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Core microbiome profiles and their modification by environmental, biological, and rearing factors in aquaculture hatcheries

Babak Najafpour [a], Patricia I.S. Pinto [a], Eric Climent Sanz [b], Juan F. Martinez-Blanch [b], Adelino V.M. Canario [a,d], Katerina A. Moutou [c], Deborah M. Power [a,d,*]

[a] CCMAR - Centre of Marine Sciences, Universidade do Algarve, Campus de Gambelas, Edifício 7, 8005-139 Faro, Portugal
[b] ADM Biopolis, Parc Cientific Universidad De Valencia, Paterna, Spain
[c] Department of Biochemistry & Biotechnology, University of Thessaly, Ploutonos 26, Larissa, Greece
[d] Shanghai Ocean University International Center for Marine Studies, Shanghai, China

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ABSTRACT

16S rRNA gene sequencing and bacteria- and genus-specific quantitative PCR was used to profile microbial communities and their associated functions in water, live feed (microalgae, *Artemia*, and rotifer), and European sea bass and gilthead sea bream larvae from hatcheries in Greece and Italy. The transfer to larvae of genus containing potential pathogens of fish was more likely with *Artemia* and rotifer than with microalgae or water, irrespective of geographic location. The presence of potentially pathogenic bacteria (*Vibrio* and *Pseudoalteromonas*) in the core microbiota of water, live feed, and fish larvae, the enrichment of different bacterial resistance pathways and biofilm formation, and the overall low beneficial bacteria load during larval ontogeny emphasizes the risk for disease outbreaks. The present data characterizing microbiota in commercial aquaculture hatcheries provides a baseline for the design of strategies to manage disease and to model or remediate potential adverse environmental impacts.

1. Introduction

Aquaculture is an industry of increasing importance for food security and the blue economy across the world. The European sea bass (*Dicentrarchus labrax*) and the gilthead sea bream (*Sparus aurata*) are the main marine aquaculture fish species in the Mediterranean. Greece, Turkey, Spain, and Italy are major producers of these two species under intensive cage culture (FAO, 2018). Despite improvements in intensive culture conditions, the survival rate of European sea bass and gilthead sea bream during production is still variable, and bacterial infections cause production losses ranging from 15 to 40 % (Lane et al., 2014). The *Vibrio* spp. are at the top of the list of known potential pathogens in mariculture, and pathogenic *Vibrio* spp. and *Photobacterium* spp. have been associated with mass mortalities of gilthead sea bream and European sea bass (Abdel-Aziz et al., 2013; Kahla-Nakbi et al., 2006; Snoussi et al., 2008). High interactions between fish and microbiota are proposed since fish live immersed in water and are influenced by multiple environmental factors in aquaculture sites (Austin, 2006; Ringø et al., 2010). An example is the effect of geographical location and species on the microbial community of European sea bass and gilthead sea bream eggs (Najafpour et al., 2021a). Larval production is a critical period and a bottleneck in fish hatcheries because of the high vulnerability of larvae to environmental stressors. However, there is a growing recognition of the importance of the fish microbiome, particularly in early developmental stages, because initial bacterial colonization in organs such as the gut can potentially affect microbiota establishment at later stages and have long-term effects on host development (Deng et al., 2022; Du et al., 2021).

The microorganisms that colonize animals and plants can have both positive and negative effects on their growth and health (Vandenkoornhuyse et al., 2015; Yukgehnaish et al., 2020). Fish like other organisms (e.g., human) host both pathogenic and non-pathogenic bacteria and an imbalance (dysbiosis) can shift the microbiota composition towards pathogenic species and facilitate disease outbreaks (Brugman et al., 2018; Wynne et al., 2020). The global characterization of fish microbiota can provide information about the relative abundance of potentially pathogenic and non-pathogenic bacteria and their interaction with the host, contributing to the development of measures for

* Corresponding author at: CCMAR - Centre of Marine Sciences, Universidade do Algarve, Campus de Gambelas, Edifício 7, 8005-139 Faro, Portugal.
E-mail address: dpower@ualg.pt (D.M. Power).

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the prevention and control of disease (Egerton et al., 2018). This explains the interest in probiotics that can modulate the microbiota and in this way positively influence the immune system, growth and digestion (Belkaid and Hand, 2014; Zorriehzahra et al., 2016; Tanaka and Nakayama, 2017). For example, in European sea bass and gilthead sea bream administration of bacterial probiotics (e.g., *Lactobacillus* spp.) via the diet had a positive effect on growth and the immune system (Carnevali et al., 2006; Suzer et al., 2008; Abelli et al., 2009; Cordero et al., 2015). Thus a particular focus has been placed on the gut microbiome due to its importance in nutritional provisioning, metabolic homeostasis and immune defense (Gómez and Balcázar, 2007; Sabree et al., 2009; Sullam et al., 2012; Egerton et al., 2018).

In recent years, understanding of the composition, complexity and contribution of the microbiota to host biology (plants or animals) has been greatly facilitated by advances in next-generation sequencing (NGS) using common DNA markers like the bacterial 16S ribosomal RNA (rRNA) gene (Ghanbari et al., 2015). The advantages of 16S rRNA gene sequencing are that it is a culture-free method and gives a snapshot of microbial communities even in complex ecosystems (Petti et al., 2005; Simon and Daniel, 2011). Analysis of the core gut microbiota has been established under standard laboratory conditions for zebrafish (*Danio rerio*) and has provided insight into host-microbe interactions (Roeselers et al., 2011). The skin and gills are also identified niche of microbiota and a core component of the host mucosal barrier (Merrifield and Rodiles, 2015). During the development of fish, the water microbiota appears to influence the gut microbiota in early larval stages, and is later modified by the introduction of different food types (Dimitroglou et al., 2010; Navarrete et al., 2013; Ingerslev et al., 2014). Fish age is also proposed as a factor influencing the diversity of the gut microbiota in smolts and adult stages of farmed chinook salmon, *Oncorhynchus tshawytscha* (Zhao et al., 2020). However, comprehensive studies of marine aquaculture species under routine production conditions are infrequent.

The overall aim of the present study was to understand the microbiome composition, and its establishment and development during early ontogeny and highlight mechanisms that influence bacterial load or enrich specific pathways in European sea bass and gilthead sea bream under hatchery conditions. For this purpose, a comprehensive assessment of the microbiota of European sea bass and gilthead sea bream larvae was carried out to identify: a) the core and diverse microbiota composition in larvae, food and water from different production sites; b) the effect of fish species, larval stage, and season on larval microbiota; c) the contribution of different foods (microalgae, rotifer and *Artemia*) and water sources (environmental water before and after UV treatment and rearing tank water) on the fish larvae microbiota in winter and spring; and d) the differentially abundant pathways with larval age (start feeding vs mid-metamorphosis), sample type (larvae vs food vs water), and food (microalgae, rotifer, *Artemia*) to identify the functional dynamics of the larval microbiota during early ontogeny and microbial-host interactions driven by food or water.

2. Material and methods

2.1. Sample collection

Samples were collected from aquaculture hatcheries in Greece and Italy, as part of their usual routine sampling procedures to verify production performance and animal welfare. The aquaculture hatcheries operated in compliance with the recommendations of the Directive 2009/58/EC of the European Parliament and of the Council of 13 July 2009 (protection of animals kept for farming). The companies held GLOBAL G.A.P. (Good Agricultural Practice) Certification, which included the demonstration of compliance with good animal welfare practices. Sampling was supervised by a qualified veterinary surgeon and was performed by hatchery technicians.

Fish larvae, food and water were sampled from eight marine hatcheries on the coast of Greece, with their approximate localization represented in Fig. 1 and detailed in Suppl. Table 1. Samples from the Greek hatcheries were obtained in winter (January 2018) and late spring (May 2018) and designated Greece Sampling 1 (GrS1) and Greece Sampling 2 (GrS2), respectively. The first sampling exercise was carried out in four hatcheries in GrS1, designated A1 to D1, and the second sampling was performed from seven hatcheries in GrS2, named A2, B2, D2 to H2 (three hatcheries were common between GrS1 and GrS2 as indicated in Fig. 1).

Samples were also obtained from one marine hatchery in Italy (Adriatic Sea) at the beginning of summer (June 2018) and were designated Italy Sampling (ItS). When possible, three types of water were collected from each sampling site: untreated water from the source (seawater or well) before ultra-violet (UV) disinfection; UV-disinfected water and water from the rearing tanks where the European sea bass or gilthead sea bream larval samples were collected (see Fig. 1 and Suppl. Table 1). Approximately 400 ml of water was collected per sample into sterile 500-ml flasks containing 50 ml of DESS preserving solution (0.25 M disodium EDTA pH 8.0, 20 % dimethyl sulphoxide and NaCl to saturation). For the collection of the water microbiota, aliquots of 50 ml were sequentially centrifuged in the same falcon tube for 15 min at 15,500 ×*g* at 4 °C, and the supernatant (water) was discarded after each centrifugation. The cumulative pellet for each sample was resuspended in 2 to 5 ml of RNA*later* (Sigma-Aldrich, Madrid, Spain) transferred to 2-ml microcentrifuge tubes and stored at −20 °C.

Samples of microalgae (single or multiple species), rotifer and *Artemia* were collected from most hatcheries as they are the main feeds used in European sea bass and gilthead sea bream hatcheries (Fig. 1 and Suppl. Table 1). These samples were collected on the same day as the larvae from their suspension cultures into 15-ml tubes, gently centrifuged, resuspended in RNA*later* and stored at −20 °C. European sea bass and gilthead sea bream larvae, ranging in age from 5 to 77 dph (from first feeding to mid-metamorphosis) were collected directly from their tanks into 15-ml tubes containing 10 volumes of RNA*later* and stored at −20 °C (Fig. 1 and Suppl. Table 1). All samples (water, food items and larvae) were shipped refrigerated to CCMAR (Faro, Portugal), where they were stored at −20 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from whole larvae, different food sources (microalgae, rotifer and *Artemia*) and water types (before and after UV treatment and fish tank water) using a DNeasy Blood & Tissue Kit (Qiagen, Germany) with some modifications.

For the water samples, the total volume received in RNA-later from the hatcheries (2–5 ml) was centrifuged for 10 min at 16,100 ×*g* at room temperature in 2-ml sterile extraction tubes (Sarstedt, Germany). The water samples were sequentially added to the same tubes to concentrate collected material in a single tube. The collected pellet from the water was extracted in 400 μl of lysis mix (200 μl of lysis buffer 20 mM Tris-HCl, pH 8, 2 mM sodium EDTA; 1.2 % Triton X-100; 40 mg/ml lysozyme mixed with 200 μl of AL buffer from the Qiagen kit) and approx. 400 mg of 0.1 mm zirconia/silica beads (Biospec) were added. The tubes were maintained on ice until mechanical disruption, which was carried out at room temperature in a Tissue Lyser (Qiagen, Germany) for 3 cycles of 5 min at 25 Hz.

For food samples, 0.3–2 ml of microalgae, rotifer or *Artemia* suspensions (20–300 mg wet weight) stored in RNA*later* were centrifuged for 10 min at 16,100 ×*g* at room temperature. Lysis mix (400 μl) was added to the pelleted material and one iron bead (Qiagen stainless steel beads of 5 mm) per tube was used for the initial mechanical disruption carried out for 3 cycles of 30 s at 30 Hz in a Tissue Lyser (Qiagen). After removal of the iron beads, 400 mg of 0.1 mm zirconia/silica beads were added and the disruption of the bacteria proceeded for 3 cycles of 5 min at 25 Hz.

