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The early life microbiome of giant grouper (*Epinephelus lanceolatus*) larvae in a commercial hatchery is influenced by microorganisms in feed

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Abstract

Fish health, growth and disease is intricately linked to its associated microbiome. Understanding the influence, source and ultimately managing the microbiome, particularly for vulnerable early life-stages, has been identified as one of the key requirements to improving farmed fish production. One tropical fish species of aquaculture importance farmed throughout the Asia-Pacific region is the giant grouper (*Epinephelus lanceolatus*). Variability in the health and survival of *E. lanceolatus* larvae is partially dependent on exposure to and development of its early microbiome. Here, we examined the development in the microbiome of commercially reared giant grouper larvae, its surrounding environment, and that from live food sources to understand the type of bacterial species larvae are exposed to, and where some of the sources of bacteria may originate. We show that species richness and microbial diversity of the larval microbiome significantly increased in the first 4 days after hatching, with the community composition continuing to shift over the initial 10 days in the hatchery facility. The dominant larval bacterial taxa appeared to be predominantly derived from live cultured microalgae and rotifer feeds and included *Marixanthomonas*, *Candidatus Hepatincola*, *Meridianimaribacter* and *Vibrio*. In contrast, a commercial probiotic added as part of the hatchery's operating procedure failed to establish in the larvae microbiome. Microbial source tracking indicated that feed was the largest influence on the composition of the giant grouper larvae microbiome (up to 55.9%), supporting attempts to modulate fish microbiomes in commercial hatcheries through improved diets. The marked abundances of *Vibrio* (up to 21.7% of 16S rRNA gene copies in larvae) highlights a need for rigorous quality control of feed material.

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Introduction

Groupers are a highly valued and sought after food fish with annual global wild harvest more than doubling over the past 20 years (approximately 184,000 tons in 2001 to 462,000 tons in 2021) [1]. However, with increased fishing pressure there is concern that many grouper species are at risk due to overexploitation [2]. The giant grouper (GG) (*Epinephelus lanceolatus*) is the largest known ray-finned fish that inhabits coral reefs. Wild populations of GG have been in decline from overfishing [3] owing to their protogynous hermaphroditism (i.e. females develop into males in later life) and late sexual development with an estimated population doubling time of 14 years [4]. As such GG were classified as a vulnerable species (International Union for Conservation of Nature, 1996 and 2006), and they are protected in several countries including Australia [2, 5].

Against the background of diminishing wild stocks, in recent years there has been increased production in farming groupers to supplement production from capture fisheries. In 2019–2020 aquaculture production of groupers accounted for 46.0% of annual global grouper supply compared to just 6.5% in 2001 [1]; however, the grouper aquaculture industry faces several challenges including a lack of reliable methods in housing and conditioning broodstock, difficulties in production of high-quality eggs, poor larval survival rates, requirements for specialized diets, occurrence of spinal deformities and susceptibility to diseases [6, 7]. Furthermore, the survival of grouper larvae in hatcheries is generally lower compared to other finfish [8]. GG larvae are sensitive to their environment including water quality and environmental changes [8, 9], and have small mouth gape requiring specialized diets to ensure adequate nutrition [8]. Although not generally associated with larval rearing, there have been documented cases of groupers in aquaculture systems infected with diverse bacterial pathogens, including well known examples such as *Streptococcus* [10, 11], *Vibrio* [12–15] and *Pseudomonas* [16].

Recently, the study of animal microbiomes has gained traction and the microbiome is now recognized as an important factor contributing to host health, growth, and nutrition [17]. The fish microbiome has been implicated in various functions ranging from feeding and metabolism, stress, and immune responses, to reproduction and development (reviewed in [18]). Unlike the vertical transmission of microbiomes evident in mammals, aquatic fish predominantly recruit their microbiomes from the surrounding environment, wherein site [19] and diet [20] have been reported to significantly influence composition of the fish's microbiome beginning from early larval stages [21]. Since site and diet are typically controlled under aquaculture settings, these findings raise the potential of exploiting the fish microbiome as an avenue to enhance aquaculture productivity. In this study, we partnered with The Company One Pty Ltd (TCO), a commercial GG hatchery producer, to profile the microbiome of GG larvae using high throughput 16S rRNA gene amplicon sequencing with the aim of generating microbiome baselines during the first 10 days post hatching. In addition, we generated microbial profiles from tank water, fish feed and other additives to track the source of microorganisms recruited into the GG larvae microbiota. As healthy development of GG, and ultimately aquaculture production success, is heavily dependent on their early life larval stages [8], results from this study will inform aquaculture management decisions by providing insight into the formation of the GG larvae microbiome during early life stages.

Materials and methods

Larval husbandry and hatchery setup

This study was performed in partnership with TCO (Cairns, Australia), the only commercial captive GG hatchery and aquaculture facility in Australia that houses wild broodstock under controlled conditions that allow the broodfish to spawn regularly. As per the company's operating procedure, fertilized eggs were exposed to ozonated water (75 ppm for 80 s) and stocked into four 10 kL tanks at a volume of 150 mL eggs per tank. The four tanks were within a recirculating aquaculture system (RAS) with a sump connected for mechanical and biological filtration and ultraviolet (UV) treatment (Fig. S1). All tanks were housed in the same room and water temperature maintained at 28 °C. For the first three days, individual tanks were kept static without water exchange. From day 4, water exchange started at 200 L/h/tank, discharging to the central sump for treatment and recirculation. The exchange rate was increased daily to maintain tank water quality. Additionally, new filtered, ozone and UV treated seawater was added to the RAS starting from day 6 and increased daily to maintain overall water quality within the RAS. Measurements including dissolved oxygen, ammonia concentration, pH and temperature were recorded daily.

