Aquaculture 532 (2021) 736072

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Probiotic *Carnobacterium divergens* increase growth parameters and disease resistance in farmed Atlantic cod (*Gadus morhua*) larvae without influencing the microbiota.

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ARTICLE INFO

Keywords:
Atlantic cod larvae
Probiotic
Carnobacterium divergens
Vibrio anguillarum
16S rRNA sequencing
Microbiota

ABSTRACT

Aquaculture represents the fastest growing food sector and the second largest export commodity in Norway. Disease in aquaculture causes mortality and huge losses in farmed fish. Probiotic treatment of farmed fish, i.e. feeding of fish with live, beneficial microorganisms, may represent one part of a sustainable solution to these problems. Some carnobacteria of fish origin have shown promising results as probiotics for fish. In this study, two *Carnobacterium divergens* strains were fed to Atlantic cod (*Gadus morhua*) larvae through intermittent feeding with *Artemia franciscana* containing the bacterial strains. Cod larvae fed with the carnobacteria showed a significantly higher growth and survival during the larval rearing stage compared to the larvae fed *A. franciscana* with no carnobacteria (control treatment). Following the probiotic feeding period, the cod larvae were challenged with a pathogenic bacterium, *Vibrio anguillarum*. Cod larvae from probiotic treatment showed significantly better disease resistance compared to the control. Microbiota analysis by 16S rRNA amplicon sequencing verified that enrichment of carnobacteria in the *A. franciscana* feed. Analysis of the whole larvae detected only minor relative levels of carnobacteria in the probiotic treated larvae, which was non-significant compared to the control larvae ($p = 0.062$). The probiotic treatment had no significant impact on the overall microbiota diversity or composition in the larvae during the probiotic feeding period or after the disease challenge. Despite this, significantly improved growth and survival during larval rearing and post-pathogen challenge suggest probiotic effects of *C. divergens* mixture on cod larval fish performance and welfare. Studies examining the mode of action should be carried out to get more insight to lead to the commercial application of *C. divergens* in Atlantic cod larviculture.

1. Introduction

Bacterial diseases are a major cause of mortality in marine finfish larviculture (Planas and Cunha, 1999). Commonly, the control of bacterial problems in fish hatcheries has relied on the use of antimicrobials, but their continued, and sometimes uncontrolled use, has resulted in the development of resistant bacterial strains (Smith et al., 1994). With increased restriction on the use of antimicrobials, the use of probiotic bacteria to control potential pathogens has been seen as an alternative method (Gatesoupe, 1999). Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). A probiotic strain may not necessarily be a natural enemy of pathogens, but they can indirectly prevent damage to the host caused by the pathogens through several potential mechanisms. These mechanisms are not fully understood, but competition for nutrients and attachment sites, production of inhibitory substances, and immunological effects have been proposed (Vine et al., 2004; Balcázar et al., 2006). Probiotics are not growth promoters per se,

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https://doi.org/10.1016/j.aquaculture.2020.736072
Received 16 June 2020; Received in revised form 20 August 2020; Accepted 16 October 2020
Available online 20 October 2020

however, growth promotion may be an indirect positive effect of probiotic administration (Balcázar et al., 2006).

Use of probiotics in fish hatcheries has been shown to enhance the immune parameters of the fish larvae and enhance the survival (Balcázar et al., 2006). The adaptive immune system is poorly developed in early developmental stages of marine finfish (Seppola et al., 2009) and using probiotics could possibly increase survival of larvae against microbial pathogens (Vadstein, 1997). Oral administration of immunostimulants to larval fish may be a promising strategy to combat pathogenic infections because vaccination is not possible due to their smaller size (Vadstein, 1997). Various studies have documented positive effects of probiotics in fish regarding increased weight, survival and enhanced activity of immunological parameters (Suzer et al., 2008; Sun et al., 2010). Some other studies have documented no effect of oral administration of different probiotic bacterial strains through live feed and rearing water for protection against diseases in different fish species (Gildberg and Mikkelsen, 1998; Ramos et al., 2017).

The Atlantic cod (*Gadus morhua*) has great potential as an aquaculture species because they are relatively easy to culture and has high fecundity and growth rates (Brown et al., 2003). Atlantic cod rearing protocols are well developed and the larval growth during early larval stages and quality of the juveniles have improved during the last decades (Hansen et al., 2011). Although survival is substantially improved, early larval stages of cod still encounter high mortality (Hansen et al., 2018). As in many marine fish larvae, cod larviculture also depends on live feed, which can introduce microbes to the rearing environment and imbalance the microbial communities in water and the intestine of the larvae (McIntosh et al., 2008). Unbalanced microbial communities could give way to the opportunistic bacteria to flourish and affect the health status of the developing larvae resulting in mass mortalities (McIntosh et al., 2008). Oral and dip vaccines are available to combat the bacterial and viral diseases in larger juveniles and adults of Atlantic cod (Mikkelsen et al., 2011), however, no vaccines are available for the early larval stages of cod.

Lactic acid bacteria (LAB), including (among others) the genera - *Lactobacillus*, *Bifidobacterium, Streptococcus* and *Carnobacterium*, are major components of the microbiota in the gastrointestinal tract of healthy fish (Ringø and Gatesoupe, 1998). Several studies have shown that LAB are beneficial when fed with diet (live feed and/or dry diet) in several aquatic species (red seabream - Dawood et al., 2015; Atlantic cod juveniles – Gildberg and Mikkelsen, 1998; gilthead seabream – Suzer et al., 2008; white shrimp – Kongnum and Hongpattarakere, 2012; Japanese eel – Lee et al., 2013). Carnobacteria have been shown to be effective in providing protection against several pathogenic bacteria through producing inhibitory compounds in salmonid fishes (Jöborn et al., 1997; Robertson et al., 2000). *C. divergens* has also been shown to be effective against *Vibrio anguillarum* in Atlantic cod juveniles (Gildberg et al., 1997), but no studies are available on the effects on cod larvae.

