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Long-term adaptation to dietary shifts of gut microbiota in gilthead seabream (*Sparus aurata*)

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In many meta-analyses and literature reviews on fish microbiota, the provenance of the animals (farmed vs. wild) is often overlooked. Given the well-established role of diet as a key factor in shaping gut microbiota, this study investigates the impact of dietary nature by comparing the microbiota of gilthead seabream (*Sparus aurata*) fed a commercial diet versus a wild-type diet, all reared within a recirculating aquaculture system. Over a 60-day period, we tracked changes in gut bacterial diversity, structure, and composition following a shift from a commercial feed to a diet exclusively based on pink shrimp (*Parapenaeus longirostris*). Gut bacterial communities were assessed using 16S rRNA gene sequencing (Illumina MiSeq platform) with primers targeting the V3-V4 hypervariable regions. Twenty days after the dietary change, microbial diversity (Shannon index) increased in fish fed the shrimp diet compared to those fed the commercial diet, while Dominance index values decreased. Additionally, inter-individual (beta-) diversity based on Bray-Curtis distances also differed between dietary treatments. These results support further that microbiota comparisons between farmed/captive and wild fish are challenging due to the unpredictable feeding regimes and dietary variations in wild fish. However, the diet impact on microbiota diminished over time, with the differences in intra- and inter-individual diversity being reduced after 40 days, which suggests an adaptation of microbial communities to dietary changes. At this point, gut microbial communities also showed a similar taxonomical composition. Moreover, a core microbiota consisting of species belonging to the genera *Ralstonia*, *Paraburkholderia*, *Fulvimonas*, *Pseudomonas*, and *Cutibacterium* was maintained in all sampling times under both dietary treatments. Overall, this study serves as a conceptual approach that shows a long-term adaptation of the gut microbiota after a radical dietary change, probably driven by host-inherent factors. Furthermore, these results may be a valuable insight for feed manufacturers aiming to develop sustainable and cost-effective ingredients since they suggest that some alternative feeds and ingredients do not have adverse long-term effects on fish gut microbiota.

KEYWORDS

diet change, wild-type diet, commercial diet, gut microbiota, time course, core microbiota, aquafeeds

1 Introduction

The fish gut microbiome plays an important role in numerous aspects of fish life-history and physiology, including digestion, nutrient absorption, immunity, and even behavior or stress response (Diwan et al., 2022). In recent years, understanding the microbial diversity and their metabolic function has garnered increasing attention due to its profound implications for fish condition and health, aquaculture practices, and ecosystem sustainability. The composition and diversity of the fish gut microbiota are influenced by various factors, such as habitat, host genetics, environmental conditions, and particularly, the diet-associated factors (Silva et al., 2011; Ghanbar et al., 2015; Degregori et al., 2024). Different dietary components, such as carbohydrates, proteins, lipids, and fiber, serve as specific substrates for microbial metabolism, that together with the feed-accompanying microbes may influence microbial species diversity (Li et al., 2014; Pan et al., 2023; Viver et al., 2023).

The growing interest in optimizing the utilization of fish meal and fish oil in aquafeeds has prompted to the search for sustainable and economically viable alternative ingredients. This search aims to identify substitutes that not only align with environmental sustainability but also contribute positively to fish health, with a particular focus on understanding the potential influence of dietary components on the gut microbiome (Rimoldi et al., 2018a; Niu et al., 2019; Xie et al., 2022). In addition, the importance of studying the diet-microbiome modulation also lies in the fact that dietary alternatives cannot only have health-promoting effects, but may also cause dysbiosis, impairing the composition and/or functions of the gut microbiota, which subsequently can have a physiological effect, such as the generation of an intestinal injury, inflammation or a reduced immune response (Infante-Villamil et al., 2021). Hence, examining microbial fluctuations over time after a diet change can offer valuable insights into the diet impact on the fish microbiota and intestinal health.

During the last years, numerous studies have highlighted that diet is a primary factor influencing gut microbial communities (Ringø et al., 2016; Egerton et al., 2018; Ruiz et al., 2024). However, the scientific literature often compares gut bacterial taxa in fish without accounting for whether they are reared under wild or cultured conditions. In this regard, several comparison studies have shown significant differences in gut microbial diversity, structure, and composition between farmed and wild species. This was the case for gilthead seabream (*Sparus aurata*) (Kormas et al., 2014), fine flounder (*Paralichthys adspersus*) (Ramírez and Romero, 2017), Nile tilapia (*Oreochromis niloticus*) (Bereded et al., 2021), common carp (*Cyprinus carpio*) (Ruzauskas et al., 2021), Korean rockfish (*Sebastes schlegelii*) (Yu et al., 2021), zig-zag eel (*Mastacembelus armatus*) (Liu et al., 2022), red cusk-eel (*Genypterus chilensis*) (Romero et al., 2022), brook trout (*Salvelinus fontinalis*) (Mugetti et al., 2023) and pike perch (*Sander lucioperca*) (Wang et al., 2024), among many other fish species. In addition, in our previous study we observed that two distinct wild fish species, the pearly razorfish (*Xyrichtys novacula*) and gilthead seabream, exhibited a more similar microbial profile

than the wild and farmed gilthead seabream, suggesting that environmental and dietary factors significantly shape microbial composition (Viver et al., 2023). In this sense, numerous environmental factors influence microbial modulation, including the water temperature, salinity, and the microbial composition of the environment (Roeselers et al., 2011; Rudi et al., 2018). Furthermore, the ingredient formulation and composition of the commercial feeds differs significantly from the fish diets in natural environments. However, the aforementioned studies reflect that is challenging to discern between the impacts caused by the environmental conditions and by the diet on the gut microbial communities.

To elucidate the impact of the diet type on fish gut microbiota over time, this study aims to evaluate how the microbial communities of gilthead seabream are modulated over a 60-day period following a dietary shift from commercial feed to a diet exclusively composed of deep-water pink shrimp (*Parapenaeus longirostris*), which more closely mimics the fish diet under natural conditions. Gilthead seabream was selected as a biological model for this study due to its status as the second most farmed fish in the European Union (APROMAR, 2023) and its importance in aquafeed research within the framework of the search for novel sustainable ingredients (Matos et al., 2017; Porcino and Genovese, 2022). The current approach seeks to determine whether there is an adaptation period during which the microbiota adjusts to the diet change, and how the effect of the wild-type *vs.* commercial diet varies over time. The results of this study may provide valuable insights into the impact of the diet type on the frequently observed differences in microbiota composition when comparing wild and farmed fish, as well as supporting the exploration of novel aquafeeds formulated with more sustainable, cost-effective, and readily available ingredients.

