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Food determines ephemerous and non-stable gut microbiome communities in juvenile wild and farmed Mediterranean fish

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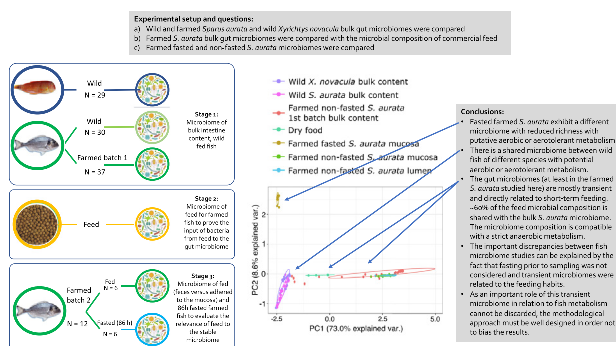
HIGHLIGHTS

- Fish gut microbiomes may be ephemerous and directly linked to short-term food ingestion.
- Most microorganisms in the fish intestines studied were transient.
- The important discrepancies between studies of the same species may be due to feeding habits.
- As transient microbiomes may be relevant to fish metabolism, studies must be well designed.
- Wild fish shared microbiomes, with the most representative being a *Ralstonia* and a *Micrococcus* species.

GRAPHICAL ABSTRACT



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ABSTRACT

Novel insights were provided by contrasting the composition of wild and farmed fish gut microbiomes because the latter had essentially different environmental conditions from those in the wild. This was reflected in the gut microbiome of the wild *Sparus aurata* and *Xyrichtys novacula* studied here, which showed highly diverse microbial community structures, dominated by *Proteobacteria*, mostly related to an aerobic or microaerophilic metabolism, but with some common shared major species, such as *Ralstonia* sp. On the other hand, farmed non-fasted *S. aurata* individuals had a microbial structure that mirrored the microbial composition of their food source, which was most likely anaerobic, since several members of the genus *Lactobacillus*, probably revived from the feed and enriched in the gut, dominated the communities. The most striking observation was that after a short fasting period (86 h), farmed gilthead seabream almost lost their whole gut microbiome, and the resident community associated with the mucosa had a very much reduced diversity that was highly dominated by a single potentially aerobic species *Micrococcus* sp., closely related to *M. flavus*. The results pointed to the fact that, at least for the juvenile *S. aurata* studied, most of the microbes in the gut were transient and highly dependent on the feed source, and that only after fasting for at least 2 days could the resident microbiome in the intestinal mucosa be determined. Since an important role of this transient microbiome in relation to fish metabolism could not be discarded, the methodological approach needs to be well designed in order not to bias the results. The results have important implications for fish gut studies that could explain the diversity and occasional contradictory results published in relation to the stability of marine fish gut microbiomes, and might provide important information for feed formulation in the aquaculture industry.

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1. Introduction

Fish gut microbiomes are partially responsible for the digestion of ingested food that plays an important role in host fitness. The composition and stability of gut microbiomes remains an open question for marine fish, as the literature reveals numerous discrepancies in this area. As in many other organisms, the gut microbiota of fish may be fundamental for their physiological functions that, in turn, will ultimately be relevant for their health and animal performance. Therefore, understanding its taxonomic composition is the first step towards revealing the role of microbes in maintaining the host's homeostasis. Furthermore, for pragmatic reasons, revealing the interactions between microbiomes and fish will help to improve and manage aquaculture practices, since balanced microbiomes may play a key role in the health, welfare, and disease amelioration of farmed fish (Diwan et al., 2022). Thus, fish gut microbiomes have been exhaustively studied in both farmed and wild animals for a wide range of families, and an important amount of information has been generated by both culture-dependent and -independent methodologies (e.g. Egerton et al., 2018; Nayak, 2010; Ghanbari et al., 2015; Clements et al., 2014; Cahil, 1990). Many studies have not only been descriptive but have also used an experimental approach in order to try and understand the modulation of the gut microbiomes by both biotic and abiotic factors, such as the diet (e.g. Kormas et al., 2014; De Paula Silva et al., 2011; Estruch et al., 2015), water salinity (e.g. Rudi et al., 2018) and fish origin (wild vs. captivity) (e.g. Dhanasiri et al., 2011), as well as even the sex and age of fish (e.g. Piazzon et al., 2020).

Despite being generally in agreement, the results to date have shown many differences between species and between rearing conditions. For example, only for gilthead seabream (*Sparus aurata*) the dominant phyla reported in order of relevance were *Firmicutes, Proteobacteria* and *Bacteroidetes* (De Paula Silva et al., 2011), whereas in another study *Firmicutes, Proteobacteria* and *Actinobacteria* were dominant (Estruch et al., 2015) and in a third one, the most dominant were *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* (Kormas et al., 2014). Such discrepancies in taxonomic composition have been considered inevitable given the high diversity of the fish species and types of treatment (Egerton et al., 2018), as well as the different composition of the diets (Kormas et al., 2014; Ringø et al., 2006). Therefore, understanding the role that microorganisms play in the functioning of the fish gut is highly relevant, especially as new species are increasingly being incorporated into the farming procedures of the aquaculture sector and diet management becomes pertinent for improving the health and performance of farmed fish (Egerton et al., 2018; Diwan et al., 2022).

By studying different species of wild fish, and wild and farmed individuals of the same species, novel insights into the stability of gut microbiome communities can be provided, since different environments and different food sources can be compared at the same ontogenetic stage. Here, we have studied the gut microbiome composition of the wild fish *Xyrichtys novacula* (Labridae) and *Sparus aurata* (Sparidae) and compared the results with farmed *S. aurata* specimens. *X. novacula* is a widely-distributed wrasse that inhabits shallow sandy habitats of temperate waters of the Mediterranean, Atlantic and Caribbean (Alós et al., 2012). This species mainly feeds on benthic food items dependent on the community of well-sorted fine sands mostly comprised of *Mollusca* and *Echinodermata* species (Castriota et al., 2005). *S. aurata* is a relevant inshore fish for coastal fisheries in the Mediterranean Sea and the North-east Atlantic Ocean. Individuals of wild *S. aurata* feed preferentially on macrobenthos (*Polychaeta* and *Amphipoda*) and macrophyte detritus (Ferrari and Chieregato, 1981). In addition, it is the most important Mediterranean aquaculture fish species in terms of volume and economic value (FAO, 2022).

The current study aimed to evaluate the bacterial diversity in the gut of wild individuals of two fish species, *S. aurata* and *X. novacula*, thriving in the coastal waters of Mallorca (Balearic Islands, Spain). Their different feeding habits were compared with the well-studied gut microbiomes of farmed *S. aurata*, and we expected to find an exclusive community depending on feeding habits and environmental conditions (Egerton et al., 2018). Nevertheless, although differences were confirmed, they were not as anticipated and therefore we further evaluated the effect of diet and fasting on farmed fish. The microbial load of feed can be readily determined, which allowed us to evaluate the contribution of ingested microbes in the gut microbiomes. The study was conducted using 16S rRNA gene amplicon sequencing followed by the operational phylogenetic unit (OPU) approach (Mora-Ruiz et al., 2016; Viver et al., 2015) because this methodology renders very accurate fine-tuned results with a resolution that allows the occurrence of single species that form the community structure to be identified. In addition, it is anticipated that the results can contribute to harmonizing the sampling protocols applied to fish gut microbiome studies.