For larger juvenile and adult fish, the probiotic bacteria can be incorporated with the dry diet (Gildberg et al., 1997; Gildberg and Mikkelsen, 1998); however, smaller marine finfish larvae feed on live zooplankton, which necessitates the probiotic bacteria to be given through live feed (Gomez-Gil et al., 2000; Suzer et al., 2008). For these reasons, the aim of our study was to investigate the effects of *C. divergens* administrated to Atlantic cod larvae by live feed *Artemia franciscana* (hereafter referred as artemia) on growth and survival of larvae, larval microbiota and their disease resistance to *V. anguillarum*.

2. Materials and methods

2.1. Bacterial strains and growth medium

C. divergens Lab01 and Lab19, isolated from salmon and cod intestine, respectively (Ringø et al., 2001), were selected based on the results from previous studies on Atlantic cod juveniles using these strains as potential probiotics (Gildberg et al., 1997; Gildberg and Mikkelsen, 1998). These studies indicated positive effects on the growth, survival and disease resistance against *V. anguillarum*. In addition, the strains were shown to possess some antimicrobial activity. Carnobacteria were grown on Tryptic Soy Agar, (TSA; Sigma-Aldrich, St Louis, MO, USA) and a Modified All Purpose Tween (MAPT) medium at 20 °C. The MAPT medium was optimized for growth of the carnobacteria by replacing bacteriological peptone in the standard All Purpose Tween recipe (Sigma-Aldrich) with an equal amount of peptone prepared from fish slaughter waste. The fish peptone was prepared from hydrolysis of salmon backbone essentially according to Safari et al. (2012), except that the inactivation step for endogenous enzymes was not performed. Briefly, salmon backbones were mixed with water (1:1) and heated to 45 °C before 1% enzyme were added. Time of hydrolysis was 2 h. *V. anguillarum* O2b ID4299 (Schrøder et al., 2009) was grown in Marine Broth (MBr; Sigma-Aldrich) at 12 °C.

The two *C. divergens* strains used in the live artemia feed were first grown on TSA plates for 4 days. A small number of bacteria from the agar plates were inoculated in several pre-cultures of 10 mL MAPT for each strain and incubated at 20 °C for 20-24 h without shaking. Then 5 mL from each of the two pre-cultures were added to two new bottles with 500 mL fresh MAPT broth. These were incubated overnight at 20 °C for 20–24 h and the cultures were subsequently mixed, giving 1 L in total. The concentration of bacteria in this mixture was around 4.5×10^9 CFU mL^{-1} and was used to enrich the artemia before feeding to the larvae.

The bacterial isolate for the disease challenge trial, *V. anguillarum* O2b, was grown on blood agar plates with 2% saline. After 5 days on agar plates, pre-cultures (5 mL) were grown in MBr at 12 °C for 22 h. At this point, the pre-culture had reached OD600 ≈ 1.0 (approximately 10^9 CFU mL^1). Then 1 mL of the pre-culture was added to several bottles with 25 mL MBr and incubated to OD600 ≈ 1.0 again. At challenge, 20 mL of this bacterial solution mix was added to each challenge bucket, resulting in approximately 1×10^7 CFU mL^{-1} (see below).

2.2. Live artemia feed with carnobacteria

Artemia (INVE Aquaculture NV, Belgium) cysts were decapsulated and incubated for 24 h at 28 °C under strong illumination and aeration. On the second day after hatching, artemia nauplii were rinsed and transferred to a bucket containing one-litre filtered UV sterilized sea water. *C. divergens* Lab01 and Lab19 strains at equal quantities along with nutrient enrichments were added to the bucket containing rinsed artemia (giving about 1×10^7 CFU mL^{-1}*C. divergens*). After 45 mins, artemia nauplii fed with probiotic (hereafter PB artemia) were filtered and rinsed with filtered UV sterilized sea water. Similarly, a control enrichment of artemia without the probiotic (hereafter noPB artemia) was also prepared. Artemia with PB were fed to the cod larvae on four days during the experimental period (at 30, 35, 40 and 50 days post hatch - dph) and on the other days, larvae were fed with artemia without probiotic bacterium (Fig. 1A). However, for the noPB treatment, larvae were fed with artemia without probiotic bacterium throughout the experimental period. Larvae were fed three times on 30, 35, 40 and 50 dph and new enrichment was done for each feeding.

2.3. Fish and rearing conditions

Eggs and sperm were stripped from captive cod broodstock kept at the Centre for Marine Aquaculture (CMA), Nofima in Tromsø and were fertilized using standard protocol and the experiments were conducted in May–June 2016 (Hansen and Puvanendran, 2010). Fertilized eggs were incubated in 25 L conical silos with aeration and water flow rate of 1.5 L $mins^{-1}$ at 4 °C. At 100% hatch, cod larvae were transferred to 190 L circular fibre glass tanks. The temperature was gradually increased from 4 °C to 10 °C from 4 to 10 dph, respectively. Larvae were reared using standard rearing protocols used at the CMA (Hansen et al., 2016). Cod larvae were fed with rotifers from 2 to 29 dph and 25–53 dph with