2 Materials and methods

2.1 Experimental setup

The experiment was conducted at Insitute of Agrefood Research and Technology (IRTA) facilities, in La Ràpita (Tarragona, Spain). Gilthead seabream juveniles (1.6 g in body weight, BW) were purchased from a commercial fish hatchery in the East coast of Spain and transported by road to IRTA. A subset of these fish was used in our previous assay, so details about their acclimation may be found there (Viver et al., 2023). Fish with an average initial body weight (BW_i) of 25.29 ± 4.90 g (standard deviation, SD) were randomly distributed in two tanks, each with a volume of 400 L (n = 30 per tank), connected to an IRTAmar^{TM} water recirculation system, where they were maintained for 4 months. During three months the fish fed with the commercial feed D-2 Optibream AE 1P (2.2 mm pellet size, Skretting; Nutreco N.V.), and in the fourth month, the commercial feed was progressively switched to D-4 Optibream AE 3P (4 mm pellet size, Skretting) in order to adjust pellet size to fish growth. The macronutrient proximate composition of D-2 Optibream AE 1P was 48.5% crude protein,

18% lipids and 18.5 MJ kg^{-1} digestible energy, while D-4 Optibream AE 3P contained 44% crude protein, 20% lipids and 18 MJ kg^{-1} digestible energy (Serie D2-D5 Fish meal composition is: fish oil, vegetable oils, cereal products and by-products, legume products and by-products, oilseed products and by-products, vitamins and minerals). After 4 months of being exclusively fed with commercial diets, the fish from the second tank were switched to a diet exclusively based on deep-water pink shrimp (T0), while the fish from the first tank continued being fed with D-4 Optibream AE 3P (Skretting), in order to compare the microbial dynamics induced by the diet change with respect to a control group over time. For the diet shift assay, 25 kg of entire individuals of deep-water pink shrimp (total length: 4-6 cm) which had been frozen only once just after capture with no further manipulation, were purchased from a local fishing company. However, commercial crustaceans normally contain sodium metabisulfite (E223), which is a synthetic antioxidant preservative added to avoid melanosis after removal from the water with a slight antimicrobial activity (Miraglia et al., 2021). To avoid the potential penetration of sodium metabisulfite into the shrimp tissues, before thawing, shrimps were cleaned with abundant sterile seawater. Then, shrimps were cut into pieces of between 2-3 cm length to facilitate their ingestion. Fish from both tanks were manually fed ad libitum with their respective diets three times per day during all the experiment, which extended for a period of 60 days after the diet change of the second tank (T0). Specimens from both tanks were collected at 20, 40, and 60 days after T0, (T20, T40, and T60, respectively). In each sampling time, eight specimens were collected from each condition (Supplementary Figure S1, Supplementary Table S1). To monitor the tanks' water quality at the microbiological level during the experiment, each week an aliquot of 100 μL was spread onto TSA (Trypticase Soy Agar) + 0.6% NaCl, and TCBS (Thiosulphate Citrate Bile Salts Sucrose) Agar medium and incubated at 22°C for 48 h to obtain the Total Viable Counts (CFU) of heterotrophic microorganism and *Vibrionaceae*, respectively.

The water temperature (18–22°C) and dissolved oxygen (6.4 ± 0.6 mg L^{-1}; OXI330, Crison Instruments) were daily measured and controlled, and pH (7.4 ± 0.2; pHmeter 507, Crison Instruments), salinity (36‰; MASTER-20 T, ATAGO Co. Ltd), ammonia (0.14 ± 0.1 mg NH$_4^+$ L^{-1}) and nitrite (0.2 ± 0.1 mg NO$_2^-$ L^{-1}) levels (HACH DR9000 Colorimeter, Hach, Spain) were weekly monitored. Just after T20, the fish were transferred to two other 400 L tanks for the cleaning and maintenance of the tanks in this module, but the water quality and environmental conditions among modules were maintained.

2.2 Sample processing

For each sampling point, fish were manipulated as detailed previously (Viver et al., 2023). Briefly, all fish in each tank were deprived of feed for 86 h before the sampling in order to collect only the autochthonous microbiota (Hao and Lee, 2004; Naya-Català et al., 2021). Among them, fish were euthanized with an overdose of the buffered anesthetic (tricaine methanesulfonate, MS-222, Sigma-

Aldrich; 300 mg L^{-1}). The entire intestinal tract was aseptically extracted and the perivisceral fat surrounding the intestine was gently removed with a scalpel; then, a 3 cm section of the anterior intestine and another of the posterior intestine with the same length, were dissected using sterile scissors, and opened lengthwise. The mucosal content was gently scraped with a round edge spatula and immediately frozen at -80 °C for further microbial analysis. The rest of the individuals were fed again after the fasting period until the next sampling point when the above-mentioned process was repeated for the next 8 fish. The fish was considered as the experimental unit of this study since it is the smallest entity that is randomly assigned to a different treatment group (Wilson et al., 2023). In this sense, all fish used for the experiment had similar characteristics (fish age, size, origin), and they were exposed to the same controlled environmental conditions (temperature, dissolved oxygen, pH, salinity, nitrite, and ammonia levels) within the IRTAmar^{TM} water recirculation system. The use of the fish as the experimental unit is a common practice in microbiota studies (Rimoldi et al., 2018b; Kokou et al., 2019; Naya-Català et al., 2021; Viver et al., 2023) which takes into account the inter-individual variation in the microbial communities (Legrand et al., 2020; Panteli et al., 2020) and aligns with the 3Rs principle (Zemanova, 2020).

2.3 Microbial composition of commercial feed and shrimp

Commercial feed pellets (D-4 OptiBream AE 3P) and samples of shrimps were stored at -80 °C at each sampling time. For processing purposes, three feed pellets and three shrimps were crushed with an autoclaved mortar and cut into very small pieces with sterile scissors, respectively. A total amount of *ca.* 150 mg in wet weight from feed or shrimps were collected and used for DNA extraction by means of the FastDNA^{TM} Spin Kit for Feces (MP Biomedicals) following the manufacturer's protocol. Three extractions for feed and shrimps were performed from each sampling time.

2.4 DNA isolation, amplification, and sequencing

The FastDNA^{TM} Spin Kit for Feces (MP Biomedicals) was used to perform DNA extraction from the scraped mucosa content (up to 150 mg), following the manufacturer's instructions. Illumina amplicon sequencing was performed using the set of primers of the V3-V4 region of the 16S rRNA gene for Bacteria forward (5' - CCTACGGGNGGCWGCAG - 3') and reverse (5' - GACTACHVG GGTATCTAATCC - 3') (Herlemann et al., 2011) containing the forward (5' - TCGTCGGCAGCGTCAGATGTGTATAA GAGACAG - 3') and reverse (5' - GTCTCGTGGGCTCGGAGAT GTGTATAAGAGACAG - 3') Illumina sequencing adapters. A first PCR was performed with the following program: an initial step of 30 s at 98°C for polymerase activation and DNA denaturation, followed by 35 cycles of 10 s at 98°C, 30 s at 55°C, 30 s at 72°C, and a

final extension step of 2 min at 72°C. Another 8-cycle amplification with similar cycle conditions was then carried out to add specific dual-index barcodes to each template. After each PCR, the templates were purified using Ampure XP beads (Beckman Coulter, USA) and 1 μL of a 1:50 dilution was analyzed on a Bioanalyzer DNA 1000 Chip (Agilent Technologies, USA) to check the size of the final library product (Illumina, 2013). Then, DNA samples were pooled at equimolar concentrations and sequencing was performed using an Illumina MiSeq instrument (2x300 bp) (FISABIO, Valencia, Spain).

