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Changes in the microbiome of mariculture feed organisms after treatment with a potentially probiotic strain of *Phaeobacter inhibens*

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**Running title:** Effect of probiotics on mariculture microbiomes.

**Keywords:** 16S rRNA amplicon sequencing, taxonomics, mariculture, microbiome, microbial community composition, *Phaeobacter*, Roseobacters, tropodithietic acid
Abstract

The *Phaeobacter* genus has been explored as probiotics in mariculture as a sustainable strategy for prevention of bacterial infections. Its antagonistic effect against common fish pathogens is predominantly due to the production of the antibacterial compound tropodithietic acid (TDA) and TDA-producing strains have repeatedly been isolated from mariculture environments. Despite many *in vitro* trials targeting pathogens, little is known about its impact on host-associated microbiomes in mariculture. Hence, the purpose of this study was to investigate how the addition of a TDA-producing *P. inhibens* affects the microbiomes of live feed organisms and fish larvae. We used 16S rRNA gene sequencing to characterize the bacterial diversity associated with live-feed microalgae (*Tetraselmis suecica*), live-feed copepod nauplii (*Acartia tonsa*), and turbot (*Scophthalmus maximus*) eggs/larvae. The microbial communities were unique to the three organisms investigated and the addition of the probiotic bacterium had varying effects on the diversity and richness of the microbiomes. The structure of the live-feed microbiomes were significantly changed, while no effect was seen on the community structure associated with turbot larvae. The changes were highly targeted at particular taxa. The Rhodobacterales order were indigenous to all three microbiomes and decreased in relative abundance when *P. inhibens* was introduced in the copepod and turbot microbiomes, while they were unaffected in the microalgal microbiome. Altogether, the study demonstrates that addition of *P. inhibens* in higher concentrations, as part of a probiotic regime, does not appear to cause major imbalances in the microbiome, but the effects are particular towards closely related taxa.

Importance

This work is an essential part of the risk assessment of the application of roseobacters as probiotics in mariculture. It provides insights into the impact of TDA-producing *Phaeobacter inhibens* on the
commensal bacteria related to mariculture live-feed and fish larvae. Also, the study provides a sequencing-based characterization of the microbiomes related to mariculture-relevant microalga, copepods, and turbot larvae.
The rearing of aquatic animals and plants for human consumption, i.e. aquaculture, is a rapidly growing industry due to the increasing demand for high-quality protein to feed the growing world population (1, 2). In finfish rearing, animals undergo multiple developmental stages from eggs through larvae, to juveniles, and finally adult fish. During this process, fish are supplied with different food sources, which may include live-feed such as algae, rotifers, *Artemia*, and copepods. The live-feed itself feeds on microalgae and hence multiple trophic layers are involved. In many cases, fish farming has become a high-throughput process, with individual companies producing thousands of tons of fish annually and by 2030, fish production from aquaculture is expected to reach an annual output of 109 million tons (2). Along with the Sustainable Development Goals of the United Nations, 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 and approximately 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 concern to mariculture facilities as they 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 (6, 9, 10). Pathogenic *Vibrio* spp. are naturally associated with zooplankton (11–14) and they can also easily proliferate in cultures of phytoplankton 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). Microbial...
67. communities respond quickly to changes in environmental factors such as oxygen concentrations, nutrient levels, pH, salinity or accumulated toxic compounds (16–19). In the event of an imbalance in the system, e.g. rapid increase in nutrient levels and temperature, opportunistic pathogens proliferate. The pathogens have traditionally been controlled by disinfection of the rearing tanks (20), sterilization of the rearing water (21), and deployment of antibiotics (22), and in recent years by vaccination of the fish (23). However, the latter is not effective at the larval stage due to their underdeveloped immune systems (23). Thus, sustainable alternatives to antibiotics are sought (22–24). One proposed alternative is the use of probiotics; live microorganisms that provide a health benefit to the host when administered in adequate amounts (25). The potential application of probiotics in aquaculture as prophylactic and acute treatment of disease outbreaks has been studied, primarily focusing on the gut microbiome of the farmed animal (26). 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 trophodithietic acid (TDA)-producing members of the *Roseobacter* group have been studied extensively for their bioactivity and probiotic potential (3, 27–29) as an alternative candidate of marine origin.