Tank additions including microalgae and copepods

Three types of microalgae were added to the hatchery tanks in batches. *Tetraselmis* sp. (strain CS317) was added to the tanks on day 1, followed by Tahitian strain of *Isochrysis* sp. (strain CS177) from day 2 to 9 and *Nannochloropsis oceanica* (strain CS246) from day 3 to 9 (Fig. 1). The calanoid copepod *Parvocalanus crassirostris* was added on day 2 at a concentration of 2 adults/mL and 1 nauplii/mL into each hatchery tank. Daily average nauplii counts were recorded (Fig. 1). On days 3, 4, 6, 7, 8 and 9, the rotifer *Brachionus rotundiformis* was added into each hatchery tank at a concentration of 12.3±0.38

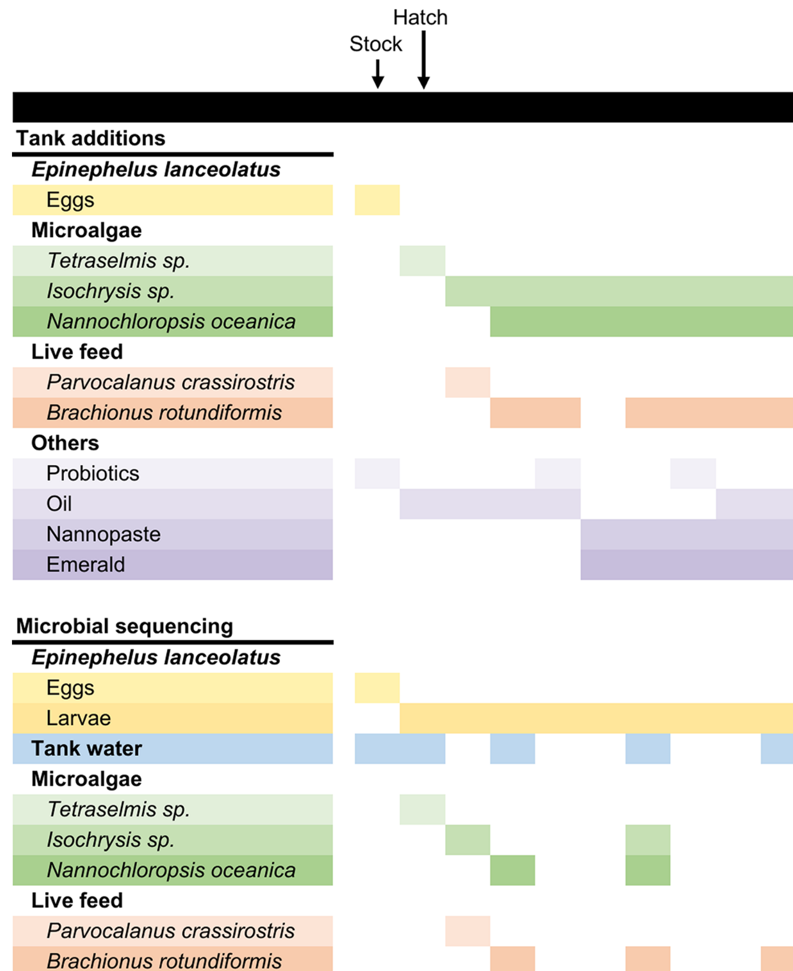

Fig. 1 Timeline of this study. Commercial, microalgae and live feed additions to the tanks are shown on top while samples processed and sent for 16S rRNA gene amplicon sequencing are shown at the bottom. dph indicates number of days after giant grouper (*Epinephelus lanceolatus*) eggs hatched

rotifers per tank (Fig. 1). A commercial live *Bacillus* probiotic, Sanolife® MIC (INVE Aquaculture, USA) was added to tank water (10 g per tank) on days 0, 4 and 7 (Fig. 1).

Sample collection

On day 0, tank water samples were collected prior to stocking the tanks with GG eggs to obtain starting microbial community profiles (Fig. 1). Tank water was collected by gently submerging a sterile 50 mL Falcon tube just below the surface of the water in each quadrant of the tank (n=4 per tank, Fig. S1). As eggs were ozonated for stocking, a sample was collected for microbial profiles before and after ozonation. The eggs hatched the next day (day 1), and larvae were collected by gently scooping into sterile 50 mL Falcon tubes. Tank water was removed from the tubes and larvae were washed in autoclaved sterile seawater and immediately frozen at -80 °C. Daily tank inputs including feed (copepods and rotifers, n=3), microalgae (n=3), tank water (n=4 per tank) and larvae samples (n=3 per tank) were collected from days one to nine (Fig. 1). To ensure reproducibility, tank water samples were collected daily after microalgae addition, but before copepod and rotifers were added to the tanks. Larvae were collected approximately 1–2 h after feeding with copepods or rotifers. Microalgae, copepod and rotifers were sampled by scooping from individual tanks containing homogenous batch preparations of each food source using sterile 50 mL Falcon tubes (n=3 per batch). All samples were stored at -80 °C immediately.

DNA extraction & quantification

Samples were pre-processed prior to DNA extraction. Tank water and microalgal samples were filtered through sterile Millex® 0.22 µm polyethersulfone syringe filters (Merck KGaA, Germany). The syringe filters were then incubated with 250 µL lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 1.2% Triton X-100, 20 mg/mL lysozyme) for at least 30 min at 37 °C to lyse bacteria captured on the membrane. After this incubation, proteinase

K was added to the syringe filters to a final concentration of 2 mg/mL and incubated overnight in a rotating oven at 56 °C. The resulting lysate was eluted with 500 μL of lysis buffer followed by 500 μL Buffer AL (part of the QIAGEN DNeasy Blood and Tissue Kit) and used for downstream DNA extraction. Copepod and rotifer samples were first pelleted at 5000 x g for 5 min at 4 °C. After removing the supernatant, the pellet was resuspended in 1 mL lysis buffer containing 2 mg/mL proteinase K and incubated at 56 °C until completely digested. Larvae were rinsed in sterile sea water, followed by overnight incubation in 500 μL lysis buffer containing 2 mg/mL proteinase K at 56 °C.