2.5 Amplicon sequence variant and operational phylogenetic unit approaches

Raw sequencing data were provided in a demultiplexed FASTQ format containing paired-end reads. The data were analyzed using the Qiime2 bioinformatic platform (Bolyen et al., 2019) with the parameters –p-trunc-len-f 280, –p-trunc-len-r 220, –p-trim-left-f 19 and –p-trim-left-r 22. Amplicon sequence variant analyses (ASV) were obtained with DADA2 software implemented in Qiime2. The representative sequence of each ASV was aligned using the non-redundant SILVA REF 138.1 database (Quast et al., 2013) and the ARB package (Ludwig et al., 2004). Sequence alignment was performed using the SINA tool implemented in the ARB program (Pruesse et al., 2012). The aligned sequences were inserted in the SILVA REF 138.1 database containing the pre-stablished operational phylogenetic units (OPUs) detected in Viver et al. (2023) for taxonomic classification, using the parsimony tool available in the ARB package. The non-affiliated sequences to the pre-stablished OPUs were inserted again in the SILVA REF 138.1 database to select the close relative sequences for phylogenetic analysis and *de novo* OPUs identification. In this context, the phylogenetic tree was manually inspected, and isolated sub-branches containing the query sequences along with at least one representative sequence were grouped into OPUs based on visual assessment of the final tree to ensure a high degree of accuracy (Mora-Ruiz et al., 2016; Viver et al., 2015). Predictive functional profiling was carried out with the R package Tax4Fun (Ref99NR) (Aßhauer et al., 2015) using the *trans_func* class function of *microeco* (Liu et al., 2021). Metagenomics functions were inferred from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and collapsed into pathways (level 1 – level 3).

2.6 Statistical analyses

Since the values of BW did not follow a normal distribution (Shapiro-Wilk test, $P < 0.05$), Wilcoxon-test was used to evaluate significant differences in BW among groups ($P \leq 0.05$). To analyze the alpha and beta diversity indices, the dataset was rarefed to the minimum sample depth (21,494 sequencing reads per sample) with the vegan R package (Oksanen et al., 2022). To assess the phylogenetic complexity of the samples, rarefaction curves, Shannon and Dominance indices were calculated using the PAST statistic tool (Hammer et al., 2001) based on the diversity and

abundance of 16S-based OPUs. The *microeco* R package (Liu et al., 2021) was used to calculate the Bray-Curtis dissimilarity values between the different community profiles of samples and periods of the assay based on OPUs. Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity was constructed using the vegan package (Oksanen et al., 2022) and plotted using the R package ggplot2 (Wickham, 2016) in R tool version 3.6.3 (R Core Team, 2020). The values of Shannon diversity index and Bray-Curtis dissimilarity were also compared between the two fish tanks at T0 (before the diet change) verifying that the tanks in which fish were initially located did not influence the microbial communities (Supplementary Figure S2). A divergence test between samples based on OPU abundance and composition was performed using non-parametric two-sample Kolmogorov-Smirnov tests (Jarek, 2015), since the data did not meet the assumptions of normality and homoscedasticity. Differences in alfa diversity indices and in the predictive functional pathways between groups were determined by Wilcoxon test ($P \leq 0.05$), with P-values adjusted by False Discovery Rate (FDR). Differences in beta diversity were evaluated by performing permutational multivariate analyses of variance (PERMANOVA), with P-values adjusted by FDR. According to the definition given in previous studies (Astudillo-García et al., 2017; Neu et al., 2021; Custer et al., 2023), in this work the core microbiota was considered as the microbial taxa shared by at least two individual samples among all the experimental conditions which was present at an average abundance of at least 2%. The cutoff for defining an OPU as highly abundant was set at 1% relative abundance.

2.7 Terminology on bacterial phyla

The authors are aware that certain phyla names have been officially proposed by Oren and Garrity (2021) as *Bacillota* (= Firmicutes), *Bacteroidota* (= Bacteroidetes), *Pseudomonadota* (= Proteobacteria), *Actinomycetota* (= Actinobacteria), *Planctomycetota* (= Planctomycetes), *Chloroflexota* (= Chloroflexi), *Fusobacteriota* (= Fusobacteria) and *Chlamydiota* (= Chlamydia), but for pragmatic reasons and comparative purposes with available published literature, the former nomenclature was used in this manuscript.

3 Results

3.1 Fish growth

Along the 60-day experimental trial, we observed a consistent BW pattern in both dietary groups. At the initial sampling day in which a group was submitted to the diet change (T0), the fish weighed an average of 158 ± 32.9 grams. At T20, the fish fed with shrimp showed a significantly higher BW (211.2 ± 23.8) than the fish fed with commercial feed (190.0 ± 25.1; $P = 0.038$). However, during T40 and T60, fish fed with both diets exhibited a similar BW (Wilcoxon-test; $P > 0.05$): an average BW of 202.1 ± 34.1 and 259 ± 48 g for those fed with commercial feed, respectively, and 220.3 ± 31.9 and 252 ± 31.8 g for those fed with deep-water pink shrimp,

respectively (P = 0.290 in T40, P = 0634 in T60; Supplementary Table S1).

3.2 Phylogenetic diversity based on OPUs

After trimming and chimera removal, all samples included in this study rendered a total of 10,957,204 sequenced reads, and the number of sequences per sample ranged between 21,494 and 160,269. All sequences were clustered in 8,072 ASVs and after phylogenetic inference a total of 1,256 OPUs were detected (ranging between 22 and 428 OPUs per sample) (Supplementary Table S2). Taking into consideration that an OPU is the smallest cluster of query sequences that affiliates with at least one reference sequence and considering that the size of each cluster typically falls within a sequence distance of less than <97% (considering the relatively short length of ~400 pb for partial sequences), we can assert with confidence that an OPU represents a single species (Mora-Ruiz et al., 2016). Each OPUs is recognized as a distinct species within genera whenever possible. To ensure statistical accuracy, the dataset was rarefied at the lowest number of sequences detected in a single sample (21,494; Supplementary Table S3), and the rarefaction curves demonstrated that the number of OPUs had reached their plateau at this number of reads for all samples (Supplementary Figure S3).