Pure TDA is a bactericidal antibiotic at high concentrations (30), but it can also act as a signaling molecule (quorum sensing) affecting behavior such as motility, biofilm formation, and secondary metabolite production in TDA-producers (31). Its antibacterial effect is due to its ability to act as an antiporter (32) and resistance does not develop easily (30, 33). Roseobacters producing TDA are indigenous to microbiomes of marine eukaryotes including micro- and macroalgae (34, 35), zooplankton (36), sponges (37) and molluscs (38, 39) where they are believed to control the abundance of pathogenic community members. However, increasing their abundance have only had subtle effects on the microbiomes associated with microalgae and oysters (40). While some
community members were unaffected, *Vibrio* spp. and *Pseudoalteromonas* spp. were shown to diminish in the presence of *P. inhibens* (40) and the presence of TDA or TDA-producers have also caused a decrease in the relative abundances of closely related members of the Rhodobacteraceae family in microalgal microbiomes (41, 42).

TDA-producing *Phaeobacter* spp. of the *Roseobacter* group have been isolated from multiple mariculture facilities rearing mollusks and various marine finfish (38, 43, 44), and they have been able to antagonize fish pathogens in live-feed cultures (9, 44, 45) without affecting growth or survival of the feed organisms (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 pathogenic vibrios (9, 46, 47).

The selective impact on host-associated microbiomes (40) along with the lack of resistance development despite their global occurrence in microbiomes (30, 33), including mariculture microbiomes, highlight the applicability of *Phaeobacter* spp. as probiotics. However, perturbations with probiotic levels of *Phaeobacter* spp. could potentially cause imbalance and thereby give rise to proliferation of other pathogens than vibrios. Furthermore, little is currently known about mariculture microbiomes and hence, the purpose of this study was to investigate how the addition of a TDA-producing *P. inhibens* affects microbiomes related to host organisms present in the food webs found in mariculture systems.

**Results**

The impact of the TDA producer *P. inhibens* DSM 17395 on the microbiomes of mariculture-relevant marine microalgae, copepods, and fish eggs/larvae was determined by sequencing 16S rRNA gene V4 region amplicons and analyzing their taxonomic composition and diversity over 4 days; each co-culture as well as control cultures without addition of the probiotic...
was sampled four times: at 0 h (T₀), 24 h (T₂₄), 48 h (T₄₈), and 96 h (T₉₆). 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.

*P. inhibens* in non-treated and treated microbial communities

Amplicon Sequence Variants (ASVs) containing the added *P. inhibens* were detectable in all three microbiomes at all time points. Relative abundances varied from 0.05 to 1.7% with the highest relative abundance in copepods (Table 1). Addition of *P. inhibens* as expected changed the relative abundances observed. In *Tetraselmis suecica* cultures, this increased from approx. 1% to 10% and was stable over the 96 hours. In copepods, *P. inhibens* constituted between 30 and 50% in relative abundances, and in turbot larvae this was approx. 20%. ASVs containing the added *P. inhibens* were removed from the data sets before the subsequent analyses to assess the potential changes in the indigenous background microbiome.

Effects of *P. inhibens* treatment on microbial community composition

The taxonomy and relative abundance of the ASVs were used to assess the community composition at different taxonomic levels, i.e. phylum and genus levels. 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 1A). Other observed orders above 2% relative abundance included Alteromonadales, Burkholderiales, Caulobacterales, Rhizobiales, Sphingomonadales, Phycisphaerales, and Cytophagales. Burkholderiales (>2%) were only present in the initial microbiomes (T₀), while Rhizobiales (>2%) appeared after 96 hours of incubation. No obvious changes occurred at order level due to addition of *P. inhibens* and hence incubation time was the...
main driver of the observed changes in community composition at this taxonomic level. Similarly, no obvious changes occurred at genus level (Figure S1).

The A. tonsa bacterial community composition was dominated by Proteobacteria, particularly members of the orders Alteromonadales and Oceanospirillales (Figure 1B). Rhodobacterales, Rhodospirillales, and Flavobacterales members were also present in all samples, though in lower relative abundances. Desulfbacterales (> 2 %) only occurred in the initial microbiome (T₀), while Caulobacterales (> 2 %) increased in relative abundance in the microbiome after 96 hours of incubation. The addition of P. inhibens decreased the relative abundance of Rhodobacterales and Rhodospirillales. Within the Rhodobacterales order, relative abundances of sequences assigned to the genera Ruegeria and Celeribacter were reduced from 2.88 % - 7.27 % to 0.60 % - 2.27 % and from 1.64 % - 3.53 % to 0.24 % - 0.54 %, respectively (Figure S1).