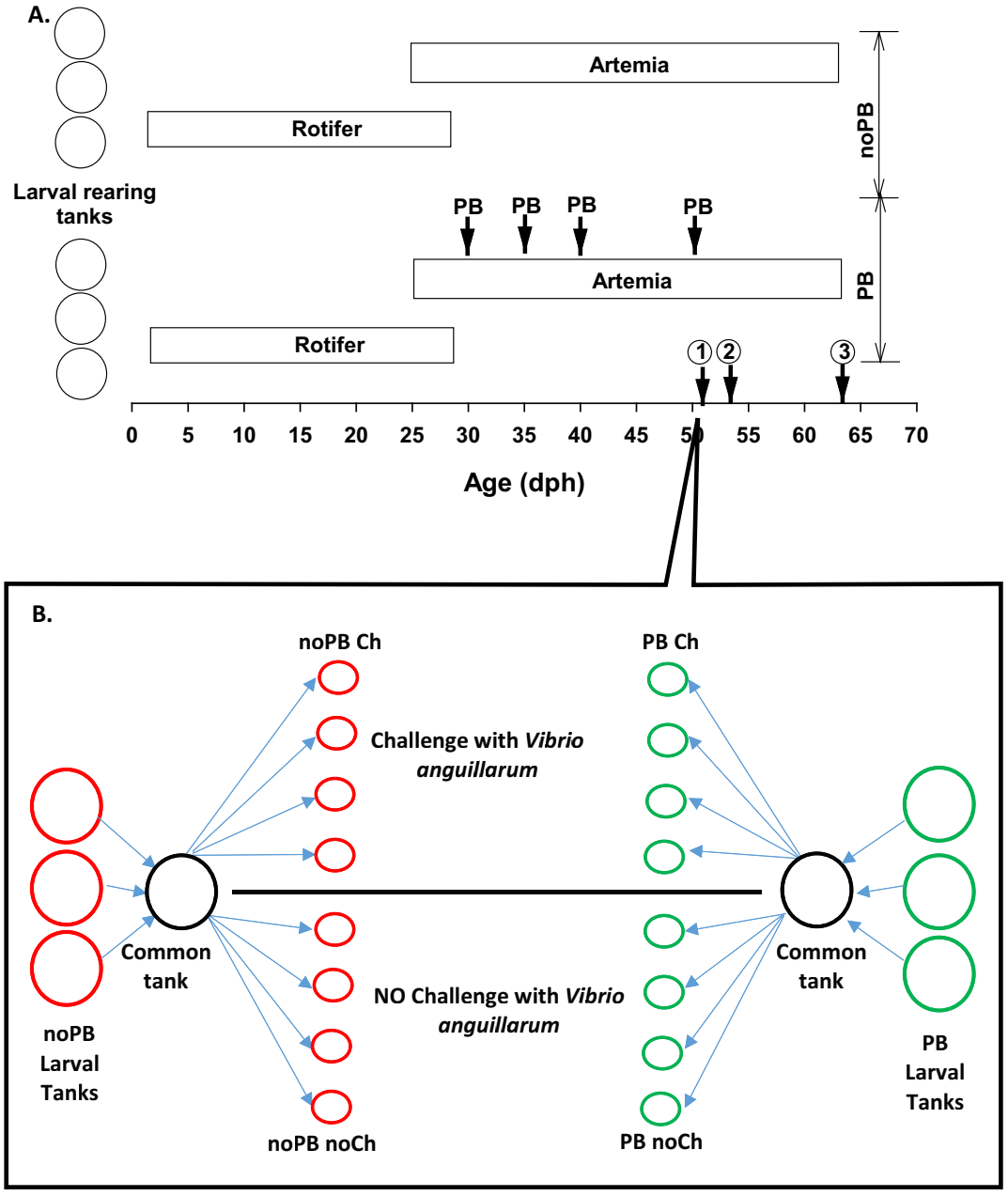

Fig. 1. A) Schematic diagram the experimental set-up. PB indicates the probiotic bacterium artemia feeding at 30, 35, 40 and 50 dph. - denotes the transfer of cod larvae to the health lab for disease challenge test at 51 dph; ② - start of the disease challenge test at 53 dph; ③ - denotes the end of the disease challenge monitoring at 63 dph. B) Disease challenge experimental set-up.

artemia (Fig. 1A; Hansen et al., 2016). *C. divergens* supplemented artemia nauplii were fed to the larvae at 30, 35, 40 and 50 dph (Fig. 1A). Two different treatment groups were established with three replicate tanks; 1) a control group where larvae were fed with enriched artemia without probiotic bacterium (noPB) and 2) a treatment group where larvae were fed with probiotic supplemented enriched artemia (PB). The feeding experiment was terminated at 80 dph. During the feeding experiment, the standard length of twenty cod larvae per tank (60 per treatment) was recorded once a week until 50 dph. Larvae were anaesthetised using MS 222 and the measurements were taken using a stereomicroscope. Wet weight and survival of the larvae were recorded at 80 dph.

2.4. Disease challenge experiment

A larval challenge test set-up and a model developed at the Fish Health Lab in the Aquaculture Research Station at Kårvika, Tromsø, were used to test the disease robustness of the larvae from both PB and noPB groups against the pathogen *V. anguillarum*, a common pathogen of Atlantic cod larvae and juveniles (Gildberg and Mikkelsen, 1998). Overview of the experimental treatment groups are indicated in Fig. 1B, with four replicate buckets per treatment group: noPB-noCh, noPB-Ch, PB-noCh and PB-Ch (Ch – challenged with *V. anguillarum* and noCh – no challenge with *V. anguillarum*). At 51 dph, larvae from all three noPB and PB rearing tanks were transferred to two common tanks (one each for noPB and PB) and then the homogenously mixed larvae were transferred to eight 20 L buckets for each treatment and each bucket was stocked with 40 larvae at the start of the challenge (Fig. 1B). Constant water flow

of sterilized seawater was used (400% exchange hr^{-1}). Larvae were acclimated to this condition for two days. During this acclimation period, any dead larvae were replaced with similar size/age larvae from the original stock. Just before the administration of *V. anguillarum*, the water was turned off. The two disease challenge groups (noPB-Ch and PB-Ch) were treated with *V. anguillarum* (one hour at a challenge dose of 1.1×10^7 CFU mL^{-1}) under continuous oxygenation and constant temperature of 10 °C. After 1 h, the water supply in all the buckets was restored. Water contamination was checked by plating the contaminated water on marine agar. The plates were dominated by fast growing white/yellow bacterial colonies. Survival of larvae was recorded twice a day for a minimum of 10 days post-infection; moribund or dead larva were removed and preserved for further microbiota analysis. During this challenge test period, the larvae were fed with standard enriched artemia 4–5 times daily.

