *Silicibacter* spp.) (Geng et al., Bruhn, 2008; Hjelm et al., 2004; Muramatsu et al., 2007), and *Phaeobacter* spp. (prev. *Roseobacter* spp.) (Breider et al., 2017, 2014; Martens et al., 2006; Ruiz-Ponte et al., 1998; Sonnenschein et al., 2017).

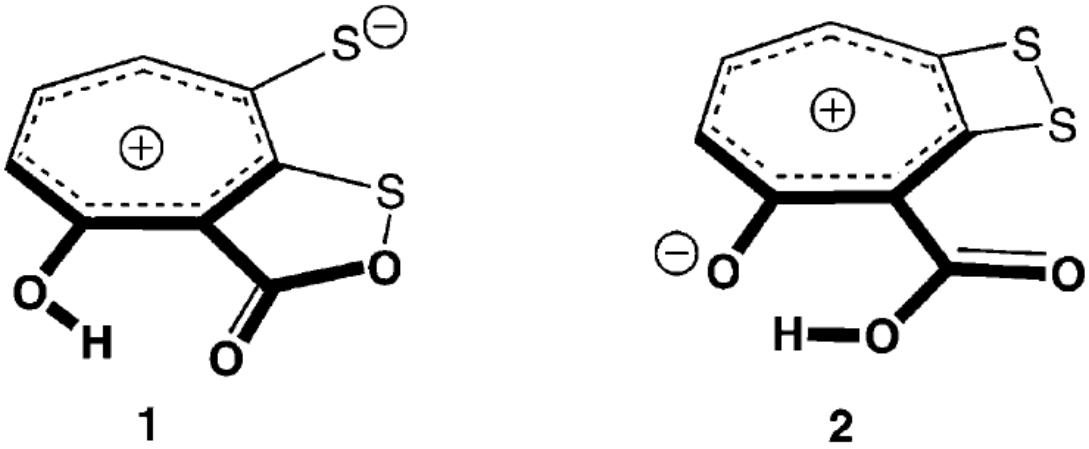


Figure 10: Chemical structure of thiotropocin (1) and tropodithietic acid (2) (Greer et al., 2008).

Biologically active TDA is produced under iron-rich conditions (D'Alvise et al., 2016) which is rather intriguing since the compound, when purified, has siderophoric activity as measured e.g. in a CAS-assay (D'Alvise et al., 2016). A non-inhibitory analogue, pre-TDA, is synthesized by TDA-producers at low iron concentrations, and it can be converted to TDA by acidifying the extract (D'Alvise et al., 2016). Interestingly, TDA can chelate iron through interactions between the disulfide bridge and iron atoms (D'Alvise et al., 2016). However, the compound is not considered a classical siderophore; 1) TDA is only synthesized when iron is present in the medium, as opposed to classical siderophores, which are produced for

iron scavenging at low iron concentrations, and 2) the binding affinity to iron is not as strong as for other siderophores (D'Alvise et al., 2016).

The role and function of TDA in nature is largely unknown. In producer strains, the TDA molecule can act as a signaling molecule impacting global gene regulation, which in turn affects the phenotype including biofilm formation, motility, and secondary metabolite production (Beyersmann et al., 2017). At high concentrations *in vitro*, TDA can act as an antimicrobial agent which works on growing as well as non-growing cells (Porsby et al., 2011).

4.2.1. Activity spectrum of TDA

TDA is bactericidal against both Gram-positive and Gram-negative bacteria; this includes common human pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica*, and *Staphylococcus aureus,* but also aquaculture-related fish pathogens like *V. anguillarum* (Porsby et al., 2011), *V. parahaemolyticus*, and *Vibrio vulnificus* (Porsby & Gram, 2016). To some extent, TDA also has anti-fungal properties towards yeast cells - *Saccharomyces cerevisiae* (Porsby, unpublished data) – and filamentous fungi (thiotropocin) (Kintaka et al., 1984), though, this field has not been investigated in-depth and remains to be understood.

Besides being a broad-range antimicrobial agent, cytotoxicity and anticancer potential has also been investigated. TDA has low toxicity to the animal model organism *Caenorhabditis elegans* (nematode) (Neu et al., 2014), but it did show pronounced toxicity towards mammalian neural cells (Wichmann et al., 2015) and human cancer cells (Wilson et al., 2016). The ambiguity concerning cytotoxicity should be further investigated if the pure compound is to be deployed in humans.

4.2.2. The mode of action for TDA

Interestingly, very few, published studies have described their observations concerning TDA, its target, and its mode of action as an antibiotic. Porsby *et al.* (2011) were some of the first to elucidate the target of TDA, which they hypothesized to be highly conserved and most likely the cell envelope based on results from a biosensor assay, as well as the fact that TDA also works on non-growing cells. A few years later, Wilson *et al.* (2016) investigated the mode of action and came to similar conclusions, though narrowed it down to the proton motive force (PMF). In comparison to other antimicrobial agents with known mode of action, the impact of TDA on *E. coli* resembles the mode of action for polyether antibiotics like salinomycin,

nigericin, and monensin, despite their distinctive structural differences. Polyether antibiotics are ionophores, which can transport ions across membranes against concentration gradients (Kevin II et al., 2009). According to their proposed mode of action, TDA disrupts the PMF by acting as an electroneutral proton antiporter importing H^+ ions and exporting metal (1+) ions (Figure 11) (Wilson et al., 2016). This will decrease the pH in the cytosol and deplete the cells for crucial metal ions, which chelate in the extracellular space.

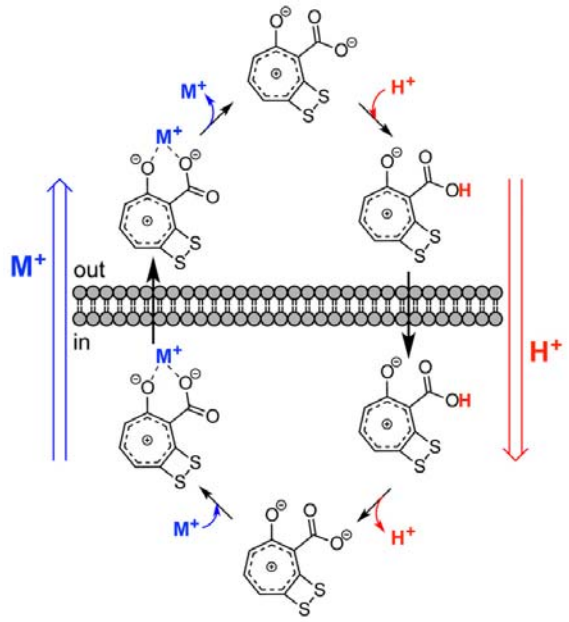


Figure 11: Proposed mechanism of action of tropodithietic acid (TDA) in *Escherichia coli* (Wilson et al., 2016).

In itself, the PMF is a highly conserved function in all cells. The current model for the mode of action of TDA does indeed explain the broad activity spectrum across taxonomic kingdoms. In addition, TDA-producing *P. inhibens* DSM 17395 also produces a less bioactive, methylated analogue of TDA – methyl-troposulfenin – which constitutes up to half of the concentration of TDA in extracts (Phippen et al., 2019). This compound lacks the labile protons, which are assumed to play a major role in the disruption of the membrane potential, according to the proposed mode of action, and concomitantly, it has little to no bioactivity.

4.2.3. Impact of TDA at sub-inhibitory concentrations and potential function

The impact and function of antibiotics at lethal concentrations are usually well-characterized. However, the *in situ* concentrations of microbially produced antibiotics are most likely much

lower, if even detectable with analytical chemistry methods. Antibiotics at sub-inhibitory concentrations can have other functions than being microbial weapons. Some can interfere with cellular regulation systems and global transcription (Linares et al., 2006; Nalca et al., 2006). Besides being a bactericidal antibiotic, TDA can also act as a signaling molecule in TDA-producing *P. inhibens*. Beyersmann *et al.* (2017) used a transcriptomic approach to assess the impact of TDA at a concentration (1.5 μM) 100-fold below the minimal inhibitory concentration of the *P. inhibens* strain DSM 17395. They observed that TDA causes the same transcriptional response as the QS molecule AHL, and both molecules are dependent on the same LuxR-type transcriptional regulator to have an effect. The influence of TDA on transcriptional regulation results in phenotypic changes including dispersal of biofilm, ceased motility, and induction of antibiotic production – traits that are important for settlement on surfaces including host-associated surfaces.

In this thesis, we applied a similar transcriptomic approach using RNA sequencing to assess the influence of a sub-inhibitory concentration (0.6 μM) of TDA on *V. vulnificus* – a marine bacterium, which does not produce TDA (Dittmann, Porsby, et al., 2019). Overall, the response of *V. vulnificus* was in accordance with the proposed mode of action for *E. coli* (Wilson et al., 2016) and the Kohanski theory on bactericidal antibiotics, which induce cell death through oxidative stress (Kohanski et al., 2007). Particularly, genes involved in cell envelope biogenesis and motility were highly affected by TDA exposure (Dittmann, Porsby, et al., 2019). This was presumably due to a phenotypic switch from motility-to-biofilm (Figure 12).

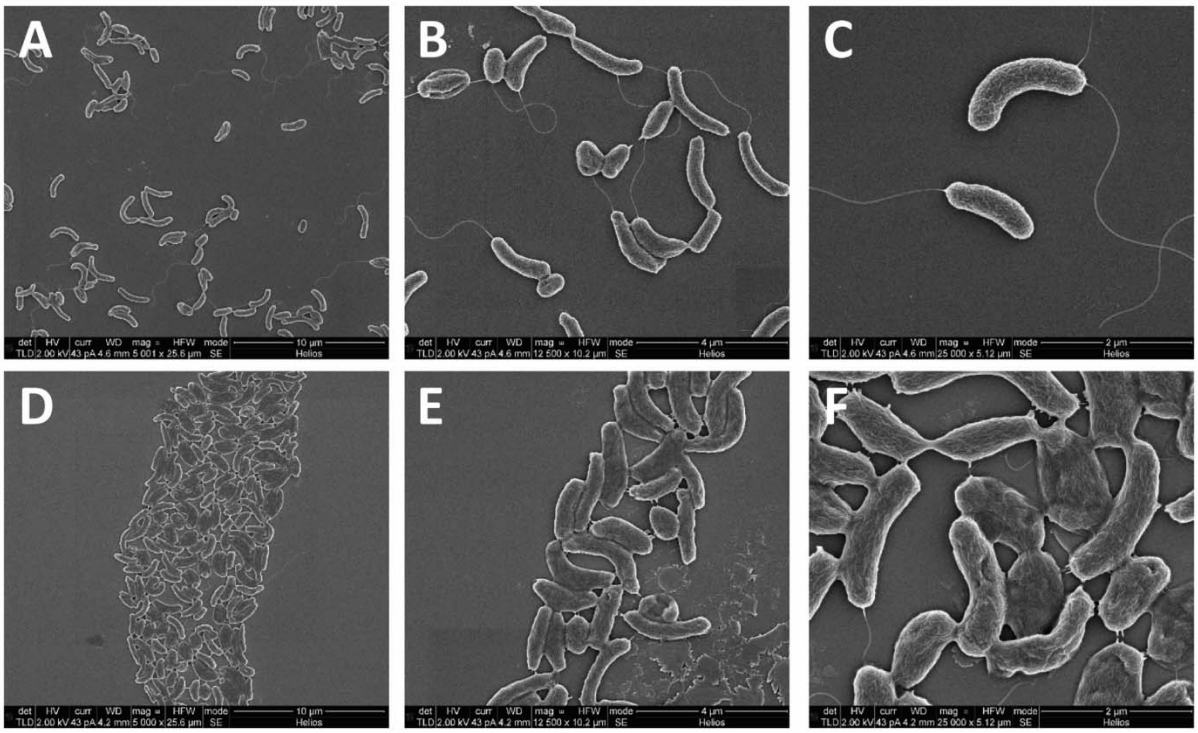


Figure 12: Scanning electron microscopy (SEM) of *Vibrio vulnificus* CMCP6 exposed to tropodithietic acid (TDA; images D-F) and the solvent of TDA (controls; A-C). The scale bars are 10 µm (A and D), 4 µm (B and E), and 2 µm (C and F) (Dittmann, Porsby, et al., 2019).

Biofilm formation is a transient, microbial protective mechanism, which enable microorganisms to survive in stressful environments, including exposure to antibiotics (Høiby et al., 2010; van der Veen & Abee, 2011). Porsby *et al.* (2011) observed transient tolerance to TDA upon long-term exposure, and they speculated that it could be due to a phenotypic switch, which reverses when TDA is absent. Our observations of TDA potentially causing a motility-to-biofilm switch in *V. vulnificus* is in concordance with a reversible, phenotypic switch, which would increase tolerance during exposure. However, not all bacteria are excellent biofilm formers and, to the best of our knowledge, it has not been investigated how TDA impacts species/strains with poor biofilm forming capabilities.

In nature, TDA-producing roseobacters are often found in microbial communities attached to surfaces. Biofilm formation can indeed offer a "herd" protective effect for non-/poor biofilm formers, which has been demonstrated in food-related biofilms (Oxaran et al., 2018), though it depends on the composition of the biofilm members. Another possibility is that the effect of TDA could be decreased or inactivated in natural microbiomes. Wichmann *et al.* (2016) demonstrated that DMSP can act as an antioxidant defense against the oxidizing effect of TDA on neuronal cells by preventing disruption of the mitochondrial membrane potential.

One could speculate that DMSP has the same function in algal microbiomes where TDA-producers are present; reducing the oxidizing effect of TDA and thereby protecting the microbial community. Similarly, this could also occur in other marine eukaryote microbiomes, where certain members of the microbiota might produce antioxidants, and thus ensure balance in the system rather than take-over by the TDA-producer.

A third option could be that TDA also acts as a signaling molecule in non-TDA-producers. TDA acts in a similar manner as AHLs through LuxIR systems in TDA-producing *P. inhibens* (Beyersmann et al., 2017). The phenotypes – motility and biofilm formation – affected by TDA in *V. vulnificus* at a sub-inhibitory concentration (Dittmann, Porsby, et al., 2019) are the same as those affected by TDA activation of the QS-system in *P. inhibens*. Furthermore, AHLs from *P. inhibens* led to down-regulation of major virulence factors – i.e. metalloproteases – in the oyster pathogen *Vibrio coralliilyticus* (Zhao et al., 2018). Though, it should be noted that the TDA "signal" induced a motility-to-biofilm switch in *V. vulnificus*, while less biofilm was produced by *P. inhibens* in the presence of TDA (Beyersmann et al., 2017). Hence, the signal might be triggering different responses in different sensing organisms. This would also explain why certain members of the microbiomes increase in abundance, while the abundance of others decrease (Dittmann et al., 2017; Dittmann, Rasmussen, Melchiorsen, et al., 2019).

Altogether, these phenotypic switches at sub-inhibitory concentrations of TDA could indicate that the probiotic/protective impact of TDA and *Phaeobacter* spp. in complex, eukaryote associated microbiotas are not necessarily solely due to killing but it can also be due to QS-mediated modulation of phenotypes, such as biofilm formation and decreased virulence. These speculations should be tested in complex microbiotas using more advanced variations of omics technologies such as metatranscriptomics and metaproteomics.

4.2.4. Resistance to TDA

While TDA-producers are found in a range of niches, sometimes at high abundances, TDA resistance is rarely observed and the tolerance mechanism is not fully understood. In TDA-producing *P. inhibens* DSM 17395, three genes – *tdaR1* to *tdaR3* – have been identified and they can increase TDA tolerance in *E. coli* when they are heterologously expressed (Wilson et al., 2016). Whether these genes can be exchanged by horizontal gene transfer in nature is uncertain. TdaR1-R2 are predicted as transmembrane proteins, while TdaR3 has similarity to a γ-glutamyl-cyclotransferase, which is involved in cation-proton exchange in *E. coli*.

Interestingly, TDA can also inhibit growth of the TDA-producing *Phaeobacter* spp. (Porsby et al., 2011; Will et al., 2017), most likely due to a high energetic demand (Will et al., 2017).

Among non-TDA-producers, genetic and phenotypic resistance to TDA have not yet been found nor developed in the laboratory. However, TDA tolerant bacteria have been isolated from a marine sponge microbiome, where a TDA-producing *Pseudovibrio* spp. had also been isolated (Harrington et al., 2014). So far, efflux pumps and porins have been out ruled as possible innate tolerance mechanisms (Porsby et al., 2011). Porsby *et al.* (2011) investigated different *in vitro* approaches to adapt and provoke mutations for inducing TDA resistance and tolerance in non-producer strains. Interestingly, several approaches were not successful in *Salmonella typhimurium*, *E. coli*, *P. aeruginosa*, and *S. aureus*. Tolerance to 2 x the minimal inhibitory concentration was transient when the adapted strains were passed through medium without TDA (Porsby et al., 2011). Rasmussen *et al.* (2016) also attempted to adapt the fish pathogen, *V. anguillarum*, to TDA through adaptive laboratory evolution, but no resistant or tolerant strains came out of it. Whole-genome sequencing revealed point mutations, though none of them were consistently due to TDA exposure (Rasmussen et al., 2016). Altogether, our lacking understanding on the TDA resistance and/or tolerance mechanism(s) come back to the insufficient knowledge on the exact target(s) and molecular mechanisms of TDA on/in a cell – particularly at concentrations resembling the levels found in nature.

4.3. Conclusions

Members of the *Roseobacter* group are found in many different environmental niches, reflecting their versatility and adaptability. Both symbiotic and pathogenic relationships are observed between roseobacters and eukaryotes. Some of the interactions with the host have been demonstrated to be influenced by the production of bioactive agents, such as TDA. The impact of TDA-producers on host-associated microbiomes is dependent on the complexity and composition of the microbial community. While the probiotic effect has been coupled to TDA production, the role of TDA in shaping microbiomes is still debatable.

TDA is a broad-spectrum antibiotic compound, which can serve as a competitive advantage for the bacterial producer by protection of its host. Resistance towards TDA is rarely observed and it is difficult to induce, while tolerance can be found, although it is reversible. Disruption of the PMF by acting as an electroneutral proton antiporter importing H^+ ions and exporting metal (1+) ions is the proposed mode of action in *E. coli*. TDA can also act as a signaling molecule regulating different phenotypes, such as biofilm formation, motility, and

secondary metabolite production, both in TDA-producers and *V. vulnificus*. This could indicate that the compound either has multiple functions or that the function *in situ* is modulation of behavior.

5. Concluding remarks and future perspectives

The aquaculture industry is growing rapidly to match the increasing demand for high-quality protein to feed the world population. High intensity farming of animals increases the stress on the animals, and thereby raises the risk of disease. A more productive and sustainable aquaculture requires measures for disease control which are minimally dependent on the antibiotic deployment due to resistance development. Vaccines have decreased the use of antibiotics, but the fish larvae with underdeveloped immune systems need to be protected by measures controlling the microbiome. Microbiome management encompasses stabilization of the rearing water quality, removal of toxic compounds, directing the community towards slow-growing heterotrophs rather than fast-growing opportunists, and addition of probiotics to improve the health of the host.

The successful management of aquaculture microbiotas require in-depth knowledge on the ecology and the microbial interactions occurring in these systems. Our current knowledge-base on marine microbiomes, including microbiotas related to aquaculture, is increasing drastically with the advances and availability of sequencing based methods. We still have a lot to unravel and understand, i.e. what is a "healthy" microbiome? And how can we manipulate and maintain the local microbiota to withstand take-over from opportunistic pathogens?

This PhD took the initial steps of this process by assessing the impact of a probiotic candidate – TDA-producing *P. inhibens* – on the microbiota associated with different trophic levels found in aquaculture and in natural aquatic systems. Previous studies had focused on the antagonistic effect, protection of fish larvae, the bioactive compound, and occurrence in nature, which indeed is important information to understand in the screening of probiotic candidates. However, in most cases, they did not account for the system and the bacterial context that the *P. inhibens* and TDA is acting in. Minor changes were observed in the microbiomes exposed to TDA-producing *P. inhibens*; the changes were highly specific and consistently targeting other roseobacters and (potentially pathogenic) vibrios. While these observations add to the positive outlook for probiotic application, there are still uncertainties regarding the long-term effects of adding the probiotic *P. inhibens*, the optimal introduction route, and how they should be monitored in the aquaculture units to ensure maximum probiotic effect. Further studies using pilot or full-scale trials over extended periods of time

in aquaculture settings are needed to answer some of these unexplored areas, which are essential in the assessment of the application potential.

Besides determining establishment, impact on the commensal microbiome, and long-term effect, it should be noted that *Phaeobacter* spp. and closely related roseobacters have been linked to pathogenicity in algae and molluscs. The virulence factors important for infection are largely unknown, although it is confirmed that certain *Phaeobacter* spp. produce algicidal compounds such as the roseobacticides and that these compounds can kill microalgal species used in aquaculture. Given that not all TDA-producers produce roseobacticides and that certain microalgal species are unaffected by certain variants, it is likely possible to find probiotic strains, which could be used in aquaculture without harming the live-feed. However, this should be further investigated and used for risk assessments of the final strains.

The other pillar of this PhD was focused on the bioactive compound TDA. The *in vitro* bactericidal effect of the pure compound on a range of Gram-positive and Gram-negative bacteria, as well as the killing effect on yeasts and the cytotoxicity to some mammalian cells, had indicated that the target and mechanism of action was essential to all living cells. The proposed mode of action – i.e. disruption of the PMF by acting as an electroneutral proton antiporter importing H^+ ions and exporting metal (1+) ions – had been studied in *E. coli*. Studies had indicated that TDA can also act as a signaling molecule in TDA-producers but interestingly, no studies had looked into mechanism in marine, non-TDA-producing bacteria. In this study, this knowledge gap was addressed. While the compound did not have a growth inhibiting effect, it still caused a pronounced metabolic shift in the *V. vulnificus* cells. Defense phenotypes such as oxidative stress defense, iron scavenging, and biofilm formation were affected. While the oxidative stress was in line with the proposed mode of action in *E. coli*, the phenotypic motility-to-biofilm switch had not been observed. If the defense against TDA is related to a transient phenotype, and that production of antioxidants (such as DMSP in algae) could limit the damage, it could explain why inherent resistance is rarely seen. Motility and biofilm formation is also QS-regulated, which could indicate that the response and impact on several genes observed in the transcriptome, might in fact be QS-induced rather than defensive. If that is the case, then TDA might also act as a behavioral modulator in the microbiomes. Therefore it would be interesting to determine if TDA affects QS-regulated phenotypes in Gram-positive bacteria as well. Furthermore, it would be relevant to assess the impact of TDA on the functionalities in the microbiomes. Pure TDA can change the community structure of the microbiota, but whether or not it modulates behavior is a

different aspect, which is not caught by DNA sequencing. Doing functional studies using metatranscriptomics, metaproteomics, or metabolomics could reveal if pure TDA, at sub-inhibitory concentrations, modulates behavior and potentially limits virulence expression for opportunistic pathogens.

6. Acknowledgements

First of all, I would like to thank my supervisors, Lone Gram and Mikkel Bentzon-Tilia, for their immense support throughout my PhD project. Your enthusiasm, support, feedback, and care has made this journey educational – both at academic and personal level.

I wish to thank Su Egan (Centre for Marine Bio-Innovation, UNSW, Sydney, Australia) for welcoming me into her group and the centre - you also made sure that I had a fantastic, inspiring research stay. Our many discussions and feedback sessions over coffee by the Library Lawn gave valuable output for two of the papers in this thesis. I also wish to thank current and past members of the Centre for Marine Bio-Innovation – particularly Professor Torsten Thomas, Dr. Priscila Goncalves, Elizabeth Copeland, Jennifer Hudson, Dr. Bernd Wemheuer, and Dr. Emmanuelle Botté – for scientific discussion and support, mentoring, inspiration, and much more.

I would like to acknowledge the funding bodies: "Oticon Fonden", "IDAs og Berg-Nielsens Studie- og Støttefond", "Augustinus Fonden", "Fabrikant P.A. Fiskers", "the scholarship Christian og Ottilia Brorsons Rejselegat for yngre videnskabsmænd og –kvinder", and "Otto Mønsted Fonden" for financial support of my research stay in Australia and partipation at the ISME17 conference.

Gratitude should also go to my colleagues in the Bacterial Ecophysiology and Biotechnology group. Our work environment with space for scientific discussion, moral support, and social interactions has been invaluable for me. Thank you goes to Jette for immense support with troublesome DNA extractions and preparation of amplicon libraries. Thanks to the three other leaves - Bastian Barker Rasmussen, Nicole Ciacotich, and Sara Skøtt Paulsen - of a four-leaved clover having their PhD projects side-by-side. We did it! A special thank you goes to Eva Sonnenschein – initially my office mate but soon became a great mentor and a dear friend.

Finally, a special thank you goes to my dear family, my partner Nicolai, and closest friends. Your loving support, encouragement, and stability has meant so much to me. Thank you for believing in me and supporting my ambitious dreams.

7. References

Araújo, C., Muñoz-Atienza, E., Nahuelquín, Y., Poeta, P., Igrejas, G., Hernández, P. E., … Cintas, L. M. (2015). Inhibition of fish pathogens by the microbiota from rainbow trout (*Oncorhynchus mykiss*, Walbaum) and rearing environment. *Anaerobe*, *32*, 7–14. https://doi.org/10.1016/j.anaerobe.2014.11.001

Araújo, C., Muñoz-Atienza, E., Poeta, P., Igrejas, G., Hernández, P., Herranz, C., & Cintas, L. (2016). Characterization of *Pediococcus acidilactici* strains isolated from rainbow trout (*Oncorhynchus mykiss*) feed and larvae: safety, DNA fingerprinting, and bacteriocinogenicity. *Diseases of Aquatic Organisms*, *119*(2), 129–143. https://doi.org/10.3354/dao02992

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

Attramadal, K. J. K., Truong, T. M. H., Bakke, I., Skjermo, J., Olsen, Y., & Vadstein, O. (2014). RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae. *Aquaculture*, *432*, 483–490. https://doi.org/10.1016/J.AQUACULTURE.2014.05.052

Austin, B., Baudet, E., & Stobie, M. (1992). Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. *Journal of Fish Diseases*, *15*(1), 55–61. https://doi.org/10.1111/j.1365-2761.1992.tb00636.x

Austin, B., Stuckey, L. F., Robertson, P. A. W., Effendi, I., & Griffith, D. R. W. (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases*, *18*(1), 93–96. https://doi.org/10.1111/j.1365-2761.1995.tb01271.x

Avnimelech, Y. (1999). Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture*, *176*(3–4), 227–235. https://doi.org/10.1016/S0044-8486(99)00085-X

Bakke, I., Coward, E., Andersen, T., & Vadstein, O. (2015). Selection in the host structures the microbiota associated with developing cod larvae (*Gadus morhua*). *Environmental Microbiology*, *17*(10), 3914–3924. https://doi.org/10.1111/1462-2920.12888

Balcázar, J. L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O., & Muzquiz, J. L. (2007). Sequencing of variable regions of the 16S rRNA gene for identification of lactic acid bacteria isolated from the intestinal microbiota of healthy salmonids. *Comparative Immunology, Microbiology and Infectious Diseases*, *30*(2), 111–118. https://doi.org/10.1016/j.cimid.2006.12.001

Balcázar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J. L., & Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*, *278*(1–4), 188–191. https://doi.org/10.1016/j.aquaculture.2008.03.014

Barbieri, E., Paster, B. J., Hughes, D., Zurek, L., Moser, D. P., Teske, A., & Sogin, M. L. (2001). Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda: Loliginidae). *Environmental Microbiology*, *3*(3), 151–167. https://doi.org/10.1046/j.1462-2920.2001.00172.x

Ben Kahla-Nakbi, A., Chaieb, K., & Bakhrouf, A. (2009). Investigation of several virulence properties among *Vibrio alginolyticus* strains isolated from diseased cultured fish in Tunisia. *Diseases of Aquatic Organisms*, *86*(1), 21–28. https://doi.org/10.3354/dao02091

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

Berger, M., Brock, N. L., Liesegang, H., Dogs, M., Preuth, I., Simon, M., … Brinkhoff, T. (2012). Genetic Analysis of the Upper Phenylacetate Catabolic Pathway in the Production of Tropodithietic Acid by *Phaeobacter gallaeciensis*. *Applied and Environmental Microbiology*, *78*(10), 3539–3551. https://doi.org/10.1128/AEM.07657-11

Beyersmann, P. G., Tomasch, J., Son, K., Stocker, R., Göker, M., Wagner-Döbler, I., … Brinkhoff, T. (2017). Dual function of tropodithietic acid as antibiotic and signaling molecule in global gene regulation of the probiotic bacterium *Phaeobacter inhibens*. *Scientific Reports*, *7*(1), 730. https://doi.org/10.1038/s41598-017-00784-7

Biondi, N., Cheloni, G., Tatti, E., Decorosi, F., Rodolfi, L., Giovannetti, L., … Tredici, M. R.

(2017). The bacterial community associated with *Tetraselmis suecica* outdoor mass cultures. *Journal of Applied Phycology*, *29*(1), 67–78. https://doi.org/10.1007/s10811-016-0966-5

Blanch, A. R., Alsina, M., Simón, M., & Jofre, J. (1997). Determination of bacteria associated with reared turbot (*Scophthalmus maximus*) larvae. *Journal of Applied Microbiology*, *82*(6), 729–734. https://doi.org/10.1046/j.1365-2672.1997.00190.x

Blancheton, J. P., Attramadal, K. J. K., Michaud, L., D'Orbcastel, E. R., & Vadstein, O. (2013). Insight into bacterial population in aquaculture systems and its implication. *Aquacultural Engineering*, *53*, 30–39. https://doi.org/10.1016/j.aquaeng.2012.11.009

Bledsoe, J. W., Peterson, B. C., Swanson, K. S., & Small, B. C. (2016). Ontogenetic Characterization of the Intestinal Microbiota of Channel Catfish through 16S rRNA Gene Sequencing Reveals Insights on Temporal Shifts and the Influence of Environmental Microbes. *PLoS ONE*, *11*(11), e0166379. https://doi.org/10.1371/journal.pone.0166379

Boardman, C. L., Maloy, A. P., & Boettcher, K. J. (2008). Localization of the bacterial agent of juvenile oyster disease (*Roseovarius crassostreae*) within affected eastern oysters (*Crassostrea virginica*). *Journal of Invertebrate Pathology*, *97*(2), 150–158. https://doi.org/10.1016/J.JIP.2007.08.007

Boettcher, K. J., Barber, B. J., & Singer, J. T. (2000). Additional Evidence that Juvenile Oyster Disease Is Caused by a Member of the *Roseobacter* Group and Colonization of Nonaffected Animals by *Stappia stellulata*-Like Strains. *Applied and Environmental Microbiology*, *66*(9), 3924–3930. https://doi.org/10.1128/AEM.66.9.3924-3930.2000

Boettcher, K. J., Geaghan, K. K., Maloy, A. P., & Barber, B. J. (2005). *Roseovarius crassostreae* sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. *International Journal of Systematic and Evolutionary Microbiology*, *55*(4), 1531–1537. https://doi.org/10.1099/ijs.0.63620-0

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., … Caporaso, J. G. (2018). QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Preprints*. https://doi.org/10.7287/peerj.preprints.27295v2

Breider, S., Freese, H. M., Spröer, C., Simon, M., Overmann, J., & Brinkhoff, T. (2017). *Phaeobacter porticola* sp. nov., an antibiotic-producing bacterium isolated from a sea harbour. *International Journal of Systematic and Evolutionary Microbiology*, *67*(7), 2153–2159. https://doi.org/10.1099/ijsem.0.001879

Breider, S., Scheuner, C., Schumann, P., Fiebig, A., Petersen, J., Pradella, S., … Göker, M. (2014). Genome-scale data suggest reclassifications in the *Leisingera-Phaeobacter* cluster including proposals for *Sedimentitalea* gen. nov. and *Pseudophaeobacter* gen. nov. *Frontiers in Microbiology*, *5*, 1–13. https://doi.org/10.3389/fmicb.2014.00416

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

Buchan, A., Gonzalez, J. M., & Moran, M. A. (2005). Overview of the Marine *Roseobacter* Lineage. *Applied and Environmental Microbiology*, *71*(10), 5665–5677. https://doi.org/10.1128/AEM.71.10.5665-5677.2005

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology*, *8*(7), 1137–1144. https://doi.org/10.1111/j.1462-2920.2006.01054.x

Cabello, F. C., Godfrey, H. P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A., & Buschmann, A. H. (2013). Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. *Environmental Microbiology*, *15*(7), 1917–1942. https://doi.org/10.1111/1462-2920.12134

Califano, G., Castanho, S., Soares, F., Ribeiro, L., Cox, C. J., Mata, L., & Costa, R. (2017). Molecular Taxonomic Profiling of Bacterial Communities in a Gilthead Seabream (*Sparus aurata*) Hatchery. *Frontiers in Microbiology*, *8*, 1–16. https://doi.org/10.3389/fmicb.2017.00204

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*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Campbell, B. J., & Kirchman, D. L. (2013). Bacterial diversity, community structure and potential growth rates along an estuarine salinity gradient. *The ISME Journal*, *7*(1), 210–220. https://doi.org/10.1038/ismej.2012.93

Cao, J., Zhang, J., Ma, L., Li, L., Zhang, W., & Li, J. (2018). Identification of fish source *Vibrio alginolyticus* and evaluation of its bacterial ghosts vaccine immune effects. *MicrobiologyOpen*, *7*(3), e00576. https://doi.org/10.1002/mbo3.576

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Carnevali, O., Zamponi, M. C., Sulpizio, R., Rollo, A., Nardi, M., Orpianesi, C., … Cresci, A. (2004). Administration of Probiotic Strain to Improve Sea Bream Wellness during Development. *Aquaculture International*, *12*(4/5), 377–386. https://doi.org/10.1023/B:AQUI.0000042141.85977.bb

Case, R. J., Longford, S. R., Campbell, A. H., Low, A., Tujula, N., Steinberg, P. D., & Kjelleberg, S. (2011). Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. *Environmental Microbiology*, *13*(2), 529–537. https://doi.org/10.1111/j.1462-2920.2010.02356.x

Chabrillón, M., Rico, R. M., Arijo, S., Díaz-Rosales, P., Balebona, M. C., & Morinigo, M. A. (2005). Interactions of microorganisms isolated from gilthead sea bream, *Sparus aurata* L., on *Vibrio harveyi*, a pathogen of farmed Senegalese sole, *Solea senegalensis* (Kaup). *Journal of Fish Diseases*, *28*(9), 531–537. https://doi.org/10.1111/j.1365-2761.2005.00657.x

Chabrillón, M., Rico, R. M., Balebona, M. C., & Morinigo, M. A. (2005). Adhesion to sole, *Solea senegalensis* Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with *Photobacterium damselae* subsp. *piscicida*. *Journal of Fish Diseases*, *28*, 229–237. https://doi.org/10.1111/j.1365-2761.2005.00623.x

Chu, W., Zhou, S., Zhu, W., & Zhuang, X. (2015). Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. *Scientific Reports*, *4*(1), 5446. https://doi.org/10.1038/srep05446

Collins, A. J., LaBarre, B. A., Wong Won, B. S., Shah, M. V., Heng, S., Choudhury, M. H.,

… Nyholm, S. V. (2012). Diversity and Partitioning of Bacterial Populations within the Accessory Nidamental Gland of the Squid *Euprymna scolopes*. *Applied and Environmental Microbiology*, *78*(12), 4200–4208. https://doi.org/10.1128/AEM.07437-11

Colwell, R. R., Huq, A., Islam, M. S., Aziz, K. M. A., Yunus, M., Khan, N. H., … Russek-Cohen, E. (2003). Reduction of cholera in Bangladeshi villages by simple filtration. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(3), 1051–1055. https://doi.org/10.1073/pnas.0237386100

Cooper, M. A., & Shlaes, D. (2011). Fix the antibiotics pipeline. *Nature*, *472*(7341), 32–32. https://doi.org/10.1038/472032a

Cooper, M. B., Smith, A. G., Paszkowski, U., & Scott, D. B. (2015). Exploring mutualistic interactions between microalgae and bacteria in the omics age. *Current Opinion in Plant Biology*, *26*, 147–153. https://doi.org/10.1016/j.pbi.2015.07.003

Cordero, H., Morcillo, P., Meseguer, J., Cuesta, A., & Esteban, M. Á. (2016). Effects of *Shewanella putrefaciens* on innate immunity and cytokine expression profile upon high stocking density of gilthead seabream specimens. *Fish & Shellfish Immunology*, *51*, 33–40. https://doi.org/10.1016/j.fsi.2016.02.008

Crab, R., Chielens, B., Wille, M., Bossier, P., & Verstraete, W. (2010). The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. *Aquaculture Research*, *41*(4), 559–567. https://doi.org/10.1111/j.1365-2109.2009.02353.x

Crab, R., Defoirdt, T., Bossier, P., & Verstraete, W. (2012). Biofloc technology in aquaculture: Beneficial effects and future challenges. *Aquaculture*, *356–357*, 351–356. https://doi.org/10.1016/j.aquaculture.2012.04.046

Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J., & Smith, A. G. (2005). Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature*, *438*(7064), 90–93. https://doi.org/10.1038/nature04056

Cytryn, E., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., & Minz, D. (2005). Identification of bacteria potentially responsible for oxic and anoxic sulfide oxidation in biofilters of a recirculating mariculture system. *Applied and Environmental*

Microbiology, *71*(10), 6134–6141. https://doi.org/10.1128/AEM.71.10.6134-6141.2005

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

D'Alvise, P. W., Lillebø, S., Wergeland, H. I., Gram, L., & Bergh, Ø. (2013). Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparison of strains and introduction times. *Aquaculture*, *384–387*, 82–86. https://doi.org/10.1016/J.AQUACULTURE.2012.12.013

D'Alvise, P. W., Melchiorsen, J., Porsby, C. H., Nielsen, K. F., & Gram, L. (2010). Inactivation of *Vibrio anguillarum* by attached and planktonic *Roseobacter* cells. *Applied and Environmental Microbiology*, *76*(7), 2366–2370. https://doi.org/10.1128/AEM.02717-09

D'Alvise, P. W., Phippen, C. B. W., Nielsen, K. F., & Gram, L. (2016). Influence of Iron on Production of the Antibacterial Compound Tropodithietic Acid and Its Noninhibitory Analog in *Phaeobacter inhibens*. *Applied and Environmental Microbiology*, *82*(2), 502–509. https://doi.org/10.1128/AEM.02992-15

Dang, H., & Lovell, C. R. (2000). Bacterial Primary Colonization and Early Succession on Surfaces in Marine Waters as Determined by Amplified rRNA Gene Restriction Analysis and Sequence Analysis of 16S rRNA Genes. *Applied and Environmental Microbiology*, *66*(2), 467–475. https://doi.org/10.1128/AEM.66.2.467-475.2000

Defoirdt, T., Sorgeloos, P., & Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*, *14*(3), 251–258. https://doi.org/10.1016/j.mib.2011.03.004

Defoirdt, T., Thanh, L. D., Van Delsen, B., De Schryver, P., Sorgeloos, P., Boon, N., & Bossier, P. (2011). N-acylhomoserine lactone-degrading *Bacillus* strains isolated from aquaculture animals. *Aquaculture*, *311*, 258–260. https://doi.org/10.1016/j.aquaculture.2010.11.046

Dhanasiri, A. K. S., Brunvold, L., Brinchmann, M. F., Korsnes, K., Bergh, Ø., & Kiron, V. (2011). Changes in the Intestinal Microbiota of Wild Atlantic cod *Gadus morhua* L.

Upon Captive Rearing. *Microbial Ecology*, *61*, 20–30. https://doi.org/10.1007/s00248-010-9673-y

Dittmann, K. K., Porsby, C. H., Goncalves, P., Mateiu, R. V., Sonnenschein, E. C., Bentzon Tilia, M., … Gram, L. (2019). Tropodithietic acid induces oxidative stress response, cell envelope biogenesis and iron uptake in *Vibrio vulnificus*. *Environmental Microbiology Reports*, 1758–2229.12771. https://doi.org/10.1111/1758-2229.12771

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

Dittmann, K. K., Rasmussen, B. B., Melchiorsen, J., Sonnenschein, E. C., Gram, L., & Bentzon-Tilia, M. (2019). Roseobacter probiotics affect lower-trophic level microbiomes in marine aquaculture. *Manuscript in Preparation*.

Dittmann, K. K., Sonnenschein, E. C., Egan, S., Gram, L., & Bentzon Tilia, M. (2019). Impact of *Phaeobacter inhibens* on marine eukaryote associated microbial communities. *Environmental Microbiology Reports*, *11*(3), 401–413. https://doi.org/10.1111/1758-2229.12698

Duerr, E. O. (1998). Cultured microalgae as aquaculture feeds. *Journal of Marine Biotechnology*, *6*(2). Retrieved from https://findit.dtu.dk/en/catalog/2272023584

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Frontiers in Microbiology*, *9*, 873. https://doi.org/10.3389/fmicb.2018.00873

FAO. (2018). *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals.* Rome.

FAO and WHO. (2001). *Probiotics in food - Health and nutrional properties ad guidelines for evaluation. Food and Nutrition Paper* (Vol. 85). Rome.

Fernandes, N., Case, R. J., Longford, S. R., Seyedsayamdost, M. R., Steinberg, P. D., Kjelleberg, S., & Thomas, T. (2011). Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*. *PloS One*,

6(12), e27387. https://doi.org/10.1371/journal.pone.0027387

Fjellheim, A. J., Playfoot, K. J., Skjermo, J., & Vadstein, O. (2012). Inter-individual variation in the dominant intestinal microbiota of reared Atlantic cod (*Gadus morhua* L.) larvae. *Aquaculture Research*, *43*(10), 1499–1508. https://doi.org/10.1111/j.1365-2109.2011.02952.x

Foesel, B. U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., … Schramm, A. (2008). *Nitrosomonas* Nm143-like ammonia oxidizers and *Nitrospira marina*-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. *FEMS Microbiology Ecology*, *63*(2), 192–204. https://doi.org/10.1111/j.1574-6941.2007.00418.x

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology*, *66*(5), 365–378. https://doi.org/10.1111/j.1365-2672.1989.tb05105.x

Garriques, D., & Arevalo, G. (1995). An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. In *Swimming Through Troubled Water. Proceedings of the Special Session on Shrimp Farming, Aquaculture '95.* (pp. 53–59).

Gatesoupe, F.-J. (1991). The effect of three strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for larval turbot, *Scophthalmus maximus*. *Aquaculture*, *96*(3–4), 335–342. https://doi.org/10.1016/0044-8486(91)90162-Z

Gatesoupe, F.-J. (1994). Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus,* against pathogenic vibrio. *Aquatic Living Resources*, *7*(4), 277–282. https://doi.org/10.1051/alr:1994030

Gatesoupe, F.-J. (1999). The use of probiotics in aquaculture. *Aquaculture*, *180*(1–2), 147–165. https://doi.org/10.1016/S0044-8486(99)00187-8

Geng, H., Bruhn, J. B., Nielsen, K. F., Gram, L., & Belas, R. (2008). Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters. *Applied and Environmental Microbiology*, *74*(5), 1535–1545. https://doi.org/10.1128/AEM.02339-07

Geng, H., Tran-Gyamfi, M. B., Lane, T. W., Sale, K. L., & Yu, E. T. (2016). Changes in the Structure of the Microbial Community Associated with *Nannochloropsis salina*

following Treatments with Antibiotics and Bioactive Compounds. *Frontiers in Microbiology*, *7*, 1155. https://doi.org/10.3389/fmicb.2016.01155

Glöckner, F. O., & Joint, I. (2010). Marine microbial genomics in Europe: current status and perspectives. *Microbial Biotechnology*, *3*(5), 523–530. https://doi.org/10.1111/j.1751-7915.2010.00169.x

Gómez-León, J., Villamil, L., Lemos, M. L., Novoa, B., & Figueras, A. (2005). Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. *Applied and Environmental Microbiology*, *71*(1), 98–104. https://doi.org/10.1128/AEM.71.1.98-104.2005

González, J. M., & Moran, M. A. (1997). Numerical dominance of a group of marine bacteria in the alpha-subclass of the class *Proteobacteria* in coastal seawater. *Applied and Environmental Microbiology*, *63*(11), 4237–4242. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4265956

González, J. M., Simó, R., Massana, R., Covert, J. S., Casamayor, E. O., Pedrós-Alió, C., & Moran, M. A. (2000). Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. *Applied and Environmental Microbiology*, *66*(10), 4237–4246. https://doi.org/10.1128/AEM.66.10.4237-4246.2000

Gram, L., Løvold, T., Nielsen, J., Melchiorsen, J., & Spanggaard, B. (2001). In vitro antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. *Aquaculture*, *199*(1–2), 1–11. https://doi.org/10.1016/S0044-8486(01)00565-8

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., & Nielsen, T. F. (1999). Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a Possible Probiotic Treatment of Fish. *Applied and Environmental Microbiology*, *65*(3), 969–973.

Greer, E. M., Aebisher, D., Greer, A., & Bentley, R. (2008). Computational Studies of the Tropone Natural Products, Thiotropocin, Tropodithietic Acid, and Troposulfenin. Significance of Thiocarbonyl−Enol Tautomerism. *The Journal of Organic Chemistry*, *73*(1), 280–283. https://doi.org/10.1021/jo7018416

Grigioni, S., Boucher-Rodoni, R., Demarta, A., Tonolla, M., & Peduzzi, R. (2000). Phylogenetic characterisation of bacterial symbionts in the accessory nidamental glands of the sepioid *Sepia officinalis* (Cephalopoda: Decapoda). *Marine Biology*, *136*(2), 217–222. https://doi.org/10.1007/s002270050679

Gromek, S. M., Suria, A. M., Fullmer, M. S., Garcia, J. L., Gogarten, J. P., Nyholm, S. V., & Balunas, M. J. (2016). *Leisingera* sp. JC1, a bacterial isolate from hawaiian bobtail squid eggs, produces indigoidine and differentially inhibits vibrios. *Frontiers in Microbiology*, *7*(SEP), 1–16. https://doi.org/10.3389/fmicb.2016.01342

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P. W., Dierckens, K., Bossier, P., & Gram, L. (2016). *Phaeobacter inhibens* as probiotic bacteria in non-axenic *Artemia* and algae cultures. *Aquaculture*, *462*, 64–69.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P. W., Dourala, N., Nielsen, K. F., & Gram, L. (2016). Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. *Systematic and Applied Microbiology*, *39*, 180–188.

Hansen, G. H., & Olafsen, J. A. (1999). Bacterial Interactions in Early Life Stages of Marine Cold Water Fish. *Microbial Ecology*, *38*(1), 1–26. https://doi.org/10.1007/s002489900158

Hari, B., Madhusoodana Kurup, B., Varghese, J. T., Schrama, J. W., & Verdegem, M. C. . (2004). Effects of carbohydrate addition on production in extensive shrimp culture systems. *Aquaculture*, *241*(1–4), 179–194. https://doi.org/10.1016/j.aquaculture.2004.07.002

Harrington, C., Reen, F., Mooij, M., Stewart, F., Chabot, J.-B., Guerra, A., … O'Gara, F. (2014). Characterisation of Non-Autoinducing Tropodithietic Acid (TDA) Production from Marine Sponge *Pseudovibrio* Species. *Marine Drugs*, *12*, 5960–5978. https://doi.org/10.3390/md12125960

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME Journal*, *5*(10), 1571–1579. https://doi.org/10.1038/ismej.2011.41

Higuera-Llantén, S., Vásquez-Ponce, F., Barrientos-Espinoza, B., Mardones, F. O., Marshall,

S. H., & Olivares-Pacheco, J. (2018). Extended antibiotic treatment in salmon farms select multiresistant gut bacteria with a high prevalence of antibiotic resistance genes. *PLoS ONE*, *13*(9), e0203641. https://doi.org/10.1371/journal.pone.0203641

Hjelm, M., Bergh, O., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., … Gram, L. (2004). Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Systematic and Applied Microbiology*, *27*, 360–371. https://doi.org/10.1078/0723-2020-00256

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., & Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*, *35*(4), 322–332. https://doi.org/10.1016/j.ijantimicag.2009.12.011

Høj, L., Bourne, D. G., & Hall, M. R. (2009). Localization, abundance and community structure of bacteria associated with *Artemia*: Effects of nauplii enrichment and antimicrobial treatment. *Aquaculture*, *293*(3–4), 278–285. https://doi.org/10.1016/j.aquaculture.2009.04.024

Hovda, M. B., Lunestad, B. T., Fontanillas, R., & Rosnes, J. T. (2007). Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *272*(1–4), 581–588. https://doi.org/10.1016/j.aquaculture.2007.08.045

Ingerslev, H. C., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M., & Madsen, L. (2014). The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture*, *424–425*, 24–34. https://doi.org/10.1016/j.aquaculture.2013.12.032

Kaneko, T., & Colwell, R. R. (1973). Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. *Journal of Bacteriology*, *113*(1), 24–32.

Kanther, M., & Rawls, J. F. (2010). Host–microbe interactions in the developing zebrafish. *Current Opinion in Immunology*, *22*(1), 10–19. https://doi.org/10.1016/J.COI.2010.01.006

Kaufman, M. R., Ikeda, Y., Patton, C., van Dykhuizen, G., & Epel, D. (1998). Bacterial Symbionts Colonize the Accessory Nidamental Gland of the Squid *Loligo opalescens* via Horizontal Transmission. *The Biological Bulletin*, *194*(1), 36–43. https://doi.org/10.2307/1542511

Kevin II, D. A., Meujo, D. A., & Hamann, M. T. (2009). Polyether ionophores: broad-spectrum and promising biologically active molecules for the control of drug-resistant bacteria and parasites. *Expert Opinion on Drug Discovery*, *4*(2), 109–146. https://doi.org/10.1517/17460440802661443

Kibenge, F. S. B., Godoy, M. G., Fast, M., Workenhe, S., & Kibenge, M. J. T. (2012). Countermeasures against viral diseases of farmed fish. *Antiviral Research*, *95*(3), 257–281. https://doi.org/10.1016/J.ANTIVIRAL.2012.06.003

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of Stomach and Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. *PLoS ONE*, *7*(12), e51475. https://doi.org/10.1371/journal.pone.0051475

Kintaka, K., Ono, H., Tsubotani, S., Harada, S., & Okazaki, H. (1984). Thiotropocin, a new sulfur-containing 7-membered-ring antibiotic produced by a *Pseudomonas* sp. *The Journal of Antibiotics*, *37*(11), 1294–1300. https://doi.org/10.7164/antibiotics.37.1294

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell*, *130*(5), 797–810. https://doi.org/10.1016/J.CELL.2007.06.049

Li, X., Yu, Y., Feng, W., Yan, Q., & Gong, Y. (2012). Host species as a strong determinant of the intestinal microbiota of fish larvae. *The Journal of Microbiology*, *50*(1), 29–37. https://doi.org/10.1007/s12275-012-1340-1

Liang, L. (2003). *Investigation of Secondary Metabolites of North Sea Bacteria: Fermentation, Isolation, Structure Elucidation and Bioactivity*. University of Göttingen.