Furthermore, Alteromonadales increased initially (T₂₄) in the probiotic group, though their dominance decreased over time. Hence, in this biological system, both time and the probiotic treatment affected the composition of the bacterial community at the order level.

The turbot egg and larval microbiomes were dominated by Proteobacteria, particularly Gammaproteobacteria of the orders Alteromonadales and Vibrionales (Figure 1C). Vibrionales were most prominent in the initial egg microbiome (T₀; relative abundance 46.2 % to 46.9 %), though their relative abundance decreased to 14.3 % - 19.2 % thereafter and remained at the same level throughout the experiment. Concurrently, the relative abundance of Alteromonadales increased in abundance after 24 hours of incubation. Rhodobacterales (> 2 %) appeared in the control samples at 24 hours. Both Rhodobacterales (only in the controls) and Oceanospirillales increased in relative abundance while Alteromonadales decreased over time. Pseudomonadales (> 2 %) occurred in the microbiome following 48 hours of incubation and remained throughout the experiment. The bacterial community associated with turbot eggs/larvae receiving probiotic
treatment did not contain members of the Rhodobacterales order (> 2 %) after the removal of the P. inhibens ASV. Altogether, the most prominent change occurred within the first 24 hours of the experiment (establishment phase) and the bacterial community was stable from this point onwards. The presence of *P. inhibens* decreased the relative abundance of other Rhodobacterales bacteria (mainly the genus *Pseudophaeobacter*; Figure S1), but otherwise the community was mainly affected by incubation time.

**Impact of *P. inhibens* treatment on bacterial microbiome richness and diversity**

The total number of observed ASVs was similar across systems with 857 in the microalgal system, 1014 in the copepod system, and 801 in the fish larval system after removal of chloroplasts and *P. inhibens* related ASVs. The richness and diversity of bacterial communities associated with the eukaryotic organisms were at the same general level with minor variations over time regardless of host organisms (Table 2). Estimated ASV (amplicon sequence variant) richness (Chao1) of the microalgal microbiome ranged from 126 to 166 in the controls and 132 to 173 in the cultures exposed to the probiotic (Table 2). The richness of the untreated copepod microbiome was initially 179 to 225 ASVs, though it dropped to 154 - 157 after 24 hours and remained at this level throughout the monitoring period (Chao1; 133 - 182; Table 2). The probiotic-treated group followed the same trend; the richness of the initial microbiome (T₀) was 132 - 153 ASVs, followed by a decrease to 110 - 126 (T₂₄) and an increase to 132 - 157 ASVs over the remaining 72 hours (T₉₆). 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, though the difference was only significant at time points 0 h (t-test, *p* = 0.014) and 24 h (t-test, *p* = 0.017). The turbot egg microbiome richness was initially 119 - 162 ASVs (T₀; Table 2). From time point 24 hours to 96
hours, both treatment groups increased richness from 122 - 154 ASVs to 172 - 199 ASVs, respectively. Altogether, these data indicate that the richness of the mariculture microbiomes is relatively low regardless of the host and treatment.

Similar patterns were observed with respect to diversity (Shannon diversity index). The microalgal microbiome diversity remained stable for the untreated controls (Shannon index 3.39 - 3.51) and cultures treated with *P. inhibens* (Shannon index 3.35 - 3.43) throughout the experiment (Table 2). The difference in diversity was significant between the controls and treatment at time points 0 h (t-test; *p* = 0.045), 24 h (t-test; *p* = 0.021), and 96 h (t-test; *p* = 0.037), although the p-value was close to the alpha level (0.05). In the copepod microbiome, the diversity was initially at the same level as the microalgal microbiome (Shannon index 3.42 - 3.43), though it dropped slightly to a Shannon index between 2.92 and 2.99 within 24 hours (Table 2). The diversity increased to the initial level after 96 hours of incubation. A similar pattern was observed for the copepod cultures supplemented with *P. inhibens* (Table 2); the initial Shannon diversity index was 2.98 - 3.04, dropping to a range of 2.61 - 2.76 and subsequently exhibiting an increase equivalent to the final level of the untreated controls (3.41 - 3.48). As for the changes observed in OTU richness estimates, the difference in diversity was significant between the controls and treatment at time points 0 h (t-test; *p* = 0.000018) and 24 h (t-test; *p* = 0.0053). The initial turbot egg microbiome diversity was lower than the microalgal and copepod microbiome diversity (Shannon 2.33 - 2.45 at T₀), though it increased steadily during the incubation period of 96 hours (in both controls and treated samples). Altogether, these observations demonstrate that *P. inhibens* has subtle, yet significant effects on the richness and diversity of the microbiomes associated with the live feed, i.e. microalgae and copepods. Due to low replication levels, further studies have to be conducted to support the observed trends in the fish larval microbiomes.
**Impact of P. inhibens on community structure**