2.5. Sampling for microbiota analysis

Artemia, larvae and water samples were collected for later microbiota analysis. On the day of PB artemia feeding (30, 35, 40 and 50 dph), both PB and noPB artemia (5 mL), were rinsed with filtered UV sterilized sea water and filtered through 200 μm sieve into vials, and stored at −80 °C until further DNA extraction. Ten larvae per tank were also sampled two days after probiotic feeding from both PB and noPB tanks (32, 37, 42 and 52 dph). In the disease challenge experiment, live larvae were sampled at the end of the challenge (day 10) and some moribund larvae were also randomly sampled during the challenge. All the collected larvae were anaesthetized with MSS 22 (100 mg L^{-1}) and surface disinfected using 0.1% benzalconiumchloride for 30 s. Then the larvae were rinsed twice with sterilized sea water and then washed in DNA-free MilliQ water before they were fixed in RNAlater. This above-mentioned sterilization procedure was done to minimize contamination of surface bacteria, thus, to detect mainly bacteria inside the larvae, e.g. the gut microbiota. The samples were left for two hours in room temperature, then kept overnight at 4 °C and frozen at −80 °C. Water samples of the larval rearing water (100 mL) were collected at 30, 35, 40 and 50 dph and filtered through 100 μm nylon filter into vials and stored at −80 °C.

2.6. DNA extraction and microbiota analysis

Bacterial DNA extraction was performed on thawed collected samples of artemia feed, larvae and water, with the following specifications: Approximately 200 mg of each artemia, all the whole larvae per sample tube (32–37-42 dph), half the tube at 52 dph, single whole larvae after challenge, and pelleted water samples. The PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories) was used for the bacterial DNA extraction following the manufacturer's protocol. The initial mechanical lysis step with bead beating was done twice using the FastPrep®-24 homogenizer (MP Biomedicals) for 60 s at 6 m s^{-1}.

The microbiota was analysed by 16S rRNA amplicon sequencing (2 × 150 bp) of the variable region 4 following our in-house protocol (Moen et al., 2016), which is presented in detail in supplementary methods of Caporaso et al. (2012). The sequencing was done on a MiSeq (Illumina) at Nofima using pooled PCR samples, which were based on duplicate PCRs per DNA sample using sample-specific barcoded reverse primers. Pooled PCR samples with low concentration of DNA (<5 ng/μL) were withdrawn before sequencing, including all water samples. DNA quantification was done using Quanti-iT Picogreen ds DNA Assay on Qubit (Invitrogen). PhiX Control v3 was included in the sequencing run and accounted for 10% of the reads. The MiSeq Control Software (MCS) version used was RTA 1.18.54.

2.7. Data processing of sequencing data

Data processing of the sequencing reads was performed using the pipelines in Quantitative Insight Into Microbial Ecology (QIIME) v.1.8 and 1.9 (Caporaso et al., 2010). Briefly, this included joining forward and reverse reads and removal of barcodes that failed to assemble. The sequences were demultiplexed into representative sample taqs and quality filtered, allowing zero barcode errors and a quality score of 30 (Q30). Reads were assigned to their respective bacterial taxonomy (Operational Taxonomic Unit: OTU) by clustering them against the Greengenes reference sequence collection (gg_13_8) using a 97% similarity threshold. Reads that did not hit a sequence in the reference sequence collection were clustered de novo. Chimeric sequences were removed using ChimeraSlayer, and all OTUs that were observed fewer than two times were discarded. This resulted in an OTU table with the final 56 samples, containing >4 million OTU counts. The OTU table was filtered to remove all OTUs with an average abundance <0.005%, narrowing the number of unique OTUs from 3841 to 212, but still containing >4 million OTU counts. All samples contained >20,000 OTU counts. The OTU table was used for alpha (within-sample diversity) and beta diversity analysis (between-sample diversity) using equal number of sequences across samples, where the OTU table was resampled to an even depth of 20,000 sequences per sample. Taxonomic summary of the OTU table was made at family level. However, OTUs assigned as family Pseudoalteromonadaceae were reassigned as Vibrionaceae when reassessing against the SILVA 132, NCBI (BLAST 16S rRNA) and RDP 11.5 databases. Erroneous annotations associated with Vibrionales and Pseudoalteromonadaceae is a known feature with the Greengenes database (Lydon and Lydon and Lipp, 2018). The OTU4339160 and OTU837040 were the OTUs most likely corresponding to the probiotic carnobacteria and the pathogenic *V. anguillarum*, assigned by the different databases as Lactobacillales, Enterococcaceae, Carnobacteriaceae or *Carnobacterium divergens*, and *Vibrio* sp. or *V. anguillarum* (Fig. S5), respectively.

2.8. Statistical analysis

The variance homogeneity of the data was performed using Levene's test. Larval growth, juvenile growth and survival, and the disease challenge survival data were compared by two-way ANOVA with age and treatment as variables, followed by Bonferroni post-hoc multiple comparison test when significant differences were found at a 0.05 level. Effect of treatment (noPB vs PB) or age (dph) on alpha diversity (number of observed OTUs) was tested using two-way ANOVA (Minitab v.19.2), while effect on beta diversity (microbiota composition) was tested using PERMANOVA (Qiime v.1.9). Principal component analysis (Unscrambler v.11) was used explorative to test for differences in microbiota at the OTU level between survived larvae after the disease challenge.