For larvae, the amount of initial material and the disruption protocol was optimized according to the larval age. Pools of approx. 10 larvae

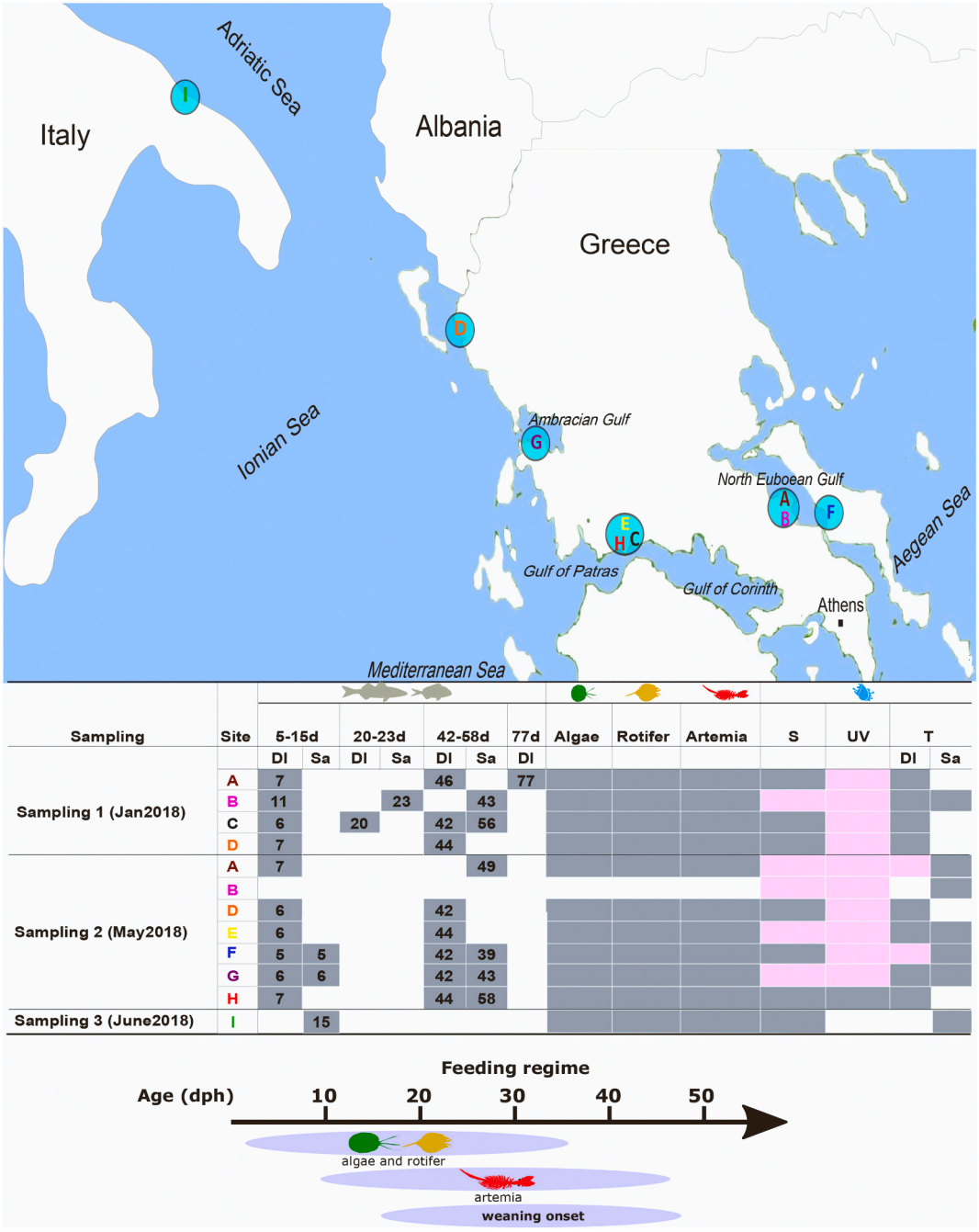

Fig. 1. Sampling scheme presenting the samples collected from Greek hatcheries. The different hatchery sites that provided samples are presented on the map and the code allocated to each sampling site is indicated in the table using capital letters (A–I). Samples were collected at three different times of the year, January, May and June (2018) and the samples collected from the same hatcheries at the two time points are shown with the same letter and colour (A, B, D). The samples collected (larvae, food and water) at each sampling time (January and May) and at each hatchery are shaded in grey in the table; in the case of the larvae samples, the age (in days post-hatch) is specified in each cell, and the species is indicated above each column (Dl - European sea bass, *Dicentrarchus labrax* and Sa - gilthead sea bream, *Sparus aurata*). Analysed water samples from the source (S), after UV treatment (UV) and the hatchery tank water (T) for each species are presented in the table and some water samples that were extracted but did not yield enough DNA for sequencing are highlighted in light pink. The feeding regime represents an approximate range of days post-hatch (blue ovals) that live feed fed to fish larvae across different hatcheries. See Suppl. Table 1 for detailed information on all collected samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from the first-feeding stages (6–11 dph) weighing between 6 and 10 mg were disrupted in 400 μl of lysis mix with iron and glass beads as described above. Pools of 8 larvae were extracted for 20–23 dph larvae, and weighed between 10 and 14 mg. Single larvae were extracted for the metamorphosis stage (40–50 dph) and weighed between 9 and 35 mg. The bigger European sea bass larvae of 77 dph, weighed approximately 79 mg and exceeded the proposed sample quantity of the extraction kit (<30 mg) and so they were extracted in a double volume of lysis mix and only 1/3 of the disrupted material was used for genomic DNA extraction.

Following mechanical disruption of samples in the lysis mix they were incubated for 30 min at 37 °C with the lysozyme (80 μl of 100 mg/ml lysozyme per sample, included in the lysis mix), followed by 30 min at 56 °C with proteinase K (25 μl of 20 mg/ml). Tubes were centrifuged for 1 min at 4300 ×*g* to pellet the beads, and the lysate was collected into

Table 1
PERMANOVA analysis (permutations = 1000) between different types of samples collected in January 2018.

	Df	Sum Sq	Mean Sq	F. model	R2	Pr (>F)
Microalgae vs *Artemia*	1	0.8713	0.87135	2.5639	0.2993	0.0309*
Microalgae vs rotifer	1	0.7674	0.7674	2.1285	0.2618	0.0219*
Microalgae vs larvae	1	1.0503	1.0503	3.0402	0.1784	0.0019**
Microalgae vs water	1	0.9153	0.9152	2.5281	0.1868	0.0019**
Artemia vs rotifer	1	0.5188	0.5188	1.4358	0.1930	0.0269*
Artemia vs larvae	1	0.8214	0.8214	2.3753	0.1450	0.0009***
Artemia vs water	1	0.8231	0.8231	2.2709	0.1711	0.0009***
Rotifer vs larvae	1	0.7019	0.7019	1.9790	0.1238	0.0089**
Rotifer vs water	1	0.7078	0.7077	1.8937	0.1468	0.0019**
Larvae vs water		0.6611	0.6611	1.8515	0.0887	0.0079**
Larvae1 vs larvae2	1	0.4533	0.4532	1.4116	0.2608	0.1333
Larvae2 vs larvae3	1	0.3920	0.3920	1.1594	0.1619	0.3037
Larvae1 vs larvae3	1	0.7800	0.7800	2.4916	0.2374	0.0069**

Df: degrees of freedom; Sq: square; significance code: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

a clean microcentrifuge tube and treated with RNase (10 μl of 10 mg/ml) for 10 min at room temperature, and then 0.5 volumes of 100 % ethanol were added. The lysate mix was then purified using the column supplied in the DNeasy Blood & Tissue kit. For the food and larvae samples DNA was eluted into two tubes, the first fraction was eluted in 30 μl of Tris-HCl (10 mM, pH 8) and the second fraction was eluted in 70 μl Tris-HCl (10 mM, pH 8). In the case of the water samples the second eluate volume was 170 μl of Tris-HCl (10 mM, pH 8). DNA quality and integrity were analysed using a Nanodrop spectrophotometer and 1 % agarose gel electrophoresis.

When the concentration of eluted DNA was low (<5–10 ng/μl), which was the case of the water samples and some of the microalgae extracts, the two fractions eluted from the column were combined and concentrated with a QIAamp DNA Micro kit (Qiagen) following the suppliers' protocol, and elution was carried out in 20 μl Tris-HCl (10 mM, pH 8).

2.3. 16S rRNA gene microbiome library preparation and sequencing

Each sequencing library was constructed using DNA from individual samples of microalgae, rotifers, *Artemia*, and water or triplicate samples corresponding to DNA pooled from 10 larvae at 1st feeding or from one older larva. Library preparation followed the 16S rRNA gene Sequencing Library Preparation protocol for the Illumina MiSeq system, using optimized primers targeting the hypervariable V3 and V4 regions of the 16S rRNA gene (Klindworth et al., 2013). Libraries were paired-ended sequenced (300 bp ×2) on an Illumina MiSeq platform at ADM Biopolis (previously Lifesequencing S.L.; Paterna, Valencia, Spain). The raw sequence data used for analysis is available in NCBI under the Sequence Read Archive (SRA) with the BioProject accession number "PRJNA608636".

2.4. Sequence processing and bioinformatics

A pipeline developed by ADM Biopolis was used for initial processing of the sequenced reads. Through this pipeline, raw reads were cleaned and merged into paired-end reads, chimeras were filtered and eliminated, and sequences were used for classification of operational taxonomic units (OTU) as previously described (Codoñer et al., 2018), using BLASTn against the NCBI 16S rRNA database with a cut-off set at 97 % identity. The software CD-HIT was used for hierarchy clustering (Fu et al., 2012).

The R Package pheatmap v. 1.0.12 was used to draw clustered heatmaps (Kolde, 2019). Nonmetric multidimensional scaling (NMDS) analysis was performed using the *metaMDS* function in the R package vegan v. 2.5-7 applying Bray–Curtis distance and data transformation (Oksanen et al., 2020). For visualizing intersecting (bacterial genera) size and the core microbiota in each sample (GrS1, GrS2 and ItS), the UpsetR package v. 1.4.0 was used (Conway et al., 2017).

To obtain standard OTU classifications for functional predictions, sequenced reads from GrS1 and GrS2 were also processed using the amplicon analysis pipeline of the SILVA project 'SILVAngs 1.4' (Quast et al., 2013). Reads shorter than 50 aligned nucleotides and reads with >2 % ambiguities, or 2 % homopolymers, respectively, were excluded from further analysis. After accomplishing initial quality control steps, identical reads were identified (dereplication), and the unique reads were clustered (OTUs) in each sample using VSEARCH v. 2.14.2 (Rognes et al., 2016) by applying identity criteria of 1.00 and 0.98, respectively. Then, the reference read of each OTU was classified by BLASTn 2.2.30+ (Camacho et al., 2009) with standard settings using the non-redundant version of the SILVA SSU Ref dataset as the classification reference (release 138.1).

