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First Evaluation of Associated Gut Microbiota in Wild Thick-Lipped Grey Mullets (*Chelon labrosus*, Risso 1827)

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Abstract: This study aimed to characterize the intestinal microbiota of wild thick-lipped grey mullets (*Chelon labrosus*) and explore its potential functionality on the host. Intestinal contents of anterior and posterior sections from wild fish were collected and DNA was extracted. Subsequently, the V3–V4 regions of 16S rRNA were sequenced using the Illumina technology and results were analyzed by bioinformatics pipeline. The functional profile of the microbial community was analyzed using PICRUSt software. Shannon and Simpson diversity indices were significantly higher in the posterior section of wild specimens. The overall taxonomic composition suggests a certain homogeneity in the anterior section of the intestine and heterogeneity in the posterior section. Due to this, no statistical differences were detected at any level among both intestinal sections. Predicted functions of intestinal microbiota showed the most abundant were those related to amino acid metabolism, carbohydrate metabolism, energy metabolism, membrane transport, and cell replication and repair. Furthermore, the analysis revealed microbial functional genes related to the elimination of environmental toxins.

Keywords: thick-lipped grey mullet; Mugilidae; gut microorganisms; PICRUSt; metabarcoding; species diversification

1. Introduction

While global aquaculture has increased significantly since the 1990s, this growth is not homogeneous globally, with Asia showing the largest increase [1]. Compared to Asia, Europe has experienced much less growth in aquaculture. One of the reasons for the limited growth of European aquaculture may lie in its most important farmed species (salmon, trout, seabream). These species are characterized by their carnivorous nature, which means that they require a certain amount of fishmeal and fish oil in their feed. Thus, 1.87 kg of wild fish are needed to produce 1 kg of salmon, 1.82 kg of wild fish are needed to produce 1 kg of trout, and 1.25 kg of wild fish are needed to produce 1 kg of marine fish [2]. This makes European aquaculture unsustainable [3,4]. As an alternative, the diversification of cultivated species has been proposed [5], with an emphasis on the cultivation of omnivorous and herbivorous species [6,7].

Within this context, it is worth noting the high potential presented by the species of the family Mugilidae (mullets). Fish belonging to this family, commonly known as the grey mullets group, comprise a great number of species, and are one of the most ubiquitous teleost families in coastal waters [8]. Since they have an extraordinary adaptability, they occur in most temperate, sub-tropical and tropical waters in both hemispheres [9], inhabiting offshore waters and coastal lagoons, lakes and rivers. Among Mediterranean Mugilidae, *Mugil cephalus* (Linnaeus, 1758), *Chelon ramada* (Risso, 1827), *Chelon labrosus*

(Risso, 1827) and *Chelon saliens* (Risso, 1810) are the main representative fish species and possess a high economic value [10]. Above all, flathead grey mullet, *M. cephalus*, is the main species produced and is highly appreciated in the food market for its salt-cured and dried eggs named "bottarga" [11]. The culture of Mugilidae species is considered a priority within the current strategies of European aquaculture [12]. As an example, it is part of the Spanish aquaculture diversification program [13]. One of the mullets with potential for use in aquaculture is the thick-lipped grey mullet (*C. labrosus*). This species presents great adaptability to different culture conditions, an omnivorous profile, high osmoregulatory capacity, and resistance to environmental variations [14,15].

There is recent information available on the natural feeding habits, sensitivity to stress, digestive physiology, or nutritional quality of *C. labrosus* [14,16,17]. In spite of that, there is a lack of information regarding the composition and functions of its intestinal microbiota. The intestinal microbiota plays a key role in host health, by increasing digestion efficiency and nutritional physiology, boosting the immune system, and preventing the colonization of opportunistic pathogens [18]. The microbiota can also act in the detoxification process by degrading xenobiotic substances [19]. However, this bacterial community can be altered by a wide range of both external and internal factors, such as water salinity, season, geographic location, as well as developmental stages, trophic levels, starvation and feeding periods, stress, and diet [20,21]. For instance, *Pseudomonas* spp. has been identified as the dominant species in the intestines of wild grey mullets in Sardinia, Italy [22]. Le and Wang [23] evaluated the structure and membership of the gut microbial communities in three cryptic species of wild grey mullet (*M. cephalus*) of different ages (adult and juvenile) under potential migratory effects, pointing out a core microbiome in all fish samples, which was dominated by Proteobacteria.

Documenting the bacteria present in healthy individuals is the first step to understanding the impacts of microbial manipulation in aquaculture systems [24]. In this sense, the knowledge of bacterial diversity from healthy wild fish in their natural environment is essential. Furthermore, the study of the functionality of the intestinal microbiota of wild fish provides information on its capacity to adapt to different culture conditions [25]. Thus, this study aimed to characterize the intestinal microbiota of wild *C. labrosus* and explore its potential functionality on the host.