The phylogenetic analysis showed that the 1,256 OPUs were classified in 620 genera and 314 families, affiliated with 38 different phyla (Supplementary Tables S4, S5). In agreement with our previous assay (Viver et al., 2023), the phylum *Proteobacteria* was the most prevalent phylum, accounting for 42.9% of the total OPUs. Within this phylum, the majority of OPUs were classified as *Gamma-* and *Alphaproteobacteria*, representing 20.7% and 18.9% of the OPUs, respectively. *Firmicutes* (22.9% of the OPUs), *Actinobacteriota* (12.7%), *Bacteroidota* (7.0%), and *Planctomycetota* (2.2%) were also predominant in the examined samples. Furthermore, the study identified a total of 620 genera, from which 514 were represented by

only one OPU. However, there was a considerable OPU diversity within some genera, such as *Vibrio* (32 distinct OPUs), *Bacillus* (29 OPUs), *Lactobacillus* (27 OPUs), *Pseudomonas* (22 OPUs), and *Paenibacillus* (15 OPUs).

3.3 Microbial composition of the feed pellets and shrimp

The microbial diversity patterns showed that the commercial feed and shrimps presented the highest diversity values, as measured by the Shannon index (average of 2.09 ± 0.22 in the commercial feed and 3.35 ± 0.58 in pink shrimps), and the lowest dominance values (average of 0.22 ± 0.04 in the commercial feed and 0.13 ± 0.1 in pink shrimps), indicating a more even distribution of species compared to the fish gut microbiomes (Figure 1; Supplementary Table S6). Shannon diversity values were positively correlated with the detection of a higher number of specific OPUs from commercial feed datasets. Out of the 424 OPUs detected in commercial feed and 810 OPUs detected in pink shrimp, a substantial proportion (30% and 35.7%, respectively) were exclusive to each set of samples. In contrast, the exclusive detected OPUs in the fish microbiomes ranged from 6.1% to 2.0% (Supplementary Table S4). The microbial diversity of the commercial feed remained relatively consistent throughout the experiment, with the lowest range of Bray-Curtis dissimilarity values (average value of 0.12 ± 0.06) (Supplementary Table S7) suggesting that storage did not significantly alter the microbial assemblage of feed pellets for at least 60 days. The OPU analysis revealed that 12 microbial species were consistently abundant (with an abundance close to 1% in all samples), and their combined abundance ranged from 89.8% to 95.02%. Among them, six of these species affiliated with phylum *Firmicutes*, specifically the class *Bacilli*, where the OPU0015, OPU0011, OPU0036 and OPU0037 affiliated with *Lactobacillus* (average abundance of 36.02 ± 5.8%,
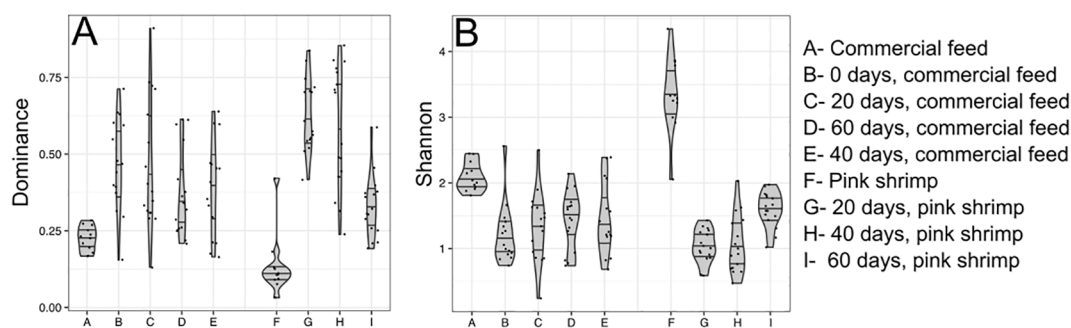

FIGURE 1
Alpha diversity variation among the experimental groups in relation to the Dominance **(A)** and Shannon **(B)** indices. Column A and F represent the direct microbial analysis of the commercial feed and deep-water pink shrimp (*Parapenaeus longirostris*), respectively. Columns B to E represent the gut microbiomes of gilthead seabream (*Sparus aurata*) collected at the beginning of the experiment and through 60 days of continuous feeding with commercial feed in chronological order. Columns G, H, and I represent the gut microbiomes of gilthead seabream fed with deep-water pink shrimp after 20, 40, and 60 days from the beginning of the experiment.

9.02 ± 1.4%, 1.75 ± 0.4% and 1.67 ± 0.3%, respectively), *Bacillus* OPU0145 (2.61 ± 0.4%) and *Staphylococcus* OPU0076 (1.49 ± 0.3%). The other highly abundant OPUs affiliated with *Ralstonia* OPU0522 (4.4 ± 3.6%) and *Photobacterium* OPU0624 (3.47 ± 0.9%). Moreover, a high abundance of chloroplasts (OPU1059) and mitochondria (OPU1038) sequences were detected in all samples (Figure 2; Supplementary Table S8). *Ralstonia* OPU0522 was found to be shared among the commercial feed (4.39 ± 3.65%), deep-water pink shrimp (18.84 ± 19.51%) and all fish gut microbiome samples (37.81 ± 29.37%) (Figure 2).

The microbial composition of shrimp exhibited a slightly higher level of heterogeneity (average Bray-Curtis dissimilarity value of 0.54 ± 0.2) than the compound feed, but all samples were closely clustered in the NMDS plot (Figure 3A). This variability could be associated to the batch selected for DNA extraction and sequencing. The high abundant OPUs detected in shrimp and absent or poorly detected in fish microbiomes affiliated with the *Flavobacterium* OPU1209 (relative abundance of 14.4 ± 11.9%) and *Chryseobacterium* OPU_R402 (2.1 ± 1.5%) of the *Bacteroidia* class; *Glutamicibacter* OPU0420 (2.4 ± 1.4%) and *Psychromicrobium* OPU0427 (2.07 ± 1.4%) of the *Actinobacteria* class; the *Psychrobacter* OPU0705 (11.9 ± 7.3%), the *Oceanimonas* OPU_P06 (1.9 ± 1.9%) and *Marinomonas* OPU0684 (3.5 ± 1.78%) of the *Gammaproteobacteria* class.