Unconstrained ordinations, i.e. Principal Coordinate Analysis (PCoA), on Bray-Curtis distances were used to assess the impact of *P. inhibens* on community structure of the microbiomes associated with the three mariculture-related eukaryotes (Figure 2). The community structure shifted during incubation time for all three microbial communities, regardless of treatment (PERMANOVA; $R^2$-values between 0.24112 and 0.44059, $p = 0.001$). The microalgal microbial community structures treated with probiotics were significantly different from the untreated controls (PERMANOVA; $R^2 = 0.31982$, $p = 0.001$; Figure 2A). This was also observed in the copepod associated microbiome (PERMANOVA; $R^2 = 0.21008$, $p = 0.001$; Figure 2B), though time was a bigger driver than treatment ($R^2$ (time) = 0.44059 > $R^2$ (treatment) = 0.21008). However, the turbot larval microbial community structure was not significantly affected by the presence of *P. inhibens* (PERMANOVA; $R^2 = 0.1021$, $p = 0.06$; Figure 2C). Hence, incubation time was a major driver of the microbial community structure across all samples and the impact of the probiotic treatment depended on the trophic layer at hand; the biggest impact occurred in the live-feed, while the fish larval community structure was unaffected.

**Impact of P. inhibens on specific taxa**

At the order level, *P. inhibens* DSM 17395 decreased the relative abundance of Rhodobacterales in two of the microbiomes (Figure 1B and 1C), while the effects on the algal microbiome were minor. Therefore, differences at ASV level (100 % sequence similarity, no clustering) were investigated to elucidate which of the most abundant members were affected. No major impact on the relative abundance of the most abundant ASVs was observed in the microbiomes of *T. suecica* due to treatment (Figure S2), and thus alterations in community structure...
was mainly confined to low-abundant ASVs. In the *A. tonsa* microbiome, the most abundant
*Halomonas* sp. were slightly lower in abundance in the *P. inhibens* treated samples, but still
dominating (Figure 3). Members of the Rhodobacteraceae family, such as *Ruegeria* sp. and
*Celeribacter* sp., decreased in relative abundance in the presence of *P. inhibens*, which is in line
with the observations in the community composition analysis (Figure 1B, S1). Members of the
Saccharospirillaceae family and *Hyphomonas* spp. were initially lower in relative abundance in both
treatment groups, but increased over time. In contrast to the microalgal and copepod microbiomes,
the samples from the more dynamic fish eggs/larvae system clustered according to time rather than
treatment (Figure S3). No major changes were observed due to treatment, but changes over time
were observed, confirming the PCoA (Figure 2C). Some *Colwellia* sp. ASVs disappeared as a
function of incubation time, while others increased in relative abundance. Other Alteromonadales
bacteria such as *Psychrobium* sp. and *Alteromonas* sp. increased. *Vibrio* spp. had high relative
abundances in the initial microbiome (*T₀*), though decreased as a function of incubation time. No
effect of treatment was observed on the vibrios. Altogether, the occurring changes due to the
presence of *P. inhibens* were unique to the eukaryotic host and the largest changes were observed in
the copepod microbiome (Figure 3).

### Discussion

TDA-producing roseobacters can function as probiotics in mariculture due to their
efficient killing of common pathogens (9, 44, 45) and protection of vibrio-challenged fish larvae (9,
46, 47). Since the natural microbiomes in these systems are imperative for the health of e.g. algae
(48), addition of probiotics should selectively remove pathogens while leaving the commensal
majority of the population unaffected. Our results demonstrate that the impact of *P. inhibens* on the
The microbiome is highly dependent on the commensal microbiome composition and the inoculation level with greater impact on the bacterial community structure at the lower levels of the food web.