2. Materials and Methods

2.1. Sample Collection

Eight wild thick-lipped grey mullets (average weight and length: 115.3 ± 5.0 g and 20.3 ± 0.6 cm, respectively) were caught in May 2021 with a net in the Puerto de la Caleta (Velez Malaga, 36°44′54.9″ N 4°03′57.6″ W, Malaga, Spain). The area corresponds to a small harbor dedicated exclusively to fishing and recreational activities. Its small size allows for water renewal and the constant flow of schools of fish from the outside to the inside and vice versa. In addition, the facilities allow the fish to be caught without the use of boats. The fish were transported alive in a 1 m³ container with marine water, constant aeration, controlled temperature and in accordance with Spanish legislation on the transport of live fish (RD 751/2006, RD 1614/2008). One hour later the fish arrived at the Centro de Experimentación de Ecología y Microbiología de Sistemas Acuáticos Controlados Grice-Hutchinson (CEMSAC) of the University of Malaga (Malaga, Spain; Spanish Operational Code REGA ES290670002043). The animals were then euthanized by immersion in water with a 2-phenoxyethanol overdose (1 mL L^{-1}, Sigma-Aldrich, ref. 77699, St. Louis, MO, USA). Specimens were then individually weighed, and intestinal samples were aseptically removed by ventral sectioning. Then, each tract was divided into two major, anterior and posterior, sections and kept at -80 °C until the subsequent analysis.

All the experimental procedures complied with guidelines of the University of Málaga (Spain) and the European Union Council (2010/63/EU) for the use of animals in research. The experimental procedures were previously approved and endorsed by the Regional Government (Junta de Andalucía reference number 11-07-2020-082).

2.2. DNA Extraction and Sequencing

DNA of anterior and posterior intestinal sections (*n* = 8) were extracted according to the protocol based on saline precipitation [26], with some modifications [27]. A sample with ddH$_2$O was used as blank control. DNA concentration was quantified fluorometrically with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and its purity and integrity were assessed using a NanoDrop™ One UV-Vis Spectrophotometer WiFi (Thermo Scientific, Wilmington, DE, USA) and through a 1% agarose gel electrophoresis, stained with GelRed Nucleic Acid Stain 20,000x (InTRON Biotechnology, Seoul, Korea).

The 16S rRNA of the samples was sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) with 2 × 300 bp paired-end sequencing at the Ultrasequencing Service of the Bioinnovation Center (University of Malaga, Spain). Sequencing was carried out using the sense primers 5′-CCTACGGGNGGCWGCAG-3′ and 5′-GACTACHVGGGTATCTAATCC-3′ [28], directed at the variable regions V3–V4 of the 16S rRNA gene.

2.3. Data Processing

All Illumina reads were analyzed using FastQC software (version 0.11.9) in order to assess the sequence quality [29]. Further data processing including trimming and 16S analysis and visualization was performed with the workflow based on the MOTHUR software package (version 1.39.5) described by our research group [30,31]. All data analysis of the intestinal microbiota was processed using phyloseq and vegan library in R statistical package [32,33]. Good coverage coefficient and ecological indices were calculated. Alpha diversity was estimated using the Chao1, Shannon, and Simpson indices. Principal coordinates analysis (PCoA) was used to evaluate the Beta diversity obtained by both weighted and unweighted UniFrac analysis. Gut microbiota composition was also compared between each sample using a permutation-based multivariate analysis of variance (PERMANOVA) of UniFrac distances (weighted and unweighted). Normal distribution was checked for all data with the Shapiro–Wilks test, while the homogeneity of the variances was obtained using the Levene test. Differences between sections were tested using Student's *t*-test. Statistical analyses were performed with XLSTAT software (version 2014.5.03) (Addinsoft, New York, NY, USA) and significance was set at $p < 0.05$.

For the taxonomical comparison, readings were normalized based on rarefaction curves and singleton and doubleton readings were removed. The taxonomical results are presented at phylum, class, and family taxonomic levels. The groups with a relative abundance of less than 1% have been considered 'ETC' according to [34]. The statistical taxonomic comparison was carried out using the R package DESeq2 at a significance level of $p < 0.05$.

2.4. Functional Analysis by PICRUSt

The functional profile of the microbial community was analyzed using PICRUSt software (version 1.1.3), which is a bioinformatic software designed to predict the functional profile of a microbial community based on the study of the 16S [35]. Sequencing files were introduced into the PICRUSt software and samples were normalized to the number of copies of the 16S rRNA. Functional metagenomes for each sample were predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [36] catalog and collapsed to a specified KEGG level. STAMP (version 2.1.3) was used to show a heatmap at KEGG 2 level. Nearest sequenced taxon index (NSTI) scores were calculated to assess the accuracy of the predicted metagenomes, and bacterial functional profiles were compared up to KEGG 3 level. Differences between sections were tested using Student's *t*-test. Statistical analyses were performed with XLSTAT software and significance was set at $p < 0.05$.

3. Results

3.1. Diversity Analysis of Microbiota of Wild C. labrosus

A total of 18,579,304 high-quality reads, corresponding to an average number of 116,120.62 reads per sample was obtained, with a minimum of 952,242 reads and a maximum of 1,404,533 reads. A total of 512 OTUs were assigned at a 97% identity threshold.

Alpha diversity indices were calculated for microbiota of anterior and posterior intestinal sections of wild *C. labrosus* (Table 1). Results showed that no statistical differences were observed for microbiota species richness (Chao1) between anterior and posterior sections. Furthermore, Shannon and Simpson's indices were found to be significantly higher in the posterior section of wild specimens.

Table 1. Alpha diversity indices of bacterial communities in anterior (IA) and posterior (IP) intestinal sections of wild *C. labrosus*.