3. Results

3.1. Probiotic carnobacteria increased growth parameters and survival of larval cod

Cod larvae receiving artemia containing carnobacteria (PB) grew faster, as manifested 12 days after commencing treatment (42 dph) and onwards (Fig. 2A). The PB artemia fed larvae were significantly bigger than the noPB artemia fed larvae at 42 and 50 dph ($p < 0.0001$). Wet weight (Fig. 2B) and survival rate (Fig. 2C) of the PB-treated cod juveniles were also significantly higher compared to the control measured at 80 dph ($p < 0.023$ and 0.035, respectively).

3.2. Increased disease resistance of probiotic fed larvae to V. anguillarum

No major mortality was observed in any of the four experimental groups until day 4 post challenge (Fig. 3). Significant difference in post challenge survival after exposure to *V. anguillarum* was evident from day 5 between PB fed larvae and noPB fed larvae ($p < 0.019$) and continued until day 10 post challenge ($p < 0.0001$), when the experiment

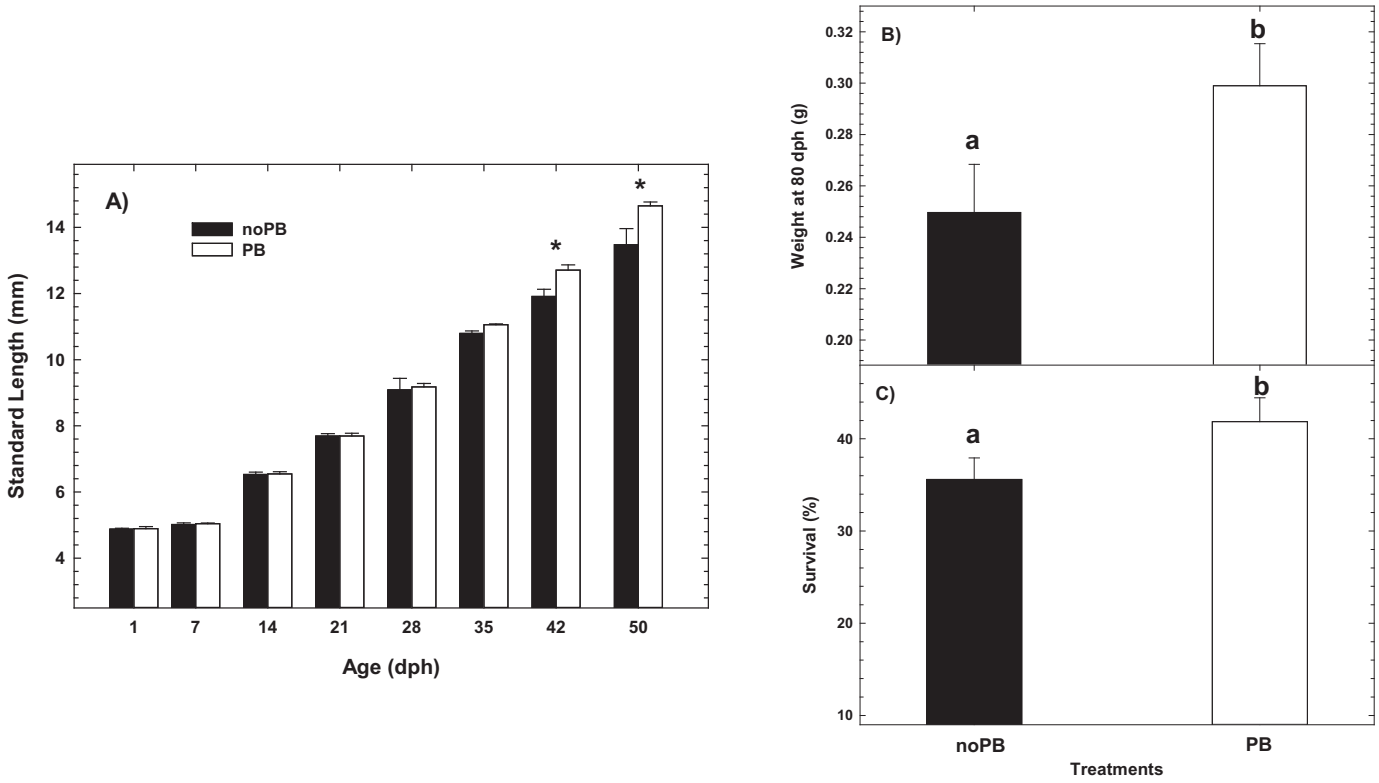

Fig. 2. A) Standard length of cod larvae from hatch to 50 dph; B) wet weight; C) survival of cod juveniles at 80 dph, fed with PB artemia and noPB artemia. Values are mean ± SD. * and different alphabets denotes significant difference at $p < 0.05$.

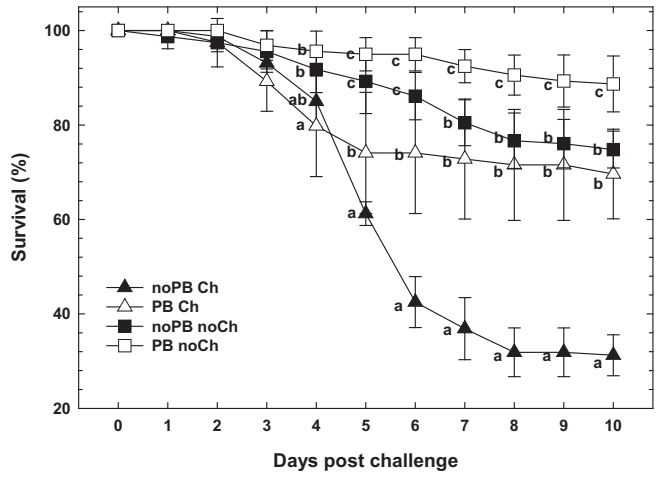

Fig. 3. Percent survival of cod larvae after exposure to *V. anguillarum* O2b ID4299. PB - larvae were fed with probiotic artemia during feeding; noPB - larvae fed with no probiotic (control) artemia during feeding; Ch - larvae were challenged with *V. anguillarum* O2b ID4299 and noCh - larvae were not challenged with *V. anguillarum* O2b ID4299. Values are mean ± SD. Different alphabets denote statistically significant differences between the different treatments at each time-point.