2. Materials and methods

2.1. Rationale of the experimental setup

The study was conducted in three different stages (Supplementary Fig. S1). A first study in 2019 compared the gut microbiomes of wild individuals of *X. novacula* and *S. aurata* with farmed *S. aurata*. Since *X. novacula* has not yet been farmed it could not be included in the comparison. As described below, complete guts were extracted from non-fasted euthanized fish, and therefore the gut biomass corresponded to autochthonous and transient microbiomes. In light of the initial different results of the farmed vs. non-farmed fish, in a second step, the microbial composition of the commercial extruded feed given to the farmed fish was sequenced. The similarities between the microbial composition of the food source and the gut microbiomes of the farmed fish led us to elaborate a third experiment in 2021 in which fasted and non-fasted farmed fish were compared in order to distinguish transient from autochthonous microbes (Naya-Català et al., 2021). The differences between the gut content and the microbiota adhered to the intestine walls, as well as the differences between the distal and proximal intestine, and the effect of fasting were also evaluated. Evaluating the food of the wild fish was not feasible as wild fish feeding habits differ greatly, and the ingested food could not be easily determined.

The two groups of farmed *S. aurata* used for this study in 2019 and 2021 were supplied by two different hatcheries located at the same latitude on the Spanish Mediterranean coast and were transported to IRTA's (Institut de Recerca i Tecnologies Agroalimentàries) facilities as fingerlings (average weight 2 g). They were fed weaning pellets for ten weeks, followed by D-2 Optibream AE according to the company's feeding protocol. All fish were fed with Skretting® feed and they were kept indoors at the IRTA facilities in recirculating aquaculture systems (RAS) (IRTAmar™). The water temperature (19–23 °C) and dissolved oxygen (6.3 ± 0.4 mg L^{-1}; OXI330, Crison Instruments) were measured daily, whereas pH (7.4 ± 0.1; pH meter 507, Crison Instruments), salinity (36 ‰; MASTER-20 T Hand-Held Refractometer, ATAGO Co. Ltd), ammonia (0.15 ± 0.1 mg NH_4^+ L^{-1}) and nitrite (0.2 ± 0.1 mg NO_2^- L^{-1}) levels (HACH DR 900 Colorimeter, Hach Company) were controlled weekly.

2.2. 1st fish batch

In July 2019, the first *S. aurata* batch (n = 37, 12.8 ± 1.1 cm total length, TL; see Table S1 for weights and further details) was transported to the facilities of the Laboratory of Marine Research and Aquaculture of the Balearic Islands (LIMIA). For the 30 days prior to the experiment, both IRTA and LIMIA used the same commercial feed (D-2 Optibream AE 1P, 2.2 mm pellet size, Skretting) that contained 48.5 % crude protein, 18 % lipids and 18.5 MJ kg^{-1} digestible energy (Serie D2-D5 Fishmeal composition is: fish oil, vegetable oils, cereal products and by-products, legume products and by-products, oilseed products and by-products, vitamins and minerals). The collection of wild individuals of *S. aurata* (n = 30, TL = 11.9 ± 1.2 cm) and *X. novacula* (n = 29, TL = 18.2 ± 2.8 cm) took place in the waters of Mallorca using a standardized fishing hook-and-line gear. No significant differences in length or weight between farmed and wild *S. aurata* were found (Table S1; ANOVA, $p > 0.05$).

Wild *X. novacula* were euthanized upon being fished, whereas wild *S. aurata* were kept for 7 days before sacrifice, since they were part of another experiment, observational in nature, and their housing conditions did not differ significantly from the other fish batches included in this study. During this short period, they were fed daily by 2.5 % of their fresh weight with live wild food (shrimp, *Palaemon serratus*). On the day of their sacrifice, the *S. aurata* intestines were visibly empty or contained a minimal amount of gut content that was too small to be quantified, whereas *X. novacula* intestines were filled with 0.38 ± 0.52 g cm^{-1} gut biomass cm^{-1} (Supplementary Fig. S2). Farmed fish were fed ad libitum with commercial feeds until they were sacrificed for gut extraction. Fish kept in captivity had been fed approximately 2 h before sacrifice, thus, the guts were still filled with 0.31 ± 0.09 g cm^{-1} gut biomass cm^{-1} (Supplementary Fig. S2).

2.3. 2nd fish batch

The second batch of fish corresponded to farmed *S. aurata* only and it was studied in 2021. The fish were kept at the IRTA facilities from arrival as fingerlings (2 g) until sacrifice. Farmed juvenile *S. aurata* ($n = 20$) were acclimatized in a 500-L tank for ten weeks while feeding with a pre-growing diet for juveniles (Skretting®). After acclimatization, fish with an initial body weight (BWi) of 25.29 ± 4.90 g were randomly distributed in two tanks each with a volume of 400 L (10 fish per tank) in a water recirculation system (IRTAmar™). During the trial, fish were manually fed three times per day with D-2 Optibream AE 1P (Skretting), following the manufacturer's instructions.

At the end of the period (62 days), fish were 88.5 ± 14.1 g final BW (BWf) and had a final standard length (SLf) of 14.9 ± 0.8 cm. The individuals from both tanks did not display significant differences in growth (ANOVA, $p > 0.05$), indicating that there was no tank effect. The animals from one tank (BWf = 88.0 ± 14.8; SLf = 14.9 ± 0.8 cm) were fasted for 3 h before sampling, while the fish from the other tank (BWf = 89.0 ± 14.0 g; SLf = 15.2 ± 0.8 cm) were fasted for 86 h before sampling. A total of six fish from each tank were then randomly hand-netted for sampling. The 86 h fasting period was determined based on several studies that used periods of >24 h to sample the autochthonous microbiota (Piazzon et al., 2019; Piazzon et al., 2020; Naya-Català et al., 2021; Solé-Jiménez et al., 2021), as well as a study by Xia et al. (2014) for Asian seabass (*Lates calcarifer*) that described a drastic change only after 12 days of fasting. We were confident that the fasting period chosen was adequate, as even long starvation periods (between 30 days to 8 weeks) have shown no negative effect on growth without compromising fish health (Favero et al., 2020; Hvas et al., 2022), and no indication of stress, based in cortisol levels, was observed for up to 7 days fasting in sea bream (Fernández-Alacid et al., 2018).

2.4. Sample processing

Fish were euthanized with an overdose of the buffered anesthetic tricaine methanesulfonate (MS-222; 1 g L^{-1}). Gut dissection was performed immediately after slaughter. Individual intestines were dissected using sterile scissors and tweezers, longitudinally opened and stored. Moreover, for batch 2 fish, after gently removing the perivisceral fat surrounding the intestine with a scalpel, a 3 cm section of the anterior intestine and another of the posterior intestine with the same length, were dissected using sterile scissors. Both sections were longitudinally opened, and the mucosal content adhered to the inner walls of the intestines was scraped with a round-edge spatula and saved into a tube. Since 3 h post-prandial fish gut still had pieces of feed in transit (chyme), the luminal content was carefully collected and saved in independent tubes. Samples were homogenized with sterile scissors and a low speed vortex. Samples were stored in RNAlater™ solution (Invitrogen, Thermo Fisher Scientific, Lithuania) at −20 °C until processed.

2.5. Microbiological composition of feed used for farmed fish

A total amount of 350 mg of the Skretting (Nutreco N.V.) commercial feed pellet used during the life of the farmed fish (MAR-PERLA MPH, MAR-PERLA HDT, MAR-PERLA MPL and D-2 OptiBream AE 1P) was directly extracted using the FastDNA Spin Kit for Feces (MP Biomedicals) following the manufacturer's protocol.