3.4 Gut microbial communities in fish fed with commercial diet

All gilthead seabream juveniles were fed with commercial pellets upon arrival to the experimental facilities and during the 4 months preceding the start of the experiment on the shift of food items. At time zero (T0), the control group continued being fed with the commercial diet over a period of 60 days and we did not observe any differences in species diversity and abundance between distal and proximal intestine for 80% of the specimens studied (n = 16 intestinal samples per diet) as indicated by the Kolmogorov-Smirnov test (Supplementary Table S9) and the NMDS plots (Supplementary Figure S4), which shows the distinction between distal and proximal intestine samples over time and diet. At T0, all samples exhibited a similar dominance and Shannon indices with T20 (with an average dominance value ranging from 0.47 ± 0.15 to 0.47 ± 0.22 and Shannon index ranging from 1.22 ± 0.46 to 1.33 ± 0.53 in T0 and T20, respectively). We detected a slight increase in diversity with a decrease in the dominance after 40 and 60 days (average dominance value ranging from 0.37 ± 0.14 to 0.39 ± 0.16 and a Shannon index ranging from 1.44 ± 0.43 to 1.46 ± 0.55 in T40 and T60, respectively) (Figure 1; Supplementary Table S6). No significant differences were observed in dominance and Shannon indices over time (Wilcoxon-test; $P > 0.05$). The NMDS plot, based on the Bray-Curtis index, indicated that the dissimilarity between the control samples from the T0 and T20 datasets was higher (averaging 0.67 ± 0.24 and 0.7 ± 0.24, respectively) compared to that within the T40 and T60 datasets (averaging 0.46 ± 0.23 and 0.53 ± 0.29, respectively), suggesting a higher species homogeneity among individuals in the final stages of the experiment (Figure 3B; Supplementary Table S7). Comparisons

between T0 and the rest of sampling times indicated that T60 showed the most significant microbial diversity changes (average Bray-Curtis dissimilarity value of 0.85 ± 0.12 between T0 and T60 samples) (Supplementary Table S7).

Commercial feed and control fish in all time points shared 206 OPUs, including the species *Ralstonia* OPU0522, *Paraburkholderia* OPU0542, *Leptospira* OPU1052, *Fulvimonas* OPU0572, *Pseudomonas* OPU0713 and *Cutibacterium* OPU0409 (Supplementary Table S8). There were microbial species exclusive in each dataset or sampling day. Out of the 319 total species detected, 5.6% were exclusive of the initial time (T0). At 20 days (T20), the percentage of exclusive species decreased to 4.6%, and at T40 and T60 the values were of 2.2% and 3.6%, respectively (Supplementary Table S4). The genus *Vibrio* exhibited the highest richness with 32 OPUs identified. In the fish, ten *Vibrio* species were identified as highly abundant (Figure 2). However, the prevalence of these species varied significantly over time. Specifically, *Vibrio* OPU0604, OPU0606, OPU0599, OPU0581 and OPU0594 were highly abundant in T0 and T20 days, and *Vibrio* OPU0600 and OPU0588 showed higher abundance in samples T20, T40 and T60. *Aliivibrio* OPU0614, OPU0615 and OPU0616 were abundant in T20. *Candidatus* Kaiserbacteria OPU1085 and *Leptospira* OPU1052 were highly abundant at T0 and T20 days, while *Psychrobacter* OPU0705, *Corynebacterium* OPU0366, *Acinetobacter* OPU0701 and *Subdoligranulum* OPU0242 were highly abundant in T40 and/or T60 days.

3.5 Gut microbial communities in fish fed with shrimp

The feed of the second group was replaced by shrimp at T0 and was maintained during 60 days. In this case, the alpha diversity values showed that the switch to a shrimp diet resulted in a reduction in microbial diversity along the first 20 days, as indicated by the non-significant, but numeral decrease in Shannon values (average from 1.22 ± 0.46 at T0 to 1.04 ± 0.25 at T20 (Wilcoxon-test; $P = 0.42$)), accompanied by a significant increase in dominance (average from 0.47 ± 0.15 at T0 to 0.63 ± 0.12 at T20 (Wilcoxon-test; $P = 0.008$)). However, after a gradual increase in diversity and a decrease in dominance occurred, reaching similar levels to the control at T60 [average Shannon value of 1.6 ± 0.27 (Wilcoxon-test; $P = 0.26$) and dominance of 0.33 ± 0.1 at T60 (Wilcoxon-test; $P = 0.28$)] (Figure 1; Supplementary Table S6).

Regarding beta diversity, the NMDS plot shows that the samples became more homogeneous after 20 days reflected by decrease in the average Bray-Curtis dissimilarity value from 0.67 ± 0.24 at T0 to 0.3 ± 0.25 at T20. This shift coincided with a significant increase in the dominance of the *Ralstonia* OPU0522, which exhibited an average abundance value of 73.59 ± 20.49% at T20. All the groups compared in this experiment were significant different among them (showing *P*-values < 0.01; PERMANOVA), except for fish fed commercial feed at T0 *vs.* fish fed commercial feed at T20 ($P = 0.137$), fish fed commercial feed at T40 *vs.* fish fed commercial feed at T60 ($P = 0.204$), and fish fed pink shrimp at T20 *vs.* fish fed pink shrimp at T40 ($P = 0.098$). A temporal shift in
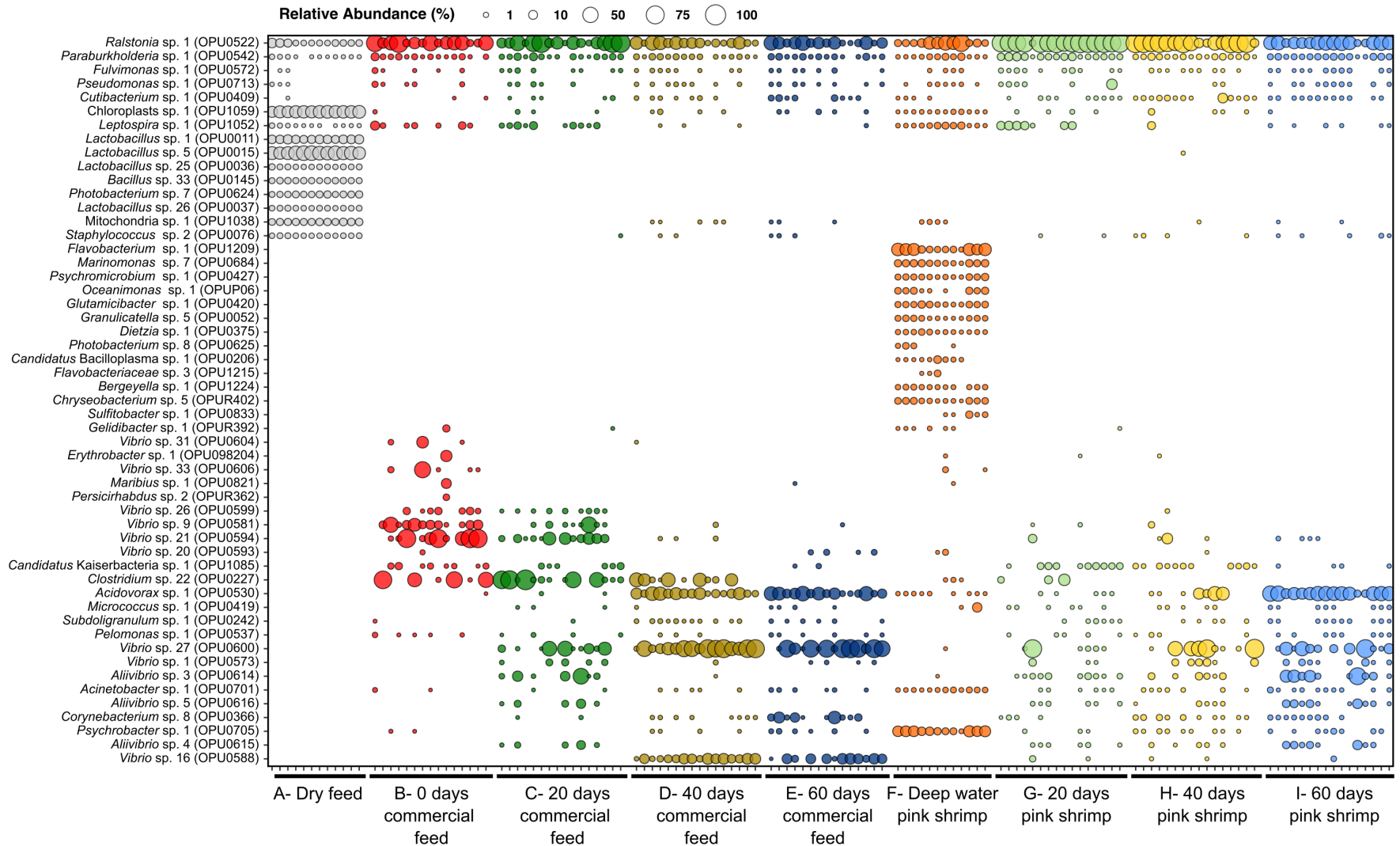
FIGURE 2