Functional annotation of the 16S rRNA gene sequencing profiles was established using the web-based platform MicrobiomeAnalyst (Chong et al., 2020) and the Tax4Fun method (Aßhauer et al., 2015). It should be kept in mind that the accuracy of these tools relies on functional information derived from available genomes, and the predicted profile of a sample with large fractions of unknown organisms may not represent the functional diversity in that sample due to the low coverage of database reference profiles (Aßhauer et al., 2015). To identify KEGG Orthologs (KO) with differential abundance, KOs with low counts and variance were filtered and normalized using the Cumulative Sum Scaling (CSS) method (Paulson et al., 2013). Two statistical methods were applied to perform differential abundance analysis and minimise bias: the RNA-seq method using the edgR algorithm (Robinson et al., 2009) and the metagenomeSeq method using a 'zero-inflated Gaussian fit' model (Paulson et al., 2016). Significant KOs associated with each variable (age and sample type including larvae, water, microalgae, rotifers, and *Artemia*) were selected for KEGG pathway analysis using the clusterProfiler R package v. 3.18.1 (Yu et al., 2012) when a confidence interval of 95 % ($p < 0.05$) was confirmed by both methods.

2.5. Validation and quantitative measurement of total bacterial load and five abundant bacterial genera using qPCR analysis

Quantitative polymerase chain reaction (qPCR) was used to quantify total bacterial load and the changes in load of some abundant bacteria during larval ontogeny and compared with the results of the 16S rRNA gene sequencing. The genomic extracts of European sea bass and gilthead sea bream larvae (from GrS1, GrS2 and ItS) of two age ranges (5–23 dph and 39–58 dph) were used in the qPCR reactions in triplicate. To enlarge the scope of the samples for the analysis of bacterial load and balance the number of samples of younger and older larvae, four additional samples (each corresponding to a pool of 10 larvae in triplicate) that were collected in February and March 2018 were also included in the qPCR analysis. The total bacterial load and five bacterial genera (*Vibrio* spp., *Massilia* spp., *Phaeobacter* spp., *Pseudomonas* spp., and *Psychrobium* spp.) were quantified using genus specific 16S rRNA primers in a Bio-Rad CFX96 qPCR Instrument (Bio-Rad Laboratories, Hercules, CA, USA). Overall, the 16S rRNA gene was quantified using the universal sense primer 16S-515fbY or 515F (Parada) 5′-GTGY-CAGCMGCCGCGGTAA-3′ and antisense primer 16S-806rbN or 806R (Apprill) 5′-GGACTACNVGGGTWTCTAAT-3 (Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016).

The qPCR amplification reactions for the 16S rRNA gene contained 30 ng of template DNA (in 2 μl), the universal primers and other reagents

as previously described (Najafpour et al., 2022). *Vibrio* genus-specific primers targeting a 120-bp region of the 16S rRNA gene were used for qPCR quantification as previously described (Tall et al., 2012; Pinto et al., 2019). The genera-specific primers for *Massilia*, *Phaeobacter*, *Pseudomonas*, and *Psychrobium* have been recently developed and validated (Najafpour et al., 2022). The amount of template DNA used in qPCR was 80 ng (in 2 μl) and optimized reaction mixtures and conditions for each primer pair were used (Najafpour et al., 2022). Bacterial genera copy number was measured by creating genera-specific standard curves ranging from 10^2 to 10^7 copies of the target gene (in 10-fold increments). Bacterial copy number based on the 16S rRNA gene was calculated as follows: copy number = $(X \times 6.022 \times 10^{23}) / (Y \times 1 \times 10^9 \times 650)$, where X is ng of the template gene, Y is the template length (bp), and 650 (Da) represented the average molecular weight of a base pair (Martyniuk et al., 2009).

2.6. Statistical analysis

The Shannon index and CHAO1 were used to estimate alpha-diversity and species richness in each sample. Data normality was checked using a Shapiro-Wilk normality test, and the data distribution for the Shannon index was normal across the samples (n = 82). To analyse CHAO1, outlier samples (n = 10, >2 standard deviations) were eliminated to obtain a normal distribution for pairwise comparison analysis (n = 70). The analysis of variance (ANOVA) and Tukey's test for multiple pairwise comparisons were used to compare the statistical difference in bacterial richness and diversity between different samples (larvae, microalgae, rotifer and *Artemia*). To evaluate beta-diversity, data homogeneity was controlled using the *betadisper* function (evaluating beta-dispersion) and an ANOVA-like test applied, using the *permutest* function in R. Based on principal coordinate analysis (PcoA) of beta-diversity data, permutational analysis of variance (PERMANOVA) using the *adonis* function and Bray–Curtis distances were used to test whether the overall microbial community differed with each variable under analysis.

The R package metagenomeSeq v 1.32.0 was used to identify differentially abundant OTUs in the microbiome data, and different models based on different variables were created using the *fitZIG* function to determine differentially abundant OTUs with an adjusted p-value < 0.05 (Paulson et al., 2016). OTUs that were significantly changed in each comparison were visualized with heatmaps using the R package superheat v 1.0.0 (Barter and Yu, 2021).

3. Results

3.1. Sequencing statistics

Of the 112 samples extracted for microbiome analysis, 89 yielded adequate DNA quality for sequencing (Fig. 1). A total of 7.5 million (M) raw read sequences were produced from the 89 libraries and generated 3.4 M paired-end reads. After quality control and trimming of low-quality sequences, 3.2 M filtered reads with an average length of 410-bp were obtained. The detailed sequencing statistics for each sample is provided in Suppl. Table 2.

The rarefaction curves indicated saturation or close to saturation coverage for diversity in each sample (Suppl. Figs. 1–3). Based on the number of detected OTUs, the rarefaction curves also revealed different bacteria richness (between different hatcheries, sample type or sampling time). In general, water samples had a higher richness (100–500 OTUs), with the greatest number of OTUs detected in the environmental source waters (SCJ, SDM) (Suppl. Figs. 1–3).

3.2. Bacterial community composition

The size of the microbiome and common/core bacteria genera were analysed by counting the number of bacterial genera across all samples (Suppl. Fig. 4). In general, the samples of source water (SCJ and SDM) had the highest number of bacteria genera and *Artemia* and microalgae (AM, GM) had the lowest number (Suppl. Fig. 4). The distribution in all samples of the 61 bacterial genera identified in at least 55 % of the samples is shown in Suppl. Fig. 4.

Table 2
PERMANOVA analysis (permutations = 1000) between different types of samples collected in May 2018.

	Df	Sum Sq	Mean Sq	F. model	R2	Pr (>F)
Microalgae vs *Artemia*	1	0.9745	0.9745	2.5766	0.1897	0.0029**
Microalgae vs rotifer	1	0.9195	0.9195	2.4826	0.1988	0.0019**
Microalgae vs larvae	1	1.0488	1.0487	2.8707	0.1255	0.0009***
Microalgae vs water	1	0.9183	0.9182	2.4226	0.1247	0.0009***
Artemia vs rotifer	1	1.0970	1.0969	3.5733	0.2452	0.0019**
Artemia vs larvae	1	1.0083	1.0083	2.9955	0.1198	0.0019**
Artemia vs water	1	1.3523	1.3522	3.9794	0.1810	0.0009***
Rotifer vs larvae	1	1.1441	1.1441	3.4575	0.1413	0.0009***
Rotifer vs water	1	1.2674	1.2673	3.8064	0.1829	0.0009***
Larvae vs water	1	1.0928	1.0927	3.1580	0.1013	0.0009***
Larvae1 vs larvae3	1	1.1686	1.1685	3.7561	0.2002	0.0009***

Df: degrees of freedom; Sq: square; significant code: ***p < 0.001, **p < 0.01.

The most abundant phyla in the sequenced libraries were *Proteobacteria* (mean = 59.6 %, range = 2.7–98.3 %), followed by *Bacteroidetes* (mean = 12.2 %, range = 0–82.6 %) and to a lesser extent *Firmicutes* (mean = 3.4 %, range = 0–44.1 %) and *Actinobacteria* (mean = 0.8 %, range = 0–10.4 %) (Fig. 2, Suppl. Tables 3–5). In general, a higher percentage of *Firmicutes* was detected in 6–23 dph larvae (mean = 8.1 %, range = 0.9–25.3 %) and *Proteobacteria* (mean = 80.4 %, range = 47.9–98.2 %) in older larvae (Suppl. Tables 3–5). The most abundant families were *Vibrionaceae* (mean = 21.4 %, range = 0–96.9 %), *Rhodobacteraceae* (mean = 8.7 %, range = 0–45.7 %), *Flavobacteriaceae* (8.1 %, range = 0–54.8), *Pseudoalteromonadaceae* (mean = 5.9 %, range = 0–45.8), *Oceanospirillaceae* (mean = 4.4 %, range = 0–28.1 %), *Alteromonadaceae* (mean = 4.3 %, range = 0–39.8 %), *Oxalobacteraceae* (mean = 3.4 %, range = 0–89.6 %), *Colwelliaceae* (mean = 2.7, range = 0–62.9 %), *Shewanellaceae* (mean = 1.7 %, range = 0–33.8 %) and *Crocinitomicaceae* (mean = 1.7 %, range = 0–80.4 %) (Fig. 2, Suppl. Tables 3–5).

The microbiota abundance was determined up to the species level (Suppl. Tables 3–5), but for higher confidence in OTU assignment, it is only discussed up to the genus level. The bacterial genera detected with an average abundance higher than 1 % are listed in Suppl. Tables 3–5. The clustered heatmaps of relative abundance only include the genera/OTUs with >8 % abundance in at least one of the 16S rRNA gene libraries (Suppl. Figs. 5–7). There was no strong clustering of samples unless the predominance of a bacterial genus led to cluster formation, e.g. *Vibrio* in fish larvae or in *Artemia* (mean = 21.5 %, range = 0–96.8 %) (Suppl. Tables 3–5, Suppl. Figs. 5–7).

The ten bacterial genera with the highest relative abundance in each of the samples are presented in Suppl. Table 6 and a graphical summary of the five most abundant genera per sample type is presented in Fig. 3. *Vibrio* was the most abundant genus in larval samples, with only a few exceptions: *Epibacterium* was more abundant in European sea bass 1BJ (29.4 %), *Massilia* in European sea bass 3AJ and gilthead sea bream 3CJ (36.1 % and 35 %, respectively) from GrS1, *Salinirepens* (80.2 %) and *Thalassotalea* (58.9 %) in European sea bass 1FM and gilthead sea bream 1FM, respectively, from GrS2 (Fig. 3, Suppl. Table 6). Some larval samples had a high percentage of "No hit" reads and retrieved no matches from the 16S rRNA gene database (e.g., European sea bass 1DM, Suppl. Table 6).

Vibrio (mean = 20.7 %, range = 0.01–88.5 %), *Alteromonas* (mean =

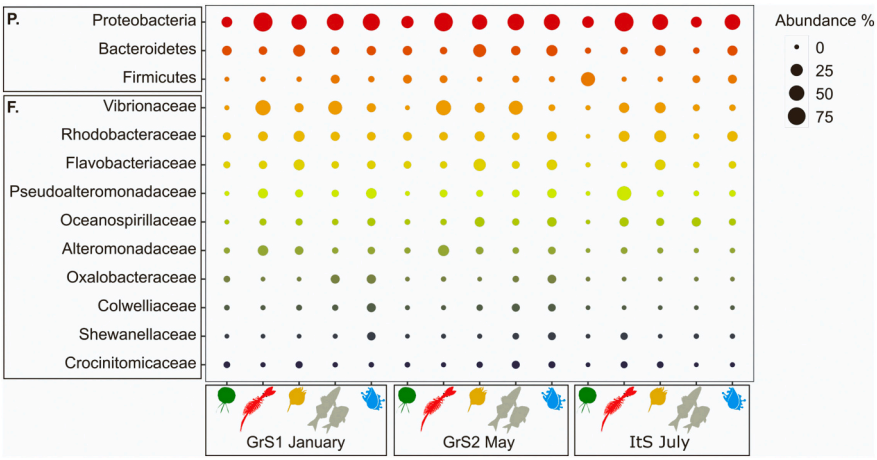

Fig. 2. A bubble plot representing the most abundant phyla (P.) and families (F.) overall by sample type collected at the two sampling timepoints, January 2018 (GrS1) and May 2018 (GrS2) and June 2018 (ItS). Different sample types are represented with different symbols: European sea bass larvae (); gilthead sea bream larvae (); microalgae (); rotifer (); *Artemia* (); water ().