2.6. DNA isolation, amplification and sequencing

Microbial DNA extraction was performed from 100 μL of mixed gut biomass, using the QIAamp DNA Microbiome Kit (Qiagen, Germany) or the FastDNA™ Spin Kit for Feces, following the manufacturer's instructions (the DNA extraction kit used for each sample is specified in Supplementary Table S2). Illumina amplicon sequencing was performed using the set of primers for Bacteria forward (5′ - CCTACGGGNGGCWGCAG - 3′) and reverse (5′ - GACTACHVGGGTATCTAATCC - 3′) (Herlemann et al., 2011) containing the forward (5′ - TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG - 3′) and reverse (5′ - GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG - 3′) Illumina sequencing adapters (Illumina, Inc.). Sequencing was performed using an Illumina MiSeq instrument (2 × 250 bp) (FISABIO, Valencia, Spain).

2.7. Amplicon sequence variant (ASV) and operational phylogenetic unit (OPU) approaches

Quality assessment of raw sequencing data was performed using the Qiime2 bioinformatic platform (Bolyen et al., 2019) with 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 longest sequence of each ASV was selected as representative, and sequences of the bacterial and archaeal ASVs were aligned using the non-redundant SILVA REF 138.1 (Quast et al., 2013) database and the ARB package (Ludwig et al., 2004). Alignment was performed using the SINA tool implemented in the ARB program (Pruesse et al., 2012). The aligned sequences were then inserted in the SILVA REF 138.1 pre-existing tree using the parsimony tool available in the ARB package. The closest relative non-type strains of an acceptable quality affiliating with the ASV representatives were selected and merged with the LTP_01_2022 (Ludwig et al., 2021) sequence database. The selected representative sequences were used for a neighbor joining reconstruction (Munoz et al., 2016) that was used as the reference tree. The partial sequences were finally inserted into the reconstructed tree using the parsimony tool implemented in ARB. For the final topology, the tree was manually supervised, and the single isolated phylogenetic sub-branches containing the query sequences and at least one representative sequence were grouped into OPUs (operational phylogenetic units) based on the visual inspection of the final tree (Mora-Ruiz et al., 2016; Viver et al., 2015).

2.8. Statistical analyses

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 vegan R package (Oksanen et al., 2022) was used to calculate the Bray-Curtis dissimilarity value between the different community profiles of samples based on OPUs. A principal coordinate analysis (PCoA) plot of the first two components based on Bray-Curtis dissimilarity was constructed using the ggplot2 R library (Wickham, 2016) in R tool version 3.6.3 (R Core Team, 2022). A divergence test between samples based on OPU abundance and composition was performed using non-parametric Kolmogorov-Smirnov tests (Jarek, 2015), since the data did not meet the assumptions of normality and homoscedasticity.

3. Results

3.1. ASVs, OPUs and diversity indices

All samples considered in this study rendered a total of 7,920,387 amplicon sequences with a mean of 81,653 ± 39,283 ranging from

16,029 to 185,060 sequences. After clustering, 26,415 ASVs were obtained (Supplementary Table S2). The amplified and sequenced data have been deposited in the NCBI under the bioproject PRJEB56884. After aligning and phylogenetic inference of representative sequences of each ASV, a total of 1803 OPUs were detected. Considering that one OPU is the smallest clade of query sequences affiliating with at least one reference sequence, and the size of each clade generally occurs within a sequence distance of <97 % (considering that the partial sequences are only ~400 pb) we were confident that one OPU correctly represented a single species (Mora-Ruiz et al., 2016). Therefore, the OPUs were considered to be different species within the detected genera and, for this purpose, the OPUs were identified at the genus level and numbered depending on their occurrence within the genus (Supplementary Table S3). As indicated in Table 1 and Supplementary Tables S3 and S4, approximately 1800 species affiliated with ~800 genera and ~ 380 families belonging to 43 distinct phyla. Notably, ~82 % of the species or OPUs detected belonged to known genera, and the remaining ~18 % to higher taxa with no clear affiliation. Very similar figures were observed for the higher taxa, indicating that most of the sequences affiliated closely with or within taxonomically described taxa. The major phyla detected in decreasing order were *Proteobacteria* (42.3 %), *Firmicutes* (26.1 %), *Actinobacteria* (11.8 %) and *Bacteroidetes* (6.6 %), which comprised ~87 % of the total diversity detected. The remaining 13 % of the OPUs affiliated with 38 distinct phyla, 1.2 % of them to non-taxonomically classified phyla. Within the *Proteobacteria*, the three major classes *Alpha-*, *Beta-* and *Gammaproteobacteria* were highly represented, especially in wild fish, with abundance values ranging from ~13 % to ~31 %. Another remarkable finding was that 61 % of the OPUs contained the sequence of a type strain of taxonomically described taxa as a reference, whereas ~19 % could be assigned to a genus but did not include any type strain sequence, and another ~19 % were OPUs affiliated to higher taxa outside any known genera. Most of the genera (641 representing ~80 % of the total) were only represented by a single OPU, whereas the remaining 157 genera with >2 OPUs (20 %) grouped ~40 % of the detected species. The genera with most detected species were *Vibrio* (with 46 distinct species) followed by *Bacillus* (42 species), *Lactobacillus* (40 species) and *Pseudomonas* (28 species).

Each sample group (i.e. wild *X. novacula,* wild *S. aurata,* farmed *S. aurata*, and feed) exhibited exclusive OPUs (Table 1) ranging from the 345 unique OPUs in *X. novacula* to 159 from the wild *S. aurata*. The different collections also showed different diversity indices. The number of OPUs in each single individual closely resembled the expected richness (Chao-1), and this was reflected by the high coverage in the samples, as indicated by the rarefaction curves (see below). The highest richness was observed in the gut samples of *X. novacula* (Chao-1 = ~192 ± 4) and in the feed (Chao-1 = ~146 ± 31) (Table 2, and Supplementary Tables S5, S6, S7 and S8). On the other hand, all groups of *S. aurata* showed lower richness values, which were less than half of *X. novacula*, ranging from the highest value for the farmed non-fasted bulk content with ~98 ± 31 and the lowest for the farmed and fasted mucosa with ~45 ± 3 species in each fish. Diversity indices mirrored the richness data, with the highest Shannon H values being for *X. novacula* (3.22) and the lowest for the farmed and fasted *S. aurata* microbiomes associated with the mucosa (0.59). For this latter very low value, the extremely high dominance (D = 0.76) shown was mostly due to the single OPU0419 *Micrococcus* sp. 1 that generated >70 % of the total amplicons. All other samples showed lower dominance values and therefore higher evenness in their prokaryote species composition. Rarefaction curves (Supplementary Fig. S3) showed that the farmed fish, with just three exceptions in the bulk content for the 1st batch of *S. aurata*, were saturated indicating that the sequencing effort embraced most of the expected diversity. On the other hand, the wild fish showed a much larger expected diversity and the curves were further away from saturation.