Linares, J. F., Gustafsson, I., Baquero, F., & Martinez, J. L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences*, *103*(51), 19484–19489. https://doi.org/10.1073/pnas.0608949103

Liu, S., Ren, H., Shen, L., Lou, L., Tian, G., Zheng, P., & Hu, B. (2015). pH levels drive bacterial community structure in sediments of the Qiantang River as determined by 454 pyrosequencing. *Frontiers in Microbiology*, *6*, 285. https://doi.org/10.3389/fmicb.2015.00285

Lozupone, C. A., & Knight, R. (2007). Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(27), 11436–

11440. https://doi.org/10.1073/pnas.0611525104

Majzoub, M. E., Beyersmann, P. G., Simon, M., Thomas, T., Brinkhoff, T., & Egan, S. (2019). *Phaeobacter inhibens* controls bacterial community assembly on a marine diatom. *FEMS Microbiology Ecology*, *95*(6). https://doi.org/10.1093/femsec/fiz060

Makridis, P., Costa, R. A., & Dinis, M. T. (2006). Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia* metanauplii. *Aquaculture*, *255*(1–4), 76–81. https://doi.org/10.1016/j.aquaculture.2005.12.010

Malmstrom, R. R., Kiene, R. P., & Kirchman, D. L. (2004). Identification and enumeration of bacteria assimilating dimethylsulfoniopropionate (DMSP) in the North Atlantic and Gulf of Mexico. *Limnology and Oceanography*, *49*(2), 597–606. https://doi.org/10.4319/lo.2004.49.2.0597

Marchesi, J. R., & Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome*, *3*, 31. https://doi.org/10.1186/s40168-015-0094-5

Martens, T., Heidorn, T., Pukall, R., Simon, M., Tindall, B. J., & Brinkhoff, T. (2006). Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al.* 1998 as *Phaeobacter gallaeciensis* gen. nov., comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* (Lafay *et al.* 1995) Uchino *et al.* 1999 as *Marinovum algicola* gen. nov., comb. nov., and emended descriptions of the genera *Roseobacter*, *Ruegeria* and *Leisingera*. *International Journal of Systematic and Evolutionary Microbiology*, *56*(6), 1293–1304. https://doi.org/10.1099/ijs.0.63724-0

Meron, D., Atias, E., Iasur Kruh, L., Elifantz, H., Minz, D., Fine, M., & Banin, E. (2011). The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*. *The ISME Journal*, *5*(1), 51–60. https://doi.org/10.1038/ismej.2010.102

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T. M., Bøgwald, J., … Ringø, E. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*, *302*(1–2), 1–18. https://doi.org/10.1016/j.aquaculture.2010.02.007

Miller, T. R., & Belas, R. (2004). Dimethylsulfoniopropionate Metabolism by *Pfiesteria*-Associated *Roseobacter* spp. *Applied and Environmental Microbiology*, *70*(6), 3383–

3391. https://doi.org/10.1128/AEM.70.6.3383-3391.2004

Miranda, C. D., Godoy, F. A., & Lee, M. R. (2018). Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. *Frontiers in Microbiology*, *9*, 1284. https://doi.org/10.3389/fmicb.2018.01284

Moisander, P. H., Sexton, A. D., & Daley, M. C. (2015). Stable Associations Masked by Temporal Variability in the Marine Copepod Microbiome. *PLoS ONE*, *10*(9), e0138967. https://doi.org/10.1371/journal.pone.0138967

Montanari, M. P., Pruzzo, C., Pane, L., & Colwell, R. R. (1999). Vibrios associated with plankton in a coastal zone of the Adriatic Sea (Italy). *FEMS Microbiology Ecology*, *29*(3), 241–247. https://doi.org/10.1111/j.1574-6941.1999.tb00615.x

Moran, M. A., Belas, R., Schell, M. A., González, J. M., Sun, F., Sun, S., … Buchan, A. (2007). Ecological Genomics of Marine Roseobacters. *Applied and Environmental Microbiology*, *73*(14), 4559. https://doi.org/10.1128/AEM.02580-06

Moran, M. A., Buchan, A., González, J. M., Heidelberg, J. F., Whitman, W. B., Kiene, R. P., … Ward, N. (2004). Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. *Nature*, *432*(7019), 910–913. https://doi.org/10.1038/nature03170

Moran, M. A., González, J. M., & Kiene, R. P. (2003). Linking a Bacterial Taxon to Sulfur Cycling in the Sea: Studies of the Marine *Roseobacter* Group. *Geomicrobiology Journal*, *20*(4), 375–388. https://doi.org/10.1080/01490450303901

Moriarty, D. J. W. (1998). Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture*, *164*(1–4), 351–358. https://doi.org/10.1016/S0044-8486(98)00199-9

Munro, P. D., Barbour, A., & Birkbeck, T. H. (1994). Comparison of the gut bacterial flora of start-feeding larval turbot reared under different conditions. *Journal of Applied Bacteriology*, *77*(5), 560–566. https://doi.org/10.1111/j.1365-2672.1994.tb04402.x

Muramatsu, Y., Uchino, Y., Kasai, H., Suzuki, K. -i., & Nakagawa, Y. (2007). *Ruegeria mobilis* sp. nov., a member of the Alphaproteobacteria isolated in Japan and Palau. *International Journal of Systematic and Evolutionary Microbiology*, *57*(6), 1304–1309. https://doi.org/10.1099/ijs.0.64572-0

Nalca, Y., Jänsch, L., Bredenbruch, F., Geffers, R., Buer, J., & Häussler, S. (2006). Quorum-

Sensing Antagonistic Activities of Azithromycin in *Pseudomonas aeruginosa* PAO1: a Global Approach. *Antimicrobial Agents and Chemotherapy*, *50*(5), 1680–1688. https://doi.org/10.1128/AAC.50.5.1680-1688.2006

Neu, A. K., Månsson, M., Gram, L., & Prol-García, M. J. (2014). Toxicity of Bioactive and Probiotic Marine Bacteria and Their Secondary Metabolites in *Artemia* sp. and *Caenorhabditis elegans* as Eukaryotic Model Organisms. *Applied and Environmental Microbiology*, *80*(1), 146–153. https://doi.org/10.1128/AEM.02717-13

Newton, R. J., Griffin, L. E., Bowles, K. M., Meile, C., Gifford, S., Givens, C. E., … Moran, M. A. (2010). Genome characteristics of a generalist marine bacterial lineage. *The ISME Journal*, *4*(6), 784–798. https://doi.org/10.1038/ismej.2009.150

Niu, Y., Defoirdt, T., Baruah, K., Van de Wiele, T., Dong, S., & Bossier, P. (2014). *Bacillus* sp. LT3 improves the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged with *Vibrio campbellii* by enhancing the innate immune response and by decreasing the activity of shrimp-associated vibrios. *Veterinary Microbiology*, *173*(3–4), 279–288. https://doi.org/10.1016/j.vetmic.2014.08.007

Norwegian Ministry of Health and Care Services. (2015). *National Strategy against Antibiotic Resistance 2015-2020*. Retrieved from https://www.regjeringen.no/contentassets/5eaf66ac392143b3b2054aed90b85210/antibiotic-resistance-engelsk-lavopploslig-versjon-for-nett-10-09-15.pdf

Olafsen, J. A. (2001). Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture*, *200*(1–2), 223–247. https://doi.org/10.1016/S0044-8486(01)00702-5

Olsen, A. I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T. H., Skjermo, J., & Vadstein, O. (2000). Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture*, *190*(1–2), 11–25. https://doi.org/10.1016/S0044-8486(00)00396-3

Oxaran, V., Dittmann, K. K., Lee, S. H. I., Chaul, L. T., Fernandes de Oliveira, C. A., Corassin, C. H., … Gram, L. (2018). Behavior of Foodborne Pathogens *Listeria monocytogenes* and *Staphylococcus aureus* in Mixed-Species Biofilms Exposed to Biocides. *Applied and Environmental Microbiology*, *84*(24), e02038-18. https://doi.org/10.1128/AEM.02038-18

Penesyan, A., Tebben, J., Lee, M., Thomas, T., Kjelleberg, S., Harder, T., & Egan, S. (2011). Identification of the Antibacterial Compound Produced by the Marine Epiphytic Bacterium *Pseudovibrio* sp. D323 and Related Sponge-Associated Bacteria. *Marine Drugs*, *9*, 1391–1402. https://doi.org/10.3390/md9081391

Petersen, J., Brinkmann, H., Bunk, B., Michael, V., Päuker, O., & Pradella, S. (2012). Think pink: photosynthesis, plasmids and the *Roseobacter* clade. *Environmental Microbiology*, *14*(10), 2661–2672. https://doi.org/10.1111/j.1462-2920.2012.02806.x

Petersen, J., Frank, O., Göker, M., & Pradella, S. (2013). Extrachromosomal, extraordinary and essential - the plasmids of the *Roseobacter* clade. *Applied Microbiology and Biotechnology*, *97*(7), 2805–2815. https://doi.org/10.1007/s00253-013-4746-8

Phippen, C. B. W., Jørgensen, C. M., Bentzon-Tilia, M., Gotfredsen, C. H., Larsen, T. O., Gram, L., & Sonnenschein, E. C. (2019). Isolation of Methyl Troposulfenin from *Phaeobacter inhibens*. *Journal of Natural Products*, acs.jnatprod.8b01097. https://doi.org/10.1021/acs.jnatprod.8b01097

Pichon, D., Gaia, V., Norman, M. D., & Boucher-Rodoni, R. (2005). Phylogenetic diversity of epibiotic bacteria in the accessory nidamental glands of squids (Cephalopoda: Loliginidae and Idiosepiidae). *Marine Biology*, *147*(6), 1323–1332. https://doi.org/10.1007/s00227-005-0014-5

Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø., & Pintado, J. (2006). Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture*, *255*, 323–333. https://doi.org/10.1016/j.aquaculture.2005.11.039

Pond, M. J., Stone, D. M., & Alderman, D. J. (2006). Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *261*(1), 194–203. https://doi.org/10.1016/j.aquaculture.2006.06.037

Porsby, C. H., & Gram, L. (2016). *Phaeobacter inhibens* as biocontrol agent against *Vibrio vulnificus* in oyster models. *Food Microbiology*, *57*, 63–70. https://doi.org/10.1016/j.fm.2016.01.005

Porsby, C. H., Nielsen, K. F., & Gram, L. (2008). *Phaeobacter* and *Ruegeria* species of the

Roseobacter clade colonize separate niches in a Danish Turbot (*Scophthalmus maximus*)-rearing farm and antagonize *Vibrio anguillarum* under different growth conditions. *Applied and Environmental Microbiology*, *74*(23), 7356–7364. https://doi.org/10.1128/AEM.01738-08

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), 1332–1337. https://doi.org/10.1128/AAC.01222-10

Prabagaran, S. R., Manorama, R., Delille, D., & Shivaji, S. (2007). Predominance of *Roseobacter*, *Sulfitobacter*, *Glaciecola* and *Psychrobacter* in seawater collected off Ushuaia, Argentina, Sub-Antarctica. *FEMS Microbiology Ecology*, *59*, 342–355. https://doi.org/10.1111/j.1574-6941.2006.00213.x

Prado, S., Montes, J., Romalde, J. L., & Barja, J. L. (2009). Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. *International Microbiology*, *12*(2), 107–114. https://doi.org/10.2436/20.1501.01.87

Ramírez, C., & Romero, J. (2017). The Microbiome of *Seriola lalandi* of Wild and Aquaculture Origin Reveals Differences in Composition and Potential Function. *Frontiers in Microbiology*, *8*, 1844. https://doi.org/10.3389/fmicb.2017.01844

Rao, D., Webb, J. S., & Kjelleberg, S. (2005). Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*. *Applied and Environmental Microbiology*, *71*(4), 1729–1736. https://doi.org/10.1128/AEM.71.4.1729-1736.2005

Rasmussen, B. B., Erner, K. E., Bentzon-Tilia, M., & Gram, L. (2018). Effect of TDA-producing *Phaeobacter inhibens* on the fish pathogen *Vibrio anguillarum* in non-axenic algae and copepod systems. *Microbial Biotechnology*, *0*(0), 1–10. https://doi.org/10.1111/1751-7915.13275

Rasmussen, B. B., Grotkjær, T., D'Alvise, P. W., Yin, G., Zhang, F., Bunk, B., … Gram, L. (2016). *Vibrio anguillarum* is genetically and phenotypically unaffected by long-term continuous exposure to the antibacterial compound tropodithietic acid. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.01047-16

Ringø, E., Løvmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., … Mayhew, T. M. (2010). Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. *Aquaculture Research*, *41*(4), 451–467. https://doi.org/10.1111/j.1365-2109.2009.02339.x

Ringø, E., Olsen, R. E., Jensen, I., Romero, J., & Lauzon, H. L. (2014). Application of vaccines and dietary supplements in aquaculture: possibilities and challenges. *Reviews in Fish Biology and Fisheries*, *24*(4), 1005–1032. https://doi.org/10.1007/s11160-014-9361-y

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S., & Krogdahl, Å. (2006). Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.). *Aquaculture*, *261*(3), 829–841. https://doi.org/10.1016/j.aquaculture.2006.06.030

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*, e2584. https://doi.org/10.7717/peerj.2584

Rørbo, N., Rønneseth, A., Kalatzis, P. G., Rasmussen, B. B., Engell-Sørensen, K., Kleppen, H. P., … Middelboe, M. (2018). Exploring the Effect of Phage Therapy in Preventing *Vibrio anguillarum* Infections in Cod and Turbot Larvae. *Antibiotics*, *7*(2), 42. https://doi.org/10.3390/antibiotics7020042

Ruiz-Ponte, C., Cilia, V., Lambert, C., & Nicolas, J. L. (1998). *Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*. *International Journal of Systematic Bacteriology*, *48*(1 998), 537–542. https://doi.org/10.1099/00207713-48-2-537

Sáenz de Rodrigáñez, M. A., Díaz-Rosales, P., Chabrillón, M., Smidt, H., Arijo, S., León-Rubio, J. M., … Moyano, F. J. (2009). Effect of dietary administration of probiotics on growth and intestine functionality of juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858). *Aquaculture Nutrition*, *15*(2), 177–185. https://doi.org/10.1111/j.1365-2095.2008.00581.x

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., … Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. https://doi.org/10.1128/AEM.01541-09

Schneider, O., Sereti, V., Eding, E. H., & Verreth, J. A. J. (2005). Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquacultural Engineering*, *32*(3–4), 379–401. https://doi.org/10.1016/j.aquaeng.2004.09.001

Schreier, H. J., Mirzoyan, N., & Saito, K. (2010). Microbial diversity of biological filters in recirculating aquaculture systems. *Current Opinion in Biotechnology*, *21*, 318–325. https://doi.org/10.1016/j.copbio.2010.03.011

Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., … Kolter, R. (2016). Dynamic metabolic exchange governs a marine algal-bacterial interaction. *ELife*, *5*, 1–28. https://doi.org/10.7554/eLife.17473

Selje, N., Simon, M., & Brinkhoff, T. (2004). A newly discovered *Roseobacter* cluster in temperate and polar oceans. *Nature*, *427*, 445–448. https://doi.org/10.1038/nature02272

Seyedsayamdost, M. R., Carr, G., Kolter, R., & Clardy, J. (2011). Roseobacticides: Small Molecule Modulators of an Algal-Bacterial Symbiosis. *Journal of the American Chemical Society*, *133*(45), 18343–18349. https://doi.org/10.1021/ja207172s

Seyedsayamdost, M. R., Case, R. J., Kolter, R., & Clardy, J. (2011). The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. *Nature Chemistry*, *3*(4), 331–335. https://doi.org/10.1038/nchem.1002

Seymour, J. R., Simó, R., Ahmed, T., & Stocker, R. (2010). Chemoattraction to Dimethylsulfoniopropionate Throughout the Marine Microbial Food Web. *Science*, *329*(5989), 342–345. https://doi.org/10.1126/SCIENCE.1188418

Silva, Y. J., Costa, L., Pereira, C., Cunha, Â., Calado, R., Gomes, N. C. M., & Almeida, A. (2014). Influence of environmental variables in the efficiency of phage therapy in aquaculture. *Microbial Biotechnology*, *7*(5), 401–413. https://doi.org/10.1111/1751-7915.12090

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., & Vadstein, O. (1997). Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. *Aquaculture International*, *5*(1), 13–28. https://doi.org/10.1007/BF02764784

Skjermo, J., & Vadstein, O. (1999). Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture*, *177*(1–4), 333–343. https://doi.org/10.1016/S0044-

8486(99)00096-4

Sochard, M. R., Wilson, D. F., Austin, B., & Colwell, R. R. (1979). Bacteria associated with the surface and gut of marine copepods. *Applied and Environmental Microbiology*, *37*(4), 750–759.

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*(1), 89–101. https://doi.org/10.1586/14760584.4.1.89

Sonnenschein, E. C., Phippen, C. B. W., Bentzon-Tilia, M., Rasmussen, S. A., Nielsen, K. F., & Gram, L. (2018). Phylogenetic distribution of roseobacticides in the *Roseobacter* group and their effect on microalgae. *Environmental Microbiology Reports*, *10*(3), 383–393. https://doi.org/10.1111/1758-2229.12649

Sonnenschein, E. C., Phippen, C. B. W., Nielsen, K. F., Mateiu, R. V., Melchiorsen, J., Gram, L., … Freese, H. M. (2017). *Phaeobacter piscinae* sp. nov., a species of the *Roseobacter* group and potential aquaculture probiont. *International Journal of Systematic and Evolutionary Microbiology*, *67*(11), 4559–4564. https://doi.org/10.1099/ijsem.0.002331

Sorokin, D. Y. (1994). Influence of thiosulphate on growth of sulphate-forming heterotrophic bacteria from the Black Sea in continuous culture. *Microbiology*, *63*, 255–259.

Sorokin, D. Y. (1995). *Sulfitobacter pontiacus* gen. nov., sp. nov — a new heterotrophic bacterium from the Black Sea, specialized on sulfite oxidation. *Microbiology*, *64*, 295–305.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., & Gram, L. (2000). Proliferation and location of *Vibrio anguillarum* during infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, *23*(6), 423–427. https://doi.org/10.1046/j.1365-2761.2000.00257.x

Summerfelt, S. T. (2003). Ozonation and UV irradiation—an introduction and examples of current applications. *Aquacultural Engineering*, *28*(1–2), 21–36. https://doi.org/10.1016/S0144-8609(02)00069-9

Sunagawa, S., Coelho, L. P., Chaffron, S., Kultima, J. R., Labadie, K., Salazar, G., … Bork, P. (2015). Ocean plankton. Structure and function of the global ocean microbiome. *Science (New York, N.Y.)*, *348*(6237), 1261359. https://doi.org/10.1126/science.1261359

Tan, D., Gram, L., & Middelboe, M. (2014). Vibriophages and their interactions with the fish

pathogen *Vibrio anguillarum*. *Applied and Environmental Microbiology*, *80*(10), 3128–3140. https://doi.org/10.1128/AEM.03544-13

Tapia-Paniagua, S. T., Vidal, S., Lobo, C., Prieto-Álamo, M. J., Jurado, J., Cordero, H., … Moriñigo, M. A. (2014). The treatment with the probiotic *Shewanella putrefaciens* Pdp11 of specimens of *Solea senegalensis* exposed to high stocking densities to enhance their resistance to disease. *Fish & Shellfish Immunology*, *41*(2), 209–221. https://doi.org/10.1016/j.fsi.2014.08.019

The review on antimicrobial resistance. (2015). *Antimicrobials in agriculture and the environment: Reducing unnecessary use and waste*. Retrieved from https://amr-review.org/sites/default/files/Antimicrobials in agriculture and the environment - Reducing unnecessary use and waste.pdf

Thompson, F. L., Iida, T., & Swings, J. (2004). Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews : MMBR*, *68*(3), 403–31, table of contents. https://doi.org/10.1128/MMBR.68.3.403-431.2004

Tinh, N. T. N., Dierckens, K., Sorgeloos, P., & Bossier, P. (2008). A Review of the Functionality of Probiotics in the Larviculture Food Chain. *Marine Biotechnology*, *10*(1), 1–12. https://doi.org/10.1007/s10126-007-9054-9

Toranzo, A. E., Magariños, B., & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, *246*(1–4), 37–61. https://doi.org/10.1016/J.AQUACULTURE.2005.01.002

Tsubotani, S., Wada, Y., Kamiya, K., Okazaki, H., & Harada, S. (1984). Structure of thiotropocin, a new sulfur-containing 7-membered antibiotic. *Tetrahedron Letters*, *25*(4), 419–422. https://doi.org/10.1016/S0040-4039(00)99900-3

United Nations, Department of Economic and Social Affairs, P. D. (2017). *World Population Prospects: The 2017 Revision, Key Findings and Advance Tables.* Working Paper No. ESA/P/WP/248.

Vadstein, O., Attramadal, K. J. K., Bakke, I., Forberg, T., Olsen, Y., Verdegem, M., … Bossier, P. (2018). Managing the Microbial Community of Marine Fish Larvae: A Holistic Perspective for Larviculture. *Frontiers in Microbiology*, *9*, 1820. https://doi.org/10.3389/fmicb.2018.01820

Vadstein, O., Attramadal, K. J. K., Bakke, I., & Olsen, Y. (2018). K-Selection as Microbial Community Management Strategy: A Method for Improved Viability of Larvae in Aquaculture. *Frontiers in Microbiology*, *9*, 2730. https://doi.org/10.3389/fmicb.2018.02730

Vadstein, O., Bergh, Ø., Gatesoupe, F.-J., Galindo-Villegas, J., Mulero, V., Picchietti, S., … Bossier, P. (2013). Microbiology and immunology of fish larvae. *Reviews in Aquaculture*, *5*(Suppl. 1), S1–S25. https://doi.org/10.1111/j.1753-5131.2012.01082.x

van der Veen, S., & Abee, T. (2011). Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. *International Journal of Food Microbiology*, *144*(3), 421–431. https://doi.org/10.1016/j.ijfoodmicro.2010.10.029

Varela, J. L., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arijo, S., León-Rubio, J. M., García-Millán, I., … Mancera, J. M. (2010). Dietary administration of probiotic Pdp11 promotes growth and improves stress tolerance to high stocking density in gilthead seabream *Sparus auratus*. *Aquaculture*, *309*, 265–271. https://doi.org/10.1016/j.aquaculture.2010.09.029

Venkat, H. K., Sahu, N. P., & Jain, K. K. (2004). Effect of feeding *Lactobacillus*-based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii* (de Man). *Aquaculture Research*, *35*, 501–507. https://doi.org/10.1111/j.1365-2109.2004.01045.x

Verner-Jeffreys, D. W., Shields, R. J., Bricknell, I. R., & Birkbeck, T. H. (2003). Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. *Aquaculture*, *219*, 21–42. https://doi.org/10.1016/S0044-8486(02)00348-4

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews : MMBR*, *64*(4), 655–671. https://doi.org/10.1128/MMBR.64.4.655-671.2000

Vestrum, R. I., Attramadal, K. J. K., Winge, P., Li, K., Olsen, Y., Bones, A. M., … 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

Vestrum, R. I., Luef, B., Forberg, T., Bakke, I., & Vadstein, O. (2018). Investigating Fish Larvae-Microbe Interactions in the 21st Century: Old Questions Studied with New Tools. In *Emerging Issues in Fish Larvae Research* (pp. 1–35). Springer International Publishing. https://doi.org/10.1007/978-3-319-73244-2_1

Vezzulli, L., Pezzati, E., Stauder, M., Stagnaro, L., Venier, P., & Pruzzo, C. (2015). Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. *Environmental Microbiology*, *17*(4), 1065–1080. https://doi.org/10.1111/1462-2920.12484

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., & Drider, D. (2019). Benefits and Inputs From Lactic Acid Bacteria and Their Bacteriocins as Alternatives to Antibiotic Growth Promoters During Food-Animal Production. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00057

Weber, B., Chen, C., & Milton, D. L. (2010). Colonization of fish skin is vital for *Vibrio anguillarum* to cause disease. *Environmental Microbiology Reports*, *2*(1), 133–139. https://doi.org/10.1111/j.1758-2229.2009.00120.x

Wegner, K., Volkenborn, N., Peter, H., & Eiler, A. (2013). Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiology*, *13*(1), 252. https://doi.org/10.1186/1471-2180-13-252

Wichmann, H., Brinkhoff, T., Simon, M., Richter-Landsberg, C., Wichmann, H., Brinkhoff, T., … Richter-Landsberg, C. (2016). Dimethylsulfoniopropionate Promotes Process Outgrowth in Neural Cells and Exerts Protective Effects against Tropodithietic Acid. *Marine Drugs*, *14*(5), 89. https://doi.org/10.3390/md14050089

Wichmann, H., Vocke, F., Brinkhoff, T., Simon, M., Richter-Landsberg, C., Wichmann, H., … Richter-Landsberg, C. (2015). Cytotoxic Effects of Tropodithietic Acid on Mammalian Clonal Cell Lines of Neuronal and Glial Origin. *Marine Drugs*, *13*(12), 7113–7123. https://doi.org/10.3390/md13127058

Wietz, M., Gram, L., Jørgensen, B., & Schramm, A. (2010). Latitudinal patterns in the abundance of major marine bacterioplankton groups. *Aquatic Microbial Ecology*, *61*(2), 179–189. https://doi.org/10.3354/ame01443

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., …

Egan, S. (2019). Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (*Seriola lalandi*). *Microbial Biotechnology*, *12*(2), 275–288. https://doi.org/10.1111/1751-7915.13323

Will, S. E., Neumann-Schaal, M., Heydorn, R. L., Bartling, P., Petersen, J., & Schomburg, D. (2017). The limits to growth - energetic burden of the endogenous antibiotic tropodithietic acid in *Phaeobacter inhibens* DSM 17395. *PloS One*, *12*(5), e0177295. https://doi.org/10.1371/journal.pone.0177295

Wilson, M. Z., Wang, R., Gitai, Z., & Seyedsayamdost, M. R. (2016). Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl cycle as a proton sink. *Proceedings of the National Academy of Sciences*, *113*(6), 1630–1635. https://doi.org/10.1073/pnas.1518034113

Wright, J. J., Konwar, K. M., & Hallam, S. J. (2012). Microbial ecology of expanding oxygen minimum zones. *Nature Reviews Microbiology*, *10*(6), 381–394. https://doi.org/10.1038/nrmicro2778

Xu, W. J., Pan, L. Q., Sun, X. H., & Huang, J. (2013). Effects of bioflocs on water quality, and survival, growth and digestive enzyme activities of *Litopenaeus vannamei* (Boone) in zero-water exchange culture tanks. *Aquaculture Research*, *44*(7), 1093–1102. https://doi.org/10.1111/j.1365-2109.2012.03115.x

Zhao, J., Chen, M., Quan, C., & Fan, S. (2015). Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. *Journal of Fish Diseases*, *38*(9), 771–786. https://doi.org/10.1111/jfd.12299

Zhao, W., Yuan, T., Piva, C., Spinard, E. J., Schuttert, C. W., Rowley, D. C., & Nelson, D. R. (2018). The Probiotic Bacterium *Phaeobacter inhibens* Downregulates Virulence Factor Transcription in the Shellfish Pathogen *Vibrio coralliilyticus* by N -Acyl Homoserine Lactone Production. *Applied and Environmental Microbiology*, *85*(2), e01545-18. https://doi.org/10.1128/AEM.01545-18

Zozaya-Valdes, E., Egan, S., & Thomas, T. (2015). A comprehensive analysis of the microbial communities of healthy and diseased marine macroalgae and the detection of known and potential bacterial pathogens. *Frontiers in Microbiology*, *6*, 146. https://doi.org/10.3389/fmicb.2015.00146

Zubkov, M. V., Fuchs, B. M., Archer, S. D., Kiene, R. P., Amann, R., & Burkill, P. H. (2001). Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea. *Environmental Microbiology*, *3*(5), 304–311. https://doi.org/10.1046/j.1462-2920.2001.00196.x

Zubkov, M. V., Fuchs, B. M., Archer, S. D., Kiene, R. P., Amann, R., & Burkill, P. H. (2002). Rapid turnover of dissolved DMS and DMSP by defined bacterioplankton communities in the stratified euphotic zone of the North Sea. *Deep Sea Research Part II: Topical Studies in Oceanography*, *49*(15), 3017–3038. https://doi.org/10.1016/S0967-0645(02)00069-3

Paper 1

Dittmann, K.K., Rasmussen, B.B., Castex, M., Gram, L. & Bentzon-Tilia, M. (2017).

The aquaculture microbiome at the centre of business creation.

(Editorial)

Microb. Biotechnol. **10**, 1279–1282.

doi:10.1111/1751-7915.12877.

microbial biotechnology

Open Access

doi:10.1111/1751-7915.12877

Editorial: The microbiome as a source of new enterprises and job creation

The aquaculture microbiome at the centre of business creation

Karen K. Dittmann,[1] Bastian B. Rasmussen,[1] Mathieu Castex,[2] Lone Gram[1] and Mikkel Bentzon-Tilia[1]

[1]*Department of Biotechnology and Biomedicine, Technical University of Denmark, Kgs. Lyngby, Denmark.*

[2]*Lallemand SAS, Blagnac Cedex, France.*

Twelve per cent of the world's population is currently securing their livelihood partly, or fully, through the fisheries and aquaculture sector (FAO Fisheries and Aquaculture Department, 2016). Most people occupied in this sector rely on wild catches; however, fish stocks are becoming depleted with 90% of stocks being fully or overexploited (FAO Fisheries and Aquaculture Department, 2016). A more productive and sustainable aquaculture sector is needed to meet the sustainable development goals (SDGs) of the UN number 2, 12 and 14 and supply a growing world population, which is expected to reach 10^{10} individuals in approximately 30 years (United Nations, Department of Economic and Social Affairs, Population Division, 2015), with high-quality protein. The aquaculture sector has, within the past few years, surpassed wild catches in the production of seafood (fish and plants combined; Bentzon-Tilia *et al.*, 2016), and overall employment in the fisheries sector has decreased by approximately one million individuals from 2010 to 2014, while the aquaculture sector saw an increase of 0.1 million individuals. In general, a shift has been seen from 1990, where 83% were employed in fisheries and 17% in aquaculture, to 2014 where 67% were employed in fisheries and 33% in aquaculture (FAO Fisheries and Aquaculture Department, 2016). The sector is projected to increase its output from 74 million tons in 2014 to 102 million tons by 2025, and up to 121 million tons by 2030 (FAO Fisheries and Aquaculture Department, 2016). Furthermore, it was recently suggested that the global biological production potential for marine aquaculture is more than 100 times the current global seafood consumption, thus suitable habitats do not seem to be a limiting factor in the growth of the sector (Gentry *et al.*, 2017). Consequently, the industry is faced with a need to significantly increase productivity while at the same time securing both livelihoods and sustainability.

Controlling the microorganisms that are associated with aquaculture systems (i.e. the aquaculture microbiome) has always been essential in high-intensity rearing of fish. Disease outbreaks caused by pathogenic bacteria are believed to be one of the most serious challenges faced by the aquaculture industry (Meyer, 1991), and consequently, extensive measures are taken to limit the introduction and proliferation of such bacteria in the aquaculture systems. Furthermore, microbial activity in these naturally eutrophied systems may produce unwanted toxic metabolites such as hydrogen sulphide (H_2S), which is formed when microorganisms reduce sulphate (SO_4^-) in anaerobic respiration and which interferes with mammalian respiration. However, microbes may also serve as a solution to an array of these very challenges. In the agriculture industry, microbiome-based products such as seed coatings that increase nutrient uptake in crops, and which antagonize plant pathogenic soil organisms, are becoming increasingly popular tools to improve productivity in a sustainable manner, and microbiome-based products may reach a market size comparable to that of chemical agro-chemicals within a few years (Singh, 2017). The very same technologies that have facilitated this development, for example advances in high-throughput sequencing and synthetic biology, have been proposed to be key in the sustainable development of the aquaculture industry in the coming years as well (Bentzon-Tilia *et al.*, 2016). However, with a few exceptions, such as studies on recirculating aquaculture systems and fish-associated microbial communities (van Kessel *et al.*, 2011; Llewellyn *et al.*, 2014), the aquaculture microbiome has not been characterized to the same degree as its terrestrial counterpart. In contrast, most studies concerning the aquaculture microbiome relies on bacterial isolation and PCR-based approaches. Hence, the implementation of microbiome-based products is in its infancy and many practices are still of a 'hope for the best' fertilization-based nature (Moriarty, 1997), where specific functional groups of the aquaculture microbiome are enriched for by adding, for example carbon-rich substrates. This is the case for most 'biofloc' approaches where molasses or an equivalent C-rich fertilizer is added as a means to increase the

C:N ratio and induce the growth of the C-limited heterotrophic fraction of the aquaculture microbiome, which in turn will remove toxic ammonia (NH_3) from the rearing water and form bioflocs (Bossier and Ekasari, 2017). Recirculated aquaculture systems (RAS) and biofilters have facilitated the rearing of fish in closed systems with a minimum of water being exchanged with the surrounding environment. This relies on the successful colonization of large-surface area structures by bacteria such as *Nitrosomonas* spp. and *Nitrospira* spp. that in combination convert NH_3 to nitrate (NO_3^-). Common for these approaches is that they in most cases have relied on modulation of the existing microbiome in the system. However, applications of targeted microbiome-based products containing a seeding microbial assemblage to aid the heterotrophic assimilation of inorganic nitrogen and/or the nitrification process are now a common practice in intensive tropical pond-based aquaculture systems (Castex *et al*., 2014). In the case of RAS technology, a similar approach to aid in the colonization of biofilters is highly desirable as it may take up to several months to obtain an efficient microbiome, specifically in marine biofilters (Manthe and Malone, 1987; Gutierrez-Wing and Malone, 2006). Seeding communities of nitrifiers for pond systems are already available, for example Pond Protect by Novozymes (Table 1), and these have been shown to mitigate increased NH_3 and nitrite (NO_2^-) levels in RAS systems as well (Kuhn *et al*., 2010). Furthermore, nitrification can be coupled with an efficient microbial denitrification process as a powerful

Table 1. Microbiome-based products for conditioning of water and pond as well as promotion of a healthy production animal microbiome (feed and feed additives).

Target environment	Company	Product	Purpose	Composition	Reference
Water and pond	AquaInTech	PRO4000X, AquaPro B, AquaPro EZ	Degrade organic matter, reduce ammonia, *Vibrio* reduction	2 Strains of Bacillus – *Bacillus subtilis, Bacillus licheniformis*	1, 2, 3
	Biomin	Aquastar	Stabilize water quality, improve pond bottom quality and support the gut health of fish and shrimp	Formula not publicly available	4
	Keeton Industries	Waste & Sludge Reducer	Improve water and bottom quality, pathogen control	*Bacillus cereus* RRRL B-30535	5, 6
	Keeton Probiotics	ShrimpShield, PondToss	Degrade organic sludge, improve feed efficiency	Formula not publicly available	7, 8
	Lallemand	Lalsea Biorem	Degrade organic matter, reduce ammonia, pathogen control, stabilize pH	7 specific bacterial strains	9
	Novozymes	Pond Plus	Pathogen control, decomposition of organic substances	Spore forming bacteria	10
	Novozymes	Pond Dtox	Hydrogen sulphide control	*Paracoccus pantotrophus*	11
	Novozymes	Pond Protect	Ammonia and nitrite reduction	*Nitrosomonas eutropha*, *Nitrobacter winogradskyi*	12
Gut microbiome (feed, feed additive)	AquaInTech	AquaPro F	Organic matter degradation, improved digestion of feed	Five strains of bacillus combined	13
	Evonik	EcoBiol	Improve gut health	*Bacillus amyloliquefaciens* CECT 5940	14
	Keeton Probiotics	FeedTreat	Degrade organic sludge and improve feed efficiency	Formula not publicly available	15
	Lallemand	Bactocell®	Reduce deformities across fish species, improve gut health across a range of fish and shrimp species	*Pediococcus acidilactici* (MA18/5M)	16, 17
	Rubinum	TOYOCERIN®	Promote growth, increase specimen homogeneity, improve intestinal mucosa	*Bacillus cereus* var. *toyoi*	18, 19

References: (1) http://www.aqua-in-tech.com/pro4000x.html; (2) http://www.aqua-in-tech.com/aquapro-b.html; (3) http://www.aqua-in-tech.com/aquapro-ez.html; (4) http://www.biomin.net/en/products/aquastar/; (5) http://keetonaquatics.com/beneficial-microbes/waste-and-sludge-reducer/; (6) Patent 'US 6878373 B2'; (7) http://keetonaqua.com/products/beneficial-microbes/shrimpshield/; (8) http://keetonaqua.com/products/beneficial-microbes/pondtoss/; (9) http://lallemandanimalnutrition.com/en/asia/products/lalsea-biorem-aquaculture/; (10) http://www.syndelasia.com/aquaculture-probiotics/pond-aquaculture-probiotics-amp-water-manage-26/pond-plus_38; (11) http://ponddtox.com/; (12) http://www.syndelasia.com/aquaculture-probiotics/pond-aquaculture-probiotics-amp-water-manage-26/pond-protect_40; (13) http://www.aqua-in-tech.com/aquapro-f.html; (14) http://animal-nutrition.evonik.com/product/feed-additives/en/products/probiotics/ecobiol/pages/default.aspx; (15) http://keetonaqua.com/products/beneficial-microbes/feedtreat/; (16) http://lallemandanimalnutrition.com/en/asia/products/bactocell-2/; (17) http://www.biomar.com/en/denmark/product-and-species/pike-perch/fry_feeds/; (18) http://www.rubinum.es/en/especies/#acuicultura; (19) http://www.rubinum.es/en/productos/.

tool in the complete removal of nitrogenous compounds from the system, and the development and application of a joined nitrification and denitrification approach for recirculated aquaculture systems, similar to the Aqua Science® concept from Camanor, likely represents an area of potential business development. The commercialization of targeted microbiome-based products containing living microorganisms, such as seeding microbial assemblages that improve water quality, has been seen for use in aquaria for decades, for example the *BIO-Spira* product from MarineLand Labs and its predecessors, which like Pond Protect and similar microbiome-based products for aquaculture systems contain bacterial assemblages that remove ammonia and nitrite. Similar microbiome-based products for use in conjunction with biofloc technology are also available now. One such product is Shrimp-Shield by Keeton Probiotics, which facilitates biofloc formation, degradation of sludge as well as microbial removal of NH_3 and NO_2^- (Table 1). Hence, such microbiome-based products aim to improve water quality and in some cases remove potential pathogens through, for example, competitive exclusion (Table 1).

Another category of microbiome-based products that is being developed for the aquaculture industry targets the gut of the animal directly (Table 1), equivalent to the more conventional probiotics for livestock and human consumption. Microbial strains evaluated as probiotics for aquaculture are from many phylogenetic lineages; however, most of them belong to two bacterial phyla, the Firmicutes (e.g. *Bacillus* spp., *Lactobacillus* spp., *Lactococcus* spp. and *Carnobacterium* spp.) and the Proteobacteria (e.g. *Vibrio* spp., *Pseudomonas* spp. and *Shewanella* spp.), while yeasts are rarely studied (Gatesoupe, 2007). The majority of the commercially available probiotic feed and feed additives for aquaculture are based on pure or mixed cultures of lactic acid bacteria and Bacilli (Merrifield *et al*., 2010; Castex *et al*., 2014). This includes Bactocell® (Lallemand; Table 1), which is based on a *Pediococcus acidilactici* strain and is, to the best of our knowledge, the only probiotic registered in Europe for use in aquaculture feed. These bacteria are usually well studied and well known for their positive effect on the human and animal gut microbiome (Cutting, 2011). Furthermore, they are Generally Regarded As Safe (GRAS) or Qualified Presumption of Safety (QPS), which makes it easier to obtain authorization for their use in food and feed products. A natural extension of this type of microbiome-based products, and a potential new avenue to be explored in aquaculture microbiome business creation, is the controlled colonization of the reared fish from larvae to adult by a microbiome that has the desired functional traits and can act as an infection barrier against pathogens and prevent major economic losses by crashes in the population (De Schryver and Vadstein, 2014).

The successful application of probiotic Firmicutes, originally applied as probiotics for humans or livestock, in aquaculture is fortunate considering the divergent niches in which these probiotics need to establish themselves and function. An avenue of potential new enterprises is to develop similar products based on bacteria of marine origin instead. Marine bacteria including members of the *Roseobacter* group and the *Vibrio* and *Shewanella* genera have been studied extensively for their probiotic potential (Austin *et al*., 1995; Ringø and Vadstein, 1998; Díaz-Rosales *et al*., 2009; D'Alvise *et al*., 2012; Lobo *et al*., 2014; Grotkjær *et al*., 2016; Bentzon-Tilia and Gram, 2017). Furthermore, these are often found as part of the indigenous microbiome of marine eukaryotes, and although their application as probiotics has been proposed, they have not yet reached a commercialization stage. To succeed with this approach, much more thorough characterizations of aquaculture and marine host microbiomes are needed. Furthermore, in most cases, the putative probiotic candidates reported in scientific publications do not go on to commercialization and industrial application. Getting a probiont to the commercial market requires many additional steps including assessments of safety, scale-up efficacy, production scale-up and pre-market registration. Consistency, efficiency and most importantly safety are key points in all large-scale productions, and they should be considered from the early stages of the discovery phase to the final application in feed products. Thus, not only does the development of a commercial product rely on substantial financial investments, but also on the contribution from a multidisciplinary team encompassing close collaborations between scientists, aquaculture experts, fermentation engineers and regulatory personnel. The latter part of the team is important for success in a regulatory landscape which varies from an absence of regulation in certain countries to a rigid regulatory framework not always adapted to the effect a probiotic can display. Despite these challenges, the aquaculture industry has already embraced the industrial application of microbiome-based products for the last two decades, and this has truly created a vast range of new enterprises especially in South East Asia, Central and South America and more recently in Europe.

Using microbiome-based products also requires developments of production, packaging and distribution technology. One must consider that the efficiency of such products only in part depends on the choice of the microbial strains that compose it (selection), but also on the way the product is produced, conditioned and finally packaged to withstand a variety of storage conditions.

In conclusion, the aquaculture industry is one of the fastest growing food producing sectors in the world and the increased productivity of this sector is essential for the fulfilment of the sustainable development goals of the UN. Microbiome-based products for application in industrial aquaculture are today a reality, but the full potential is far from exploited. Despite decades of experience and an increasing number of microbial biotechnological products, there is a large innovation potential; from the discovery of new probionts of marine origin and large-scale cultivation strategies to manoeuvering the political, regulatory landscape and disseminating the use of probiotics to ensure future, sustainable technologies for high-quality protein production.

References

Austin, B., Stuckey, L. F., Robertson, P. A. W., Effendi, I., and Griffith, D. R. W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J Fish Dis* **18:** 93–96.

Bentzon-Tilia, M., and Gram, L. (2017) Biotechnological Applications of the *Roseobacter* Clade. In *Biotechnological Applications of the Roseobacter Clade*. Paterson, R., and Lima, N. (eds). Basel: Springer International Publishing, pp. 137–166.

Bentzon-Tilia, M., Sonnenschein, E. C., and Gram, L. (2016) Monitoring and managing microbes in aquaculture – towards a sustainable industry. *Microb Biotechnol* **9:** 576–584.

Bossier, P., and Ekasari, J. (2017) Biofloc technology application in aquaculture to support sustainable development goals. *Microb Biotechnol* **10:** 1012–1016.

Castex, M., Durand, H., and Okeke, B. (2014) Issues with industrial probiotics scale-up. *Aquacult Nutr Gut Health Probiot Prebiot*. Merrifield, D. L., and Ringo, E. (Eds). Wiley-Blackwell. 347–359.

Cutting, S. M. (2011) *Bacillus* probiotics. *Food Microbiol* **28:** 214–220.

D'Alvise, P. W., Lillebø, S., Prol-Garcia, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, Ø., and Gram, L. (2012) *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS ONE* **7:** e43996.

De Schryver, P., and Vadstein, O. (2014) Ecological theory as a foundation to control pathogenic invasion in aquaculture. *ISME J* **8:** 2360–2368.

Díaz-Rosales, P., Arijo, S., Chabrillón, M., Alarcón, F. J., Tapia-Paniagua, S. T., Martínez-Manzanares, E., *et al.* (2009) Effects of two closely related probiotics on respiratory burst activity of Senegalese sole (*Solea senegalensis*, Kaup) phagocytes, and protection against *Photobacterium damselae* subsp. piscicida. *Aquaculture* **293:** 16–21.

Food and Agriculture Organization of the United Nations (FAO), Fisheries and Aquaculture Department (2016) The State of World Fisheries and Aquaculture 2016.

Gatesoupe, F. J. (2007) Live yeasts in the gut: natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture* **267:** 20–30.

Gentry, R. R., Froehlich, H. E., Grimm, D., Kareiva, P., Parke, M., Rust, M., *et al.* (2017) Mapping the global potential for marine aquaculture. *Nat Ecol Evol* **1:** 1317–1324.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P. W., Dourala, N., Nielsen, K. F., and Gram, L. (2016) Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. *Syst Appl Microbiol* **39:** 180–188.

Gutierrez-Wing, M. T., and Malone, R. F. (2006) Biological filters in aquaculture: trends and research directions for freshwater and marine applications. *Aquacult Eng* **34:** 163–171.

van Kessel, M. A., Dutilh, B. E., Neveling, K., Kwint, M. P., Veltman, J. A., Flik, G., *et al.* (2011) Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio L.*). *AMB Express* **1:** 41.

Kuhn, D. D., Drahos, D. D., Marsh, L., and Flick, G. J. (2010) Evaluation of nitrifying bacteria product to improve nitrification efficacy in recirculating aquaculture systems. *Aquacult Eng* **43:** 78–82.

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., and Derome, N. (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front Microbiol* **5:** 207.

Lobo, C., Moreno-Ventas, X., Tapia-Paniagua, S., Rodríguez, C., Moriñigo, M. A., and de La Banda, I. G. (2014) Dietary probiotic supplementation (*Shewanella putrefaciens* Pdp11) modulates gut microbiota and promotes growth and condition in Senegalese sole larviculture. *Fish Physiol Biochem* **40:** 295–309.

Manthe, D. P., and Malone, R. F. (1987) Chemical addition for accelerated biological filter acclimation in closed blue crab shedding systems. *Aquacult Eng* **6:** 227–236.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T. M., Bøgwald, J., *et al.* (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302:** 1–18.

Meyer, F. P. (1991) Aquaculture disease and health management. *J Anim Sci* **69:** 4201.

Moriarty, D. J. W. (1997) The role of microorganisms in aquaculture ponds. *Aquaculture* **151:** 333–349.

Ringø, E., and Vadstein, O. (1998) Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus L.*) larvae. *J Appl Microbiol* **84:** 227–233.

Singh, B. K. (2017) Creating new business, economic growth and regional prosperity through microbiome-based products in the agriculture industry. *Microb Biotechnol* **10:** 224–227.

United Nations, Department of Economic and Social Affairs, Population Division (2015) World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. ESA/P/WP.241.

Paper 2

Dittmann, K.K., Sonnenschein, E.C., Egan, S., Gram, L. & Bentzon-Tilia, M. (2019).

Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities.

Environ Microbiol Rep., **11**(3), 401–413.

https://doi.org/10.1111/1758-2229.12698

environmental microbiology reports

Environmental Microbiology Reports (2019) **11**(3), 401–413 doi:10.1111/1758-2229.12698

Brief Report

Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities

Karen K. Dittmann,[1] Eva C. Sonnenschein,[1] Suhelen Egan,[2] Lone Gram[1] and Mikkel Bentzon-Tilia[1]*

[1]*Department of Biotechnology and Biomedicine, Technical University of Denmark, Copenhagen, Denmark.*

[2]*School of Biological, Earth and Environmental Sciences, The University of New South Wales, Randwick, NSW, Australia.*

Summary

Bacteria–host interactions are universal in nature and have significant effects on host functionality. Bacterial secondary metabolites are believed to play key roles in such interactions as well as in interactions within the host-associated microbial community. Hence, prominent secondary metabolite-producing bacteria may be strong drivers of microbial community composition in natural host-associated microbiomes. This has, however, not been rigorously tested, and the purpose of this study was to investigate how the secondary metabolite producer *Phaeobacter inhibens* affects the diversity and composition of microbiomes associated with the microalga *Emiliania huxleyi* and the European flat oyster, *Ostrea edulis*. Roseobacters were indigenous to both communities exhibiting relative abundances between 2.8% and 7.0%. Addition of *P. inhibens* caused substantial changes in the overall structure of the low-complexity microbiome of *E. huxleyi*, but did not shape microbial community structure to the same degree in the more complex oyster microbiomes. Species-specific interactions occurred in both microbiomes and specifically the abundances of other putative secondary metabolite-producers such as vibrios and pseudoalteromonads were reduced. Thus, the impact of a bioactive strain like *P. inhibens* on host-associated microbiomes depends on the complexity and composition of the existing microbiome.

Received 23 February, 2018; accepted 25 September, 2018. *For correspondence. E-mail mibti@bio.dtu.dk; Tel. (+45) 45 25 25 18; Fax (+45) 45 88 49 22.

Introduction

In nature, microorganisms live and interact as part of complex multispecies communities. These interspecies interactions may be of a synergistic, amensal or commensal nature and can be facilitated by the exchange of metabolites in syntrophic cooperation, or by the production of bioactive secondary metabolites (Cole, 1982). In parallel to the use of secondary metabolites as defence against microbial infections by plants (Pusztahelyi *et al*., 2015), bacterial secondary metabolites with antimicrobial properties are currently believed to facilitate the success of the compound-producer by killing competitors. Not all bacteria are equally proficient in secondary metabolite production, and whereas some groups appear to produce few or no metabolites, others such as filamentous soil bacteria and marine vibrios, roseobacters and *Pseudoalteromonas* spp. produce an array of different bioactive compounds (Brinkhoff *et al*., 2004; Murphy *et al*., 2012; Rasmussen *et al*., 2014; Maansson *et al*., 2016; Sonnenschein *et al*., 2017b). Hence, if the role of these compounds is to eliminate competing microorganisms, proficient secondary metabolite producers should be strong drivers of microbial community composition in natural environments.

The *Roseobacter* group represents one of the most abundant groups of marine bacteria, constituting on average 3%–5% of microbial communities in the upper mixed layer (Wietz *et al*., 2010). On a global scale, the group exhibits a positive abundance-chlorophyll *a* correlation (Wietz *et al*., 2010) and may exhibit relative abundances of up to 20%–30% during algal blooms (González and Moran, 1997; González *et al*., 2000; Zubkov *et al*., 2001; West *et al*., 2008), suggesting association with microalgae. The paraphyletic *Roseobacter* group comprises multiple deeply branching clades (Newton *et al*., 2010; Simon *et al*., 2017), of which especially clade 1 includes prominent producers of bioactive secondary metabolites, such as the antimicrobial compounds tropodithietic acid (TDA) (Brinkhoff *et al*., 2004; Bruhn *et al*., 2005; Sonnenschein *et al*., 2017a), indigoidine (Cude *et al*., 2012; Gromek *et al*., 2016) and likely multiple other small

molecules (Machado *et al*., 2015; Bentzon-Tilia and Gram, 2017; Sonnenschein *et al*., 2018).

One conspicuous genus of *Roseobacter* clade 1 is *Phaeobacter*, which is often found in microbial communities associated with a wide variety of marine eukaryotes including micro- and macroalgae (Rao *et al*., 2005; Segev *et al*., 2016b), mesozooplankton (Freese *et al*., 2017) and larger animals such as bivalve molluscs (Ruiz-Ponte *et al*., 1998; Prado *et al*., 2009; Wegner *et al*., 2013). Recently, it was shown that the species *Phaeobacter inhibens* produces small bioactive molecules, for example, indole-3-acetic acid, which affect the metabolism of the coccolithophorid microalga *Emiliania huxleyi* (Segev *et al*., 2016a,b). It has been proposed that the interaction between *E. huxleyi* and *P. inhibens* exhibits a biphasic pattern where a mutualistic symbiosis gives way for a parasitic interaction where the bacteria accelerates algal lysis in response to algal break-down products (Seyedsayamdost *et al*., 2011). Mutualistic mechanisms have also been suggested for the symbiosis between bioactive *Roseobacter* clade 1 organisms living in association with the Hawaiian bobtail squid (*Euprymna scolopes*), specifically on the outer surface of the eggs and in the accessory nidamental gland (Collins *et al*., 2012; Gromek *et al*., 2016), where they supposedly ward off potential pathogens through the production of antimicrobials. Hence, proficient secondary metabolite-producing species, such as members of the *Phaeobacter* genus might be strong modulators of both the behaviour and the microbiome composition of their eukaryotic hosts.

For *P. inhibens*, TDA is the most studied secondary metabolite and its antimicrobial property is likely a result of the ability of TDA to act as a proton antiporter at the cytoplasmic membrane (Wilson *et al*., 2016). Hence, TDA is a broad-spectrum antibiotic affecting a wide range of both Gram-positive and Gram-negative bacteria (Porsby *et al*., 2011). Despite the fact that resistance toward TDA does not arise easily (Porsby *et al*., 2011; Rasmussen *et al*., 2016), a large fraction of bacterial isolates from eukaryote-associated microbiomes similar to those harbouring TDA-producers are tolerant toward TDA (Harrington *et al*., 2014). Such microbiomes may hence be resilient to perturbations caused by compounds such as TDA. Recently, Geng and colleagues (2016) showed that additions of the pure TDA compound had pronounced dose-dependent effects on community structure and composition of the microalgal *Nannochloropsis salina* microbiome at relatively low concentrations (31–500 nM). At these concentrations, TDA may act as an inter-microbial signalling molecule rather than an antibiotic (Beyersmann *et al*., 2017). However, the concentrations, at which secondary metabolites are produced in natural communities are currently unknown, and to what extent the presence of secondary metabolite-producing organisms directly affect the microbial communities has not been addressed. Hence, considering the scarcity of *in vivo* models exploring the effects of prominent secondary metabolite producers on natural eukaryote-associated microbiomes, the purpose of the present study was to establish co-culture model systems to investigate how TDA-producing *P. inhibens* shapes the *E. huxleyi* and *Ostrea edulis* microbiomes over time. One of our key goals was to investigate how different concentrations of TDA-producing *P. inhibens* would alter the community structure due to a gradual increase in roseobacters (dose–response) mimicking the increase in algal blooms. The algal model system was chosen as there is a marked increase in roseobacters in the natural environment during algal blooms and we used levels of *P. inhibens* DSM 17395 reflecting the *in situ* abundances (Amin *et al*., 2015; Segev *et al*., 2016b; Sonnenschein *et al*., 2018). The oyster system was chosen as a model for another trophic layer, which would be affected by algal blooms and potentially be exposed to high densities of roseobacters given its feeding mechanism.

Results

The impact of *P. inhibens* strain DSM 17395 on marine eukaryote-associated microbiomes was assessed by sequencing 16S rRNA gene V4 region amplicons from the two different hosts; *E. huxleyi* (microalga) and *O. edulis* (European flat oyster).

Emiliania huxleyi-*associated microbial community composition*

To investigate if *P. inhibens* can affect the microbiome composition and diversity of microalgae, co-cultures of *E. huxleyi* and *P. inhibens* were set up in three groups; (i) untreated controls, (ii) low density (10^4 CFU ml^{-1}) of *P. inhibens* and (iii) high density (10^6 CFU ml^{-1}) of *P. inhibens.* The added densities were equivalent to 0.4 and 40 bacterial cells per algal cell, and 0.09 and 9 *Phaeobacter* cells per bacterium of the initial microbiome, mimicking the ratios observed during algal blooms. The initial algal microbiome was sampled at time point 0 h prior to treatment and the total bacterial count was 5.48 ± 0.58 log_{10} CFU ml^{-1} (using a qPCR-based quantification). Each co-culture was sampled three times: at 24, 48 and 96 h. The most abundant bacterial OTU in the co-cultures (EH_OTU 4) was identified as a *Phaeobacter* sp. (SILVA annotation) and the representative sequence was 100% identical to *P. inhibens* strain DSM 17395 (accession no. CP002976.1). However, it is important to note that the V4 region does not allow for the discrimination between *P. inhibens* and closely related roseobacters (Supporting Information Table S1). In the amplicon

sequencing data, EH_OTU 4 was observed in all samples at relative abundances of 4.0%–4.2% in the initial microbiome, 4.2%–8.2% in the untreated controls (24–96 h), 8.3%–11.2% in the microbiomes treated with the low density of *P. inhibens* and finally 53.8%–79.9% in the microbiomes of algae treated with the high density. However, using qPCR, *P. inhibens* was below the detection limit (3.06 $\log_{10}$ CFU ml^{-1}) in the initial microbiome samples. Accordingly, EH_OTU 4 was excluded from subsequent analyses. Taxonomy and relative abundance of the 10 most abundant OTUs (excluding EH_OTU 4) across all samples can be found in Supporting Information Material (Supporting Information Tables S2 and S3).

The community was dominated by orders of the Proteobacteria and Bacteroidetes phyla regardless of treatment or incubation time (Fig. 1) and neither parameter influenced the composition at class or order level notably. Gammaproteobacteria, Flavobacteria and Alphaproteobacteria were equally dominating across samples. Co-culturing with high levels of *P. inhibens* altered the relative abundance of other Rhodobacterales members from 18% to 23% in controls, to 16% to 24% in low *P. inhibens* density co-cultures and 7.1% to 9.7% in high *P. inhibens* density co-cultures except for one high-density, 96 h co-culture (19%; Fig. 1). However, the relative abundance of the order Flavobacteriales and an unidentified Gammaproteobacteria order remained stable across treatments with relative abundances of 23.1%–50.1% and 22.0%–34.8% respectively.

Oyster-associated microbial community composition

Oysters were divided into two groups: (i) untreated controls and (ii) high density (10^7 CFU ml^{-1}) of *P. inhibens.* Two to three oysters were sacrificed before (0 h) and 48 h after treatment to assess potential changes occurring in the microbiome. The total bacterial count of the initial microbiome was 6.99 $\pm$ 0.91 $\log_{10}$ CFU ml^{-1} (using qPCR-based quantification), hence, the density of *P. inhibens* is equivalent to 0.3 *Phaeobacter* cells to 1 indigenous bacterium. The most abundant OTU in the more complex oyster microbiomes (OE_OTU 1) was identified as an unclassified member of the Rhodobacteraceae family (SILVA annotation). The OE_OTU 1 representative sequence was 100% identical to *P. inhibens* strain DSM 17395 (accession no. CP002976.1) (Supporting Information Table S4). OE_OTU 1 was observed in all samples; the relative abundance accounted for 2.8%–7.0% of the initial microbiome, decreased to 0.5%–0.6% upon 48 h incubation of the untreated control oysters but was increased to 33.1%–46.7% in the microbiomes treated with *P. inhibens.* However, *P. inhibens* was below the qPCR detection limit (3.06 $\log_{10}$ CFU ml^{-1}) in the initial microbiome samples. Accordingly, OE_OTU1 was excluded from subsequent analyses. Taxonomy and relative abundance of the 10 most abundant OTUs (excluding OE_OTU 1) across all samples can be found in Supporting Information Material (Supporting Information Tables S5 and S6).

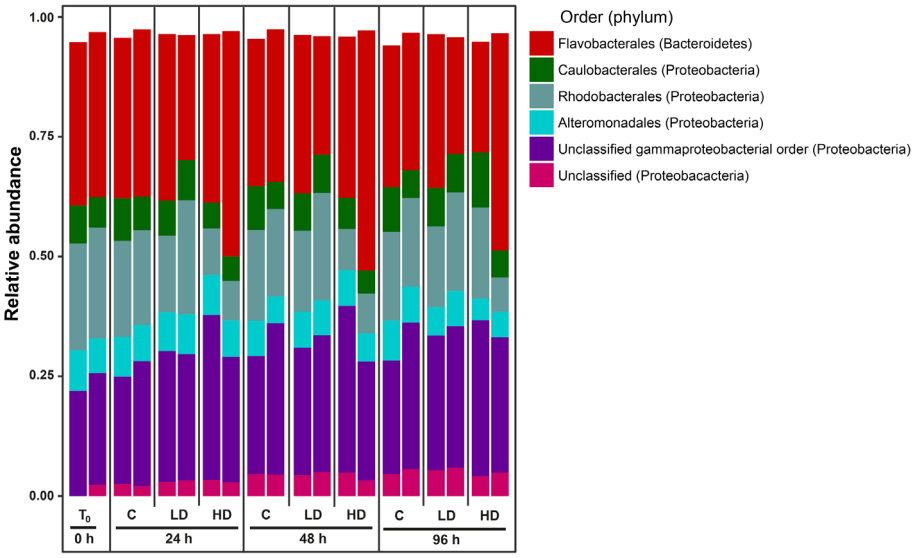


Fig. 1. The composition of bacterial communities associated with *Emiliania huxleyi* in response to the addition of *Phaeobacter inhibens* DSM 17395 at 0, 24, 48 and 96 h in duplicates. The compositions of individual microbiomes are illustrated as relative abundances of all the bacterial orders observed in co-cultures of the microalga and different densities of *Phaeobacter inhibens* DSM 17395 over time. Only orders with abundance above 2% were included (the remaining low abundance orders are represented by the distance up to 1.00). EH_OTU 4 containing the added *P. inhibens* was removed from the dataset prior to plotting. T_0: untreated time zero control, C: untreated control, LD: low density (10^4 CFU ml^{-1}), HD: high density (10^6 CFU ml^{-1}).

Five different phyla (Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes) with relative abundances above 2% were observed across the samples of the oyster microbiomes. Proteobacteria was the dominant phylum in all samples and it increased in relative abundance from 0 to 48 h. At class level, Gammaproteobacteria was the major contributor, which was mainly due to the orders Alteromonadales and Vibrionales (Fig. 2). The relative abundance of Vibrionales decreased in oysters treated with *P. inhibens* (3.1%–4.4%) compared with the 48 h control oysters (16%–29%). In contrast, Alteromonadales were more abundant in the *P. inhibens* treated samples (56%–70%) in comparison to the control (31%–47%). Tenericutes were dominant in the untreated controls after 48 h, but not in the samples treated with *P. inhibens.* At order level, this phylum consisted mainly of Mycoplasmatales.

Impact on richness and diversity of host-associated microbiomes

Microbiomes associated with the algae were less complex than the oyster microbiomes exhibiting estimated OTU richness values (Chao1) in the range of 140–493 OTUs (Fig. 3A) compared with 810 to 5746 OTUs for oysters (Fig. 3B). Some variation was observed in the estimated richness of the *E. huxleyi* microbiomes, but no clear temporal patterns or treatment effects were apparent. In contrast, the *P. inhibens* treatment of oysters resulted in a significantly increased richness index in comparison to the initial and the control populations (*t*-test, $p < 0.05$), while the latter, untreated microbiomes (0 vs. 48 h) did not significantly change during the time of incubation (*t*-test, $p > 0.05$). As indicated by the estimated richness of the microbiomes, the diversity of oyster microbiomes (Supporting Information Fig. S2B) were higher than that of the alga (Supporting Information Fig. S2A), but in contrast to the effect on species richness in the oyster microbiome, the introduction of *P. inhibens* did not affect overall diversity in any of the microbiomes (Supporting Information Fig. S2). Hence, the addition of *P. inhibens* had a significant positive effect on species richness in the oyster microbiomes, but the abundances of these species were not evenly distributed and did not affect overall microbiome diversity.