6.8 %, range = 0.01–38.4 %) and *Pseudoalteromonas* (mean = 3.5 %, range = 0.004–27.8 %) were the most abundant bacterial genera in the food samples from GrS1 and GrS2 (Fig. 3, Suppl. Table 6). In general, these genera were present at lower abundance in microalgae than in *Artemia* and rotifers (Fig. 3, Suppl. Table 6). High relative abundance of some genera was limited to a few food samples and food types, such as *Epibacterium* in *Artemia* AJ (35.4 %), *Artemia* AM (30.6 %) and *Artemia* IUJ (11.2 %), *Donghicola* in rotifer AJ and AM (15 %), *Kordia* in rotifer CJ (14.8 %) and rotifer EM (14.6 %) and *Aestuariibacter* in rotifer CJ (11.2 %), *Yoonia* in rotifer BJ (21.8 %) and rotifer IJU (23.3 %), and *Algoriphagus* in microalgae DJ and DM (10.7 % and 7.7 %; Suppl. Table 6). *Mesoflavibacter* was one of the top 10 most abundant genera in some rotifer samples and showed high abundance in the samples AJ, FM, AM, and HM (Fig. 3, Suppl. Table 6). *Marinomonas* was common in food samples (mean = 3.2 %, range = 0–24.3 %) and was found in most *Artemia* and rotifer samples from GrS1, GrS2 and ItS (Fig. 3, Suppl. Table 6). Some clusters of unknown identity had high abundance in the microalgae samples, and BLAST searches indicated they resulted from non-specific PCR amplification of microalgae chloroplast sequences.

Vibrio was also among the most abundant genera in water samples, particularly in tank water (TAJ, TBJ, TDJ, TEM) - Fig. 3 and Suppl. Table 6. *Pseudoalteromonas* (mean = 11.6 %, range = 0.01–45.8 %) was among the top 10 genera in water samples and had a 22–45 % abundance in GrS1 and GrS2 water samples (SAJ, TAJ, TBJ, TFM, and TBM). *Massilia*, *Fucophilus*, *Thalassotalea*, *Psychrobium*, *Marinomonas*, *Amphritea*, *Colwellia* and *Polaribacter* were also among the most abundant bacterial genera of water samples (Fig. 3).

The relative abundance of each of the five genera from 16S rRNA gene sequencing and their 16S rRNA gene copy number estimated by quantitative PCR in larvae samples using genus-specific primers were positively correlated (Suppl. Fig. 8). The total 16S rRNA gene copy numbers (bacterial loads quantified by qPCR with 16s rRNA universal primers, Fig. 4) had a normal distribution across the larval samples (p-value = 2.33 e^{-05}) and was not significantly different between European sea bass and gilthead sea bream samples ($p > 0.05$). A significant increase in 16S rRNA gene copy number with larvae age reflected the increase in the total bacterial load irrespective of species ($p = 0.009$, Fig. 4). When genera were considered separately, species and age did not significantly affect the *Vibrio* population ($p > 0.05$) (Fig. 4); *Massilia* was significantly more abundant in gilthead sea bream (age range = 39–58 dph) compared to European sea bass (age range = 42–46 dph). *Phaeobacter*, *Pseudomonas* and *Psychrobium* abundance was not dependent on species or age (Fig. 4).

3.3. Alpha diversity

There was a high variation in CHAO1 richness and Shannon diversity for equivalent sample types from GrS1 and GrS2 (Suppl. Fig. 9, Suppl. Table 7). Multiple pairwise comparisons showed divergent bacterial richness between rotifer - microalgae (difference = 194.5, $p < 0.05$), water - microalgae (difference = 190.1, $p < 0.05$) and larvae - microalgae (difference = 169.7, $p < 0.05$). The CHAO1 richness in *Artemia* samples was not significantly different ($p > 0.05$) from water (difference = 152.9), larvae (difference = 132.4) or rotifer (difference = 157.3).

There was a Shannon diversity trend from low to high values in food, water and larvae in both GrS1 and GrS2 (Suppl. Fig. 9 and Suppl. Table 7). The average Shannon diversity index was around 2.85 and multiple pairwise comparisons revealed that it was only significantly different between larvae and *Artemia* (difference = 0.93, $p < 0.05$). Bacterial richness and diversity did not differ significantly between samples collected in January (GrS1) and May (GrS2, Suppl. Fig. 9, Suppl. Table 7). In some samples with low Shannon diversity, there was a high CHAO1 richness index (e.g., gilthead sea bream 3BJ), suggesting that high bacterial richness does not necessarily lead to a balanced bacterial community (Suppl. Fig. 9, Suppl. Table 7).

3.4. Beta diversity

Overall, a comparison of the beta diversity composition of the microbiome between samples using non-metric multidimensional scaling (NMDS) showed sample type clustering in GrS1 and GrS2 (e.g., microalgae, *Artemia*, rotifer and larvae, Fig. 5). The effect of species/rearing practice, geographical location, and type of water sample (source water, rearing tank water and UV treatment water) caused divergence in the microbiome. Comparing the larvae samples within GrS1 (Fig. 5a) or GrS2 (Fig. 5b) showed that the younger larvae (ranging between 5 and 7 dph, code 1) were generally differentiated from older larvae (ranging between 39 and 77 dph, code 3). Larvae of 15–23 dph (code 2) tended to cluster with the younger larvae (code 1). In GrS1, gilthead sea bream larvae of 56 dph (3CJ) and European sea bass larvae of 77 dph (3AJ) were discriminated from the younger larvae (codes 1, 2). An obvious separation of the gilthead sea bream and European sea bass larvae beta diversity was not observed. PERMANOVA analysis (Bray–Curtis distance) supported the outcome of the PcoA that indicated different microbial communities in different sample types (larvae, water, microalgae, *Artemia*, and rotifer) and larval ages, irrespective of the sampling time (GrS1 and GrS2, Tables 1 and 2).

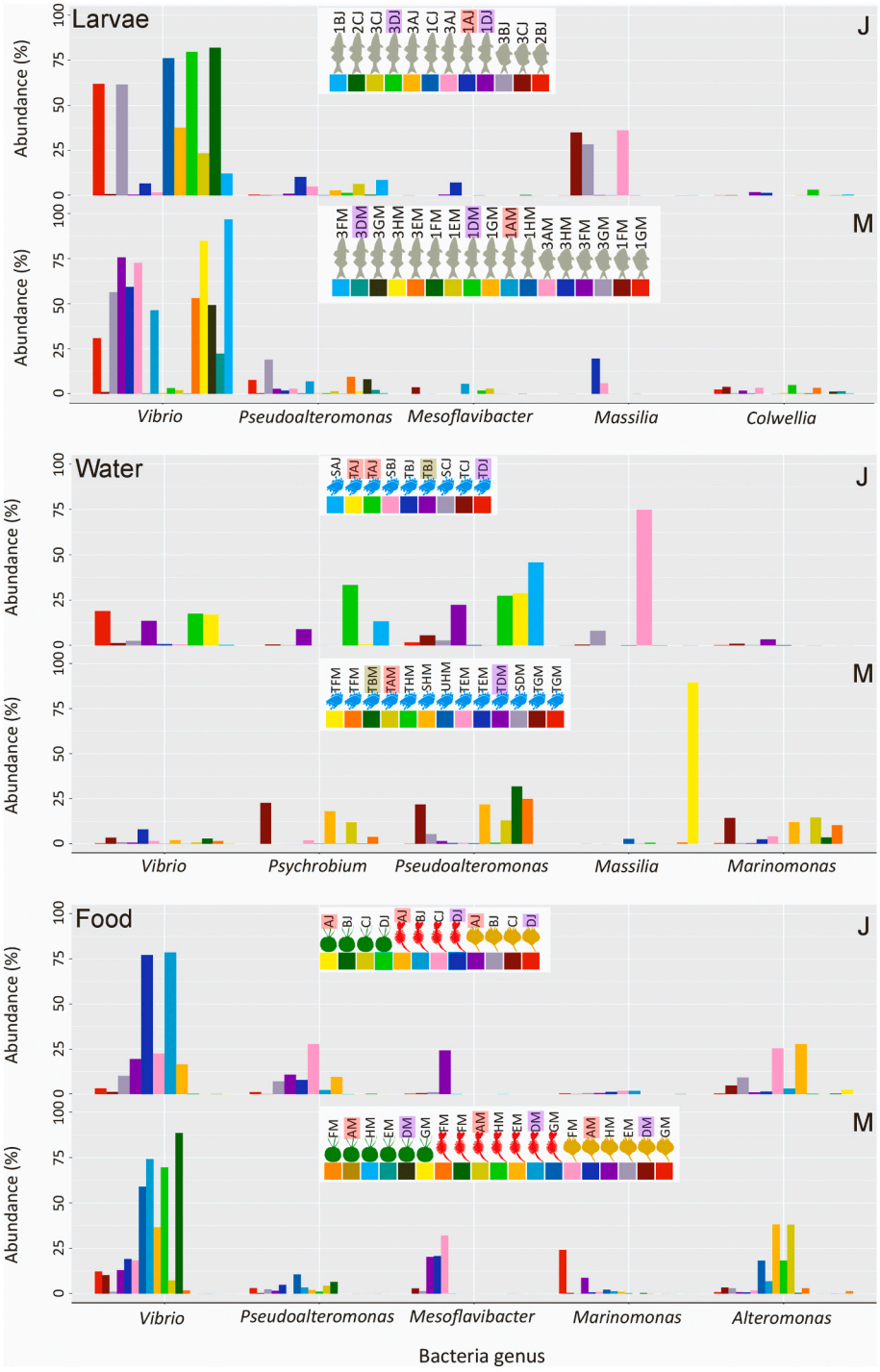

Fig. 3. Selection of the five most abundant bacterial genera in each type of sample, obtained from the detailed analysis of the ten genera represented in the highest proportion in all analysed microbiomes (Suppl. Table 6). The percentage of each identified genus relative to the total reads per library is presented for each sample, organized by sample type (larvae, water or food) for Greece Sampling 1 in January (panel J in each sample type), and Greece Sampling 2 in May (panel M). Different sample types are represented with different symbols: European sea bass larvae (); gilthead sea bream larvae (); microalgae (); rotifer (); *Artemia* (); water (). In the larvae panel, the different age ranges are represented with a code: 1 (5–11 dph), 2 (15–23 dph) and 3 (42–77 dph). Different letters (A, B, C, D, E, F, G, H, I) identify the different hatcheries and different times of sampling are shown by 'J' (January), and 'M' (May) after each site. The type of water is represented by "S" for source water "U" for water after UV treatment and "T" for water from hatchery tanks (see Suppl. Table 1 for more details). The codes of the collected samples in January and their equivalents from the same site in May are highlighted using common colours.

3.5. *Differential abundance according to sample type*

The beta diversity analysis revealed distinct microbial communities between sample types (larvae, water, rotifer, and microalgae), time of year (May and January), hatchery, larval species, and age, prompting the development of models of bacterial abundance by sample type.