	IA	IP	*p*
Chao1	124.15 ± 44.26	158.61 ± 64.87	0.235
Shannon	1.40 ± 0.23	1.91 ± 0.42 *	0.009
Simpson	0.66 ± 0.04	0.76 ± 0.07 *	0.002

Values are the mean ± SD ($n = 8$). Asterisks denote significant differences ($p < 0.05$).

Principal coordinates analysis (PCoA) scores are plotted based on the relative abundance of OTUs of intestinal microbiota from analyzed specimens. PCoA showed no grouping between anterior and posterior samples (Figure 1a,b). Furthermore, the PERMANOVA based on weighted ($p = 0.053$) and unweighted ($p = 0.834$) UniFrac metric distances revealed no significant differences between groups.
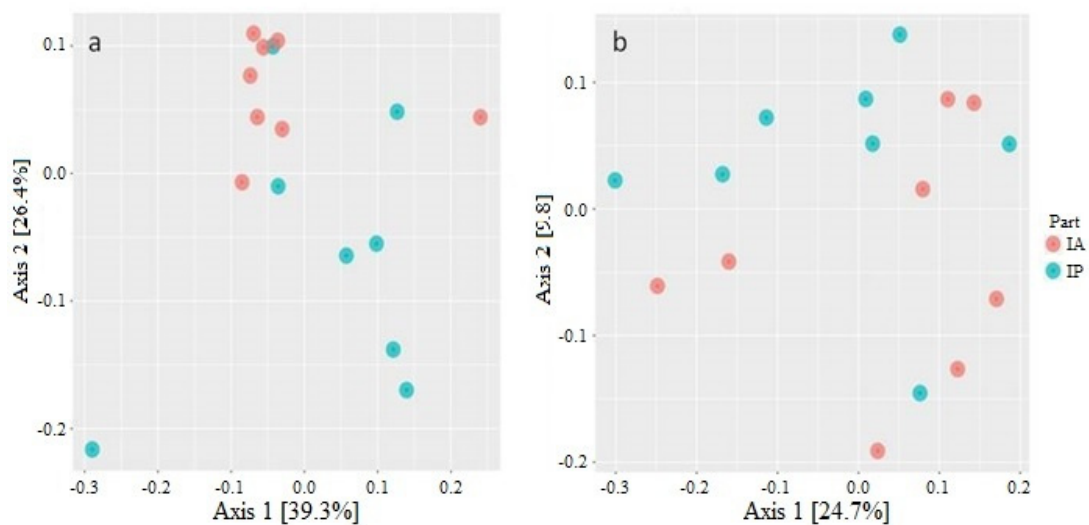

Figure 1. Principal coordinate analysis (PCoA) of the bacterial communities. (**a**) Calculated from the weighted UniFrac distance matrix and (**b**) calculated from the unweighted UniFrac distance matrix. Red circles represent samples from the intestinal anterior section and blue circles represent the intestinal posterior section of each individual of wild *C. labrosus*.

3.2. Composition of Intestinal Microbiota of Wild C. labrosus

The rarefaction curves were calculated, and it was observed that all the readings were correctly asymptotized (Supplemental Figure S1), because of that the normalization of the data to the minimum length was considered appropriate. Good's coverage coefficient >99%, indicated adequate sequencing depth. In addition, to avoid clustering errors, singleton and doubleton readings that appeared in at least 10% of the sample were removed. Finally, 222 OTUs were used for further taxonomic analysis.