Impact on community structure of host-associated microbiomes

Community structure analyses of both *E. huxleyi* and *O. edulis* microbiomes were based on Bray–Curtis dissimilarity indexes. For *E. huxleyi*, treatment-dependent clustering was observed for the microbiomes receiving the high density of *P. inhibens*, and as indicated by order-level community composition (Fig. 1), the untreated communities clustered independently of sampling time point (Fig. 4), and hence the effect of *P. inhibens* on the community was immediate (within 0–24 h). In agreement with the negligible effects on the abundance of EH_OTU 4 in communities receiving the low density, these

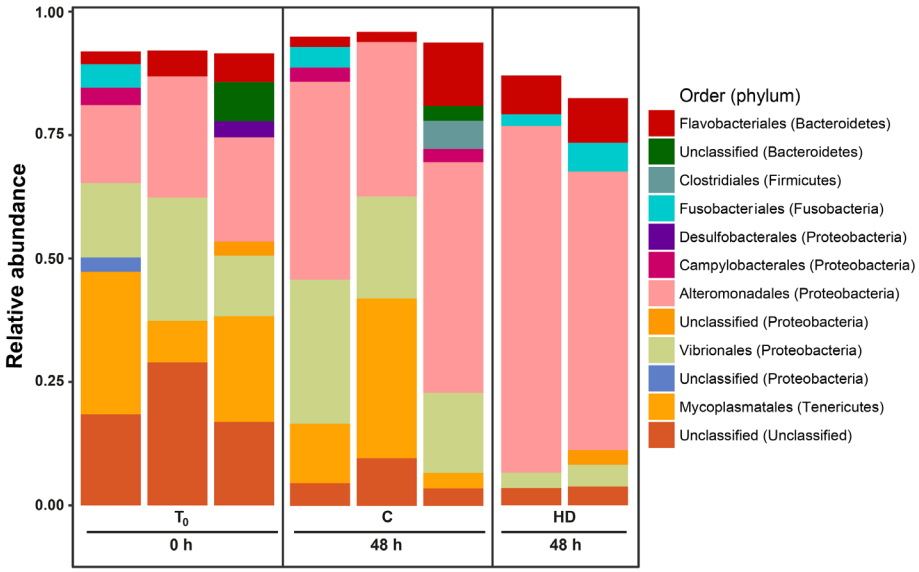


Fig. 2. The composition of bacterial communities associated with European flat oysters (*Ostrea edulis*) in response to the addition of *Phaeobacter inhibens* DSM 17395 at 0 and 48 h in triplicates (control) and duplicates (treated). The compositions of individual microbiomes are illustrated as relative abundances of bacterial orders observed in oysters over a time course of 48 h. Only orders with abundance above 2% were included (the remaining low abundance orders are represented by the distance up to 1.00). OE_OTU 1 containing the added *P. inhibens* was removed from the dataset prior to plotting. Some oyster received a high density (HD) of *Phaeobacter inhibens* DSM 17395 (10^7 CFU ml^{-1}) while others were untreated (controls; C).

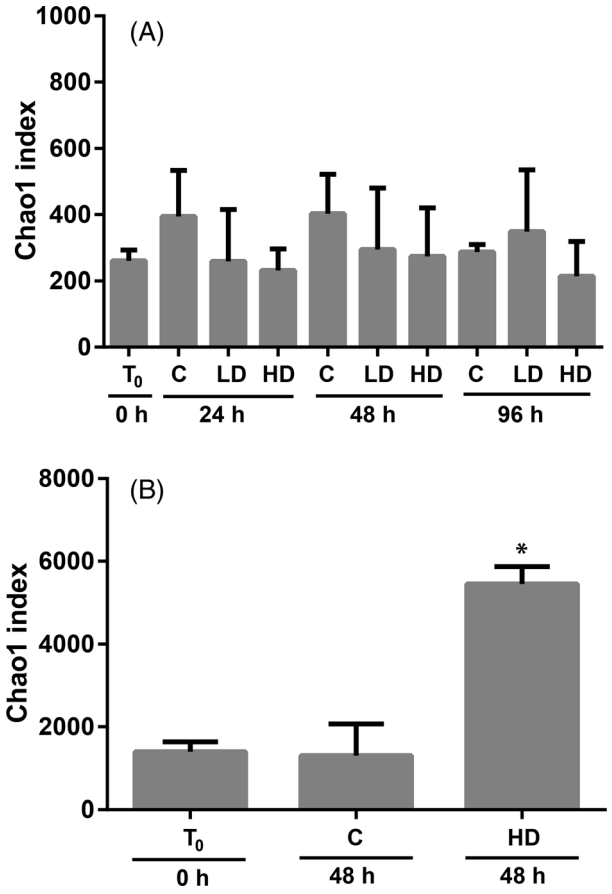


Fig. 3. Richness of bacterial microbiomes observed in (A) *Emiliania huxleyi* (microalga) and (B) *Ostrea edulis* (European flat oysters) in response to the addition of *Phaeobacter inhibens* DSM 17395. The richness is depicted as the average Chao1 richness estimate, error bars represent the standard deviation of the average. OE_OTU 1 and EH_OTU 4 containing the added *P. inhibens* were removed from the datasets prior to plotting. T_0: untreated time zero control, C: untreated control, LD: low density, HD: high density. Note the difference in the y-axis. Statistical significance of the change in the oyster microbiomes ($p \leq 0.05$) is indicated by an asterisk.

communities were interspersed between the other treatment groups. Furthermore, samples from replicate cultures of the same treatment were found in separate, individual clusters regardless of time point, thus indicating a strong 'bottle' effect in the low density co-cultures (Fig. 4). Similarly, for *O. edulis*, the microbiomes co-cultured with the high *P. inhibens* densities clustered separately from the untreated controls (Fig. 5) and the untreated microbiomes were dispersed independent of time point, suggesting that high levels of *P. inhibens* altered community structure for both microbiomes. However, there was substantial variation between some biological replicates.

Analysis of Molecular Variance (AMOVA; Bray Curtis distances; 10 000 iterations) on the *O. edulis* microbiomes indicated a significant variance among the three treatment groups (T_0, 48 h control, 48 h *P. inhibens* treatment; $p = 0.0044$), but none of the pairwise variance comparisons were significantly different from each other. AMOVA was not performed on the *E. huxleyi* microbiome given the low replication level. However, the analyses of the community structures indicate that the less complex microbiome associated with *E. huxleyi* was likely influenced by the introduction of high concentrations of *P. inhibens*, while the differences observed in the more complex oyster microbiome were not significant under the conditions tested.

Impact on individual OTUs

At the order level, the effect of *P. inhibens* on community composition in the *E. huxleyi* microbiome was subtle (Fig. 1), and hence we investigated how the differences observed in community structure came across at the species level (OTU clustering at 97% sequence similarity). Analysing the 40 most abundant OTUs from both host-associated microbiomes confirmed some variation among replicates. For the *E. huxleyi* microbiome, the relative abundances of the seven most abundant OTUs (2, 3, 5, 6, 7, 10, 8; Supporting Information Table S2) were unaffected by the presence of *P. inhibens* (Fig. 4). EH_OTU 9 (*Loktanella* sp.) and EH_OTU 12 (*Marivita* sp.) decreased only in communities treated with the high density of *P. inhibens.* Two gammaproteobacterial OTUs of the *Vibrio* and *Pseudoalteromonas* genera (EH_OTU 20 and 18) were present in the initial microbiomes and increased in relative abundance over time in control microbiomes. However, their abundance decreased in microbiomes supplemented with *P. inhibens*, irrespective of treatment density. In contrast, two OTUs, EH_OTU 33 (*Colwellia* sp.) and 56 (*Neptuniibacter* sp.), were abundant in the microbiomes co-cultured with *P. inhibens* while absent in the controls throughout the experiment. Thus, trends of co-occurring changes in relative abundance in the presence or absence of *P. inhibens* seems to be species if not strain specific in the *E. huxleyi* microbiome.

In contrast to observations from the *E. huxleyi* microbiome, OTUs related to *Colwellia* (OE_OTU 2 and 6; Supporting Information Table S5) were unaffected by the presence of *P. inhibens* in the oyster microbiome (Fig. 5). However, as for the *E. huxleyi* microbiome, species of the *Vibrio* and *Pseudoalteromonas* genera (OE_OTU 3 and 4) decreased in relative abundance in samples treated with *P. inhibens.* Furthermore, OE_OTUs 7, 9, 26 (*Mycoplasma* sp.), 5 (*Shewanella* sp.) and 77 (unclassified proteobacterial OTU) also decreased in the presence of *P. inhibens.* Finally, one *Mycoplasma*-related OTU (OE_OTU 729) increased in relative abundance in the presence of *P. inhibens.* Henceforth, strain rather than species-specific changes in relative abundances of

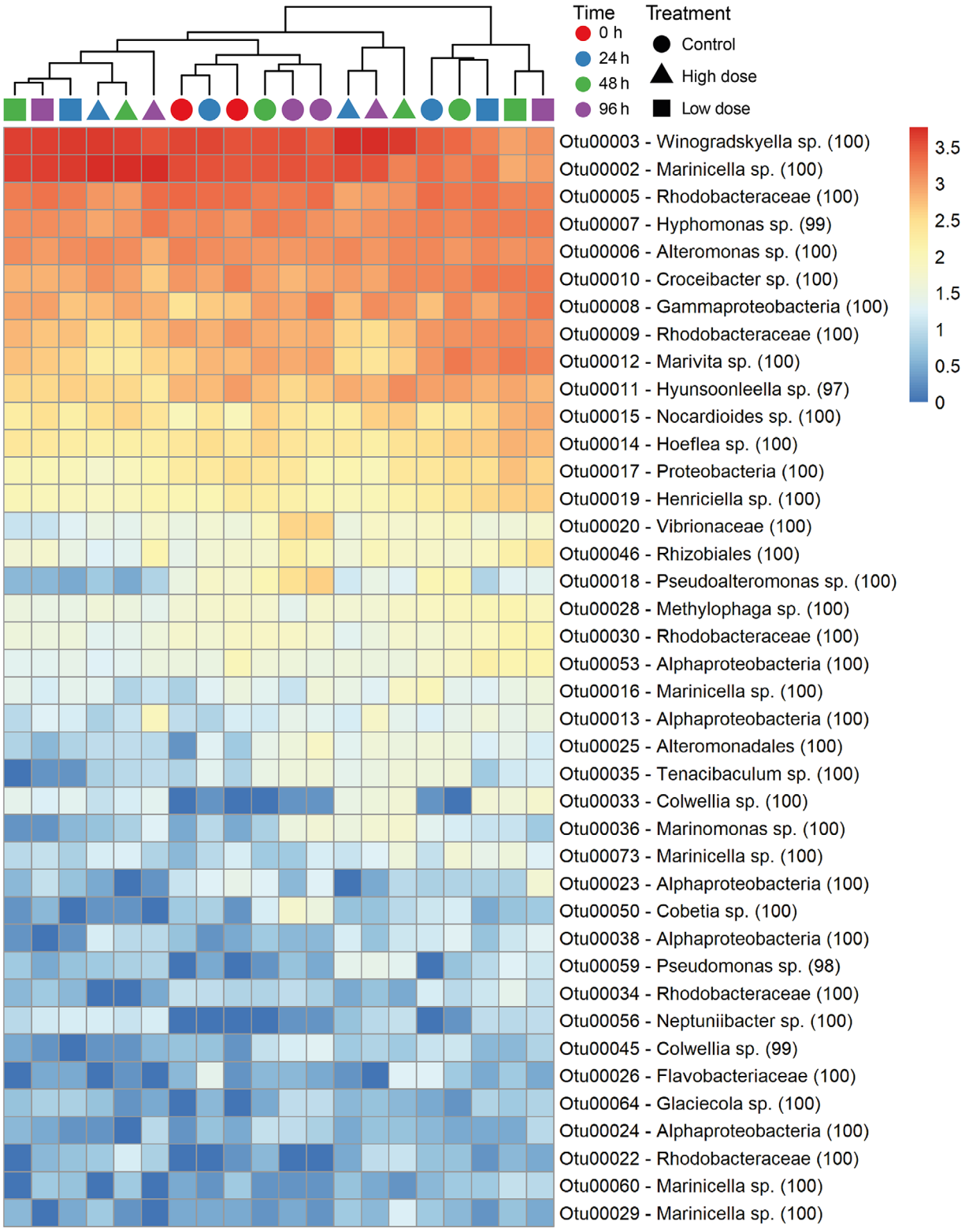


Fig. 4. Heatmap indicating the $\log_{10}(x + 1)$ transformed relative abundances of sequences of the 40 most abundant OTUs of the *Emiliania huxleyi* microbiome in response to the addition of *Phaeobacter inhibens* DSM 17395. Individual microbiomes are arranged according to the Bray–Curtis distances between samples as indicated by the tree above the heatmaps (tree not drawn to scale). The SILVA annotation with identity scores (%) are listed next to the individual OTU. OTUs, which were unclassified at genus level, were listed with their nearest classified level (family, order or class level).

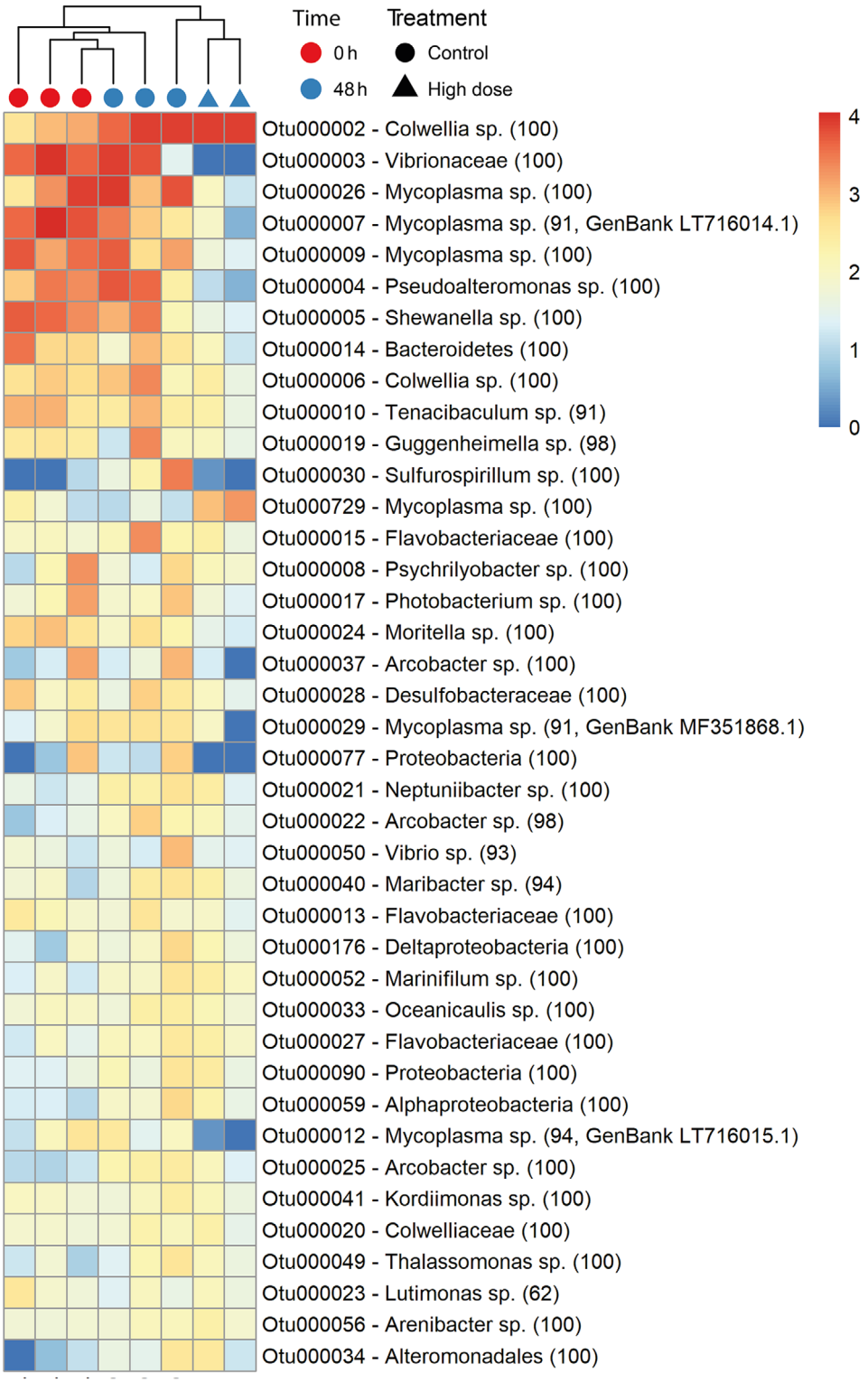


Fig. 5. Heatmap indicating the $\log_{10}(x + 1)$ transformed relative abundances of sequences of the 40 most abundant OTUs of the *Ostrea edulis* microbiome in response to the addition of *Phaeobacter inhibens* DSM 17395. Individual microbiomes are arranged according to the Bray-Curtis distances between samples as indicated by the tree above the heatmaps (tree not drawn to scale). The SILVA annotation with identity scores (%) are listed next to the individual OTU. OTUs, which were unclassified at genus level, were listed with their nearest classified level (family, order or class level). OTUs 7, 29 and 12 were only classified as 'Bacteria' according to the SILVA database and thus, the most significant alignment from NCBI's BLAST has been used instead (GenBank accession number and % identity is listed).

Mycoplasma species occur in the presence of *P. inhibens* in the oyster microbiome.

To investigate a potentially specific, amensal interaction between *P. inhibens* and vibrios, given the potential pathogenicity of certain species, we assessed *Vibrio* spp. abundances in oyster microbiomes quantitatively using quantitative real-time PCR (qPCR). As suggested by the community composition analysis (Fig. 2), the introduction of *P. inhibens* did not remove vibrios in the oyster microbiomes completely (Supporting Information Fig. S3). However, it resulted in a reduction of *Vibrio* spp. 16S rRNA genes to 5.61 $\pm$ 0.23 $\log_{10}$ copies g^{-1} as compared with the initial abundance of 5.91 $\pm$ 0.40 $\log_{10}$ copies g^{-1}, and it further reduced *Vibrio* abundances significantly with more than one order of magnitude compared with control oysters (6.89 $\pm$ 0.18; $p = 0.0328$), thus corroborating the results of the sequence analyses.

Discussion

Bacterial communities associated with eukaryotes have a significant impact on the health and function of their hosts, and investigating how microbiomes of higher organisms are formed and affected by external and internal factors, has become an area of broad and current interest (e.g., Ley *et al*., 2008; Lebeis *et al*., 2015). Some members of the host-associated microbiomes carry the capacity to produce bioactive secondary metabolites that may act in the competition with other members of the microbiomes, yet, it is currently unknown to what extent these bacteria can directly influence and shape the structure of host-associated microbial communities. Our results suggest that the TDA-producing *P. inhibens* has the capacity to influence and shape marine eukaryote-associated microbiomes, yet the effects are variable, dependent on the abundance of *P. inhibens*, and on the complexity and species composition of the host microbiome. Furthermore, the imposed changes occur within a short temporal scale (≤24 h) and are otherwise independent of time (within 5 days).

The two eukaryote model systems were used to investigate how a TDA-producing *P. inhibens* would shape eukaryote-associated microbiomes; during algal blooms versus non-bloom conditions for the microalga, and how potentially high densities of roseobacters would impact the microbiome of a filter-feeder at another trophic layer during algal blooms. Several studies have been conducted on bacteria associated with *E. huxleyi* and oysters (Zabeti *et al*., 2010; Carella *et al*., 2013; Farto Seguín *et al*., 2014; Green *et al*., 2015), but the amount of comprehensive culture-independent, diversity studies is limited. The oyster microbiomes of *Crassostrea* spp. have been characterized (King *et al*., 2012; Wegner *et al*., 2013; Ossai *et al*., 2017; Vezzulli *et al*., 2018) due to their importance in aquaculture, but the present study is to the best of our knowledge, the first culture-independent study investigating the bacterial population of *O. edulis.* Roseobacters were indigenous to both of the investigated microbiomes in agreement with previous findings of molluscan species (Ruiz-Ponte *et al*., 1998; Grigioni *et al*., 2000; Barbieri *et al*., 2001; Martens *et al*., 2006; Prado *et al*., 2009) and algae (González *et al*., 2000; Green *et al*., 2015; Segev *et al*., 2016b). The occurrence of roseobacters in both native microbiomes supports our choice of *P. inhibens* DSM 17395 as a model organism for a secondary metabolite producer in the natural environment of the eukaryotes in the event of an algal bloom.

The estimated OTU richness of the *E. huxleyi* microbiome was four to seven times lower than the richness of the complex *O. edulis* microbiome. Increasing the *P. inhibens* abundance in the low complexity microbiome of *E. huxleyi* had little to no effect on the total species richness and diversity, whereas the richness increased dramatically in oysters. If *P. inhibens* uses its bioactive compounds to kill competitors, that is, as antibiotics, a decrease in bacterial richness and diversity could be expected, but the addition of *P. inhibens* had the opposite effect in the oyster system, indicating that the bioactive compounds, such as TDA, are either not produced or serve another function. The expression of TDA-encoding genes have been shown in algal co-culture systems (D'Alvise *et al*., 2012). Furthermore, TDA is likely produced in our model systems as *P. inhibens* DSM 17395 was pre-grown at conditions known to induce TDA production, that is, nutrient/iron rich broth. While nutrient-rich medium, such as marine broth, differs from the natural environment, some heterotrophs thrive at high nutrient levels (Alonso and Pernthaler, 2006; Pohlner *et al*., 2017), which are comparable to the dense, nutrient-rich surroundings of algal cells or in oysters. Henceforth, it is most likely that TDA has another function than being a broad-spectrum defence compound, and that it is probably highly dependent on the investigated conditions.

Species-rich microbiomes are typical of healthy, marine invertebrates including oysters (King *et al*., 2012; Wegner *et al*., 2013), whereas ill and diseased oyster microbiomes are characterized by a decrease in complexity, loss of rare bacterial strains (Wegner *et al*., 2013), disruption of the community structure (Lokmer and Mathias Wegner, 2015) and increased abundance of few, specialist OTUs (Wegner *et al*., 2013; Lokmer and Mathias Wegner, 2015). Thus, it could be speculated that an increase in *P. inhibens* might be beneficial for the host by decreasing the load of potential opportunistic pathogens and allowing rare taxa to proliferate, which has been suggested as the role of various *Roseobacter* group members associated with other molluscs (Collins *et al*., 2012; Gromek *et al*., 2016) and microalgae (Seyedsayamdost *et al*., 2011).

P. inhibens influenced the bacterial community structure in a dose dependent manner, independently of temporal space. The high density of *P. inhibens* caused a shift in the community structure of the *E. huxleyi* microbiome, but no evident clustering patterns were observed for the algal microbiome exposed to the low density. While due to the low sample number these results still require further verification, similar patterns were observed in the microbiome structure of *N. salina* after the addition of pure TDA (Geng *et al*., 2016).

The oyster microbiome was richer than the algal microbiome indicating that the microalga represented a narrower niche in comparison to the larger, more differentiated bivalve. As filter feeders, oysters accumulate detritus and hence also bacteria. In combination with the higher degree of tissue differentiation, it is not surprising that the oyster microbiome comprises a broader taxonomic assortment of bacteria than that of the microalga. Furthermore, the alga has been kept under continuous laboratory cultivation since its isolation, which may have reduced the richness and diversity of its associated microbiome. Proteobacteria dominated the bacterial community of both *E. huxleyi* and *O. edulis* in agreement with previous observations in coccolithophorid microalgae and other oyster species (Wegner *et al*., 2013; Green *et al*., 2015; García Bernal *et al*., 2017). Green *et al*., 2015 used a culture-dependent approach and found that Alphaproteobacteria were dominating in their coccolithophorid cultures. Similarly, it has been reported that Gamma- and Alphaproteobacteria of the orders Alteromonadales and Rhodobacterales dominated *E. huxleyi* blooms in the North Atlantic Ocean (Segev *et al*., 2016b). In our culture-independent approach, we found both Gamma- and Alphaproteobacteria as well as Flavobacteria (Bacteroidetes) dominated in the *E. huxleyi* microbiomes.

In *O. edulis*, Tenericutes and Bacteroidetes were prominent phyla although less dominating than Proteobacteria. Wegner and colleagues (2013) similarly found Proteobacteria as the dominant phyla followed by Flavobacteria and Bacteroidetes in pacific oysters (*Crassostrea gigas*). The community composition and dominance of individual taxa depends on local environment, tissue (different organs vs. whole organism) and individual oyster genetics and physiology (King *et al*., 2012; Wegner *et al*., 2013; García Bernal *et al*., 2017; Vezzulli *et al*., 2018). The Vibrionales and Mycoplasmatales orders decreased in abundance in the presence of *P. inhibens* while Alteromonadales increased in abundance. In the microbiome of the microalga *N. salina*, the abundance of Alteromonadales also increased in the presence of pure TDA while Rhodobacteraceae decreased (Geng *et al*., 2016). We did not find that *P. inhibens* decreased other Rhodobacterales in the oyster microbiome, however, any change in relative abundance of this order would not be observed since it was below the 2% cutoff in all microbiomes. Although, the relative abundance of the Rhodobacterales order did decrease in the *E. huxleyi* microbiome when exposed to the high density of *P. inhibens* while the abundance of *Alteromonadales* was unaffected. Hence, the impact of the TDA-producer *P. inhibens* on overall community composition is likely dependent on the eukaryotic host and/or the indigenous community composition.

Given the subtle alterations at higher taxonomic levels in some microbiomes, we assessed changes at the OTU-level (species level) to identify the underlying causes. Indeed, we observed some differences in the OTUs affected by different densities of *P. inhibens,* though with noticeable variability between replicate cultures. Some species (*Colwellia* and *Sulfurospirillum*) were either unaffected or increased by the presence of *P. inhibens*, whereas others including *Vibrio* spp. and *Pseudoalteromonas* spp. were reduced in both eukaryote microbiomes. Interestingly, vibrios and pseudalteromonads are also considered proficient secondary metabolite producers and hence it is plausible that *P. inhibens* specifically and efficiently antagonizes other microorganisms occupying similar niches. The efficient inhibition of vibrios by TDA-producing roseobacters have been repeatedly demonstrated in studies targeting the fish pathogenic *Vibrio* spp. in marine eukaryotes (Porsby *et al*., 2008; Prado *et al*., 2009; D'Alvise *et al*., 2012; Grotkjær *et al*., 2016a, b; Porsby and Gram, 2016; Rasmussen *et al*., 2018). The high degree of target organism specificity is however somewhat surprising considering the spectrum of bioactivity of TDA (Porsby *et al*., 2011). Multiple *Mycoplasma* spp. decreased in abundance while a single OTU classified to the same genus increased in abundance further substantiating that the effects of *P. inhibens* are very specific (species level or below). Corroborating the observation that the majority of the species in the microbiomes exhibited minor changes in abundance due to the presence of *P. inhibens*, is the findings by Harrington and colleagues (2014) who observed a high degree of TDA tolerance in non-TDA producing bacterial isolates from marine eukaryote-associated microbiomes. Thus, the impact of increased abundances of a particular secondary metabolite producing microorganism such as *P. inhibens* seems to be highly selective and likely dependent on its specific secondary metabolite profile.

In conclusion, TDA-producing *P. inhibens* can shape host-associated microbiomes of marine eukaryotes. These alterations are subtle at the broader taxonomic levels, but seems to be highly selective and consistent at the OTU level (97% similarity) across eukaryote host systems. The effects of *P. inhibens* on the species richness and microbiome structure are multifaceted; the richness in the complex microbiome of oysters increased while it

remained constant in the microalgal model and the microbiome structure shifted only due to high densities of *P. inhibens*. Thus, the impact likely relies on the composition and complexity of the indigenous bacterial communities. This suggests that a highly diverse microbiome is more stable, though future work across more, varied eukaryote–microbiome associations with larger sample sizes would provide more knowledge to substantiate this hypothesis.

Acknowledgements

The authors wish to thank Professor Torsten Thomas for providing valuable discussions. We acknowledge the help by Cisse Hedegaard Porsby and the Danish Shellfish Center (National Institute of Aquatic Resources, Technical University of Denmark, Denmark) with providing oysters and setting up experiments with oysters.

The present study was funded by the Technical University of Denmark (PhD grant for KKD), the Villum Kann Rasmussen foundation (grant VKR023285), the Danish Council for Strategic Research, Committee for Strategic Research in Health, Food and Welfare (grant 0603-00515B), the Danish National Research Foundation (grant DNRF137), the Oticon foundation (Personal travel grant for KKD), IDAs og Berg-Nielsens Studie- og Støttefond (Personal travel grant for KKD), the Augustinus foundation (Personal travel grant for KKD), the Fabrikant P.A. Fiskers foundation (Personal travel grant for KKD), and the scholarship Christian og Ottilia Brorsons Rejselegat for yngre videnskabsmænd og –kvinder (Personal travel grant for KKD).

References

Alonso, C., and Pernthaler, J. (2006) *Roseobacter* and SAR11 dominate microbial glucose uptake in coastal North Sea waters. *Environ Microbiol* **8**: 2022–2030.

Amin, S.A., Hmelo, L.R., van Tol, H.M., Durham, B.P., Carlson, L.T., Heal, K.R., *et al*. (2015) Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature* **522**: 98–101.

Barbieri, E., Paster, B.J., Hughes, D., Zurek, L., Moser, D.P., Teske, A., and Sogin, M.L. (2001) Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid Loligo pealei (Cephalopoda: Loliginidae). *Environ Microbiol* **3**: 151–167.

Bentzon-Tilia, M., and Gram, L. (2017) Biotechnological Applications of the Roseobacter Clade. In *Biotechnological applications of the roseobacter clade*. Paterson, R., and Lima, N. (eds). Basel, Switzerland: Springer International Publishing, pp. 137–166.

Beyersmann, P.G., Tomasch, J., Son, K., Stocker, R., Göker, M., Wagner-Döbler, I., *et al*. (2017) Dual function of tropodithietic acid as antibiotic and signaling molecule in global gene regulation of the probiotic bacterium *Phaeobacter inhibens*. *Sci Rep* **7**: 730.

Brinkhoff, T., Bach, G., Heidorn, T., Liang, L., Schlingloff, A., and Simon, M. (2004) Antibiotic production by a *Roseobacter* clade-affiliated species from the German Wadden Sea and its antagonistic effects on indigenous isolates. *Appl Environ Microbiol* **70**: 2560–2565.

Bruhn, J.B., Nielsen, K.F., Hjelm, M., Hansen, M., Bresciani, J., Schulz, S., and Gram, L. (2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. *Appl Environ Microbiol* **71**: 7263–7270.

Carella, F., Carrasco, N., Andree, K.B., Lacuesta, B., Furones, D., and De Vico, G. (2013) Nocardiosis in Mediterranean bivalves: first detection of *Nocardia crassostreae* in a new host *Mytilus galloprovincialis* and in *Ostrea edulis* from the Gulf of Naples (Italy). *J Invertebr Pathol* **114**: 324–328.

Cole, J.J. (1982) Interactions between bacteria and algae in aquatic ecosystems. *Annu Rev Ecol Syst* **13**: 291–314.

Collins, A.J., LaBarre, B.A., Wong Won, B.S., Shah, M.V., Heng, S., Choudhury, M.H., *et al*. (2012) Diversity and partitioning of bacterial populations within the accessory Nidamental gland of the squid *Euprymna scolopes*. *Appl Environ Microbiol* **78**: 4200–4208.

Cude, W.N., Mooney, J., Tavanaei, A.A., Hadden, M.K., Frank, A.M., Gulvik, C.A., *et al*. (2012) Production of the antimicrobial secondary metabolite Indigoidine contributes to competitive surface colonization by the marine Roseobacter *Phaeobacter* sp. strain Y4I. *Appl Environ Microbiol* **78**: 4771–4780.

D'Alvise, P.W., Lillebø, S., Prol-Garcia, M.J., Wergeland, H. I., Nielsen, K.F., Bergh, Ø., and Gram, L. (2012) *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS One* **7**: e43996.

Farto Seguín, R.M., Bermúdez, M.B., Rivera, L., and Nieto, T.P. (2014) Increased survival of juvenile turbot *Scophthalmus maximus* by using bacteria associated with cultured oysters. *J Aquat Anim Health* **26**: 251–262.

Freese, H.M., Methner, A., and Overmann, J. (2017) Adaptation of surface-associated bacteria to the Open Ocean: a Genomically distinct subpopulation of *Phaeobacter gallaeciensis* colonizes Pacific Mesozooplankton. *Front Microbiol* **8**: 1–12.

García Bernal, M., Trabal Fernández, N., Saucedo Lastra, P. E., Medina Marrero, R., and Mazón-Suástegui, J.M. (2017) *Streptomyces* effect on the bacterial microbiota associated to *Crassostrea sikamea* oyster. *J Appl Microbiol* **122**: 601–614.

Geng, H., Tran-Gyamfi, M.B., Lane, T.W., Sale, K.L., and Yu, E.T. (2016) Changes in the structure of the microbial community associated with *Nannochloropsis salina* following treatments with antibiotics and bioactive compounds. *Front Microbiol* **7**: 1155.

González, J.M., and Moran, M.A. (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class *Proteobacteria* in coastal seawater. *Appl Environ Microbiol* **63**: 4237–4242.

González, J.M., Simó, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedrós-Alió, C., and Moran, M.A. (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. *Appl Environ Microbiol* **66**: 4237–4246.

Green, D.H., Echavarri-Bravo, V., Brennan, D., and Hart, M. C. (2015) Bacterial diversity associated with the

Coccolithophorid algae *Emiliania huxleyi* and *Coccolithus pelagicus* f. *braarudii*. *Biomed Res Int* **2015**: 1–15.

Grigioni, S., Boucher-Rodoni, R., Demarta, A., Tonolla, M., and Peduzzi, R. (2000) Phylogenetic characterisation of bacterial symbionts in the accessory nidamental glands of the sepioid *Sepia officinalis* (Cephalopoda: Decapoda). *Mar Biol* **136**: 217–222.

Gromek, S.M., Suria, A.M., Fullmer, M.S., Garcia, J.L., Gogarten, J.P., Nyholm, S.V., and Balunas, M.J. (2016) *Leisingera* sp. JC1, a bacterial isolate from hawaiian bobtail squid eggs, produces indigoidine and differentially inhibits vibrios. *Front Microbiol* **7**: 1–16.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P.W., Dierckens, K., Bossier, P., and Gram, L. (2016a) *Phaeobacter inhibens* as probiotic bacteria in non-axenic *Artemia* and algae cultures. *Aquaculture* **462**: 64–69.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P.W., Dourala, N., Nielsen, K.F., and Gram, L. (2016b) Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. *Syst Appl Microbiol* **39**: 180–188.

Harrington, C., Reen, F., Mooij, M., Stewart, F., Chabot, J.-B., Guerra, A., *et al*. (2014) Characterisation of non-autoinducing Tropodithietic acid (TDA) production from marine sponge *Pseudovibrio* species. *Mar Drugs* **12**: 5960–5978.

King, G.M., Judd, C., Kuske, C.R., and Smith, C. (2012) Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One* **7**: e51475.

Lebeis, S.L., Paredes, S.H., Lundberg, D.S., Breakfield, N., Gehring, J., McDonald, M., *et al*. (2015) Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* **349**: 860–864.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., and Gordon, J.I. (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* **6**: 776–788.

Lokmer, A., and Mathias Wegner, K. (2015) Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J* **9**: 670–682.

Maansson, M., Vynne, N.G., Klitgaard, A., Nybo, J.L., Melchiorsen, J., Nguyen, D.D., *et al*. (2016) An integrated Metabolomic and genomic mining workflow to uncover the biosynthetic potential of bacteria. *mSystems* **1**: e00028-15.

Machado, H., Sonnenschein, E.C., Melchiorsen, J., and Gram, L. (2015) Genome mining reveals unlocked bioactive potential of marine gram-negative bacteria. *BMC Genom* **16**: 158.

Martens, T., Heidorn, T., Pukall, R., Simon, M., Tindall, B.J., and Brinkhoff, T. (2006) Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al*. 1998 as *Phaeobacter gallaeciensis* gen. Nov., comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* (Lafay *et al*. 1995) Uchino *et al*. 1999 as *Marinovum algicola* gen. Nov., comb. nov., and emended descriptions of the genera *Roseobacter*, *Ruegeria* and *Leisingera*. *Int J Syst Evol Microbiol* **56**: 1293–1304.

Murphy, B., Jensen, P., and Fenical, W. (2012) The chemistry of marine bacteria. In *Handbook of Marine Natural Products*. Fattorusso, E., Gerwick, W., and Taglialatela-Scafati, O. (eds). New York, NY: Springer, pp. 153–190.

Newton, R.J., Griffin, L.E., Bowles, K.M., Meile, C., Gifford, S., Givens, C.E., *et al*. (2010) Genome characteristics of a generalist marine bacterial lineage. *ISME J* **4**: 784–798.

Ossai, S., Ramachandran, P., Ottesen, A., Reed, E., DePaola, A., and Parveen, S. (2017) Microbiomes of American oysters (*Crassostrea virginica*) harvested from two sites in the Chesapeake Bay. *Genome Announc* **5**: 1–12.

Pohlner, M., Degenhardt, J., von Hoyningen-Huene, A.J.E., Wemheuer, B., Erlmann, N., Schnetger, B., *et al*. (2017) The biogeographical distribution of benthic *Roseobacter* group members along a Pacific transect is structured by nutrient availability within the sediments and primary production in different oceanic provinces. *Front Microbiol* **8**: 2550.

Porsby, C.H., and Gram, L. (2016) *Phaeobacter inhibens* as biocontrol agent against *Vibrio vulnificus* in oyster models. *Food Microbiol* **57**: 63–70.

Porsby, C.H., Nielsen, K.F., and Gram, L. (2008) *Phaeobacter* and *Ruegeria* species of the *Roseobacter* clade colonize separate niches in a Danish turbot (*Scophthalmus maximus*)-rearing farm and antagonize *Vibrio anguillarum* under different growth conditions. *Appl Environ Microbiol* **74**: 7356–7364.

Porsby, C.H., Webber, M.A., Nielsen, K.F., Piddock, L.J.V., and Gram, L. (2011) Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. *Antimicrob Agents Chemother* **55**: 1332–1337.

Prado, S., Montes, J., Romalde, J.L., and Barja, J.L. (2009) Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. *Int Microbiol* **12**: 107–114.

Pusztahelyi, T., Holb, I.J., and Pócsi, I. (2015) Secondary metabolites in fungus-plant interactions. *Front Plant Sci* **6**: 573.

Rao, D., Webb, J.S., and Kjelleberg, S. (2005) Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*. *Appl Environ Microbiol* **71**: 1729–1736.

Rasmussen, B., Nielsen, K., Machado, H., Melchiorsen, J., Gram, L., and Sonnenschein, E. (2014) Global and phylogenetic distribution of quorum sensing signals, acyl Homoserine lactones, in the family of Vibrionaceae. *Mar Drugs* **12**: 5527–5546.

Rasmussen, B.B., Erner, K.E., Bentzon-Tilia, M., and Gram, L. (2018) Effect of TDA-producing *Phaeobacter inhibens* on the fish pathogen *Vibrio anguillarum* in non-axenic algae and copepod systems. *J Microbial Biotechnol* **0**: 1–10.

Rasmussen, B.B., Grotkjær, T., D'Alvise, P.W., Yin, G., Zhang, F., Bunk, B., *et al*. (2016) *Vibrio anguillarum* is genetically and phenotypically unaffected by long-term continuous exposure to the antibacterial compound tropodithietic acid. *Appl Environ Microbiol* **82**: 4802–4810.

Ruiz-Ponte, C., Cilia, V., Lambert, C., and Nicolas, J.L. (1998) *Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*. *Int J Syst Bacteriol* **48**: 537–542.

Segev, E., Castañeda, I.S., Sikes, E.L., Vlamakis, H., and Kolter, R. (2016a) Bacterial influence on alkenones in live microalgae. *J Phycol* **52**: 125–130.

Segev, E., Wyche, T.P., Kim, K.H., Petersen, J., Ellebrandt, C., Vlamakis, H., *et al*. (2016b) Dynamic metabolic exchange governs a marine algal-bacterial interaction. *Elife* **5**: 1–28.

Seyedsayamdost, M.R., Case, R.J., Kolter, R., and Clardy, J. (2011) The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. *Nat Chem* **3**: 331–335.

Simon, M., Scheuner, C., Meier-Kolthoff, J.P., Brinkhoff, T., Wagner-Döbler, I., Ulbrich, M., *et al*. (2017) Phylogenomics of *Rhodobacteraceae* reveals evolutionary adaptation to marine and non-marine habitats. *ISME J* **11**: 1483–1499.

Sonnenschein, E.C., Nielsen, K.F., D'Alvise, P., Porsby, C. H., Melchiorsen, J., Heilmann, J., *et al*. (2017a) Global occurrence and heterogeneity of the Roseobacter-clade species Ruegeria mobilis. *ISME J* **11**: 569–583.

Sonnenschein, E.C., Phippen, C.B.W., Bentzon-Tilia, M., Rasmussen, S.A., Nielsen, K.F., and Gram, L. (2018) Phylogenetic distribution of roseobacticides in the *Roseobacter* group and their effect on microalgae. *Environ Microbiol Rep* **10**: 383–393.

Sonnenschein, E.C., Stierhof, M., Goralczyk, S., Vabre, F. M., Pellissier, L., Hanssen, K.Ø., *et al*. (2017b) Pseudochelin a, a siderophore of Pseudoalteromonas piscicida S2040. *Tetrahedron* **73**: 2633–2637.

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L., and Pruzzo, C. (2018) Comparative 16S rDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microb Ecol* **75**: 495–504.

Wegner, K., Volkenborn, N., Peter, H., and Eiler, A. (2013) Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiol* **13**: 252.

West, N.J., Obernosterer, I., Zemb, O., and Lebaron, P. (2008) Major differences of bacterial diversity and activity inside and outside of a natural iron-fertilized phytoplankton bloom in the Southern Ocean. *Environ Microbiol* **10**: 738–756.

Wietz, M., Gram, L., Jørgensen, B., and Schramm, A. (2010) Latitudinal patterns in the abundance of major marine bacterioplankton groups. *Aquat Microb Ecol* **61**: 179–189.

Wilson, M.Z., Wang, R., Gitai, Z., and Seyedsayamdost, M. R. (2016) Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl cycle as a proton sink. *Proc Natl Acad Sci* **113**: 1630–1635.

Zabeti, N., Bonin, P., Volkman, J.K., Guasco, S., and Rontani, J.-F. (2010) Fatty acid composition of bacterial strains associated with living cells of the haptophyte *Emiliania huxleyi*. *Org Geochem* **41**: 627–636.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2001) Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea. *Environ Microbiol* **3**: 304–311.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1: Species determination of OTU classified as *Phaeobacter* sp. in *Emiliania huxleyi* microbiome. The identity percentage, E-value and GenBank accession number listed for sequences producing significant alignments with the representative sequence of OTU 4 in NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide).

Table S2: The ten most abundant OTUs (excluding the OTU of the added *Phaeobacter* sp.) in the *Emiliania huxleyi* microbiome. The SILVA taxonomy with identity scores (%) are listed next to the individual OTU. *: OTU which was unclassified at genus level and listed with its nearest classified level (family, order or class level). Further genus determination using NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide) can be found in Table S3.

Table S3: Genus determination of OTUs unclassified at this level in *Emiliania huxleyi* microbiome. The identity percentage, E-value and GenBank accession number listed for sequences producing the ten most significant alignments with the representative sequences in NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide).

Table S4: Species determination of abundant OTU classified to the Rhodobacteraceae family in *Ostrea edulis* microbiome. The identity percentage, E-value and GenBank accession number listed for sequences producing significant alignments with the representative sequence of OTU 1 in NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide).

Table S5: The ten most abundant OTUs (excluding the OTU of the added *Phaeobacter* sp.) in the *Ostrea edulis* microbiome. The SILVA taxonomy with identity scores (%) are listed next to the individual OTU. *: OTU which was unclassified at genus level and listed with its nearest classified level (family, order or class level). Further genus determination using NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide) can be found in Table S6.

Table S6: Genus determination of highly abundant OTU unclassified at this level in *Ostrea edulis* microbiome. The identity percentage, E-value and GenBank accession number listed for sequences producing the ten most significant alignments with the representative sequences in NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide).

Table S7: List of indexed used for PCR amplification and sequencing of V4 16S rRNA amplicons from the bacterial microbiomes of *Emiliania huxleyi* and *Ostrea edulis*.

Table S8: Primer combinations for PCR amplification and sequencing of V4 16S rRNA amplicons from the bacterial microbiomes of *Emiliania huxleyi* and *Ostrea edulis*.

Fig. S1: Rarefaction curves for all sequenced samples from the *Emiliania huxleyi* (A) and *Ostrea edulis* (B) microbiomes. After quality filtering, 1.3×10^6 and 2.5×10^6 V4 sequences were obtained from the *E. huxleyi*- and *O. edulis*-associated microbiomes respectively. Sequences were clustered at a 97% sequence similarity, which resulted in 1,346 and 6,706 unique operational taxonomic units (OTUs) for the *E.huxleyi*- and *O. edulis*-associated microbiomes respectively. In the community structure analyses, 17,000 (*E. huxleyi*) and

41,000 (*O. edulis*) sequences from each sample were analysed.

Fig. S2: Diversity of bacterial microbiomes observed in A) *Emiliania huxleyi* (microalga) and B) *Ostrea edulis* (European flat oysters) in response to the addition of *Phaeobacter inhibens* DSM 17395. The diversity is expressed as the average Shannon diversity index value, error bars represent the standard deviation of the average. OE_OTU 1 and EH_OTU 4 containing the added *P. inhibens* were removed from the datasets prior to plotting. T_0: untreated time zero control, C: untreated control, LD: low density, HD: high density.

Fig. S3: Changes in *Vibrio* abundances (*Vibrio* 16S rRNA gene copies/g oyster) in the European flat oyster microbiome as a function of the addition of *Phaeobacter inhibens* DSM 17395. O: untreated time zero control, C: untreated control, HD: high density. Error bars represent the standard deviation of the mean. Statistically significant differences in *Vibrio* abundances ($P \leq 0.05$) are indicated by Tukey groupings.

Appendix S1: Supporting information

Supplementary File 1

Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities

Karen K. Dittmann, Eva C. Sonnenschein, Suhelen Egan, Lone Gram, Mikkel Bentzon-Tilia*

* Address correspondence to Mikkel Bentzon-Tilia: mibti@bio.dtu.dk

Experimental procedures

Cultivation of bacterial isolates

Phaeobacter inhibens DSM 17395 (Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006; Buddruhs *et al.*, 2013) was grown in half-strength Yeast extract, Tryptone, Sea Salts broth (½YTSS, 2 g/L Bacto Yeast extract, 1.25 g/L Bacto Tryptone, 20 g/L Sigma Sea Salts) (Sobecky *et al.*, 1997), or in Marine broth (MB, Difco 2216), while *Pseudoalteromonas tunicata* $D2^T$ (Holmström *et al.*, 1998) and *Vibrio anguillarum* 90-11-287 (Skov *et al.*, 1995) were only grown in MB. When grown on solid substrates, ½YTSS agar (½YTSS broth, 15.0 g/L agar) or Marine Agar (MA, Difco 2216) was used for *P. inhibens*, MA was used for *P. tunicate*, and Tryptone Soya Agar (TSA, Oxoid CM0131) was used for *V. anguillarum*. Liquid cultures were incubated under agitation (200 rpm) at 25° C.

Cultivation of non-axenic Emiliania huxleyi

The non-axenic *E. huxleyi* strain K-1565 was obtained from the Scandinavian Culture Collection of Algae and Protozoa (SCCAP, Copenhagen, Denmark). K-1565 was originally isolated from French Mediterranean coastal water (43°34.46742' N, 007°07.53144' E) on November 3rd, 2010 and maintained in L1 medium (Guillard and Hargraves, 1993) at SCCAP. In our laboratory, the strain was subsequently transferred to f/2 medium (Guillard, 1975) containing the following: 0.88 mM $NaNO_3$, 36 µM $NaH_2PO_4 \times H_2O$, 12 µM $FeCl_3 \times 6$ H_2O, 12 µM $Na_2EDTA \times 2$ H_2O, 39 nM $CuSO_4 \times 5$ H_2O, 26 nM $Na_2MoO_4 \times 2$ H_2O, 77 nM $ZnSO_4 \times 7$ H_2O, 42 nM $CoCl_2 \times 6$ H_2O, 0.91 mM $MnCl_2 \times 4$ H_2O, 0.30 µM thiamine HCl, 2.1 nM biotin, 0.37 nM cyanocobalamin in 1 L of 3 % Instant Ocean® Sea Salt (Aquarium Systems Inc., Sarrebourg, France).

E. huxleyi-Phaeobacter *co-cultivation*

The concentration of an *E. huxleyi* stock culture was determined using an improved Neubauer counting chamber and re-inoculated into 6 × 400 mL f/2 medium in 1 L Schott flasks at a concentration of approximately 10^5 cells mL^{-1}. An overnight culture of *P. inhibens* DSM 17395 grown in ½YTSS was washed one time in f/2 medium (3,000 × g, 3 min). In duplicates, co-cultures were inoculated with *P. inhibens* DSM 17395 at final concentrations of 4×10^4 CFU mL^{-1} (low density) or 4×10^6 CFU mL^{-1} (high density), verified by plate spreading dilutions on MA. The inoculum levels are equivalent to 0.4 and 40 *P. inhibens* cells per algal cell. Two cultures were treated with sterile medium and served as controls. The co-cultures were incubated horizontally, rolling (20 rpm) at 18° C and white fluorescent light (24 µmol m^{-2} s^{-1} photosynthetically active radiation; PAR). The cultures were sampled for biomass for DNA extractions (see below) and for algal abundance determinations at 0 h, 24 h, 48 h and, 96 h. For abundance measures 1 mL co-culture was fixed in 1 % 0.2 µm-filtered glutaraldehyde (final conc.) and the cell numbers were determined using an improved Neubauer counting chamber.

Oyster exposure to Phaeobacter

European flat oysters (*Ostrea edulis*) were exposed to *Phaeobacter inhibens* DSM 13795 as described previously (Porsby and Gram, 2016). In brief, oysters were harvested at Dansk Skaldyrscenter in the Limfjord, Denmark (56°47.27712' N, 008°52.73022' E) in March 2015. Following cleaning and acclimation, oysters were placed into two tanks (15 oysters per tank) containing 7.5 L 3 % Instant Ocean® (Aquarium systems Inc., Sarrebourg, France), and incubated at 15° C with aeration. The oysters were either exposed to 10^7 CFU mL^{-1} *P. inhibens* (verified by plate spreading dilutions on MA), or a volume of marine broth (MB) equivalent to the inoculum volume used in the other tank. The oysters were exposed to two doses on two consecutive days (day 0 and day 1). Tissue (whole animal without shell) was obtained from three oysters sacrificed just prior to the division into the two tanks (T_0 oysters)

as well as from two oysters from the tank inoculated with *P. inhibens* DSM 17395 after 48 h of incubation and three oysters from the MB control tank after 48 h of incubation. The tissue was homogenized with PBS (1:1) using an Ultra Turrex (IKA Werke, Staufen, Germany).

DNA extraction and PCR

DNA was extracted from 100 mL pelleted algal cultures (centrifugation at 8,000 × g, 5 min, 25° C), or 500 mg homogenized oyster tissue. The biomass was resuspended in 1 mL lysis buffer (400 mM sodium chloride, 750 mM sucrose, 20 mM EDTA, 50 mM Tris-HCl, 1 mg mL^{-1} lysozyme, pH 8.5) (Boström *et al.*, 2004) and stored at -80° C until extraction.

Extractions were performed using a phenol/chloroform-based protocol adapted from Boström *et al.* (Boström *et al.*, 2004). Samples were thawed and incubated at 37° C for 30 minutes. Subsequently, proteinase K (Sigma; St. Louis, MO, USA) and sodium dodecyl sulfate (SDS) were added to final concentrations of 100 µg mL^{-1} and 1 % (vol/vol), respectively, followed by overnight incubation at 55° C with slow agitation (60 rpm). Extractions were performed in two steps. Initially with one volume of phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol/vol; Sigma, St. Louis, MO, USA), and subsequently the aqueous phase was extracted with one volume of chloroform:isoamyl alcohol (24:1 vol/vol; Sigma, St. Louis, MO, USA). The phases were separated by centrifugation (20,000 × g, 4° C, 5 min) and the DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.6) and 0.6 volumes of ice-cold isopropanol followed by incubation at -20° C for 1 hour. The precipitated DNA was pelleted by centrifugation (20,000 × g, 4° C, 20 min), washed with ice-cold 70 % ethanol, pelleted again by centrifugation (20,000 × g, 4° C, 20 min), and dissolved in pre-warmed (56° C) TE buffer. The gDNA quality and quantity were assessed by absorption (DeNovix DS-11+, DeNovix Inc., Wilmington, DE, USA) and fluorescence (Qubit™ dsDNA BR assay; Invitrogen by Thermo Fisher Scientific Inc., Eugene, OR, USA) spectroscopy.

DNA was diluted to the same concentration for all samples within experiments – i.e. 27 ng/µL for the *E. huxleyi* microbiome and 120 ng/µL for the oyster microbiome – prior to application in a nested PCR reaction of the 16S rRNA V4 region (Kozich *et al.*, 2013; Staley *et al.*, 2015) using the TEMPase Hot Start 2 × Master Mix Blue II [Ampliqon, 290806]. The universal primers 27F and 1492R (Lane, 1991) were applied for the initial amplification of the 16S rRNA gene using 1.2 µg and 0.135 µg gDNA per sample as template from the oysters and *E. huxleyi*, respectively. The PCR products were used as templates in the subsequent PCR amplification of the V4 region using indexed primers (Table S7, for combinations see Table S8) according to previously described procedures (Kozich *et al.*, 2013). PCRs were run in duplicates and pooled prior to purification (AmPure XP PCR purification; Agencourt Bioscience Corporation, Beverly, MA, USA) and subsequent quality and quantity assessment (as described above).

Amplicon sequencing and bioinformatic analyses

Amplicons were pooled in equal amounts prior to 250PE Illumina MiSeq sequencing at BGI Genomics Co. Ltd., Hong Kong. The raw, de-multiplexed reads were assembled into contigs, processed and analyzed using mothur (v. 1.33.3) (Schloss *et al.*, 2009). Upon assembly, each dataset was denoised by removing the sequences that were poorly assembled, had a length differing from the intended fragment length (275 bp), contained ambiguous bases or contained homopolymers longer than eight nucleotides. The sequences were aligned to the SILVA database (v. 123, bacterial 16S rRNA V4 subfraction) (Pruesse *et al.*, 2007) and poor alignments were excluded from the dataset. UCHIME (Edgar *et al.*, 2011) identified chimeras which were subsequently excluded along with all the sequences classified outside the bacterial domain (i.e. Eukaryota, Archaea, Chloroplasts, Mitochondria and unknown classification). The OTU table was manually curated for algal plastids, chloroplasts, and mitochondria, which had initially been misclassified as “Bacteria”. EH_OTU 4 and OE_OTU

1 were excluded in the datasets used in subsequent analyses in order to dismiss any effects of the increased *Phaeobacter* abundance on composition, and alpha- and beta-diversity measures, hence focusing the analyses on the background microbiota.

The cleaned sequences were analyzed with an Operational Taxonomic Unit (OTU) approach. First, the sequences were clustered into OTUs and classified with a 97 % nucleotide sequence similarity cut-off (species-level). Measures of alpha (Chao1, Shannon) and beta (Bray-Curtis distances) diversity were calculated based on a subsampling of sequences; 17,000 and 41,000 sequences for each sample in the *E. huxleyi* and the *O. edulis* datasets, respectively (rarefaction curves are shown in Figure S2). Community composition analyses and visualizations were performed in R (v. 3.4.2) using the phyloseq and ggplot2 packages (Wickham, 2009; McMurdie and Holmes, 2013). The rarefaction curves and alpha diversity measures were visualized in GraphPad Prism 6. Microbial community structures (beta-diversity) were visualized as trees using the iTOL web-based tool (https://itol.embl.de/itol.cgi). Abundances of specific OTUs were log10(x+1) transformed and heatmaps were made using the pheatmap package in R. Using the built-in mothur functions, the significance of the differences in microbial community structure in the oyster microbiome (Bray-Curtis distances) were assessed using AMOVA (10,000 iterations, significance level $\alpha = 0.05$). Statistics were not applied to the algal microbiome due to the low number of replicates ($n = 2$).