3.2. Analysis of the bulk gut content of farmed S. aurata, wild S. aurata, and wild X. novacula

The gut contents for the three groups of fish were different (Supplementary Fig. S2). Wild *S. aurata* showed a significantly reduced gut content, similar to that of the fasted farmed fish (see below). On the contrary, both the farmed *S. aurata* and wild *X. novacula* groups showed an average gut content of 0.31 ± 0.09 g cm^{-1} and 0.38 ± 0.52 g cm^{-1}, respectively, although for the latter the inorganic content (i.e. sand grains and mussel shells) accounted for an average of 0.037 ± 0.03 g cm^{-1}, whereas for the former it was 0.007 ± 0.002 g cm^{-1} (Supplementary Fig. S2). In general terms, the microbiomes of the three fish groups in the 1st batch (farmed *S. aurata*, wild *S. aurata*, and wild *X. novacula*) already showed remarkable differences (Fig. 1 and Supplementary Tables S5, S6 and S7). *X. novacula* was the fish species with the largest number of OPUs (1019), followed by the farmed *S. aurata* (935) and the wild *S. aurata* with the lowest number of OPUs (695). Farmed *S. aurata* also exhibited a very different community structure than that of their wild congeners (Figs. 1 and 2A), with a notable dominance of members of the genus *Lactobacillus* (Fig. 1 and Supplementary Tables S5, S6 and S7), especially OPU0036 *Lactobacillus* sp. 25 closely related to L. *aviaries.* In the most extreme case, this OPU comprised ~55.4 % of the total reads, and the group showed a mean of ~38 % for the total amplicons. On the other hand, both wild fish groups showed a very different pattern, as well as between themselves, although they had a common exceptional dominance of the betaproteobacterial OPU0522 *Ralstonia* sp. 1, closely related to *R. mannitolilytica*, that could comprise >82 % of the total amplicons in both wild fish groups. These abundances did not occur evenly among the fish, although they were present in all of them. OPU0522 *Ralstonia* sp. 1 was nearly absent (<1.1 % in only 4 out of 37 individuals) in the farmed *S. aurata* group. The PCoA

Table 1
Values for the number of taxa observed, as well as the values of the number of OPUs present in each single sample group and shared between them.

Taxa numbers			
Phyla	43		
Classes	100		
Orders	228		
Families	382		
Genera	798		
Species/OPUs	1803		
Total number of OPUs:	OPUs	%	Total
Farmed *S. aurata* 1st batch	935	51.9	1803
Farmed *S. aurata* non-fasted intestinal scrape	282	15.6	1803
Farmed *S. aurata* non-fasted stomach content	236	13.1	1803
Farmed *S. aurata* non-fasted	1074	59.6	1803
Farmed *S. aurata* fasted	180	10.0	1803
Farmed *S. aurata* fasted and non-fasted	1148	63.7	1803
Wild *S. aurata*	695	38.5	1803
Wild and farmed *S. aurata*	1401	77.7	1803
Wild *X. novacula*	1019	56.5	1803
Dry food	291	16.1	1803
Exclusive OPUs (sample unique)	OPUs	%	Total
Farmed *S. aurata* 1st batch	244	26.1	935
Farmed *S. aurata* non-fasted intestinal scrape	19	6.7	282
Farmed *S. aurata* non-fasted intestine content	29	12.3	236
Farmed *S. aurata* non-fasted (all)	350	32.6	1074
Farmed *S. aurata* fasted	27	15.0	180
Farmed *S. aurata* fasted and non-fasted	106	9.2	1148
Wild *S. aurata*	159	22.9	695
Wild and farmed *S. aurata*	516	36.8	1401
Wild *X. novacula*	345	33.9	1019
Dry food	38	13.1	291
OPUs shared by:	OPUs	%	Total
Farmed *S. aurata* non-fasted 1st batch vs intestinal scrape	192	17.9	1074
Farmed *S. aurata* 1st batch non-fasted vs intestine content	159	14.8	1074
Farmed *S. aurata* intestinal scrape non-fasted vs stomach content	147	13.7	1074
Farmed *S. aurata* non-fasted (all) vs fasted	106	9.2	1148
Farmed *S. aurata* non-fasted (all) vs dry food	198	17.0	1167
Farmed *S. aurata* non-fasted (all) vs wild *S. aurata*	422	30.1	1401
Farmed *S. aurata* non-fasted (all) vs wild *X. novacula*	530	33.2	1595
Farmed *S. aurata* fasted vs wild *S. aurata*	99	7.3	1360
Farmed *S. aurata* fasted vs wild *X. novacula*	134	9.9	1360
Wild *S. aurata* vs wild *X. novacula*	391	28.8	1360
Common to all fish	73	4.1	1762

Table 2
Diversity indices and values of the seven sample groups. The values given are the mean and standard deviation, and the median.

	Dry food			Farmed non-fasted *S. aurata* 1st batch			Farmed non-fasted *S. aurata* lumen			Farmed non-fasted *S. aurata* mucosa			Farmed and fasted *S. aurata*			Wild *S. aurata*			Wild *X. novacula*		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Taxa_S	146.25	31.42	137.00	97.97	31.57	88.00	78.08	21.99	72.00	71.83	16.52	69.00	45.33	10.15	43.00	78.33	22.05	75.00	192.42	72.56	214.50
Dominance_D	0.16	0.03	0.17	0.28	0.10	0.32	0.15	0.02	0.15	0.16	0.02	0.16	0.76	0.11	0.79	0.20	0.23	0.10	0.16	0.17	0.07
Simpson_1-D	0.84	0.03	0.83	0.72	0.10	0.68	0.85	0.02	0.85	0.84	0.02	0.84	0.24	0.11	0.21	0.80	0.23	0.90	0.84	0.17	0.93
Shannon_H	2.43	0.28	2.41	1.85	0.80	1.50	2.43	0.16	2.46	2.23	0.14	2.22	0.59	0.25	0.51	2.80	0.94	3.18	3.22	1.00	3.55
Evenness_e^H/S	0.08	0.01	0.08	0.10	0.14	0.05	0.15	0.04	0.15	0.13	0.02	0.13	0.04	0.01	0.04	0.29	0.17	0.30	0.17	0.09	0.17
Chao-1	146.25	31.42	137.00	98.00	31.56	88.00	78.08	21.99	72.00	71.83	16.52	69.00	45.33	10.15	43.00	78.33	22.05	75.00	192.42	72.56	214.50

distribution of the samples clearly showed that the microbiome of the farmed fish was very different than that of both wild species, which, despite the important differences, resembled each other much more than the wild *S. aurata* group and its counterpart farmed group (Figs. 1 and 2A). A high presence of chloroplasts (OPU1059) and cyanobacteria (OPU1060) was found in both wild fish species. Farmed *S. aurata* also had a relatively high content of chloroplast sequences that could probably have originated from the dry feed (see below).

3.3. Microbial composition of the feed pellets

The feed samples showed a different pattern of bacteria than the farmed fish intestines but with some shared taxa and a remarkable diversity (Fig. 3A). In addition, the dominance in the samples was low (Fig. 3B), indicating a good distribution of abundances among the different OPUs detected. In the first instance, 291 OPUs could be detected, from which only 38 were exclusive (Fig. 1, Table 1). The occurrence and abundance patterns of the OPUs from the feed pellets were different from those of both groups of farmed *S. aurata*, but approximately 200 OPUs were shared between both types of samples. The farmed *S. aurata* shared only ~17 % OPUs with the feed pellets, but the shared OPUs represented ~69 % of the feed pellet richness. The major components of the farmed non-fasted *S. aurata* were *Lactobacillus* sp. OPUs and the OPU0145 *Bacillus* sp. 33, which were also present in the feed pellets with lower relative abundances, but still highly present (values that could be as high as ~8 %; Supplementary Tables S5, S6 and S7). The comparisons using the PCoA (Fig. 2A) showed that despite the differences, feed pellets and the farmed *S. aurata* microbiomes were closer than any of them with respect to wild fish.