Anterior and posterior intestinal sections of specimens primarily present representatives of the phyla Proteobacteria (98% and 81%, respectively) (Figure 2). However, other phyla such as Tenericutes, Spirochaetes and Cyanobacteria appeared in posterior sections with low relative abundance.
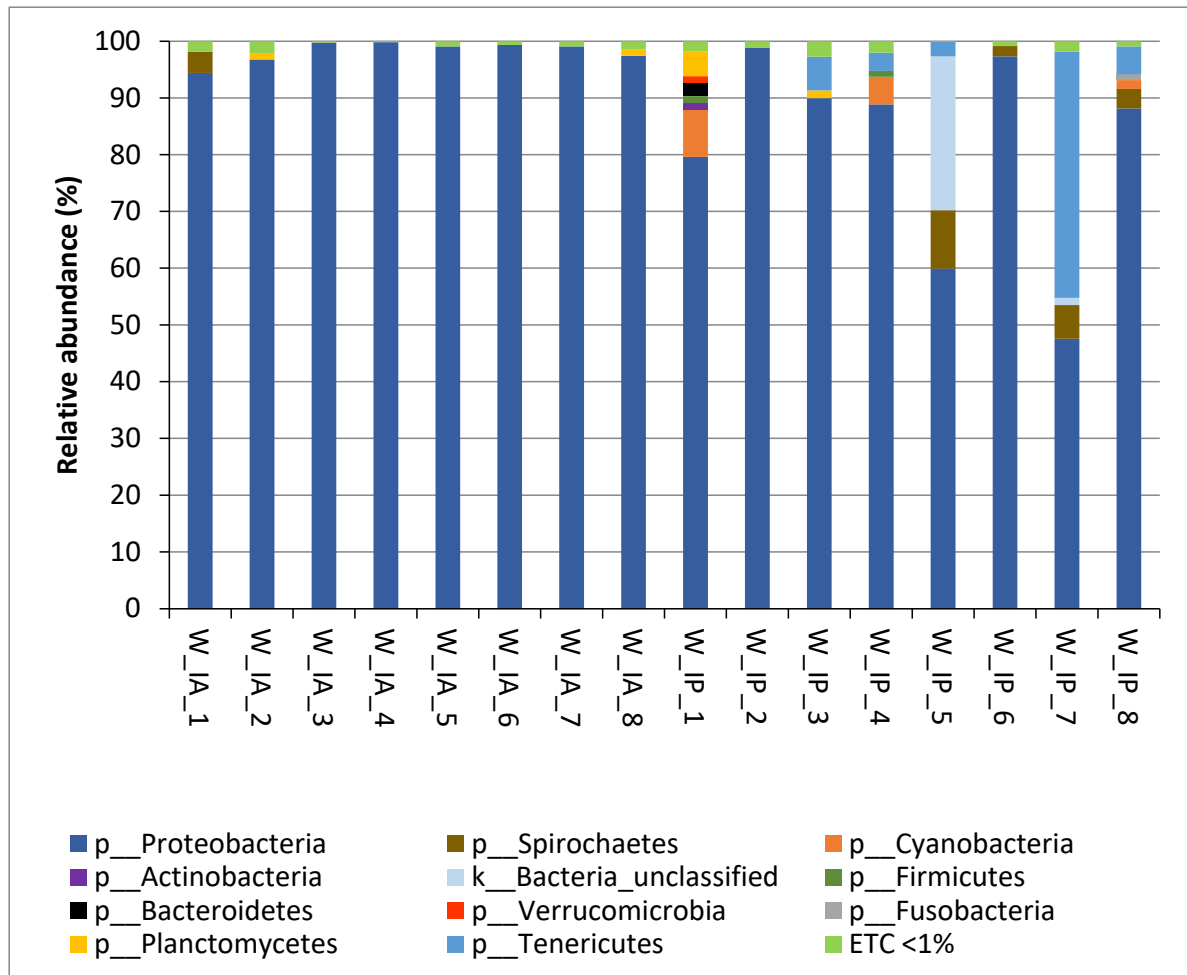

Figure 2. Relative abundance (percentage) at *phylum* level for each sample of intestinal microbiota from anterior and posterior sections of wild *C. labrosus*. In the figure, W_IA corresponds to the anterior intestinal section of wild fish, and W_IP corresponds to the posterior intestinal section of wild fish. ETC: phylum <1%.

At the class level, the anterior section of specimens was dominated by Gammaproteobacteria (96%) and Alphaproteobacteria (1%); whereas the posterior presented Gammaproteobacteria (79%) and Mollicutes (7%), followed by the Alphaproteobacteria, and Brevinematae in low relative abundance (Figure 3).

The bacterial families found in the anterior and posterior section of wild *C. labrosus*, although in different quantities, were predominantly Vibrionaceae, Pseudomonadaceae, and Gammaproteobacteria class and Vibrionales order, which were not identified at family level. Furthermore, Brevinemataceae and Mycoplasmataceae were present in the posterior section but not in the anterior of the specimens (Figure 4).
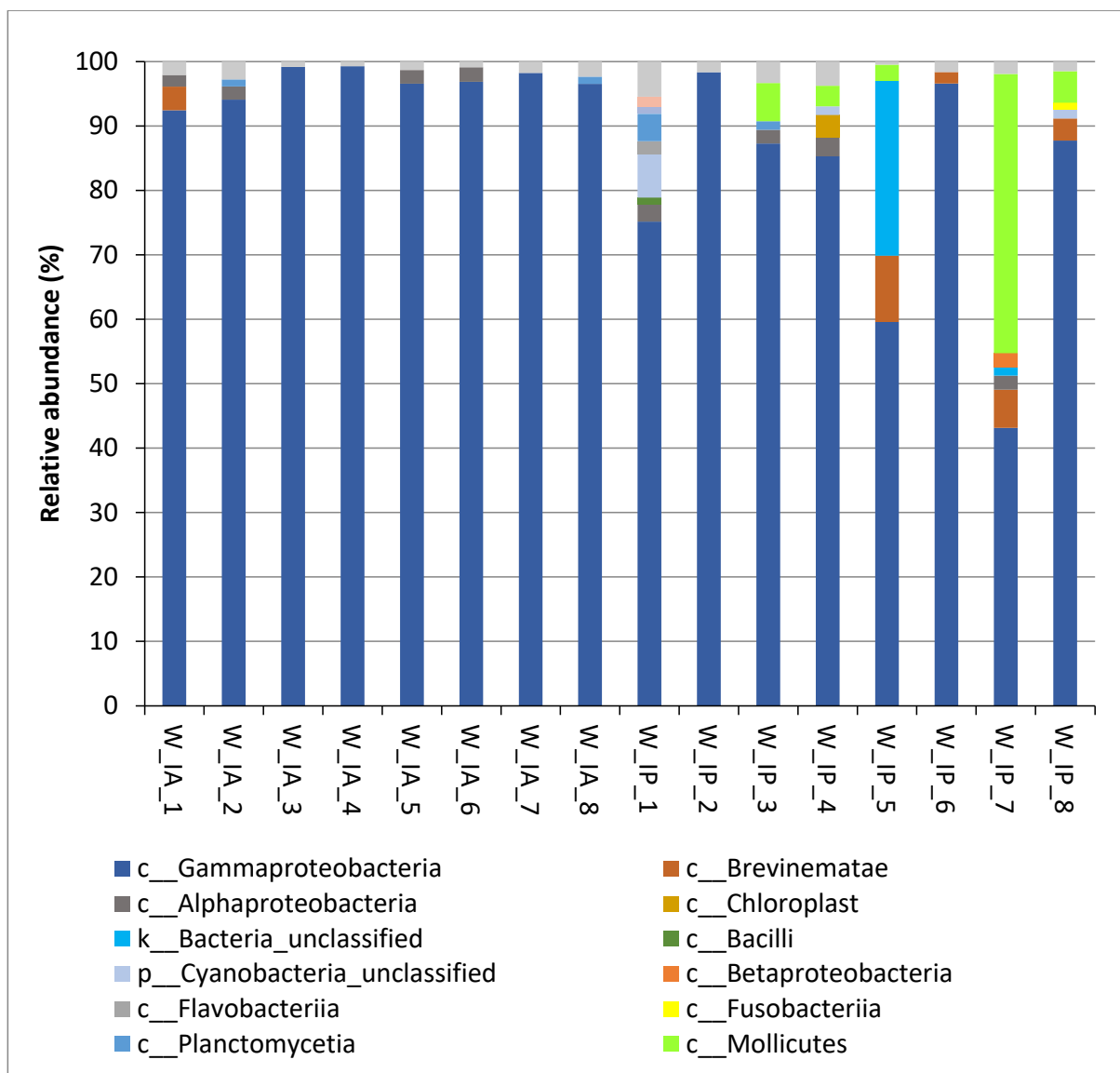
Figure 3. Relative abundance (percentage) at class level for each sample of intestinal microbiota from anterior and posterior sections of wild *C. labrosus*. In the figure, W_IA corresponds to the anterior intestinal section of wild fish, and W_IP corresponds to the posterior intestinal section of wild fish.