After the digestion steps described above, bacterial genomic DNA was extracted from all sample lysates using the DNeasy Blood and Tissue kit (QIAGEN, United States) according to manufacturer's instructions with DNA eluted in TE buffer. DNA purity was examined by gel electrophoresis (1.2% agarose) and concentration determined using a NanoDrop One UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States). To confirm successful bacterial DNA extraction, DNA from a random subset of samples were tested in a PCR using 16S rRNA primers targeting the prokaryotic V3-V4 region (341F: 5'-CCTACGGGNGGCWGCAG-3'and 806R: 5'-GGACTACNVGGGTWTCTAAT-3' [22, 23], to check for amplification. PCR was performed using Platinum II Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific, United States) and the presence of PCR amplicons checked on a 1% agarose gel. Concentration of PCR amplicons was measured using a QuantiFluor ONE dsDNA System and Quantus Fluorometer (Promega, United States).

Droplet digital PCR analysis

The QX200 Droplet Digital Polymerase Chain Reaction (ddPCR) System (Bio-Rad Laboratories, United States) was used to determine bacterial load. Universal bacterial 16S rRNA gene primers were used (1406F: 5'-GYACWCACCGCCCGT-3'; 1525R: 5'-AAGGAGGTGWTCCARCC-3') to quantify total bacterial DNA concentration [24]. Primers specific for the genus *Vibrio* (567 F: 5'- GGCGTAAAGCGCATGCAGGT-3'; 680R: 5'-GAAATTCTACCCCCCTCTACAG-3') were used to quantify DNA concentration of *Vibrio spp.* [25]. Multiplex ddPCR was performed in 25 μL volumes with each reaction consisting of 1X QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, United States), 300 nM of each primer and 5 μL template DNA. For DNA negative controls, 5 μL DNase/RNase free water was added instead of extracted DNA. After preparing the PCR reactions, 20 μL of each reaction mixture was loaded into a sample well of an 8-well cartridge of a droplet generator (Bio-Rad Laboratories, United States) to generate 20,000 droplets according to the manufacturer's instructions. After generating droplets, PCR amplification was performed using the C1000 Touch Thermal Cycler (Bio-Rad Laboratories, United States) with the following thermocycling conditions: initial enzyme activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s and primer annealing at 56 °C for 1 min; followed by a final inactivation step at 98 °C for 10 min. Droplet fluorescence was measured using a QX200 droplet reader (Bio-Rad Laboratories, United States) and data analysed with the QuantaSoft analysis software (version 1.0.596, Bio-Rad Laboratories, United States). Droplets were considered positive when fluorescence was above the background threshold of the negative droplets of the same sample and of negative controls. The number of molecules of target DNA present in the 20 μL reaction mixture was determined from the ratio of positive/total droplets. Quality controls included the presence of >10,000 droplets of size and structure checked by the QuantaSoft software, <10 positive droplets in negative controls and >5 positive droplets in positive controls. In addition, the lower limit of detection was defined as at least 5 positive droplets, and the upper limit of detection was defined as at least 5 negative droplets [26].

16S rRNA gene amplicon sequencing

PCR amplification of the 16S rRNA gene for microbial community profiling and the subsequent sequencing library preparation for amplicon sequencing was performed by the Australian Genome Research Facility (AGRF, Melbourne, Australia). Briefly, extracted genomic DNA were first PCR amplified using 16S rRNA primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'), targeting the V3-V4 regions of the 16S rRNA gene. Sequencing libraries were then constructed using the Illumina Nextera XT Index Kit, pooled, and sequenced on an Illumina MiSeq platform (300 bp paired-end sequencing). Raw data are available at the Sequence Read Archive under accession PRJNA1120584.

16S rRNA amplicon sequence data processing and statistical analyses

Demultiplexed sequence reads were provided by the AGRF in fastq format. All reads were imported into QIIME™ 2 v2022.2 [27] for quality filtering and processing using the DADA2 workflow [28]. Adapter, primer, and low-quality sequences (median quality score<20) were trimmed, and remaining reads subsequently denoised, merged and checked for chimeras using default settings in DADA2. Alpha diversity metrics (species richness and Shannon's Index) were calculated based on subsampling sequence counts at a depth of 2930 reads 100 times (Fig. S2). To infer taxonomy of the 16S rRNA gene reads, a classifier was first trained on V3-V4 regions of reference

16S sequences downloaded from the SILVA 16S database v138.1 [29]. The trained classifier was then used to infer taxonomies of the representative 16S sequences output by DADA2. The final 16S counts table based on amplicon sequence variants (ASVs) was exported from QIIME2 and used as input into R v4.2.1 for statistical analyses [30]. All QIIME2 scripts are included in supplementary file 1. A centered log ratio (CLR) transformation was first applied to the counts data. Permutational multivariate analysis of variance (PERMANOVA) and linear models were then used to assess associations between community composition and experiment variables using the CLR transformed counts as Euclidean distances. Principal component analysis (PCA) was used to visualize the clustering of samples based on compositional similarities of their microbial communities. Relative abundances were calculated by dividing each ASV count by total library size (total sum scaling). Microbial source tracking was performed using fast expectation-maximization microbial source tracking (FEAST) [31] on the 16S counts to estimate the proportion of the microbial communities attributable to respective input sources. PERMANOVA and PCA are implemented in the vegan R package v2.4–6 [32]. Figures were edited in Inkscape v0.94 for clarity.