Quantification of total bacterial abundance, Phaeobacter inhibens and vibrios in oysters by Quantitative PCR (qPCR)

Total bacterial abundance of the initial microbiome was estimated by using a previously described quantitative PCR method with universal primers (Bernbom *et al.*, 2013). In brief, standard curves based on gDNA from dilution series of three marine bacterial species,

Phaeobacter inhibens DSM 17395, *Pseudoalteromonas tunicata* D2^T, and *Vibrio anguillarum* 90-11-287, were used to relate the threshold cycle (C_T-value) to CFU/mL. The gDNA was extracted by the same phenol-chloroform-based method as described above and CFU/mL was determined by plate spreading on MA and TSA. SYBR® Green Master Mix (Applied Biosystems, Warrington, UK; 4309155) was used for the qPCR with 0.7µM (final concentration) of each universal primer; 338F (ACT CCT ACG GGA GGC AGC AG) and 518R (ATT ACC GCG GCT GCT GG). The standard curve was based on qPCR performed on 1 µL of gDNA template from each dilution of each strain in triplicates. qPCR on the microbiome samples was performed in triplicates on 1.6875 ng of gDNA from the *E. huxleyi* microbiome samples (T_0) and 60 ng of gDNA from the *O. edulis* microbiome samples (T_0 oysters). MilliQ water was included as non-template controls. The final reaction volume was 15 µL for all reactions. The 2-step PCR amplifications followed by a melting curve were performed with a MX3000P instrument (Stratagene, La Jollla, CA); SYBR was detected as the fluorescent tag, while ROX was the reference dye. The annealing/elongation temperature was 60□.

Detection of *P. inhibens* was performed according to the method described for the total bacterial count though with specific primers: Pi_Fw (GTG TGT TGC GGT CTT TCA CC) and Pi_Rev (AGG ACC ATG TCC CCT CTA CC). Pi_Fw and Pi_Rev were designed based on the *P. inhibens* DSM 17395 genome (GenBank accession CP002976.1) using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers align to the positions 44271-44290 and 44447-44428, resulting in a fragment length of 177 bp. We applied 60□ as annealing/elongation temperature. A standard curve for relating C_T-values to CFU/mL was based on *P. inhibens* DSM 17395 as described above.

Vibrio spp. were quantified in the *O. edulis* microbiome using a previously described qPCR approach (Thompson *et al.*, 2004). Briefly, 20 µL qPCR reactions contained 0.5 µM of each

of the primers 567F and 680R (Thompson *et al.*, 2004) as well as 10 µL 2 × SYBR® Green Master Mix (Applied Biosystems) and 60 ng of DNA extracted from *O. edulis*. A standard dilution series containing 10 – 10^7 16S rRNA genes from *V. anguillarum* 90-11-286 was included. All standards and samples were run in triplicates alongside three No Template Controls (NTCs) receiving sterile water instead of DNA. Thermal cycling was done in a Stratagene Mx3000P series thermal cycler with the following conditions: one cycle of 95° C for 10 min followed by 40 cycles of 95° C for 15 s and 58° C for 1 min. A dissociation curve was included at the end of the program (95° C for 1 min, 58° C for 30 s, 95° C for 1 min).

Accession numbers.

The demultiplexed sequencing reads were deposited in the sequencing read archive (SRA) at NCBI under the project number SRP132348.

References.

Bernbom, N., Ng, Y.Y., Olsen, S.M., and Gram, L. (2013) *Pseudoalteromonas* spp. Serve as Initial Bacterial Attractants in Mesocosms of Coastal Waters but Have Subsequent Antifouling Capacity in Mesocosms and when Embedded in Paint. *Appl. Environ. Microbiol.* **79**: 6885–6893.

Boström, K.H., Simu, K., Hagström, Å., and Riemann, L. (2004) Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. *Limnol. Oceanogr. Methods* **2**: 365–373.

Buddruhs, N., Pradella, S., Goker, M., Pauker, O., Pukall, R., Sproer, C., et al. (2013) Molecular and phenotypic analyses reveal the non-identity of the *Phaeobacter gallaeciensis* type strain deposits CIP 105210T and DSM 17395. *Int. J. Syst. Evol. Microbiol.* **63**: 4340–4349.

177 Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME
178 improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194–2200.

179 Guillard, R.R.L. (1975) Culture of Phytoplankton for Feeding Marine Invertebrates. In,
180 *Culture of Marine Invertebrate Animals*. Springer US, Boston, MA, pp. 29–60.

181 Guillard, R.R.L. and Hargraves, P.E. (1993) *Stichochrysis immobilis* is a diatom, not a
182 chrysophyte. **32**: 234–236.

183 Holmström, C., James, S., Neilan, B.A., White, D.C., and Kjelleberg, S. (1998)
184 *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. *Int.*
185 *J. Syst. Bacteriol.* **48 Pt 4**: 1205–12.

186 Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013)
187 Development of a dual-index sequencing strategy and curation pipeline for analyzing
188 amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ.*
189 *Microbiol.* **79**: 5112–5120.

190 Lane, D. (1991) 16S/23S rRNA sequencing. In, Stackebrandt,E. and Goodfellow,M. (eds),
191 *Nucleic Acid Techniques in Bacterial Systematics*. J.Wiley and Sons, Amsterdam, pp.
192 115–175.

193 Martens, T., Heidorn, T., Pukall, R., Simon, M., Tindall, B.J., and Brinkhoff, T. (2006)
194 Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al.* 1998 as *Phaeobacter*
195 *gallaeciensis* gen. nov., comb. nov., description of *Phaeobacter inhibens* sp. nov.,
196 reclassification of *Ruegeria algicola* (Lafay *et al.* 1995) Uchino *et al.* 1999 as
197 *Marinovum algicola* gen. nov., comb. nov., and emended descriptions of the genera
198 *Roseobacter*, *Ruegeria* and *Leisingera*. *Int. J. Syst. Evol. Microbiol.* **56**: 1293–1304.

199 McMurdie, P.J. and Holmes, S. (2013) phyloseq: An R Package for Reproducible Interactive

200 Analysis and Graphics of Microbiome Census Data. *PLoS One* **8**: e61217.

201 Porsby, C.H. and Gram, L. (2016) *Phaeobacter inhibens* as biocontrol agent against *Vibrio*
202 *vulnificus* in oyster models. *Food Microbiol.* **57**: 63–70.

203 Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glockner, F.O.
204 (2007) SILVA: a comprehensive online resource for quality checked and aligned
205 ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* **35**: 7188–
206 7196.

207 Ruiz-Ponte, C., Cilia, V., Lambert, C., and Nicolas, J.L. (1998) *Roseobacter gallaeciensis* sp.
208 nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten*
209 *maximus*. *Int. J. Syst. Bacteriol.* **48**: 537–542.

210 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al.
211 (2009) Introducing mothur: open-source, platform-independent, community-supported
212 software for describing and comparing microbial communities. *Appl. Environ.*
213 *Microbiol.* **75**: 7537–41.

214 Skov, M.N., Pedersen, K., and Larsen, J.L. (1995) Comparison of Pulsed-Field Gel
215 Electrophoresis, Ribotyping, and Plasmid Profiling for Typing of *Vibrio anguillarum*
216 Serovar O1. *Appl. Environ. Microbiol.* **61**: 1540–1545.

217 Sobecky, P.A., Mincer, T.J., Chang, M.C., and Helinski, D.R. (1997) Plasmids isolated from
218 marine sediment microbial communities contain replication and incompatibility regions
219 unrelated to those of known plasmid groups. *Appl. Environ. Microbiol.* **63**: 888–95.

220 Staley, C., Gould, T.J., Wang, P., Phillips, J., Cotner, J.B., and Sadowsky, M.J. (2015)
221 Evaluation of water sampling methodologies for amplicon-based characterization of
222 bacterial community structure. *J. Microbiol. Methods* **114**: 43–50.

223 Thompson, J.R., Randa, M.A., Marcelino, L.A., Tomita-Mitchell, A., Lim, E., and Polz, M.F.
224 (2004) Diversity and Dynamics of a North Atlantic Coastal *Vibrio* Community. *Appl.*
225 *Environ. Microbiol.* **70**: 4103–4110.

226 Wickham, H. (2009) ggplot2: Elegant Graphics for Data Analysis Springer New York, New
227 York, NY.

228

229

230

Supplementary File 2

Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities

Karen K. Dittmann, Eva C. Sonnenschein, Suhelen Egan, Lone Gram, Mikkel Bentzon-Tilia*

* Address correspondence to Mikkel Bentzon-Tilia: mibti@bio.dtu.dk

Table S1: Species determination of OTU classified as *Phaeobacter* sp. in *Emiliania huxleyi* microbiome. The identity percentage, E-value and GenBank accession number listed for sequences producing significant alignments with the representative sequence of OTU 4 in NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide).

OTU no.	BLAST hit scores			
	Species - strain description	**Identity**	**E-value**	**GenBank Accession**
4	Phaeobacter inhibens strain DOK1-1, complete genome	100%	4.00E-128	CP019307.1
	Phaeobacter inhibens strain P59 chromosome, complete genome	100%	4.00E-128	CP010741.1
	Phaeobacter inhibens strain P72 chromosome, complete genome	100%	4.00E-128	CP010735.1
	Phaeobacter inhibens strain P88 chromosome, complete genome	100%	4.00E-128	CP010725.1
	Phaeobacter inhibens strain P66 chromosome, complete genome	100%	4.00E-128	CP010705.1
	Phaeobacter inhibens strain P24 chromosome, complete genome	100%	4.00E-128	CP010696.1
	Phaeobacter inhibens strain P57 chromosome, complete genome	100%	4.00E-128	CP010668.1
	Phaeobacter inhibens strain P74 chromosome, complete genome	100%	4.00E-128	CP010661.1
	Phaeobacter inhibens strain P54 chromosome, complete genome	100%	4.00E-128	CP010650.1
	Phaeobacter inhibens strain P78 chromosome, complete genome	100%	4.00E-128	CP010629.1
	Phaeobacter inhibens strain P51 chromosome, complete genome	100%	4.00E-128	CP010623.1
	Phaeobacter inhibens strain P30 isolate M4-3.1A chromosome, complete genome	100%	4.00E-128	CP010617.1
	Phaeobacter inhibens strain P83 chromosome, complete genome	100%	4.00E-128	CP010599.1
	Phaeobacter inhibens strain P92 chromosome, complete genome	100%	4.00E-128	CP010610.1
	Phaeobacter inhibens strain P80 chromosome, complete genome	100%	4.00E-128	CP010756.1
	Phaeobacter inhibens strain P70 chromosome, complete genome	100%	4.00E-128	CP010749.1
	Phaeobacter inhibens strain P48 isolate M21-2.3 chromosome, complete genome	100%	4.00E-128	CP010745.1
	Phaeobacter inhibens strain P10 chromosome, complete genome	100%	4.00E-128	CP010595.1
	Phaeobacter inhibens strain P93 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357447.1
	Phaeobacter inhibens strain P92 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357446.1
	Phaeobacter inhibens strain P88 clone 3 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357444.1
	Phaeobacter inhibens strain P88 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357443.1
	Phaeobacter inhibens strain P88 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357442.1
	Phaeobacter inhibens strain P87 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357441.1

Phaeobacter inhibens strain P84 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357440.1
Phaeobacter inhibens strain P83 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357439.1
Phaeobacter inhibens strain P83 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357438.1
Phaeobacter inhibens strain P82 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357437.1
Phaeobacter inhibens strain P82 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357436.1
Phaeobacter inhibens strain P81 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357435.1
Phaeobacter inhibens strain P81 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357434.1
Phaeobacter inhibens strain P80 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357433.1
Phaeobacter inhibens strain P80 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357432.1
Phaeobacter inhibens strain P79 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357431.1
Phaeobacter inhibens strain P78 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357430.1
Phaeobacter inhibens strain P74 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357426.1
Phaeobacter inhibens strain P72 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357424.1
Phaeobacter inhibens strain P70 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357422.1
Phaeobacter inhibens strain P66 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357418.1
Phaeobacter inhibens strain P62 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357414.1
Phaeobacter inhibens strain P61 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357413.1
Phaeobacter inhibens strain P60 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357412.1
Phaeobacter inhibens strain P59 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357411.1
Phaeobacter inhibens strain P58 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357410.1
Phaeobacter inhibens strain P57 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357409.1
Phaeobacter inhibens strain P56 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357408.1
Phaeobacter inhibens strain P55 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357407.1
Phaeobacter inhibens strain P54 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357406.1
Phaeobacter inhibens strain P53 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357405.1
Phaeobacter inhibens strain P52 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357404.1
Phaeobacter inhibens strain P51 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357403.1
Phaeobacter inhibens strain P50 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357402.1
Phaeobacter inhibens strain P49 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357401.1
Phaeobacter inhibens strain P48 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357400.1
Phaeobacter inhibens strain P47 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357399.1
Phaeobacter inhibens strain P46 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357398.1
Phaeobacter inhibens strain P30 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357382.1

Phaeobacter inhibens strain P24 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357376.1
Phaeobacter inhibens strain P10 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357362.1
Sulfitobacter sp. strain WHOIMSCC93816RPlateKeller02201702231716RB01 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600285.1
Sulfitobacter sp. strain WHOIMSCC86516RPlateKeller02201702231716RA07 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600235.1
Pseudoseohaeicola sp. strain WHOIMSCC84316RPlateR77284C03 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600223.1
Alteromonas sp. strain WHOIMSCC96616RRedo1PlateR77272E02 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF599717.1
Phaeobacter sp. H8 gene for 16S ribosomal RNA, partial sequence	100%	4.00E-128	LC230096.1
Phaeobacter sp. H6 gene for 16S ribosomal RNA, partial sequence	100%	4.00E-128	LC230095.1
Sulfitobacter pseudonitzschiae strain SMR1 plasmid pSMR1-2, complete sequence	100%	4.00E-128	CP022417.1
Sulfitobacter pseudonitzschiae strain SMR1, complete genome	100%	4.00E-128	CP022415.1
Bacterium strain 7002-268 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770694.1
Bacterium strain 7002-208 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770634.1
Bacterium strain 7002-140 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770567.1
Ponticoccus sp. strain 7002-056 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770484.1
Ponticoccus sp. strain 7002-055 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770483.1
Ponticoccus sp. strain 7002-029 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770458.1
Ponticoccus sp. strain 1334-337 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770361.1
Sulfitobacter sp. SAG13 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KX268604.1
Marinovum algicola partial 16S rRNA gene, isolate 130-UT	100%	4.00E-128	LK022238.1
Sulfitobacter sp. ER-48 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT325155.1
Sulfitobacter sp. BR-58 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT325042.1
Sulfitobacter sp. BR-46 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT325030.1
Phaeobacter gallaeciensis strain HQB346 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT758505.1
Phaeobacter inhibens strain HQB345 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT758504.1
Phaeobacter gallaeciensis strain HQB255 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT758453.1
Seohaeicola sp. SS011A0-7#2-2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KF312716.1
Rhodobacteraceae bacterium DG1572 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM279025.1
Sulfitobacter sp. SA56 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033276.1
Sulfitobacter sp. SA53 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033273.1
Sulfitobacter sp. SA52 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033272.1
Sulfitobacter sp. SA51 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033271.1
Sulfitobacter sp. SA49 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033269.1
Sulfitobacter sp. SA48 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033268.1

Sulfitobacter sp. SA47 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033267.1
Sulfitobacter sp. SA43 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033264.1
Sulfitobacter sp. SA35 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033257.1
Sulfitobacter sp. SA30 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033254.1
Sulfitobacter sp. S19SW 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KF418804.1
Sulfitobacter sp. KMM 6719 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KC247329.1
Phaeobacter sp. SH4H2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205636.1
Phaeobacter sp. SH4a 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205634.1
Phaeobacter sp. SH4b 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205633.1
Phaeobacter sp. SH4H1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205632.1

Table S2: The ten most abundant OTUs (excluding the OTU of the added *Phaeobacter* sp.) in the *Emiliania huxleyi* microbiome. The SILVA taxonomy with identity scores (%) are listed next to the individual OTU. *: OTU which was unclassified at genus level and listed with its nearest classified level (family, order or class level). Further genus determination using NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide) can be found in table S3.

OTU no.	Abundance (%)	SILVA annotation	Identity (%)
EH_OTU 2	6.5 - 29.1	*Marinicella sp.*	100
EH_OTU 3	3.9 - 26.3	*Winogradskyella sp.*	100
EH_OTU 5	2.1 - 15.0	Unclassified Rhodobacteraceae *	100
EH_OTU 6	0.9 - 7.9	*Alteromonas sp.*	100
EH_OTU 7	1.9 - 8.4	*Hyphomonas sp.*	99
EH_OTU 10	0.5 - 6.0	*Croceibacter sp.*	100
EH_OTU 8	0.8 - 5.4	Unclassified Gammaproteobacterium *	100
EH_OTU 9	0.5 - 4.8	Unclassified Rhodobacteraceae *	100
EH_OTU 12	0.3 - 3.4	*Marivita sp.*	100
EH_OTU 11	0.2 - 3.7	*Hyunsoonleella sp.*	97

15 **Table S3:** Genus determination of OTUs unclassified at this level in *Emiliania huxleyi* microbiome. The identity percentage, E-value and GenBank accession
16 number listed for sequences producing the ten most significant alignments with the representative sequences in NCBI's Basic Local Alignment Search Tool
17 (BLAST, nucleotide).

	BLAST hit scores			
OTU no.	**Species - strain description**	**Identity**	**E-value**	**GenBank Accession**
5	Aestuariivita sp. strain 7002-232 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770658.1
	Aestuariivita sp. strain 7002-179 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770605.1
	Aestuariivita sp. strain 7002-146 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770573.1
	Aestuariivita sp. strain 7002-091 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770519.1
	Rhodobacteraceae Bacterium R11M1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KC439177.1
	Alpha proteobacterium SY190 partial 16S rRNA gene, strain SY190	100%	4.00E-128	HE589557.1
	Roseobacter gallaeciensis clone SE84 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	AY771774.1
	Ruegeria sp. TCg-9 partial 16S rRNA gene, isolate TCg-9	100%	4.00E-128	AJ515042.1
	Ruegeria sp. WRs-12 partial 16S rRNA gene, isolate WRs-12	100%	4.00E-128	AJ515040.1
	Ruegeria sp. LTs-2 partial 16S rRNA gene, isolate LTs-2	100%	4.00E-128	AJ515039.1
8	Litorivivens aequoris strain KMU-37 16S ribosomal RNA, partial sequence	97%	8.00E-115	NR_149215.1
	Litorivivens aequoris gene for 16S ribosomal RNA, partial sequence	97%	8.00E-115	LC167346.1
	Gamma proteobacterium NAMAF009 gene for 16S ribosomal RNA, partial sequence	97%	8.00E-115	AB377223.1
	Litorivivens lipolytica strain HJTF-7 16S ribosomal RNA gene, partial sequence	96%	2.00E-111	KM017973.1
	Spongiibacter sp. CC-AMW-B 16S ribosomal RNA gene, partial sequence	95%	2.00E-106	KC169814.1
	Bacterium ectosymbiont of Cladonema sp. isolate SA 16S ribosomal RNA gene, partial sequence	94%	8.00E-105	KJ493944.1
	Spongiibacter sp. HME8849 16S ribosomal RNA gene, partial sequence	94%	4.00E-103	KC153058.1
	Cellvibrio sp. J113 16S ribosomal RNA gene, partial sequence	94%	4.00E-103	EU143370.1
	Gamma proteobacterium NEP4 gene for 16S rRNA, partial sequence	94%	4.00E-103	AB212803.1
	Ectosymbiont of Gianthauma karukerense partial 16S rRNA gene	94%	2.00E-101	FN398075.1
9	Loktanella sp. S4079 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	FJ460047.1
	Silicibacter sp. S1-6 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	FJ218376.1
	Ruegeria sp. strain S51 16S ribosomal RNA gene, partial sequence	99%	2.00E-126	KX989367.1
	Ruegeria sp. strain 7002-314 16S ribosomal RNA gene, partial sequence	99%	2.00E-126	KY770740.1
	Ruegeria sp. strain 1334-246 16S ribosomal RNA gene, partial sequence	99%	2.00E-126	KY770270.1

Ruegeria mobilis strain NIOSSD020#22 16S ribosomal RNA gene, partial sequence	99%	2.00E-126	KY616198.1
Ruegeria sp. strain ST329 16S ribosomal RNA gene, partial sequence	99%	2.00E-126	KY474029.1
Ruegeria sp. URN111 gene for 16S ribosomal RNA, partial sequence	99%	2.00E-126	AB916877.1
Ruegeria sp. URN65 gene for 16S ribosomal RNA, partial sequence	99%	2.00E-126	AB916874.1
Ruegeria sp. URN43 gene for 16S ribosomal RNA, partial sequence	99%	2.00E-126	AB916873.1

19 **Table S4:** Species determination of abundant OTU classified to the Rhodobacteraceae family in *Ostrea edulis* microbiome. The identity percentage, E-value
20 and GenBank accession number listed for sequences producing significant alignments with the representative sequence of OTU 1 in NCBI's Basic Local
21 Alignment Search Tool (BLAST, nucleotide).

OTU no.	BLAST hit scores			
	Species - strain description	**Identity**	**E-value**	**GenBank Accession**
1	Phaeobacter inhibens strain DOK1-1, complete genome	100%	4.00E-128	CP019307.1
	Phaeobacter inhibens strain P59 chromosome, complete genome	100%	4.00E-128	CP010741.1
	Phaeobacter inhibens strain P72 chromosome, complete genome	100%	4.00E-128	CP010735.1
	Phaeobacter inhibens strain P88 chromosome, complete genome	100%	4.00E-128	CP010725.1
	Phaeobacter inhibens strain P66 chromosome, complete genome	100%	4.00E-128	CP010705.1
	Phaeobacter inhibens strain P24 chromosome, complete genome	100%	4.00E-128	CP010696.1
	Phaeobacter inhibens strain P57 chromosome, complete genome	100%	4.00E-128	CP010668.1
	Phaeobacter inhibens strain P74 chromosome, complete genome	100%	4.00E-128	CP010661.1
	Phaeobacter inhibens strain P54 chromosome, complete genome	100%	4.00E-128	CP010650.1
	Phaeobacter inhibens strain P78 chromosome, complete genome	100%	4.00E-128	CP010629.1
	Phaeobacter inhibens strain P51 chromosome, complete genome	100%	4.00E-128	CP010623.1
	Phaeobacter inhibens strain P30 isolate M4-3.1A chromosome, complete genome	100%	4.00E-128	CP010617.1
	Phaeobacter inhibens strain P83 chromosome, complete genome	100%	4.00E-128	CP010599.1
	Phaeobacter inhibens strain P92 chromosome, complete genome	100%	4.00E-128	CP010610.1
	Phaeobacter inhibens strain P80 chromosome, complete genome	100%	4.00E-128	CP010756.1
	Phaeobacter inhibens strain P70 chromosome, complete genome	100%	4.00E-128	CP010749.1
	Phaeobacter inhibens strain P48 isolate M21-2.3 chromosome, complete genome	100%	4.00E-128	CP010745.1
	Phaeobacter inhibens strain P10 chromosome, complete genome	100%	4.00E-128	CP010595.1
	Phaeobacter inhibens strain P93 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357447.1
	Phaeobacter inhibens strain P92 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357446.1
	Phaeobacter inhibens strain P88 clone 3 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357444.1
	Phaeobacter inhibens strain P88 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357443.1
	Phaeobacter inhibens strain P88 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357442.1
	Phaeobacter inhibens strain P87 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357441.1

Phaeobacter inhibens strain P84 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357440.1
Phaeobacter inhibens strain P83 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357439.1
Phaeobacter inhibens strain P83 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357438.1
Phaeobacter inhibens strain P82 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357437.1
Phaeobacter inhibens strain P82 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357436.1
Phaeobacter inhibens strain P81 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357435.1
Phaeobacter inhibens strain P81 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357434.1
Phaeobacter inhibens strain P80 clone 2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357433.1
Phaeobacter inhibens strain P80 clone 1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357432.1
Phaeobacter inhibens strain P79 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357431.1
Phaeobacter inhibens strain P78 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357430.1
Phaeobacter inhibens strain P74 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357426.1
Phaeobacter inhibens strain P72 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357424.1
Phaeobacter inhibens strain P70 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357422.1
Phaeobacter inhibens strain P66 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357418.1
Phaeobacter inhibens strain P62 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357414.1
Phaeobacter inhibens strain P61 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357413.1
Phaeobacter inhibens strain P60 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357412.1
Phaeobacter inhibens strain P59 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357411.1
Phaeobacter inhibens strain P58 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357410.1
Phaeobacter inhibens strain P57 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357409.1
Phaeobacter inhibens strain P56 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357408.1
Phaeobacter inhibens strain P55 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357407.1
Phaeobacter inhibens strain P54 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357406.1
Phaeobacter inhibens strain P53 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357405.1
Phaeobacter inhibens strain P52 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357404.1
Phaeobacter inhibens strain P51 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357403.1
Phaeobacter inhibens strain P50 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357402.1
Phaeobacter inhibens strain P49 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357401.1
Phaeobacter inhibens strain P48 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357400.1
Phaeobacter inhibens strain P47 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357399.1
Phaeobacter inhibens strain P46 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357398.1
Phaeobacter inhibens strain P30 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357382.1

Phaeobacter inhibens strain P24 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357376.1
Phaeobacter inhibens strain P10 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY357362.1
Sulfitobacter sp. strain WHOIMSCC93816RPlateKeller02201702231716RB01 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600285.1
Sulfitobacter sp. strain WHOIMSCC86516RPlateKeller02201702231716RA07 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600235.1
Pseudoseohaeicola sp. strain WHOIMSCC84316RPlateR77284C03 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600223.1
Alteromonas sp. strain WHOIMSCC96616RRedo1PlateR77272E02 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF599717.1
Phaeobacter sp. H8 gene for 16S ribosomal RNA, partial sequence	100%	4.00E-128	LC230096.1
Phaeobacter sp. H6 gene for 16S ribosomal RNA, partial sequence	100%	4.00E-128	LC230095.1
Sulfitobacter pseudonitzschiae strain SMR1 plasmid pSMR1-2, complete sequence	100%	4.00E-128	CP022417.1
Sulfitobacter pseudonitzschiae strain SMR1, complete genome	100%	4.00E-128	CP022415.1
Bacterium strain 7002-268 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770694.1
Bacterium strain 7002-208 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770634.1
Bacterium strain 7002-140 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770567.1
Ponticoccus sp. strain 7002-056 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770484.1
Ponticoccus sp. strain 7002-055 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770483.1
Ponticoccus sp. strain 7002-029 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770458.1
Ponticoccus sp. strain 1334-337 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KY770361.1
Sulfitobacter sp. SAG13 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KX268604.1
Marinovum algicola partial 16S rRNA gene, isolate 130-UT	100%	4.00E-128	LK022238.1
Sulfitobacter sp. ER-48 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT325155.1
Sulfitobacter sp. BR-58 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT325042.1
Sulfitobacter sp. BR-46 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT325030.1
Phaeobacter gallaeciensis strain HQB346 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT758505.1
Phaeobacter inhibens strain HQB345 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT758504.1
Phaeobacter gallaeciensis strain HQB255 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KT758453.1
Seohaeicola sp. SS011A0-7#2-2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KF312716.1
Rhodobacteraceae bacterium DG1572 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM279025.1
Sulfitobacter sp. SA56 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033276.1
Sulfitobacter sp. SA53 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033273.1
Sulfitobacter sp. SA52 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033272.1
Sulfitobacter sp. SA51 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033271.1
Sulfitobacter sp. SA49 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033269.1
Sulfitobacter sp. SA48 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033268.1

Sulfitobacter sp. SA47 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033267.1
Sulfitobacter sp. SA43 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033264.1
Sulfitobacter sp. SA35 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033257.1
Sulfitobacter sp. SA30 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KM033254.1
Sulfitobacter sp. S19SW 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KF418804.1
Sulfitobacter sp. KMM 6719 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KC247329.1
Phaeobacter sp. SH4H2 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205636.1
Phaeobacter sp. SH4a 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205634.1
Phaeobacter sp. SH4b 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205633.1
Phaeobacter sp. SH4H1 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	KJ205632.1

Table S5: The ten most abundant OTUs (excluding the OTU of the added *Phaeobacter* sp.) in the *Ostrea edulis* microbiome. The SILVA taxonomy with identity scores (%) are listed next to the individual OTU. *: OTU which was unclassified at genus level and listed with its nearest classified level (family, order or class level). Further genus determination using NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide) can be found in table S6.

OTU no.	Abundance (%)	SILVA annotation	Identity (%)
OE_OTU 2	0.9 – 21.4	*Colwellia sp.*	100
OE_OTU 4	1.9 - 20.4	*Pseudoalteromonas sp.*	100
OE_OTU 3	1.5 - 27.0	Unclassified Vibrionaceae *	100
OE_OTU 5	2.9 - 14.3	*Shewanella sp.*	100
OE_OTU 6	1.1 - 6.1	*Colwellia sp.*	100
OE_OTU 9	0.3 - 19.8	*Mycoplasma sp.*	100
OE_OTU 7	0.2 - 14.2	Unclassified Bacteria *	100
OE_OTU 8	0.04 - 4.4	*Psychrilyobacter sp.*	100
OE_OTU 26	0.1 - 17.9	*Mycoplasma sp.*	100
OE_OTU 10	0.6 - 3.1	*Tenacibaculum sp.*	91

Table S6: Genus determination of highly abundant OTU unclassified at this level in *Ostrea edulis* microbiome. The identity percentage, E-value and GenBank accession number listed for sequences producing the ten most significant alignments with the representative sequences in NCBI's Basic Local Alignment Search Tool (BLAST, nucleotide).

	BLAST hit scores			
OTU no.	**Species - strain description**	**Identity (%)**	**E-value**	**GenBank Accession**
3	Vibrio alginolyticus strain K08M4 chromosome 1, complete sequence	100%	4.00E-128	CP017916.1
	Vibrio alginolyticus strain K08M4 chromosome 2, complete sequence	100%	4.00E-128	CP017917.1
	Vibrio sp. strain NFH.MB010 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MG788349.1
	Vibrio sp. strain E517-9 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF975605.1
	Vibrio sp. strain E425-6 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF975586.1
	Vibrio sp. strain E425-5 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF975585.1
	Vibrio sp. strain E425-4 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF975584.1
	Vibrio sp. strain WHOIMSCC36516RPlateR7729116RA10 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600122.1
	Vibrio sp. strain WHOIMSCC21316RPlateR77284C10 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600072.1
	Vibrio sp. strain WHOIMSCC208 16S ribosomal RNA gene, partial sequence	100%	4.00E-128	MF600069.1
7	Bacterium WH8-10 16S ribosomal RNA gene, partial sequence	94%	2.00E-102	JQ269320.1
	Mycoplasma sp. PE partial 16S rRNA gene, strain PE	91%	3.00E-89	LT716014.1
	Mycoplasma phocicerebrale strain 1049 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	JN935885.1
	Mycoplasma phocicerebrale strain ATCC 51405 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	JN935879.1
	Mycoplasma phocicerebrale strain Cheryl 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	JN935876.1
	Mycoplasma sp. Sgv2e 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	GQ150568.1
	Mycoplasma sp. Sgv2d 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	GQ150567.1
	Mycoplasma sp. Sgv2b 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	GQ150565.1
	Mycoplasma sp. Sgv2a 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	GQ150564.1
	Mycoplasma phocicerebrale strain CSL 5195S2 16S ribosomal RNA gene, partial sequence	90%	2.00E-87	DQ840513.1

Table S7: List of indexed used for PCR amplification and sequencing of V4 16S rRNA amplicons from the bacterial microbiomes of *Emiliania huxleyi* and *Ostrea edulis*.

Primer	Index	Sequence (5' - 3')*
Forward		
v4.SA501	ATCGTACG	AATGATACGGCGACCACCGAGATCTACAC**ATCGTACG**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA502	ACTATCTG	AATGATACGGCGACCACCGAGATCTACAC**ACTATCTG**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA503	TAGCGAGT	AATGATACGGCGACCACCGAGATCTACAC**TAGCGAGT**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA504	CTGCGTGT	AATGATACGGCGACCACCGAGATCTACAC**CTGCGTGT**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA505	TCATCGAG	AATGATACGGCGACCACCGAGATCTACAC**TCATCGAG**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA506	CGTGAGTG	AATGATACGGCGACCACCGAGATCTACAC**CGTGAGTG**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA507	GGATATCT	AATGATACGGCGACCACCGAGATCTACAC**GGATATCT**TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
Reverse		
v4.SA701	AACTCTCG	CAAGCAGAAGACGGCATACGAGAT**AACTCTCG**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA702	ACTATGTC	CAAGCAGAAGACGGCATACGAGAT**ACTATGTC**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA703	AGTAGCGT	CAAGCAGAAGACGGCATACGAGAT**AGTAGCGT**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA704	CAGTGAGT	CAAGCAGAAGACGGCATACGAGAT**CAGTGAGT**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA705	CGTACTCA	CAAGCAGAAGACGGCATACGAGAT**CGTACTCA**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA706	CTACGCAG	CAAGCAGAAGACGGCATACGAGAT**CTACGCAG**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

*Index sequences are shown in bold.

35 **Table S8:** Primer combinations for PCR amplification and sequencing of V4 16S rRNA amplicons from the

36 bacterial microbiomes of *Emiliania huxleyi* and *Ostrea edulis*.

Host	Time point (h)	Treatment	Sample ID	Forward primer	Reverse primer
Emiliania huxleyi	0	Control	EH-T0-0.1	v4.SA501	v4.SA701
	0	Control	EH-T0-0.2	v4.SA501	v4.SA702
	24	Control	EH-T24-0.1	v4.SA502	v4.SA701
	24	Control	EH-T24-0.2	v4.SA502	v4.SA702
	24	Low dose	EH-T24-104.1	v4.SA502	v4.SA703
	24	Low dose	EH-T24-104.2	v4.SA502	v4.SA704
	24	High dose	EH-T24-106.1	v4.SA502	v4.SA705
	24	High dose	EH-T24-106.2	v4.SA502	v4.SA706
	48	Control	EH-T48-0.1	v4.SA503	v4.SA701
	48	Control	EH-T48-0.2	v4.SA503	v4.SA702
	48	Low dose	EH-T48-104.1	v4.SA503	v4.SA703
	48	Low dose	EH-T48-104.2	v4.SA503	v4.SA704
	48	High dose	EH-T48-106.1	v4.SA503	v4.SA705
	48	High dose	EH-T48-106.2	v4.SA503	v4.SA706
	96	Control	EH-T96-0.1	v4.SA504	v4.SA701
	96	Control	EH-T96-0.2	v4.SA504	v4.SA702
	96	Low dose	EH-T96-104.1	v4.SA504	v4.SA703
	96	Low dose	EH-T96-104.2	v4.SA504	v4.SA704
	96	High dose	EH-T96-106.1	v4.SA504	v4.SA705
	96	High dose	EH-T96-106.2	v4.SA504	v4.SA706
Ostrea edulis	0	Control	T0-1	v4.SA505	v4.SA703
	0	Control	T0-2	v4.SA506	v4.SA703
	0	Control	T0-3	v4.SA501	v4.SA704
	48	Control	C-T48-1	v4.SA503	v4.SA706
	48	Control	C-T48-2	v4.SA504	v4.SA706
	48	Control	C-T48-3	v4.SA505	v4.SA706
	48	High dose	P-T48-1	v4.SA505	v4.SA704
	48	High dose	P-T48-2	v4.SA506	v4.SA704

Impact of *P. inhibens* on microbial communities

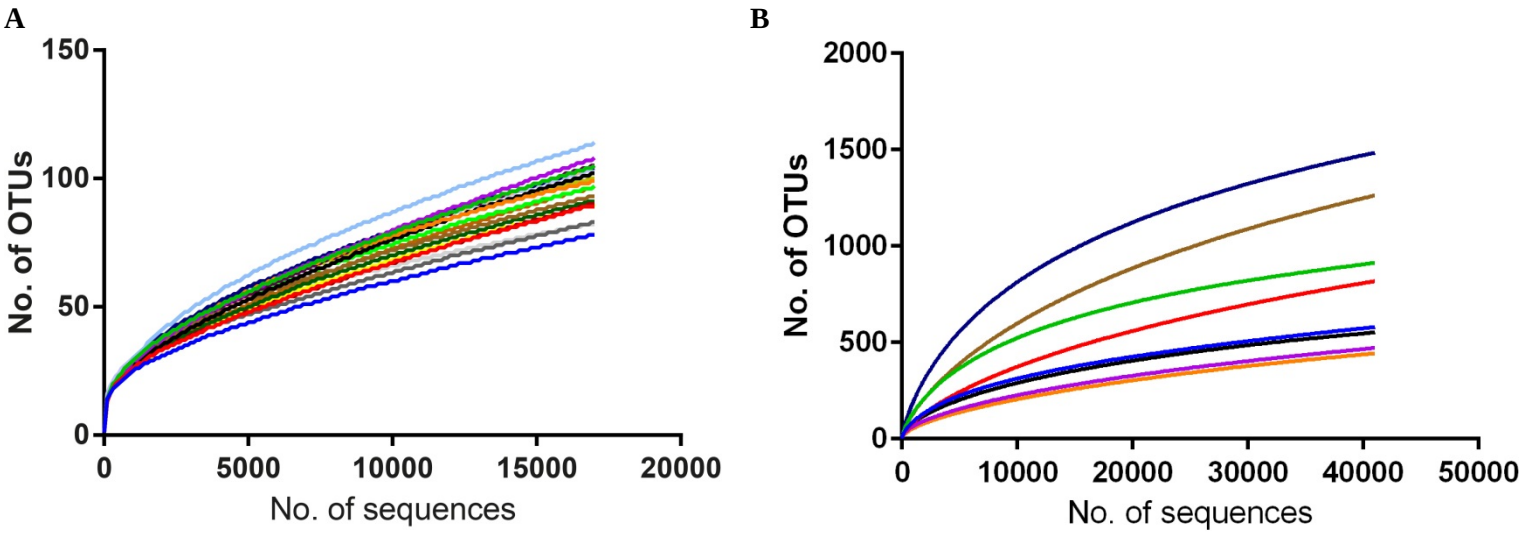


Figure S1: Rarefaction curves for all sequenced samples from the *Emiliania huxleyi* (A) and *Ostrea edulis* (B) microbiomes. After quality filtering, 1.3×10^6 and 2.5×10^6 V4 sequences were obtained from the *E. huxleyi*- and *O. edulis*-associated microbiomes, respectively. Sequences were clustered at a 97 % sequence similarity, which resulted in 1,346 and 6,706 unique operational taxonomic units (OTUs) for the *E. huxleyi*- and *O. edulis*-associated microbiomes, respectively. In the community structure analyses, 17,000 (*E. huxleyi*) and 41,000 (*O. edulis*) sequences from each sample were analyzed.

Impact of *P. inhibens* on microbial communities

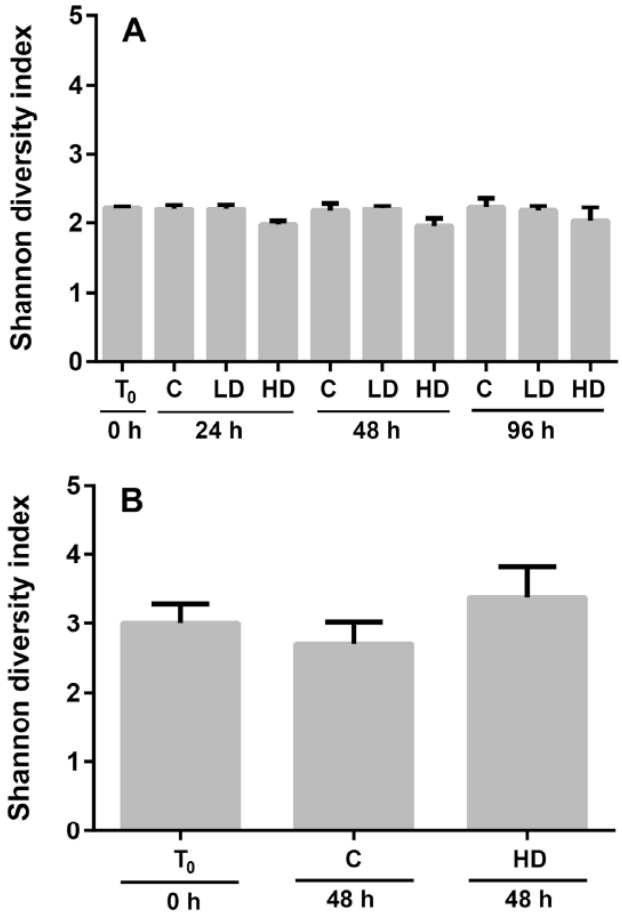


Figure S2: Diversity of bacterial microbiomes observed in A) *Emiliania huxleyi* (microalga) and B) *Ostrea edulis* (European flat oysters) in response to the addition of *Phaeobacter inhibens* DSM 17395. The diversity is expressed as the average Shannon diversity index value, error bars represent the standard deviation of the average. OE_OTU 1 and EH_OTU 4 containing the added *P. inhibens* were removed from the datasets prior to plotting. T_0: untreated time zero control, C: untreated control, LD: low density, HD: high density.

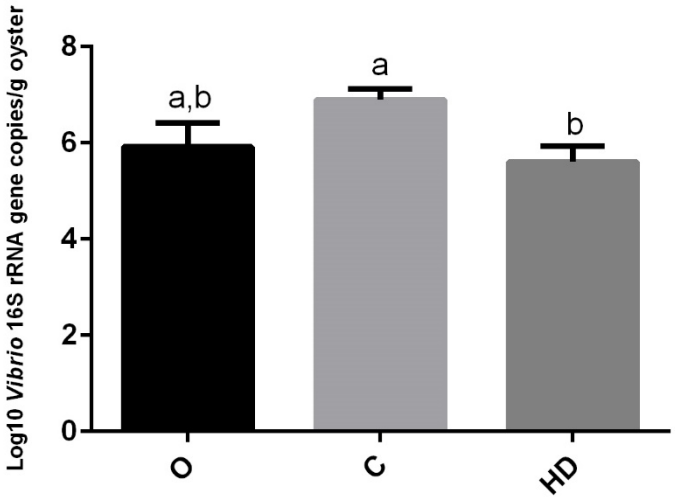


Figure S3: Changes in *Vibrio* abundances (*Vibrio* 16S rRNA gene copies/g oyster) in the European flat oyster microbiome as a function of the addition of *Phaeobacter inhibens* DSM 17395. O: untreated time zero control, C: untreated control, HD: high density. Error bars represent the standard deviation of the mean. Statistically significant differences in *Vibrio* abundances ($P \leq 0.05$) are indicated by Tukey groupings.

Paper 3

Dittmann, K.K., Porsby, C.H., Goncalves, P., Mateiu, R.V., Sonnenschein, E.C., Bentzon-Tilia, M., Egan, S. & Gram, L. (2019).

Tropodithietic acid induces oxidative stress response, cell envelope biogenesis and iron uptake in *Vibrio vulnificus*.

Environ Microbiol Rep. **Accepted**.

https://doi.org/10.1111/1758-2229.12771

Tropodithietic acid induces oxidative stress response, cell envelope biogenesis and iron uptake in *Vibrio vulnificus*

Karen K. Dittmann[a,b], Cisse H. Porsby[a]*, Priscila Goncalves[b], Ramona Valentina Mateiu[c], Eva C. Sonnenschein[a], Mikkel Bentzon-Tilia[a], Suhelen Egan[b] & Lone Gram[a#]

[a] Department of Biotechnology and Biomedicine, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.

[b] School of Biological, Earth and Environmental Sciences, The University of New South Wales, Randwick NSW 2052, Australia.

[c] Center for Electron Nanoscopy, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.

Corresponding author: Lone Gram, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads Bldg. 221, DK-2800 Kgs. Lyngby, Denmark, Tel.: (+45) 45 25 25 86, Fax: (+45) 45 25 25 01, gram@bio.dtu.dk,

*Current address: Chr. Hansen A/S, DK-2970 Hørsholm, Denmark.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1758-2229.12771

Running title: Impact of TDA on gene expression of *V. vulnificus*

Keywords: Transcriptomics, marine bacteria, biofilm, roseobacters, secondary metabolites, tropodithietic acid, TDA, motility

Originality-Significance Statement

The natural role of the antibiotic tropodithietic acid (TDA) and its impact on non-producing marine bacteria at *in situ* concentrations is currently unknown. Here we determine how a sub-lethal concentration of TDA affects the opportunistic fish and human pathogen *Vibrio vulnificus*, providing insight into the mechanism of action of TDA and its potential effects on both pathogenic and commensal bacteria.

Abstract

The *Roseobacter* group is a widespread marine bacterial group, of which some species produce the broad-spectrum antibiotic tropodithietic acid (TDA). A mode of action for TDA has previously been proposed in *Escherichia coli*, but little is known about its effect on non-producing marine bacteria at *in situ* concentrations. The purpose of this study was to investigate how a sub-lethal level of TDA affects *Vibrio vulnificus* at different time points (30 min and 60 min) using a transcriptomic approach. Exposure to TDA for as little as 30 min resulted in the differential expression of genes associated with cell regeneration, including the up-regulation of those involved in biogenesis of the cell envelope. Defense mechanisms including oxidative stress defense proteins and iron uptake systems were also up-regulated in response to TDA, while motility-related genes were down-regulated. Gene expression data and scanning electron microscopy imaging revealed a switch to a biofilm phenotype in the

presence of TDA. Our study shows that a low concentration of this antibiotic triggers a defense response to reactive oxygen species and iron depletion in *V. vulnificus*, which indicates that the mode of action of TDA is likely more complex in this bacterium than what is known for *E. coli*.

Introduction

The *Roseobacter* group is one of the most widespread marine bacterial groups and is often associated with eukaryotes such as algae (González *et al.*, 2000; Buchan *et al.*, 2005; Sonnenschein *et al.*, 2017; Dittmann *et al.*, 2018) and molluscs (Ruiz-Ponte *et al.*, 1998; Prado *et al.*, 2009; Wegner *et al.*, 2013). Members of this group have also been repeatedly detected in aquaculture systems (Hjelm, Riaza, *et al.*, 2004; Porsby *et al.*, 2008). Some species, including *Phaeobacter inhibens* and *Ruegeria mobilis*, produce the potent antibacterial compound tropodithietic acid (TDA) (Bruhn *et al.*, 2005; Porsby *et al.*, 2008), which is active against a range of Gram-positive and Gram-negative bacteria (Kintaka *et al.*, 1984; Porsby *et al.*, 2011; Rabe *et al.*, 2014). TDA has low toxicity to the roundworm *Caenorhabditis elegans* (Neu *et al.*, 2014), which is often used as a model organism for testing cytotoxicity (Sese *et al.*, 2009; Sprando *et al.*, 2009) resembling the effects induced in mammalian model organisms (Sprando *et al.*, 2009). However, TDA has also shown anticancer activity (Wilson *et al.*, 2016) and the compound can be cytotoxic to mammalian neuronal cells (Wichmann *et al.*, 2015), which indicates that the effect of TDA on eukaryotic cells and eukaryotes is dependent on the cell type and target organism. The activity towards neuronal cells is believed to be driven by disruption of the mitochondrial membrane potential and activation of oxidative stress response (Wichmann *et al.*, 2015). Altogether, the broad target range and toxicity towards mammalian cells should be taken into consideration if TDA and TDA-producers are to be used as treatment in humans as well as in rearing of fish and shellfish.

TDA-producing bacteria have potential as probiotics in aquaculture systems as they can prevent fish larvae mortality caused by pathogenic *Vibrio* spp. (Planas *et al.*, 2006a; D'Alvise *et al.*, 2013) without causing adverse effects on either the larvae (Hjelm, Bergh, *et al.*, 2004; Planas *et al.*, 2006b; D'Alvise *et al.*, 2010, 2012) or the live-feed organisms such as *Artemia* (Neu *et al.*, 2014; Grotkjær *et al.*, 2016), copepods (Rasmussen *et al.*, 2018), rotifers, and microalgae (D'Alvise *et al.*, 2012). The prospect of using antibiotic-producing bacteria in aquaculture raises the concern of resistance development in target organisms. However, previous studies have shown that human and fish pathogens are genetically and phenotypically unaffected by long-term exposure to TDA (Porsby *et al.*, 2011; Rasmussen *et al.*, 2016). Although TDA-tolerant bacteria have been co-isolated with TDA-producing *Pseudovibrio* spp. from marine sponges (Harrington *et al.*, 2014), their tolerance/susceptibility mechanisms and long-term stability remain unclear. Understanding such processes is crucial for a broader implementation of TDA-producing strains in the aquaculture industry.

The antagonistic effect of TDA against a wide range of prokaryotes, in conjunction with the fact that resistance to this compound is rarely observed, suggest that TDA has multiple targets in the cell, and that at least one of the targets has a vital function, which is conserved and sensitive to mutations. Wilson *et al.* (2016) proposed that TDA in *Escherichia coli* acts as an electroneutral proton-antiporter creating an acidic cytosol by the import of H^+ ions while exporting metal ions, which would be chelated in the extracellular space. As a result, the proton motive force (PMF) is disrupted and the cells are killed. The natural role, mode of action, and effect of TDA on bacteria in marine environments remain to

be understood. We have previously shown that the impact of TDA-producers on marine microbiomes is dependent on the complexity and composition of the established microbial community, with stronger influence on specific community members (Dittmann *et al.*, 2018). Particularly, genera known to include fast-growing opportunistic fish pathogens, such as *Vibrio* spp. and *Pseudoalteromonas* spp., decrease in abundance in the presence of TDA (Dittmann *et al.*, 2018). Within TDA-producers, TDA can also act as a quorum sensing molecule regulating motility, biofilm formation, and antibiotic production (Beyersmann *et al.*, 2017). Henceforth, TDA may have multiple functions and induce different responses depending on the sensing organism.

Altogether, TDA and TDA-producing bacteria are promising candidates for controlling pathogenic bacteria in aquaculture. However, it is crucial to understand the impact of TDA and the potential consequences of bacterial exposure to the compound – particularly how TDA-susceptible pathogens compensate metabolically to avoid mortality and if TDA exposure triggers expression of genes related to undesirable phenotypes such as virulence. Thus, the purpose of this study was to determine how a sub-lethal concentration of TDA affects the transcriptome of the human and fish pathogenic bacterium *Vibrio vulnificus* upon 30 min and 60 min exposure to the compound. This species was chosen as a model organism for vibrios, which are some of the most common causes of bacterial diseases in aquaculture. *V. vulnificus* is one of a few species causing major economic losses in rearing of several fish species (Thompson *et al.*, 2004; Toranzo *et al.*, 2005). Furthermore, vibrios are known to be particularly susceptible to TDA (Porsby *et al.*, 2011).

Results and Discussion

Impact of TDA on gene expression of *V. vulnificus*

The effect of TDA on the transcriptional profiles of *V. vulnificus* CMCP6 was assessed by mRNA sequencing. A sub-lethal concentration of TDA (0.6 µM; 260 times lower than the determined MIC value of 15.6 µM) was chosen based on repeated growth experiments (data not included). This concentration had only a very marginal effect on growth (Supplementary File 2, Figure S1) and, hence, is likely to produce a metabolic effect. Cells exposed to TDA for 30 min and 60 min showed distinct gene expression profiles compared to control groups, i.e. cells exposed to DMSO (TDA solvent) for 30 and 60 min (Figure 1). Such a difference in gene expression between TDA-treated and control groups became more pronounced with increasing exposure time (TDA vs. control at 60 min compared to TDA vs. control at 30 min). Differential gene expression analysis comparing the transcriptome of the TDA-treated cells to the controls (FDR < 0.05, absolute $\log_2$FC > 1) revealed 164 genes which were differentially expressed (DE) at 30 min of exposure (139 up-regulated, 25 down-regulated) and 687 DE genes at 60 min of exposure (417 up-regulated, 270 down-regulated). A total of 140 genes were DE at both time points; of these, 122 were up-regulated and 18 were down-regulated by TDA exposure. A full list of the DE genes with annotations can be found in Supplementary File 2 (Supplementary Table S1).

TDA exposure induced the expression of genes related to amino acid, carbohydrate, and lipid metabolism along with genes involved in biogenesis of the cell envelope, wall, and membrane (Figure 2). The up-regulation of these genes occurred regardless of the exposure time, being observed at both time points. In contrast, genes related to energy generation and conversion were down-regulated. Of the 547 DE genes unique for time point 60 min, 3.8% were involved in cell motility (down-regulated) and 1.3% in defense

mechanisms (up-regulated). Between 41% and 46% of the annotated protein sequences across the different time points were functionally annotated as "unknown" or had no comparable hit in the eggNOG database. This may not be surprising as proteins and protein domains of unknown function encompass a large fraction of the entries in biological data repositories (Nadzirin and Firdaus-Raih, 2012). The need for accurate functional annotation tools becomes inevitable with increasing amounts of –omics data being generated, and, while some open-source candidates exist, e.g. eggNOG (Huerta-Cepas *et al.*, 2016), BlastKOALA (Kanehisa *et al.*, 2016), and PANNZER2 (Törönen *et al.*, 2018), experimental validation of these predictions are still required.

It has recently been demonstrated that TDA can act as an electroneutral proton-antiporter disrupting the PMF by the import of H^+ ions and export of 1^+ metal ions in *E. coli* (Wilson *et al.*, 2016). The transcriptomic assessment of *V. vulnificus* CMCP6 performed in the current study indicates that the final parts of the electron transport chain were affected by TDA exposure, as well as genes related to oxidative stress and iron starvation. Genes encoding the Cytochrome C oxidase complex IV (*ccoN, ccoO, ccoP, ccoQ*) were down-regulated by 2.8 to 3.0-fold at 30 min and by 4.6 to 5.3-fold at 60 min, while the cytochrome bd oxidoreductase complex encoding genes (*cydA, cydB, cydX*) were down-regulated by 2.3 to 2.6-fold at 60 min of TDA exposure (Figure 3; Supplementary File 2, Supplementary Table S1). In general, blocking the electron transport chain, and particularly the terminal oxidases, results in energy depletion and increased levels of intracellular superoxide radicals (Poole and Cook, 2000). Our findings show that a predicted oxidative stress defense protein (WP_011079481.1) as well as a superoxide dismutase (WP_011079237.1) were highly up-

regulated in the presence of TDA at both time points (FC = 13.0 to 22.6 and 2.8 to 3.2, respectively). Furthermore, a gene annotated as a tellurite resistance TerB family protein was also up-regulated in response to TDA (WP_011079743.1, FC = 5.3 at 30 min, 14.9 at 60 min) and proteins from this functional category are also known to alleviate oxidative stress (Chasteen *et al.*, 2009). Given that TDA is a bactericidal antibiotic, these observations are in line with the theory by Kohanski *et al.* (2007); for *E. coli*, they proposed that bactericidal antibiotics induce cell death by stimulating the Fenton-mediated production of reactive oxygen species (ROS) through hyperactivation of the electron transport chain. This mode of action has been confirmed for other species (Thomas *et al.*, 2013; Van Acker *et al.*, 2013), and it might explain why components of the ROS stress-response are triggered by TDA. Alternatively, the mode of action of TDA proposed by Wilson *et al.* (2016), involving the export of protons, could explain why the down-regulation of cytochrome oxidases would be counteracting the change in membrane potential. Hence, even sub-lethal concentrations of TDA with insignificant effect on bacterial growth (Supplementary File 2, Figure S1) can induce a metabolic stress response that negatively affects the PMF, though the exact molecular interactions remain uncertain.