3.4. Effect of short-term fasting on farmed fish

The gut microbiomes of the second batch of fish led to several relevant observations that could clarify the origin of the microbial communities, at least in the farmed fish. In the first instance, the microbiomes of the gut content of the non-fasted *S. aurata* did not differ from that adhered to the intestine wall, nor was there a difference between the distal and proximal intestine as revealed by the Kolmogorov test (Supplementary Table S9), which was also supported by the PCoA (Fig. 2B). In addition, despite not having identical profiles to the 1st group of farmed *S. aurata*, the most relevant OPUs coincided, especially for the *Lactobacillus* species that showed very similar abundance patterns (Fig. 1 and Supplementary Tables S5, S6 and S7). These results were seen in the PCoA (Fig. 2A) where the three microbiomes of non-fasted individuals appeared to be very closely related. In the latter experimental setup, the abundance of OPU0036 *Lactobacillus* sp. 25 was approximately 20 % lower, but it was still the most abundant OPU within the microbiome. On the other hand, OPU0145 *Bacillus* sp. 33, which is closely related to *B. firmus*, appeared to be enhanced in the fed individuals. It was present in almost all farmed fish from the 1st batch, although it was not especially relevant with values ranging from 5 % to 15 %. This latter OPU did not appear in any of the wild fish or in the samples of the farmed fish subjected to short-term fasting. The 1st batch of farmed and fed *S. aurata* showed a higher variation in the diversity trends of each individual microbiome (Fig. 3A), as reflected in the largest dispersion of values in the PCoA (Fig. 2A and C).

The short-term fasting of the farmed *S. aurata* promoted an important drop in diversity (Fig. 3A) and richness, with a severe increase in dominance, since only 180 OPUs could be detected, 27 of which were sample-exclusive. The 86 h fasting period resulted in a completely different community structure, with a remarkable dominance of the single OPU0419 *Micrococcus* sp. 1, most closely related to *M. flavus*, which comprised 76.5 % to 94.3 % of the reads. This OPU was consistently found in most of the fish samples from which the bulk microbiomes were studied, but in none of the farmed *S. aurata* gut content that was previously separated from the gut mucosa-adhered microbiomes. From the different groups of individuals, the microbiomes of the fasted fish showed the highest dominance values and lowest diversity (Fig. 3A and B). In the fasted fish, and similarly with

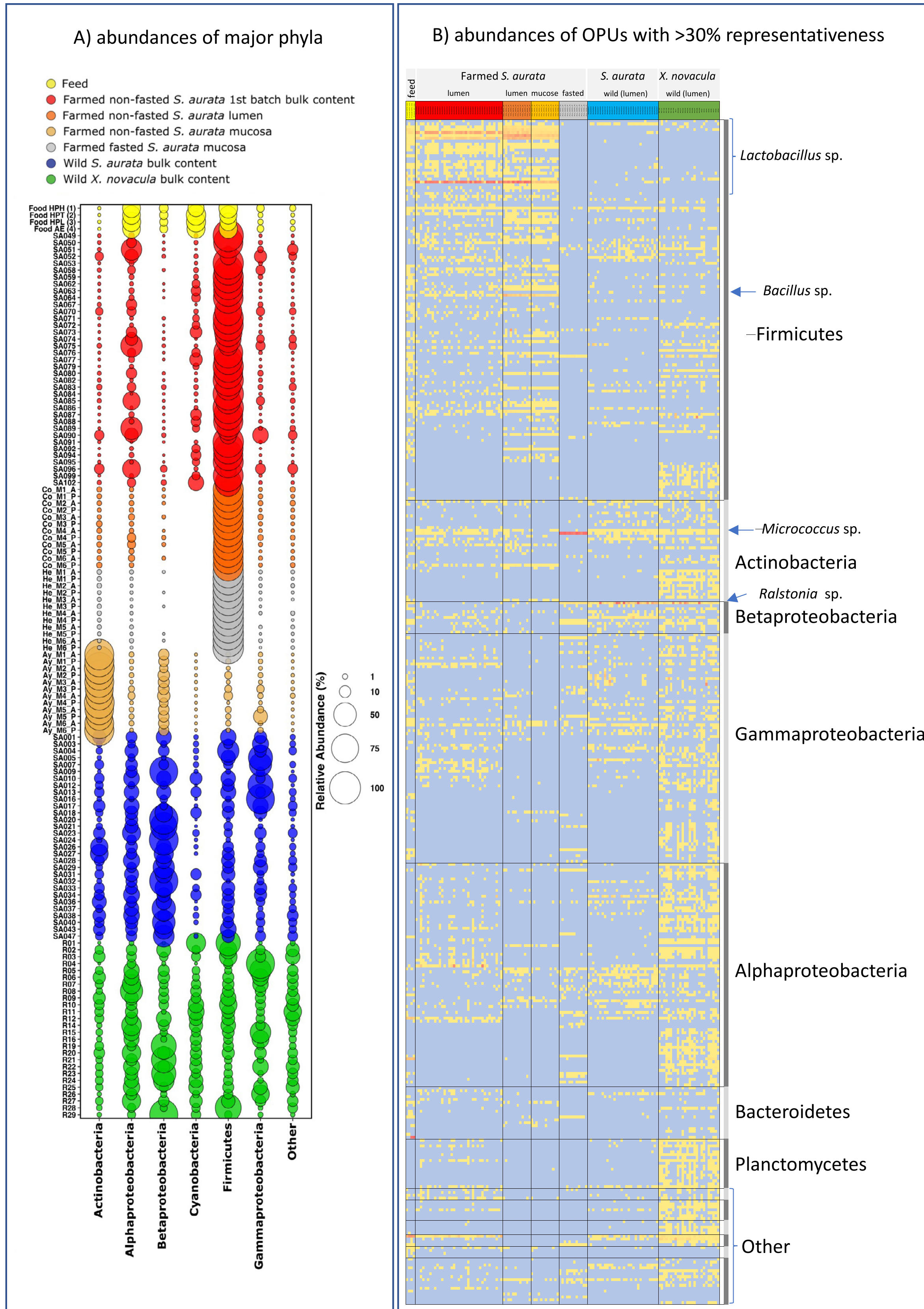

Fig. 1. (A) Relative abundance of the major phyla Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Cyanobacteria, Firmicutes and Gammaproteobacteria in each sample type highlighted by different colors: feed (yellow), bulk content of farmed non-fasted *S. aurata* (red), lumen (brown) and mucosa-adhered (orange) content of non-fasted farmed *S. aurata*, adhered microbiome of farmed fasted *S. aurata* (grey), bulk content of wild *S. aurata* (blue), and bulk content of wild *X. novacula* (green). The panel shows how Firmicutes dominated the farmed non-fasted *S. aurata*, whereas Actinobacteria dominated the mucosa of the fasted farmed *S. aurata*, and the profiles are clearly different for the wild fish with a more even distribution among phyla. (B) Heatmap indicating the relative abundances of the 408 most relevant OPUs present in >30 % of each sample type (following the same color code as above). The most relevant species detected due to their high abundances are highlighted (*Lactobacillus* sp., *Bacillus* sp., *Ralstonia* sp. and *Micrococcus* sp.). The heatmap colors indicate blue as absence of reads, and yellow to red as a progressive increase in the relative presence of each OPU read.

the wild individuals, the presence of OPU0522 *Ralstonia* sp. 1 was also detected, with relative abundances ranging between ~4.4 and ~ 11 %.