Construction of *Vibrio spp*. phylogenetic tree

Representative 16S rRNA gene sequences from ASVs classified as *Vibrio* were used to search for closely related sequences within the SILVA 16S reference database using the online search tool available at https://www.arb-silva.de/aligner/. Sequences reported by the search tool were then aligned with the *Vibrio* ASVs using MAFFT v7.508 [33], and the resulting sequence alignment was trimmed in silico according to the 341 F and 806R 16S primers using SeqKit v2.6.0 [34]. The trimmed sequence alignment was used to infer a phylogenetic tree using IQTREE2 v2.2.0.3 [35] with 2000 bootstraps. The output tree was midpoint rooted using GenomeTreeTk v0.1.8 (https://github.com/donovan-h-parks/GenomeTreeTk) and visualized in ITOL v6 [36]. All scripts are included in supplementary file 1.

Results

Stable water quality metrics and conditions during study duration

Fluctuations in water temperature, pH, dissolved oxygen, and ammonia concentration were largely consistent across all four tanks over the nine days of sampling. Average water temperature in the four tanks ranged between 27.5 °C and 27.6 °C. Tanks T3 and T4 experienced a drop of about 1.5 °C over a 12 h duration on day 4 due to a water heater malfunction (Fig. S3A), although this did not affect dissolved oxygen, pH or ammonia concentration (Fig. S3B-D). Average dissolved oxygen ranged between 6.75 mg/L and 7.77 mg/L, while pH ranged between 8.11 and 8.46 over the course of the study. Average ammonia concentration over the nine days was slightly higher in tank T1 compared with tanks T2, T3 and T4 (0.29 mg/L vs. 0.21–0.22 mg/L), with all values within the acceptable range of the hatchery SOP.

Development of the larvae-associated microbiota

Amplicon sequencing of DNA generated an average of 6866 sequences per sample. Identical sequences were clustered resulting in 2738 ASVs overall. Each sample was rarefied to 2930 sequences to calculate alpha diversity metrics including microbial community richness, Shannon diversity and Faith's phylogenetic diversity. Eggs washed in ozonated water prior to stocking, as part of hatchery operational procedures, showed that microbial community richness, but not diversity, was significantly reduced in post-washed eggs compared to before washing ($p < 0.05$, Kruskal-Wallis test) (Fig. 2A-C). This implies that the population load of some bacterial species is unequally impacted by ozone treatment compared to other species, although treatment does not eliminate them completely. For instance, the most notable change in community composition was a reduced overall proportion of *Pseudoalteromonas* from an average 69.6% relative abundance to 57.3%, and an increase in *Tenacibaculum* from 9.6 to 18.4% after washing (Fig. S4A) (Table S1). After stocking washed eggs in the four tanks, larvae were collected daily for nine days to monitor development of the larvae microbiota. Using PCA to visualize compositional shifts in the larvae microbiota (Fig. 2D), we observed a developmental trajectory characterized by rapid increases in microbial species richness and diversity over the first four days post hatching ($p < 0.05$, linear mixed model). While community composition continued to develop beyond four days post hatching as indicated by the continued shift of samples in the PCA ($p < 0.05$, pairwise PERMANOVA; $p < 0.05$, PERMDISP2 for beta dispersion) (Table S2), richness and Shannon diversity largely plateaued around 150 unique ASVs and ~6.5, respectively. Absolute bacterial load in larvae measured using ddPCR indicated that it remained largely stable throughout the study (Fig. S5B).

Larvae microbiota is primarily comprised of microbes from feed

Although sequences affiliated with *Pseudoalteromonas* and *Tenacibaculum* were detected at high relative abundance in washed eggs (57.3% and 18.4%, respectively), they were detected at markedly lower relative abundance in larvae one day post hatching (average 4.1% and 2.4%, respectively) and continued to decline in proportion throughout the study duration ($p < 0.01$, generalized linear model [GLM]) (Fig. 3) (Table S1). Instead,