3.3. Functional Inference of the Gut Microbiota

The changes in the presumptive functions of the intestinal microbiota of wild *C. labrosus* were examined by predicting the metagenomes using PICRUSt. The accuracy of the prediction, evaluated by computing the nearest sequenced taxon index (NSTI), was 0.09 ± 0.02 (NSTI values within the expected range for this organism [35]). PICRUSt analyzed 330 functional KEGG pathways (Supplemental Table S1), grouped into 31 KEGG 2 categories. Figure 5 details the general metabolic pathways, comparing microbiota functions from anterior and posterior sections, which highlights how the functions had a similar abundance between the two sections, the most abundant being those related to amino acid metabolism, carbohydrate metabolism, energy metabolism, membrane transport and cell replication and repair.
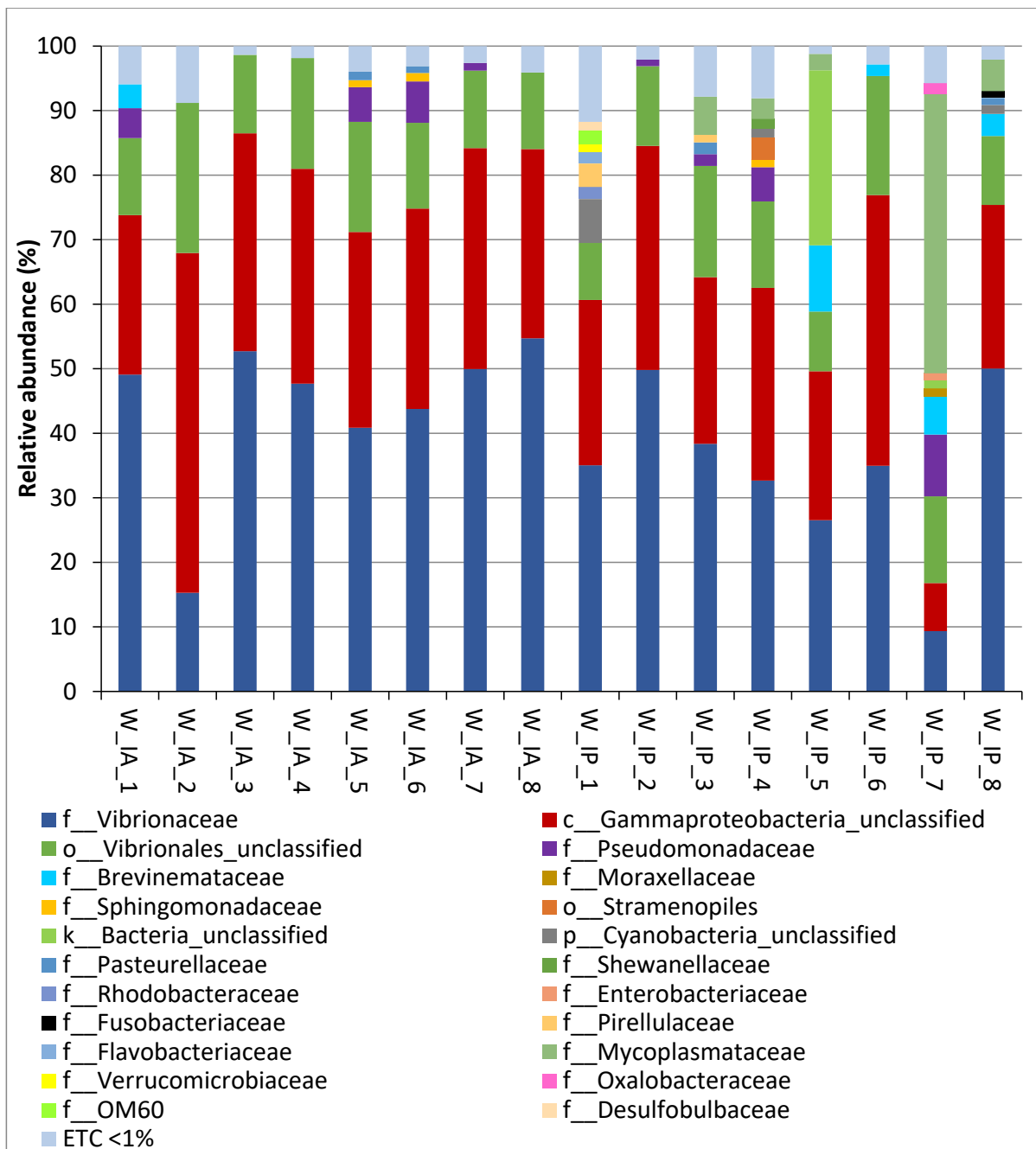
Figure 4. Relative abundance (percentage) at family level for each sample of intestinal microbiota from anterior and posterior sections of wild *C. labrosus*. In the figure, W_IA corresponds to the anterior intestinal section of wild fish, and W_IP corresponds to the posterior intestinal section of wild fish. ETC: families < 1%.