Microalgae and rotifer bacterial populations were more similar between themselves compared to *Artemia*, water or larvae samples (as is revealed by the heatmaps and differential abundance analyses in Fig. 6) and this was confirmed by the clear separation of microalgae and rotifer microbial communities in the PcoA analyses. For example, the *Planctomycetaceae* and *Phyllobacteriaceae* families were more abundant in

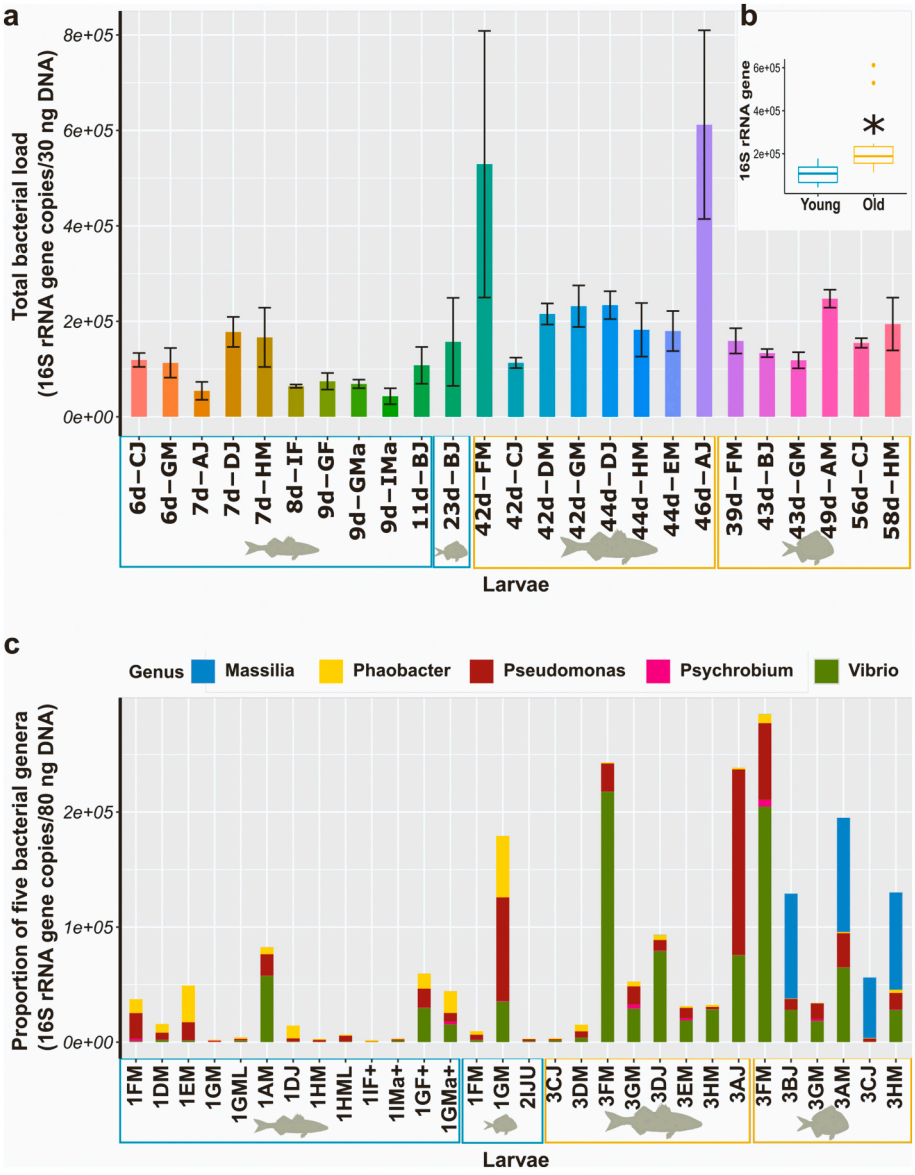

Fig. 4. Results from quantitative PCR (qPCR). The graphs show the quantitative measurements of the total bacterial load and five bacterial genera during gilthead sea bream (🐟) and European sea bass (🐟) larval ontogeny using specific primers. a) The total bacterial load was measured by quantifying the 16S rRNA gene in larvae of two age ranges: younger larvae (6–23 dph) and older larvae (42–58 dph); b) the significant change identified between the total bacterial load of younger and older larvae (younger larvae were mainly related to European sea bass species); c) the relative proportion of the five bacterial genera quantified using genus-specific primers amplifying bacterial 16s rRNA genes in the different larvae samples. The age range of the European sea bass and gilthead sea bream larvae are denoted with a code: 1 (5–11 dph), 2 (15–23 dph) and 3 (39–77 dph). The letters A, B, C, D, E, F, G, H, and I identify the different hatchery sites (see Fig. 1 for more details). The main sampling times of the material used for 16S rRNA gene metabarcoding analysis and qPCR are shown with J (January), and M (May). A larvae sample obtained at the beginning of summer (June 2018) from one aquaculture site in Italy (Adriatic Sea) is shown by JU. Additional samples (+) from other sampling time points that were analysed only with the genus specific primers and 16S rRNA primers are indicated by F (related to February 2018) and Ma (related to March 2018); for more details and sample codes see Suppl. Table 1.

microalgae and rotifer samples than in *Artemia*, larvae and water samples ($p < 0.05$, Fig. 6a). Lower abundance of *Vibrionaceae* and *Pseudoalteromonadaceae* and higher abundance of *Rhodospirillaceae* were also observed in microalgae compared to the other sample types rotifer, *Artemia*, water and larvae ($p < 0.05$, Fig. 6a). *Erysipelotrichaceae*, *Coriobacteriaceae*, *Bifidobacteriaceae* were of significantly higher abundance ($p < 0.05$) in younger larvae (age range = 5–23 dph) compared to older larvae (age range = 39–77 dph; Fig. 6b).

Some bacterial genera were more abundant (e.g., *Streptococcus*, *Bifidobacterium*, *Lactobacillus*, *Bacteroides* and *Blautia*) and others less abundant (e.g., *Alkalimarinus*, *Agarivorans*, *Leucothrix* and *Pseudomonas*) in younger larvae (5–23 dph) compared to older larvae (39–77 dph, Fig. 7a). Most of the bacterial genera that were differentially distributed between larvae and water samples had a higher abundance in water (e.g., *Psychrobium*, *Marinomonas*, *Amphritea*, *Pseudofulvibacter*, Fig. 7b). Relatively few genera had lower abundance in water (e.g., *Blautia*, *Bacteroides*, *Bifidobacterium*) than in larvae (Fig. 7b). Generally, in larvae, there was a higher abundance of *Polaribacter*, *Colwellia*, *Blautia*, *Bacteroides*, *Alkalimarinus*, *Leucothrix* and *Psychrobium* and less abundance of *Alteromonas*, *Roseovarius*, *Winogradskyella*, and *Aestuariibacter* compared to food irrespective of type (Fig. 7c; Suppl. Fig. 10). Marker bacterial genera that separated the microbial community of different food types were identified in differential abundance analysis. There was a differential distribution of bacterial genera between the food type microalgae, *Artemia*, and rotifer ($p < 0.05$, Fig. 8), and some more abundant genera in each food type were more likely to be transferred to larvae (Figs. 7 and 8, Suppl. Tables 3–5).

There were no significant differences in the microbial abundance of the rearing water of gilthead sea bream and European sea bass. Differential abundance was present, however, between source water and tank rearing water: *Maliponia*, *Ichthyenterobacterium*, *Oleispira*, *Marivita*, and *Phaeobacter* were more abundant in larval rearing water and a higher abundance of *Psychrobium*, *Bifidobacterium*, and *Escherichia* was found in source water of some hatcheries.

3.6. Functional prediction

Common KEGG orthologs (KOs) were identified by both the RNA-seq method-edgR algorithm and metagenomeSeq used in KEGG enrichment analysis (Suppl. Table 8). Enriched KEGG pathways were identified by

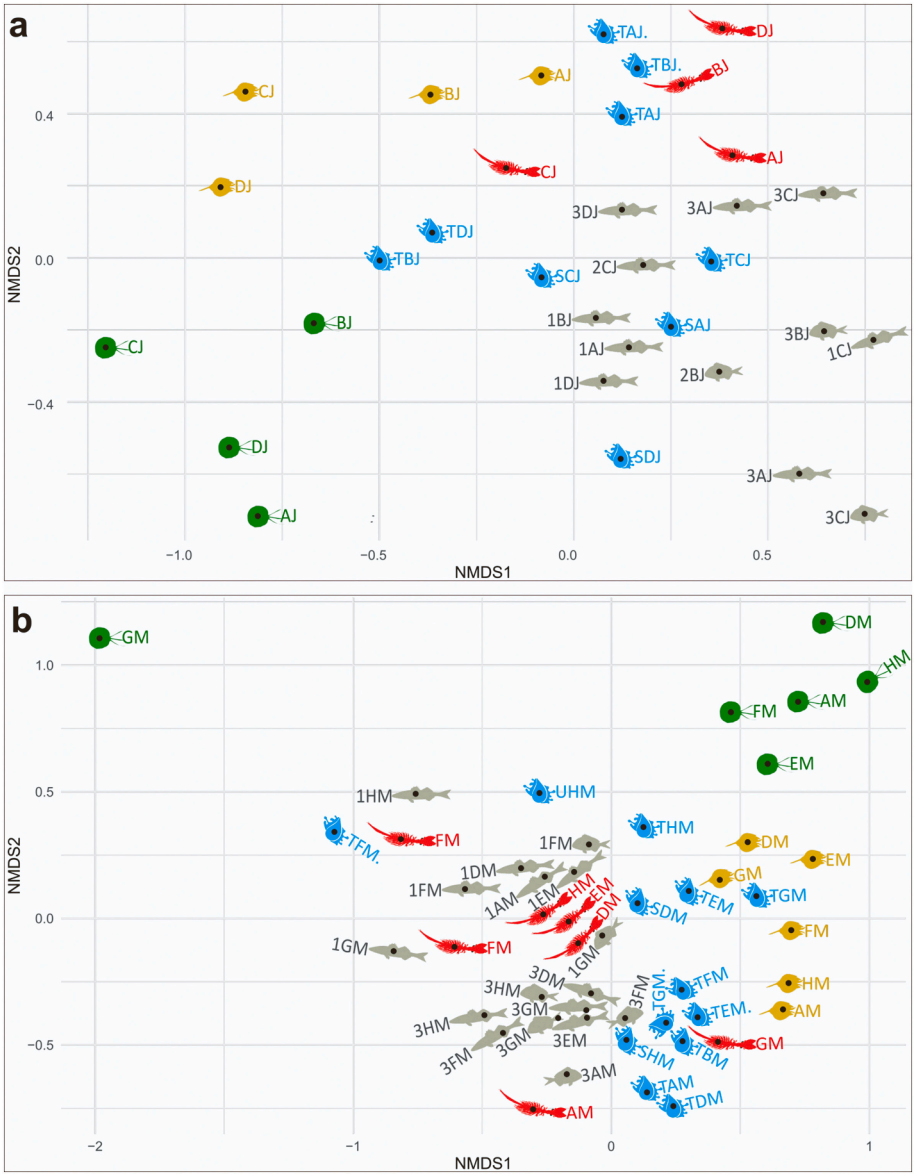

Fig. 5. Nonmetric multidimensional scaling (NMDS) analysis of the samples collected in January (panel a, Greece Sampling 1) and May (b, Greece Sampling 2). Bray–Curtis distance and data transformation were applied for the analyses. Different types of samples are shown with different symbols: European sea bass larvae (); gilthead sea bream larvae (); microalgae (); rotifer (); *Artemia* (); water (). Samples are coded so that age, site, and time of sampling are perceptible. Larvae of different ages are indicated as follows: 1 (5–11 dph), 2 (20–23 dph) and 3 (39–77 dph). The letters A, B, C, D, E, F, G, H, and I identify the different hatchery sites (see Fig. 1 for more details). The letters J and M represent the different sampling times, January, and May, respectively. Water from source (S), after UV treatment (U) and in hatchery tanks (T) are also specified (see also Suppl. Table 1).