In addition to disruption of the PMF, TDA is potentially able to chelate +1 charged metal ions in the extracellular space of *E. coli* (Wilson *et al.*, 2016). Exposure of *V. vulnificus* CMCP6 to sub-lethal levels of TDA resulted in the up-regulation of several genes that play a role in iron transport and utilization. Three out of five core genes - a peptide synthetase (WP_011081748.1), an amino acid adenylation domain-containing protein (WP_052298478.1), and an isochorismatase (WP_011081755.1) - involved in production of

the siderophore vulnibactin were up-regulated by > 2-fold after 60 min of TDA exposure (Figure 3; Supplementary File 2, Supplementary Table S1). Additionally, several iron transporters and iron utilization systems were up-regulated by 2.3 to 4.6-fold (e.g. WP_043921119.1, WP_011081918.1, WP_011081754.1, WP_011082460.1). TDA is produced under iron-enriched conditions (D'Alvise *et al.*, 2016), and is therefore not considered to be a siderophore despite its iron-chelating ability. However, TDA production by roseobacters in marine broth is accompanied by the formation of a characteristic brown pigment (Prol García *et al.*, 2014), which is a TDA-iron complex (D'Alvise *et al.*, 2016). This complex is produced as a result of TDA's capacity to chelate ferric iron (D'Alvise *et al.*, 2016). Henceforth, the iron chelating effect of TDA could potentially trigger an iron-starvation response or induce iron scavenging as a defense mechanism in *V. vulnificus.*

In *E. coli*, disruption of the PMF by TDA exposure leads to several phenotypic changes, including decreased or eliminated motility (Wilson *et al.*, 2016). Our study shows that several flagella biogenesis-related genes are down-regulated by 2.0 to 2.5-fold in *V. vulnificus* due to TDA exposure. Motility assays confirmed decreased motility of *V. vulnificus* in the presence of a sub-lethal dose of TDA (Supplementary File 2, Figure S2). Our transcriptomic data revealed that the expression of genes involved in motility-to-biofilm phenotype (e.g. the outer membrane protein OmpU - WP_011079605.1 - and pilus assembly proteins - WP_011080200.1, WP_052298469.1, WP_011081080.1; Figure 3) was increased in response to TDA exposure (FC = 4.3 to 5.7 and 3.0 to 22.6). SEM analysis showed lack of flagella as well as pili-mediated cell-cell aggregation and cell-surface attachment following TDA exposure (Figure 4). Collectively, this indicates that TDA-exposed cells could have

switched to a biofilm phenotype. The biofilm matrix produced by vibrios consists mainly of extracellular polysaccharides (EPS) (Yildiz and Visick, 2009) and *V. vulnificus* also produces capsular polysaccharides when the cell density is high (Hayat *et al.*, 1993; Lee *et al.*, 2013). Multiple genes encoding capsular polysaccharide biosynthesis proteins (WP_011080173.1 and WP_011080171.1) or polysaccharide export protein (WP_043920971.1) were up-regulated by fold change above 5.7 at both tested time points, which further supports the shift of *V. vulnificus* to a biofilm phenotype in the presence of TDA (Figure 3; Supplementary File 2, Supplementary Table S1). Biofilm formation is one of many phenotypes that enable *V. vulnificus* to survive and proliferate in a variety of ecosystems, both during infection and when naturally occurring in the environment (Jones and Oliver, 2009; Yildiz and Visick, 2009). The phenotype is regulated by quorum sensing (McDougald *et al.*, 2006; Lee *et al.*, 2013), but its induction by antibiotics is, to the best of our knowledge, not described in this species. In TDA-producing species, TDA can act as a signaling molecule at low concentrations, regulating motility, biofilm formation, and antibiotic production (Beyersmann *et al.*, 2017). Therefore, it is possible that this molecule also induces biofilm formation in other marine species, and further studies should be performed to confirm this hypothesis.

In conclusion, a sub-lethal concentration of TDA has substantial effects on the transcriptome of *V. vulnificus*, particularly altering the expression of genes associated with a range of defense responses, such as oxidative stress response and biofilm formation. Our data support the previously reported model whereby TDA acts by disrupting the PMF in *V. vulnificus*, though some cellular reactions (e.g. lack of an acid response) do not fully concur with the

effect of TDA observed in *E. coli*. This suggests that the TDA mode of action is likely more complex than currently understood.

Funding

This work was supported by the Danish Research Council for Technology and Production [grant number 0602-01740b]; a DTU PhD Stipend for KKD, Oticon foundation (travel grant for KKD); IDAs og Berg-Nielsens Studie- og Støttefond (travel grant for KKD); the Augustinus foundation (travel grant for KKD); the Fabrikant P.A. Fiskers foundation (travel grant for KKD); Otto Mønsted Foundation (travel grant for KKD) and the scholarship Christian og Ottilia Brorsons Rejselegat for yngre videnskabsmænd og –kvinder (travel grant for KKD).

Acknowledgements

The authors wish to thank Professor Torsten Thomas, Dr. Bernd Wemheuer, and Jennifer Hudson for providing valuable discussions. Bioinformatic analysis reported in this study was performed using the Linux computational cluster Katana supported by the Faculty of Science, UNSW Australia.

The authors declare no conflict of interests.

References

Van Acker, H., Sass, A., Bazzini, S., De Roy, K., Udine, C., Messiaen, T., et al. (2013) Biofilm-Grown *Burkholderia cepacia* Complex Cells Survive Antibiotic Treatment by Avoiding Production of Reactive Oxygen Species. *PLoS One* **8**: e58943.

Beyersmann, P.G., Tomasch, J., Son, K., Stocker, R., Göker, M., Wagner-Döbler, I., et al. (2017) Dual function of tropodithietic acid as antibiotic and signaling molecule in global gene regulation of the probiotic bacterium *Phaeobacter inhibens*. *Sci Rep* **7**: 730.

Bruhn, J.B., Nielsen, K.F., Hjelm, M., Hansen, M., Bresciani, J., Schulz, S., and Gram, L. (2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. *Appl Environ Microbiol* **71**: 7263–7270.

Buchan, A., Gonzalez, J.M., and Moran, M.A. (2005) Overview of the Marine *Roseobacter* Lineage. *Appl Environ Microbiol* **71**: 5665–5677.

Chasteen, T.G., Fuentes, D.E., Tantaleán, J.C., and Vásquez, C.C. (2009) Tellurite: history, oxidative stress, and molecular mechanisms of resistance. *FEMS Microbiol Rev* **33**: 820–832.

D'Alvise, P.W., Lillebø, S., Prol-Garcia, M.J., Wergeland, H.I., Nielsen, K.F., Bergh, Ø., and Gram, L. (2012) *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS One* **7**: e43996.

D'Alvise, P.W., Lillebø, S., Wergeland, H.I., Gram, L., and Bergh, Ø. (2013) Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparison of strains and introduction times. *Aquaculture* **384**–**387**: 82–86.

D'Alvise, P.W., Melchiorsen, J., Porsby, C.H., Nielsen, K.F., and Gram, L. (2010) Inactivation of Vibrio anguillarum by attached and planktonic Roseobacter cells. *Appl Environ Microbiol* **76**: 2366–70.

D'Alvise, P.W., Phippen, C.B.W., Nielsen, K.F., and Gram, L. (2016) Influence of Iron on Production of the Antibacterial Compound Tropodithietic Acid and Its Noninhibitory Analog in *Phaeobacter inhibens*. *Appl Environ Microbiol* **82**: 502–9.

Dittmann, K.K., Sonnenschein, E.C., Egan, S., Gram, L., and Bentzon-Tilia, M. (2018) Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities. *Environ Microbiol Rep* **00**:.

González, J.M., Simó, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedrós-Alió, C., and Moran, M.A. (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. *Appl Environ Microbiol* **66**: 4237–46.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P.W., Dourala, N., Nielsen, K.F., and Gram, L. (2016) Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. *Syst Appl Microbiol* **39**: 180–188.

Harrington, C., Reen, F., Mooij, M., Stewart, F., Chabot, J.-B., Guerra, A., et al. (2014) Characterisation of Non-Autoinducing Tropodithietic Acid (TDA) Production from Marine Sponge *Pseudovibrio* Species. *Mar Drugs* **12**: 5960–5978.

Hayat, U., Reddy, G.P., Bush, C.A., Johnson, J.A., Wright, A.C., and Morris, J.G. (1993) Capsular Types of *Vibrio vulnificus*: An Analysis of Strains from Clinical and Environmental Sources. *J Infect Dis* **168**: 758–762.

Hjelm, M., Bergh, O., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., et al. (2004)

Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Syst Appl Microbiol* **27**: 360–371.

Hjelm, M., Riaza, A., Formoso, F., Melchiorsen, J., and Gram, L. (2004) Seasonal incidence of autochthonous antagonistic *Roseobacter* spp. and *Vibrionaceae* strains in a turbot larva (*Scophthalmus maximus*) rearing system. *Appl Environ Microbiol* **70**: 7288–7294.

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M.C., et al. (2016) EGGNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res* **44**: D286–D293.

Jones, M.K. and Oliver, J.D. (2009) *Vibrio vulnificus*: Disease and Pathogenesis. *Infect Immun* **77**: 1723–1733.

Kanehisa, M., Sato, Y., and Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J Mol Biol* **428**: 726–731.

Kintaka, K., Ono, H., Tsubotani, S., Harada, S., and Okazaki, H. (1984) Thiotropocin, a new sulfur-containing 7-membered-ring antibiotic produced by a *Pseudomonas* sp. *J Antibiot (Tokyo)* **37**: 1294–1300.

Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007) A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell* **130**: 797–810.

Lee, K.-J., Kim, J.-A., Hwang, W., Park, S.-J., and Lee, K.-H. (2013) Role of capsular polysaccharide (CPS) in biofilm formation and regulation of CPS production by quorum-sensing in *Vibrio vulnificus*. *Mol Microbiol* **90**: 841–857.

McDougald, D., Lin, W.H., Rice, S.A., and Kjelleberg, S. (2006) The role of quorum sensing and the effect of environmental conditions on biofilm formation by strains of *Vibrio vulnificus*. *Biofouling* **22**: 161–172.

Nadzirin, N. and Firdaus-Raih, M. (2012) Proteins of unknown function in the Protein Data Bank (PDB): an inventory of true uncharacterized proteins and computational tools for their analysis. *Int J Mol Sci* **13**: 12761–72.

Neu, A.K., Månsson, M., Gram, L., and Prol-García, M.J. (2014) Toxicity of Bioactive and Probiotic Marine Bacteria and Their Secondary Metabolites in *Artemia* sp. and *Caenorhabditis elegans* as Eukaryotic Model Organisms. *Appl Environ Microbiol* **80**: 146–153.

Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø., and Pintado, J. (2006a) Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture* **255**: 323–333.

Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø., and Pintado, J. (2006b) Probiotic effect in vivo of Roseobacter strain 27-4 against Vibrio (Listonella) anguillarum infections in turbot (Scophthalmus maximus L.) larvae. *Aquaculture* **255**: 323–333.

Poole, R.K. and Cook, G.M. (2000) Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation., pp. 165–224.

Porsby, C., Nielsen, K., and Gram, L. (2008) *Phaeobacter* and *Ruegeria* species of the *Roseobacter* clade colonize separate niches in a Danish Turbot (*Scophthalmus maximus*)-rearing farm and antagonize *Vibrio anguillarum* under different growth conditions. *Appl Environ Microbiol* **74**: 7356–7364.

Porsby, C.H., Webber, M. a, Nielsen, K.F., Piddock, L.J. V, and Gram, L. (2011) Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. *Antimicrob Agents Chemother* **55**: 1332–7.

Prado, S., Montes, J., Romalde, J.L., and Barja, J.L. (2009) Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. *Int Microbiol* **12**: 107–114.

Prol García, M.J., D'Alvise, P.W., Rygaard, A.M., and Gram, L. (2014) Biofilm formation is not a prerequisite for production of the antibacterial compound tropodithietic acid in *Phaeobacter inhibens* DSM17395. *J Appl Microbiol* **117**: 1592–1600.

Rabe, P., Klapschinski, T.A., Brock, N.L., Citron, C.A., D'Alvise, P., Gram, L., and Dickschat, J.S. (2014) Synthesis and bioactivity of analogues of the marine antibiotic tropodithietic acid. *Beilstein J Org Chem* **10**: 1796–801.

Rasmussen, B.B., Erner, K.E., Bentzon-Tilia, M., and Gram, L. (2018) Effect of TDA-producing *Phaeobacter inhibens* on the fish pathogen *Vibrio anguillarum* in non-axenic algae and copepod systems. *Microb Biotechnol* **11**: 1070–1079.

Rasmussen, B.B., Grotkjær, T., D'Alvise, P.W., Yin, G., Zhang, F., Bunk, B., et al. (2016) *Vibrio anguillarum* is genetically and phenotypically unaffected by long-term continuous exposure to the antibacterial compound tropodithietic acid. *Appl Environ Microbiol*.

Ruiz-Ponte, C., Cilia, V., Lambert, C., and Nicolas, J.L. (1998) *Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*. *Int J Syst Bacteriol* **48**: 537–542.

Sese, B.T., Grant, A., and Reid, B.J. (2009) Toxicity of Polycyclic Aromatic Hydrocarbons to the Nematode *Caenorhabditis elegans*. *J Toxicol Environ Heal Part A* **72**: 1168–1180.

Sonnenschein, E.C., Nielsen, K.F., D'Alvise, P., Porsby, C.H., Melchiorsen, J., Heilmann, J., et al. (2017) Global occurrence and heterogeneity of the *Roseobacter*-clade species *Ruegeria mobilis*. *ISME J* **11**: 569–583.

Sprando, R.L., Olejnik, N., Cinar, H.N., and Ferguson, M. (2009) A method to rank order water soluble compounds according to their toxicity using Caenorhabditis elegans, a Complex Object Parametric Analyzer and Sorter, and axenic liquid media. *Food Chem Toxicol* **47**: 722–728.

Thomas, V.C., Kinkead, L.C., Janssen, A., Schaeffer, C.R., Woods, K.M., Lindgren, J.K., et al. (2013) A Dysfunctional Tricarboxylic Acid Cycle Enhances Fitness of *Staphylococcus epidermidis* During β-Lactam Stress. *MBio* **4**: e00437-13.

Thompson, F.L., Iida, T., and Swings, J. (2004) Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**: 403–31, table of contents.

Toranzo, A.E., Magariños, B., and Romalde, J.L. (2005) A review of the main bacterial fish diseases in mariculture systems. *Aquaculture* **246**: 37–61.

Törönen, P., Medlar, A., and Holm, L. (2018) PANNZER2: a rapid functional annotation web server. *Nucleic Acids Res* **46**: W84–W88.

Wegner, K., Volkenborn, N., Peter, H., and Eiler, A. (2013) Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiol* **13**: 252.

Wichmann, H., Vocke, F., Brinkhoff, T., Simon, M., Richter-Landsberg, C., Wichmann, H., et al. (2015) Cytotoxic Effects of Tropodithietic Acid on Mammalian Clonal Cell Lines of Neuronal and Glial Origin. *Mar Drugs* **13**: 7113–7123.

Wilson, M.Z., Wang, R., Gitai, Z., and Seyedsayamdost, M.R. (2016) Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl cycle as a proton sink. *Proc Natl Acad Sci* **113**: 1630–1635.

Yildiz, F.H. and Visick, K.L. (2009) *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol* **17**: 109–118.

Figure legends

Figure 1. Gene expression profiles of *Vibrio vulnificus* CMCP6 upon exposure to 0.6 µM tropodithietic acid (TDA). The multi-dimensional scaling plot depicts distances as leading log-fold-changes (logFC); the root-mean-square of the largest absolute log-fold-changes between each sample pair. Circles indicate samples exposed to TDA, triangles indicate controls exposed to DMSO, the solvent of TDA. Each triplicate culture was sampled upon 30 min (green) and 60 min (blue) exposure. The plot is based on the top 500 genes.

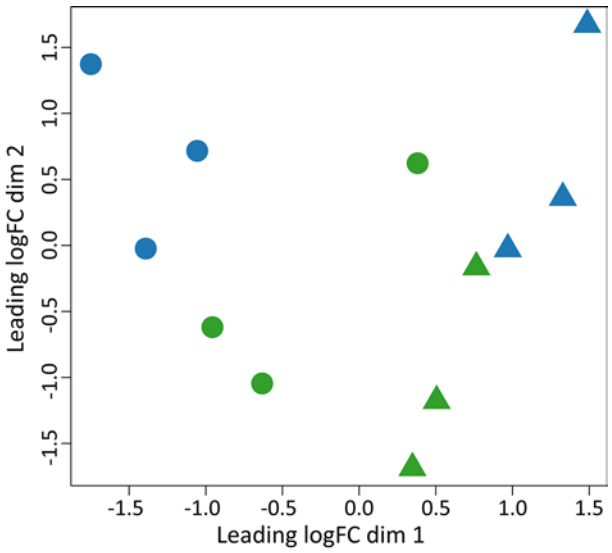
Figure 2. Functional categories of differentially expressed genes in *Vibrio vulnificus* CMCP6 upon exposure to 0.6 µM tropodithietic acid (TDA). The bars represent the percentage of annotated, differentially expressed genes (FDR < 0.05, absolute $\log_2$FC > 1) upon 30 min (green) and 60 min (blue) exposure to TDA. The up-regulated genes are represented by the lighter shade of color, the down-regulated genes are represented by the darker shade of color. The functional categories were assigned using the eggNOG 4.5.1 tool (Huerta-Cepas *et al.*, 2016) on protein sequences of the annotated genes. Genes with multiple functions have been excluded from the diagram.

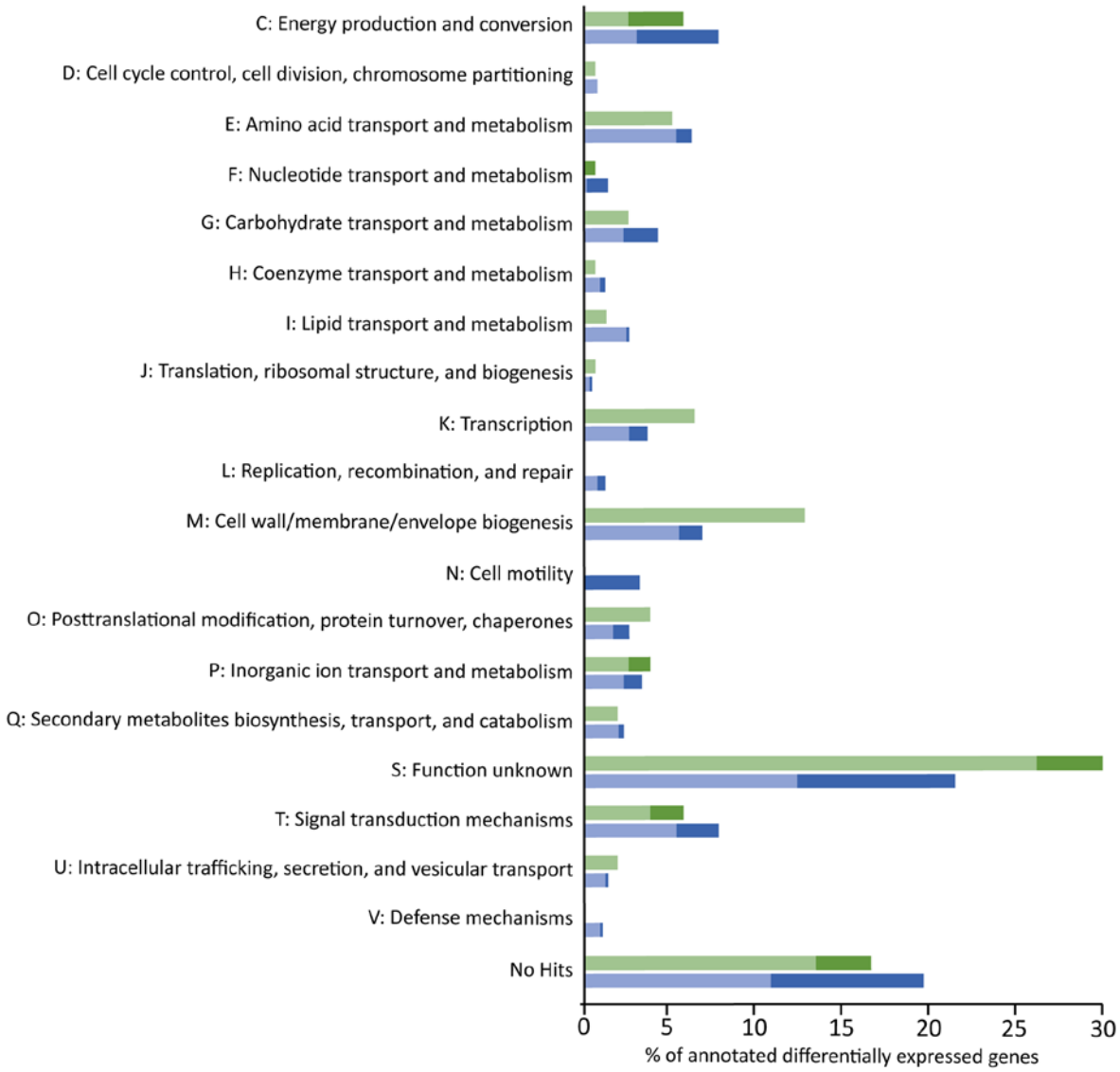
Figure 3. Expression levels of a set of target genes in *Vibrio vulnificus* CMCP6 upon exposure to 0.6 µM tropodithietic acid (TDA) or solvent (DMSO) acting as control at time point 30 min and 60 min. The heatmaps visualize Z-scores calculated from the normalised

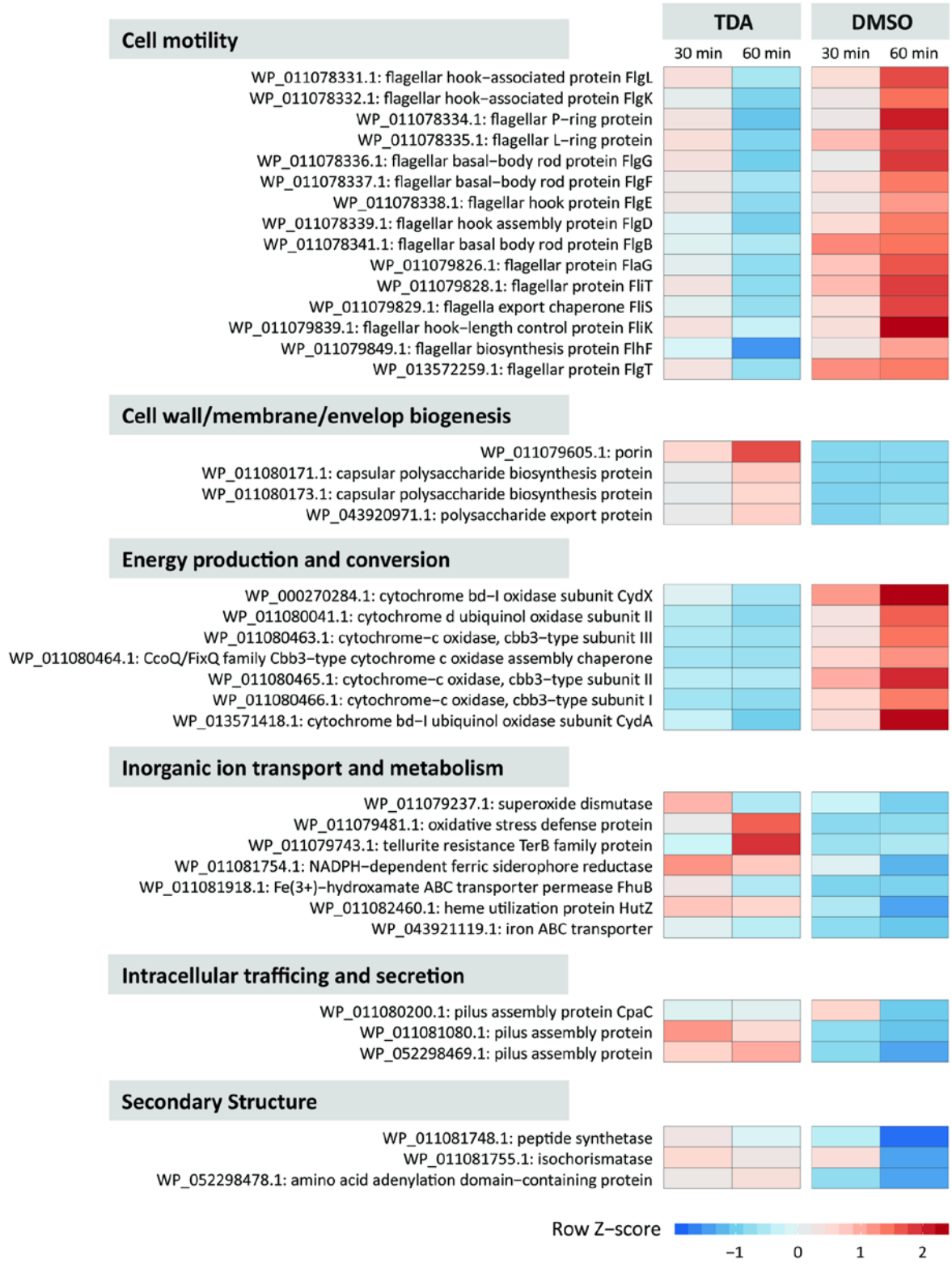
counts per million (CPM). Each treatment-time point column is based on mRNA sequencing data from three biological replicates.

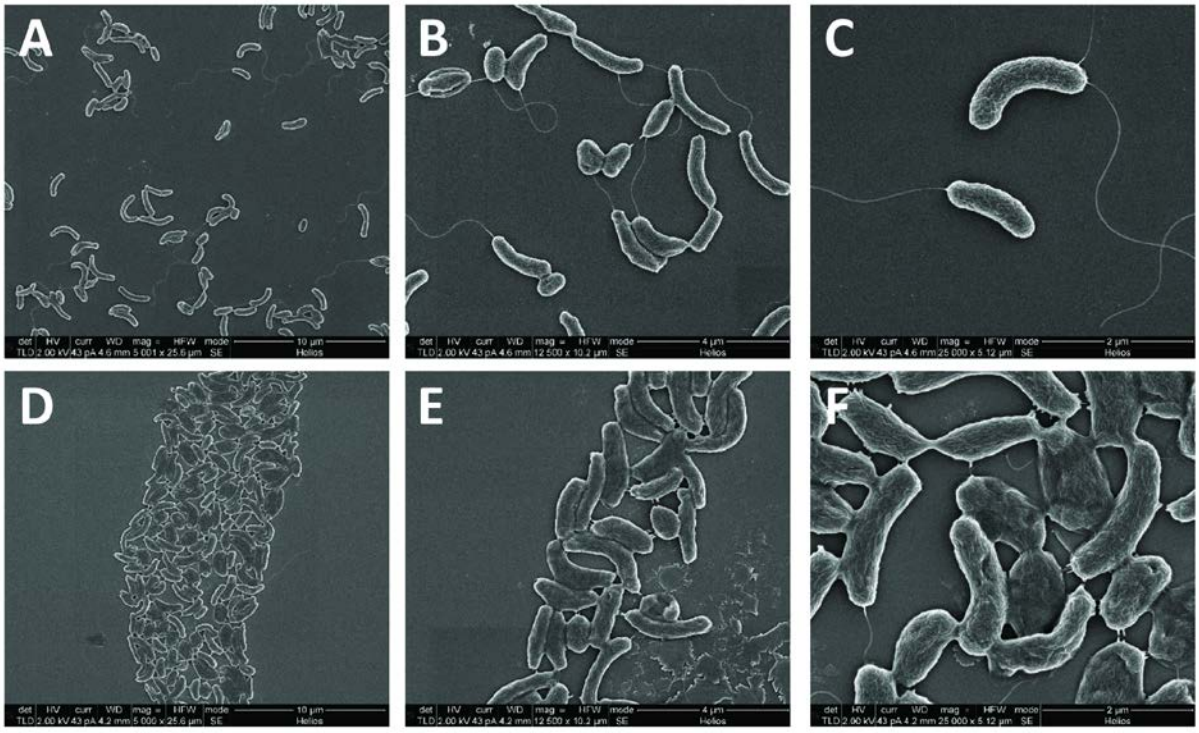
Figure 4. Scanning electron microscopy of *Vibrio vulnificus* CMCP6 upon exposure to 0.03 mM TDA tropodithietic acid (TDA). A-C) Controls exposed to solvent (DMSO); D-F) Cells exposed to TDA. The scale bars are 10 µm (A and D), 4 µm (B and E), and 2 µm (C and F).

Impact of TDA on gene expression of *V. vulnificus*









Supplementary File 1

Tropodithietic acid induces oxidative stress response, cell envelope biogenesis and iron uptake in *Vibrio vulnificus*

Karen K. Dittmann, Cisse H. Porsby, Priscila Goncalves, Ramona Valentina Mateiu, Eva C. Sonnenschein, Mikkel Bentzon-Tilia, Suhelen Egan & Lone Gram[#]

Address correspondence to: Lone Gram (gram@bio.dtu.dk)

Experimental Procedures

Bacterial strain and culture media

Vibrio vulnificus CMCP6 (Kim *et al.*, 2003) was used in this study. CMCP6 was stored in freeze storage medium (Gibson and Khoury, 1986) at -80 °C. Unless otherwise stated, the strain was grown at 37 °C overnight on Mueller Hinton II agar (Cation-Adjusted) (211438; BD) (MHA) or in 5 ml Mueller Hinton II broth (Cation-Adjusted) (212322; BD) (MHB) at 300 rpm.

Dilution of tropodithietic acid (TDA).

Stock solutions of TDA (BioViotica, Germany) were dissolved in 100% dimethyl sulfoxide (D8418; Sigma-Aldrich, Missouri, USA) (DMSO) to 8 mM and stored at -20 °C. Working solutions were prepared by diluting TDA stock solutions in demineralized H_2O to a final concentration of 1 mM and sonicating the solution 2 x 30 s in an ultrasound bath (Aerosec, France).

Transcriptomic analyses following TDA exposure.

Overnight cultures of *V. vulnificus* CMCP6 were inoculated at 1% level in pre-warmed (37 °C) MHB and incubated at 37 °C in a shaking water bath (SW22; Julabo, Pennsylvania, USA) at 200 rpm. At OD_{600} 0.5 ± 0.02 (Novaspec III; Amersham Biosciences, Piscataway, USA), cultures were divided into two, and pre-warmed MHB (1:1 v/v) was added. When OD_{600} reached 0.5 ± 0.02, 0.6 µl of 1 mM TDA was added per ml of culture, giving a final concentration of 0.6 µM (260 x lower than the minimal inhibitory concentration - MIC - value of 15.6 µM). The solvent of TDA was added to control cultures to a final concentration of 0.007%. Samples for transcriptomic analysis were taken after 30 and 60 min of exposure. All controls and treatments were performed in biological triplicates (n = 3 per treatment per

time point). Cell densities were determined by serial ten-fold dilution and spread-plating on MHA.

RNA extraction.

Samples were mixed with RNA protect (76506; Qiagen, Netherlands) and incubated 5 min at room temperature. Samples were centrifuged for 10 min at 10,000 x *g* and pellets stored at -80 °C prior to treatment with proteinase K (P8102S; New England Biolabs Inc., MA, USA) and lysozyme (L6876; Sigma). RNA was purified using RNeasy Mini Kit (74104; Qiagen) including an additional DNase treatment using RNase-free DNase (79254; Qiagen) according to the manufacturer's instruction. Quality and quantity of total RNA was assessed using NanoDrop (ND-1000; Thermo Fisher Scientific Inc., Delaware, USA) and Bioanalyzer (2100; Agilent, California, USA) combined with RNA 6000 Nano kit (5067-1511; Agilent). Five µg of total RNA (RIN ≥ 8.3) were precipitated with ethanol, dissolved in 10 µl RNase-free water and used in the MICROBExpress™ Bacterial mRNA Enrichment Kit (AM1905; Ambion) by which 16S and 23S rRNA were removed. Qubit RNA assay kit (Q32852; Invitrogen) and a Bioanalyzer (RNA 6000 Nano kit) were used for evaluation of quantity and quality of rRNA-depleted RNA.

cDNA library construction and sequencing.

The TruSeq RNA Sample preparation Kit v2 (set A: RS-122-2001 and set B: RS-122-2001; Illumina, California, USA) was used according to the manufacturer's protocol for preparing libraries for RNA sequencing. In brief, fragments of cDNA of approx. 180 bp were made from RNA (rRNA-depleted) and specific adaptors were ligated to each of the 12 samples. Quantity and quality of the libraries were verified using Qubit dsDNA BR assay kit (Q32850; Invitrogen) and a Bioanalyzer on DNA-1000 (r 5067-1504; Agilent) or High Sensitivity (5067-4626; Agilent) chips. Each library was diluted to 10 nM and pooled. The average size

was 270 bp. Sequencing (100 bp paired ends reads) was performed on pooled libraries by BGI-Hong Kong using the HiSeq2000 platform.

RNA sequence analyses.

Quality assessment of the reads before and after trimming was performed using FastQC (v. 0.11.6, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Number of reads, PHRED-score, % GC, nucleotide contribution, quality distribution and enriched 5mers sequences were used for quality assessment of RNA sequencing data. Trimmomatic (v. 0.33, Bolger et al. 2014) was used for quality-based trimming of the reads, which included adapter removal, leading/trailing low quality bases (below quality 3) removal, 4-base sliding window and trim when average dropped below 20, and filtering of sequences with length below 36 bp. The trimmed reads were processed using the HISAT2-StringTie pipeline (Pertea et al., 2016). In brief, the reads were aligned to the reference genome (downloaded from NCBI; NC_004459 and NC_004460 for *V. vulnificus* CMCP6), transcripts were assembled and quantified, transcripts from individual sample were merged and transcript abundances were estimated for generation of a count table (ballgown element). The raw counts were extracted by using the prepDE.py script from the StringTie manual. Differential expression analysis was performed on the raw counts using edgeR (Robinson *et al.*, 2010) by fitting a quasi-likelihood negative binomial generalized log-linear model to the counts. Genes were considered to be significantly differentially expressed if absolute $\log_2$-fold change ($\log_2$FC) between conditions > 1 (either up- or down-regulation), at p-value < 0.05, and false discovery rate (FDR) < 0.05. Significantly differentially expressed genes were functionally annotated using EggNOG (Huerta-Cepas *et al.*, 2016) and BlastKOALA (Kanehisa *et al.*, 2016). Functional categories were based on cluster of orthologous groups (COGs). The expression levels of a set of target genes were visualised by heatmaps; row Z-scores were calculated

from the normalized counts per million (CPM, computed by edgeR) and plotted using R package ggplot2.

Transcriptomic data.

Raw sequencing reads can be accessed through the Sequencing Read Archives (SRA) under PRJNA516163.

Motility assay.

A swimming motility assay was used to investigate if a sub-lethal concentration of TDA affects the motility of *V. vulnificus* CMCP6 cells. TDA or the solvent (DMSO) were added to MHB supplemented with 0.25 % agar resulting in the final concentrations of 0.6 µM and 0.007 % respectively. The assay was conducted in 24-well Nunc Nuclon^{TM} Surface plates (142475). CMCP6 was inoculated in the center of the agar and incubated at 30 °C. The diameter of the colonies was measured after 5 h of exposure. The viable cell count was determined by diluting the content of each well in PBS and subsequent plate spreading on MHA.

Scanning electron microscopy (SEM).

SEM was used to inspect if TDA induces any visible damages to *V. vulnificus* CMCP6 cells and cell envelope at concentrations above the MIC. A total of 225 µl of overnight culture was treated with 25 µl TDA or DMSO at final concentrations of 30 µM (2 x MIC) and 0.3%, respectively. Samples were incubated at 37 °C, 300 rpm for 60 min. Fixation of the cells was done in two steps. In the first step, 25 µL of 25 % glutaraldehyde (G5882; Sigma-Aldrich) was added to each sample (final concentrations of 2.5 %), samples were incubated for 10 min at room temperature and pelleted at 2,000 x *g* for 10 min. In the second step, glutaraldehyde diluted to 2 % in phosphate buffered saline (BR0014R; Oxoid) (PBS) was added to each cell pellet and the mixture was incubated overnight at 5 °C. Samples were then centrifuged at

2000 x *g*, 5 min and the cell pellets were dried in ethanol series (50, 60, 70, 80, 90, 99.9%) for 10 minutes each step. Cells were placed onto a square piece of silicon wafer and left to dry overnight in the fume hood. The silicon substrate with the sample was attached onto an aluminum stub with a carbon tape. The uncoated sample was imaged in a FEI Helios dual beam SEM. For imaging, an accelerating voltage of 2 keV and a current of 43 pA was used and the secondary electron was detected with the lens detector.

References

Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–20.

Gibson, L.F. and Khoury, J.T. (1986) Storage and survival of bacteria by ultra-freeze. *Lett. Appl. Microbiol.* **3**: 127–129.

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M.C., et al. (2016) EGGNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.* **44**: D286–D293.

Kanehisa, M., Sato, Y., and Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J. Mol. Biol.* **428**: 726–731.

Kim, Y.R., Lee, S.E., Kim, C.M., Kim, S.Y., Shin, E.K., Shin, D.H., et al. (2003) Characterization and Pathogenic Significance of *Vibrio vulnificus* Antigens Preferentially Expressed in Septicemic Patients. *Infect. Immun.* **71**: 5461–5471.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**:

127 139–40.

128

Supplementary File 2

Tropodithietic acid induces oxidative stress response, cell envelope biogenesis and iron uptake in *Vibrio vulnificus*

Karen K. Dittmann, Cisse H. Porsby, Priscila Goncalves, Ramona Valentina Mateiu, Eva C. Sonnenschein, Mikkel Bentzon-Tilia, Suhelen Egan & Lone Gram[#]

Address correspondence to: Lone Gram (gram@bio.dtu.dk)

Table S1:

Complete list of differentially expressed genes in *Vibrio vulnificus* CMCP6 exposed to sub-lethal levels of tropodithietic acid (TDA). This includes gene ID (assigned by the HISAT2-Stringtie pipeline), log_2-fold change (FC) at 30 min and 60 min of exposure (comparing TDA-exposed and non-exposed cells per time point), as well as genomic information (chromosome ID, gene name, protein ID, and protein annotation according to NCBI). Gene IDs with prefix "MSTRG" were annotated by the pipeline due to mapping of transcripts to a non-annotated part of the CMCP6 genome. NS (not significant) is used when the difference in gene expression was not statistically significant at a particular time point (e.g. gene2 was differently expressed between TDA-treated and non-treated cells at 60 min, but not at 30 min). NA indicates unidentified entries. Genes highlighted in bold are shown in the heatmaps in Figure 3.

Gene ID	Log_2FC (30 min)	Log_2FC (60 min)	Chromosome	Gene name	Protein product	Protein annotation
gene2	NS	-1.03	NC_004459.3	VV1_RS00015	WP_011078131.1	D-glutamate deacylase
gene24	NS	-1.32	NC_004459.3	VV1_RS00125	WP_011078153.1	DUF3857 domain-containing protein
gene30	NS	2.32	NC_004459.3	VV1_RS00155	WP_011078159.1	sugar ABC transporter permease
gene35	NS	1.07	NC_004459.3	VV1_RS00180	WP_011078164.1	methyl-accepting chemotaxis protein
gene38	NS	-1.05	NC_004459.3	VV1_RS00190	WP_011078166.1	MULTISPECIES: NAD(P)/FAD-dependent oxidoreductase
gene117	NS	1.08	NC_004459.3	VV1_RS00585	WP_011078244.1	hypothetical protein
gene128	NS	1.65	NC_004459.3	VV1_RS00640	WP_011078255.1	starvation lipoprotein Slp
gene164	NS	1.36	NC_004459.3	VV1_RS00820	WP_011078291.1	MULTISPECIES: DUF4442 domain-containing protein
gene173	NS	1.40	NC_004459.3	VV1_RS00865	WP_011078300.1	MFS transporter
gene178	1.04	NS	NC_004459.3	VV1_RS00890	WP_011078305.1	transcriptional regulator
gene179	1.20	NS	NC_004459.3	VV1_RS00895	WP_011078306.1	MULTISPECIES: hypothetical protein
gene183	NS	-1.73	NC_004459.3	VV1_RS00915	WP_011078310.1	MULTISPECIES: CBS domain-containing protein
gene184	NS	-1.37	NC_004459.3	VV1_RS00920	WP_013572243.1	MULTISPECIES: P-II family nitrogen regulator
gene193	NS	-1.55	NC_004459.3	VV1_RS00965	WP_011078320.1	hypothetical protein
gene201	NS	-2.12	NC_004459.3	VV1_RS01005	WP_011078328.1	flagellin
gene202	NS	-1.92	NC_004459.3	VV1_RS01010	WP_011078329.1	flagellin
gene204	NS	-1.21	NC_004459.3	VV1_RS01020	WP_011078330.1	MULTISPECIES: flagellin
MSTRG.2743.1	NS	-1.51	NC_004459.3	NA	NA	NA

gene205	**NS**	**-1.18**	**NC_004459.3**	**VV1_RS01025**	**WP_011078331.1**	**flagellar hook-associated protein FlgL**
gene206	**NS**	**-1.30**	**NC_004459.3**	**VV1_RS01030**	**WP_011078332.1**	**flagellar hook-associated protein FlgK**
gene208	**NS**	**-1.09**	**NC_004459.3**	**VV1_RS01040**	**WP_011078334.1**	**MULTISPECIES: flagellar P-ring protein**
gene209	**NS**	**-1.28**	**NC_004459.3**	**VV1_RS01045**	**WP_011078335.1**	**flagellar L-ring protein**
gene210	**NS**	**-1.32**	**NC_004459.3**	**VV1_RS01050**	**WP_011078336.1**	**MULTISPECIES: flagellar basal-body rod protein FlgG**
gene211	**NS**	**-1.18**	**NC_004459.3**	**VV1_RS01055**	**WP_011078337.1**	**flagellar basal-body rod protein FlgF**
gene212	**NS**	**-1.05**	**NC_004459.3**	**VV1_RS01060**	**WP_011078338.1**	**flagellar hook protein FlgE**
gene213	**NS**	**-1.23**	**NC_004459.3**	**VV1_RS01065**	**WP_011078339.1**	**MULTISPECIES: flagellar hook assembly protein FlgD**
gene215	**NS**	**-1.11**	**NC_004459.3**	**VV1_RS01075**	**WP_011078341.1**	**MULTISPECIES: flagellar basal body rod protein FlgB**
gene221	NS	-1.29	NC_004459.3	VV1_RS01105	WP_011078347.1	MULTISPECIES: hypothetical protein
gene222	NS	-1.33	NC_004459.3	VV1_RS01110	WP_011078348.1	membrane protein
gene223	**NS**	**-1.12**	**NC_004459.3**	**VV1_RS01115**	**WP_013572259.1**	**MULTISPECIES: flagellar protein FlgT**
gene225	NS	1.24	NC_004459.3	VV1_RS01125	WP_086016749.1	Fe3+-citrate ABC transporter substrate-binding protein
gene234	NS	-2.75	NC_004459.3	VV1_RS23025	WP_011078358.1	MULTISPECIES: DUF3149 domain-containing protein
gene238	NS	-1.25	NC_004459.3	VV1_RS01190	WP_011078362.1	bifunctional metallophosphatase/5\'-nucleotidase
gene339	NS	-1.66	NC_004459.3	VV1_RS01685	WP_011078446.1	MULTISPECIES: aminobenzoyl-glutamate transporter
gene351	NS	3.14	NC_004459.3	VV1_RS01745	WP_011078459.1	dicarboxylate/amino acid:cation symporter
gene402	1.56	2.58	NC_004459.3	VV1_RS02000	WP_011078508.1	MULTISPECIES: DedA family protein
gene405	NS	1.01	NC_004459.3	VV1_RS02015	WP_080553392.1	LysR family transcriptional regulator
gene406	NS	1.29	NC_004459.3	VV1_RS02020	WP_011078512.1	D-amino acid dehydrogenase small subunit
gene407	NS	1.67	NC_004459.3	VV1_RS02025	WP_011078513.1	alanine:cation symporter family protein
gene409	NS	1.53	NC_004459.3	VV1_RS02035	WP_011078515.1	carbohydrate-binding protein
gene443	NS	1.85	NC_004459.3	VV1_RS02205	WP_086016912.1	malate synthase A
gene456	NS	1.52	NC_004459.3	VV1_RS02270	WP_011078562.1	polyphosphate kinase
gene475	1.74	1.37	NC_004459.3	VV1_RS02365	WP_011078575.1	MULTISPECIES: ribosome-associated translation inhibitor RaiA
gene479	NS	1.66	NC_004459.3	VV1_RS02385	WP_011078579.1	murein transglycosylase
gene532	1.00	1.11	NC_004459.3	VV1_RS02650	WP_011078632.1	bifunctional aspartate kinase/homoserine dehydrogenase I
gene534	NS	1.14	NC_004459.3	VV1_RS02660	WP_011078634.1	MULTISPECIES: ribonuclease
gene536	NS	-1.41	NC_004459.3	VV1_RS02670	WP_011078635.1	MULTISPECIES: two-component system response regulator ArcA
gene564	NS	1.20	NC_004459.3	VV1_RS02810	WP_011078663.1	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase
gene565	NS	1.26	NC_004459.3	VV1_RS02815	WP_013572432.1	MULTISPECIES: cell division protein FtsW
gene566	NS	1.12	NC_004459.3	VV1_RS02820	WP_011078665.1	UDP-N-acetylmuramoyl-L-alanine--D-glutamate ligase
gene567	NS	1.12	NC_004459.3	VV1_RS02825	WP_011078666.1	phospho-N-acetylmuramoyl-

						pentapeptide-transferase
gene568	NS	1.34	NC_004459.3	VV1_RS02830	WP_011078667.1	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase
gene569	NS	1.44	NC_004459.3	VV1_RS02835	WP_011078668.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase
gene570	1.03	1.72	NC_004459.3	VV1_RS02840	WP_011078669.1	peptidoglycan glycosyltransferase FtsI
gene571	NS	1.34	NC_004459.3	VV1_RS02845	WP_011078670.1	MULTISPECIES: cell division protein FtsL
gene572	NS	1.35	NC_004459.3	VV1_RS02850	WP_011078671.1	MULTISPECIES: ribosomal RNA small subunit methyltransferase H
gene575	NS	1.29	NC_004459.3	VV1_RS02860	WP_011078674.1	penicillin-binding protein activator
gene576	NS	1.37	NC_004459.3	VV1_RS02865	WP_011078675.1	MULTISPECIES: YraN family protein
gene578	NS	1.09	NC_004459.3	VV1_RS02875	WP_011078677.1	BON domain-containing protein
gene582	NS	-1.04	NC_004459.3	VV1_RS02895	WP_011078681.1	MULTISPECIES: cytochrome b
gene583	NS	-1.10	NC_004459.3	VV1_RS02900	WP_011078682.1	MULTISPECIES: ubiquinol-cytochrome c reductase iron-sulfur subunit
gene589	NS	1.02	NC_004459.3	VV1_RS02925	WP_011078687.1	DegQ family serine endoprotease
gene625	NS	-1.14	NC_004459.3	VV1_RS03105	WP_011078721.1	glutamine--fructose-6-phosphate aminotransferase
gene628	NS	-1.68	NC_004459.3	VV1_RS03120	WP_011078724.1	GntR family transcriptional regulator
gene634	2.35	2.94	NC_004459.3	VV1_RS03150	WP_011078729.1	MULTISPECIES: transcriptional regulator LeuO
gene664	NS	1.03	NC_004459.3	VV1_RS03300	WP_011078758.1	MULTISPECIES: BolA family transcriptional regulator
gene665	NS	1.21	NC_004459.3	VV1_RS03305	WP_011078759.1	MULTISPECIES: STAS domain-containing protein
gene666	1.09	1.33	NC_004459.3	VV1_RS03310	WP_043920913.1	phospholipid-binding protein MlaC
gene667	NS	1.14	NC_004459.3	VV1_RS03315	WP_011078761.1	MULTISPECIES: outer membrane lipid asymmetry maintenance protein MlaD
gene699	NS	1.09	NC_004459.3	VV1_RS03475	WP_011078793.1	DUF2796 domain-containing protein
gene700	NS	1.04	NC_004459.3	VV1_RS03480	WP_011078794.1	MULTISPECIES: ABC transporter ATP-binding protein
gene701	NS	1.02	NC_004459.3	VV1_RS03485	WP_011078795.1	ABC transporter permease
gene713	1.16	NS	NC_004459.3	VV1_RS03545	WP_011149350.1	MULTISPECIES: lysine transporter LysM
gene751	NS	1.16	NC_004459.3	VV1_RS03715	WP_011078838.1	nucleotide sugar dehydrogenase
gene760	-1.32	NS	NC_004459.3	VV1_RS03760	WP_011078847.1	hypothetical protein
MSTRG.3230.1	-1.18	-2.02	NC_004459.3	NA	NA	NA
gene807	NS	-1.56	NC_004459.3	VV1_RS03985	WP_011078895.1	JAB domain-containing protein
gene808	NS	-1.59	NC_004459.3	VV1_RS03990	WP_011078896.1	methyl-accepting chemotaxis protein
gene815	NS	2.55	NC_004459.3	VV1_RS04025	WP_011078903.1	DMT family transporter
gene816	2.77	2.95	NC_004459.3	VV1_RS04030	WP_011078904.1	hypothetical protein
gene835	-1.25	-1.63	NC_004459.3	VV1_RS04125	WP_011078923.1	uracil-xanthine permease
gene839	NS	1.35	NC_004459.3	VV1_RS04145	WP_011078927.1	RNA-binding transcriptional accessory protein

gene840	NS	-1.02	NC_004459.3	VV1_RS04150	WP_011078928.1	MULTISPECIES: hypothetical protein
gene860	NS	-1.31	NC_004459.3	VV1_RS04250	WP_011078948.1	phosphoenolpyruvate carboxykinase (ATP)
MSTRG.3345.9	NS	19.90	NC_004459.3	NA	NA	NA
gene906	-1.37	NS	NC_004459.3	NA	NA	NA
gene910	NS	1.13	NC_004459.3	VV1_RS04495	WP_011078985.1	hypothetical protein
gene937	NS	1.15	NC_004459.3	VV1_RS04630	WP_011079012.1	multicopper oxidase family protein
gene949	NS	1.63	NC_004459.3	VV1_RS04690	WP_011079018.1	fatty acid oxidation complex subunit alpha FadB
gene950	NS	1.55	NC_004459.3	VV1_RS04695	WP_011079019.1	acetyl-CoA C-acyltransferase FadA
gene953	NS	1.34	NC_004459.3	VV1_RS04710	WP_011079022.1	MULTISPECIES: hypothetical protein
gene959	-1.28	-2.92	NC_004459.3	VV1_RS04740	WP_011079028.1	hypothetical protein
gene962	1.13	NS	NC_004459.3	VV1_RS04755	WP_011079031.1	heat-shock protein Hsp20
gene999	NS	1.09	NC_004459.3	VV1_RS04925	WP_011079065.1	MULTISPECIES: acetolactate synthase 2 small subunit
gene1000	NS	1.27	NC_004459.3	VV1_RS04930	WP_011079066.1	acetolactate synthase 2 catalytic subunit
gene1006	NS	-1.15	NC_004459.3	VV1_RS04960	WP_011079072.1	MULTISPECIES: cytochrome c oxidase accessory protein CcoG
gene1007	NS	-1.13	NC_004459.3	VV1_RS04965	WP_011079073.1	DUF1040 family protein
gene1043	-1.18	-1.50	NC_004459.3	VV1_RS05140	WP_039552050.1	cytochrome c5 family protein
gene1049	NS	1.51	NC_004459.3	VV1_RS05170	WP_011079103.1	ABC transporter ATP-binding protein
gene1050	NS	1.61	NC_004459.3	VV1_RS05175	WP_011079104.1	MULTISPECIES: ABC transporter substrate-binding protein
gene1051	NS	1.25	NC_004459.3	VV1_RS05180	WP_011079105.1	MULTISPECIES: ABC transporter permease
gene1052	NS	1.31	NC_004459.3	VV1_RS05185	WP_011079106.1	ABC transporter permease
gene1054	-1.69	-1.91	NC_004459.3	VV1_RS05195	WP_011079108.1	hypothetical protein
gene1057	NS	-1.19	NC_004459.3	VV1_RS05210	WP_011079111.1	DNA-directed RNA polymerase subunit sigma
gene1075	1.01	2.88	NC_004459.3	VV1_RS05305	WP_011079129.1	hypothetical protein
gene1105	3.21	3.79	NC_004459.3	VV1_RS05455	WP_011079158.1	acyltransferase
gene1106	1.87	1.53	NC_004459.3	VV1_RS05460	WP_011079159.1	DUF2500 domain-containing protein
gene1116	NS	1.34	NC_004459.3	VV1_RS05505	WP_011079168.1	MULTISPECIES: cell division protein FtsX
gene1117	NS	1.01	NC_004459.3	VV1_RS05510	WP_011079169.1	RNA polymerase sigma factor RpoH
gene1126	NS	1.33	NC_004459.3	VV1_RS05550	WP_011079177.1	class I SAM-dependent methyltransferase
gene1184	NS	2.59	NC_004459.3	VV1_RS05835	WP_011079223.1	acetate--CoA ligase
gene1186	NS	1.70	NC_004459.3	VV1_RS05845	WP_011079225.1	cyclic nucleotide-binding/CBS domain-containing protein
gene1194	NS	1.10	NC_004459.3	VV1_RS05885	WP_011079230.1	MarC family protein
gene1196	NS	1.05	NC_004459.3	VV1_RS05895	WP_011079232.1	protein-disulfide reductase DsbD
gene1197	NS	-1.21	NC_004459.3	VV1_RS05900	WP_011079233.1	anaerobic C4-dicarboxylate transporter
gene1201	**1.53**	**1.66**	**NC_004459.3**	**VV1_RS05920**	**WP_011079237.1**	**superoxide dismutase**
gene1204	NS	1.70	NC_004459.3	VV1_RS05935	WP_080553404.1	periplasmic repressor CpxP
gene1210	1.99	1.66	NC_004459.3	VV1_RS05965	WP_013570978.1	MULTISPECIES: hypothetical protein

gene1215	NS	-2.05	NC_004459.3	VV1_RS05990	WP_011079251.1	fumarate reductase subunit D
gene1216	NS	-2.12	NC_004459.3	VV1_RS05995	WP_011079252.1	MULTISPECIES: fumarate reductase subunit C
gene1218	NS	-2.20	NC_004459.3	VV1_RS06005	WP_011079254.1	fumarate reductase (quinol) flavoprotein subunit
gene1232	NS	-1.12	NC_004459.3	VV1_RS06070	WP_011079267.1	SLC13 family permease
gene1247	NS	1.09	NC_004459.3	VV1_RS06145	WP_011079273.1	MULTISPECIES: LysM peptidoglycan-binding domain-containing protein
gene1256	NS	-1.20	NC_004459.3	VV1_RS06190	WP_011079282.1	MULTISPECIES: sel1 repeat family protein
gene1257	1.39	NS	NC_004459.3	VV1_RS06195	WP_011079283.1	flavohemoprotein
gene1259	NS	-1.02	NC_004459.3	VV1_RS06205	WP_013570995.1	amidohydrolase
gene1267	NS	1.41	NC_004459.3	VV1_RS06245	WP_011079293.1	aspartate aminotransferase family protein
gene1268	NS	1.49	NC_004459.3	VV1_RS06250	WP_011079294.1	arginine N-succinyltransferase
gene1269	NS	1.22	NC_004459.3	VV1_RS06255	WP_043920934.1	N-succinylglutamate 5-semialdehyde dehydrogenase
gene1274	NS	1.34	NC_004459.3	VV1_RS06280	WP_011079300.1	hydrolase
gene1275	1.17	1.41	NC_004459.3	VV1_RS06285	WP_011079301.1	MULTISPECIES: TIGR02444 family protein
gene1277	NS	1.42	NC_004459.3	VV1_RS06295	WP_013571005.1	glutathione-regulated potassium-efflux system ancillary protein KefG
gene1283	NS	1.15	NC_004459.3	VV1_RS06325	WP_013571009.1	MULTISPECIES: hypothetical protein
gene1284	NS	1.35	NC_004459.3	VV1_RS06330	WP_011079310.1	MULTISPECIES: FKBP-type peptidyl-prolyl cis-trans isomerase
gene1293	NS	1.85	NC_004459.3	VV1_RS06375	WP_011079319.1	MULTISPECIES: bacterioferritin
gene1294	NS	1.50	NC_004459.3	VV1_RS06380	WP_011079320.1	MULTISPECIES: bacterioferritin
gene1301	NS	-1.05	NC_004459.3	VV1_RS06415	WP_011079327.1	class II fructose-bisphosphatase
gene1319	NS	1.29	NC_004459.3	VV1_RS06505	WP_011079345.1	N-acetyl-gamma-glutamyl-phosphate reductase
gene1320	NS	1.08	NC_004459.3	VV1_RS06510	WP_011079346.1	MULTISPECIES: acetylglutamate kinase
gene1328	NS	-1.33	NC_004459.3	VV1_RS06550	WP_011079354.1	fimbrial protein
gene1340	2.31	3.29	NC_004459.3	VV1_RS06610	WP_011079365.1	MULTISPECIES: DUF481 domain-containing protein
gene1404	NS	1.30	NC_004459.3	VV1_RS06930	WP_011079423.1	MULTISPECIES: ornithine carbamoyltransferase
gene1405	NS	2.14	NC_004459.3	VV1_RS06935	WP_011079424.1	arginine deiminase
gene1407	NS	1.21	NC_004459.3	VV1_RS06945	WP_011079426.1	hypothetical protein
gene1410	NS	-2.45	NC_004459.3	VV1_RS06960	WP_011079429.1	DUF898 domain-containing protein
gene1411	NS	-2.69	NC_004459.3	VV1_RS06965	WP_011079430.1	Zn-dependent protease
gene1421	NS	-1.14	NC_004459.3	VV1_RS07015	WP_011079439.1	MULTISPECIES: PTS sugar transporter subunit IIC
gene1436	NS	1.20	NC_004459.3	VV1_RS07090	WP_011079446.1	MULTISPECIES: MBL fold metallo-hydrolase
gene1437	NS	1.11	NC_004459.3	VV1_RS07095	WP_011079447.1	hypothetical protein
gene1445	NS	-1.04	NC_004459.3	VV1_RS07130	WP_011079448.1	chemotaxis protein
gene1468	NS	-2.25	NC_004459.3	VV1_RS07245	WP_043921085.1	hypothetical protein
gene1469	NS	1.56	NC_004459.3	VV1_RS07250	WP_011079469.1	endonuclease
gene1481	**3.70**	**4.50**	**NC_004459.3**	**VV1_RS07310**	**WP_011079481.1**	**MULTISPECIES: oxidative stress defense protein**
gene1483	NS	-1.07	NC_004459.3	VV1_RS07320	WP_011079483.1	ribose-5-phosphate isomerase