Figure 5. General metabolic pathways of the intestinal microbiota from both sections of wild *C. labrosus*. In the figure, W_IA corresponds to the anterior intestinal section of wild fish, and W_IP corresponds to the posterior intestinal section of wild fish.

Only those pathways at KEGG level 3 that could be related to *C. labrosus* gut microbiota functions have been studied independently (Table 2). Statistical differences were found between intestinal sections. In this sense, pantothenate and CoA biosynthesis pathway was statistically higher in the posterior section, while inositol phosphate metabolism, phenylalanine metabolism, biosynthesis of siderophore group non ribosomal peptides, glycosphingolipid biosynthesis–globo series, fluorobenzoate degradation, and styrene degradation pathways were significantly higher in the anterior intestinal section.

Table 2. Relative abundance (%) of the functional predictions on the microbial composition of anterior (IA) and posterior (IP) intestinal sections of wild *C. labrosus* at KEGG level 3. In the table, functions with a relative abundance ≤0.020% have been removed.

KEGG Pathways	IA	IP	*p*
Lipid metabolism			
Biosynthesis of unsaturated fatty acids	0.355 ± 0.006	0.308 ± 0.062	0.080
Fatty acid biosynthesis	0.496 ± 0.016	0.466 ± 0.084	0.360
Lipid biosynthesis proteins	0.750 ± 0.017	0.744 ± 0.073	0.827
Carbohydrate metabolism			
Inositol phosphate metabolism	0.228 ± 0.008 *	0.203 ± 0.002	0.032
Amino acid metabolism			
Lysine biosynthesis	0.603 ± 0.014	0.560 ± 0.096	0.252
Lysine degradation	0.377 ± 0.009	0.384 ± 0.060	0.736
Phenylalanine metabolism	0.388 ± 0.020 *	0.345 ± 0.016	0.013
Phenylalanine, tyrosine and tryptophan biosynthesis	0.713 ± 0.017	0.670 ± 0.104	0.280
Valine, leucine and isoleucine biosynthesis	0.663 ± 0.020	0.658 ± 0.025	0.653
Valine, leucine and isoleucine degradation	0.758 ± 0.027	0.734 ± 0.096	0.512
Metabolism of cofactors and vitamin			
Folate biosynthesis	0.476 ± 0.015	0.442 ± 0.056	0.137
Pantothenate and CoA biosynthesis	0.479 ± 0.012	0.507 ± 0.017 *	0.023
Ubiquinone and other terpenoid-quinone biosynthesis	0.398 ± 0.007	0.368 ± 0.069	0.255
Energy metabolism			
Carbon fixation in photosynthetic organisms	0.380 ± 0.007	0.463 ± 0.108	0.069
Carbon fixation pathways in prokaryotes	0.992 ± 0.014	0.986 ± 0.126	0.896
Photosynthesis	0.274 ± 0.008	0.361 ± 0.093	0.063
Metabolism of terpenoids and polyketides			
Biosynthesis of ansamycins	0.040 ± 0.002	0.052 ± 0.015	0.054
Biosynthesis of siderophore group non ribosomal peptides	0.092 ± 0.004 *	0.077 ± 0.013	0.049
Biosynthesis of vancomicyn group antibiotics	0.047 ± 0.003	0.047 ± 0.010	0.936
Geraniol degradation	0.301 ± 0.010	0.299 ± 0.081	0.948
Limonene and pinene degradation	0.252 ± 0.009	0.267 ± 0.036	0.280
Polyketide sugar unit biosynthesis	0.133 ± 0.004	0.126 ± 0.023	0.463
Terpenoid backbone biosynthesis	0.399 ± 0.008	0.456 ± 0.072	0.058
Tetracycline biosynthesis	0.143 ± 0.005	0.133 ± 0.023	0.280
Zeatin biosynthesis	0.029 ± 0.001	0.036 ± 0.012	0.139
Glycan biosynthesis and metabolism			
Glycosaminoglycan degradation	0.038 ± 0.004	0.037 ± 0.014	0.827
Glycosphingolipid biosynthesis–ganglio series	0.035 ± 0.001	0.028 ± 0.008	0.069
Glycosphingolipid biosynthesis–globo series	0.082 ± 0.007 *	0.066 ± 0.011	0.028
Lipopolysaccharide biosynthesis	0.392 ± 0.010	0.357 ± 0.068	0.190
Lipopolysaccharide biosynthesis proteins	0.543 ± 0.013	0.487 ± 0.083	0.097
N-Glycan biosynthesis	0.027 ± 0.003	0.029 ± 0.011	0.697
Other glycan degradation	0.103 ± 0.003	0.096 ± 0.012	0.167
Peptidoglycan biosynthesis	0.622 ± 0.012	0.576 ± 0.098	0.229
Biosynthesis of other secondary metabolites			
beta-Lactam resistance	0.045 ± 0.003	0.037 ± 0.011	0.105
Butirosin and neomycin biosynthesis	0.046 ± 0.004	0.043 ± 0.009	0.434
Isoquinoline alkaloid biosynthesis	0.045 ± 0.002	0.053 ± 0.012	0.110
Novobiocin biosynthesis	0.133 ± 0.002	0.129 ± 0.006	0.104
Penicillin and cephalosporin biosynthesis	0.071 ± 0.004	0.062 ± 0.014	0.146
Phenylpropanoid biosynthesis	0.108 ± 0.006	0.109 ± 0.017	0.965
Streptomycin biosynthesis	0.235 ± 0.004	0.223 ± 0.039	0.384
Tropane, piperidine and pyridine alkaloid biosynthesis	0.127 ± 0.001	0.125 ± 0.003	0.063