Three eukaryotes, green microalgae, copepods, and turbot larvae, were selected to represent different important levels in the food web: feed for live-feed, live-feed, and reared fish, found in mariculture. Several studies have been conducted on microalgal microbiomes and how roseobacters interact with these unicellular eukaryotes (49–51). Despite *T. suecica* being widely used and produced in hatcheries, the microbial community associated with this microalga is not well studied. Biondi *et al.* (52) observed that the *T. suecica* microbiome was dominated by Proteobacteria—particularly members of the *Roseobacter* group, but also Rhizobiales and Bacteroidetes (Flavobacteriales). This is similar to our findings and also to findings for another mariculture-relevant microalgal genus, *Nannochloropsis* (53). The *A. tonsa* microbiome was also dominated by Proteobacteria, particularly Gammaproteobacteria in this study. This has previously been observed in copepods from the North Atlantic Ocean (54). Cultivation-based methods have found that *Vibrio* spp. were dominating (14, 55), however, the order of Vibrionales was below the 2% relative abundance cut-off in our community composition analysis, indicating that the relative abundance of these bacteria is likely overestimated in cultivation-dependent studies. Moisander *et al.* (54) also observed that Rhodobacteraceae dominated the transient food microbiome and proposed that they contribute to copepod nutrition. Members of the Rhodobacterales order were also found at high relative abundances in our copepod system, though Alteromonadales and Oceanospirillales bacteria exhibited the highest relative abundances. These differences in the composition of the copepod-associated community is likely due to differences in the composition of the bacterial community in the immediate environment (natural vs. laboratory cultivation), and to different methodologies applied (cultivation-dependent vs cultivation-independent).
The culturable bacteria of turbot eggs and larvae have been studied for decades, and the isolates have been dominated by members of the Vibrionales and Aeromonadales orders (56, 57). While we observed Vibrionales in the egg microbial community, the Aeromonadales were not abundant (below the 2% relative abundance cut-off) in any of the samples. By contrast, we observed high relative abundances of Alteromonadales. However, most studies on aquaculture microbiomes are cultivation-based and poor correlations between culture-dependent and -independent microbiome investigations was also observed by Fjellheim et al. in cod larval microbiomes (58).

In concordance with a previous study of the microalgal E. huxleyi microbiome (40), we found that effects of probiotic treatment did not cause major changes to the community across orders. Interestingly, addition of P. inhibens reduced the relative abundance of closely related taxa from the Rhodobacterales order in the copepod and fish larvae microbiomes, while they were unaffected in the microbiome of T. suecica. Effects on closely related Rhodobacterales members have been observed in the haptophyte E. huxleyi microbiome exposed to the same P. inhibens strain (40). Several genera of the Rhodobacteraceae family – namely Sulfitobacter, Phaeobacter, Pelagibacter, and Loktanella – were reduced or absent in the presence of another TDA-producing strain of P. inhibens (2.10) in the diatom Thalassiosira rotula microbiome (42). In our study, among the 30 most abundant taxa in the copepod and turbot microbiomes, unclassified genera of the Rhodobacteraeae family, Ruegeria spp., Celeribacter spp., and Pseudophaeobacter spp. decreased in relative abundance. Addition of pure TDA to cultures of Nannochloropsis salina has also been shown to decrease the relative abundance of Rhodobacteraeae at relatively low concentrations (31.25 – 500 nM; 74), which could indicate that TDA is causing the observed decrease of Rhodobacteraeae members in the copepod and turbot microbiomes. Potentially, production and/or sensing of TDA is involved in the interspecies competition within the Roseobacter group where...
specific species or strains are more susceptible than others. Vibrio spp. and Pseudoalteromonas spp. were reduced by the presence of P. inhibens DSM 17395 in the microalgal E. huxleyi microbiome (59), though in the present study, the orders Vibrionales and Alteromonadales were unaffected as compared to the controls. Majzoub et al. (42) found that the T. rotula microbiome exposed to a P. inhibens 2.10 variant (NCV12a1) with reduced antagonistic effect, developed in the same way as microbiomes exposed to the original bioactive strain and factors other than TDA could be responsible for our observations. 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 microbiome. However, as the sampling of the co-culture systems was different between the tested systems, planktonic bacteria could be washed away from the copepods and fish eggs/larvae during sampling and therefore not captured as highly abundant in the sequences, while more planktonic bacteria would remain in the pelleting of the microalgal culture. Further studies should reveal how specific interactions determine which species prevail.