gene1496	NS	1.43	NC_004459.3	VV1_RS07380	WP_011079494.1	MULTISPECIES: RNA polymerase sigma factor RpoE
gene1497	1.03	1.27	NC_004459.3	VV1_RS07385	WP_011079495.1	MULTISPECIES: anti-sigma E factor
gene1498	NS	1.08	NC_004459.3	VV1_RS07390	WP_011079496.1	sigma-E factor regulatory protein RseB
gene1508	NS	-1.63	NC_004459.3	VV1_RS07440	WP_011079506.1	MULTISPECIES: hypothetical protein
gene1509	NS	-1.03	NC_004459.3	VV1_RS07445	WP_052298468.1	sensor domain-containing diguanylate cyclase
gene1522	NS	1.18	NC_004459.3	VV1_RS07510	WP_011079520.1	LysM peptidoglycan-binding domain-containing protein
gene1523	NS	1.38	NC_004459.3	VV1_RS07515	WP_011079521.1	RNA polymerase sigma factor RpoS
rna107	-1.02	NS	NC_004459.3	NA	NA	NA
gene1542	1.25	1.40	NC_004459.3	VV1_RS07610	WP_011079532.1	NADP-dependent oxidoreductase
gene1543	1.05	1.65	NC_004459.3	VV1_RS07615	WP_011079533.1	MULTISPECIES: DedA family protein
gene1544	1.09	1.68	NC_004459.3	VV1_RS07620	WP_011079534.1	insulinase family protein
gene1564	NS	1.31	NC_004459.3	VV1_RS07720	WP_011079554.1	MULTISPECIES: pyruvate dehydrogenase complex transcriptional repressor PdhR
gene1565	NS	1.17	NC_004459.3	VV1_RS07725	WP_011079555.1	MULTISPECIES: pyruvate dehydrogenase (acetyl-transferring), homodimeric type
gene1566	NS	1.08	NC_004459.3	VV1_RS07730	WP_011079556.1	pyruvate dehydrogenase complex dihydrolipoyllysine-residue acetyltransferase
gene1591	NS	2.03	NC_004459.3	VV1_RS07855	WP_011079581.1	iron ABC transporter substrate-binding protein
gene1608	NS	1.18	NC_004459.3	VV1_RS07940	WP_011079598.1	iron-sulfur cluster insertion protein ErpA
gene1615	**2.14**	**2.54**	**NC_004459.3**	**VV1_RS07975**	**WP_011079605.1**	**porin**
gene1642	NS	-3.26	NC_004459.3	VV1_RS08110	WP_043920944.1	U32 family peptidase
gene1643	NS	-3.57	NC_004459.3	VV1_RS08115	WP_011079631.1	U32 family peptidase
gene1645	NS	-2.44	NC_004459.3	VV1_RS08125	WP_011079633.1	MULTISPECIES: SCP2 domain-containing protein
gene1653	NS	-1.39	NC_004459.3	VV1_RS08165	WP_011079641.1	NupC/NupG family nucleoside CNT transporter
gene1668	NS	1.28	NC_004459.3	VV1_RS08240	WP_011079655.1	AMP-dependent synthetase
gene1679	**NS**	**2.55**	**NC_004459.3**	**VV1_RS08295**	**WP_052298469.1**	**pilus assembly protein**
gene1683	2.54	2.15	NC_004459.3	VV1_RS08315	WP_011151038.1	type II secretion system F family protein
gene1702	NS	-1.96	NC_004459.3	VV1_RS22975	WP_011079688.1	porin family protein
gene1716	NS	1.37	NC_004459.3	VV1_RS08480	WP_080553406.1	DUF3413 domain-containing protein
gene1723	1.61	2.31	NC_004459.3	VV1_RS08515	WP_011079709.1	murein transglycosylase A
gene1727	NS	-1.29	NC_004459.3	VV1_RS08535	WP_011079713.1	hypothetical protein
gene1752	2.18	2.42	NC_004459.3	VV1_RS08660	WP_011079738.1	DUF2884 family protein
gene1753	1.29	2.34	NC_004459.3	VV1_RS08665	WP_011079739.1	chitinase
gene1755	1.38	2.83	NC_004459.3	VV1_RS08675	WP_011079741.1	MULTISPECIES: DUF4250 domain-containing protein
gene1756	1.47	2.86	NC_004459.3	VV1_RS08680	WP_011079742.1	MULTISPECIES: hypothetical protein
gene1757	**2.45**	**3.86**	**NC_004459.3**	**VV1_RS08685**	**WP_011079743.1**	**tellurite resistance TerB family protein**
gene1763	NS	1.17	NC_004459.3	VV1_RS08710	WP_011079748.1	ATP-grasp domain-containing protein
gene1765	NS	1.60	NC_004459.3	VV1_RS08720	WP_011079750.1	MBL fold metallo-hydrolase

gene1766	NS	1.85	NC_004459.3	VV1_RS08725	WP_011079751.1	NAD-dependent epimerase/dehydratase family protein
gene1767	NS	1.61	NC_004459.3	VV1_RS08730	WP_043920951.1	ketoacyl-ACP synthase III
gene1788	NS	1.05	NC_004459.3	VV1_RS08835	WP_017419561.1	MULTISPECIES: molecular chaperone
gene1797	NS	-1.75	NC_004459.3	VV1_RS08880	WP_011079780.1	MULTISPECIES: cytochrome c
gene1812	NS	-1.19	NC_004459.3	VV1_RS08955	WP_011079795.1	hypothetical protein
gene1813	NS	2.41	NC_004459.3	VV1_RS08960	WP_011079796.1	acyl-CoA dehydrogenase
gene1819	NS	-1.55	NC_004459.3	VV1_RS08990	WP_011079802.1	uracil-xanthine permease
gene1835	NS	-2.17	NC_004459.3	VV1_RS09075	WP_011079818.1	MULTISPECIES: hypothetical protein
gene1840	NS	-1.54	NC_004459.3	VV1_RS09100	WP_011079823.1	flagellin
gene1841	NS	-1.71	NC_004459.3	VV1_RS09105	WP_011079824.1	flagellin
gene1842	NS	-1.64	NC_004459.3	VV1_RS09110	WP_011079825.1	MULTISPECIES: flagellin
gene1843	**NS**	**-1.34**	**NC_004459.3**	**VV1_RS09115**	**WP_011079826.1**	**MULTISPECIES: flagellar protein FlaG**
gene1845	**NS**	**-1.34**	**NC_004459.3**	**VV1_RS09125**	**WP_011079828.1**	**flagellar protein FliT**
gene1846	**NS**	**-1.36**	**NC_004459.3**	**VV1_RS09130**	**WP_011079829.1**	**MULTISPECIES: flagella export chaperone FliS**
gene1856	**NS**	**-1.07**	**NC_004459.3**	**VV1_RS09180**	**WP_011079839.1**	**flagellar hook-length control protein FliK**
gene1866	**NS**	**-1.00**	**NC_004459.3**	**VV1_RS09230**	**WP_011079849.1**	**flagellar biosynthesis protein FlhF**
gene1870	NS	-1.16	NC_004459.3	VV1_RS09250	WP_011079853.1	MULTISPECIES: protein phosphatase CheZ
gene1871	NS	-1.17	NC_004459.3	VV1_RS09255	WP_011079854.1	MULTISPECIES: chemotaxis protein CheA
gene1875	NS	-1.13	NC_004459.3	VV1_RS09275	WP_011079858.1	MULTISPECIES: chemotaxis protein CheW
gene1876	NS	-1.27	NC_004459.3	VV1_RS09280	WP_011079859.1	DUF2802 domain-containing protein
gene1892	2.24	3.49	NC_004459.3	VV1_RS09360	WP_011079875.1	fatty acid oxidation complex subunit alpha FadJ
gene1916	NS	2.68	NC_004459.3	VV1_RS24270	WP_011150877.1	hypothetical protein
gene1917	NS	2.36	NC_004459.3	VV1_RS23145	WP_047109460.1	MULTISPECIES: DUF454 domain-containing protein
gene1928	-1.08	NS	NC_004459.3	VV1_RS09525	WP_011079909.1	YcgN family cysteine cluster protein
gene1929	1.51	NS	NC_004459.3	VV1_RS09530	WP_011079910.1	antibiotic biosynthesis monooxygenase
gene1930	1.89	NS	NC_004459.3	VV1_RS09535	WP_086016946.1	lactoylglutathione lyase
gene1933	NS	-1.75	NC_004459.3	VV1_RS09550	WP_011079914.1	hypothetical protein
gene1934	NS	1.27	NC_004459.3	VV1_RS09555	WP_011079915.1	iron-regulated protein
gene1938	NS	1.09	NC_004459.3	VV1_RS09575	WP_043920956.1	phosphatidylinositol kinase
gene1939	NS	1.08	NC_004459.3	VV1_RS09580	WP_011079919.1	SAVED domain-containing protein
gene1940	NS	1.52	NC_004459.3	VV1_RS09585	WP_011079920.1	hypothetical protein
gene1941	NS	1.45	NC_004459.3	VV1_RS09590	WP_043920957.1	nucleotidyltransferase
gene1965	NS	-2.75	NC_004459.3	VV1_RS09700	WP_011079944.1	DUF2846 domain-containing protein
gene1966	NS	-1.85	NC_004459.3	VV1_RS09705	WP_011079945.1	methyl-accepting chemotaxis protein
gene1978	NS	6.43	NC_004459.3	VV1_RS09760	WP_043920960.1	lipoprotein
gene1998	NS	1.77	NC_004459.3	VV1_RS09860	WP_011079976.1	SpoVR family protein
gene2000	NS	1.85	NC_004459.3	VV1_RS09870	WP_011079978.1	MULTISPECIES: PrkA family serine protein kinase
gene2004	NS	1.62	NC_004459.3	VV1_RS09890	WP_011079982.1	methyl-accepting chemotaxis

						protein
gene2008	NS	-1.59	NC_004459.3	NA	NA	NA
gene2009	NS	1.14	NC_004459.3	VV1_RS09915	WP_011079987.1	MULTISPECIES: histidine/lysine/arginine/ornithine ABC transporter ATP-binding protein HisP
gene2010	NS	1.54	NC_004459.3	VV1_RS09920	WP_013571389.1	MULTISPECIES: ABC transporter substrate-binding protein
gene2011	1.44	2.17	NC_004459.3	VV1_RS09925	WP_011079989.1	MULTISPECIES: ABC transporter permease subunit
gene2013	1.21	1.31	NC_004459.3	VV1_RS09935	WP_043920961.1	hypothetical protein
gene2015	NS	1.01	NC_004459.3	VV1_RS09945	WP_011079993.1	methyl-accepting chemotaxis protein
gene2020	NS	-4.83	NC_004459.3	VV1_RS09970	WP_011079998.1	porin
gene2030	NS	1.07	NC_004459.3	VV1_RS10020	WP_043921092.1	MULTISPECIES: glycine zipper 2TM domain-containing protein
gene2034	NS	-1.15	NC_004459.3	NA	NA	NA
gene2035	NS	-1.09	NC_004459.3	VV1_RS10040	WP_011080011.1	glucose-1-phosphate adenylyltransferase
gene2040	2.50	2.36	NC_004459.3	VV1_RS10065	WP_011080016.1	MULTISPECIES: DNA transformation protein
gene2043	NS	-1.45	NC_004459.3	VV1_RS10080	WP_011080019.1	MULTISPECIES: two-component system response regulator TorR
gene2050	NS	1.97	NC_004459.3	VV1_RS10115	WP_011080026.1	coniferyl aldehyde dehydrogenase
gene2051	NS	1.56	NC_004459.3	NA	NA	NA
gene2052	1.35	1.52	NC_004459.3	VV1_RS10125	WP_011080029.1	alpha-L-glutamate ligase-like protein
gene2053	1.31	1.36	NC_004459.3	VV1_RS10130	WP_011080030.1	gonadoliberin III
gene2054	1.27	1.51	NC_004459.3	VV1_RS10135	WP_011080031.1	ATP-dependent Zn protease
gene2064	**NS**	**-1.43**	**NC_004459.3**	**VV1_RS10180**	**WP_013571418.1**	**MULTISPECIES: cytochrome bd-I ubiquinol oxidase subunit CydA**
gene2065	**NS**	**-1.23**	**NC_004459.3**	**VV1_RS10185**	**WP_011080041.1**	**cytochrome d ubiquinol oxidase subunit II**
gene2066	**NS**	**-1.21**	**NC_004459.3**	**VV1_RS23165**	**WP_000270284.1**	**MULTISPECIES: cytochrome bd-I oxidase subunit CydX**
gene2067	NS	-1.19	NC_004459.3	VV1_RS10195	WP_011080042.1	cyd operon protein YbgE
gene2069	1.47	1.45	NC_004459.3	VV1_RS10205	WP_011080044.1	MULTISPECIES: protein TolQ
gene2070	1.62	1.84	NC_004459.3	VV1_RS10210	WP_011080045.1	MULTISPECIES: ExbD/TolR family protein
gene2071	1.46	1.95	NC_004459.3	VV1_RS10215	WP_011080046.1	MULTISPECIES: cell envelope integrity protein TolA
gene2082	NS	1.68	NC_004459.3	VV1_RS10270	WP_011080057.1	MULTISPECIES: L-alanine exporter AlaE
gene2086	NS	-1.14	NC_004459.3	VV1_RS10290	WP_011080060.1	ATP-binding protein
gene2088	NS	-1.02	NC_004459.3	NA	NA	NA
gene2094	NS	-1.86	NC_004459.3	VV1_RS10330	WP_011080068.1	hypothetical protein
gene2095	NS	-1.42	NC_004459.3	VV1_RS10335	WP_013571435.1	GNAT family acetyltransferase
gene2096	NS	-1.49	NC_004459.3	VV1_RS10340	WP_011080070.1	ATP-binding protein
gene2097	NS	-1.21	NC_004459.3	VV1_RS10345	WP_011080071.1	hypothetical protein
gene2098	NS	-1.23	NC_004459.3	VV1_RS10350	WP_011080072.1	nitrate reductase
gene2100	NS	-1.19	NC_004459.3	VV1_RS10360	WP_080553412.1	HAD family phosphatase

gene2102	NS	1.75	NC_004459.3	VV1_RS10370	WP_011080076.1	2-hydroxyacid dehydrogenase
gene2122	NS	1.49	NC_004459.3	VV1_RS10460	WP_011080096.1	ABC transporter substrate-binding protein
gene2123	NS	-1.87	NC_004459.3	VV1_RS10465	WP_011080097.1	MULTISPECIES: ABC transporter permease
gene2124	NS	-1.02	NC_004459.3	VV1_RS10470	WP_011080098.1	MULTISPECIES: ATP-binding cassette domain-containing protein
gene2126	NS	1.00	NC_004459.3	VV1_RS10480	WP_080553414.1	sensor domain-containing diguanylate cyclase
gene2130	-1.00	-1.79	NC_004459.3	VV1_RS10500	WP_011080104.1	Na+/H+ antiporter NhaB
gene2152	-1.34	-3.35	NC_004459.3	VV1_RS10610	WP_011080126.1	methyl-accepting chemotaxis protein
gene2157	NS	-1.18	NC_004459.3	VV1_RS10635	WP_011080131.1	septum formation initiator
gene2164	NS	1.48	NC_004459.3	VV1_RS10670	WP_011080137.1	MULTISPECIES: hypothetical protein
gene2165	1.83	2.59	NC_004459.3	VV1_RS10675	WP_011080138.1	YcjX family protein
gene2166	1.84	2.60	NC_004459.3	VV1_RS10680	WP_011080139.1	TIGR01620 family protein
gene2171	NS	-3.47	NC_004459.3	VV1_RS10705	WP_011080144.1	SLC13/DASS family transporter
gene2172	NS	-3.05	NC_004459.3	VV1_RS10710	WP_011080145.1	GHKL domain-containing protein
gene2173	NS	-4.37	NC_004459.3	VV1_RS10715	WP_011080146.1	MULTISPECIES: response regulator
gene2174	NS	-1.88	NC_004459.3	VV1_RS10720	WP_011080147.1	sensor domain-containing diguanylate cyclase
gene2182	4.39	5.09	NC_004459.3	VV1_RS10760	WP_011080155.1	DUF4382 domain-containing protein
gene2197	3.02	3.39	NC_004459.3	VV1_RS10835	WP_011080170.1	undecaprenyl-phosphate glucose phosphotransferase
gene2198	**2.71**	**2.81**	**NC_004459.3**	**VV1_RS10840**	**WP_011080171.1**	**capsular polysaccharide biosynthesis protein**
gene2199	**2.76**	**2.63**	**NC_004459.3**	**VV1_RS10845**	**WP_043920971.1**	**polysaccharide export protein**
gene2200	**2.52**	**2.83**	**NC_004459.3**	**VV1_RS10850**	**WP_011080173.1**	**capsular polysaccharide biosynthesis protein**
gene2201	2.63	2.77	NC_004459.3	VV1_RS10855	WP_011080174.1	capsular polysaccharide biosynthesis protein
gene2202	2.07	2.09	NC_004459.3	VV1_RS10860	WP_011080175.1	hypothetical protein
gene2203	1.88	2.48	NC_004459.3	VV1_RS10865	WP_011080176.1	glycosyltransferase
gene2204	2.06	2.68	NC_004459.3	VV1_RS10870	WP_011080177.1	glycosyltransferase family 1 protein
gene2205	1.49	2.43	NC_004459.3	VV1_RS10875	WP_086016921.1	glycosyltransferase family 1 protein
gene2206	1.79	2.16	NC_004459.3	VV1_RS10880	WP_011080179.1	hypothetical protein
gene2207	1.42	2.24	NC_004459.3	VV1_RS10885	WP_011080180.1	DUF4832 domain-containing protein
gene2210	NS	1.17	NC_004459.3	VV1_RS10900	WP_011080183.1	diguanylate cyclase
gene2222	1.33	NS	NC_004459.3	VV1_RS10965	WP_011080196.1	DUF1566 domain-containing protein
gene2226	**NS**	**4.54**	**NC_004459.3**	**VV1_RS10985**	**WP_011080200.1**	**pilus assembly protein CpaC**
gene2248	NS	-1.51	NC_004459.3	VV1_RS11090	WP_011080221.1	hypothetical protein
gene2257	NS	1.03	NC_004459.3	VV1_RS11135	WP_043920977.1	ATPase
gene2258	NS	1.51	NC_004459.3	VV1_RS11140	WP_011080231.1	hypothetical protein
gene2263	-1.08	-2.88	NC_004459.3	VV1_RS11165	WP_011080236.1	GGDEF domain-containing protein
gene2270	2.68	3.18	NC_004459.3	VV1_RS11200	WP_011080243.1	MULTISPECIES: phosphoribosylglycinamide formyltransferase
gene2276	2.73	3.38	NC_004459.3	VV1_RS11230	WP_026050560.1	glycine zipper 2TM domain-containing protein

gene2281	NS	-1.54	NC_004459.3	VV1_RS11255	WP_011080254.1	serine protease
gene2326	NS	-1.04	NC_004459.3	VV1_RS11390	WP_011080279.1	hypothetical protein
gene2341	NS	-1.29	NC_004459.3	VV1_RS11430	WP_043920987.1	hypothetical protein
gene2346	NS	-1.34	NC_004459.3	VV1_RS11455	WP_011080286.1	GNAT family N-acetyltransferase
gene2365	NS	-1.15	NC_004459.3	VV1_RS11510	WP_011080297.1	hypothetical protein
gene2366	NS	-1.13	NC_004459.3	VV1_RS11515	WP_043920994.1	MULTISPECIES: hypothetical protein
gene2408	NS	-1.12	NC_004459.3	VV1_RS23450	WP_011080314.1	hypothetical protein
gene2414	NS	-1.16	NC_004459.3	VV1_RS11635	WP_043921005.1	hypothetical protein
gene2421	NS	-1.27	NC_004459.3	VV1_RS11660	WP_080553440.1	hypothetical protein
gene2423	NS	-1.93	NC_004459.3	NA	NA	NA
gene2424	NS	-1.40	NC_004459.3	VV1_RS11665	WP_011080319.1	hypothetical protein
gene2425	NS	-1.40	NC_004459.3	VV1_RS11670	WP_011080320.1	MULTISPECIES: VOC family protein
gene2426	NS	-1.36	NC_004459.3	VV1_RS23480	WP_080553496.1	DUF3265 domain-containing protein
gene2428	NS	1.48	NC_004459.3	VV1_RS11680	WP_011080322.1	MULTISPECIES: DNA-binding protein
gene2429	NS	1.50	NC_004459.3	VV1_RS11685	WP_011080323.1	hypothetical protein
gene2430	NS	1.53	NC_004459.3	VV1_RS11690	WP_011080324.1	replication endonuclease
gene2441	NS	-1.27	NC_004459.3	VV1_RS11710	WP_011080328.1	class I SAM-dependent methyltransferase
gene2449	NS	-1.30	NC_004459.3	VV1_RS23540	WP_080553443.1	DUF3265 domain-containing protein
gene2451	NS	-1.15	NC_004459.3	NA	NA	NA
gene2453	NS	-1.08	NC_004459.3	VV1_RS11725	WP_043921010.1	MULTISPECIES: hypothetical protein
gene2454	NS	-1.05	NC_004459.3	VV1_RS23555	WP_080553444.1	DUF3265 domain-containing protein
gene2455	NS	-1.03	NC_004459.3	VV1_RS11730	WP_011080330.1	VOC family protein
gene2462	NS	-1.67	NC_004459.3	VV1_RS23570	WP_079856094.1	MULTISPECIES: DUF3265 domain-containing protein
gene2463	NS	-1.18	NC_004459.3	VV1_RS23575	WP_045596915.1	YceK/YidQ family lipoprotein
gene2468	NS	-1.30	NC_004459.3	VV1_RS11765	WP_011080336.1	hypothetical protein
gene2473	NS	-1.24	NC_004459.3	VV1_RS11770	WP_011080339.1	GNAT family N-acetyltransferase
gene2476	NS	-1.20	NC_004459.3	VV1_RS11780	WP_043921013.1	hypothetical protein
gene2480	NS	-1.00	NC_004459.3	NA	NA	NA
gene2490	NS	-1.20	NC_004459.3	VV1_RS11810	WP_011080347.1	hypothetical protein
gene2539	NS	-1.28	NC_004459.3	NA	NA	NA
gene2544	NS	-1.51	NC_004459.3	VV1_RS11940	WP_011080368.1	N-acetyltransferase
gene2546	NS	-1.97	NC_004459.3	VV1_RS23775	WP_080553457.1	DUF3265 domain-containing protein
gene2547	-1.16	-2.23	NC_004459.3	VV1_RS11945	WP_011080369.1	GTP pyrophosphokinase
gene2549	NS	-1.24	NC_004459.3	VV1_RS23790	WP_080553511.1	DUF3265 domain-containing protein
gene2550	NS	-1.12	NC_004459.3	VV1_RS11950	WP_043921021.1	hypothetical protein
gene2553	NS	-1.53	NC_004459.3	VV1_RS11960	WP_039465016.1	MULTISPECIES: aldehyde-activating protein
gene2568	NS	-1.42	NC_004459.3	VV1_RS12000	WP_011080377.1	hypothetical protein
gene2571	NS	-1.16	NC_004459.3	VV1_RS12005	WP_026050567.1	MULTISPECIES: hypothetical protein, partial
gene2575	NS	-1.64	NC_004459.3	VV1_RS12015	WP_043921023.1	hypothetical protein
gene2598	NS	-1.64	NC_004459.3	VV1_RS12070	WP_011080387.1	toll/interleukin-1 receptor domain-containing protein

gene2599	NS	-1.42	NC_004459.3	VV1_RS23890	WP_080553467.1	DUF3265 domain-containing protein
gene2608	NS	-1.48	NC_004459.3	VV1_RS12095	WP_043921030.1	hypothetical protein
gene2618	NS	-1.23	NC_004459.3	VV1_RS12130	WP_043921032.1	hypothetical protein
gene2660	NS	-1.43	NC_004459.3	VV1_RS12305	WP_039553740.1	HAMP domain-containing protein
gene2661	1.18	1.88	NC_004459.3	VV1_RS12310	WP_011080429.1	MULTISPECIES: potassium transporter KefA
gene2662	1.37	2.50	NC_004459.3	VV1_RS12315	WP_039555168.1	ATP-binding protein
gene2664	NS	1.62	NC_004459.3	VV1_RS12325	WP_011080432.1	HAMP domain-containing protein
gene2666	NS	-1.27	NC_004459.3	VV1_RS12335	WP_011080434.1	DUF3305 domain-containing protein
gene2667	NS	-1.05	NC_004459.3	VV1_RS12340	WP_011080435.1	DUF3306 domain-containing protein
gene2694	NS	-1.11	NC_004459.3	VV1_RS12475	WP_011080462.1	MULTISPECIES: hypothetical protein
gene2695	**-1.47**	**-2.24**	**NC_004459.3**	**VV1_RS12480**	**WP_011080463.1**	**MULTISPECIES: cytochrome-c oxidase, cbb3-type subunit III**
gene2696	**-1.52**	**-2.31**	**NC_004459.3**	**VV1_RS12485**	**WP_011080464.1**	**MULTISPECIES: CcoQ/FixQ family Cbb3-type cytochrome c oxidase assembly chaperone**
gene2697	**-1.60**	**-2.28**	**NC_004459.3**	**VV1_RS12490**	**WP_011080465.1**	**MULTISPECIES: cytochrome-c oxidase, cbb3-type subunit II**
gene2698	**-1.58**	**-2.36**	**NC_004459.3**	**VV1_RS12495**	**WP_011080466.1**	**MULTISPECIES: cytochrome-c oxidase, cbb3-type subunit I**
gene2703	NS	-1.36	NC_004459.3	VV1_RS12515	WP_011080469.1	HDOD domain-containing protein
gene2709	NS	1.65	NC_004459.3	VV1_RS12545	WP_011080475.1	MULTISPECIES: ribosome modulation factor
gene2714	1.65	1.67	NC_004459.3	VV1_RS12570	WP_011080480.1	cell division protein ZapC
gene2719	NS	1.87	NC_004459.3	VV1_RS12590	WP_011080485.1	MULTISPECIES: hypothetical protein
gene2720	NS	1.38	NC_004459.3	VV1_RS12595	WP_011080486.1	carboxy terminal-processing peptidase
gene2728	NS	1.76	NC_004459.3	VV1_RS12635	WP_011080494.1	MULTISPECIES: hypothetical protein
gene2729	NS	1.51	NC_004459.3	VV1_RS12640	WP_011080495.1	MULTISPECIES: DUF4442 domain-containing protein
gene2739	NS	1.51	NC_004459.3	VV1_RS12690	WP_011080505.1	hybrid sensor histidine kinase/response regulator
gene2740	NS	1.34	NC_004459.3	VV1_RS12695	WP_011080506.1	sigma-54-dependent Fis family transcriptional regulator
gene2741	NS	1.97	NC_004459.3	VV1_RS12700	WP_011080507.1	glycosyltransferase family 1 protein
gene2756	NS	1.12	NC_004459.3	VV1_RS12770	WP_011080521.1	MULTISPECIES: hypothetical protein
MSTRG.771.1	NS	1.91	NC_004459.3	NA	NA	NA
gene2757	NS	1.39	NC_004459.3	VV1_RS12775	WP_011080523.1	hypothetical protein
gene2758	NS	1.05	NC_004459.3	VV1_RS12780	WP_011080524.1	MULTISPECIES: phosphogluconate dehydrogenase (NADP(+)-dependent, decarboxylating)
gene2759	NS	1.09	NC_004459.3	VV1_RS12785	WP_011080525.1	6-phosphogluconolactonase
gene2760	NS	1.36	NC_004459.3	VV1_RS12790	WP_011080526.1	glucose-6-phosphate dehydrogenase
gene2763	1.00	1.14	NC_004459.3	VV1_RS12805	WP_011080529.1	MULTISPECIES: hypothetical protein
gene2769	NS	1.44	NC_004459.3	VV1_RS12835	WP_011080535.1	chromosome segregation

						ATPase
gene2770	NS	1.66	NC_004459.3	VV1_RS24445	WP_011150215.1	hypothetical protein
gene2777	NS	-1.69	NC_004459.3	VV1_RS12875	WP_039553533.1	MULTISPECIES: ferredoxin-type protein NapG
gene2787	-1.06	-1.52	NC_004459.3	VV1_RS12925	WP_011080552.1	methyl-accepting chemotaxis protein
gene2803	1.75	3.46	NC_004459.3	VV1_RS13005	WP_011080567.1	hypothetical protein
gene2811	1.80	2.02	NC_004459.3	VV1_RS13045	WP_011080575.1	tripartite tricarboxylate transporter substrate binding protein
gene2812	NS	2.76	NC_004459.3	VV1_RS13050	WP_011080576.1	tripartite tricarboxylate transporter TctB family protein
gene2813	NS	1.90	NC_004459.3	VV1_RS13055	WP_011080577.1	MULTISPECIES: tripartite tricarboxylate transporter permease
gene2819	NS	1.46	NC_004459.3	VV1_RS13085	WP_043921038.1	GlyGly-CTERM sorting domain-containing protein
gene2822	-1.05	-1.46	NC_004459.3	VV1_RS13100	WP_011080586.1	HD domain-containing protein
gene2839	NS	1.39	NC_004459.3	VV1_RS13185	WP_011080601.1	MULTISPECIES: maleylacetoacetate isomerase
gene2840	1.39	1.33	NC_004459.3	VV1_RS13190	WP_011080602.1	MULTISPECIES: FAA hydrolase family protein
gene2841	NS	2.15	NC_004459.3	VV1_RS13195	WP_011080603.1	homogentisate 1,2-dioxygenase
gene2842	2.27	3.46	NC_004459.3	VV1_RS13200	WP_011080604.1	4-hydroxyphenylpyruvate dioxygenase
gene2843	1.71	NS	NC_004459.3	VV1_RS13205	WP_011080605.1	M20/M25/M40 family metallo-hydrolase
gene2869	NS	-1.90	NC_004459.3	VV1_RS13335	WP_011080631.1	MULTISPECIES: hypothetical protein
gene2870	NS	-1.46	NC_004459.3	VV1_RS13340	WP_011080632.1	MULTISPECIES: phospholipase A
gene2873	1.34	1.17	NC_004459.3	VV1_RS13355	WP_086016956.1	SanA protein
gene2877	1.82	3.12	NC_004459.3	VV1_RS13375	WP_011080639.1	cysteine desulfurase-like protein
gene2878	NS	5.04	NC_004459.3	VV1_RS13380	WP_011080640.1	agglutination protein
gene2884	NS	-1.53	NC_004459.3	VV1_RS13410	WP_011080646.1	ATPase
gene2885	NS	-1.19	NC_004459.3	NA	NA	NA
gene2893	NS	-2.54	NC_004459.3	VV1_RS13455	WP_011080655.1	MULTISPECIES: iron-containing alcohol dehydrogenase
gene2897	NS	1.14	NC_004459.3	VV1_RS13475	WP_011080659.1	acyl-CoA dehydrogenase
gene2909	1.58	2.44	NC_004459.3	VV1_RS13535	WP_011080671.1	MULTISPECIES: membrane protein
gene2910	1.15	2.15	NC_004459.3	VV1_RS13540	WP_011080672.1	DNA-binding response regulator
gene2911	1.05	1.83	NC_004459.3	VV1_RS13545	WP_080553476.1	ATP-binding protein
gene2916	NS	2.82	NC_004459.3	VV1_RS13570	WP_011080679.1	hypothetical protein
gene2918	3.09	2.93	NC_004459.3	VV1_RS13580	WP_011080681.1	MULTISPECIES: META domain-containing protein
gene2923	NS	-1.41	NC_004459.3	VV1_RS13605	WP_011080686.1	nitric oxide reductase transcriptional regulator NorR
gene2928	NS	-1.06	NC_004459.3	VV1_RS13630	WP_043921044.1	methyl-accepting chemotaxis protein
gene2953	NS	-1.49	NC_004459.3	VV1_RS13755	WP_011080716.1	trimethylamine-N-oxide reductase TorA
gene2954	NS	-1.75	NC_004459.3	VV1_RS13760	WP_011080717.1	MULTISPECIES: pentaheme c-type cytochrome TorC, partial
gene2955	NS	-1.74	NC_004459.3	VV1_RS13765	WP_011080718.1	MULTISPECIES: trimethylamine N-oxide reductase system protein TorE

gene2957	-1.30	NS	NC_004459.3	VV1_RS13775	WP_011080720.1	formate transporter FocA
gene2958	-1.28	-2.16	NC_004459.3	VV1_RS13780	WP_011080721.1	DNA repair ATPase
gene2976	NS	1.03	NC_004459.3	VV1_RS13870	WP_011080739.1	histidinol dehydrogenase
gene2977	1.14	1.19	NC_004459.3	VV1_RS13875	WP_011080740.1	ATP phosphoribosyltransferase
gene2998	NS	1.14	NC_004459.3	VV1_RS13970	WP_011080760.1	8-amino-7-oxononanoate synthase
gene3000	NS	1.32	NC_004459.3	VV1_RS13980	WP_039553314.1	MULTISPECIES: adenosylmethionine--8-amino-7-oxononanoate transaminase
gene3004	NS	1.03	NC_004459.3	VV1_RS14000	WP_011080766.1	DNA translocase FtsK
gene3012	NS	-1.04	NC_004459.3	VV1_RS14040	WP_011080776.1	MULTISPECIES: hypothetical protein
gene3015	1.49	1.35	NC_004459.3	VV1_RS14055	WP_011080779.1	MULTISPECIES: DUF882 domain-containing protein
gene3017	NS	1.04	NC_004459.3	VV1_RS14065	WP_011080781.1	MULTISPECIES: DUF1513 domain-containing protein
gene3019	NS	2.36	NC_004459.3	VV1_RS14075	WP_011080783.1	thiol oxidoreductase
gene3020	NS	3.04	NC_004459.3	NA	NA	NA
gene3047	5.82	6.20	NC_004459.3	VV1_RS14210	WP_011080810.1	hypothetical protein
gene3048	NS	-1.19	NC_004459.3	VV1_RS14215	WP_011080811.1	MULTISPECIES: PTS glucose transporter subunit IIBC
gene3068	1.49	2.01	NC_004459.3	VV1_RS14315	WP_011080831.1	AsmA family protein
gene3077	NS	-2.22	NC_004459.3	VV1_RS14360	WP_011080838.1	MULTISPECIES: cytochrome c nitrite reductase subunit NrfD
gene3078	NS	-2.36	NC_004459.3	NA	NA	NA
gene3079	NS	-1.95	NC_004459.3	VV1_RS14370	WP_043921054.1	MULTISPECIES: cytochrome c nitrite reductase pentaheme subunit
gene3080	NS	-2.44	NC_004459.3	VV1_RS14375	WP_011080841.1	MULTISPECIES: ammonia-forming cytochrome c nitrite reductase subunit c552
gene3086	NS	-1.21	NC_004459.3	VV1_RS14405	WP_011080846.1	MULTISPECIES: ribonucleoside-diphosphate reductase subunit alpha
gene3088	NS	-1.00	NC_004459.3	VV1_RS14415	WP_011080848.1	MULTISPECIES: (Fe-S)-binding protein
gene3091	NS	-1.13	NC_004459.3	VV1_RS14430	WP_011080851.1	transcriptional regulator
gene3120	NS	-1.21	NC_004459.3	VV1_RS14570	WP_011080874.1	hypothetical protein
gene3125	NS	1.12	NC_004459.3	VV1_RS14595	WP_011080879.1	glutathione S-transferase family protein
gene3135	NS	1.06	NC_004459.3	VV1_RS14645	WP_039553216.1	MULTISPECIES: GTP 3\',8-cyclase MoaA
gene3151	NS	-1.09	NC_004459.3	VV1_RS14725	WP_011080904.1	MULTISPECIES: OmpA family protein
gene3157	NS	-2.49	NC_004459.3	VV1_RS14755	WP_011080910.1	hypothetical protein
gene3162	1.71	2.54	NC_004459.3	VV1_RS14780	WP_011080915.1	prepilin-type N-terminal cleavage/methylation domain-containing protein
gene3164	NS	-3.16	NC_004459.3	VV1_RS14790	WP_011080917.1	MULTISPECIES: Na+/H+ antiporter NhaC
gene3165	2.33	1.47	NC_004459.3	VV1_RS14795	WP_080553481.1	chemotaxis protein
gene3180	NS	1.64	NC_004459.3	VV1_RS14870	WP_086016957.1	NADPH-dependent 2,4-dienoyl-CoA reductase
gene3188	NS	-1.77	NC_004459.3	VV1_RS14910	WP_011080940.1	chemotaxis protein
gene3196	2.38	2.04	NC_004459.3	VV1_RS14945	WP_011080947.1	hypothetical protein
gene3197	NS	1.13	NC_004459.3	VV1_RS14950	WP_011080948.1	acyl-CoA thioesterase II
gene3200	NS	1.23	NC_004459.3	VV1_RS14965	WP_011080951.1	PLP-dependent cysteine synthase family protein

gene3227	NS	1.09	NC_004459.3	NA	NA	NA
gene3245	NS	2.42	NC_004459.3	VV1_RS15190	WP_011080993.1	response regulator
gene3246	NS	1.65	NC_004459.3	VV1_RS15195	WP_011080994.1	response regulator
gene3256	4.30	4.84	NC_004460.2	VV1_RS15250	WP_011081003.1	hypothetical protein
gene3257	5.49	7.53	NC_004460.2	VV1_RS15255	WP_043921112.1	hypothetical protein
gene3266	NS	-3.20	NC_004460.2	VV1_RS15300	WP_011081013.1	L-threonine dehydrogenase
gene3277	NS	-1.31	NC_004460.2	VV1_RS15355	WP_011081024.1	hypothetical protein
gene3284	NS	1.09	NC_004460.2	VV1_RS15390	WP_011081032.1	aspartate aminotransferase family protein
gene3285	NS	2.60	NC_004460.2	VV1_RS15395	WP_011081033.1	N-acetylglucosamine-binding protein GbpA
gene3286	NS	1.03	NC_004460.2	VV1_RS15400	WP_011081034.1	EamA family transporter RarD
gene3296	NS	1.49	NC_004460.2	VV1_RS15450	WP_011081044.1	MULTISPECIES: DUF3763 domain-containing protein
gene3312	NS	2.24	NC_004460.2	VV1_RS15525	WP_011081060.1	PAS domain S-box protein
gene3313	NS	1.44	NC_004460.2	VV1_RS15530	WP_011081061.1	STAS domain-containing protein
gene3316	NS	1.04	NC_004460.2	VV1_RS15545	WP_011081064.1	serine phosphatase
gene3317	NS	1.01	NC_004460.2	VV1_RS15550	WP_011081065.1	hybrid sensor histidine kinase/response regulator
MSTRG.1272.1	NS	1.12	NC_004460.2	NA	NA	NA
gene3325	NS	1.94	NC_004460.2	VV1_RS15590	WP_011081072.1	hypothetical protein
gene3333	**NS**	**1.57**	**NC_004460.2**	**VV1_RS15630**	**WP_011081080.1**	**pilus assembly protein**
gene3337	4.14	3.06	NC_004460.2	VV1_RS15650	WP_011081084.1	pyruvate dehydrogenase
gene3341	NS	-1.84	NC_004460.2	VV1_RS15670	WP_011081086.1	MULTISPECIES: hypothetical protein
gene3346	NS	-1.16	NC_004460.2	VV1_RS15695	WP_015727796.1	MULTISPECIES: sugar O-acetyltransferase
gene3347	NS	-1.17	NC_004460.2	VV1_RS15700	WP_011081092.1	NUDIX domain-containing protein
gene3348	NS	-2.34	NC_004460.2	VV1_RS15705	WP_011081093.1	nucleotide pyrophosphohydrolase
gene3350	**1.18**	**2.22**	**NC_004460.2**	**VV1_RS15710**	**WP_043921119.1**	**iron ABC transporter**
gene3354	NS	1.62	NC_004460.2	VV1_RS15730	WP_011081099.1	MULTISPECIES: membrane protein
gene3356	1.55	2.82	NC_004460.2	VV1_RS15740	WP_011081101.1	3-hydroxy-3-methylglutaryl-CoA reductase
gene3357	3.49	2.93	NC_004460.2	VV1_RS15745	WP_011081102.1	MULTISPECIES: hypothetical protein
gene3358	4.28	3.58	NC_004460.2	VV1_RS15750	WP_017790802.1	MULTISPECIES: hypothetical protein
gene3377	NS	-2.12	NC_004460.2	VV1_RS15845	WP_011081122.1	MULTISPECIES: hypothetical protein
gene3387	1.12	NS	NC_004460.2	VV1_RS15895	WP_011081132.1	LysR family transcriptional regulator
gene3396	1.43	1.25	NC_004460.2	VV1_RS15940	WP_011081141.1	LysR family transcriptional regulator
gene3400	NS	1.29	NC_004460.2	VV1_RS15960	WP_011081145.1	hypothetical protein
gene3407	NS	2.38	NC_004460.2	VV1_RS15995	WP_011081151.1	HlyD family secretion protein
gene3420	NS	-1.00	NC_004460.2	VV1_RS16060	WP_011081164.1	deoxycytidylate deaminase
gene3440	NS	1.01	NC_004460.2	VV1_RS16160	WP_011152050.1	MULTISPECIES: EAL domain-containing protein
gene3448	1.07	2.57	NC_004460.2	VV1_RS16200	WP_011081192.1	chitinase
gene3449	NS	1.37	NC_004460.2	VV1_RS16205	WP_011081193.1	glucose-1-phosphate adenylyltransferase
gene3458	1.24	1.30	NC_004460.2	VV1_RS16245	WP_011081201.1	DUF479 domain-containing protein

gene3460	NS	-1.05	NC_004460.2	VV1_RS16255	WP_011152067.1	EAL domain-containing protein
gene3462	NS	1.43	NC_004460.2	VV1_RS16265	WP_011081205.1	cytochrome b
gene3464	NS	-1.53	NC_004460.2	VV1_RS16275	WP_011081207.1	formate transporter FocA
gene3468	NS	1.55	NC_004460.2	VV1_RS16295	WP_043921125.1	bifunctional metallophosphatase/5\'-nucleotidase
gene3475	NS	-1.54	NC_004460.2	VV1_RS16335	WP_011081218.1	Fe-S type, tartrate/fumarate subfamily hydro-lyase subunit alpha
gene3492	NS	-1.58	NC_004460.2	VV1_RS16420	WP_011081235.1	trans-2-enoyl-CoA reductase family protein
gene3502	NS	1.12	NC_004460.2	NA	NA	NA
gene3506	NS	1.02	NC_004460.2	VV1_RS16490	WP_011081249.1	D-alanine--D-alanine ligase
gene3511	NS	1.59	NC_004460.2	VV1_RS16515	WP_011081254.1	hypothetical protein
gene3512	NS	1.58	NC_004460.2	VV1_RS16520	WP_011081255.1	hypothetical protein
gene3513	NS	1.58	NC_004460.2	VV1_RS16525	WP_011081256.1	hypothetical protein
gene3514	NS	1.49	NC_004460.2	VV1_RS16530	WP_011081257.1	hypothetical protein
gene3515	NS	1.51	NC_004460.2	VV1_RS16535	WP_052298474.1	hypothetical protein
gene3516	NS	1.58	NC_004460.2	VV1_RS16540	WP_011081259.1	hypothetical protein
gene3517	NS	1.51	NC_004460.2	VV1_RS16545	WP_011081260.1	hypothetical protein
gene3518	NS	1.55	NC_004460.2	VV1_RS16550	WP_011081261.1	hypothetical protein
gene3519	NS	1.63	NC_004460.2	VV1_RS16555	WP_011081262.1	hypothetical protein
gene3520	NS	1.62	NC_004460.2	VV1_RS16560	WP_011081263.1	hypothetical protein
gene3521	NS	1.17	NC_004460.2	VV1_RS16565	WP_043921129.1	DUF87 domain-containing protein
gene3522	NS	1.34	NC_004460.2	VV1_RS16570	WP_011081265.1	hypothetical protein
gene3524	NS	1.22	NC_004460.2	VV1_RS16580	WP_011081267.1	hypothetical protein
gene3525	NS	1.56	NC_004460.2	VV1_RS16585	WP_011081268.1	hypothetical protein
gene3526	NS	1.34	NC_004460.2	VV1_RS16590	WP_011081269.1	hypothetical protein
gene3527	NS	1.34	NC_004460.2	VV1_RS16595	WP_011081270.1	hypothetical protein
gene3528	NS	1.46	NC_004460.2	VV1_RS16600	WP_011081271.1	hypothetical protein
gene3532	NS	1.38	NC_004460.2	VV1_RS16615	WP_011081275.1	DUF2861 family protein
gene3533	1.01	1.89	NC_004460.2	VV1_RS16620	WP_011081276.1	MULTISPECIES: DNA-binding response regulator
gene3534	NS	1.51	NC_004460.2	VV1_RS16625	WP_040110783.1	MULTISPECIES: DUF3404 domain-containing protein
gene3549	3.56	5.04	NC_004460.2	VV1_RS16700	WP_011081292.1	hypothetical protein
gene3554	NS	-2.94	NC_004460.2	VV1_RS16725	WP_011081297.1	anaerobic ribonucleoside-triphosphate reductase-activating protein
gene3555	NS	-4.10	NC_004460.2	VV1_RS16730	WP_011081298.1	anaerobic ribonucleoside-triphosphate reductase
gene3572	-1.53	NS	NC_004460.2	VV1_RS16815	WP_011081315.1	DUF2986 domain-containing protein
gene3573	NS	-1.70	NC_004460.2	VV1_RS16820	WP_011081316.1	methyl-accepting chemotaxis protein
gene3574	NS	-3.21	NC_004460.2	VV1_RS16825	WP_011081317.1	MULTISPECIES: anaerobic C4-dicarboxylate transporter
gene3579	NS	1.20	NC_004460.2	VV1_RS16850	WP_011081322.1	flagellar motor protein MotA
gene3580	NS	1.30	NC_004460.2	VV1_RS16855	WP_011081323.1	DUF3450 domain-containing protein
gene3587	NS	-3.80	NC_004460.2	VV1_RS16890	WP_011081330.1	PAS domain-containing protein
gene3596	1.21	2.72	NC_004460.2	VV1_RS16935	WP_026130873.1	IclR family transcriptional regulator

gene3602	NS	1.02	NC_004460.2	VV1_RS16965	WP_011081344.1	diguanylate cyclase
gene3610	NS	1.37	NC_004460.2	VV1_RS17005	WP_011081352.1	dienelactone hydrolase family protein
gene3615	NS	-2.37	NC_004460.2	VV1_RS17030	WP_011081357.1	alpha-amylase
gene3617	NS	-1.31	NC_004460.2	VV1_RS17040	WP_011081359.1	methyl-accepting chemotaxis protein
gene3618	NS	2.47	NC_004460.2	VV1_RS17045	WP_080553528.1	cytolysin secretion protein
gene3619	NS	2.61	NC_004460.2	VV1_RS17050	WP_011081361.1	cytolysin
gene3620	NS	-1.42	NC_004460.2	VV1_RS17055	WP_011081362.1	glycerol-3-phosphate transporter
gene3621	NS	-1.18	NC_004460.2	VV1_RS17060	WP_011081363.1	glycerophosphodiester phosphodiesterase
gene3623	NS	1.05	NC_004460.2	VV1_RS17070	WP_011081365.1	TldD/PmbA family protein
gene3625	NS	-2.67	NC_004460.2	VV1_RS17080	WP_015728001.1	MULTISPECIES: anaerobic C4-dicarboxylate transporter DcuC
gene3659	1.81	NS	NC_004460.2	VV1_RS17250	WP_011081401.1	glutathione S-transferase
gene3666	NS	1.93	NC_004460.2	VV1_RS17285	WP_011081408.1	MULTISPECIES: 4a-hydroxytetrahydrobiopterin dehydratase
gene3667	NS	2.18	NC_004460.2	VV1_RS17290	WP_011081409.1	MULTISPECIES: phenylalanine 4-monooxygenase
gene3668	NS	1.97	NC_004460.2	VV1_RS17295	WP_080553531.1	acetoacetate--CoA ligase
gene3673	NS	-1.48	NC_004460.2	VV1_RS17320	WP_011081415.1	isoprenoid biosynthesis protein ElbB
gene3675	NS	1.39	NC_004460.2	VV1_RS17330	WP_011081417.1	two-component sensor histidine kinase
gene3679	NS	2.52	NC_004460.2	VV1_RS17350	WP_011081422.1	MULTISPECIES: pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha
gene3680	1.88	2.69	NC_004460.2	VV1_RS17355	WP_011081423.1	alpha-ketoacid dehydrogenase subunit beta
gene3681	NS	4.91	NC_004460.2	VV1_RS17360	WP_086016960.1	2-oxo acid dehydrogenase subunit E2
gene3683	NS	2.53	NC_004460.2	VV1_RS17370	WP_011081426.1	electron transfer flavoprotein subunit beta/FixA family protein
gene3684	NS	1.05	NC_004460.2	VV1_RS17375	WP_011081427.1	electron transfer flavoprotein-ubiquinone oxidoreductase
gene3685	NS	-1.25	NC_004460.2	VV1_RS17380	WP_011081428.1	methyl-accepting chemotaxis protein
gene3686	NS	-1.54	NC_004460.2	VV1_RS17385	WP_011081429.1	alanine racemase
gene3693	NS	1.08	NC_004460.2	VV1_RS17420	WP_043921138.1	MULTISPECIES: amino acid ABC transporter substrate-binding protein
gene3696	NS	1.14	NC_004460.2	VV1_RS17435	WP_011081439.1	SDR family NAD(P)-dependent oxidoreductase
gene3697	NS	1.63	NC_004460.2	VV1_RS17440	WP_011081440.1	3-hydroxyisobutyrate dehydrogenase
gene3698	NS	1.74	NC_004460.2	VV1_RS17445	WP_011081441.1	enoyl-CoA hydratase/isomerase family protein
gene3699	NS	2.24	NC_004460.2	VV1_RS17450	WP_011081442.1	enoyl-CoA hydratase
gene3701	NS	1.85	NC_004460.2	VV1_RS17460	WP_011081444.1	methylmalonate-semialdehyde dehydrogenase (CoA acylating)
gene3702	NS	2.96	NC_004460.2	VV1_RS17465	WP_011081445.1	acetyl-CoA C-acyltransferase
gene3703	NS	2.91	NC_004460.2	VV1_RS17470	WP_011081446.1	MULTISPECIES: MerR family DNA-binding transcriptional regulator

gene3708	NS	1.43	NC_004460.2	VV1_RS17495	WP_011081451.1	alkyl hydroperoxide reductase subunit C
gene3709	NS	1.26	NC_004460.2	VV1_RS17500	WP_011081452.1	alkyl hydroperoxide reductase subunit F
gene3716	NS	1.30	NC_004460.2	VV1_RS17535	WP_011081458.1	MULTISPECIES: DUF3012 domain-containing protein
gene3725	NS	-1.95	NC_004460.2	VV1_RS24025	WP_011081466.1	alternative ribosome-rescue factor A
gene3730	NS	-1.37	NC_004460.2	VV1_RS17600	WP_011081471.1	OmpA family protein
gene3738	NS	-2.57	NC_004460.2	VV1_RS17640	WP_011081479.1	peptidase T
gene3743	NS	-3.38	NC_004460.2	VV1_RS24030	WP_011081484.1	hypothetical protein
gene3744	-1.15	-3.38	NC_004460.2	VV1_RS17665	WP_011081485.1	VWA domain-containing protein
gene3753	NS	1.03	NC_004460.2	VV1_RS17705	WP_011081494.1	EAL domain-containing protein
gene3754	NS	-1.32	NC_004460.2	VV1_RS17710	WP_011081495.1	MULTISPECIES: chitinase
gene3761	NS	1.25	NC_004460.2	VV1_RS17745	WP_011081502.1	3-deoxy-7-phosphoheptulonate synthase
gene3762	NS	-1.74	NC_004460.2	VV1_RS17750	WP_011081503.1	MULTISPECIES: OmpA family protein
gene3772	NS	1.60	NC_004460.2	VV1_RS17800	WP_011081512.1	phospho-sugar mutase
gene3781	NS	2.73	NC_004460.2	VV1_RS17845	WP_011081521.1	porin
gene3810	1.29	NS	NC_004460.2	VV1_RS17985	WP_011081550.1	VOC family protein
gene3814	1.24	NS	NC_004460.2	VV1_RS18005	WP_011081554.1	hypothetical protein
gene3826	NS	-1.06	NC_004460.2	VV1_RS18065	WP_011081567.1	hypothetical protein
gene3827	NS	-1.22	NC_004460.2	VV1_RS18070	WP_011081568.1	hypothetical protein
gene3852	1.05	1.77	NC_004460.2	VV1_RS18195	WP_043921148.1	GTPase
gene3875	NS	2.35	NC_004460.2	VV1_RS18300	WP_011081616.1	MFS transporter
gene3876	NS	1.30	NC_004460.2	VV1_RS18305	WP_011081617.1	HEXXH motif domain-containing protein
gene3882	NS	2.52	NC_004460.2	VV1_RS18335	WP_011081623.1	sulfate ABC transporter permease subunit CysW
gene3885	1.31	1.72	NC_004460.2	VV1_RS18350	WP_011081626.1	hypothetical protein
gene3891	NS	-1.06	NC_004460.2	VV1_RS18375	WP_043921155.1	GTP-binding protein
gene3905	NS	-1.49	NC_004460.2	VV1_RS18440	WP_011081645.1	membrane protein
gene3916	NS	-1.02	NC_004460.2	VV1_RS18490	WP_011081656.1	MULTISPECIES: cytochrome c
gene3926	NS	-3.13	NC_004460.2	VV1_RS18535	WP_011081664.1	N-acetylmannosamine kinase
gene3935	NS	1.43	NC_004460.2	VV1_RS18580	WP_043921161.1	hypothetical protein
gene3941	NS	1.25	NC_004460.2	VV1_RS18605	WP_011081677.1	DMT family transporter
gene3945	NS	-2.94	NC_004460.2	NA	NA	NA
gene3950	NS	1.66	NC_004460.2	VV1_RS18650	WP_011081686.1	MATE family efflux transporter
gene3951	NS	1.28	NC_004460.2	VV1_RS18655	WP_011081687.1	hybrid sensor histidine kinase/response regulator
gene3955	3.43	4.09	NC_004460.2	VV1_RS18675	WP_011081691.1	MULTISPECIES: hypothetical protein
gene3956	1.69	3.04	NC_004460.2	VV1_RS18680	WP_086016971.1	LysR family transcriptional regulator
gene3958	1.41	1.77	NC_004460.2	VV1_RS18690	WP_011081694.1	MULTISPECIES: peroxiredoxin
gene3968	-1.29	-1.29	NC_004460.2	VV1_RS18740	WP_015728181.1	MULTISPECIES: DNA-binding response regulator
gene3969	NS	-1.69	NC_004460.2	VV1_RS18745	WP_011081704.1	hypothetical protein
gene3974	NS	-1.00	NC_004460.2	VV1_RS18770	WP_080553538.1	AraC family transcriptional regulator
gene3979	NS	1.36	NC_004460.2	VV1_RS18795	WP_011081714.1	ABC transporter ATP-binding protein

gene3991	NS	-1.23	NC_004460.2	VV1_RS18855	WP_011081726.1	MULTISPECIES: porin family protein
gene3997	NS	1.43	NC_004460.2	VV1_RS18885	WP_011081730.1	EAL domain-containing protein
gene3999	2.94	2.88	NC_004460.2	VV1_RS18895	WP_040110995.1	phenylalanine--tRNA ligase
gene4000	5.81	6.43	NC_004460.2	VV1_RS18900	WP_011081733.1	copper resistance protein NlpE
gene4009	NS	1.07	NC_004460.2	VV1_RS18945	WP_011081742.1	amino acid ABC transporter
gene4015	**NS**	**1.10**	**NC_004460.2**	**VV1_RS18975**	**WP_011081748.1**	**peptide synthetase**
gene4016	**1.03**	**2.51**	**NC_004460.2**	**VV1_RS18980**	**WP_052298478.1**	**amino acid adenylation domain-containing protein**
gene4017	NS	2.34	NC_004460.2	VV1_RS18985	WP_011081750.1	3-deoxy-7-phosphoheptulonate synthase
gene4021	**NS**	**1.15**	**NC_004460.2**	**VV1_RS19005**	**WP_011081754.1**	**NADPH-dependent ferric siderophore reductase**
gene4022	**NS**	**2.08**	**NC_004460.2**	**VV1_RS19010**	**WP_011081755.1**	**isochorismatase**
gene4023	NS	2.03	NC_004460.2	VV1_RS19015	WP_011081756.1	MULTISPECIES: isochorismate lyase
gene4024	NS	2.07	NC_004460.2	VV1_RS19020	WP_011081757.1	(2,3-dihydroxybenzoyl)adenylate synthase
gene4026	NS	1.47	NC_004460.2	VV1_RS19030	WP_011081759.1	siderophore ABC transporter substrate-binding protein
MSTRG.1900.1	NS	1.23	NC_004460.2	NA	NA	NA
gene4043	NS	-2.35	NC_004460.2	VV1_RS19110	WP_011081774.1	hypothetical protein
gene4047	NS	-1.21	NC_004460.2	VV1_RS19130	WP_040111024.1	PTS sugar transporter subunit IIB
gene4059	3.30	4.29	NC_004460.2	VV1_RS19190	WP_080553540.1	PhzF family phenazine biosynthesis protein
gene4060	1.09	1.22	NC_004460.2	VV1_RS19195	WP_011081791.1	LuxR family transcriptional regulator
gene4064	2.99	4.21	NC_004460.2	VV1_RS19215	WP_043921259.1	LysR family transcriptional regulator
gene4077	NS	1.15	NC_004460.2	VV1_RS19285	WP_043921262.1	hypothetical protein
gene4078	NS	-4.18	NC_004460.2	VV1_RS19290	WP_011081809.1	M20 family peptidase
gene4079	NS	-4.08	NC_004460.2	VV1_RS19295	WP_011081810.1	MULTISPECIES: membrane protein
gene4080	NS	-3.39	NC_004460.2	VV1_RS19300	WP_011081811.1	MULTISPECIES: membrane protein
gene4081	NS	1.32	NC_004460.2	VV1_RS19305	WP_011081812.1	MULTISPECIES: DUF302 domain-containing protein
gene4095	NS	-1.02	NC_004460.2	VV1_RS19375	WP_011081825.1	MULTISPECIES: DNA-binding transcriptional regulator KdgR
gene4105	NS	-1.50	NC_004460.2	VV1_RS19425	WP_011081835.1	hypothetical protein
gene4110	NS	1.96	NC_004460.2	VV1_RS19445	WP_011081839.1	hybrid sensor histidine kinase/response regulator
gene4120	NS	-1.31	NC_004460.2	VV1_RS19495	WP_011081848.1	MULTISPECIES: GNAT family N-acetyltransferase
gene4128	1.28	2.04	NC_004460.2	VV1_RS19530	WP_011081855.1	GlpM family protein
gene4139	2.10	2.48	NC_004460.2	VV1_RS19585	WP_011081866.1	DUF2057 domain-containing protein
gene4140	NS	2.19	NC_004460.2	VV1_RS19590	WP_011081867.1	DMT family transporter
gene4153	NS	2.57	NC_004460.2	VV1_RS19655	WP_086016964.1	hemagglutinin
gene4158	NS	1.40	NC_004460.2	VV1_RS19680	WP_086016965.1	methyl-accepting chemotaxis protein
gene4159	NS	2.11	NC_004460.2	VV1_RS19685	WP_011081886.1	response regulator
gene4160	NS	2.04	NC_004460.2	VV1_RS19690	WP_011081887.1	two-component system response regulator
gene4161	6.02	6.96	NC_004460.2	VV1_RS19695	WP_015728355.1	DUF3316 domain-containing protein