3.5. Core gut microbial communities

From the 1803 OPUs detected, only 408 (~23 %) were present in >30 % of each sample group (Fig. 1; Supplementary Table S7). However, the total amplicon abundances of these shared OPUs were between 84.6 % (± 11.4 %) in the wild *S. aurata* gut content, and 99.8 % (± 0.7 %) in the farmed and fasted *S. aurata*. Therefore, these OPUs could be considered to be the core microbiomes of each sample. The most relevant genera, given the number of different species or OPUs observed, were *Lactobacillus* (23 OPUs), followed by *Vibrio* (12 OPUs), *Bacillus* (10 OPUs), *Staphylococcus* (9 OPUs) and *Clostridium* (8 OPUs), all of them closely related to known and taxonomically described species. However, the most representative single OPUs were as mentioned above: OPU0036 *Lactobacillus* sp. 25 representative of the farmed non-fasted *S. aurata*, OPU0522 *Ralstonia* sp. 1 representative of the wild fish, and OPU0419 *Micrococcus* sp. 1 representative of the mucosa-resident microbiota of the fasted *S. aurata*. Most of the taxa detected in the wild fish and the fasted fish were more compatible with an aerobic or microaerophilic gut system, with species from genera such as *Ralstonia*, *Micrococcus*, *Aequorivita*, *Rubinisphaera* or *Labrenzia*, among others, known to thrive in aerobic systems. On the other hand, the non-fasted farmed *S. aurata* gut microbiomes were seemingly more compatible with an anaerobic environment given the occurrence of strict anaerobe genera, such as *Clostridium*, *Peptococcus* or *Weissella*, or the aerotolerant fermenting organisms of the genera *Lactobacillus* and *Fructobacillus*.

4. Discussion

The initial goal of the study was to reveal the gut microbiomes of two distinct fish *S. aurata* and *X. novacula*, which frequently occur free-living in the coastal waters of the Balearic Islands, and compare them with *S. aurata* specimens kept in captivity under farming conditions. The expectations of finding an exclusive microbial structure depending on the feeding habits and on the environmental conditions (wild vs farmed; Egerton et al., 2018) were confirmed, but not as anticipated. As described below, the microbiomes of each different study group showed high internal similarities and important differences between groups, but not due to trophic-level variations related to their evolutionary development (Egerton et al., 2018; Miyake et al., 2015), phylogeny (Miyake et al., 2015), or even due to being wild or captive (Bano et al., 2007). Apparently, only the feed quality or habits in the immediate timeframe when the fish had been sacrificed seemed to be relevant. The results pointed to the fact that the gut microbiome, at least in the juvenile *S. aurata*, was only a transient situation depending on what had been eaten recently, but also, wild fish showed remarkable similarities in their microbial composition that were compatible with a common wild-type microbiome.

Species richness and diversity of the gut microbiomes were higher for the wild than the farmed animals, a fact that had already been observed for wild Atlantic cod (*Gadus morhua*), whose microbiome diversity was reduced when fed with commercial food with respect to wild cod fed under natural conditions (Dhanasiri et al., 2011). However, this contradicted the results observed for Atlantic salmon (*Salmo salar*), where the highest diversity was observed for the farmed fish instead (Holben et al., 2002). The reasons for these variations could reside in the differences related to the DNA extraction methods used (Kashinskaya et al., 2017) or to the diversity of fish species that could lead to important contradictions between studies, as indicated in a review by Egerton and co-workers (Egerton et al., 2018). In light of the results of the current study, the differences related to the DNA extraction methods can be discarded, since no biases could be detected for the same batches of fish (Supplementary Table S2), and the differences observed may be more likely related to short-term feed quality.

Our approach allowed each unique OPU to be designated as a different species, and showed that ~82 % were species of described genera and only ~18 % affiliated with unclassified environmental sequences. The fact that most of the diversity detected was covered by known taxonomic groups was not surprising, as animal-related microbiomes, especially human, are the most explored by both culture-dependent and -independent approaches, for which most of the culture media have been designed, and this is a phenomenon already observed in human-related gut microbiomes (Vidal et al., 2015). Therefore, the results agreed with the trends of isolation of bacteria by cultivation that have mostly recovered members of the four major phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Rossello-Mora and Whitman, 2019), together with some minor phyla of less abundance. This composition was compatible with what had already been reported for various species of fish, as well as the composition heterogeneity between samples of the same species (Egerton et al., 2018; Kormas et al., 2014). Some genera included a high number of species, but with the exception of *Lactobacillus* (40 species), others such as *Vibrio* (46 species) or *Pseudomonas* (28 species), showed testimonial abundances, and were probably not as relevant as hypothesized in some previous studies (e.g. De Paula Silva et al., 2011; Ray et al., 2012; Nayak, 2010; Egerton et al., 2018).

The first set of experiments, which only compared the bulk intestinal content, indicated that wild fish from unrelated species, ecology and feeding habits resembled each other more than the farmed vs. wild *S. aurata*. The fact that the two wild species shared ~400 OPUs contrasted with other observations that different species (i.e. *S. aurata*, *Dicentrarchus labrax*, *Diplodus puntazzo*, *Pagrus pagrus* and *Argyrosomus regius*) reared in the same aquaculture facilities with similar ages and diets hardly shared operational taxonomic units (OTUs), as shown in the study of Nikouli et al. (2021). The wild fish microbiomes in the current study, mostly dominated by *Proteobacteria*, shared one relevant *Ralstonia* species, closely related to *R. mannitolilytica*, which was nearly absent in the farmed fish. This finding was similar to the highly abundant OTU identified as *Diaphorobacter* sp. found in wild *S. aurata* (Kormas et al., 2014), which could comprise up to 50 % of their amplicon reads. In our collection, *Diaphorobacter* sp. (OPU0529) was also detected but it was neither abundant nor present in all wild samples. However, it cannot be discarded that this OTU (Kormas et al., 2014) could perhaps equate to our OPU0522. Both genera *Ralstonia* and *Diaphorobacter* are phylogenetically related, and the latter may need some revision due to its polyphyletic nature within the *Burkholderiaceae* (Ludwig et al., 2021). There are not many reports on the occurrence of *Ralstonia* in fish intestines, but by both culture-dependent and -independent methods, members of this genus have been detected (e.g. Nayak, 2010; Piazzon et al., 2020). It had been speculated that these might show antimicrobial activity or biosynthesis of bioactive compounds, and could produce beneficial secondary metabolites for the host (Cerezo-Ortega et al., 2021). In addition, the taxa composition of wild fish was more compatible with an aerobic or microaerophilic system and it was especially dominated by *Proteobacteria* with strict or facultative aerobic metabolisms. The presence of cyanobacterial OPUs (mostly chloroplasts) indicated that, despite *X. novacula* mostly feeding on benthic food items (mainly *Mollusca* and *Echinodermata* (Castriota et al., 2005)), and *S. aurata* preferentially feeding on macrobenthos (*Polychaeta* and *Amphipoda*) and macrophyte detritus (Ferrari and Chieregato, 1981), these wild fish had consumed vegetal biomass that could come from direct or indirect ingestion when feeding on herbivorous microbenthic invertebrates.

On the other hand, the farmed fish, with high biomass contents, were dominated by members of the *Firmicutes,* but especially by lactobacilli that were nearly absent in wild fish, with putative anaerobic fermentative metabolism. The food accumulated in the gut with high organic content could probably be responsible for establishing conditions where oxygen is depleted due to the enhanced microbial activity leading to fermentation. The presence of *Lactobacillus* species in fish microbiomes is not unusual, but conspicuously all cases found in the literature were described using farmed animals with commercial feeding regimes (e.g. Rimoldi et al., 2020a; Rimoldi et al., 2020b; Hovda et al., 2007; Rudi et al., 2018; Estruch et al., 2015; Jang et al., 2022; Cui et al., 2022). The microbial feed profiles showed a similar microbial composition to the farmed fish being fed. The fact that ~66 % of the OPUs from the feed was shared