All microbiomes had similar richness and diversity indices. Bakke et al. (60) 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. (60). 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’) were much higher than observed in the larval microbiome (60) 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. (60) was conducted in large-scale, aquaculture flow-through systems. The addition of the probiotic treatment in this study had a slight, yet significant effect on alpha-diversity of the live-feed. Dittmann et al. (40) observed that
treatment with, and the inoculation density of, *P. inhibens* DSM 17395 did not impact the richness and diversity of the low-complexity microalgal *E. huxleyi* microbiome. In contrast, the more complex oyster microbiome increased in richness when *P. inhibens* had been added to the system, though the diversity was unaffected. How the observed changes in alpha-diversity of host associated microbiomes translate to the overall health state of the host organism needs further scrutiny.

The microbial communities associated with the three microbiomes were generally dynamic and changed over time, which is in concordance with previous studies (42, 60, 61). The addition of *P. inhibens* had significant impact on the microbiome structure of *T. suecica* and *A. tonsa*. In contrast, the microbiome associated with the turbot larvae was more affected by incubation time compared to probiotic treatment. All 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 would not cause dysbiosis in a healthy larval microbiome and a subsequent population crash. To determine this fully, longer trials would have to be conducted monitoring the overall health of the larvae. The minor impact on the larval microbiome 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 microbiome (56, 57). In this study, the high relative abundance of Vibrionales in the turbot microbiome 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 concentrations where a probiotic effect has been observed in previous challenge trials (9, 47, 62). In those trials, vibrios were reduced in numbers if not kept at inoculation level (9, 47, 62), depending on the initial concentration of *Vibrio* spp. (9).
Combined, these results would suggest that addition of *P. inhibens*, or the presence of inherent, closely related taxa can keep vibrios in the fish microbiome 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 microalgal *E. huxleyi* microbiome (40). Altogether, these data emphasize the need for investigating the optimal addition of probiotic *P. inhibens* – in relation to dose and which part of the food web to add the probiotic treatment to – in order to obtain the most efficient protection against opportunistic pathogens while keeping the effects on the commensal microbiome to a minimum. The addition of the probiotic is likely more efficient at the live feed stages such as microalgae or zooplankton, where *P. inhibens* establishes itself and changes the structure. However, results based on nested PCRs on low biomass samples, as those presented in this study, are prone to biases and vulnerable to contaminations. Hence, it is not possible to conclude yet, whether any changes would be beneficial or detrimental to the microbiome or its function. Broader –omics studies should elucidate this in the future.

In conclusion, addition of TDA-producing *P. inhibens* caused significant changes to the microbiome structure of the live feed, but had little effect on the order-level composition, and varying effects on diversity and richness of the microbial communities associated with microalgae and copepods. No effect was seen on the community structure associated with turbot larvae. Particularly, the relative 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 microbiomes. *Vibrio* spp. were highly abundant in the turbot microbiome 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 microbiome of mariculture-related eukaryotes is not likely to cause major perturbations to the existing microbial communities.
Materials and Methods

Bacterial cultivation

*Phaeobacter inhibens* DSM 17395 (38, 63, 64) 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) (65). 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 microalgae *Tetraselmis suecica*, was obtained from the aquaculture facility Selonda Aquaculture SA, Athens, Greece. It was grown in sterilized f/2 medium (66) without Na₂SiO₃ but with 5 mM NH₄Cl 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 × 10⁵ algae mL⁻¹ before splitting into six cultures of 600 mL in 1 L Erlenmeyer flasks. Three overnight cultures of *P. inhibens* DSM 17395 in ½YTSS were adjusted to Optical Density at 600 nm (OD₆₀₀nm) = 1.0 and washed once 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 × 10⁶ ± 1.05 × 10⁶ 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 (1623 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, 1 mg mL⁻¹ lysozyme, pH 8.5) (67) and stored at -80°C until extraction.