Table 2. *Cont.*

KEGG Pathways	IA	IP	*p*
Xenobiotics biodegradation and metabolism			
Aminobenzoate degradation	0.323 ± 0.005	0.307 ± 0.063	0.507
Atrazine degradation	0.032 ± 0.009	0.029 ± 0.008	0.528
Benzoate degradation	0.408 ± 0.043	0.384 ± 0.090	0.509
Bisphenol degradation	0.045 ± 0.005	0.056 ± 0.019	0.163
Caprolactam degradation	0.192 ± 0.006	0.192 ± 0.047	0.983
Chloroalkane and chloroalkene degradation	0.284 ± 0.009	0.282 ± 0.020	0.737
Chlorocyclohexane and chlorobenzene degradation	0.094 ± 0.006	0.083 ± 0.020	0.174
Dioxin degradation	0.043 ± 0.011	0.035 ± 0.008	0.104
Drug metabolism–cytochrome P450	0.220 ± 0.013	0.192 ± 0.040	0.099
Ethylbenzene degradation	0.076 ± 0.003	0.069 ± 0.012	0.130
Fluorobenzoate degradation	0.057 ± 0.001 *	0.046 ± 0.001	0.037
Metabolism of xenobiotics by cytochrome P450	0.218 ± 0.013	0.189 ± 0.041	0.089
Naphthalene degradation	0.215 ± 0.007	0.202 ± 0.035	0.337
Nitrotoluene degradation	0.059 ± 0.004	0.064 ± 0.021	0.563
Polycyclic aromatic hydrocarbon degradation	0.025 ± 0.006	0.036 ± 0.017	0.113
Styrene degradation	0.104 ± 0.005 *	0.088 ± 0.012	0.017
Toluene degradation	0.198 ± 0.003	0.172 ± 0.033	0.061
Xylene degradation	0.042 ± 0.011	0.034 ± 0.008	0.083
Membrane transport			
ABC transporters	2.520 ± 0.199	2.654 ± 0.242	0.245
Bacterial secretion system	0.849 ± 0.034	0.821 ± 0.041	0.153
Secretion system	2.423 ± 0.070	2.213 ± 0.293	0.083
Transporters	4.286 ± 0.280	4.660 ± 0.625	0.144
Cell motility			
Bacterial chemotaxis	0.895 ± 0.025	0.855 ± 0.139	0.451
Bacterial motility proteins	2.668 ± 0.082	2.422 ± 0.431	0.156
Flagellar assembly	0.980 ± 0.025	0.893 ± 0.164	0.179
Other			
Bacterial toxins	0.124 ± 0.007	0.115 ± 0.013	0.147
Cell cycle–Caulobacter	0.381 ± 0.008	0.402 ± 0.023	0.088
Two-component system	2.787 ± 0.032	2.569 ± 0.295	0.076
Pertussis	0.062 ± 0.004	0.054 ± 0.008	0.072
Tuberculosis	0.138 ± 0.002	0.151 ± 0.013	0.091
Vibrio cholerae infection	0.031 ± 0.006	0.027 ± 0.007	0.267
Vibrio cholerae pathogenic cycle	0.335 ± 0.020	0.285 ± 0.061	0.060

Values are the mean ± SD (*n* = 8). Asterisks denote significant differences (*p* < 0.05).