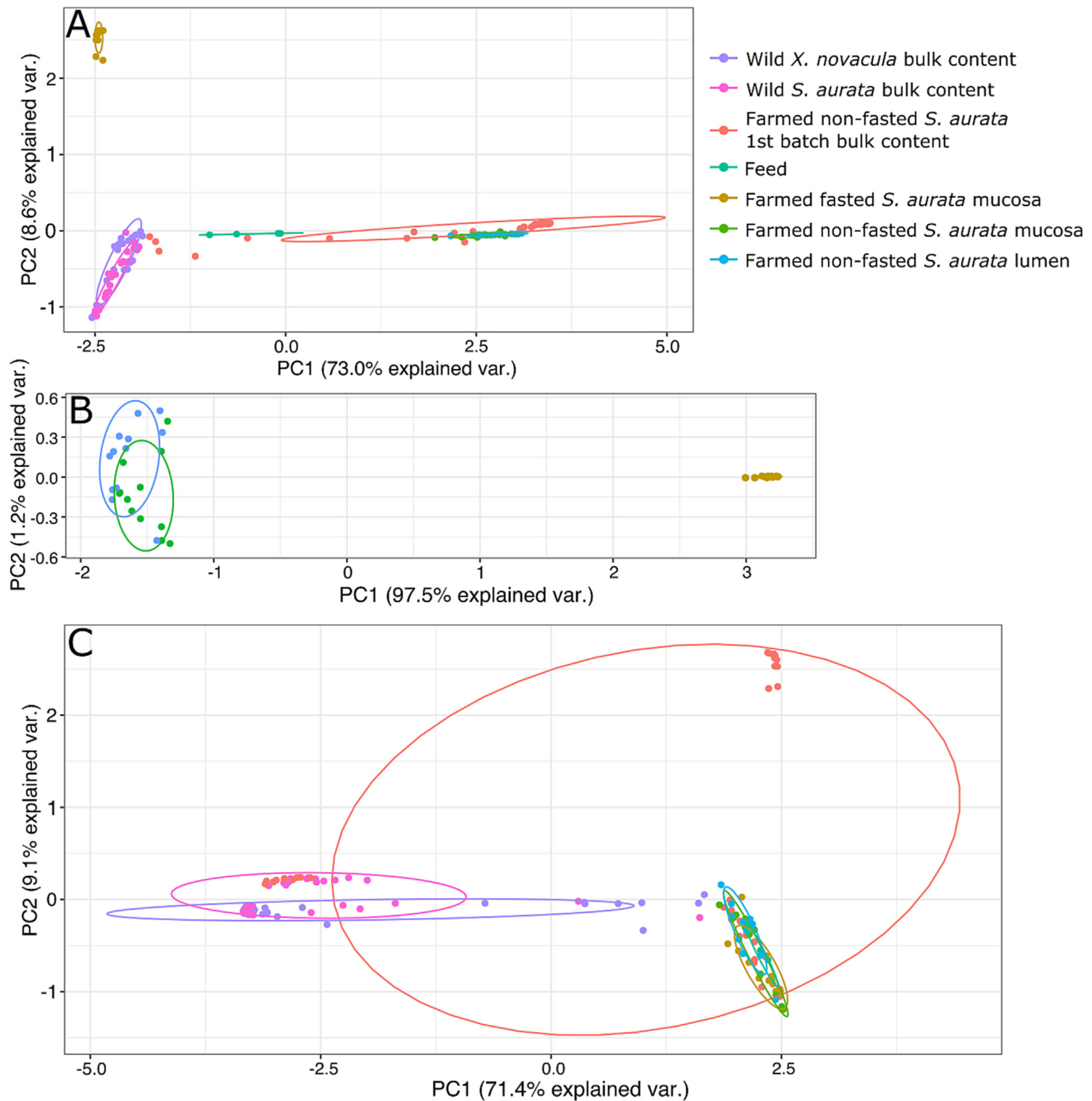

Fig. 2. Principal component analyses of the microbiomes of: (A) all samples and all OPUs detected in this study showing how wild gut microbiomes clustered together (pink and violet labels; lower left) and are well discriminated from the farmed non-fasted and the feed microbial composition (light and dark green labels, and red; middle right), and from the farmed and fasted mucosa microbiome (brown labels, upper left). (B) farmed *S. aurata* gut bulk (blue label) and mucosa (dark green label) contents are very similar (left) and highly different from the mucosa microbiome after 86 h fasting (brown label, right). (C) considering only the 408 OPUs present in at least 30 % of all samples analyzed showing a similar discriminant structure as (A).

with the farmed *S. aurata*, and that the major most relevant *Lactobacillus* species were present in both groups, were clear indications that the microorganisms accompanying the dry feed strongly influenced the fish's microbiome. The fact that an important part of the ingested microbes showed an increased relative abundance in the intestines could only be explained either by an unlikely selective digestion that would enhance the presence of lactobacilli, or, and most plausibly, the lactobacilli present in the feed occurred in a viable state that colonized the intestine and metabolized the available substrates. The important abundance of lactobacilli in the farmed fish gut may certainly promote benefits for the fish during transit through the digestive system, which is a process that has been previously studied (e.g. Carnevali et al., 2004; Ige, 2013; Rimoldi et al., 2020a).

The early results seemed to indicate that we were mainly observing the allochthonous microbiota generated by the transient effect of the feed in the intestine. To clarify this hypothesis, the experiments were repeated but by comparing fasted and non-fasted individuals. The results were revealing, since, in the first instance, there were no differences between the distal and proximal intestine, which was contrary to other studies (e.g. Hovda et al., 2007; Ringø et al., 2006). This lack of diversification along the intestine may be due to the fact that carnivorous fish have a shorter digestive tract compared to species displaying other feeding habits (e.g. omnivores, herbivores or detritivores) (Egerton et al., 2018) and that the fish in the current study were juveniles with relatively short intestines. No differences were found between the intestinal content of the non-fasted fish

