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Phaeobacter inhibens as probiotic bacteria in non-axenic *Artemia* and algae cultures

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ABSTRACT

The growing aquaculture industry is in need for non-antibiotic based disease control strategies to reduce risk of bacteria developing and spreading antibiotic resistance. We have previously, in axenic model systems of live larval feed, demonstrated that bacteria from the *Roseobacter* clade can antagonize fish pathogens such as *Vibrio anguillarum* and *Vibrio harveyi* and that they can reduce larval mortality in challenge trials. However, in the aquaculture production, a natural microbiota is present at all stages and may affect the efficacy of the probiotic bacteria. The purpose of the present study was to determine if marine roseobacters in non-axenic systems were capable of antagonizing fish pathogenic vibrios. We added a controlled background microbiota of four bacterial strains to axenic *Artemia* and algae (*Duniella*) and these bacteria had a marginal but significant reducing effect on inoculated *Vibrio anguillarum* that grew to 10^7 in control samples but to a level 1–2 log lower in samples with background microbiota. The addition of the *Roseobacter*-clade bacteria, *Phaeobacter inhibens*, caused a significant reduction in growth of the pathogen that reached levels 3–4 log lower than in the control. In non-axenic natural *Artemia* and algae (*Tetraselmis*) received from an aquaculture unit, *Vibrio anguillarum* grew to 10^7 CFU/ml but only reached 10^4 CFU/ml when *P. inhibens* was also added. *P. inhibens* was added at a concentration 10^6 CFU/ml in all systems and remained at this concentration at the end of the study, irrespective of the background microbiota. We therefore conclude that *P. inhibens* are indeed promising as probiotic bacteria in marine larvi-culture where it in natural live feed can suppress fish larval pathogens.

Statement of relevance: We and others have in several studies demonstrated the potential effect of probiotic bacteria in axenic or gnotobiotic models. Here, we for the first time present data that demonstrate that probiotic roseobacters also are efficient in non-axenic systems; even in commercial algae and Artemia from a commercial fish farmer. This adds further promise to probiotics as disease control in aquaculture.

1. Introduction

A major constraint in aquaculture is the sudden reduction (crash) in a number of fish larval cultures caused by pathogenic bacteria (Reid et al., 2009). Antibiotics can control these crashes, however, widespread use of antibiotics is not sustainable because it selects for antibiotic resistant bacteria, and this resistance can be transferred to human pathogens (Rhodes et al., 2000). Several more sustainable disease control measures are being explored in aquaculture. This includes vaccination, immune-stimulants, prebiotics and probiotics. Vaccination requires a functioning immune system with memory, which is not yet developed at the fish larval stage. Non-antibiotic-based strategies include immune-stimulating compounds, quorum sensing inhibiting compounds, bacteriophages and fish probiotic bacteria. Marine *Roseobacter* clade bacteria can antagonize fish pathogenic bacteria in axenic systems of live larval feed and they can also, in controlled model systems, improve the survival of infected fish larvae (D'Alvise et al., 2012, 2013; Grotkjær et al., 2016). The roseobacters are marine alpha-Proteobacteria that are widely distributed across climate zones (Buchan et al., 2005; Segev et al., 2015; Sonnenshein et al., 2016). Especially the genera *Phaeobacter* and *Ruegeria* have repeatedly been isolated from aquaculture units (Grotkjær et al., 2016; Hjelm et al., 2004b; Porsby et al., 2008) and recently, *Phaeobacter inhibens* was repeatedly isolated from biofilms in Danish habour areas (Gram et al., 2015).

To be able to control and manipulate the composition of bacteria in aquaculture live feed it is important to understand the complex interactions between the microbes and the live feed (Berland et al., 1970; Grossart and Simon, 2007; Nakase and Eguchi, 2007; Salvesen et al., 2000) and to determine if the probiotic bacteria are capable of pathogen suppression also in systems with a complex microbiota. Bacteria grow well in algal cultures as they are supplied with organic compounds from the algal organisms (Cole, 1982). Also, bacteria may be beneficial for the algae by decomposing organic material or by producing