Relative abundance of the OPUs having a total average abundance >2%. Samples A and F represent the direct microbial analysis of the commercial feed and deep-water pink shrimp (*Parapenaeus longirostris*), respectively. Samples B to E represent the gut microbiomes of gilthead seabream (*Sparus aurata*) collected at the beginning of the experiment and through 60 days of continuous feeding with commercial feed in chronological order. Samples G, H, and I represent the gut microbiomes of gilthead seabream fed with deep-water pink shrimp after 20, 40, and 60 days from the beginning of the experiment.
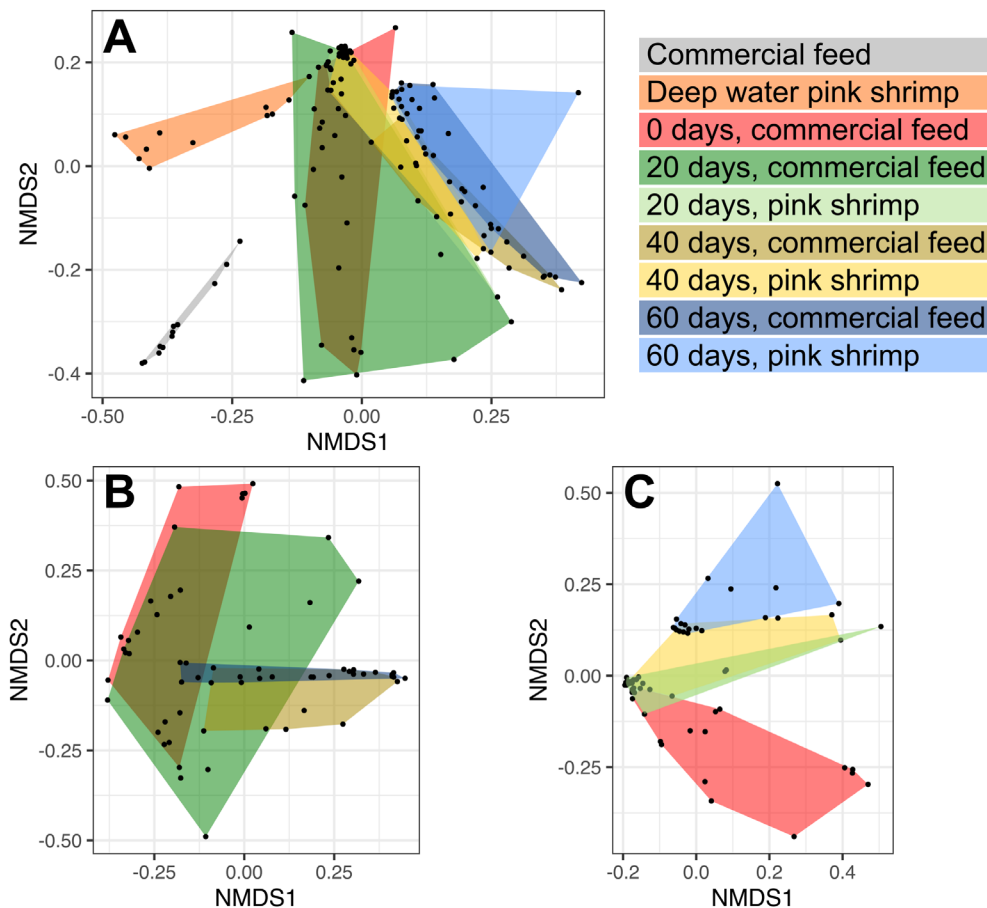
FIGURE 3
NMDS analyses based on Bray-Curtis distances of the microbiota of: **(A)** all samples included in this study; **(B)** gilthead seabream (*Sparus aurata*) fed with commercial feed; **(C)** gilthead seabream fed with deep-water pink shrimp (*Parapenaeus longirostris*). "Commercial feed" (grey) and "Pink shrimp" (orange) represents the direct microbial analysis of the commercial feed and deep-water pink shrimp, respectively. The dataset includes samples labeled by colors according to the day of the experiment followed by the type of feed provided to the fish.

beta diversity was observed during the experiment, with the most significant changes occurring in the samples collected at T60, as shown by the differences in beta diversity observed during the rest of timepoints ($P < 0.01$) and represented in the NMDS (Figure 3C). We observed a similar pattern for the control dataset in the percentage of exclusive OPUs, with 6.1%, 6%, and 2% of OPUs being unique to T20, T40, and T60, respectively (Supplementary Table S4). A total of 324 OPUs shared between the shrimps and the gut microbiota of the fish fed with them were also observed. Among the most abundant OPUs from shrimp, very few were shared between the fish gut microbiome and the shrimp, such as *Paraburkholderia* OPU0542 and *Pseudomonas* OPU0713 (Figure 2). The *Candidatus* Kaiserbacteria OPU1085 was manly present in samples T20 and *Vibrio* OPU0600 and OPU0573 were mainly detected in specimens collected at T40 and T60. On the other hand, *Acidovorax* OPU0530 and *Aliivibrio* OPU0614, OPU0615 and OPU0616 were most abundant in T60 (Figure 2).