MSTRG.2028.1	4.47	6.54	NC_004460.2	NA	NA	NA
MSTRG.2029.1	4.96	6.50	NC_004460.2	NA	NA	NA
gene4162	NS	-3.06	NC_004460.2	VV1_RS19700	WP_011081889.1	MULTISPECIES: L-2-hydroxyglutarate oxidase
gene4169	NS	2.60	NC_004460.2	VV1_RS19735	WP_011081896.1	MULTISPECIES: DUF3316 domain-containing protein
gene4170	NS	2.18	NC_004460.2	VV1_RS19740	WP_011081897.1	MULTISPECIES: DNA-binding response regulator
gene4171	1.11	1.81	NC_004460.2	VV1_RS19745	WP_052298480.1	HAMP domain-containing protein
gene4175	NS	1.60	NC_004460.2	VV1_RS19765	WP_011081902.1	methionine synthase
gene4184	NS	1.04	NC_004460.2	VV1_RS19810	WP_011081911.1	ABC transporter substrate-binding protein
gene4189	NS	1.49	NC_004460.2	VV1_RS19835	WP_011081915.1	siderophore ferric iron reductase
gene4190	NS	4.29	NC_004460.2	VV1_RS19840	WP_011081916.1	ATP-binding cassette domain-containing protein
gene4191	NS	3.54	NC_004460.2	VV1_RS19845	WP_011081917.1	iron-siderophore ABC transporter substrate-binding protein
gene4192	**NS**	**1.94**	**NC_004460.2**	**VV1_RS19850**	**WP_011081918.1**	**Fe(3+)-hydroxamate ABC transporter permease FhuB**
gene4221	NS	1.37	NC_004460.2	VV1_RS19990	WP_015728397.1	methyl-accepting chemotaxis protein
gene4222	NS	-1.21	NC_004460.2	VV1_RS19995	WP_043921185.1	MULTISPECIES: hypothetical protein
gene4257	NS	-1.09	NC_004460.2	VV1_RS20165	WP_011081978.1	MULTISPECIES: L-ascorbate 6-phosphate lactonase
gene4260	NS	-2.13	NC_004460.2	VV1_RS20180	WP_011081980.1	PTS ascorbate-specific subunit IIBC
gene4266	NS	1.95	NC_004460.2	VV1_RS20210	WP_040111258.1	hypothetical protein
gene4267	1.10	1.21	NC_004460.2	VV1_RS20215	WP_086016972.1	amidinotransferase
gene4270	NS	-1.70	NC_004460.2	VV1_RS20230	WP_011081990.1	1,3-beta-galactosyl-N-acetylhexosamine phosphorylase
gene4271	NS	-1.32	NC_004460.2	VV1_RS20235	WP_011081991.1	MULTISPECIES: glycosyhydrolase
gene4284	NS	-1.52	NC_004460.2	VV1_RS20295	WP_011082004.1	DUF2264 domain-containing protein
gene4292	2.34	1.93	NC_004460.2	VV1_RS24480	WP_015728451.1	hypothetical protein
gene4293	2.56	1.55	NC_004460.2	VV1_RS24125	WP_011152832.1	MULTISPECIES: hypothetical protein
MSTRG.2152.1	3.89	4.72	NC_004460.2	NA	NA	NA
gene4294	NS	1.30	NC_004460.2	VV1_RS20335	WP_011082013.1	sodium/proline symporter PutP
gene4296	NS	1.56	NC_004460.2	VV1_RS20345	WP_011082015.1	bifunctional proline dehydrogenase/L-glutamate gamma-semialdehyde dehydrogenase PutA
gene4298	NS	-1.11	NC_004460.2	VV1_RS20355	WP_011082017.1	helix-turn-helix transcriptional regulator
gene4306	1.98	NS	NC_004460.2	VV1_RS20395	WP_011082025.1	flavodoxin family protein
gene4324	NS	-2.51	NC_004460.2	VV1_RS20485	WP_011082043.1	hypothetical protein
gene4333	NS	-1.03	NC_004460.2	VV1_RS20525	WP_011082051.1	DUF342 domain-containing protein
gene4336	NS	1.16	NC_004460.2	VV1_RS20540	WP_011082054.1	chemotaxis protein
gene4337	NS	1.39	NC_004460.2	VV1_RS20545	WP_080553544.1	chemotaxis response regulator protein-glutamate methylesterase
gene4341	NS	1.17	NC_004460.2	VV1_RS20565	WP_011082059.1	chemotaxis protein CheW

gene4343	NS	1.18	NC_004460.2	VV1_RS20575	WP_011082061.1	chemotaxis protein CheA
gene4372	1.02	2.33	NC_004460.2	VV1_RS20715	WP_011082091.1	sodium/glutamate symporter
gene4390	NS	2.01	NC_004460.2	VV1_RS20805	WP_011082109.1	chitinase
gene4395	NS	1.01	NC_004460.2	VV1_RS20830	WP_011082114.1	GGDEF domain-containing protein
gene4396	NS	-1.75	NC_004460.2	VV1_RS20835	WP_011082115.1	hypothetical protein
gene4414	1.21	NS	NC_004460.2	VV1_RS20920	WP_043921199.1	ribosome small subunit-dependent GTPase A
gene4425	NS	1.58	NC_004460.2	VV1_RS20975	WP_011082142.1	methyl-accepting chemotaxis protein
gene4427	NS	1.59	NC_004460.2	VV1_RS20985	WP_011082144.1	LysE family translocator
gene4430	NS	1.35	NC_004460.2	VV1_RS21000	WP_043921203.1	spindolin
gene4448	NS	1.82	NC_004460.2	VV1_RS21090	WP_011082164.1	amino acid ABC transporter
gene4464	NS	-1.25	NC_004460.2	VV1_RS21165	WP_011082178.1	MULTISPECIES: ABC transporter substrate-binding protein
gene4473	NS	-1.18	NC_004460.2	VV1_RS21210	WP_011082187.1	maltoporin
gene4477	NS	-1.02	NC_004460.2	VV1_RS21225	WP_011082191.1	MATE family efflux transporter
gene4506	NS	1.73	NC_004460.2	VV1_RS21370	WP_043921211.1	AraC family transcriptional regulator
gene4521	NS	-1.36	NC_004460.2	VV1_RS21445	WP_011082235.1	MULTISPECIES: PTS fructose transporter subunit IIB
gene4529	NS	-2.82	NC_004460.2	VV1_RS21485	WP_011082243.1	formate dehydrogenase subunit alpha
gene4530	2.10	2.19	NC_004460.2	VV1_RS21490	WP_043921213.1	glycoside hydrolase family 2 protein
gene4531	NS	-1.14	NC_004460.2	VV1_RS21495	WP_011082245.1	LysR family transcriptional regulator
gene4536	2.88	3.03	NC_004460.2	VV1_RS21520	WP_011082250.1	penicillin-binding protein 2
gene4545	NS	1.37	NC_004460.2	VV1_RS21565	WP_011082259.1	hypothetical protein
gene4547	1.24	NS	NC_004460.2	VV1_RS21575	WP_011082261.1	LysR family transcriptional regulator
gene4553	NS	-1.68	NC_004460.2	VV1_RS21605	WP_011082267.1	MULTISPECIES: hypothetical protein
MSTRG.2396.1	NS	-1.03	NC_004460.2	NA	NA	NA
gene4559	NS	-1.98	NC_004460.2	VV1_RS21635	WP_011082273.1	LuxR family transcriptional regulator
gene4560	NS	-1.13	NC_004460.2	VV1_RS21640	WP_011082274.1	hypothetical protein
gene4563	1.17	NS	NC_004460.2	VV1_RS21655	WP_011082277.1	peptidase M3
gene4568	NS	1.69	NC_004460.2	VV1_RS21680	WP_011082282.1	amino acid ABC transporter substrate-binding protein
gene4571	1.56	1.70	NC_004460.2	VV1_RS21695	WP_011151663.1	MULTISPECIES: YccF domain-containing protein
gene4584	NS	1.09	NC_004460.2	VV1_RS21755	WP_011082298.1	hypothetical protein
gene4587	NS	-1.44	NC_004460.2	VV1_RS21770	WP_011082301.1	3,4-dihydroxy-2-butanone-4-phosphate synthase
gene4592	NS	1.79	NC_004460.2	VV1_RS21795	WP_011082307.1	phosphatase PAP2 family protein
gene4615	NS	-1.27	NC_004460.2	VV1_RS21910	WP_086016897.1	DUF3103 family protein
gene4619	NS	1.59	NC_004460.2	VV1_RS21930	WP_011082326.1	HD domain-containing protein
gene4622	1.83	2.66	NC_004460.2	VV1_RS21945	WP_011082329.1	MULTISPECIES: soluble cytochrome b562
gene4633	NS	1.79	NC_004460.2	VV1_RS21995	WP_011082338.1	hypothetical protein
gene4640	NS	-1.22	NC_004460.2	VV1_RS22030	WP_011082345.1	hypothetical protein
gene4641	NS	-1.34	NC_004460.2	VV1_RS22035	WP_011082346.1	MULTISPECIES: hypothetical protein
gene4656	2.15	2.48	NC_004460.2	VV1_RS22105	WP_011082360.1	DNA-binding response

						regulator
gene4661	NS	1.17	NC_004460.2	VV1_RS22130	WP_080553552.1	hypothetical protein
gene4668	NS	1.58	NC_004460.2	VV1_RS22165	WP_080553553.1	carbonic anhydrase
gene4670	2.09	2.23	NC_004460.2	VV1_RS22175	WP_011082374.1	hypothetical protein
gene4679	-1.32	NS	NC_004460.2	VV1_RS22220	WP_011082383.1	MULTISPECIES: ECF-type riboflavin transporter substrate-binding protein
gene4690	1.11	1.31	NC_004460.2	VV1_RS22275	WP_011082394.1	MULTISPECIES: membrane protein
gene4702	NS	-2.25	NC_004460.2	VV1_RS22335	WP_011151766.1	MULTISPECIES: phosphate ABC transporter ATP-binding protein
gene4704	NS	1.01	NC_004460.2	VV1_RS22345	WP_011082408.1	protein BatD
gene4705	NS	1.02	NC_004460.2	VV1_RS22350	WP_011082409.1	VWA domain-containing protein
gene4710	NS	-1.35	NC_004460.2	VV1_RS22375	WP_011082414.1	MULTISPECIES: methyl-accepting chemotaxis protein
gene4712	NS	1.34	NC_004460.2	VV1_RS22385	WP_080553554.1	helix-turn-helix transcriptional regulator
gene4733	NS	1.01	NC_004460.2	VV1_RS22485	WP_011082436.1	DMT family transporter
gene4735	1.22	1.65	NC_004460.2	VV1_RS22495	WP_011082438.1	MULTISPECIES: DUF2817 domain-containing protein
gene4736	1.72	1.84	NC_004460.2	VV1_RS22500	WP_011082439.1	DUF808 domain-containing protein
gene4755	NS	2.23	NC_004460.2	VV1_RS22595	WP_080553556.1	heme anaerobic degradation radical SAM methyltransferase ChuW/HutW
gene4756	1.38	2.04	NC_004460.2	VV1_RS22600	WP_011082459.1	heme utilization cystosolic carrier protein HutX
gene4757	**1.02**	**2.22**	**NC_004460.2**	**VV1_RS22605**	**WP_011082460.1**	**MULTISPECIES: heme utilization protein HutZ**
gene4764	NS	-1.33	NC_004460.2	VV1_RS22640	WP_011082467.1	methyl-accepting chemotaxis protein
gene4774	NS	1.38	NC_004460.2	VV1_RS22685	WP_043921227.1	hypothetical protein
gene4781	3.44	3.30	NC_004460.2	VV1_RS22720	WP_011082483.1	DUF3541 domain-containing protein
gene4813	NS	-1.27	NC_004460.2	VV1_RS22880	WP_011082516.1	MFS transporter
gene4819	NS	-1.38	NC_004460.2	VV1_RS22910	WP_043921231.1	chitinase
gene4825	NS	1.06	NC_004460.2	VV1_RS22945	WP_011082527.1	MULTISPECIES: choline ABC transporter substrate-binding protein

21

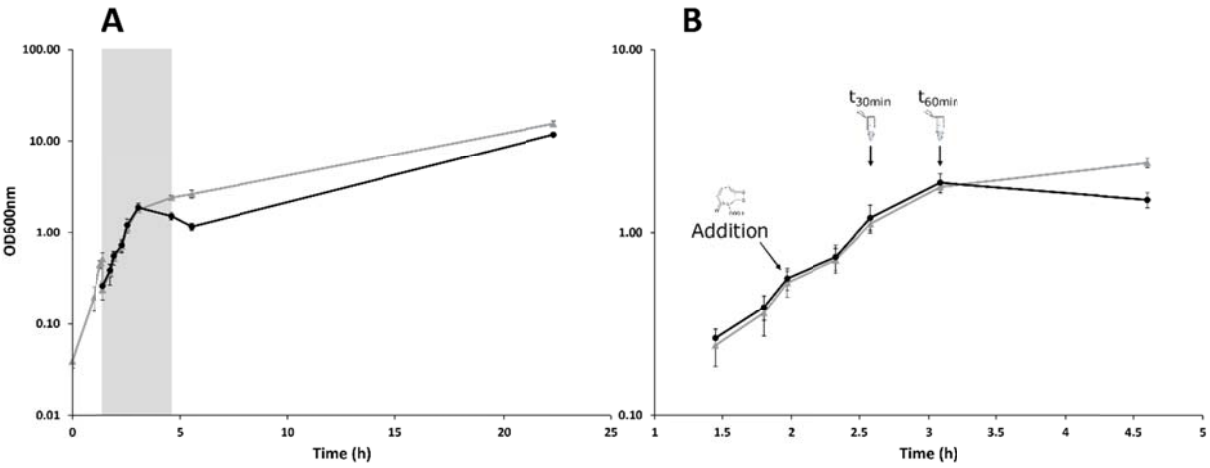


Figure S1: Growth of *Vibrio vulnificus* CMCP6 exposed to sub-lethal levels of tropodithietic acid (TDA). Black circles/line depict samples exposed to TDA, while grey triangles/line depict controls exposed to solvent (DMSO). A) growth curve of CMCP6 cultures, depicted as optical density at 600 nm (OD_{600nm}) over time (h). B) depicts a subset of data points from the late exponential phase (OD_{600nm} = 0.5 ± 0.02, grey area in graph A), when TDA was added. Samples for RNA extraction were taken upon 30 min and 60 min exposure. Please note the difference in the scale of the y-axis between plots. Each data point is the average of three biological replicates, error bars represent the standard deviation.

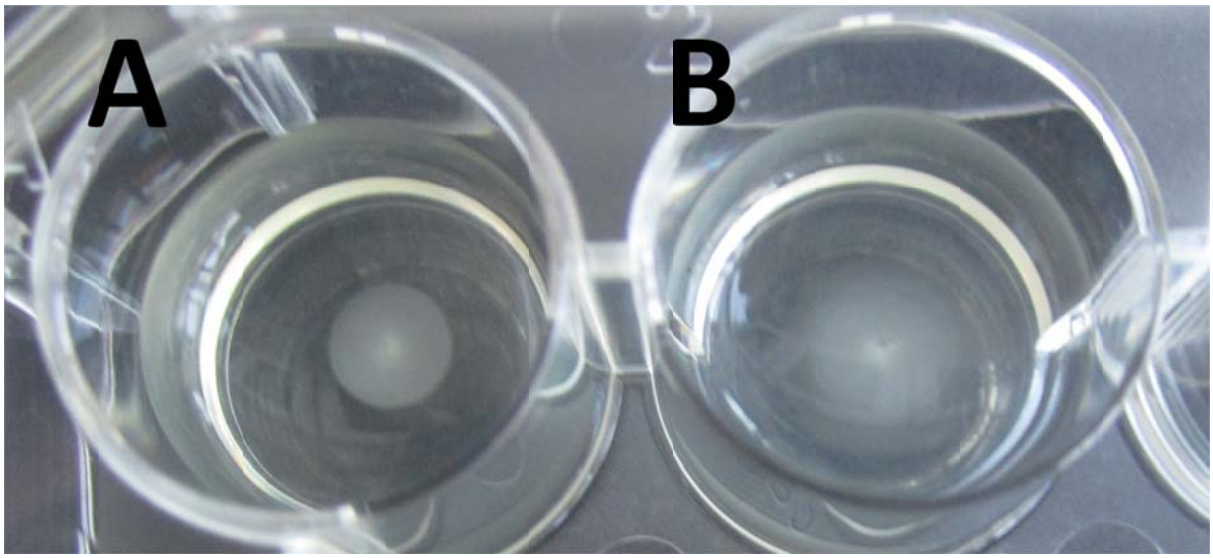


Figure S2: Illustration of motility assay of *Vibrio vulnificus* CMCP6. A) shows motility of cells exposed to sub-lethal levels of tropodithietic acid (TDA) – zone diameter 6 mm, 1.68 × 10^8 CFU mL^{-1} ; B) shows control exposed to solvent (DMSO) – zone diameter 9 mm, 2.59 × 10^8 CFU mL^{-1}.

Paper 4

Dittmann, K.K., Rasmussen, B.B., Melchiorsen, J., Sonnenschein, E.C., Gram, L. & Bentzon-Tilia, M. (2019).

Roseobacter probiotics affect lower-trophic level microbiomes in marine aquaculture

Manuscript in preparation

Roseobacter probiotics affect lower-trophic level microbiomes in marine aquaculture

Karen K. Dittmann, Bastian Barker Rasmussen, Jette Melchiorsen, Eva C. Sonnenschein, Lone Gram, Mikkel Bentzon-Tilia#

Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads Bldg. 221, DK-2800 Kgs. Lyngby.

#Address correspondence to Mikkel Bentzon-Tilia: mibti@bio.dtu.dk

Running title: Effect of probiotics on aquaculture microbiotas.

Keywords: 16S rRNA amplicon sequencing, taxonomics, Aquaculture, microbiome, Microbial community composition, *Phaeobacter*, Roseobacters, tropodithietic acid

Abstract

As a sustainable strategy for prevention of bacterial infections in aquaculture, the *Phaeobacter* genus has been developed as probiotics in aquaculture. Its antagonistic effect against common fish pathogens is predominantly due to the production of the antibiotic tropodithietic acid (TDA) and strains have repeatedly been isolated from aquaculture environments. Despite many *in vitro* trials targeting pathogens, little is known about the impact on the commensal microbiota in these systems. Hence, the purpose of this study was to investigate how the addition of a TDA-producing *P. inhibens* affects microbiotas at different trophic levels found in aquacultures. We used 16S rRNA gene taxonomics to characterize the bacterial diversity associated with microalgae (*Tetraselmis suecica*), live-feed copepod nauplii (*Acartia tonsa*), and turbot (*Scophthalmus maximus*) eggs/larvae. We observed that microbial communities at all trophic levels were highly dynamic. The addition of the probiotic bacterium caused significant changes to the structure of the microbial communities associated with lower trophic level organisms i.e. microalga and copepods. Members of the Rhodobacterales order were indigenous to all three microbiotas but in varying abundances. The addition of the probiotic *P. inhibens* decreased the abundance of closely related taxa from the *Roseobacter* group in the copepod and turbot microbiotas, while they were unaffected in the microalgal microbiota. *Vibrio* spp., comprising common fish pathogens, were kept at a stable low level, though they were not eliminated in the turbot microbiome. Altogether, the inherent roseobacters and addition of probiotics suppressed the proliferation of vibrios, while causing minor changes to the commensal microbiota.

Importance

This work is an essential part of the risk assessment of the application of roseobacters as probiotics in aquaculture; providing an understanding of the impact of TDA-producing *Phaeobacter inhibens* on the commensal bacteria related to aquaculture live-feed and fish larvae. Furthermore, these characterizations elucidate the composition and diversity of microbiotas related to aquaculture-relevant microalga, copepods, and turbot larvae, which have been scarcely studied using Next-Generation Sequencing technologies.

Introduction

Aquaculture is a rapidly growing industry due to the increasing demand for high-quality protein to feed the growing world population (1, 2). By 2030, fish production from aquaculture is expected to reach 109 million tons (2) and along with the United Nation's Sustainability Development Goals, there is an increasing focus on sustainable production of food; ending hunger while protecting wild fish populations (3).

One of the major bottlenecks in fish production is disease outbreaks. About 55 % of infections are caused by pathogenic bacteria (4) that are typically introduced with supply water (5), broodstock, humans, and plankton feed (6). Particularly, vibrios, such as *Vibrio splendidus, V. harveyi, V. vulnificus,* and *V. anguillarum*, are of major economical concern to aquacultures because they can cause severe fish diseases and mortalities (7, 8). This is predominantly an issue related to marine fish larvae where several species are reared in nutrient-rich greenwater tanks, feeding on live-feed, such as *Artemia*, rotifers, and copepods (6, 9, 10). Pathogenic *Vibrio* spp. are naturally associated with zooplankton (11–14) and they can also easily proliferate in cultures of phytoplankton, which are used as feed for the live-feed (9). Thus, live-feed organisms can act as vectors of opportunistic pathogenic vibrios.

Major crashes of fish larval populations are most likely due to detrimental interactions (dysbiosis) in the microbial communities associated with the fish larvae (15–18). Microbial communities respond and adapt quickly to environmental changes. Oxygen levels, pH, and salinity are strong drivers of microbial community composition in aquatic environments (19–24). In aquacultures, these parameters are controlled in the rearing tanks (1) to minimize stress on the fish caused by environmental imbalance. Other factors such as the levels of nutrients and accumulated toxic compounds can also impact the balance. To reduce the risk of self-pollution, recirculating

aquaculture systems (RAS) often use mechanic filters and biofilters to clean the rearing water for dissolved organic matter and accumulated inorganic nitrogen and phosphorus (1). In the event of an imbalance in the system, e.g. rapid increase in nutrient levels and temperature increase, the result can be proliferation of opportunistic pathogens. The pathogens have traditionally been controlled by disinfection of the rearing tanks (25, 26), sterilization of the rearing water (27), and deployment of antibiotics (28, 29) and in recent years by vaccination of the fish (30, 31), but the latter does not work on fish larvae due to their underdeveloped immune systems (30). Thus, sustainable alternatives to antibiotics are sought, given the severe impact on the commensal microbiome and the increasing problem of spreading antimicrobial resistance genes (29, 30, 32). One proposed alternative is the use of probiotics; live microorganisms that provide a health benefit to the host when administered in adequate amounts (33). The potential application of probiotics in aquaculture as prophylactic and acute treatment of disease outbreaks has been studied for decades; most of them focusing on the gut microbiome of the farmed animal (34–36). Currently, the majority of commercially available probiotics for aquaculture are based on mono- or mixed cultures of Firmicutes (3), which have been successful in humans and livestock, though not adapted to the diverse aquatic environments. Proteobacteria such as *Shewanella* spp. and tropodithietic acid (TDA)-producing members of the *Roseobacter* group have been studied extensively for their bioactivity and probiotic potential (3, 37–39) as an alternative solution of marine origin.

TDA-producers are often found in microbiomes of marine eukaryotes including micro- and macroalgae (40, 41), zooplankton (42), sponges (43) and molluscs (39, 44, 45). Mutualistic interactions between the microalga *Emiliania huxleyi* and *Phaeobacter inhibens* have been proposed due to the production of TDA and algal-growth promoting auxins (40, 46), though this relation can turn parasitic, when *P. inhibens* responds to algal break-down products and accelerates the lysis process by production of algaecides known as roseobacticides (47). TDA is a

bactericidal antibiotic against a wide range of both Gram-negative and Gram-positive bacteria (48). In low concentration, it can act as a signaling molecule affecting global gene regulation and behavior including motility, biofilm formation, and secondary metabolite production (49). The antibiotic mode of action is highly complex and still not fully understood, though the compound is most likely disrupting the proton motive force by changing the membrane potential (50, 51). Resistance towards TDA does not develop easily (48, 52), but tolerance of non-TDA producing bacteria has been observed among isolates from deep sea sponges (43). Furthermore, the presence of TDA-producers has varying impact on marine microbiomes depending on the composition and complexity of the existing community (53). While some community members are unaffected, *Vibrio* spp. and *Pseudoalteromonas* spp. diminish in the presence of *P. inhibens* (53). Thus, individual tolerance or cross-protective mechanisms may result in microbiome resilience to perturbations caused by TDA, while potential pathogens are kept down.

Phaeobacter spp. have been isolated from multiple aquacultures (44, 54, 55), which indicates that they are already an inherent part of the microbiome in some farms. Several studies have demonstrated their antagonistic effect against fish pathogens in live-feed cultures (9, 55, 56) without noticeable adverse effect on the eukaryote (9, 10). Most importantly, addition of 10^6 to 10^7 CFU mL^{-1} can decrease mortality of turbot and cod larvae when challenged with vibrios (9, 57, 58). The selective impact on microbiomes along with the lack of resistance development despite their global occurrence in microbiomes, including aquaculture microbiomes, highlight the applicability of *Phaeobacter* as probiotic. However, perturbations with probiotic levels of *Phaeobacter* could potentially cause imbalance and thereby give rise to proliferation of other pathogens than vibrios. Furthermore, little is currently known about aquaculture microbiomes at the different trophic levels and hence, the purpose of this study was to investigate how the addition of a TDA-producing *P. inhibens* affects microbiotas related to different trophic levels found in aquaculture systems.

Results

The impact of probiotic *P. inhibens* strain DSM 17395 on the microbiotas of aquaculture-relevant, marine microalga, copepods, and fish eggs/larvae was assessed by sequencing 16S rRNA gene V4 region amplicons and analyzing their taxonomic composition and diversity over 4 days; each co-culture as well as a control cultures without addition of the probiotic was sampled four times: at 0 h (T_0), 24 h (T_{24}), 48 h (T_{48}), and 96 h (T_{96}). The co-culture experiment with turbot was initiated with eggs, which all hatched within 48 hours of incubation; up to 2 % of the eggs were hatched at time point 24 h. Amplicon Sequence Variants (ASVs) containing the added *P. inhibens* were identified and removed from the data sets in the subsequent analysis to assess the potential changes in the inherent background microbiota.

Effects of probiotic treatment on microbial community composition

The community of the microalga *T. suecica* was dominated by bacteria from the phyla Proteobacteria, particularly members of the Rhodobacterales order, and Bacteroidetes, particularly members of the Flavobacteriales order (Figure 1). Other observed orders above 2 % relative abundance included Alteromonadales, Burkholderiales, Caulobacterales, Rhizobiales, Sphingomonadales, Phycisphaerales, and Cytophagales. Burkholderiales were only present in the initial microbiomes (T_0), while Rhizobiales appeared after 96 hours of incubation. The communities were stable and no obvious changes occurred due to probiotic treatment at the order level. Hence, incubation time was the main driver of the observed changes in community composition at this trophic level.

140 The *A. tonsa* bacterial community composition was dominated by Proteobacteria,
141 particularly members of the orders Alteromonadales and Oceanospirillales (Figure 2). Members of
142 the Rhodobacterales, Rhodospirillales, and Flavobacteriales orders were also present in all samples,
143 though in lower abundance. Desulfobacterales only occurred in the initial microbiota (T_0), while
144 Caulobacterales turned up in the microbiota following 96 hours of incubation. The addition of *P.*
145 *inhibens* decreased the abundance of Rhodobacterales and Rhodospirillales. Furthermore,
146 Alteromonadales increased initially (T_{24}) in the probiotic group, though their dominance decreased
147 over time. Hence, at this trophic level, both time and the probiotic treatment affected the
148 composition of the bacterial community.

149 The turbot egg and larval microbiotas were dominated by Proteobacteria, particularly
150 Gammaproteobacteria order such as members of the orders Alteromonadales and Vibrionales
151 (Figure 3). Vibrionales were most prominent in the initial egg microbiome (T_0; relative abundance
152 46.2 % to 46.9 %), though their relative abundance decreased to 14.3 % to 19.2 % after 24 hour
153 incubation and remained at the same level throughout the experiment. Concurrently, the relative
154 abundance of Alteromonadales and Rhodobacterales increased in abundance after 24 hours of
155 incubation. Both Rhodobacterales and Oceanospirillales increased in abundance while
156 Alteromonadales decreased over time. Pseudomonadales occurred in the microbiota after 48 hour
157 incubation and remained throughout the experiment. The bacterial community associated with
158 turbot eggs/larvae receiving probiotic treatment did not contain members of the Rhodobacterales
159 order (relative abundance above 2 %). Altogether, the biggest shift occurred within the first 24
160 hours of the experiment (establishment phase) and the bacterial community was stable throughout
161 the monitored time. The presence of *P. inhibens* decreased the abundance of other Rhodobacterales
162 bacteria, but otherwise the community was mainly affected by incubation time.

163 **Impact of probiotic treatment on bacterial microbiota richness and diversity**

The richness and diversity of the bacterial communities associated with the eukaryotic organisms were constant regardless of trophic level (Figure 4). The estimated ASV (amplicon sequence variant) richness (Chao1) values of the microalgal microbiota ranged from 126 to 166 in the controls and 132 to 173 in the cultures exposed to the probiotic (Figure 4A). The richness of the untreated copepod microbiota was initially 179 to 225 ASVs, though it dropped to 154 to 157 after 24 hours and remained at this level throughout the monitoring period (Chao1; 133 to 182) (Figure 4B). The probiotic-treated group followed the same trend; the richness of the initial microbiota (T_0) was 132 to 153 ASVs, followed by a decrease to 110 to 126 (T_{24}) and an increase to 132 to 157 ASVs over the remaining 72 hours (T_{96}). A slight effect of probiotic treatment was observed in this microbiome as the estimated richness was lower in treated copepods as compared to the controls at all time points. The turbot egg microbiota richness was initially 119 to 162 ASVs (T_0) (Figure 4C). From time point 24 hours to 96 hours, both treatment groups increased richness from 122 to 154 ASVs to 172 to 199 ASVs, respectively. Altogether, these data indicate that the richness is relatively low regardless of the trophic level and treatment.

Similar patterns were observed with respect to the diversity (Shannon diversity index). The microalgal microbiota diversity remained stable for the untreated controls (Shannon index 3.39 to 3.51) and cultures treated with the probiotic (Shannon index 3.35 to 3.43) throughout the experiment (Figure 4D). The diversity of the copepod microbiota was initially at the same level as the microalgal microbiota (Shannon index 3.42 to 3.43), though dropped to a Shannon index between 2.92 and 2.99 within 24 hours (Figure 4E). The diversity increased to the initial level after 96 hours incubation. A similar pattern was observed for the copepod cultures receiving probiotics (Figure 4F); the initial Shannon diversity index was 2.98 to 3.04, which dropped to a range of 2.61 to 2.76 and increased to the final level of the untreated controls (3.41 to 3.48). The initial turbot egg microbiota diversity was lower than the microalgal and copepod microbiota diversity (Shannon 2.33

188 to 2.45 at T_0), though increased steadily to a Shannon index between 3.38 and 3.54 (both controls
189 and probiotics treated) upon incubation for 96 hours. Altogether, these observations demonstrate
190 that probiotic *P. inhibens* has little to no impact on the richness and diversity of the microbiotas
191 associated with microalgae, copepods, and fish larvae.

192 **Impact of probiotic treatment on community structure**

193 Unconstrained ordinations – i.e. Principal Coordinate Analysis (PCoA) - on Bray-
194 Curtis distances were used to assess the impact of *P. inhibens* on community structure of the
195 microbiotas associated with the three aquaculture-related eukaryotes (Figure 5). The community
196 structure shifted during incubation time for all three microbial communities, regardless of treatment.
197 The microalgal microbial community structures treated with probiotics were significantly different
198 from the untreated controls (PERMANOVA; $p = 0.001$) (Figure 5A). This was also observed in the
199 copepod associated microbiota (PERMANOVA; $p = 0.001$) (Figure 5B). However, the turbot larval
200 microbial community structure was not significantly affected by the presence of *P. inhibens*
201 (PERMANOVA; $p = 0.279$) (Figure 5C). Hence, incubation was the major driver of the microbial
202 community structure and the impact of the probiotic treatment depends on the trophic layer at hand;
203 the biggest impact occurs at the lower tropic levels, while the fish larval community structure is
204 unaffected.

205 **Probiotic impact on specific taxa**

206 At the order level, the probiotic *P. inhibens* DSM 17395 decreased the abundance of
207 Rhodobacterales in two of the assessed microbiotas (Figure 2, Figure 3), while other effects of the
208 treatment were minor. Therefore, differences at ASV level (100 % sequence similarity, no
209 clustering) were investigated to elucidate which of the most abundant members were affected. No
210 major impact on individual taxa was observed in the microbiota associated with *T. suecica* due to

211 treatment (Figure 6). In the *A. tonsa* microbiota, the most abundant *Halomonas* sp. were slightly
212 lower in abundance in the probiotic treated samples, but still dominating (Figure 7). Members of the
213 Rhodobacteraceae family, such as *Ruegeria* sp. and *Celeribacter* sp., decreased in the presence of
214 *P. inhibens*, which is in line with the observations in the community composition analysis (Figure
215 2). Members of the Saccharospirillaceae family and *Hyphomonas* spp. were initially lower in
216 abundance in both treatment groups, but increased over time. In contrast to the microalgal and
217 copepod microbiotas, the samples from fish eggs and larvae clustered according to time rather than
218 treatment (Figure 8). No major changes were observed due to treatment, but changes over time were
219 observed, confirming the PCoA (Figure 5C). Some *Colwellia* sp. ASVs disappeared with time,
220 while others increased in abundance. Other Alteromonadales bacteria such as *Psychrobium* sp. and
221 *Alteromonas* sp. increased over time. *Vibrio* spp. were highly abundant in the initial microbiota
222 (T_0), though decreased as a function of incubation time. Altogether, the occurring changes in the
223 presence of *P. inhibens* were unique to the eukaryotic host and the largest changes were observed in
224 the copepod microbiota.

225 **Discussion**

226 Characterizing what a healthy microbiome is, determining the course of disease at the
227 bacterial population level, and assessing the impact of measures for disease control, such as
228 probiotics, is of crucial importance to increase sustainable aquaculture production without
229 increasing the risk of economic losses due to disease. *P. inhibens* has potential as probiotic in
230 aquaculture given efficient killing of common pathogens (9, 55, 56) and protection of vibrio-
231 challenged fish larvae (9, 57, 58) without noticeable adverse effect on the live-feed or the larvae (9,
232 10). Their natural association with eukaryotes (39, 44, 45, 53, 59–61) and aquaculture systems (54,
233 57) along with their efficient killing of opportunistic pathogens and no resistance development are
234 some of the key features highlighting their applicability in the aquaculture industry. However, it is

currently not known how probiotic concentrations of *P. inhibens* influence and shape the structure the commensal microbiota of aquaculture-related eukaryotes. The results of this study suggest that the microbial communities of all trophic levels in aquaculture are highly dynamic, in all cases changing over time. The impact of adding *P. inhibens* to the system is overall minor and highly dependent on the commensal microbiota with greater impact on the bacterial communities of the lower trophic levels.

Three eukaryotes – *T. suecica*, *A. tonsa*, and turbot – were selected to represent different trophic levels – feed for live-feed, live-feed, and reared fish – found in aquacultures. Several studies have been conducted on microalgal microbiomes and how roseobacters interact with these unicellular eukaryotes (47, 62, 63). Despite *T. suecica* being widely used and produced in hatcheries, the microbial community associated with this microalga is not well studied. Biondi *et al.* (64) observed that the microbiota of *T. suecica* was dominated by Proteobacteria – particularly members of the *Roseobacter* group, but also Rhizobiales and Bacteroidetes (Flavobacteriales). This is in concordance with the community composition observed in the *T. suecica* cultures assessed in this study as well as for another aquaculture-related microalgal genus, *Nannochloropsis* (65). In addition, we also observed a relatively high abundance of Phycisphaerales (Planctomycetes). The microbiome of *A. tonsa* was also dominated by Proteobacteria, particularly Gammaproteobacteria in this study. This has previously also been observed in copepods from the North Atlantic Ocean (66). Cultivation-based methods have found that *Vibrio* spp. were dominating (14, 67), however, the order of Vibrionales was below the 2 % relative abundance cutoff in our community composition analysis, indicating that these bacteria were not abundant in the cultured copepods. Moisander *et al.* (66) also observed that Rhodobactereceae dominated the transient food microbiome and proposed that they might contribute to copepod nutrition. Members of the Rhodobacterales order were also abundant in our copepod system, though Alteromonadales and Oceanospirillales bacteria were the

most abundant. These differences in the composition of the copepod-associated community most likely due to differences in the composition of the bacterial community in the immediate environment (natural vs. laboratory cultivation).

The culturable microbiota of turbot eggs and larvae has been studied for decades, however, it remains poorly understood. Culture-dependent studies have isolated multiple members of the Vibrionales and Aeromonadales orders (68, 69). While we observed that Vibrionales dominated the egg microbial community, the Aeromonadales were not abundant (below the 2 % cutoff) in any of the samples. By contrast, we observed high abundances of Alteromonadales. Poor correlation between culture-dependent and -independent investigations of the microbiotas has also been observed by Fjellheim *et al.* in cod larval microbiomes (70). Hence, there is a need for studies characterizing the microbiome of aquaculture-related fish species, particularly at the larval stages, where detrimental bacterial-fish interactions can lead to population crashes and economic losses.

The addition of probiotic *P. inhibens* had minor effects on the overall bacterial community composition at the higher taxonomic levels (order level and above). This was previously observed in the microbiome of the microalga *E. huxleyi* as well (53). Interestingly, closely related taxa from the Rhodobacterales order decreased in abundance in the microbiotas associated with the copepod and fish larvae, though they were unaffected in the microalgal, *T. suecica*, microbiota, in the presence of *P. inhibens*. This was also observed in the microbiota of the microalga *E. huxleyi* exposed to the same *P. inhibens* strain (53). Several genera of the Rhodobacteraceae family – namely *Sulfitobacter, Phaeobacter, Pelagicola,* and *Loktanella* – were reduced or absent in the presence of another strain of *P. inhibens* (2.10) in the microbiota of the diatom *Thalassiosira rotula* (71). Among the 30 most abundant taxa in the copepod and turbot microbiotas, unclassified genera of the Rhodobacteraceae family, *Ruegeria* spp., *Celeribacter* spp., and *Pseudophaeobacter* spp. decreased in abundance. *Roseobacter* spp. closely related to *Phaeobacter gallaeciencis* (originally

283 *Roseobacter gallaeciencis* (72)) have previously been isolated from copepods (73), though the
284 taxonomic resolution on the V4 region would not be sufficient to tell closely related *Roseobacter*
285 spp. and *Phaeobacter* spp. apart. Both *P. inhibens* DSM 17395 and 2.10 produce the broad-
286 spectrum antimicrobial agent, TDA and addition of pure TDA to cultures of *Nannochloropsis*
287 *salina* have been shown to decrease the relative abundance of Rhodobacteraceae at relatively low
288 concentrations (31.25 – 500 nM; 74), which could indicate that this molecule is causing the
289 observed decrease. Potentially, production and / or sensing of TDA is involved in the interspecies
290 competition within the *Roseobacter* group, although TDA production has not been reported in
291 *Pseudophaeobacter* spp. and *Celeribacter* spp.. However, if TDA acted as a broad spectrum
292 antibiotic, it would be expected that TDA also affected the remaining commensal microbiome.
293 *Vibrio* spp. and *Pseudoalteromonas* spp. diminished in the microbiota of the microalga *E. huxleyi* in
294 the presence of *P. inhibens* DSM 17395 (75), though in this study, the orders Vibrionales and
295 Alteromonadales were unaffected by the presence of *P. inhibens* in comparison to the controls.
296 Furthermore, Majzoub *et al.* (71) also saw that the microbiome exposed to a *P. inhibens* 2.10
297 variant (NCV12a1) with reduced antagonistic effect, developed in the same way as the microbiomes
298 exposed to the original, bioactive strain. Another possibility is that the decrease is due to quorum
299 sensing (QS). *P. inhibens* produces TDA and N-acyl homoserine lactones (AHLs), which both can
300 modulate motility, biofilm formation, and production of secondary metabolites in producer strains
301 (49). Beyersmann *et al.* (49) proposed that the induction of QS reduced attachment and induced
302 dispersal of the biofilm to associate with a new host. If a high dose of *P. inhibens* resembles the cue
303 to dispersal from the host, it would mean that the Rhodobacterales were in a planktonic state in the
304 water and therefore not captured as highly abundant in the sequences from eukaryotes. However,
305 this does not explain why the addition of *P. inhibens* had minor effect on the high abundance of
306 Rhodobacterales in the microalgal, *T. suecica*, microbiome. Altogether, these results indicate that

closely related roseobacters compete for the same niches and that the impact is dependent on the eukaryotic host as well as the abundance of the roseobacters present in the commensal microbiota. From an applied point of view, it is promising that a potential probiotic, such as *P. inhibens*, enters the microbiome and establishes itself rather than being out-competed by the inherent and closely related taxa. Further studies should reveal how interactions determine which species take over, and which ones disappear in the microbiome.

All microbiotas had similar richness and diversity indexes. Bakke *et al.* (76) reported that richness and diversity varied throughout the life-stages of cod larvae. While the turbot larvae in this study were younger, the alpha diversity measure were similar to the observations by Bakke *et al.* (76). Interestingly, the richness and diversity of the rearing water (i.e., green water prepared with algal, *Nannochloropsis oculata*, paste) and live-feed (copepod, *A. tonsa*, and rotifers, *Brachionus* 'Nevada') was much higher than observed in the larval microbiome (76) and the live-feed assessed in this study. However, this is most likely due to experimental differences; this study was conducted in laboratory, small-scale cultures, while the study by Bakke *et al.* (76) was conducted in large-scale, aquaculture flow-through systems. The addition of the probiotic treatment in this study had only a slight effect on richness, though a decrease, and it only occurred in the copepod microbiomes. Dittmann *et al.* (53) observed that treatment with *P. inhibens* DSM 17395 did not impact the richness and diversity of the microbiota of the microalga *E. huxleyi*. In contrast, the oyster microbiota increased in richness when *P. inhibens* had been added to the system, though the diversity was unaffected. Together with the minor impact on diversity and community composition, these observations indicate that a perturbation of the microbiota with high loads of the probiotic treatment does not have major impact on the overall diversity and taxa present, thereby likely causes minor imbalance, which is mediated within the first 24 hours of incubation.

The microbial communities associated with the three investigated microbiotas were generally very dynamic and changed over time, which is in concordance with previous studies (71, 76, 77). The addition of probiotic *P. inhibens* had significant impact on the microbiome structure of *T. suecica* and *A. tonsa*. In contrast, the microbiota associated with the turbot larvae was more affected by incubation time compared to probiotic treatment. The eggs hatched within the first 48 hours of the experiment and thereby, a sudden increase in nutrients has likely occurred. In contrast, no nutrients were added to the microalgal and copepod systems, and thus, nutrients from the medium and the eukaryotic hosts were slowly consumed and competition likely increased. The minor impact of *P. inhibens* addition to the turbot egg and larval microbiome would indicate that addition of probiotics at this trophic level would not cause dysbiosis in a healthy larval microbiota and a subsequent population crash. However, it might also mean that the probiotic is less efficient at this level. *Vibrio* spp. are commonly reported as detrimental pathogens to fish larvae (7, 8), while they are also part of the commensal microbiota (68, 69). In this study, the high abundance of Vibrionales in the turbot microbiota was due to relatively few ASVs belonging to the *Vibrio* genus and the abundance of these ASVs did not change regardless of treatment (from day one through day four). We added *P. inhibens* at probiotic concentrations similar to those used for protection of fish larvae against vibriosis in challenge trials (9, 58, 78). In those trials, vibrios were reduced in numbers if not kept at inoculation level (9, 58, 78), depending on the initial concentration of *Vibrio* spp. (9). Combined, these results would suggest that addition of probiotic *P. inhibens,* or the presence of inherent, closely related taxa can keep vibrios in the fish microbiota at a stable level, however, this does not necessarily eliminate potential pathogens from the system. The effect is likely dose-dependent, which was observed in a previous study of the *E. huxleyi* microbiome (53). Altogether, these data emphasize the need for investigating the optimal addition of probiotic *P. inhibens* – in relation to dose and which trophic level to add the probiotic treatment to – in order to obtain the most efficient

protection against opportunistic pathogens with minor effects on the commensal microbiota. The addition of the probiotic is likely more efficient at the lower trophic levels such as the microalgal or live-feed level, where *P. inhibens* establishes itself and changes the structure. However, it is not possible to say whether the changes would be beneficial or detrimental to the microbiome function. Broader –omics studies should elucidate this in the future.

In conclusion, addition of probiotic *P. inhibens* caused significant changes to the structure of the microbial communities associated with the microalgae and copepods, though no effect was seen on the community associated with turbot larvae. Particularly, the abundances of closely related taxa from the *Roseobacter* group were reduced as a function of probiotic treatment, but only in the copepod and turbot larval microbiotas. *Vibrio* spp. were highly abundant in the turbot microbiota and these were kept at a stable level, though not eliminated, which indicates that the probiotic effect towards vibrios is likely dose-dependent. Hence, the effect of adding a probiotic bacterium such as *P. inhibens* to the microbiota of aquaculture-related eukaryotes is dependent on the commensal microbiota composition and the eukaryotic host with greater impact at the lower trophic levels.

Materials and Methods

Bacterial cultivation

Phaeobacter inhibens DSM 17395 (44, 72, 79) was routinely grown in half-strength Yeast extract, Tryptone, Sea Salts broth (½YTSS, 2 g/L Bacto Yeast extract, 1.25 g/L Bacto Tryptone, 20 g/L Sigma Sea Salts) (80). Liquid cultures were incubated under agitation (250 rpm) at 25° C or room temperature. When grown on solid substrates, Marine Agar (MA, Difco 2216) or ½YTSS agar (½YTSS with 15 g/L agar) was used.

Algae-*Phaeobacter* co-culturing.

A non-axenic strain of the green algae *Tetraselmis suecica*, was obtained from the aquaculture facility Selonda Aquaculture SA, Athens, Greece. It was grown in f/2 medium (81) without Na_2SiO_3 but with 5 mM NH_4Cl in 1 L of 3 % Instant Ocean® Sea Salt (Aquarium Systems Inc., Sarrebourg, France). This modified f/2 will from this point be referred to as f/2.

The cell density of *T. suecica* in the stock culture was determined using an improved Neubauer counting chamber. The cells were re-inoculated in f/2 medium at a final concentration of approximately 5×10^5 algae mL^{-1} before splitting into six cultures of 600 mL in 1 L Erlen-Meyer flasks. Three overnight cultures of *P. inhibens* DSM 17395 in ½YTSS were adjusted to Optical Density at 600 nm (OD_{600nm}) = 1.0 and washed one time in f/2 medium (7,000 × rpm, 3 min). In triplicates, co-cultures of *T. suecica* were inoculated with *P. inhibens* DSM 17395 at a final concentration of $4.06 \times 10^6 \pm 1.05 \times 10^6$ CFU mL^{-1} (equivalent to 8 *P. inhibens* cells per algal cell), verified by plate spreading dilutions on MA. The remaining three cultures of *T. suecica* were treated with sterile 2% Instant Ocean and served as controls. The cultures were incubated stagnant, at 18°C with white fluorescent light (24 µmol m^{-2} s^{-1} photosynthetically active radiation; PAR). The cultures were sampled at 0 h, 24 h, 48 h and, 96 h for algal abundance determinations and for biomass to be used in DNA extractions. For abundance measures, 1 mL co-culture was fixed in 1 % 0.2 µm-filtered glutaraldehyde (final conc.) and the cell numbers were determined using an improved Neubauer counting chamber. For DNA extraction, 100 mL of each culture was pelleted (8000 x g, 5 minutes, 25°C) and resuspended in 1 mL lysis buffer (400 mM sodium chloride, 750 mM sucrose, 20 mM EDTA, 50 mM Tris-HCl, 1 mg mL^{-1} lysozyme, pH 8.5) (82) and stored at -80°C until extraction.

Copepod-*Phaeobacter* co-culturing.

400 *A. tonsa* eggs were kindly provided by Prof. B. W. Hansen, Roskilde University and stored at 5°C
401 until use. Three days before the experiment, eggs were inoculated in 3 % Instant Ocean and
402 incubated at 18°C with white fluorescent light (24 µmol m^{-2} s^{-1} PAR). The density of *A. tonsa*
403 nauplii in the culture was determined using Sedgewick rafter counting cell and the culture was
404 adjusted to 2 nauplii per mL using 3 % Instant Ocean. Seven cultures of 30 mL adjusted nauplii
405 culture were set-up in 50-mL Falcon tubes. In triplicates, overnight cultures of *P. inhibens* DSM
406 17395 in ½YTSS was inoculated into the *A. tonsa* nauplii culture to a level of 0.5 % (equivalent to
407 5×10^6 CFU mL^{-1}, verified by plate spreading on MA). Three *A. tonsa* cultures were treated with
408 sterile ½YTSS and served as controls. The last culture was used untreated for quantification of live
409 *A. tonsa*. All co-cultures were incubated horizontally with shake (60 rpm) at 18°C with white
410 fluorescent light (24 µmol m^{-2} s^{-1} PAR) and sampled at day 0, 1, 2, and 4. Before sampling, each
411 tube was mixed by inversion and 5 mL culture (equivalent to 10 *A. tonsa* nauplii) was taken out for
412 filtration onto a MontaMil Polycarbonate membrane filter (pore size 0.2µm, diameter 47mm). The
413 filters were transferred to cryo tubes, flooded in sucrose lysis buffer, and stored at -80°C until
414 extraction.

415 **Turbot egg and larvae - *Phaeobacter* co-culturing.**

416 Non-axenic turbot eggs were received from France Turbot, hatchery L'Epine (Noirmoutier Island,
417 France), with 24 h of transport before conducting the experiment. One-hundred eggs were
418 transferred to four Petri dishes (20 cm diameter, glass) containing sterile-filtered (0.22 µm filter)
419 sea water adjusted to salinity 34 ‰ with Sigma sea salts [S9883, Sigma] and pre-tempered to 15°C.
420 The final volume was 200 mL. An overnight culture of *P. inhibens* DSM 17395 in ½YTSS was
421 washed one time in 2 % sterile Instant Ocean (7,000 × rpm, 3 min). In duplicates, co-cultures of
422 turbot eggs were inoculated with *P. inhibens* DSM 17395 at a final concentration of 1×10^7 CFU
423 mL^{-1} in the sea water (equivalent to 2×10^7 *P. inhibens* cells per egg), verified by plate spreading

dilutions on ½YTSS agar. The remaining two cultures of eggs were treated with an equivalent volume of sterile 2% Instant Ocean and served as controls. The experiment was initiated with 0 % of the eggs being hatched. After 24 h incubation, 0 % to 2 % of the eggs were hatched, while all the eggs were hatched after 48 hours of incubation. Biomass samples for DNA extraction were taken at day 0, 1, 2 and, 4 by transferring 15 eggs from each culture to a cryo tube. Transferred sea water was removed, the eggs were resuspended in sucrose lysis buffer and stored at 80°C until extraction. At each sampling time point, the number of eggs that had hatched was noted.

DNA extraction and PCR amplification.

Extractions were performed using the phenol/chloroform-based protocol described by Dittmann *et al.* (53).The gDNA was eluted in TE buffer and incubated at 4° C overnight. Quality and quantity were assessed by absorption (DeNovix DS-11+, DeNovix Inc., Wilmington, DE, USA) and fluorescence (Qubit™ dsDNA BR assay; Invitrogen by Thermo Fisher Scientific Inc., Eugene, OR, USA) spectroscopy. The DNA was diluted to the same concentration (15 ng/µL) for all samples – except samples with lower DNA yield, which were used undiluted – prior to application in a nested PCR reaction of the 16S rRNA V4 region. The universal primers 27F and 1492R (83) were applied for the initial amplification of the 16S rRNA gene using the TEMPase Hot Start 2 × Master Mix Blue II [Ampliqon, 290806];75 ng gDNA was used as template for each reaction except for samples with lower yield, where the added amount was down to 10 ng. The PCR products were used as templates in the subsequent PCR amplification of the V4 region using the primers 515F-Y (GTGYCAGCMGCCGCGGTAA) (84) and 806R (GGACTACNVGGGTWTCTAAT) (85). The V4 PCRs were run in duplicates using the KAPA HiFi HotStart ReadyMix [Roche, 07958935001] and pooled prior to purification (AmPure XP PCR purification; Agencourt Bioscience Corporation, Beverly, MA, USA) and subsequent quality and quantity assessment (as described above).

Amplicon sequencing and bioinformatics data analysis.

Amplicons were indexed and prepared for 250PE Illumina MiSeq sequencing at the sequencing core at the Novo Nordisk Foundation Center for Biosustainability, Kgs. Lyngby, Denmark. The raw, de-multiplexed reads were checked for quality and trimmed using AfterQC (86) default settings; i.e. trim front and tail based on auto-detected quality, per-base quality trimming using phred-score ≥ 20, minimum sequence length 35 bp, maximum number of N = 5, and filtering of sequences with phred-score below 20. The trimmed reads were processed through the QIIME2 pipeline (v. 2019.1) (87) run in a Docker virtual machine (https://www.docker.com/). In brief, the reads were imported along with metadata. The DADA2 (88) plugin for QIIME2 was used for removing PhiX, denoising, merging of paired reads, merging duplicate sequences, removal of chimeric sequences, and construction of the amplicon sequence variant (ASV) table. Taxonomy of the ASVs was assigned by global alignment against the SILVA database (v132 SSU release, V4 fraction extracted reference sequences using the primers applied in this study) using the VSEARCH consensus taxonomy classifier (89). The ASV table and taxonomy was extracted from the QIIME2 format using the qiime tools "export" and "convert", followed by import into R (v. 3.5.2) along with the metadata. ASVs classified as chloroplasts were filtered using the dplyr and tidyr packages for R. ASVs containing the added *P. inhibens* DSM 17395 were classified as Rhodobacteraceae by VSEARCH; these were identified based on their relative abundances in the "probiotic" treated samples compared to the "controls" as well as 100 % similarity of the representative sequence to *P. inhibens* strain DSM 17395 (accession no. CP002976.1). Two ASVs - relative abundances of 0.02 % to 0.1 % in controls, 3.2 % to 7.6 % in samples treated with probiotic - were determined to contain the added *P. inhibens* bacteria in the *T. suecica* microbiota. Four (ASVs) - relative abundances of 0 % to 2.3 % in controls, 0.2 % to 32.4 % in samples treated with probiotic - were determined to contain the added *P. inhibens* bacteria in the *A. tonsa* microbiota. Five ASVs -

relative abundances of 0 % to 0.1 % in controls, 0.08 % to 11.9 % in samples treated with probiotic - were determined to contain the added *P. inhibens* bacteria in the turbot microbiota. To reduce any biasing effects of the increased abundance of the added probiont, these ASVs were excluded in subsequent analyses of composition, and alpha- and beta-diversity measures, thus focusing the analyses on the background microbiota.