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secondary metabolites (Berland et al., 1970; Seyedsayamdost et al., 2011a). The use of live feed such as algae and rotifers in aquaculture improves the growth and health of fish larvae during their first feeding since the nutritional quality is improved (Reitan et al., 1993) and because of the bacterial composition (Skjermo and Vadstein, 1993). The microbiota of fish larvae is changed when algae are added (Bergh et al., 1994), where a gradual shift of nonfermentative bacteria of the *Cytophaga/Flexibacter/Flavobacteriurn* group is to be dominated by the fermentative *Vibrio/Aeromonas* group. Newly hatched fish larvae ingest algae but are often not able to digest the cell contents and therefor need bacteria and the extracellular material produced by the algae as a significant source of nutrition (Daume, 2006). The nauplii of the brine shrimp *Artemia* are the most commonly used live feed organism in aquaculture industry (Van Stappen, 1996). Unfortunately the *Artemia* nauplii are also a vector for introducing bacteria into the hatchery systems (Austin and Allen, 1982; Benavete and Gatesoupe, 1988) herein opportunistic pathogenic bacteria (Gomez-Gil et al., 1994; López-Torres and Lizárraga-Partida, 2001). The bacterial community of newly hatched nauplii is dominated by uncultured members of Gammaproteobacteria and Planctomycetales situated in the gut rather than at their external surfaces (Høj et al., 2009), and can reach densities of up to 10^7 within 24 h (Austin and Allen, 1982).

The use of *Roseobacter* clade bacteria as probiotic bacteria in larviculture requires consideration of how to introduce the bacteria to the aquaculture system. It is not feasible to produce the quantities of probiotics that would be needed to add them on a continuous basis to the larvae tanks, and we and others (D'Alvise et al., 2012, 2013; Grotkjær et al., 2016) have suggested introducing the probiont via the live feed which would require lower quantities of probiont culture. Also, the live feed is potentially the source of pathogens and thus a logic step in the process to intervene. Usually, pathogens are not introduced with the intake water (Olafsen, 2001; Pintado et al., 2010; Tinh et al., 2007), but are likely carried by the live feed where concentrations of nutrients from microalgae or *Artemia* are high. These dense cultures allow the opportunistic pathogenic bacteria to proliferate. In addition to pathogens thriving in these live feed cultures, other commensal background bacteria also proliferate (Grossart and Simon, 2007; Grossart et al., 2005), and this raises the question whether they will interfere with the growth and colonization of the probiont and, more importantly, how they affect the antagonism of the pathogens by the probionts. Also, the probiotic bacteria may affect the composition of the natural microbiota and since this community interacts in several ways with the algae (Grossart and Simon, 2007; Jensen et al., 1996; Seyedsayamdost et al., 2011a), it is important to know how addition of a probiotic bacteria potentially affects the natural microbiota.

Most studies testing the potential of different probiotic strategies have been based on axenic systems. The purpose of the present study was to determine if the probiotic effect by roseobacters that we have previously demonstrated (D'Alvise et al., 2012, 2013; Grotkjær et al., 2016; Planas et al., 2006; Porsby et al., 2008) could be transferred to a non-axenic system. We first mimick a natural live-feed setup by adding, in a controlled manner, bacterial strains isolated from aquaculture to *Artemia* in parallel to the addition of pathogen and probionts. We finally demonstrate that in natural, non-axenic live feed cultures, the probiotic *Phaeobacter inhibens* is capable of inhibiting the fish pathogen *Vibrio anguillarum*.

2. Material and methods

2.1. Bacterial strains and media

All bacterial strains are listed in Table 1. The pathogenic *Vibrio anguillarum* strain NB10 was isolated from the Gulf of Bothnia and has caused disease in rainbow trout (Norqvist et al., 1989, 1990). The strain used is a variant that has been tagged by insertion of plasmid pNQFlaC4-gfp27 (*cat*, *gfp*) into an intergenic region on the chromosome, and was kindly provided by D. Milton, University of Umeå (Croxatto et al., 2007). The *gfp*-tagged *Vibrio anguillarum* NB10 was counted using a flow cytometer (BD Accuri C6, Becton, Dickinson and Company) and on selective medium (Tryptone Soy Agar (TSA, Difco 212185) supplemented with 10 mg/l chloramphenicol). The *Phaeobacter inhibens* DSM17395 gentamicin resistant (GM^r) has been tagged chromosomally with a miniTn7(Gm)PA1/04/03*DsRedExpress-a* cassette, using a mini-Tn7 tagging system (D'Alvise et al., 2012). The four bacterial strains that were used as a controlled microbiota were isolated from *Artemia* cultures and shrimp or fish in aquaculture and were provided by The Artemia Reference Center. These had been identified by 16S rRNA gene sequence analyses. Bacteria from frozen stock cultures (−80 °C) were streaked on MA and MA with 25 mg/l gentamicin was used for counting *P. inhibens* DSM17395 GM^r. The bacterial pre-cultures for the *Artemia* and algae experiments were grown in 20 ml of 1/2 YTSS (2 g Bacto Yeast extract, 1.25 g Bacto Tryptone, 20 g Sigma Sea Salts, 1 l deionized water) (Sobecky et al., 1997) at 25 °C under aerated conditions.