Despite significant differences between the two dietary treatments in all the timepoints ($P < 0.01$), the NMDS based on Bray-Curtis dissimilarity showed a higher overlapping in the microbial structure of fish fed both diets at the final stage of the

experiment (T60) with respect to T20 groups (Figure 3A). Moreover, when evaluating dominance and Shannon index values at T60, no statistically significant differences were observed between fish fed with commercial feed and shrimp (Wilcoxon-test; $P = 0.05$).

4 Discussion

In this study, we examined the impact of a dietary shift (from a compound diet to a wild type shrimp-based diet) on the resident (autochthonous) gut microbiota of a commercially significant fish species. In all cases, bacteria accompanying the feed had a limited influence on the diversity in the gut, which was evidenced by the independent clustering of the diets with respect to the fish as well as by the different Shannon diversity and dominance indices. For instance, highly abundant species in the commercial feed or in shrimps did not establish in fish gut. Other studies also described different bacterial compositions between feed and intestinal autochthonous microbiota of many teleosts as gilthead seabream (Rimoldi et al., 2018b), European sea bass (*Dicentrarchus labrax*) (Rimoldi et al., 2020) or rainbow trout (*Oncorhynchus mykiss*) (Rimoldi et al., 2021). Our

findings are also supported by similar studies using experimental diets for gilthead seabream with increasing levels of *Firmicutes* that not only did not establish, but rather decreased in abundance over time (Moroni et al., 2021), and by a previous study in European sea bass (Rimoldi et al., 2021), where the feed pellets were dominated by *Lactobacillus* that did not either establish in their guts (Rimoldi et al., 2021). In this sense, while some studies have shown the presence of feed-associated bacteria in the fish digesta (Wilkes Walburn et al., 2019; Gaudioso et al., 2021; Ruiz et al., 2024), these bacteria may not establish in the gut as part of the autochthonous commensal microbiota, as suggested by previous works (Karlsen et al., 2022; Viver et al., 2023). In addition, commercial feeds are submitted to an extrusion process reaching high temperatures (> 100°C) that would washout the vast majority of viable bacteria (Encarnação, 2016; Konovalenko et al., 2022), but some species, as the observed *Lactobacillus* sp. may develop as a consequence of fermentative conditions during feed processing, manipulation or storage. These organisms or their residual DNA can be identified through amplicon sequencing, even in cases where they are non-viable, signifying their transient presence in the fish gut.

One of the most relevant species detected was *Ralstonia* sp. 1, which was found in remarkable abundance in the gut of gilthead seabream in our previous study (Viver et al., 2023). Members of this genus are normally found as part of the autochthonous gut microbiota in several fish species, such as rainbow trout (Kim et al., 2007), yellow catfish (*Pelteobagrus fulvidraco*) (Wu et al., 2010), European sea bass (Carda-Diéguez et al., 2014), yellowtail kingfish (*Seriola lalandi*) (Dam et al., 2020), large yellow croaker (*Larimichthys crocea*) (Zhang et al., 2022), pearly razorfish, and gilthead seabream (Viver et al., 2023). In the present study, *Ralstonia* was present in all samples, but its abundance was clearly enhanced in fish fed with shrimps. Contrarily to the commercial feed, beyond freezing, shrimps were not submitted to a pre-processing to destroy or minimize the accompanying bacteria. Thus, the increased amounts of *Ralstonia* in the fish fed with shrimps may partially respond to the colonization of this genus having its origin in the feed. Despite the commercial feed also contained *Ralstonia*, probably originated from fish meal, these bacteria may be not viable and this fact would explain the lack of their growth in fish guts. Although further research is needed to unravel the role of the members of this aerobic genus in the fish intestine, they may play an important role in marine animals, and their presence in the guts of fish may respond to a symbiotic or mutualistic relation (Cerezo-Ortega et al., 2021). Indeed, some *Ralstonia* species have been suggested to have antimicrobial activity biosynthesize bioactive compounds, and produce beneficial secondary metabolites for the host fish (Cerezo-Ortega et al., 2021).

Altogether, here the type of feed does not seem to be a key factor as a source of bacteria, as no remarkable changes were found between the two groups for the rest of represented species (> 2%). However, the diet ingredients could modulate the gut autochthonous microbiomes. Utilizing diverse protein, lipid, and carbohydrate sources, or incorporating varying levels of dietary supplements, can induce a spectrum of pronounced effects on the microbial profile of the fish. These effects may be directly and/or indirectly initiated by alterations in host physiology (Ringø et al.,

2016). For example, here, the effect of the diet was reflected in the differential relative abundances of some *Aliivibrio* species in the group fed shrimps, especially at T60, that were not detected when using the commercial diet. These species related to *A. finisterrensis*, *A. fischeri*, and *A. logei*, seem to be established in the gut of marine teleosts (Dunn, 2012; Hatje et al., 2014; Bazhenov et al., 2019). Some *Aliivibrio* species in the gut of the Atlantic cod (*Gadus morhua*) has been reported to be stimulated by the supplementation with chitin (Ringø et al., 2012), one of the main components of exoskeletal shells from crustaceans (Kurita, 2006). In aquafeed, chitin is considered as a prebiotic, able to increase bacterial intestinal richness by favoring the growth of beneficial and chitin-degrading bacteria (Mohan et al., 2023; Rimoldi et al., 2023). In this sense, many *Aliivibrio* species have chitinase activity (Raimundo et al., 2021), which may aid in the digestion of this ingredient since many marine fish, such as gilthead seabream, have shown a low chitin digestibility (Piccolo et al., 2017; Belghit et al., 2018; Basto et al., 2020). Then, it seems plausible that the higher growth of *Aliivibrio* in fish fed with shrimp compared to those fed with commercial feed was due to the probiotic effect of chitin. However, no differences on the KEGG pathway "Other glycan degradation" were observed at T60 between both groups (fish fed commercial feed: 0.04%; fish fed pink shrimp: 0.03%; *P* = 0.31). Nonetheless, this metabolic pathway does not only reflect degradation of chitin, but also of other glycans, such as starch, cellulose, and xylans, which are likely present in the commercial feed and may be degraded by the fish gut microbiota (Pedrotti et al., 2015; Maas et al., 2020). Further research on transcriptomic and/or activity characterization is needed to confirm whether the absence of changes in this metabolic pathway responded to the ability of different taxa to degrade the distinct types of glycans. In addition, we cannot discard that the transient feed-associated microbes that do not establish in the gut, if viable, may also influence the gut autochthonous microbiota (e.g., the probiotic effects in modulating microbial functionality and exerting a beneficial effect on the fish health; Rimoldi et al., 2021; Marco, 2019; Schoultz and Keita, 2019).