comparison of larvae at different ages (Fig. 9a), different foods (Fig. 9b) and larvae - food comparisons (Suppl. Fig. 11). "Quorum sensing", "peptidoglycan biosynthesis", "starch and sucrose metabolism", "oxidative phosphorylation", and "terpenoid backbone biosynthesis" were enriched in younger larvae, while "biofilm formation", "bacterial secretion system", "lipopolysaccharide biosynthesis", "cationic antimicrobial peptide (CAMP) resistance", "biosynthesis of amino acids", "homologous recombination and bacterial invasion of epithelial cells" were enriched in older larvae (Fig. 9a). Generally, a greater number of enriched pathways were found in *Artemia* compared to rotifer and microalgae. Enriched pathways in *Artemia* included "two−component system", "*Staphylococcus aureus* infection", "peptidoglycan biosynthesis", "quorum sensing", "fructose and mannose metabolism", "starch and sucrose metabolism", and "galactose metabolism". "Bacterial secretion system" was enriched in rotifers and microalgae compared to *Artemia* (Fig. 9b). "Two-component system", "phosphotransferase system (PTS)", "peptidoglycan biosynthesis", "biosynthesis of amino acids", "starch and sucrose metabolism", "fructose and mannose metabolism", "beta-lactam resistance", "ubiquinone and other terpenoid-quinone biosynthesis", "terpenoid backbone biosynthesis" were enriched in larvae compared to rotifer and microalgae (Suppl. Fig. 11). "Quorum sensing" was enriched in larvae compared to rotifer and "oxidative phosphorylation" was enriched in larvae compared to microalgae (Suppl. Fig. 11). "Legionellosis", "cell cycle – *Caulobacter*", and "arginine and proline metabolism" were enriched in microalgae compared to larvae (Suppl. Fig. 11). There was no significant pathway enrichment when larvae were compared to *Artemia*.

4. Discussion

The present 16S rRNA gene metabarcoding study shows that there are significant differences in microbial communities between larvae development stages, food items and source of water, and that some communities are abundant irrespective of sample type, geographical location or time of year. The reported large-scale approach has the advantage of identifying a common core microbiota independent of

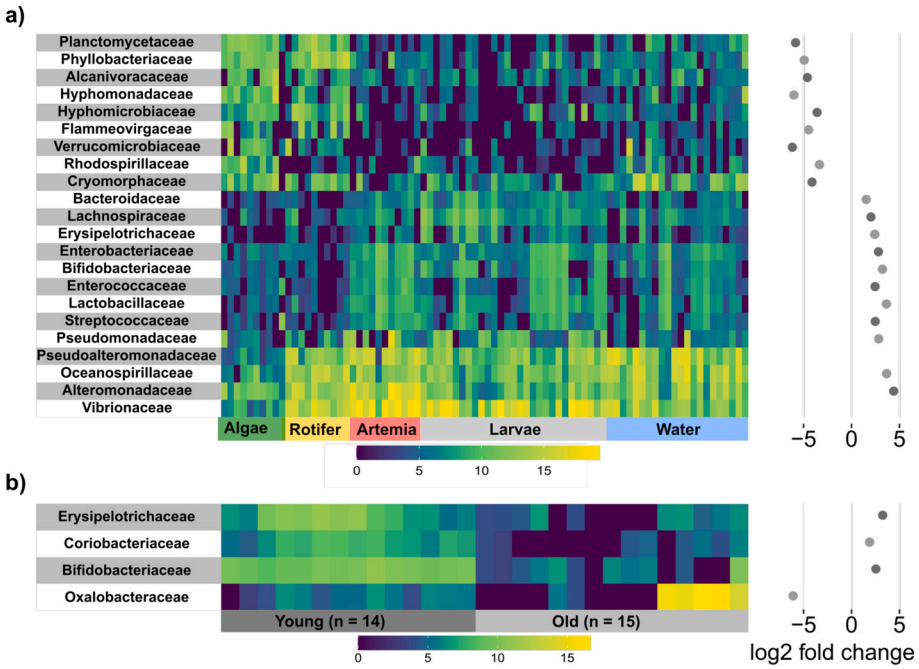

Fig. 6. Differentially abundant bacterial families across different types of samples and larvae; a) the model factors included site, time of sampling (January and May) and sample type (microalgae, rotifer, *Artemia*, larvae); b) the factors included in the model included species (European sea bass and gilthead sea bream), site, time of sampling (January and May) and larval age range (younger = 5–23 dph and older = 39–77 dph). The R package metagenomeSeq v 1.32.0 was used to identify differentially abundant OTUs with an adjusted p-value < 0.05 (Paulson et al., 2016). Bacterial families with significant changes in each comparison are visualized with heatmaps using the R package superheat v 1.0.0 (Barter and Yu, 2021).

rearing conditions in each hatchery. The most abundant or core bacteria identified in the current study will be a valuable guide for understanding microbiome development during gilthead sea bream and European sea bass larval ontogeny and the modulation of the microbiota by the fish or external factors. Moreover, the diverse microbiota abundance and function across different sample types provides the basis for a robust tool for microbiome management in aquaculture.

4.1. Core and most abundant bacterial communities

The most abundant and common phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, detected across all samples, including larval species, food and water in the present study, may be considered core or common bacterial phyla in aquaculture (seawater or freshwater), since they have also been identified among the topmost bacterial phyla in other studies of fish microbiomes. Previous studies that pointed to all or some of these phyla include the microbiomes of gilthead sea bream and European sea bass eggs and water (Najafpour et al., 2021a), adult skin and gills (Pimentel et al., 2017; Rosado et al., 2019, 2021), gilthead sea bream larvae and live feeds (Califano et al., 2017), cod larvae (*Gadus morhua*) and their live feeds (Bakke et al., 2015) and adult rainbow trout (*Oncorhynchus mykiss*) gut (Ingerslev et al., 2014). The presence in almost all samples of the present study (larvae, rotifer, *Artemia*, microalgae, water) of *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Escherichia*, *Marinomonas*, *Phaeobacter*, *Bifidobacterium*, *Pseudomonas*, *Lactobacillus* and *Bacteroides* is most likely explained by their high adaptability to marine environments. This may stem from the fact they are symbiotic and contribute to nutrient exchange between the host and microbiota, e.g., some species of the genera *Bifidobacterium* (Turroni et al., 2008), *Lactobacillus* (Walter et al., 2011), *Bacteroides* (Comstock, 2009) and *Phaeobacter* (Fuentes et al., 2016).

The high abundance of *Vibrio* and *Pseudoalteromonas*, is in line with other studies and indicates that these two genera are among the most successful in marine environments (Baker-Austin et al., 2018; Parrilli et al., 2021). The identification of potentially pathogenic *Vibrio* and *Pseudoalteromonas* in hatcheries may be problematic if dysbiosis occurs, as it may lead to disease outbreaks. Together with commonalities, we also found discrepancies with previous studies of fish microbiota. For example, a study of gilthead sea bream food microbiomes identified *Loktanella* and *Paracoccus* as dominant in rotifer and *Artemia*, respectively (Califano et al., 2017). In contrast, in our data *Loktanella* had a maximum abundance of 1 % in one *Artemia* sample from ItS (*Artemia*-IUJ) and *Paracoccus* was among the top bacteria in only two *Artemia* samples (*Artemia*-HM and GM, GrS2). These differences emphasize the need when studying fish microbiota from an aquaculture perspective to have samples with sufficient breadth and scope to overcome bias driven by specific and restricted environmental conditions, particularly if the aim is to develop comprehensive disease management tools. For example, the use of the most efficient probiotics in diverse hatchery conditions, the identification of the core microbiota, or potential beneficial interactions between bacteria and the host in different environments should be specified by comprehensive studies that consider multiple variables across multiple sites.

4.2. Diverse bacteria across larvae, food, and water

Although a common core microbiota existed across different samples there was divergence in the global microbiome of the European sea bass and gilthead sea bream larvae that suggested the sample type (microalgae, *Artemia*, rotifer, and water), larval age, and geographical location influenced the composition of the microbiota, as has previously been observed (Bakke et al., 2015; Califano et al., 2017). A higher richness and lower Shannon diversity was reported for rearing water compared to gilthead sea bream (Califano et al., 2017) although in our study the relationship did not achieve statistical significance. The difference in microbial abundance and composition of different types of food is suggested as a potential modulator of the larval microbiome and highlights one of the challenges of feeding larva with live food in hatcheries. For example, the high bacterial richness or diversity of live feeds (marine invertebrates) means that in addition to providing essential nutrients for larvae they may also have positive benefits for the establishment of the larval microbiota (Vázquez-Salgado et al., 2020). The lower bacterial richness of microalgae compared to other live foods and their capacity to disrupt bacterial communication or quorum sensing (Natrah et al., 2011) may be advantageous in relation to potential pathogens. The lower abundance of the families *Vibrionaceae* and *Pseudoalteromonadaceae* in microalgae compared to *Artemia* and rotifers may indicate that microalgae have a minor contribution to the establishment