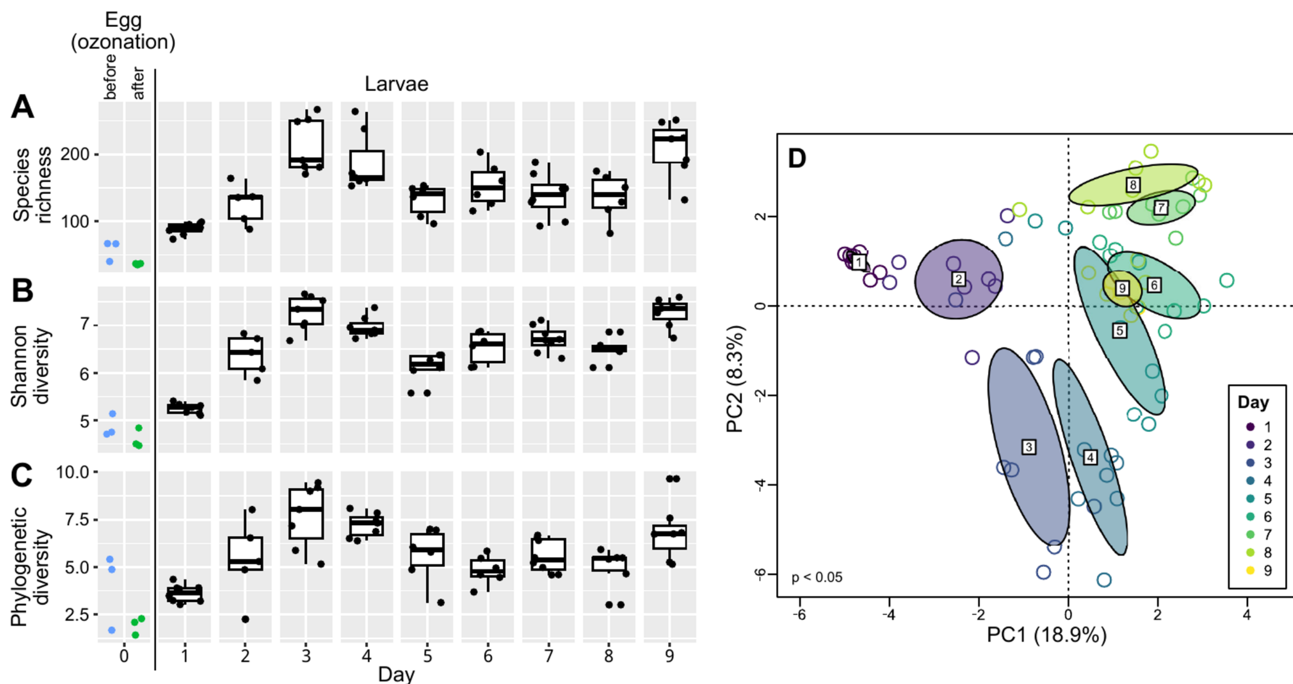

Fig. 2 Microbial community alpha diversity and composition in giant grouper eggs (before and after ozone wash) and larvae. Box and whisker plots of alpha diversity metrics including (**A**) number of observed species, (**B**) Shannon diversity, and (**C**) Faith's phylogenetic diversity calculated from rarefying samples to a depth of 2930 sequences 100 times. The thick centre within each box represents median values, and the upper and lower boundaries of the boxes represent the upper and lower quartiles, respectively. Whiskers extend to 1.5x interquartile range. (**D**) Principal component analysis (PCA) visualization of larvae-associated community composition over nine days. The PCA was performed on centered log ratio-transformed 16S counts as Euclidean distances. Each ring represents one replicate coloured by day of sampling. Coloured ellipses represent the 95% confidence limits of the group (sampling day) centroids

Alteromonas was the dominant genus in the larvae microbiota on day 1 (84.3% average relative abundance), and it declined in relative abundance ($p<0.01$, GLM) and was replaced by *Marixanthomonas* on day 4 as the most dominant genus (26.3% vs. 8.9% average relative abundance). *Alteromonas* was also present at high relative abundances in tank water (average 57.9% relative abundance) on day 1, although it failed to establish as its relative abundance declined to <1% in both tank water and larvae by day 6 ($p<0.01$, GLM). By day 9, several other genera in the larvae microbiota had increased in relative abundance, the top five including *Marixanthomonas* (16.7%), *Marivivens* (6.6%), *Algicola* (5.5%) ($p<0.01$, GLM), *Vibrio* (4.6%), and *Pseudofulvibacter* (4.4%) accounting for 37.8% of the larval microbiota affiliated reads (Fig. 3) (Table S1). Although a *Bacillus* probiotic product was added to the tanks on days 0, 4 and 7, *Bacillus* ASVs were detectable in the larvae for only one day after addition at 0.03%, 0.51% and 0.29% on days 1, 5 and 8, respectively (Table S1).

To estimate the contribution of the surrounding environment (tank water, feed) to composition of the larvae microbiota, we generated microbial community profiles from tank water, feed and additives, including live cultures of copepods, rotifers, and the alga *Tetraselmis*, *Nannochloropsis* and *Isochrysis* raised at the hatchery (Fig. S4B-E). The resulting tank water and feed microbial community profiles were then used as input sources in a microbial source tracking analysis. The analysis indicated that the larvae microbiota was primarily derived from microorganisms present in feed, with a maximum of 55.9% of the larvae community contributed from feed on day 5 (Fig. 4) (Table S3). The main taxa can be traced, for example, to increasing relative abundances of (i) *Marixanthomonas* in larvae beginning day 2 ($p<0.01$, GLM) coinciding with the addition of *Isochrysis* feed in which *Marixanthomonas* was detected at 42.5% relative abundance, (ii) *Candidatus Hepatincola* and *Meridianimaribacter* from day 4 and 7, respectively ($p<0.01$, GLM), coinciding with addition of rotifer feed in which the two genera were detected at 41.5% and 22.8% relative abundance, respectively, and (iii) *Vibrio* from day 3 ($p<0.01$, GLM) coinciding with addition of copepod and rotifer feed in which *Vibrio* was detected at 1.1% and 1.6%, respectively (Fig. 3, S3) (Table S1). In contrast, additives consisting of commercial probiotic products did not substantially contribute to development of the larvae microbiome. The contribution from tank water was estimated at an average 9.8%, which was comparatively lower than the contribution from feed likely due to UV treatment