Table 1
Bacterial strains used in the study.

Strain	Isolated from	Source or reference
Vibrio anguillarum NB10	*Oncorhynchus mykiss*	Croxatto et al. (2007)
Phaeobacter inhibens DSM17395 GM^r	*Pecten maximus*	D'Alvise et al. (2012)
LT3 (*Bacillus* sp.)	*Penaeus vannamei*	Defoirdt et al. (2011)
B7 (*Acinetobacter* sp.)	*Dicentrarchus labrax*	Liu et al. (2010)
M 13 (*Ochrobactrum* sp)	*Macrobrachium rosenbergi*	Liu et al. (2010)
M 15 (*Ochrobactrum* sp)	*Macrobrachium rosenbergi*	Liu et al. (2010)

2.2. Preparation of Artemia for pathogen-probiont experiments with defined background bacteria

Axenic *Artemia* were prepared by disinfecting 1 g of *Artemia* cysts (Yik Sung et al., 2007) and decapsulating them in 90 ml deionized water for 1 h. 3.3 ml 30% NaOH, 50 ml NaOCl and 70 ml 1% $Na_2S_2O_3$ were added for 2 min. The *Artemia* cysts were filtered and washed with 1 l 3.5% Instant Ocean sea saltwater (3% Instant Ocean sea salts; Aquarium Systems Inc., Sarrebourg, France). The cysts were transferred to a 1-liter blue cap bottle with 1 l 3.5% IO sea saltwater and incubated for 24 h, aerated at 30 °C. For bacterial mono- or co-culture combinations, 20 individual live *Artemia* were pipetted into a 50 ml falcon tube containing 20 ml 3.5% IO. For the non-axenic setup, cultures with 20 mg of non-disinfected *Artemia* cysts were hatched in 20 ml 3.5% IO and incubated at 20 °C shaken at 200 rpm. The cysts hatched at this temperature within approx. 24 h.

2.3. Preparation of axenic Dunaliella tertiolecta for pathogen-probiont experiments with defined background microbiota

The *Dunaliella tertiolecta* algae stock was grown in 20 ml algae medium (1 ml WALNE's medium for algal cultures, 0.1 ml vitamin solution, 1 l autoclaved seawater) (Walne, 1970). The algae culture was incubated at 20 °C in an algae growth room on a rotator at 50 rpm for 48 h. The *Dunaliella tertiolecta* was treated with a cocktail of five antibiotics (gentamicin, tetracycline, chloramphenicol, streptomycin and penicillin G) of 50 mg/l for 48 h at 20 °C in an algae growth room on a rotator. The antibiotic treated and an untreated *Dunaliella tertiolecta* culture were centrifuged at 6000 ×*g* and washed with sterile 3.5% IO three times to remove residual antibiotics. Bacterial concentrations of the cultures were determined after 48 h of incubation by plating on MA plates.

2.4. Preparation of non-axenic Tetraselmis suecica for pathogen-probiont experiments

Non-axenic *Tetraselmis suecica* cultures in f/2 medium (Guillard and Ryther, 1962) in 3% IO was acquired from a commercial aquaculture

unit. The concentration of *Tetraselmis suecica* was determined using a Neubauer-improved counting chamber. *Tetraselmis suecica* was added to 50 ml falcon tubes with 20 ml 3.5% IO aiming at 10^5 cells/ml as the initial concentration. All cultures were incubated at 20 °C shaken at 200 rpm with a light intensity on the tubes of 2000 lx.