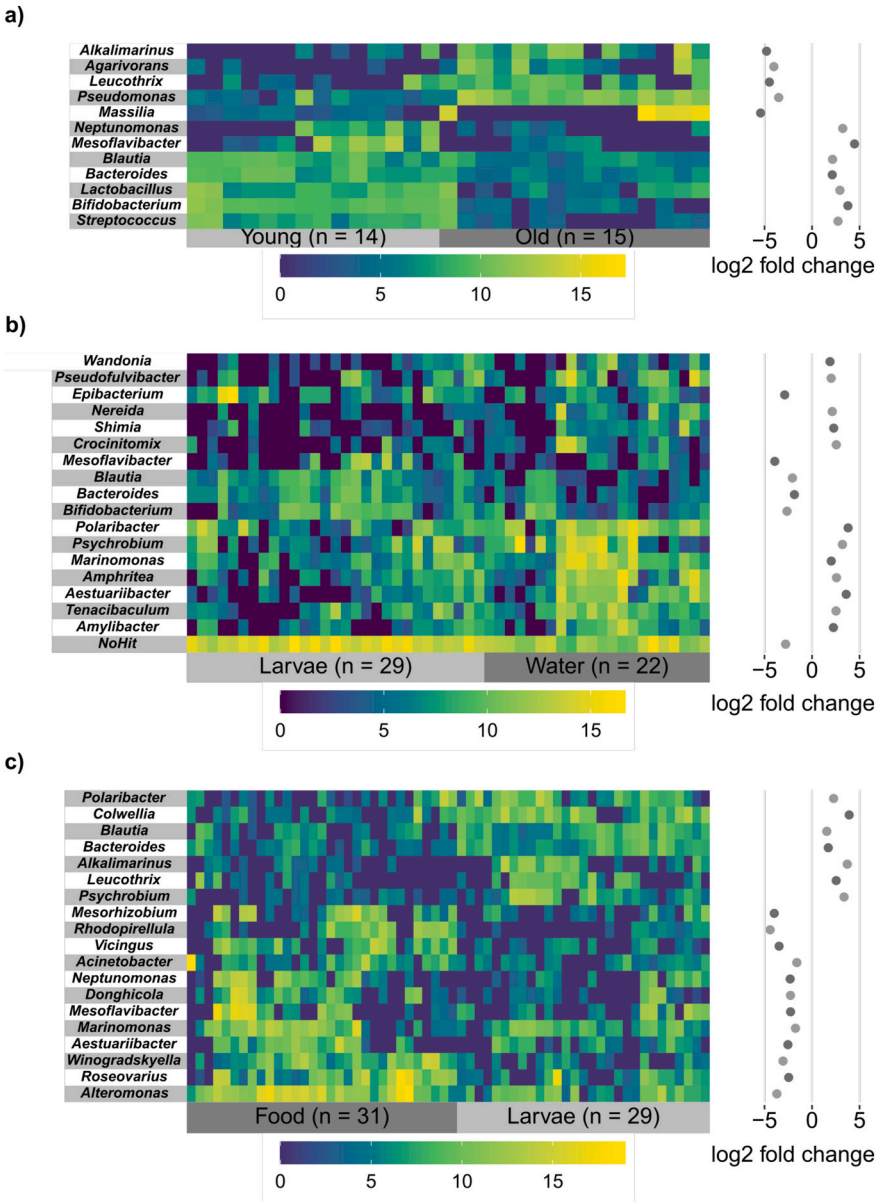

Fig. 7. Differentially abundant bacterial genera comparing younger and older larvae, larvae and water, and larvae and food; a) the model factors included species (European sea bass and gilthead sea bream), site, time of sampling (January and May), and larvae age range (younger = 5–23 dph and older = 39–77 dph); b) the model factors included site, time of sampling (January and May) and sample type (larvae and water); c) the model factors included site, time of sampling (January and May) and sample type (larvae and food), the separate analysis of differentially abundant genera between larvae and each food type (microalgae, rotifer, and *Artemia*) is presented in Suppl. Fig. 10. The R package metagenomeSeq v 1.32.0 was used to identify differentially abundant OTUs with an adjusted p-value < 0.05 (Paulson et al., 2016). Bacterial families with significant changes in each comparison are visualized in heatmaps generated with the R package superheat v 1.0.0 (Barter and Yu, 2021).

of these two bacterial genera in the larval microbiome. The lifecycle and culture condition of rotifers, such as very high organic loads due to rotifer feed, dead rotifers, and faecal matter (Dhont et al., 2013), may explain the higher bacterial richness of rotifers compared to microalgae and *Artemia*. The contribution of *Artemia* for the establishment of beneficial gut microbiota in fish larvae such as the genera *Bifidobacterium* spp. (Turroni et al., 2008), *Lactobacillus* spp. (Walter et al., 2011) and *Enterococcus* spp. (Ringø et al., 2010) is probably high since these bacteria are more abundant in *Artemia* compared to rotifer and microalgae.

4.3. Seasonal effects on the microbial community

The influence of season and temperature change on the gut microbiota has been reported in several studies (Hagi et al., 2004; Dulski et al., 2020; Bereded et al., 2021). Overall, in the present study there were small changes in the microbial community between January and May, possibly because of the consistent and controlled environmental parameters (e.g., temperature, oxygen) in closed circuits/semi-closed circuits of the aquaculture units of the gilthead sea bream and European sea bass hatcheries. Nonetheless, a significant change in abundance of some bacterial genera was observed such as the increase in May compared to January of the relative abundance of *Aestuariibacter* (mainly in water, larvae, and *Artemia*) and *Amphritea* (mainly in water and larvae) or the decrease of *Blautia* (mainly in water, larvae, microalgae, and rotifers).

4.4. Effect of geographical location on the microbial community

The divergence in bacterial communities between sites was less pronounced, possibly because of the limited number of replicated samples from each site or the higher differences in the microbiol community of different sample types that are included in a single PcoA. However, the differences in the relative abundance of bacteria in samples from different sites and the separation between specimens that belonged to the same group (e.g., the separation between microalgae samples in January) suggest an effect of the geographical location on the abundance of microbial communities. An effect of geographical location on the microbial community of water and eggs of European sea bass and gilthead sea bream has previously been reported (Najafpour et al.,

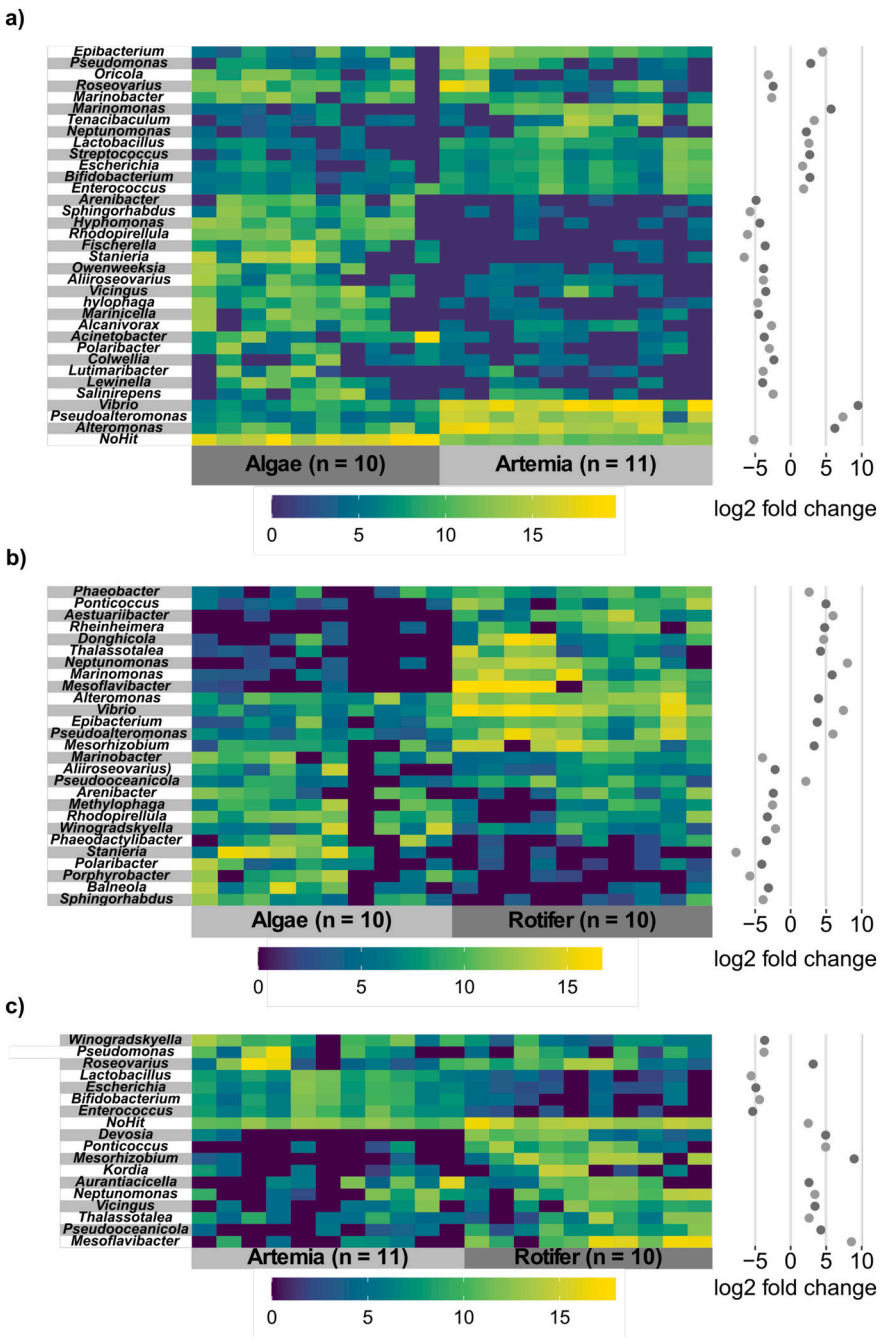

Fig. 8. Differentially abundant bacterial genera comparing different types of foods; a) the factors in the model included site, time of sampling (January and May), and food type (microalgae and *Artemia*); b) the model factors included site, time of sampling (January and May), and food type (microalgae and rotifer); c) the model factors included site, time of sampling (January and May), and food type (*Artemia* and rotifer). The R package metagenomeSeq v 1.32.0 was used to identify differentially abundant OTUs with an adjusted p-value < 0.05 (Paulson et al., 2016). Bacterial families with significant changes in each comparison are visualized with heatmaps using the R package superheat v 1.0.0 (Barter and Yu, 2021).

2021a). The presence of a different abundance of bacterial genera in the rearing tank water and source water in some sites is most likely a consequence of the water treatment regime, management practices and the introduction of feed and even larvae.

4.5. Age related microbial community

Developmental stage, diet, and rearing water are three factors previously reported to modulate the gut microbiome with age (Wong and Rawls, 2012; Llewellyn et al., 2014; Bakke et al., 2015) and the difference in the microbial composition of European sea bass and gilthead sea bream larvae at different developmental stages in this study corroborated previous observations in zebrafish adults (Yan et al., 2012), cod larvae (Bakke et al., 2015), and gilthead sea bream and European sea bass adults (Rosado et al., 2021). In the present study some bacterial genera typical of the adult gut, such as *Lactobacillus*, *Streptococcus* and *Blautia* (*Firmicutes*), and *Bifidobacterium* and *Bacteroides* were at higher abundance in early larval stages, and we speculate this may indicate a significant change in the gut microbiota occurs with age.

Host selection of beneficial bacteria to establish a mutualistic relationship may explain the higher abundance of genera containing beneficial bacteria (e.g., *Bifidobacterium* and *Lactobacillus*) during early development (Sullam et al., 2012; Bakke et al., 2015), and provides the selective pressure needed to boost the host immune system (Swain et al., 2006). Live foods with a high abundance of beneficial bacteria may play an important role in modulating the larval microbiome composition. An example of this is the genera *Mesoflavibacter*, which was abundant in larvae at start of feeding compared to mid-metamorphosis and presumably originated from the rotifers where they are also abundant and, which are fed to early larvae. Most of the abundant bacteria in early