Beyond dietary factors, the gut microbiome is also influenced by the environmental conditions such as the temperature which is a paramount driver determining fish microbiomes (Rudi et al., 2018; Soriano et al., 2018; Kim et al., 2021). In this context, we observed and increase in abundance of *Staphylococcus*, *Acidovorax*, *Vibrio*, *Corynebacterium*, and *Psychrobacter* in both groups regardless the diet provided just after T20 when we relocated the fish in the new tanks for the cleaning and maintenance. Although we maintained the environmental conditions between tanks after the change, the differential microbiota in the water and on the tanks' wall biofilms might have been the source of these taxonomic changes (Minich et al., 2020; Zeng et al., 2020). Unfortunately, we did not collect samples from water or biofilms from the tanks to compare their taxonomic profiles, and therefore, we cannot confirm whether these bacteria were present in the new tanks. Additionally, some of the changes induced by the relocation were more marked in gilthead seabream fed with the commercial diet, such as the differential abundances of some *Vibrio* species and *Clostridium* sp. 22 before and after T20. The higher impact of the relocation in fish fed with the commercial diet in comparison to those fed with pink shrimp

may be attributed to their longer adaptation period to the diet before the relocation. In this context, at the moment of the transference fish fed the commercial feed had been already adapting to this diet type for 7 weeks plus other 3 previous months when they were fed with a similar commercial diet. In this sense, a microbial adaptation over time can be expected in response to external changing factors, such as shifts in the diet (Michl et al., 2017, 2019), the feeding regime (Sherif et al., 2020), an antibiotic administration (Payne et al., 2022), or an induced stress (Webster et al., 2021). Considering that the period of adaptation to a diet can last for some weeks (up to 12 weeks in some cases), with variations depending on the fish species, size and diet formulation among other experimental factors (Heikkinen et al., 2006; Ingerslev et al., 2014; Keating et al., 2021), the changes in the gut microbial communities of fish fed with pink shrimp observed from T20 on may reflect the combined or synergetic effect of the adaptation to the diet change and to the relocation.

Under current experimental conditions, the most striking observation was to find that there were some bacteria which were present in all groups of individuals regardless the sampling time and diet provided, such as *Ralstonia* sp. 1 (15.6-73.6%), *Paraburkholderia* sp. 1 (1.6-16.3%), *Fulvimonas* sp. 1 (0.2-1.4%), *Pseudomonas* sp. 1 (0.1-0.7%) and *Cutibacterium* sp. 1 (0.1-5.2%). Thus, this bacterial members could be considered as part of the core microbiota of this farmed species (Astudillo-García et al., 2017; Roeselers et al., 2011; Rudi et al., 2018; Kokou et al., 2019). In addition, the same OPUs identified as *Ralstonia* sp. 1, *Paraburkholderia* sp. 1, *Pseudomonas* sp. 1 and *Cutibacterium* sp. 1 were found at high abundances in our previous study in the intestine of farmed and wild fasted gilthead seabream (Viver et al., 2023), which reinforces the hypothesis that these genera may be considered as part of the core microbiota of this species regardless the external conditions, such as the type of diet and/or the animal lifestyle. The similarities in terms of species composition and abundance among individuals were reflected in a similar diversity (Shannon diversity and Dominance indices) and structure (representation of Bray-Curtis distances in NMDS, despite significant differences) of both dietary groups at the later stages of the experiment (T40 and T60). It seems that the core microbiome prevalence is not affected by the environmental changes after the adaptation processes in the tanks. In contraposition, studies in juveniles of rainbow trout (Michl et al., 2017) and brown trout (*Salmo trutta*) (Michl et al., 2019) have shown that the microbiome composition is directly dependent in the actual diet provided after a dietary shift, but barely influenced by the initial diet provided before the change. However, these juveniles were younger and with much lesser body weight (around two orders of magnitude smaller) than ours at the end of the assay, which pinpoints that the differential plasticity of the fish gut microbiota to adapt to diet changes depends on the animal age as it was previously postulated in gilthead seabream (Piazzon et al., 2019) as well as in mammals' gut (Quercia et al., 2014; Barreto et al., 2020) and rumen (Yin et al., 2021).

Previous results have already showed that there are host-inherent factors which act as paramount modulators of the fish gut microbiota, such as the species, sex, age, and genetic background (Navarrete et al., 2012; Piazzon et al., 2019). Overall, the present results suggest that a diet change have much less weight in the

modulation of the gut microbiota than these host-inherent factors, as demonstrated by the presence of a core microbiome in all fish regardless the diet provided, leading to gut bacterial communities with a similar diversity, structure, and composition in the long-term. These findings are consistent with those of Keating et al. (2021), who observed that the effect of time on the hindgut microbiota of Atlantic cod was significantly higher than the impact of different dietary treatments which resulted in similar microbial communities over time. In addition, these authors also identified a core microbiome that persisted throughout the 12-week duration of the experiment. Furthermore, previous studies in rainbow trout have shown that its gut microbiota is highly sensitive to new diets, but stabilizes over time (Ingerslev et al., 2014). Similarly, Niu et al. (2020) observed that when comparing the gut microbial profiles of olive flounder (*Paralichthys olivaceus*) fed two diets with different replacement levels of fish meal by plant-based protein sources over an 8-month period, the effect of the growth stage on the gut microbiota was much higher than the diet effect. Moreover, Kokou et al. (2019) reported a common core microbiome over time in European seabass fed with different diets over a 6-week period. Our hypothesis is also in consistency with the existing evidence of a host-specific shaping of the fish gut microbiota regardless the environmental conditions (Nikouli et al., 2021). However, in the present trial the diversity, structure, and composition of the microbial communities of gilthead seabream fed each type of diet was very different in the first 20 days after the dietary shift. Given that there are many variables that may affect the fish feeding regimes in natural conditions (i.e., dietary changes based on prey availability), our results indicate that even when considering the specific environmental conditions of each study, it is not advisable to establish comparisons of the microbiota of wild and farmed fish. Furthermore, the relevance of these findings lies in the adaptation of the microbial communities to the diets over time, which offers the possibility to explore novel feeds formulated with more sustainable, cost-effective, and readily available ingredients and which may not have a long-term effect on the fish microbiota.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB64773.

Ethics statement

The animal study was approved by the Ethical Committee of Animal Experimentation of the Institute of Agrifood Research and Technology (Spain) and the Generalitat of Catalunya (Ref. CEEA 219/2020). The study was carried out by trained competent personnel in accordance with European Directive 2010/63/UE and Spanish Royal Decree RD53/2013 to ensure good practices for animal care, health, and welfare. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JA: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision. EG: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing, Resources, Validation. DF: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. TV: Conceptualization, Validation, Writing – original draft, Writing – review & editing, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this larticle can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1498892/full#supplementary-material

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