2.5. Pathogen-probiont experiments in Artemia and Dunaliella tertiolecta cultures

Pre-cultures of *V. anguillarum* NB10 and the microbiota strains (Table 1) were adjusted to $OD_{600} = 0.5$ by diluting with 1/2 YTSS, and 10-fold serially diluted in artificial seawater (3.5% Instant Ocean sea salts). Fifty μl of the appropriate bacterial dilutions were added to the *Artemia* and *Dunaliella tertiolecta* cultures, aiming at 10^4 CFU/ml as the initial concentration. One hundred μl of overnight *P. inhibens* DSM17395 GM^r culture was added to the *Artemia* and *Dunaliella tertiolecta* cultures, aiming at 10^6 CFU/ml as the initial concentration. One hundred μl of 1/2 YTSS medium was added to the axenic *Artemia* control cultures and to the monoxenic *Vibrio* and background controls. The *Artemia* cultures were incubated at 30 °C on a rotary shaker at 200 rpm. The *Dunaliella tertiolecta* cultures were incubated at 20 °C, on a rotary shaker at 50 rpm in an algae growth room. All challenge cultures were done in independent duplicates. Bacterial concentrations were determined daily. *P. inhibens* DSM17395 GM^r numbers were determined on selective medium (MA with 25 mg/l gentamicin). Samples from cultures without *P. inhibens* DSM17395 GM^r were also plated as controls. Concentrations of the *gfp* tagged *V. anguillarum* NB10 were determined by using a flow cytometer (BD Accuri C6, Becton, Dickinson and Company), using a 533 ± 30 filter for green fluorescence. For none fluorescent protein tagged strains SYBR green staining was used together with the 533 ± 30 filter.

2.6. Pathogen-probiont experiments in non-axenic Artemia and Tetraselmis suecica cultures

V. anguillarum NB10 pre-cultures were adjusted to $OD_{600} = 0.5$ by diluting with 1/2 YTSS, and 10-fold serially diluted in 3.5% IO. Fifty μl of the appropriate bacterial dilutions were added to the non-axenic *Artemia* and *Tetraselmis suecica* cultures, aiming at 10^4 CFU/ml as the initial concentration. One hundred μl of overnight *P. inhibens* DSM17395 GM^r culture was added to the non-axenic *Artemia* and *Tetraselmis suecica* cultures, aiming at 10^6 CFU/ml as the initial concentration. One hundred μl of 1/2 YTSS medium was added to the non-axenic *Artemia* and *Tetraselmis suecica* control cultures and to the cultures with *V. anguillarum* NB10 together with the non-axenic *Artemia* or the non-axenic *Tetraselmis suecica.* Bacterial concentrations of *V. anguillarum* NB10 were determined daily by plating on selective medium (TSA with 10 mg/l chloramphenicol).

2.7. Statistical analysis

Bacterial counts were log-transformed, and differences e.g. between *V. anguillarum* counts under different treatments were tested for significance using ANOVA on GraphPad Prism 5.00 (GraphPad Software, San Diego CA). Tukey's multiple comparisons test was used for pairwise comparisons. Values from day 0, which are merely a proof of the inoculated concentrations, were omitted in the analysis.

3. Results

3.1. Artemia and Dunaliella tertiolecta pathogen-probiont experiments with defined background bacteria

P. inhibens DSM 17395 GM^r colonized all of the *Artemia* and *Dunaliella tertiolecta* cultures reaching densities of 10^6 to 10^8 CFU/ml after 48 h irrespective of addition of a background microbiota (Figs. 1 and 2). *V. anguillarum* grew in *Artemia* cultures that were inoculated with the background bacteria separately and reached densities of 10^5 to 10^6 (Fig. 3). The four background bacteria were enumerated as the non-fluorescent counts and all were inoculated at approx. 10^3 cells/ml and grew in two days to 10^6 cells/ml (data not shown).

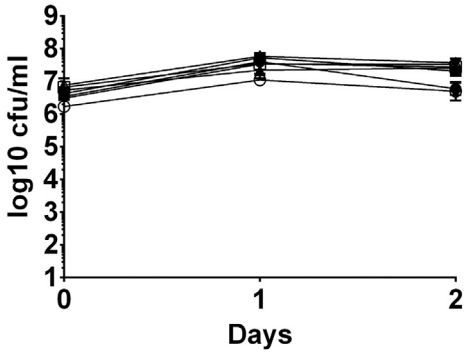

Fig. 1. Counts of *P. inhibens* DSM 17395 GM^r in axenic *Artemia* cultures. *P. inhibens* DSM 17395 GM^r inoculated at 10^6 CFU/ml alone (○), or in the presence of *V. anguillarum* (□), *V. anguillarum* and background strains separately (▲, ▼, ●, ■) or *V. anguillarum* and all background strains (Δ). Points are average of two biological replicates and errorbars are standard deviation of the mean.