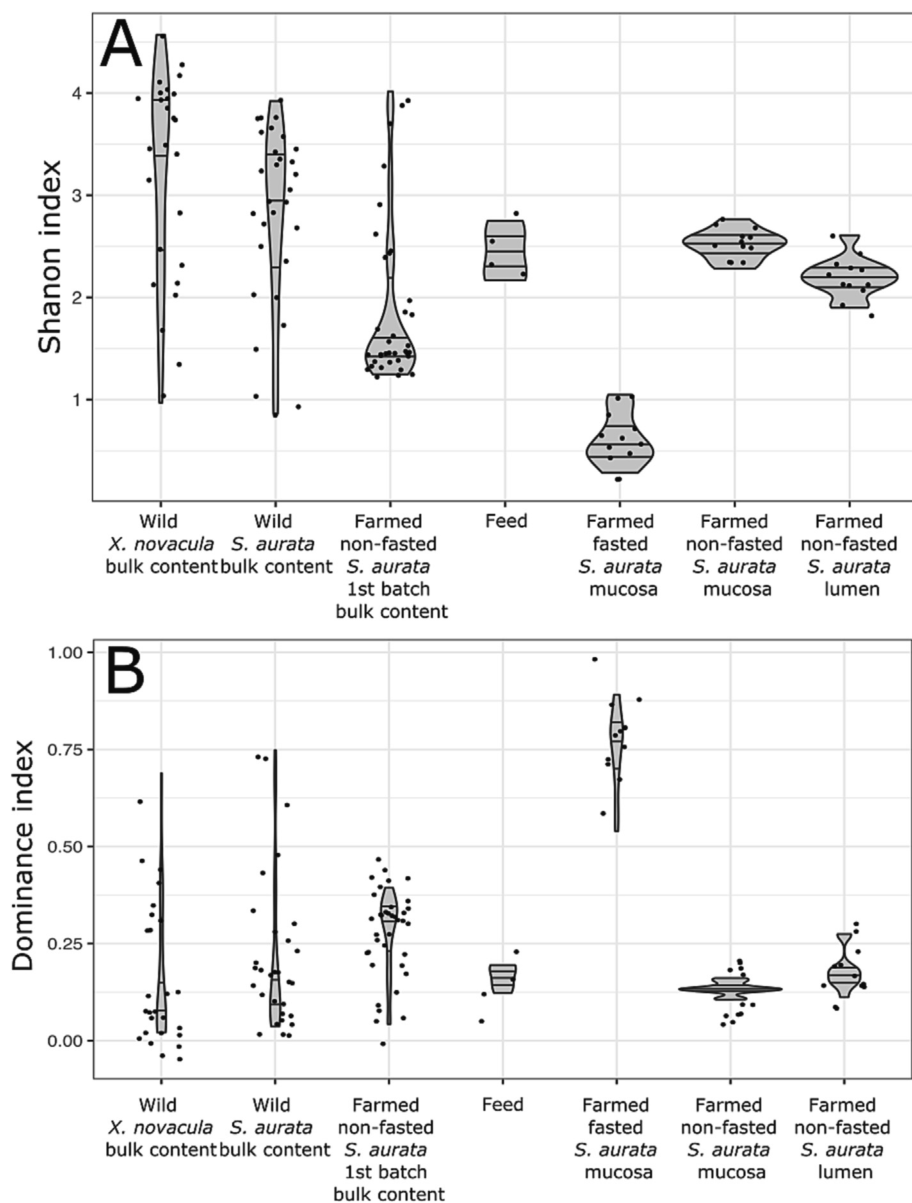

Fig. 3. Variation among the samples related to the Shannon (A) and Dominance (B) indices of all samples. The indices show how wild fish were more variable than the homogeneous guts of farmed fish, and also that the fasted microbiomes were of much lower diversity and higher dominance than the rest.

and the microbiome adhered to the mucosa, a fact that, on the one hand, was similar to other studies (Zhou et al., 2009) and, on the other hand, also validated our previous results on the initial analyses of bulk intestinal content. Another major relevant observation came from the fasted *S. aurata* individuals that showed a remarkable drop in diversity and richness after fasting, indicating that most bacteria detected in the non-fasted individuals were transient. In all cases, fasting for 86 h promoted a lack of transient biomass in the lumen and a radical drop in species richness and diversity in the intestinal mucosa, with a severe dominance of a single *Micrococcus* species most closely related to *M. flavus*. Cultivation has shown that members of this *Micrococcus* can occur in both the gut-adhered and transient microbiota of fish, such as Atlantic salmon (*S. salar*) (Bakke-McKellep et al., 2007), Puget Sound rockfish (*Sebastes emphaeus*) (Colwell, 1962) or European plaice (*Pleuronectes platessa*) (Gilmour et al., 1976). The role of this described (potentially strict) aerobic, heterotrophic member of the phylum *Actinobacteria* (Liu et al., 2007) remains unknown, but as for *Ralstonia,* it may also be a resident species producing bioactive molecules beneficial for the host (Cerezo-Ortega et al., 2021). Another hypothesis that can be derived from these findings is that given *Micrococcus* is (theoretically) a strict aerobe (Busse, 2015), and the remaining bacterial species shared with the wild fish are either strict or facultative aerobes, perhaps the standard conditions in the juvenile fish intestines are not permanently oxygen depleted.

Our major observation was that the farmed fish, which were > 7 months old, lost all major key species and almost the complete microbiome after fasting, contrasting with the observation that, for example, Atlantic cod establishes a stable core microbiome during the first 50 days of development (McIntosh et al., 2008). Additional fasting studies with wild fish and other species may be needed to ensure that this was not an exclusive trait of the farmed *S. aurata* used in this study. In general, very few microbiome studies have been conducted on short-term fasted individuals, or at least this step was not reported in published reports (e.g. Rimoldi et al., 2020a; Rimoldi et al., 2020b; Hovda et al., 2007; Rudi et al., 2018; Estruch et al., 2015; Jang et al., 2022; Cui et al., 2022). Most studies that considered fasting were either conducted using culture-dependent techniques (e.g. Dhanasiri et al., 2011; Gilmour et al., 1976), and thus with results that were difficult to translate to the real situation (Amann et al., 1995). Recently, fasting has been included in the sampling protocols, but times differ from just one day

fasting (e.g. Liu et al., 2021; Deng et al., 2021) to two days (e.g. Solé-Jiménez et al., 2021; Piazzon et al., 2019; Piazzon et al., 2020). Conspicuously, the taxonomic microbial composition after only one day of fasting (dominated by *Cetobacterium*, *Plesiomonas*, *Escherichia-Shigella*, *Ruminiclostridium*, *Lachnospiraceae* and other strict or facultative anaerobes) was more compatible with an anaerobic metabolism, whereas after two days of fasting (dominated by *Kokuria*, *Micrococcus*, *Afipia*, *Pseudoalteromonas*, *Psychrobacter*, *Paracoccus*, *Arthrobacter*, and other strict aerobic or facultative anaerobic bacteria) most taxa were compatible with an aerobic lifestyle. In this current study, the fish were fasted for 3 days (86 h), and the fact that our observations of a putative aerobic metabolism for the resident microbes in the mucosa were congruent with the previous two-day fasting analysis, reinforced the idea that in order to study the resident microbial communities, a fasting pre-manipulation is necessary.

Our observations have relevant implications. Firstly, almost all studies carried out in the past with fish gut microbiomes would have addressed a potentially transient, non-stable microbiome that is highly dependent on the quality of the supplied food in the case of farmed fish and feeding habits of the wild fish. This could explain the many discrepancies observed among the studies (Egerton et al., 2018). Secondly, it is highly possible that the quality of the feed supplied in the case of farmed fish and the feeding habits of the wild fish only temporarily influence their health and performance in the short term, and that the food-accompanying microbes may play a relevant role during their transient saprophytic metabolic activity. For wild fish, a suboptimal microbiome could be optimized by migrating to better environmental and food source conditions or by changing the feeding habits. However, captive farmed fish will always depend on the quality of the feed from the suppliers. As an important role of this transient microbiome in relation to fish metabolism cannot be discarded, the methodological approach must be well designed in order not to bias the results. Therefore, standardization of the sampling protocol is paramount to achieve comparative observations.

4.1. NB

In the current manuscript, we used the commonly applied terminology for the higher taxa as in the past. We 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). However, for the current best interpretation we preferred to retain the former widely used names in the text.

CRediT authorship contribution statement

RRM, IC, JA, DF and AR designed the experiments. RRM and TV analyzed the data and wrote the manuscript. MMB, MS, MBS, EA, AP, JA, and IC collected and processed the wild fish and the first batch of farmed fish. AR, EB, DF and EG provided all farmed fish, executed the experiments of the second batch and prepared the samples at IRTA La Ràpita. AG maintained the fish at LIMIA in Mallorca. MU technically supported all molecular studies. TV isolated, amplified and sequenced the DNAs. All coauthors also read, commented, and corrected the manuscript, and in addition CR as a native English speaker edited the final draft.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that there are no competing interests.

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Animal welfare statement

All animal care procedures were approved by the Ethical Committee of Animal Experimentation (CEEA-UIB, Spain; Ref. CEEA 97/07/18 and CEEA 98/07/18) and were 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 Department of Environment, Agriculture and Fisheries of the Government of the Balearic Islands granted permission for capturing the wild animals (ref. L30S15749/2018). The IRTA facilities are certified and have obtained the necessary authorization for the breeding and husbandry of animals for scientific purposes. Experimental procedures were conducted following the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and the guidelines of the relevant Spanish laws (Law 32/2007 and RD 1201/2015).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164080.

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