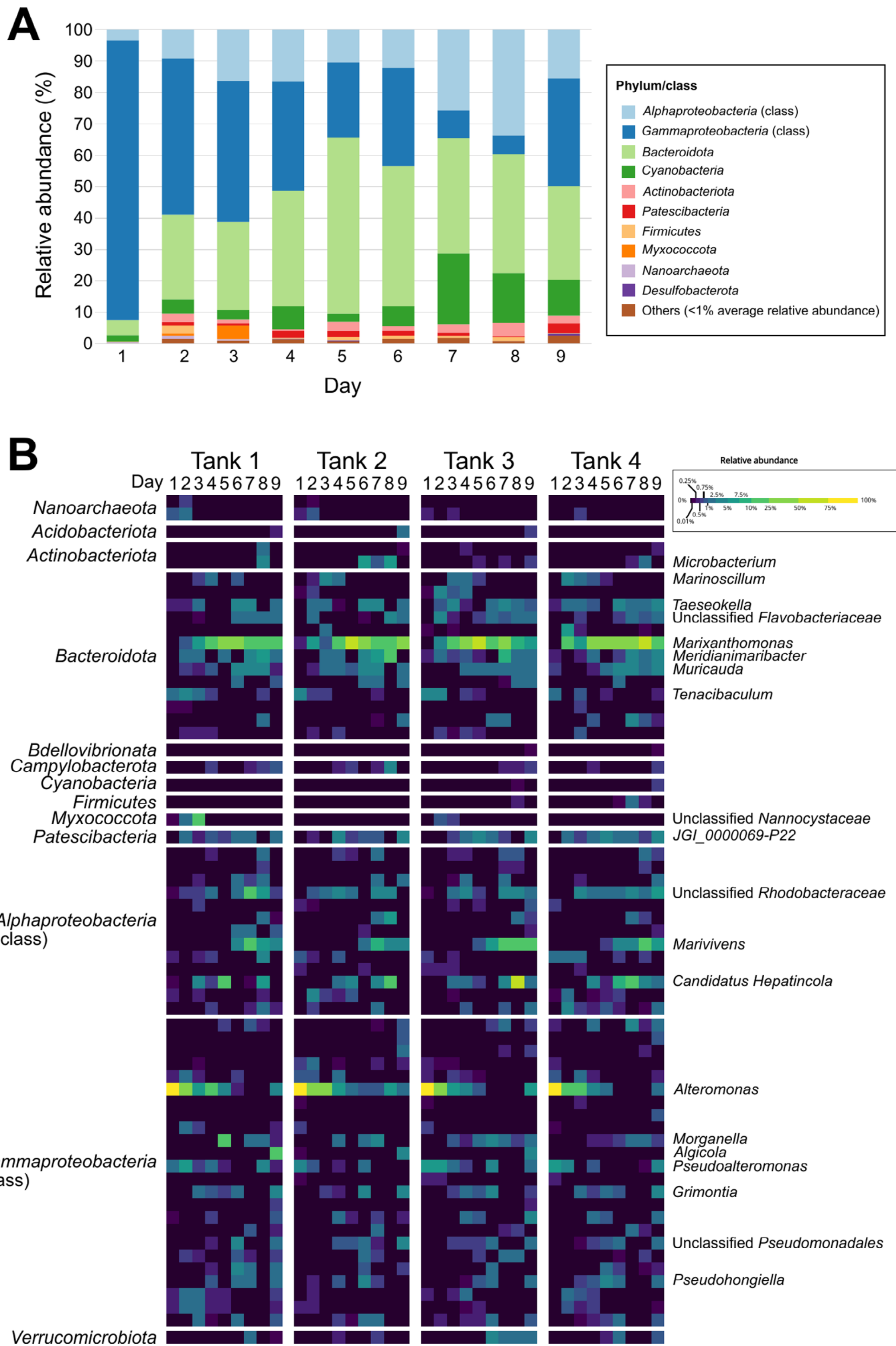

Fig. 3 (**A**) Average relative abundances of microbial phyla detected in the larvae over the study duration. (**B**) Heat map of giant grouper larvae microbial community composition over the study duration. Labels to the left of the heat map indicate phyla (class for *Alphaproteobacteria* and *Gammaproteobacteria*) while labels on the right indicate genera. Only genera whose relative abundances significantly vary over the study duration are included in the heat map (generalized linear model, $p < 0.05$ and false discovery rate < 0.2). Genera labels are listed for genera with average relative abundance > 0.5% for clarity. See Table S1 for all relative abundance values

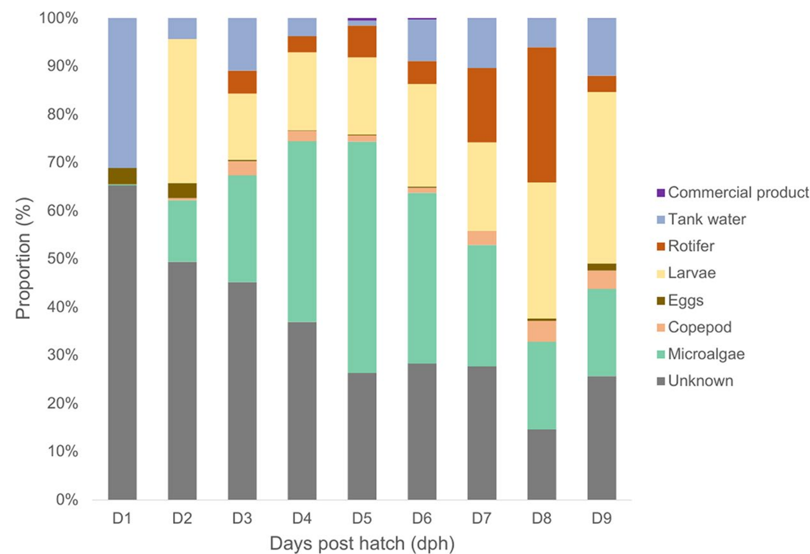

Fig. 4 Proportion of larvae microbial communities contributed from potential sources inferred using fast expectation-maximization microbial source tracking (FEAST) [31]. See Table S3 for numerical output from FEAST. Commercial product includes all commercial additions including probiotic, oil, nannopaste and emerald while microalgae include *Tetraselmis sp.*, *Nannochlropsis oceanica* and *Isochrysis sp*

and filtration implemented in the RAS to control microbial load in tank water.

Vibrio detected in GG larvae possibly related to putative pathogens

Since *Vibrio* are often implicated in zoonoses in fish [37] and were detected in larvae at appreciable abundances through 16S rRNA gene amplicon sequencing (up to 12.9% of average bacterial load across tanks) and ddPCR (up to 21.7%) (Fig. 3, S5, S6), we thus examined whether the GG larvae *Vibrio* ASVs were related to known *Vibrio* pathogens. By comparing the *Vibrio* ASVs with closely related sequences obtained from the SILVA database sequence search tool, a phylogenetic tree inferred from these sequences indicated several ASVs formed monophyletic clades with putatively pathogenic *Vibrio* species such as *V. coralliilyticus* [38], *V. tubiashii* [39], *V. fortis* [40], *V. proteolyticus* [41] and *Vibrio* sp. 5-BBD-M4 (coral black band disease isolate) (Fig. 5). However, the phylogenetic tree lacked strong bootstrap support (<95% UFBoot) [42], likely due to the short 16S V3-V4 ASVs used. These results indicate that while the *Vibrio* ASVs in GG larvae are potentially closely related to putative *Vibrio* pathogens as reported by the SILVA sequence search tool, their exact species identity requires longer 16S rRNA sequences or genome sequence data to be resolved.