The background microbiota strains caused a slight reduction approximately one log unit of the *V. anguillarum* as compared to the control without background microbiota strains. Addition of *P. inhibens* 17395 GM^r, caused a significant ($p < 0.001$) reduction of *V. anguillarum* of 3 log units in all combinations (Figs. 4 and 5) as compared to the *Artemia and Dunaliella tertiolecta* where only *V. anguillarum* was added.

3.2. Non-axenic Artemia and Tetraselmis suercica pathogen-probiont experiments

Attempts were made to use FACS to enumerate *V. anguillarum*, however, numbers were too low compared to the level of the overall background microbiota and despite several attempts to adjust the gating parameters, it was not possible to get reliable counts. Therefore, *V. anguillarum* were enumerated on chloramphenicol plates. *V. anguillarum* NB10 was able to colonize and grew well in non-axenic *Artemia* and *Tetraselmis* cultures and reached concentrations of 10^7 (Figs. 6 and 7). The concentration of *V. anguillarum* NB10 decreased significantly ($p < 0.001$) in the presence of *P. inhibens* DSM 17395 GM^r in

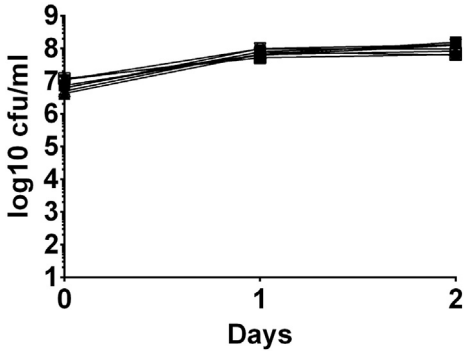

Fig. 2. Counts of *P. inhibens* DSM 17395 GM^r in antibiotic treated *D. tertiolecta*. *P. inhibens* DSM 17395 GM^r inoculated at 10^6 CFU/ml alone (○) or in the presence of *V. anguillarum* (□), *V. anguillarum* and background strains separately (▲, ▼, ●, ■) or *V. anguillarum* and all background strains (Δ). Counts of *P. inhibens* DSM 17395 GM^r in *D. tertiolecta* with natural microbiota in presence of *V. anguillarum* and all of the background strains (▽). Points are average of two biological replicates and errorbars are standard deviation of the mean.

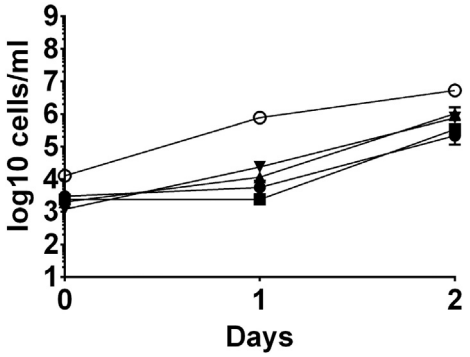

Fig. 3. Counts of *V. anguillarum* NB10 in axenic *Artemia*. *Gfp* tagged cells of *V. anguillarum* inoculated at 10^4 CFU/ml alone (○), or in the presence of background strain LT3 (▲), background strain B7 (▼), background strain M13 (●), or background strain M15 (■). Points are average of two biological replicates and errorbars are standard deviation of the mean.