Copepod-Phaeobacter co-culturing. Acartia tonsa eggs were kindly provided by Prof. B. W. Hansen, Roskilde University and stored at 5°C until use. Three days before the experiment, eggs were inoculated in 3% Instant Ocean and incubated at 18°C with white fluorescent light (1623 µmol m⁻² s⁻¹ PAR). The density of A. tonsa nauplii in the culture was determined using Sedgewick-Rafter counting cell and the culture was adjusted to 2 nauplii per mL using 3% Instant Ocean. Seven cultures of 30 mL adjusted nauplii culture were set-up in 50 mL Falcon tubes. In triplicates, overnight cultures of P. inhibens DSM 17395 in ½YTSS was inoculated into the A. tonsa nauplii culture to a level of 0.5% (equivalent to 5 × 10⁶ CFU mL⁻¹, verified by plate spreading on MA). Three A. tonsa cultures were treated with sterile ½YTSS and served as controls. The last culture was used untreated for quantification of live A. tonsa. All co-cultures were incubated horizontally with shake (60 rpm) at 18°C with white fluorescent light (1623 µmol m⁻² s⁻¹ PAR) and sampled at day 0, 1, 2, and 4. Before sampling, each tube was mixed by inversion and 5 mL culture (equivalent to 10 A. tonsa nauplii) was taken out for filtration onto a MontaMil Polycarbonate membrane filter.
(pore size 0.2 µm, diameter 47 mm). The filters were transferred to cryo tubes, flooded in sucrose lysis buffer, and stored at -80°C until extraction.

**Turbot egg and larvae - Phaeobacter co-culturing.** Non-axenic turbot eggs were received from France Turbot, hatchery L’Epine (Noirmoutier Island, France), with 24 h of transport before conducting the experiment. One-hundred eggs were transferred to four Petri dishes (20 cm diameter, glass) containing sterile-filtered (0.22 µm filter) sea water adjusted to salinity 34‰ with Sigma sea salts [S9883, Sigma] and pre-tempered to 15°C. The final volume was 200 mL. An overnight culture of *P. inhibens* DSM 17395 in ½YTSS was washed one time in 2% sterile Instant Ocean (7,000 × rpm, 3 min). In duplicates, co-cultures of turbot eggs were inoculated with *P. inhibens* DSM 17395 at a final concentration of $1 \times 10^7$ CFU mL$^{-1}$ in the sea water (equivalent to 2 $\times 10^7$ *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.* (40). 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 (68) were applied for the initial amplification of
the 16S rRNA gene using the TEMPase Hot Start 2 x 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 program was: 1 cycle of denaturation for 15 min at 95°C,
35 cycles of denaturation (95°C for 30 s), annealing (51°C for 30 s), and elongation (72°C
for 2 min), and finally 1 cycle of extended elongation (72°C for 5 min). The PCR products were
used as templates in the subsequent PCR amplification of the V4 region using the primers 515F-Y
(GTGYCAGCMGCCGCGGTAA) (69) and 806R (GGACTACNVGGGTWTCTAAT) (70). The
V4 PCRs were run in duplicates using the KAPA HiFi HotStart ReadyMix [Roche, 07958935001]
and the PCR program: 1 cycle of denaturation for 3 min at 95°C, 25 cycles of denaturation (98°C
for 20 s), annealing (53°C for 15 s), and elongation (72°C for 15 s), and finally 1 cycle of
extended elongation (72°C for 1 min). The PCR products of the duplicates were 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 (71) 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) (72) run in a Docker virtual machine (https://www.docker.com/). In brief, the reads were imported along with metadata. The DADA2 (73) 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 (74). 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 (publically available at NCBI under 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* microbiome. 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* microbiome. 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 microbiome. To reduce any biasing effects of the increased abundance of the added probiont, these ASVs were excluded from all samples in analyses of composition, and alpha- and beta-diversity measures, thus focusing the analyses on the background microbiome. For relative abundances of *P. inhibens* in all samples, see Table 1.
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 the unfiltered data set using 100 iterations and statistical significant difference was determined using F- and t-tests. Multivariate analysis was conducted on unfiltered data using unconstrained ordinations - i.e. Principle Coordinate Analysis, PCoA – on Bray-Curtis distances (community dissimilarities) and Permutational Analysis Of Variance (PERMANOVA) using the adonis function from the vegan R package on the Bray-Curtis distances was applied to test significance of treatment and time (control vs. probiotics, time in days, 999 permutations).