Discussion

The microbiome of fish bred in captivity is distinct compared with their wild counterparts [43–45]; however, the implications on host health and productivity, if any, are still unknown. Research of captive bred fish predominantly focuses on diseases and pathogenic microbes, although in recent years there is growing interest in exploiting the microbiome to guide breeding practices and increase production yields [46–48]. In the present study, we examined the microbiome of GG larvae raised in a commercial hatchery over the first 10 days of larval culture and show that the GG's microbiome composition is primarily influenced by microorganisms from feed sources. While our larvae microbiome community profiles represent that in the entire larvae (as they were too small to subsample individual body sites like the gut), our observations were consistent with microbiome surveys conducted in aquaculture of Atlantic salmon (*Salmo salar*) [20], yellowtail kingfish (*Seriola lalandi*) [49] and Nile tilapia (*Oreochromis niloticus*) [50] in which gut microbiome composition of the fishes reflected microorganisms found in their respective diets, indicating that the fish gut microbiome is markedly influenced by microorganisms in feed. It has been suggested that the influence of diet on fish gut microbiomes is a carry-over effect of bacterial DNA from feed present in the gut [20], and our sampling strategy designed around hatchery operations does not preclude this proposed carry-over as we sampled larvae 2 h after feeding. Nevertheless, in the microbial source tracking analysis we identified a consistent source proportion of the larvae microbiome attributed to larvae (i.e. ASVs shared with larvae-associated microbial communities from the previous day), which suggests that these feed-derived microbes were becoming established in the larvae. In this batch of GG larvae, several bacterial taxa including *Marixanthomonas*, *Candidatus Hepatincola*, *Meridianimaribacter* and *Vibrio* were inferred to originate from the algae and rotifer feed,

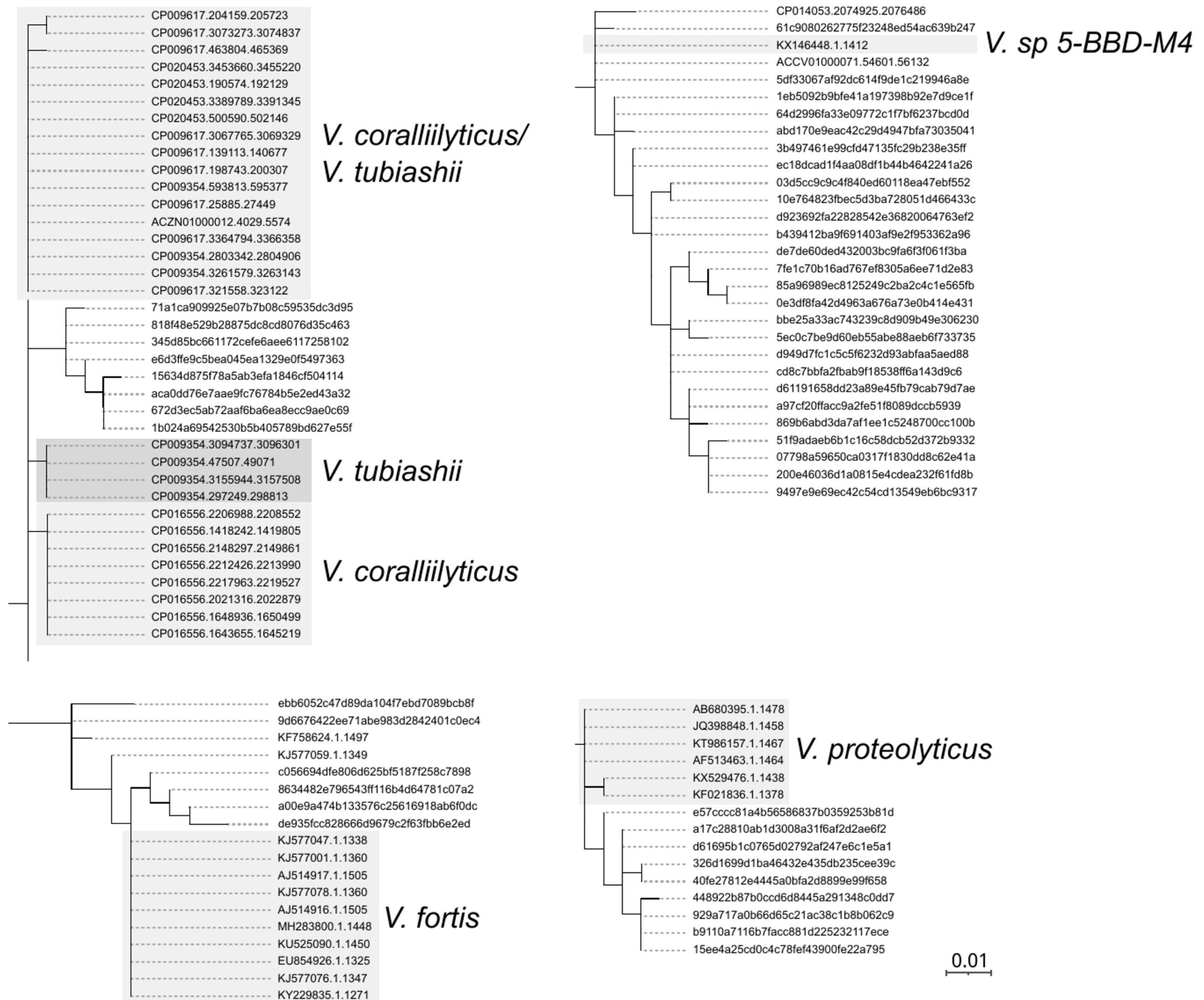

Fig. 5 Phylogenetic tree of *Vibrio* amplicon sequence variants (ASVs) detected in this study and their closest neighbours retrieved from the SILVA 16S database v138.1 (https://www.arb-silva.de/). Reference *Vibrio* sequences from SILVA are indicated by a grey background, while ASVs from this study are indicated by their respective ASV IDs (string of lowercase alphanumeric characters) generated in QIIME2. Sequences were aligned using MAFFT and phylogenetic tree was inferred using IQTREE2