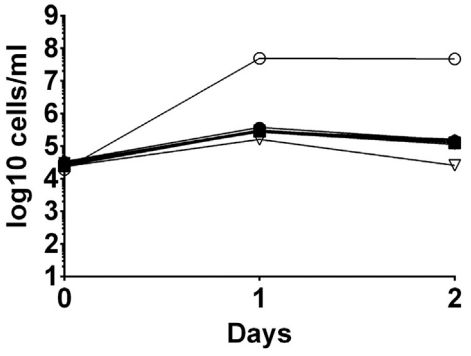

Fig. 5. Counts *V. anguillarum* in *D. tertiolecta* cultures. *Gfp* tagged cells of *V. anguillarum* inoculated in antibiotic treated *D. tertiolecta* at 10^4 CFU/ml alone (○), or in the presence of *P. inhibens* 17395 GM^r (□), or *P. inhibens* 17 395 GM^r and background strains separately (▲, ▼, ●, ■), or in the presence of all the strains (Δ). Counts of *V. anguillarum* in *D. tertiolecta* (▽) with natural microbiota and added probiont. Points are average of two biological replicates and errorbars are standard deviation of the mean.

both culture settings (Figs. 6 and 7). The reduction was in order of 4 log units in the non-axenic *Artemia* cultures and 3 log units in non-axenic *Tetraselmis suercica* cultures as compared to the non-axenic controls with only added *V. anguillarum* NB10. In both the *Artemia* and the *Tetraselmis suercica* with no added pathogen or probiont, an increase in chloramphenicol resistant bacteria was seen in the order of almost 2 log units (Figs. 6 and 7).

4. Discussion

To circumvent the need for prophylactic use of antibiotics in aquaculture, the application of probiotics seems to hold great potential and previous studies have demonstrated a clear probiotic effect of the *Roseobacter* clade bacterium *Phaeobacter inhibens* in axenic model systems (D'Alvise et al., 2012, 2013; Grotkjær et al., 2016; Prol-García and Pintado, 2013). Our results demonstrate that moving *P. inhibens* from an axenic model system to a defined non-axenic, and ultimately to a completely non-axenic, live feed model system, does not interfere with the establishment of the potential probiont, nor with its antagonistic effects on the fish pathogenic bacterium *Vibrio anguillarum*.

In the application of probiotics it is imperative that the probiont is able to establish itself in the aquaculture environment. This may potentially be a problem when supplying probionts through the live feed as different kinds of live feed already have different established microbiotas (Austin and Allen, 1982; Berland et al., 1970; Grossart and Simon, 2007; López-Torres and Lizárraga-Partida, 2001; Nakase and Eguchi, 2007; Salvesen et al., 2000; Van Stappen, 1996). *P. inhibens* have previously been shown to colonize axenic algae (Seyedsayamdost et al., 2011b, 2014) as well as other live feed subjects such as *Artemia* and rotifers (D'Alvise et al., 2012; Grotkjær et al., 2016), but our data suggest that this is also the case when *P. inhibens* is co-cultured with an existing microbiota. Irrespective of whether one or more of the background strains were added, *P. inhibens* maintained cell densities of around 10^6 CFU/ml, or grew an additional order of magnitude. This is congruent with the fact that members of the *Phaeobacter* genus, as well as other genera of the *Roseobacter* clade, are readily isolated from the aquaculture environments (Grotkjær et al., 2016; Hjelm et al., 2004a).

We observed the largest increase in *P. inhibens* density in non-axenic *D. tertiolecta* cultures compared to the non-axenic *Artemia* cultures. This may be due to nutritional differences in the respective media since *D. tertiolecta* were grown in WALNE's medium, which is rich in ammonium, iron, and phosphate, while the experiments with *P. inhibens* and *Artemia* were performed in pure Instant Ocean®. However, it has been suggested that *P. inhibens* lives in association with phytoplankton in its natural environment (Seyedsayamdost et al., 2011a), and hence live feed in the form of algae may be particularly suited to accommodate *Phaeobacter* probionts.

Similarly, the pathogenic *V. anguillarum* strain NB10 was able to establish itself in the *Artemia* cultures where it swiftly increased three orders of magnitude. This is in line with previous findings where the organism grows well in axenic algae, rotifer and *Artemia* cultures (D'Alvise et al., 2012, 2013; Grotkjær et al., 2016). However, when each of the background bacterial strains were added in conjunction with the

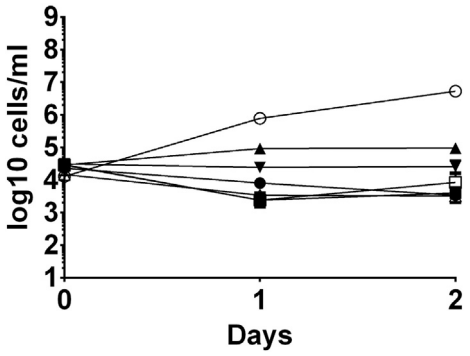

Fig. 4. Counts of *V. anguillarum* in axenic *Artemia*. *Gfp* tagged cells of *V. anguillarum* inoculated at 10^4 CFU/ml alone (○), or in the presence of *P. inhibens* 17395 GM^r (□), *P. inhibens* 17395 GM^r and background strains separately (▲, ▼, ●, ■), or in the presence of all strains (Δ). Points are average of two biological replicates and errorbars are standard deviation of the mean.