**Accession numbers.** The demultiplexed sequencing reads were deposited in the Sequencing Read Archive (SRA) at NCBI under the accession number PRJNA558217.

**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 and France Turbot hatchery L’Epine for turbot eggs.

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The authors declare no conflict of interests.
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Figure legends

Figure 1: The composition of bacterial communities associated with A) *Tetraselmis suecica*, B) *Acartia tonsa*, and C) *Scophthalmus maximus* eggs/larvae in response to the addition of probiotic *Phaeobacter inhibens* DSM 17395. All eggs were hatched after 48 h of incubation while 98 – 100% was at the egg stage prior to the 48 h sampling. Each bar represents a culture which was sampled upon 0, 24, 48 and 96 hours incubation; the *T. suecica* and *A. tonsa* cultures were in triplicates, the *S. maximus* were in duplicates. 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*.

Figure 2: Community structure of microbial communities associated with three mariculture-relevant host organisms. Principal Coordinate Analysis (PCoA) on Bray Curtis distances between samples from microbiomes 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 3: 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 96 h. The VSEARCH classified SILVA annotation are listed next to the individual ASV and the Bray-Curtis distances are represented as dendrograms.
Table 1: Relative abundance of *Phaeobacter inhibens* in microbiomes associated with *Tetraselmis suecica*, *Acartia tonsa*, and *Scophthalmus maximus* eggs/larvae over time. The relative abundances were pooled Amplicon Sequence Variants (ASVs) identified as *P. inhibens* in each culture prior to calculation of average and standard deviation for two to three replicate cultures.

<table>
<thead>
<tr>
<th>Eukaryotic host</th>
<th>Relative abundance of <em>Phaeobacter inhibens</em> (%)</th>
<th>Non-treated controls</th>
<th>Probiotic treated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T₀</td>
<td>T₂₄</td>
</tr>
<tr>
<td><em>Tetraselmis suecica</em> (microalga)</td>
<td>0.09 ± 0.05</td>
<td>0.06 ± 0.01</td>
<td>0.11 ± 0.07</td>
</tr>
<tr>
<td><em>Acartia tonsa</em> (copepod)</td>
<td>1.66 ± 2.49</td>
<td>0.22 ± 0.02</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td><em>Scophthalmus maximus</em> (turb)</td>
<td>0.15 ± 0.06</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

NT: Not tested
Table 2. Alpha diversity measures (Chao1 richness and Shannon diversity) for microbiomes of *Tetraselmis suecica*, *Acartia tonsa* nauplii, and turbot eggs and larvae treated with *Phaeobacter inhibens* DSM 17395 and without treatment (controls).

<table>
<thead>
<tr>
<th>Eukaryotic host</th>
<th>Time point (h)</th>
<th>Chao1 richness</th>
<th>Shannon diversity index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>p-value</td>
</tr>
<tr>
<td></td>
<td>Average Std.</td>
<td>Average Std.</td>
<td></td>
</tr>
<tr>
<td><em>Tetraselmis suecica</em> (microalgae)</td>
<td>0</td>
<td>148.08 15.80</td>
<td>148.14 4.44</td>
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<tr>
<td></td>
<td>24</td>
<td>147.68 16.69</td>
<td>145.08 5.47</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>140.42 13.91</td>
<td>158.47 14.30</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>156.18 2.52</td>
<td>141.01 7.81</td>
</tr>
<tr>
<td><em>Acartia tonsa</em> (copepod)</td>
<td>0</td>
<td>202.07 22.93</td>
<td>141.28 10.64</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>155.97 1.71</td>
<td>119.30 8.14</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>149.89 13.95</td>
<td>134.04 5.85</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>169.11 13.42</td>
<td>147.22 13.29</td>
</tr>
<tr>
<td><em>Scophthalmus maximus</em> (turbot; eggs and larvae)</td>
<td>0</td>
<td>140.78 30.01</td>
<td>NT NT NT</td>
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<tr>
<td></td>
<td>24</td>
<td>128.17 7.36</td>
<td>141.20 18.30</td>
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<td>48</td>
<td>184.01 10.09</td>
<td>141.73 25.55</td>
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<td></td>
<td>96</td>
<td>186.42 19.07</td>
<td>190.96 12.65</td>
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</tbody>
</table>

NT: Not tested