but other than *Vibrio* not much is known about their relevance to fish health and physiology. *Marixanthomonas*, first isolated from a deep sea brittle star [51], have been reported to be enriched in the gut of giant prawns fed a diet of *Thalassiosira weissflogii* microalga [52]. *Candidatus Hepatincola* is likely a parasitic microorganism associated with isopods, insects, marine invertebrates [53] and possibly rotifers as our data suggests, while *Meridianimaribacter* is likely a lignocellulose degrader largely described in marine sediment [54] and soils [55], but have also been found in healthy shrimp larvae in aquaculture and were thus suggested to be a beneficial microbe [56]. In contrast, the *Vibrio* genus consists of widely known marine pathogens, many of which are linked to mortality in aquaculture of fishes, shrimp, oysters and other marine invertebrates [57–59]. Although it is tempting to ascribe the *Vibrio* ASVs found here to pathogenic species based on their close sequence similarities, the 16S rRNA gene amplicon data used in this study does not have sufficient length to confidently resolve species identities, nor does it differentiate between live and dead bacterial cells. Other potentially pathogenic taxa such as *Pseudomonas* and *Aeromonas* [60] were also detected at low abundances in the larvae. As such, follow up surveys including isolation of pure cultures, phenotyping and animal inoculation assays are needed to investigate the roles and effects of these *Vibrio* and other microorganisms of interest in GG aquaculture.

As our findings in GG larvae indicate that their microbiome is largely influenced by feed, a universal

microbiome-guided aquaculture management strategy is therefore unlikely due to differences in feed and environment between hatcheries. However, it raises the possibility of modulating the GG microbiome through diet. A commercial probiotic consisting of *Bacillus* was added to tank water on days 1, 4 and 7 as part of the hatchery's operating procedure, however, *Bacillus* were only detectable in larvae for one day following application indicating that it likely failed to establish in the larvae microbiome. Although certain probiotics are comprised of inactivated bacteria [61] and are not expected to result in colonization of animal hosts, the *Bacillus* probiotic used in this study was marketed as viable and tested to germinate within two hours of application (see methods section for product details). It is possible that the nine-day study duration was insufficient for colonization, however, the non-detection in both larvae and tank water samples two days after application indicates that the probiotics product was likely to have been removed from the RAS either by UV and/or filtration. These findings suggest that either: (i) more regular applications are necessary to introduce and maintain microbes of interest into the larvae microbiome (as seen by the transfer of microbes from feed), (ii) microbes have to be ingested to become part of the larvae microbiome, and/or (iii) only specific microbes are able to establish and persist in these fish [62]. Conversely, another implication of the larvae microbiome being largely derived from microorganisms in feed is a need for rigorous quality control of feed material to avoid introducing pathogens into aquaculture systems. For example, the close sequence similarity of *Vibrio* ASVs in rotifers fed to the GG larvae to pathogenic species warrants further examination to establish whether these specific *Vibrio* species/strains pose risks to the fish as the presence of opportunistic pathogens does not necessarily result in disease [63]. Rotifers as carriers of putative pathogens could be a widespread issue in aquaculture. A survey of microbiomes of farmed European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) similarly reported high relative abundances of *Vibrio* in fish larvae and their rotifer/artemia feed (>20% relative abundance) and postulated that the *Vibrio* was transferred from feed to larvae [64], although there was no supporting microbial source tracking data. The increased load of *Vibrio* and other pathogens in aquaculture systems could pose problems as they can lead to mortality and production losses, and more crucially could result in disease if transmitted to consumers [65]. A previous aquaculture experiment demonstrated that feeding gilthead seabream larvae with live rotifer pretreated with algae-derived antibacterials resulted in reduced relative abundances of known pathogens including *Pseudomonas*, *Klebsiella* and *Stenotrophomonas* in the larvae microbiome [66]. Correspondingly, there was an increase in potentially beneficial alphaproteobacterial taxa such as *Paracoccus* and *Polymorphum*, thus highlighting potential benefits of optimizing feeding strategy and quality control over feed material in aquaculture production.

In conclusion, to successfully exploit the microbiome as an avenue to increase agriculture productivity, breeding programs need to be tailored accordingly to accommodate variation across hatcheries, feed and likely also host genetics [67]. As such, longer duration microbiome surveys encompassing independent aquaculture batches are needed to address which microbes establish and persist in GG through to maturity. This includes assessing whether the microbiome from distinct batches of larvae converges as the GG host and its genetics could select for a conserved set of taxa in later life (e.g. akin to a core microbiome), how diet, feeding strategies and probiotics influence the microbiomes through to maturity, and how features of the microbiome (e.g. abundance of known beneficial microorganisms, putative pathogens) translate to host traits relevant to aquaculture production such as animal health, size, flesh colour and texture. Future experiments should include the necessary comparisons (e.g. controlled diets and probiotic treatments in isolated tanks, tank water vs. tank biofilm microbial community composition) to identify the exact microbes and host-microbe interactions that contribute to production. In addition, stringent quality control over feed material will be necessary to control bacterial loads [68]. This information will be crucial for the incorporation of microbiome data into aquaculture management strategies.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

JYL: conceptualization, sample collection & processing, experimental design, methodology development & execution, data & statistical analyses, manuscript – original draft & edits. YKY: data analysis, manuscript review. MC: sample collection, manuscript review. RK: acquired funding, provided hatchery resources and fish for study, manuscript review. DRJ: acquired funding & resources, manuscript review. DGB: conceptualization, acquired funding & resources, supervision, manuscript review.

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Data availability
The dataset supporting the conclusions of this article are available at the Sequence Read Archive under accession PRJNA1120584.

Declarations

Conflict of interest
The authors declare no competing conflict of interest.

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