terminated. Higher survival rate (~70%) was observed for larvae fed with PB artemia compared to noPB fed larvae (~30%). Interestingly, survival of the PB-treated *Vibrio*-challenged larvae was similar to the non-challenged noPB-fed larvae (Fig. 3). PB-treatment also had a positive effect on survival of the non-challenged larvae population (Fig. 3) and a significant difference in survival between these two groups existed from day 7 onwards ($p < 0.034$).

3.3. No effect of probiotic feeding on larval microbiota

Beta diversity analysis showed that the larvae and artemia had different microbiota composition (Fig. 4A), explained by PC1, while differences between larvae pre-challenge and post-challenge was explained by PC2. Vibrionaceae was the main dominating family in all the larvae, while Rhodobacteraceae among others also dominated in the artemia (Fig. 4B). Differences in pre- and post-challenged larvae was reflected by presence of Shewanellaceae in pre-challenged, with higher presence of Pseudoalteromonadaceae, Rhodobacteraceae and Oleiphilaceae in larvae that survived the challenge period (Fig. 4B).

High abundance of the OTU4339160 (OTU with highest sequence match with *C. divergens,* Fig. S5, thereof referred to as carnobacteria), was detected in the live feed artemia exposed to PB at 30, 35, 40 and 50 dph, especially at the latest feeding at 50 dph (>60% of the total microbiota) (Figs. 4B and 5A). Only a slight relative increase of the carnobacteria OTU4339160 was detected in the PB-fed larvae two days after each artemia feeding, and the relative abundance was not significantly different compared to noPB-fed larvae ($p = 0.062$) (Fig. 5B). Impact of PB-feeding was indeed affecting the microbiota in the artemia, in terms of both alpha diversity ($p = 0.039$) and beta diversity ($p = 0.011$) (Fig. S1). However, PB-feeding had no significant impact on the microbiota in the pre-challenged larvae (Fig. S2). Instead age (dph) of the larvae was significantly affecting both the alpha and beta diversity of the larvae ($p = 0.000$ and $p = 0.001$), respectively (Fig. 4B and S2).

Larvae that survived the 10-day challenge period had no detectable levels of carnobacteria and there were no differences in microbiota composition between the PB-fed vs noPB-fed larvae (Fig. S3). Principal component analysis (PCA) of the dominating OTUs in all the post-challenged larvae revealed a difference in microbiota composition between the *V. anguillarum* challenged vs the non-challenged larvae at PC2 and PC4 (Fig. S4A). The challenged larvae were associated with higher relative abundance of OTUs assigned to *Vibrio* or *Allivibrio* species