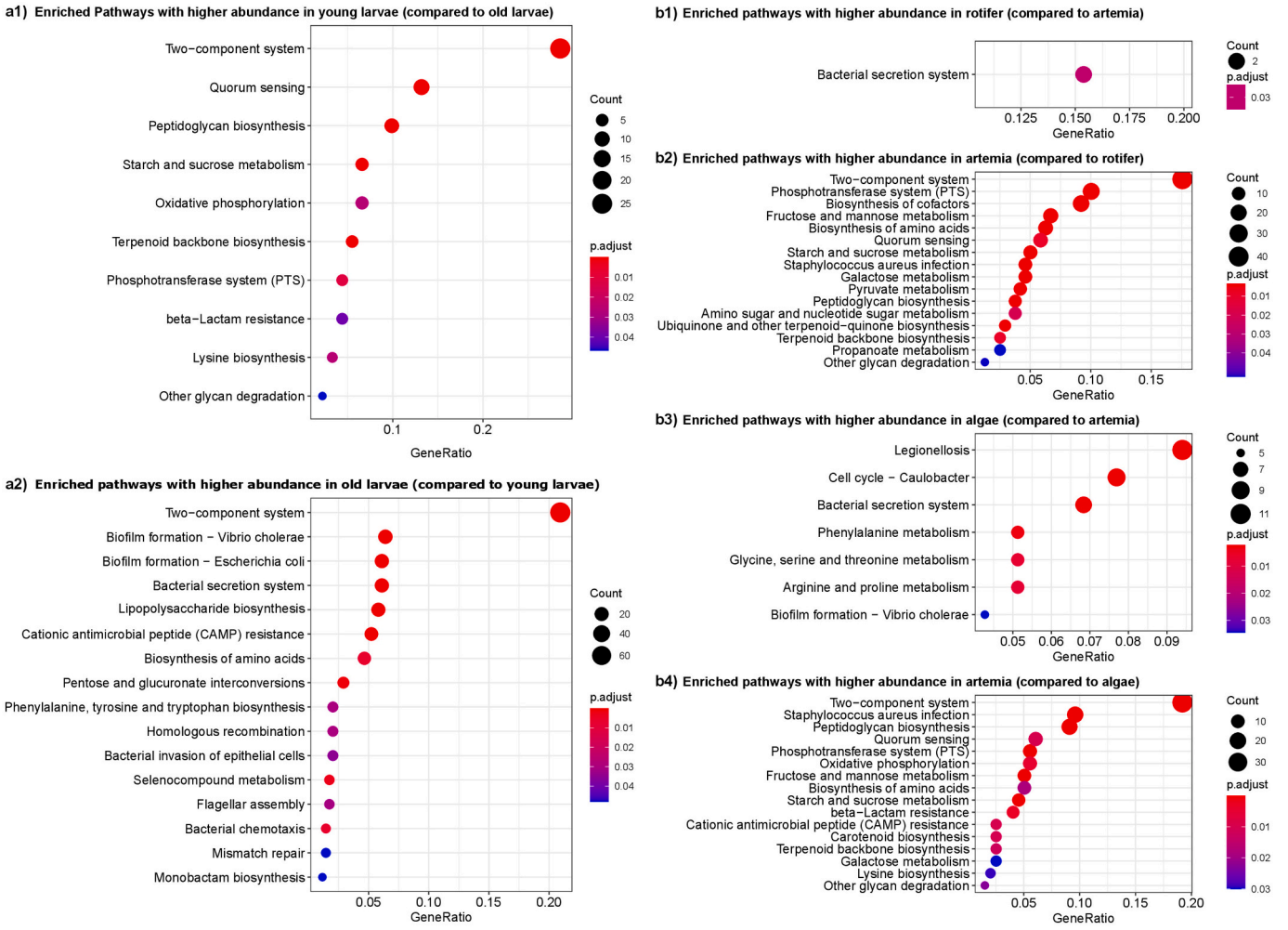

Fig. 9. The enriched KEGG pathway analyses. a1) The pathways enriched in younger larvae (age range = 5–23 dph), a2) the pathways enriched in older larvae (age range = 39–77 dph). The larvae samples included both European sea bass and gilthead sea bream species. b1–b4) The enriched KEGG in each food sample using pairwise comparisons of different types of food (microalgae, rotifer, *Artemia*). Differentially abundant KOs were found using two methods, the RNA-seq method-edgR algorithm and metagenomeSeq. Based on age and food type variables, the common KOs obtained by the two methods and that showed higher abundance in each age group (a = younger, b = older) or food (microalgae, rotifer, *Artemia*) were used in KEGG enrichment analysis.

larval stages are characteristic of the gut microbiota and are anaerobic, while the shift in dominance to other genera (e.g., *Alkalimarinus*, *Leucothrix*, *Pseudomonas*, and *Agarivorans*) in older larvae (e.g., metamorphosis) may reflect modulation of the microbiota by species-specific morpho-physiochemical changes (e.g., increasing intestinal folds) (Najafpour et al., 2021b) and the change in feed regime that shifts from microalgae to *Artemia*. The abundance of bacteria genera in larvae, e.g. *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Blautia* suggests they may colonize larvae at early developmental stages and prevent host intestinal colonization by other microorganisms such as the anaerobic bacteria, *Blautia producta* and *Clostridium bolteae*, and in this way prevent infection by vancomycin-resistant enterococci (Caballero et al., 2017; Barbeiro et al., 2020). The consequence of the high abundance of *Streptococcus* when larvae start to feed needs to be further studied since this genus includes species that may be potential pathogens or probiotics.

4.6. Host-bacteria interactions during larval development

Bacteria use quorum sensing to regulate a diversity of physiological activities, e.g., symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller and Bassler, 2001). Since the most abundant bacteria at start feeding (compared to mid-metamorphosis) were associated with the gut, the "quorum sensing" enrichment in younger larvae (5–23 dph) may be indicative of symbiosis and the colonization of the gut environment by bacteria (Jimenez and Sperandio, 2019). Enrichment of "biofilm formation" pathways in mid-metamorphosis (39–77 dph) larvae may be related to the increased surface area and villosity of the gut as it matures. Although this process is expected to be beneficial it is also associated with infections and pathogenesis in humans (Hall-Stoodley and Stoodley, 2009; Bjarnsholt, 2013). Considering the abundance of *Vibrio* and that some species are pathogenic biofilm forming species (Tan et al., 2015) they could pose a disease risk in dysbiosis. Enrichment of the "bacterial invasion of epithelial cells" pathway further emphasizes the potential for pathogenic bacteria colonization in early larval stages. It should be noted, however, that bacterial metabolic pathways are mainly named based on human nomenclature. For instance, biofilm formation during gilthead sea bream and European sea bass larval development in this study is likely to be by other bacteria species rather than *V. cholerae* and *E. coli*, which are associated with the biofilm pathway in humans.

Enrichment in fish larvae microbiota of "peptidoglycan biosynthesis" and "beta−Lactam resistance" pathways in younger larvae and "lipopolysaccharide biosynthesis" and "cationic antimicrobial peptide (CAMP) resistance" pathways at mid-metamorphosis is suggestive of bacterial resistance caused by major modifications in the microbial cell

wall and defense against potential host antimicrobial peptides or antibiotic usage in aquaculture (Ryu et al., 2012; Anaya-López et al., 2013; Nikolaidis et al., 2014; Chen et al., 2018; Rebl and Goldammer, 2018). Although, a further interesting possibility suggested by the enrichment terms that requires further investigation is the developmental status of the larval immune system. Different resistance mechanisms of bacteria across larval stages may be a consequence of changes in bacterial composition, e.g., a change in gram-positive and gram-negative bacterial communities. Exposure of bacteria to the host oxidative stress defenses may lead to DNA damage followed by homologous recombination DNA repair (Michod et al., 2008), as suggested by enrichment of the "homologous recombination" pathway at the mid-metamorphosis stage.

The enrichment of the "starch and sucrose metabolism" pathway in younger larval stages (5–23 dph) and "biosynthesis of amino acids" and "pentose and glucuronate interconversions" pathways at the mid-metamorphosis stage suggests a potential trade-off in the metabolism of the host and microbiota during larval development. "Enrichment of phenylalanine, tyrosine, and tryptophan biosynthesis" pathways at mid-metamorphosis suggest that an increase in biosynthesis of specific amino acids could be a key factor in increasing bacterial load/abundance and colonization of the host. Biosynthesis of several amino acids was previously reported to be enriched in older age groups of European sea bass and gilthead sea bream, when comparing the gill and skin microbiota of mature and juvenile fish (Rosado et al., 2021) although not directly comparable with our study of larvae it may suggest an increasing trend in amino acid biosynthesis with age. Enhanced biosynthesis and metabolism pathways for carbohydrates, amino acids, and lipids in ryegrass-fed grass carp was proposed as a response to a low-protein diet (Ni et al., 2014). The pairwise comparison in the present study of the functional predictions for microbiota in microalgae, *Artemia* and rotifer suggests diverse impacts of different live foods on the fish microbiota. The *Artemia* microbiota was enriched in the "biosynthesis of amino acids" pathway, which is essential for bacterial growth and survival (Amorim Franco and Blanchard, 2017) and allows pathogen growth in amino acid-deficient environments (Zhang and Rubin, 2013; Li et al., 2019). Potential deficiencies in amino acids may result from a host cell response to deplete intracellular amino acids (e.g., tryptophan) during pathogen invasion (Silva et al., 2002) or result from the lack of essential amino acids (e.g., phenylalanine and tryptophan) in fish food.

Overall, our data shows that microbial abundance in larvae and *Artemia* are more similar and that more pathways are enriched in *Artemia* than the other live feeds. Both beneficial and harmful effects on larval microbiota are predicted from the enriched pathways in *Artemia* (compared to other live feeds). Beneficial would be supporting the host with carbohydrate metabolism (e.g., the enrichment of "fructose and mannose metabolism", "starch and sucrose metabolism", and "galactose metabolism" pathways). Harmful would be potential vectors to increase bacterial resistance or transfer of potential pathogens (e.g., the enrichment in "peptidoglycan biosynthesis" or "beta-lactum resistance" pathways).

5. Conclusions

A large-scale field study of aquaculture hatcheries geographically separated in the Mediterranean identified the core (e.g., *Vibrio* and *Pseudoalteromonas*) and most abundant bacterial genus in different types of samples (larvae, microalgae, rotifer, *Artemia*, water). Season (January vs May) had a minor effect on the microbial community possibly because physico-chemical conditions are kept constant in the closed recirculating systems of hatcheries. Geographical location influenced the abundance of some bacteria. The microbiota of European sea bass and gilthead sea bream larvae was similar with some exceptions while the microbial community of their tank water did not differ. Both live foods and rearing water contributed to the larval microbiota during larval ontogeny, but with differing importance. There was a higher possibility of *Vibrio* transfer to larvae with *Artemia* and rotifer than with microalgae and water. This study suggests strong host selection of beneficial bacteria to establish a mutualistic relationship in very early larvae (at the start of feeding) compared to mid-metamorphosis larvae and suggested bacteria of the *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Blautia* genera are part of the beneficial communities which colonize larvae at early stages. Functional analyses of significantly higher abundance bacterial genera predicted a potential interaction between the host and microbiota during larval development that may be linked to requirements of bacteria for growth and resistance and to changes in host demand, physiology, immune system and morphology. In respect to the effects of feeds, *Artemia* microbiota seemed to have a higher contribution to biosynthesis of amino acids and carbohydrate metabolism. The low relative abundance of beneficial bacteria in live feeds and tank water in the present study indicates manipulation of bacteria in aquaculture facilities using food or water enrichment techniques (e.g., probiotics) may be an effective way to counter negative effects of abundant and potentially pathogenic bacteria of the *Vibrio* and *Pseudoalteromonas* genus.

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CRediT authorship contribution statement

Babak Najafpour: Methodology, Formal analysis, Validation, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Patricia I.S. Pinto:** Formal analysis, Validation, Investigation, Visualization, Writing – review & editing. **Eric Climent Sanz:** Formal analysis, Validation, Investigation, Writing – review & editing. **Juan F. Martinez-Blanch:** Formal analysis, Validation, Investigation, Writing – review & editing. **Adelino V.M. Canario:** Resources, Writing – review & editing, Supervision, Project administration. **Katerina A. Moutou:** Investigation, Resources, Writing – review & editing, Funding acquisition. **Deborah M. Power:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

We have deposited the raw data in NCBI and indicate the reference code in the methods (under the Sequence Read Archive (SRA) with the BioProject accession number PRJNA608636)

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