The community composition of each microbiome was analyzed and visualized using the functions of the phyloseq and qqplot2 packages. These packages were also used to calculate measures of alpha diversity – Chao1 estimated richness and Shannon diversity index – and Beta diversity – Bray Curtis distances – on data rarefied to even sampling depth: 68,163 for the *T. suecica* data set, 62,049 for the copepod data set, and 85,621 for the turbot egg / larval data set. The richness and diversity estimates were calculated based on 100 iterations. Multivariate analysis was conducted using unconstrained ordinations - i.e. Principle Coordinate Analysis, PCoA – on Bray Curtis distances and Permutational Analysis Of Variance (PERMANOVA) using the vegan package was applied to test significance of treatment (control vs. probiotics, 999 permutations). Statistics were not applied to the turbot egg / larval microbiome due to the low number of replicates (n = 2).

Accession numbers.

The demultiplexed sequencing reads will be deposited in the Sequencing Read Archive (SRA).

Acknowledgements

The authors wish to thank Nancy Dourala, Selonda Aquaculture, Greece, for providing cultures of *T. suecica* and Professor Benni W. Hansen, Department of Science and Environment, Roskilde University, Denmark for providing copepod eggs.

492 The present study was funded by the Technical University of Denmark (PhD grant for KKD) and
493 The Danish Council for Strategic Research, Programme Commission on Health, Food and Welfare
494 (12-132390; ProAqua).

495 The authors declare no conflict of interests.

496 **References**

497 1. Bentzon-Tilia M, Sonnenschein EC, Gram L. 2016. Monitoring and managing microbes in
498 aquaculture - Towards a sustainable industry. Microb Biotechnol 9:576–584.

499 2. FAO. 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable
500 development goals. Rome.

501 3. Dittmann KK, Rasmussen BB, Castex M, Gram L, Bentzon-Tilia M. 2017. The aquaculture
502 microbiome at the centre of business creation. Microb Biotechnol 10:1279–1282.

503 4. Kibenge FSB, Godoy MG, Fast M, Workenhe S, Kibenge MJT. 2012. Countermeasures
504 against viral diseases of farmed fish. Antiviral Res 95:257–281.

505 5. Douillet P., Pickering P. 1999. Seawater treatment for larval culture of the fish Sciaenops
506 ocellatus Linnaeus (red drum). Aquaculture 170:113–126.

507 6. Eddy SD, Jones SH. 2002. Microbiology of summer flounder Paralichthys dentatus
508 fingerling production at a marine fish hatchery. Aquaculture 211:9–28.

509 7. Thompson FL, Iida T, Swings J. 2004. Biodiversity of vibrios. Microbiol Mol Biol Rev
510 68:403–31, table of contents.

511 8. Toranzo AE, Magariños B, Romalde JL. 2005. A review of the main bacterial fish diseases in
512 mariculture systems. Aquaculture 246:37–61.

513 9. D'Alvise PW, Lillebø S, Prol-Garcia MJ, Wergeland HI, Nielsen KF, Bergh Ø, Gram L.
514 2012. *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and
515 rotifers, and prevents vibriosis in cod larvae. PLoS One 7:e43996.

516 10. Rasmussen BB, Erner KE, Bentzon-Tilia M, Gram L. 2018. Effect of TDA-producing

517 *Phaeobacter inhibens* on the fish pathogen *Vibrio anguillarum* in non-axenic algae and
518 copepod systems. Microb Biotechnol 0:1–10.

519 11. Kaneko T, Colwell RR. 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J
520 Bacteriol 113:24–32.

521 12. Colwell RR, Huq A, Islam MS, Aziz KMA, Yunus M, Khan NH, Mahmud A, Sack RB, Nair
522 GB, Chakraborty J, Sack DA, Russek-Cohen E. 2003. Reduction of cholera in Bangladeshi
523 villages by simple filtration. Proc Natl Acad Sci U S A 100:1051–5.

524 13. Vezzulli L, Pezzati E, Stauder M, Stagnaro L, Venier P, Pruzzo C. 2015. Aquatic ecology of
525 the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. Environ Microbiol 17:1065–
526 1080.

527 14. Sochard MR, Wilson DF, Austin B, Colwell RR. 1979. Bacteria associated with the surface
528 and gut of marine copepods. Appl Environ Microbiol 37:750–9.

529 15. Vadstein O, Bergh Ø, Gatesoupe F-J, Galindo-Villegas J, Mulero V, Picchietti S, Scapigliati
530 G, Makridis P, Olsen Y, Dierckens K, Defoirdt T, Boon N, De Schryver P, Bossier P. 2013.
531 Microbiology and immunology of fish larvae. Rev Aquac 5:S1–S25.

532 16. Kanther M, Rawls JF. 2010. Host–microbe interactions in the developing zebrafish. Curr
533 Opin Immunol 22:10–19.

534 17. Vadstein O, Attramadal KJK, Bakke I, Forberg T, Olsen Y, Verdegem M, Giatsis C, Skjermo
535 J, Aasen IM, Gatesoupe F-J, Dierckens K, Sorgeloos P, Bossier P. 2018. Managing the
536 Microbial Community of Marine Fish Larvae: A Holistic Perspective for Larviculture. Front
537 Microbiol 9:1820.

538 18. Vestrum RI, Luef B, Forberg T, Bakke I, Vadstein O. 2018. Investigating Fish Larvae-
539 Microbe Interactions in the 21st Century: Old Questions Studied with New Tools, p. 1–35. *In*
540 Emerging Issues in Fish Larvae Research. Springer International Publishing, Cham.

541 19. Lozupone CA, Knight R. 2007. Global patterns in bacterial diversity. Proc Natl Acad Sci U S
542 A 104:11436–40.

543 20. Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF. 2011.
544 Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea.
545 ISME J 5:1571–1579.

546 21. Meron D, Atias E, Iasur Kruh L, Elifantz H, Minz D, Fine M, Banin E. 2011. The impact of
547 reduced pH on the microbial community of the coral Acropora eurystoma. ISME J 5:51–60.

548 22. Wright JJ, Konwar KM, Hallam SJ. 2012. Microbial ecology of expanding oxygen minimum
549 zones. Nat Rev Microbiol 10:381–394.

550 23. Campbell BJ, Kirchman DL. 2013. Bacterial diversity, community structure and potential
551 growth rates along an estuarine salinity gradient. ISME J 7:210–220.

552 24. Liu S, Ren H, Shen L, Lou L, Tian G, Zheng P, Hu B. 2015. pH levels drive bacterial
553 community structure in sediments of the Qiantang River as determined by 454
554 pyrosequencing. Front Microbiol 6:285.

555 25. Skjermo J, Vadstein O. 1999. Techniques for microbial control in the intensive rearing of
556 marine larvae. Aquaculture 177:333–343.

557 26. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. 2000. Probiotic bacteria as biological
558 control agents in aquaculture. Microbiol Mol Biol Rev 64:655–71.

559 27. Summerfelt ST. 2003. Ozonation and UV irradiation—an introduction and examples of
560 current applications. Aquac Eng 28:21–36.

561 28. Cabello FC. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem
562 for human and animal health and for the environment. Environ Microbiol 8:1137–1144.

563 29. Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dölz H, Millanao A, Buschmann AH.
564 2013. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance
565 and to animal and human health. Environ Microbiol 15:1917–1942.

566 30. Defoirdt T, Sorgeloos P, Bossier P. 2011. Alternatives to antibiotics for the control of
567 bacterial disease in aquaculture. Curr Opin Microbiol 14:251–258.

568 31. Ringø E, Olsen RE, Jensen I, Romero J, Lauzon HL. 2014. Application of vaccines and
569 dietary supplements in aquaculture: possibilities and challenges. Rev Fish Biol Fish
570 24:1005–1032.

571 32. Higuera-Llantén S, Vásquez-Ponce F, Barrientos-Espinoza B, Mardones FO, Marshall SH,
572 Olivares-Pacheco J. 2018. Extended antibiotic treatment in salmon farms select multiresistant
573 gut bacteria with a high prevalence of antibiotic resistance genes. PLoS One 13:e0203641.

574 33. FAO and WHO. 2001. Probiotics in food - Health and nutrional properties ad guidelines for
575 evaluation.Food and Nutrition Paper. Rome.

576 34. Vine NG, Leukes WD, Kaiser H. 2006. Probiotics in marine larviculture. FEMS Microbiol
577 Rev 30:404–427.

578 35. Wang Y-B, Li J-R, Lin J. 2008. Probiotics in aquaculture: Challenges and outlook.
579 Aquaculture 281:1–4.

580 36. Balcázar JL, Blas I de, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Múzquiz JL. 2006. The
581 role of probiotics in aquaculture. Vet Microbiol 114:173–186.

582 37. Tapia-Paniagua ST, Vidal S, Lobo C, Prieto-Álamo MJ, Jurado J, Cordero H, Cerezuela R,
583 García de la Banda I, Esteban MA, Balebona MC, Moriñigo MA. 2014. The treatment with
584 the probiotic Shewanella putrefaciens Pdp11 of specimens of Solea senegalensis exposed to
585 high stocking densities to enhance their resistance to disease. Fish Shellfish Immunol
586 41:209–221.

587 38. Porsby CH, Gram L. 2016. *Phaeobacter inhibens* as biocontrol agent against *Vibrio*
588 *vulnificus* in oyster models. Food Microbiol 57:63–70.

589 39. Prado S, Montes J, Romalde JL, Barja JL. 2009. Inhibitory activity of *Phaeobacter* strains
590 against aquaculture pathogenic bacteria. Int Microbiol 12:107–114.

591 40. Segev E, Wyche TP, Kim KH, Petersen J, Ellebrandt C, Vlamakis H, Barteneva N, Paulson
592 JN, Chai L, Clardy J, Kolter R. 2016. Dynamic metabolic exchange governs a marine algal-
593 bacterial interaction. Elife 5.

594 41. Rao D, Webb JS, Kjelleberg S. 2005. Competitive interactions in mixed-species biofilms
595 containing the marine bacterium *Pseudoalteromonas tunicata*. Appl Environ Microbiol
596 71:1729–1736.

597 42. Freese HM, Methner A, Overmann J. 2017. Adaptation of Surface-Associated Bacteria to the
598 Open Ocean: A Genomically Distinct Subpopulation of *Phaeobacter gallaeciensis* Colonizes
599 Pacific Mesozooplankton. Front Microbiol 8:1–12.

600 43. Harrington C, Reen F, Mooij M, Stewart F, Chabot J-B, Guerra A, Glöckner F, Nielsen K,
601 Gram L, Dobson A, Adams C, O'Gara F. 2014. Characterisation of Non-Autoinducing

602 Tropodithietic Acid (TDA) Production from Marine Sponge *Pseudovibrio* Species. Mar
603 Drugs 12:5960–5978.

604 44. Ruiz-Ponte C, Cilia V, Lambert C, Nicolas JL. 1998. *Roseobacter gallaeciensis* sp. nov., a
605 new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*.
606 Int J Syst Bacteriol 48:537–542.

607 45. Wegner K, Volkenborn N, Peter H, Eiler A. 2013. Disturbance induced decoupling between
608 host genetics and composition of the associated microbiome. BMC Microbiol 13:252.

609 46. Segev E, Castañeda IS, Sikes EL, Vlamakis H, Kolter R. 2016. Bacterial influence on
610 alkenones in live microalgae. J Phycol 52:125–130.

611 47. Seyedsayamdost MR, Case RJ, Kolter R, Clardy J. 2011. The Jekyll-and-Hyde chemistry of
612 *Phaeobacter gallaeciensis*. Nat Chem 3:331–335.

613 48. Porsby CH, Webber M a, Nielsen KF, Piddock LJ V, Gram L. 2011. Resistance and
614 tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. Antimicrob
615 Agents Chemother 55:1332–7.

616 49. Beyersmann PG, Tomasch J, Son K, Stocker R, Göker M, Wagner-Döbler I, Simon M,
617 Brinkhoff T. 2017. Dual function of tropodithietic acid as antibiotic and signaling molecule
618 in global gene regulation of the probiotic bacterium *Phaeobacter inhibens*. Sci Rep 7:730.

619 50. Wilson MZ, Wang R, Gitai Z, Seyedsayamdost MR. 2016. Mode of action and resistance
620 studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl
621 cycle as a proton sink. Proc Natl Acad Sci 113:1630–1635.

622 51. Dittmann KK, Porsby CH, Goncalves P, Mateiu RV, Sonnenschein EC, Bentzon□Tilia M,

623 Egan S, Gram L. 2019. Tropodithietic acid induces oxidative stress response, cell envelope
624 biogenesis and iron uptake in *Vibrio vulnificus*. Environ Microbiol Rep 1758–2229.12771.

625 52. Rasmussen BB, Grotkjær T, D'Alvise PW, Yin G, Zhang F, Bunk B, Spröer C, Bentzon-
626 Tilia M, Gram L. 2016. *Vibrio anguillarum* is genetically and phenotypically unaffected by
627 long-term continuous exposure to the antibacterial compound tropodithietic acid. Appl
628 Environ Microbiol.

629 53. Dittmann KK, Sonnenschein EC, Egan S, Gram L, Bentzon□Tilia M. 2019. Impact of
630 Phaeobacter inhibens on marine eukaryote□associated microbial communities. Environ
631 Microbiol Rep 11:401–413.

632 54. Porsby CH, Nielsen KF, Gram L. 2008. *Phaeobacter* and *Ruegeria* species of the
633 *Roseobacter* clade colonize separate niches in a Danish Turbot (*Scophthalmus maximus*)-
634 rearing farm and antagonize *Vibrio anguillarum* under different growth conditions. Appl
635 Environ Microbiol 74:7356–64.

636 55. Grotkjær T, Bentzon-Tilia M, D'Alvise PW, Dourala N, Nielsen KF, Gram L. 2016.
637 Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their
638 probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. Syst Appl Microbiol
639 39:180–188.

640 56. Grotkjær T, Bentzon-Tilia M, D'Alvise PW, Dierckens K, Bossier P, Gram L. 2016.
641 *Phaeobacter inhibens* as probiotic bacteria in non-axenic *Artemia* and algae cultures.
642 Aquaculture 462:64–69.

643 57. Hjelm M, Bergh O, Riaza A, Nielsen J, Melchiorsen J, Jensen S, Duncan H, Ahrens P,
644 Birkbeck H, Gram L. 2004. Selection and identification of autochthonous potential probiotic

bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. Syst Appl Microbiol 27:360–371.

58. D'Alvise PW, Lillebø S, Wergeland HI, Gram L, Bergh Ø. 2013. Protection of cod larvae from vibriosis by Phaeobacter spp.: A comparison of strains and introduction times. Aquaculture 384–387:82–86.

59. Buchan A, Gonzalez JM, Moran MA. 2005. Overview of the Marine *Roseobacter* Lineage. Appl Environ Microbiol 71:5665–5677.

60. González JM, Simó R, Massana R, Covert JS, Casamayor EO, Pedrós-Alió C, Moran MA. 2000. Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. Appl Environ Microbiol 66:4237–46.

61. Sonnenschein EC, Nielsen KF, D'Alvise P, Porsby CH, Melchiorsen J, Heilmann J, Kalatzis PG, López-Pérez M, Bunk B, Spröer C, Middelboe M, Gram L. 2017. Global occurrence and heterogeneity of the Roseobacter-clade species Ruegeria mobilis. ISME J 11:569–583.

62. Seyedsayamdost MR, Carr G, Kolter R, Clardy J. 2011. Roseobacticides: Small Molecule Modulators of an Algal-Bacterial Symbiosis. J Am Chem Soc 133:18343–18349.

63. Sonnenschein EC, Phippen CBW, Bentzon-Tilia M, Rasmussen SA, Nielsen KF, Gram L. 2018. Phylogenetic distribution of roseobacticides in the *Roseobacter* group and their effect on microalgae. Environ Microbiol Rep 10:383–393.

64. Biondi N, Cheloni G, Tatti E, Decorosi F, Rodolfi L, Giovannetti L, Viti C, Tredici MR. 2017. The bacterial community associated with Tetraselmis suecica outdoor mass cultures. J Appl Phycol 29:67–78.

666 65. Nakase G, Eguchi M. 2007. Analysis of bacterial communities in *Nannochloropsis* sp.
667 cultures used for larval fish production. Fish Sci 73:543–549.

668 66. Moisander PH, Sexton AD, Daley MC. 2015. Stable Associations Masked by Temporal
669 Variability in the Marine Copepod Microbiome. PLoS One 10:e0138967.

670 67. Montanari MP, Pruzzo C, Pane L, Colwell RR. 1999. Vibrios associated with plankton in a
671 coastal zone of the Adriatic Sea (Italy). FEMS Microbiol Ecol 29:241–247.

672 68. Munro PD, Barbour A, Birkbeck TH. 1994. Comparison of the gut bacterial flora of start-
673 feeding larval turbot reared under different conditions. J Appl Bacteriol 77:560–566.

674 69. Blanch AR, Alsina M, Simón M, Jofre J. 1997. Determination of bacteria associated with
675 reared turbot (*Scophthalmus maximus*) larvae. J Appl Microbiol 82:729–734.

676 70. Fjellheim AJ, Playfoot KJ, Skjermo J, Vadstein O. 2012. Inter-individual variation in the
677 dominant intestinal microbiota of reared Atlantic cod (*Gadus morhua* L.) larvae. Aquac Res
678 43:1499–1508.

679 71. Majzoub ME, Beyersmann PG, Simon M, Thomas T, Brinkhoff T, Egan S. 2019.
680 Phaeobacter inhibens controls bacterial community assembly on a marine diatom. FEMS
681 Microbiol Ecol.

682 72. Martens T, Heidorn T, Pukall R, Simon M, Tindall BJ, Brinkhoff T. 2006. Reclassification of
683 *Roseobacter gallaeciensis* Ruiz-Ponte *et al.* 1998 as *Phaeobacter gallaeciensis* gen. nov.,
684 comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria*
685 *algicola* (Lafay *et al.* 1995) Uchino *et al.* 1999 as *Marinovum algicola* gen. nov., comb. nov.,
686 and emended descriptions of the genera *Roseobacter*, *Ruegeria* and *Leisingera*. Int J Syst
687 Evol Microbiol 56:1293–1304.

688 73. Venmathi Maran BA, Iwamoto E, Okuda J, Matsuda S, Taniyama S, Shida Y, Asakawa M,
689 Ohtsuka S, Nakai T, Boxshall GA. 2007. Isolation and characterization of bacteria from the
690 copepod *Pseudocaligus fugu* ectoparasitic on the panther puffer *Takifugu pardalis* with the
691 emphasis on TTX. Toxicon 50:779–790.

692 74. Geng H, Tran-Gyamfi MB, Lane TW, Sale KL, Yu ET. 2016. Changes in the Structure of the
693 Microbial Community Associated with *Nannochloropsis salina* following Treatments with
694 Antibiotics and Bioactive Compounds. Front Microbiol 7:1155.

695 75. Dittmann KK, Sonnenschein EC, Egan S, Gram L, Bentzon-Tilia M. 2018. Impact of
696 *Phaeobacter inhibens* on marine eukaryote-associated microbial communities. Environ
697 Microbiol Rep 00.

698 76. Bakke I, Coward E, Andersen T, Vadstein O. 2015. Selection in the host structures the
699 microbiota associated with developing cod larvae (*G adus morhua*). Environ Microbiol
700 17:3914–3924.

701 77. Wilkes Walburn J, Wemheuer B, Thomas T, Copeland E, O'Connor W, Booth M, Fielder S,
702 Egan S. 2019. Diet and diet-associated bacteria shape early microbiome development in
703 Yellowtail Kingfish (*Seriola lalandi*). Microb Biotechnol 12:275–288.

704 78. Planas M, Pérez-Lorenzo M, Hjelm M, Gram L, Uglenes Fiksdal I, Bergh Ø, Pintado J. 2006.
705 Probiotic effect in vivo of Roseobacter strain 27-4 against Vibrio (Listonella) anguillarum
706 infections in turbot (Scophthalmus maximus L.) larvae. Aquaculture 255:323–333.

707 79. Buddruhs N, Pradella S, Goker M, Pauker O, Pukall R, Sproer C, Schumann P, Petersen J,
708 Brinkhoff T. 2013. Molecular and phenotypic analyses reveal the non-identity of the
709 *Phaeobacter gallaeciensis* type strain deposits CIP 105210T and DSM 17395. Int J Syst Evol

Microbiol 63:4340–4349.

80. Sobecky PA, Mincer TJ, Chang MC, Helinski DR. 1997. Plasmids isolated from marine sediment microbial communities contain replication and incompatibility regions unrelated to those of known plasmid groups. Appl Environ Microbiol 63:888–95.

81. Guillard RRL. 1975. Culture of Phytoplankton for Feeding Marine Invertebrates, p. 29–60. *In* Culture of Marine Invertebrate Animals. Springer US, Boston, MA.

82. Boström KH, Simu K, Hagström Å, Riemann L. 2004. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. Limnol Oceanogr Methods 2:365–373.

83. Lane D. 1991. 16S/23S rRNA sequencing, p. 115–175. *In* Stackebrandt, E, Goodfellow, M (eds.), Nucleic Acid Techniques in Bacterial Systematics. J.Wiley and Sons, Amsterdam.

84. Parada AE, Needham DM, Fuhrman JA. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ Microbiol 18:1403–1414.

85. Apprill A, McNally S, Parsons R, Weber L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat Microb Ecol 75:129–137.

86. Chen S, Huang T, Zhou Y, Han Y, Xu M, Gu J. 2017. AfterQC: automatic filtering, trimming, error removing and quality control for fastq data. BMC Bioinformatics 18:80.

87. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ,

731 Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope E, Silva R Da, Dorrestein
732 PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J,
733 Gauglitz JM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H,
734 Huttenhower C, Huttley G, Janssen S, Jarmusch AK, Jiang L, Kaehler B, Kang K Bin, Keefe
735 CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MG, Lee J,
736 Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D,
737 McIver LJ, Melnik A V, Metcalf JL, Morgan SC, Morton J, Naimey AT, Navas-Molina JA,
738 Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E,
739 Rasmussen LB, Rivers A, Michael S Robeson I, Rosenthal P, Segata N, Shaffer M, Shiffer
740 A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A,
741 Turnbaugh PJ, Ul-Hasan S, Hooft JJ van der, Vargas F, Vázquez-Baeza Y, Vogtmann E,
742 Hippel M von, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CH, Willis
743 AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG. 2018. QIIME 2:
744 Reproducible, interactive, scalable, and extensible microbiome data science. PeerJ Prepr.

745 88. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2:
746 High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583.

747 89. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. 2016. VSEARCH: a versatile open source
748 tool for metagenomics. PeerJ 4:e2584.

749

750

Figures

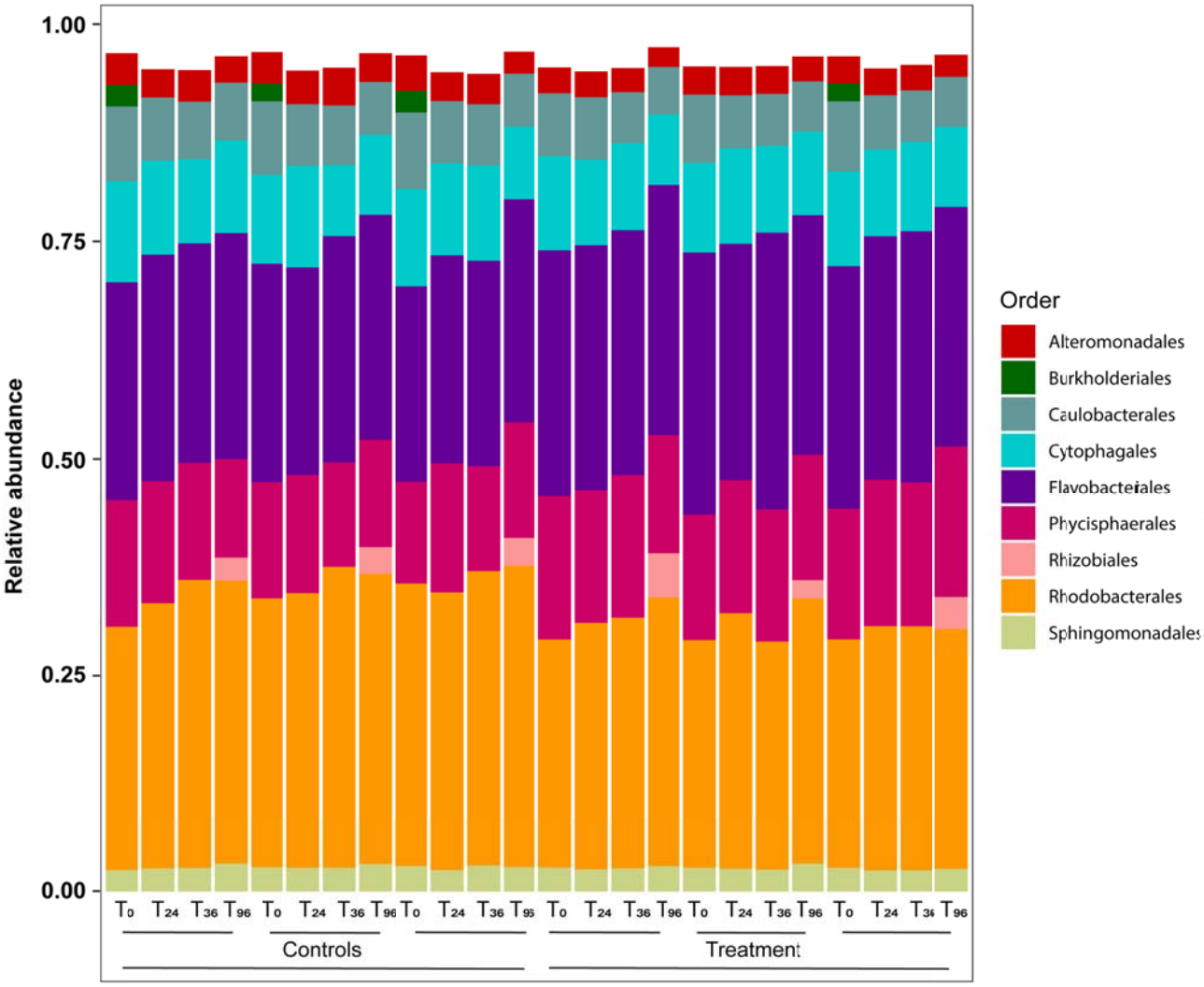
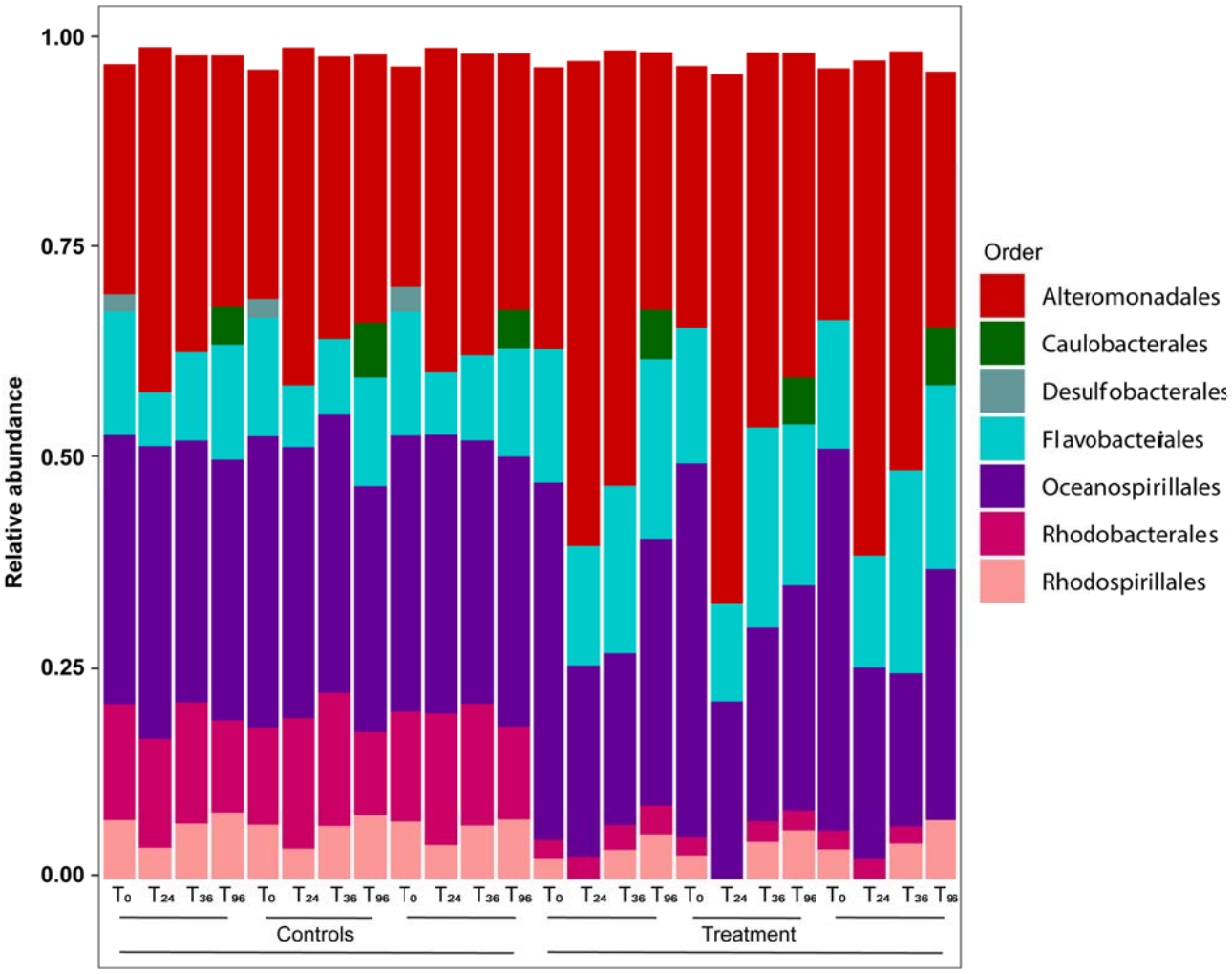


Figure 1: The composition of bacterial communities associated with *Tetraselmis suecica* in response to the addition of probiotic *Phaeobacter inhibens* DSM 17395 at 0, 24, 48 and 96 hours in triplicates. The compositions of individual microbiomes are illustrated as relative abundances of all the bacterial orders observed in the cultures of microalga with or without *P. inhibens*. Only orders with abundance above 2 % were included (the remaining low abundance orders are represented by the distance up to 1.00). Amplicon Sequence Variants (ASVs) containing the added *P. inhibens* was removed from the dataset prior to plotting. Controls: untreated controls, Treatment: probiotic *P. inhibens*.



761

762 **Figure 2:** The composition of bacterial communities associated with *Acartia tonsa* in response to
763 the addition of probiotic *Phaeobacter inhibens* DSM 17395 at 0, 24, 48 and 96 hours in triplicates.
764 The compositions of individual microbiomes are illustrated as relative abundances of all the
765 bacterial orders observed in cultures of copepods with or without *P. inhibens*. Only orders with
766 abundance above 2 % were included (the remaining low abundance orders are represented by the
767 distance up to 1.00). Amplicon Sequence Variants (ASVs) containing the added *P. inhibens* was
768 removed from the dataset prior to plotting. Controls: untreated controls, Treatment: probiotic *P.*
769 *inhibens*.

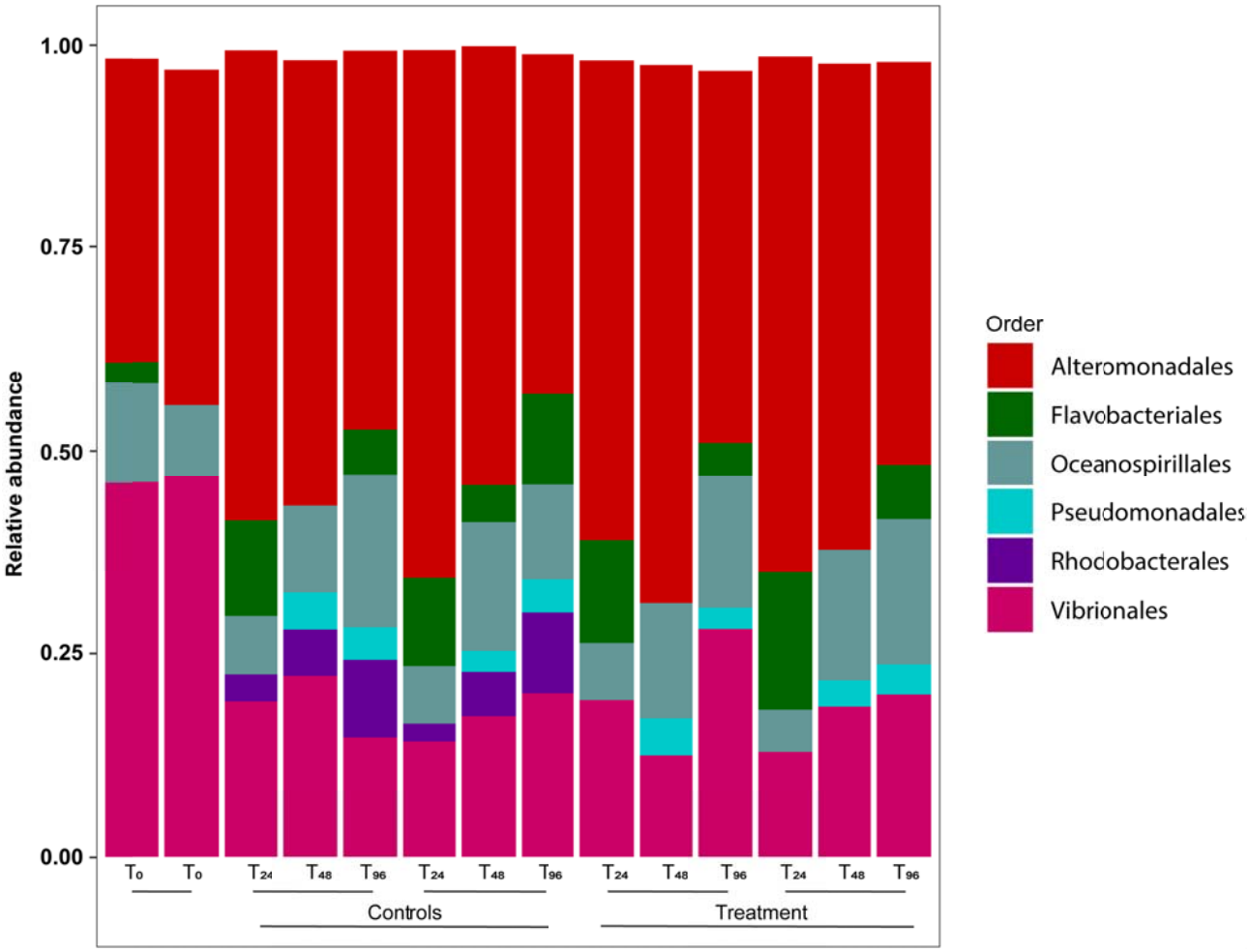


Figure 3: The composition of bacterial communities associated with turbot eggs and larvae in response to the addition of probiotic *Phaeobacter inhibens* DSM 17395 at 0, 24, 48 and 96 hours in duplicates. The compositions of individual microbiomes are illustrated as relative abundances of all the bacterial orders observed in cultures of turbot eggs / larvae with or without *P. inhibens*. Only orders with abundance above 2 % were included (the remaining low abundance orders are represented by the distance up to 1.00). Amplicon Sequence Variants (ASVs) containing the added *P. inhibens* was removed from the dataset prior to plotting. T_0: untreated time zero control, Controls: untreated controls, Treatment: probiotic *P. inhibens*.

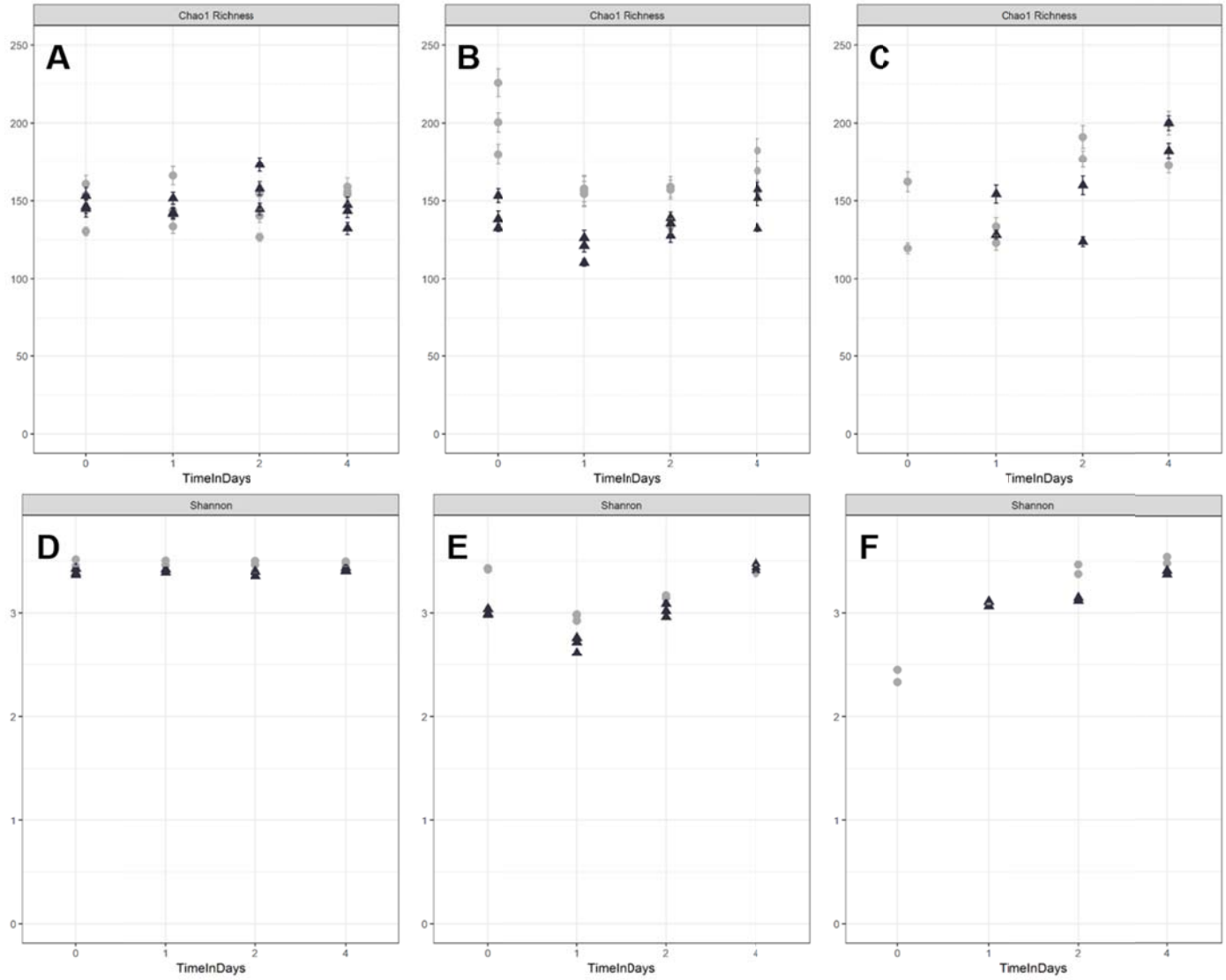
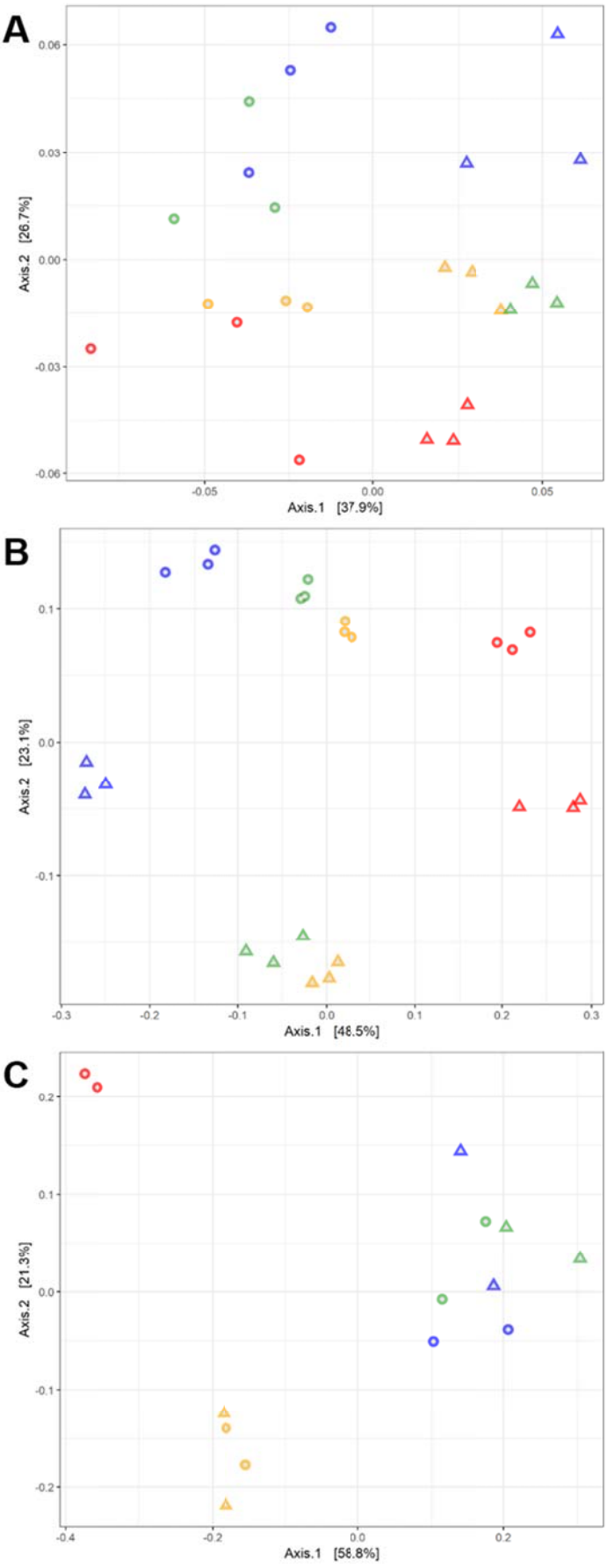


Figure 4: Alpha diversity measures for microbiotas related to *Tetraselmis suecica* (A and D), *Acartia tonsa* nauplii (B and E), and turbot eggs and larvae (C and F). The black triangles depict the cultures receiving probiotic treatment with *Phaeobacter inhibens* DSM 17395. Untreated controls are depicted as grey circles. Each data point is the mean of alpha measures – i.e. Chao1 richness estimate and Shannon diversity index – calculated on 100 iterations. The error bars reflect the standard deviations.


A
Axis.2 [26.7%]
Axis.1 [37.9%]
B
Axis.2 [23.1%]
Axis.1 [48.5%]
C
Axis.2 [21.3%]
Axis.1 [58.8%]

787

Figure 5: Community structure of microbial communities associated with three aquaculture trophic levels. Principal Coordinate Analysis (PCoA) on Bray Curtis distances between samples from microbiotas associated with *Tetraselmis suecica* (A), *Acartia tonsa* nauplii (B), and turbot eggs and larvae (C). The shape of the data point indicates treatment; microbial communities exposed to probiotic *Phaeobacter inhibens* DSM 17395 (triangles) or sterile media (untreated control, circles). Each community was sampled at time point 0 h (red), 24 h (yellow), 48 h (green), and 96 h (blue).

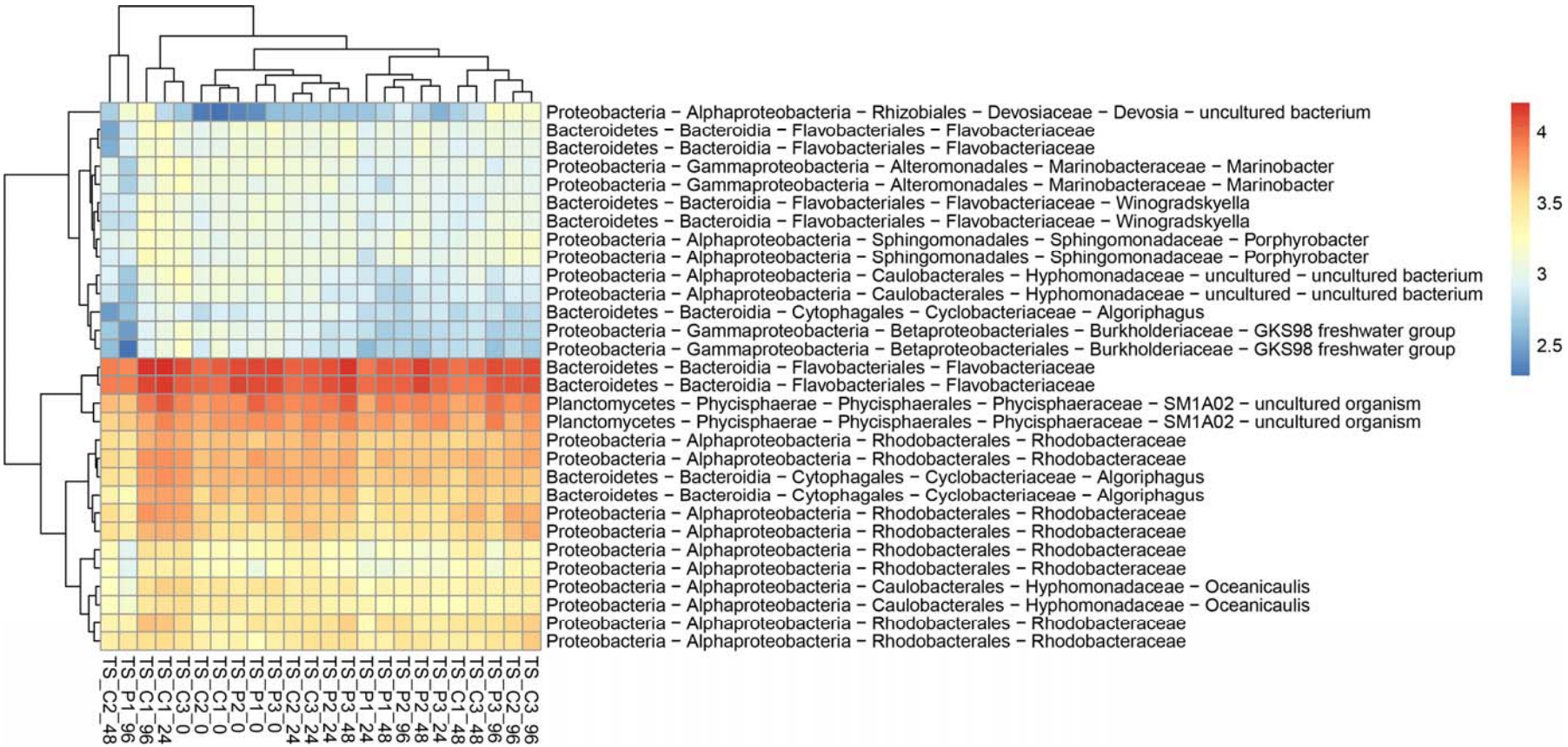


Figure 6: Heatmap indicating the $\log_{10}(x+1)$ transformed relative abundances of the 30 most abundant Amplicon Sequence Variants (ASVs) in the *Tetraselmis suecica* (TS) microbiome in response to the addition of probiotic *Phaeobacter inhibens* DSM 17395 (P). Untreated controls are included (C). Each microbiome was sampled at time point 0 h, 24 h, 48 h, and 96h. The VSEARCH classified SILVA annotation are listed next to the individual ASV.

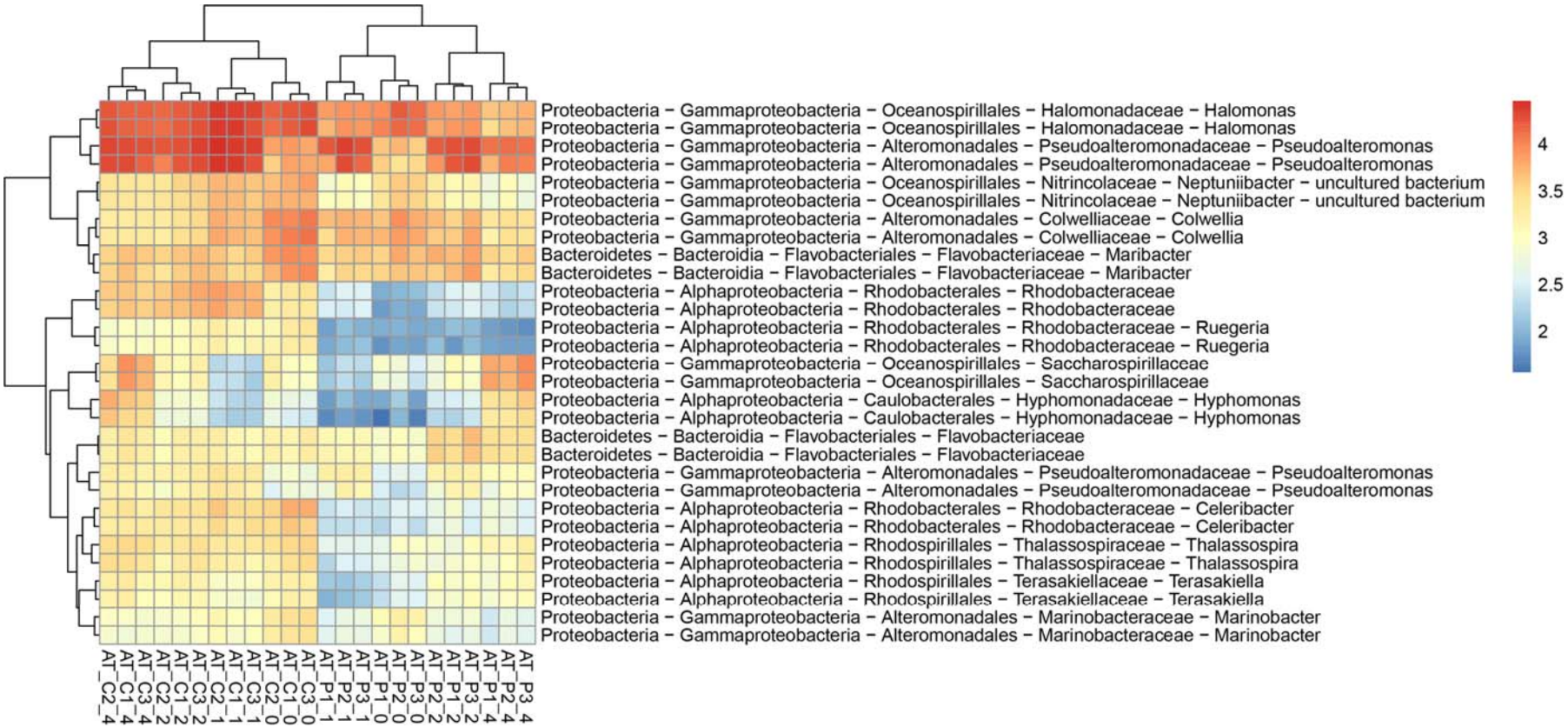


Figure 7: Heatmap indicating the $\log_{10}(x+1)$ transformed relative abundances of the 30 most abundant Amplicon Sequence Variants (ASVs) in the *Acartia tonsa* (AT) microbiome in response to the addition of probiotic *Phaeobacter inhibens* DSM 17395 (P). Untreated controls are included (C). Each microbiome was sampled at time point 0 h, 24 h, 48 h, and 96h. The VSEARCH classified SILVA annotation are listed next to the individual ASV.

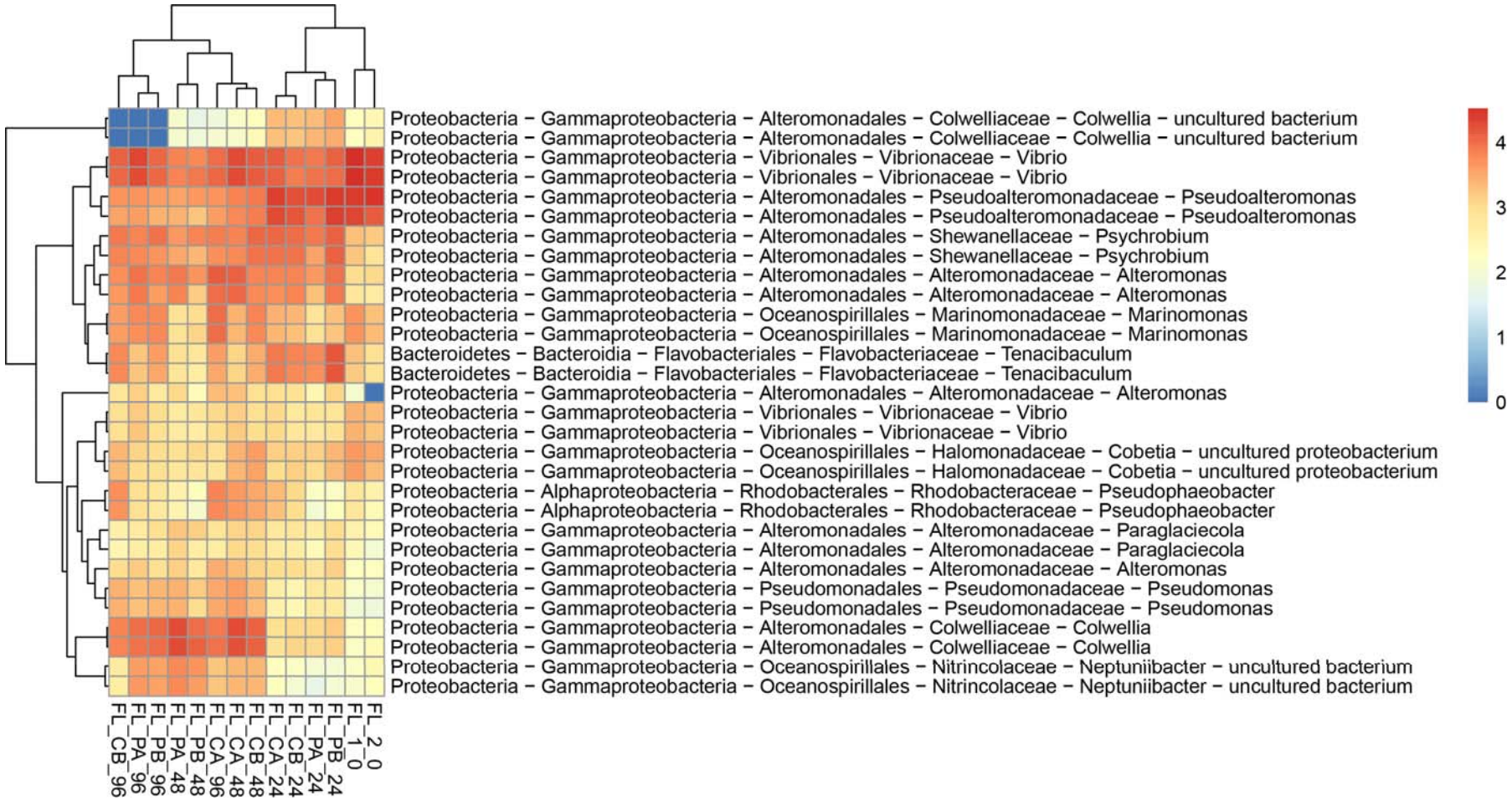


Figure 8: Heatmap indicating the $\log_{10}(x+1)$ transformed relative abundances of the 30 most abundant Amplicon Sequence Variants (ASVs) in the turbot egg and larval (FL) microbiome in response to the addition of probiotic *Phaeobacter inhibens* DSM 17395 (P). Untreated controls are included (C). Each microbiome was sampled at time point 0 h, 24 h, 48 h, and 96h. The VSEARCH classified SILVA annotation are listed next to the individual ASV.