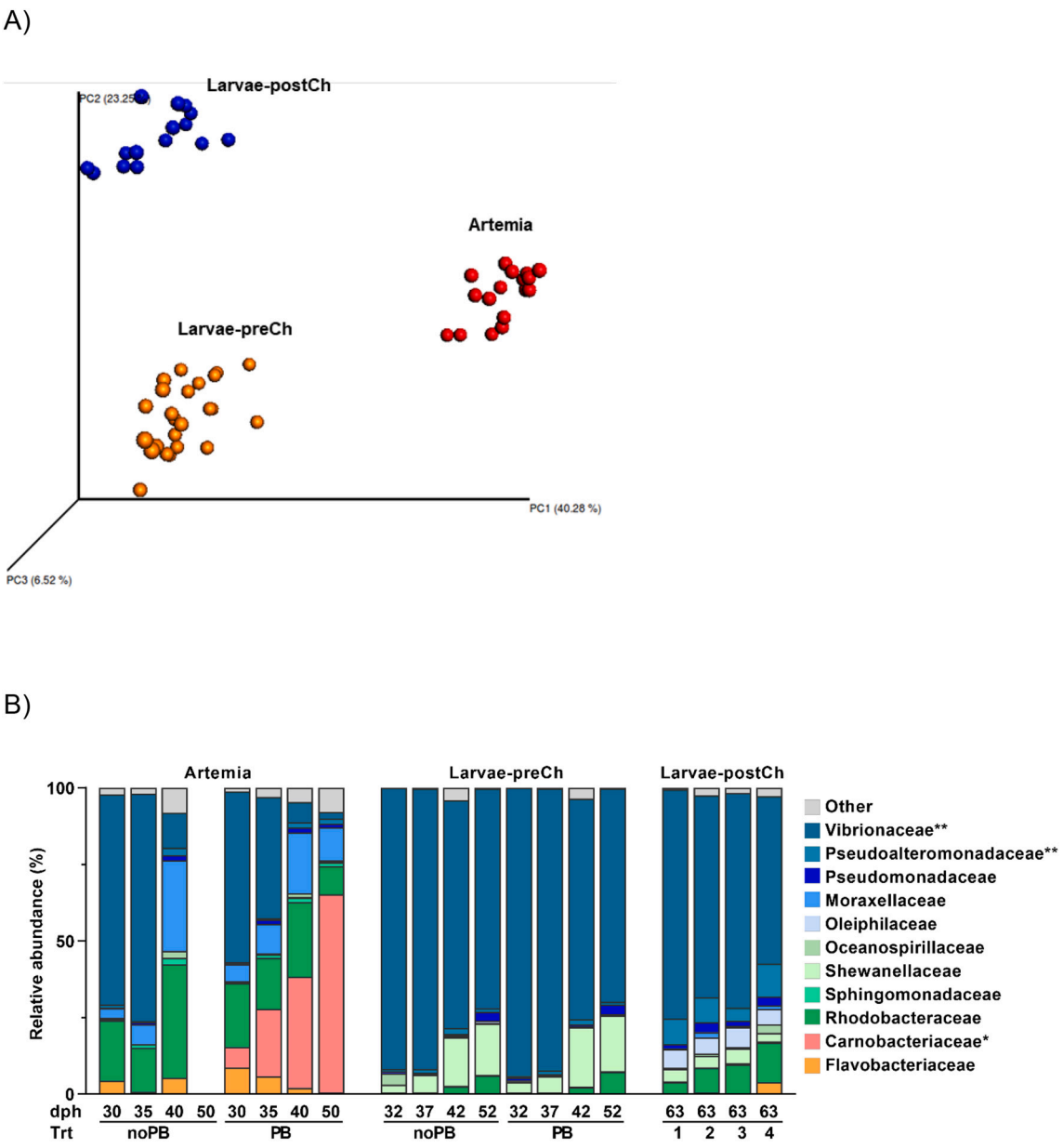

Fig. 4. Overview of microbiota in live feed artemia and larvae (pre- and post-disease challenge). A) Unweighted beta diversity of all samples. Red circles: artemia, orange: larvae pre-challenged, blue: larvae post-challenge. B) Relative abundance (%) of dominating families at different time points (dph) per treatment group (Trt) pre-challenge (preCh) (noPB vs PB) and post challenge (postCh): 1:Ch-noPB, 2: noCh-noPB, 3: Ch-PB, 4: noCh-PB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. S4B and S5), but not to the OTU with 100% sequence match with *V. anguillarum* (OTU837040). The OTU837040 was not among the dominating OTUs of the larvae that survived the challenge period but was highly present in several of the *V. anguillarum* challenged larvae that died during the challenge period (Fig. S4C).

4. Discussion

In our experiment, cod larvae fed with probiotic *C. divergens* in live feed artemia had better growth, survival and improved disease resistance against *V. anguillarum*. The latter has previously been demonstrated in cod fry fed *C. divergens* (Gildberg et al., 1997, Gildberg & Mikkelsen, 1998), but this is the first study showing this in cod larvae, as well as demonstrating a growth promoting effect of the probiotic.

The most common strategies of administering probiotics in marine finfish larvae are by incorporating the probiotic bacteria to live feed or by directly adding the probiotics to the rearing water or a combination of both methods (Lobo et al., 2014), and by continuous feeding throughout the study period (Suzer et al., 2008; Lauzon et al., 2010; Lobo et al., 2014; Dawood et al., 2015). In our study, we fed the cod larvae with probiotics through live artemia, intermittently four times (30, 35, 40 and 50 dph) during the 50-day feeding experiment, which indeed improved the performance of the larval cod. The intermittent feeding might be the reason why *C. divergens* not were detected as the dominant part of the larval microbiota neither before or after the challenge test, nor seemed to have any influence on the overall larval microbiota profile. Indeed, the intestinal microbiota in aquatic animals changes rapidly with the constant influx of microbes coming from water and feed, thus the intestinal microbiota will be transient in nature. Another reason might be due to the low detection of carnobacteria in the artemia used in the first feedings, thus assuming less successful enrichment in the artemia, further causing low detection of the carnobacteria

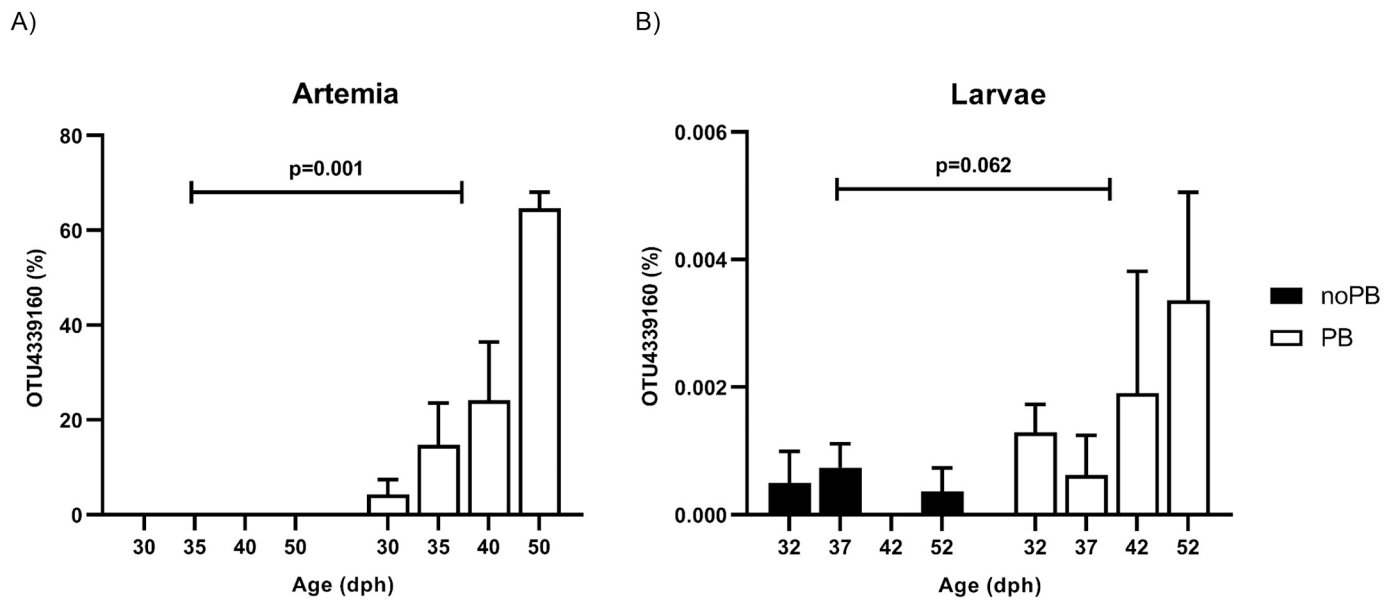

Fig. 5. Relative abundance (%) of OTU4339160 (*C. divergens*) in A) live feed artemia and B) larvae per PB-treatment group (PB vs noPB) pre-disease challenge. No detection of this OTU in the live larvae post challenge.