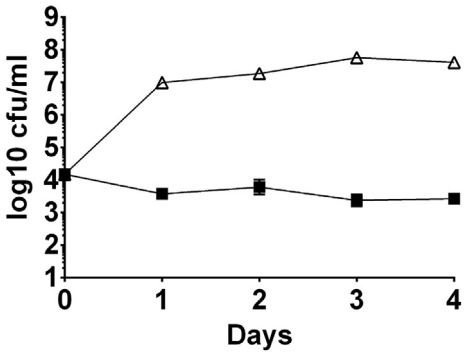

Fig. 6. Counts of *V. anguillarum* in *Artemia* with natural microbiota. *V. anguillarum* inoculated at 10^4 CFU/ml alone (Δ), or in the presence of *P. inhibens* 17395 GM^r (■). Points are average of two biological replicates and errorbars are standard deviation of the mean.

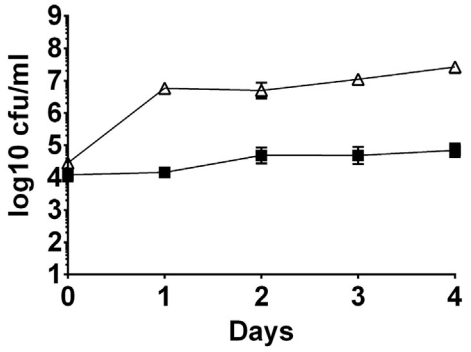

Fig. 7. Counts of *V. anguillarum* in *Tetraselmis suecica* with natural microbiota. *V. anguillarum* inoculated at 10^4 CFU/ml alone (△), or in the presence of *P. inhibens* 17395 GM^r (■). Points are average of two biological replicates and errorbars are standard deviation of the mean.

pathogen, its growth was slightly impaired. Despite the fact that this reduction in the number of vibrios was not significantly different from control cultures where *V. anguillarum* was colonizing *Artemia* alone, these data may suggest that the natural microbiota present in the aquaculture environment impose some kind of restriction on growth of pathogens. Interestingly, *Bacillus* sp. strain LT3 has previously been shown to have probiotic effects in *Artemia* cultures both affecting the pathogen (*V. campbellii*) and the innate immune system of the brine shrimp (Niu et al., 2014). *Ochrobactum* sp. strains M13 and M15 have also been shown to have probiotic effects, yet not through antagonism of pathogens, but rather through degradation of poly-β-hydroxybutyrate (Liu et al., 2010), which was not supplied in our experiments. The effect of the background microbiota was most pronounced after 24 h of incubation and then declined and we attribute the slight reduction rather to an interspecies competition for nutrients and not to a direct bactericidal effect. The difference after 48 h is the same as the start inoculation at time 0 corresponding with the expected outcome derived from former studies (Liu et al., 2010; Niu et al., 2014).

In the live feed cultures obtained from aquaculture units, both the pathogen and the probiotic bacteria when added as mono-cultures established themselves and grew well. This demonstrates that if pathogens are introduced into the live feed with no control measures, they are able to grow rapidly to high densities and are a likely source of infection. *P. inhibens* antagonized the pathogen in these non-axenic systems and numbers remained at the inoculum level. In previous studies (D'Alvise et al., 2012; Grotkjær et al., 2016), we have seen a probiont mediated killing of *V. anguillarum* independently of the initial inoculum (D'Alvise et al., 2012). It is possible that the natural microbiota had a protective effect on the pathogen and potential absorbed some of the antibacterial compound, tropodithietic acid, produced by the probiont.

In conclusion, we have demonstrated that the potential probiotic bacterium, *P. inhibens*, is capable of antagonizing *V. anguillarum* in several live feed systems with natural or controlled microbiota. This is promising as it brings the probiotic concept, and hence the reduction in antibiotic usage, a step forward.

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