in the corresponding larvae (Fig. 5A and B). It should be taken into account that the detection of carnobacteria was based on 16S rRNA sequencing, which only gives relative abundance to the total microbiota and no information on the number of live carnobacteria. Gildberg et al. (1997) showed for instance by plate counts that cod juveniles fed with *C. divergens* dominated the intestinal microbiota of fished that survived the challenges. Skjermo et al. (2015) have also demonstrated low colonization success of probiotic bacteria in Atlantic cod larvae (also based on relative estimates), when administered intermittently (0, 2, 4, 8, 16, 30 and 45 dph) through live feed and the water. Similar as for Skjermo et al. (2015), the microbiology analysis in our study was based on DNA extracted from the whole larvae, due to the small sizes of the larvae. Even though, the larvae were surface disinfected and rinsed with sea water, the results will not completely represent the intestinal microbiota, and might be also reflected by the skin microbiota and the surrounding water.

We treated the Artemia nauplii with probiotic *C. divergens* for 45 mins before feeding nauplii to cod larvae. This short period makes it unlikely that *C. divergens* could have improved the condition of the nauplii and subsequently the performance of the cod larvae, although we cannot completely rule this out. Giarma et al. (2017) and Niu et al. (2014) have suggested such effects of probiotic bacteria fed to Artemia, but this involved much longer exposure times (24–144 h).

It has also been suggested that absence of substrates could be the reasons for the failure of probiotic bacteria establishing in the gut after discontinuing the probiotic feeding (Rurangwa et al., 2009), and that supplementation of non-digestible fibres and prebiotics may help the probiotic bacteria to establish in the gut. Gobeli et al. (2009) reported that a transient colonization of *Pseudomonas chlororaphis* strain JF3835 has reduced the mortality caused by *Aeromonas sobria* in juvenile perch *Perca fluviatilis*. Similar results have also been reported in giant freshwater prawn, *Macrobrachium rosenbergii* where transient colonization of *Lactobacillus plantarum* strain has improved the immune parameters (Dash et al., 2014). It can only be speculated that the better growth performance of the probiotic feed cod larvae, were related to nutritional benefits caused by *C. divergens*, as probiotic bacteria in the gut of the fish can secrete digestive enzymes and can help the host in digestion exogenously by producing digestive enzymes or endogenously by stimulating the host to produce enzymes (Lauzon et al., 2010; Lazado et al., 2012). Increased production of fermentation-derived short-chain fatty acids (SCFAs) by the probiotic bacteria during the transient colonization of the fish GI tract could also exert positive effects (Tyagi et al., 2019). In aquaculture, SCFAs have been used as feed supplements to promote fish immunity and growth (Hoseinifar et al., 2017).

It has been suggested in a review on the probiotic research on Atlantic cod (Lazado and Caipang, 2014b), that the positive performance of cod larvae by probiotic treatment may result from the production of inhibitory compounds, competition for nutrients and adhesion sites and protection against pathogens. Supernatants from *C. divergens* Lab01 and Lab19 broth cultures have previously been shown to possess some antimicrobial activity (Gildberg and Mikkelsen, 1998). However, these results could not be reproduced when neutralized supernatants and a well-buffered experimental system was used (unpublished observations), indicating that the activity could merely be due to organic acid production. In addition, since no major changes in microbiota could be detected with the PB feeding, there is no indication of competition for nutrients and adhesion sites between *C. divergens* and the microbiota members of cod larvae.

Another possible mechanism of the probiotic benefits of the cod larvae, may be of immunological nature. Cod larval immunity can be stage dependent, where larger larvae have a better developed immune system compared to smaller larvae. Information on immunostimulatory capabilities of probiotics in fish, and in cod particularly, is limited and fragmentary (Lazado and Caipang, 2014b). Though, candidate probiotics were shown to modulate the cutaneous immune response of epidermal cells of cod during interaction with pathogenic *V. anguillarum* (Lazado and Caipang, 2014a).

5. Conclusions

Feeding Atlantic cod larvae with probiotic carnobacteria strains improved growth parameters and disease resistance. The probiotics did not persist in the larvae and had no impact on the overall microbiota composition of the larvae, which rather changed over time due to other factors. Larval performance may not be the direct causative mechanism of the probiotic feed. Rather, the mechanism could be a direct interaction of *C. divergens* with the larval immunological or physiological mechanisms, leading to increased robustness and growth. Further research is needed to elucidate the mechanism(s) behind the positive effects of probiotic treatment in Atlantic cod.

Authors statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We would like to thank staff at the Centre for Marine Aquaculture and the Aquaculture Research Station Health Laboratory in Tromsø for their help in running the experiment. We also thank Merete R. Jensen and Ane Meisland for excellent technical assistance with the microbiota analyses, Dr. Carlo Lazado for his valuable comments on the manuscript and Nivetha Puvanendran for the grammatical editing of this manuscript. This work was supported by the Research Council of Norway (Project no. 227356).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.736072.

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