4. Discussion

Identifying the bacterial diversity of healthy wild animals in their natural habitat is essential to understanding the impact of fish microbiota in aquaculture [37]. Fish microbiota is strongly influenced by biotic (e.g., host genotype, life-stage cycle, trophic level) and abiotic (water quality, temperature, salinity) factors [20,21].

One of the objectives of our study was to establish the diversity of microbes among different sections of the *C. labrosus* gut. In this sense, *C. labrosus* intestine is arranged in several convolutions and its length is 3.6–3.9 times longer than the total body length [38]. The position of the anterior, central, and posterior intestinal segments is assumed to be one of the variables influencing the gut microbiota composition of the fish [21]. In our study, higher diversity was reported in the posterior intestine than in the anterior intestine of *C. labrosus*. In this sense, Ringø et al. [39] identified the posterior intestine as the most diverse community among the intestinal segments. However, different studies have reported higher, equal, and lower diversity in the posterior intestine than the other intestinal segments in different fish species. For instance, Amillano-Cisneros et al. [40] reported higher bacterial diversity in the anterior versus the posterior intestine of cultured

Mexican pike silverside (*Chirostoma estor*). In contrast, the anterior gut of hatchery-reared rainbow trout (*Oncorhynchus mykiss*) had a significantly lower diversity index than the posterior intestine [41]. For wild Atlantic cod (*Gadus morhua*), a general increase in bacterial diversity from the mid intestine to the posterior intestine was recorded, however, this difference was not statistically different [42]. To resume, the varied diversity reported along the gut could be attributed to numerous factors, including geographical location, developmental stages, and fish species, among many others [20,21].

The overall taxonomic composition of wild *C. labrosus* suggests a certain homogeneity in the anterior section of the intestine and heterogeneity in the posterior section, although no statistical differences were detected at any level among both intestinal sections. Based on taxonomic composition results, the intestinal microbiota of wild *C. labrosus* was dominated by the phylum Proteobacteria, whereas Tenericutes, Spirochaetes, and Cyanobacteria appeared in the posterior section with low relative abundance. Proteobacteria has been described as the most predominant phylum in wild fish [43–45]. A previous study by Le and Wang [23] evaluated the structure and membership of the gut microbial communities in three cryptic species of wild grey mullet (*Mugil cephalus*) of different ages (adult and juvenile) under potential migratory effects. These authors point out that, although distinct bacterial communities were observed in the adult fish of different *M. cephalus* cryptic species, there was still a core microbiome in all fish samples, which was dominated by Proteobacteria. In this respect, despite the fluctuations of bacterial relative abundance, the gut of grey mullets appeared to maintain the same specific bacterial groups in their microbiota. This is consistent with recent observations in other fish families, such as the *Sparidae* family [46].

The presence of cyanobacteria (formerly known as blue-green algae) in the posterior section of the intestine and the existence of photosynthesis-related genes, could be related to the normal feeding of wild grey mullets. In this sense, Crosetti and Blaber [9] reviewed the nutritional preferences of the grey mullet in the wild, which is composed of green and blue-green algae, epiphytic diatoms, and macro-and meiofauna.

In the abovementioned reports of wild fish microbiota [43–45], the most abundant class identified within the Proteobacteria phylum were Gammaproteobacteria, which is similar to the results of the current study. However, although Gammaproteobacteria was the predominant class in both segments, the posterior intestine contained differentially represented classes, such as Mollicutes (mainly family Mycoplasmataceae) or Brevinematae (mainly family Brevinemataceae). In this sense, Mycoplasmataceae species have been reported as consistently present in the gut microbiota of both wild and farmed species [47–49]. Li et al. [50] suggested that *Mycoplasma* species could utilize cytoplasmatic secretions from their wild salmon host to produce lactic and acetic acids, which could be subsequently used by other bacteria. Members of this family are also known to be fish pathogenic bacteria [51,52].

Brevinemataceae have been identified in the gut of several farmed fish species [53,54], including rainbow trout with genetic susceptibility to some pathogens (e.g., *Flavobacterium psychrophylum*) [55]. However, some members of the genus *Brevinema* found in Atlantic salmon have been reported to produce butyrate [56], which may support intestinal barrier function and mucosal immunity [57].

The family Pseudomonadaceae was present in both sections of the *C. labrosus* gut. Pseudomonas is a diverse bacterial group showing a wide variety of metabolic abilities, broad ecological distribution, and adaptability to a range of environmental niches [58]. In this sense, Pseudomonas strains have been used as probiotics in aquaculture, improving the response to pathogens in different hosts [59,60].

Vibrionaceae are an extended family of the phylum Proteobacteria, and it is composed of a great number of bacterial species that are very important in the aquatic environment. Regarding the *Vibrio* genus, some species have been reported as abundant taxons in the intestinal microbiota of marine fish species [18,27,61]. Many of *Vibrio* spp. such as *V. anguillarum*, *V. harveyi* or *V. parahaemolyticus* had been described as opportunistic pathogenic,

causing ulcerative diseases in fish [62]. However, some *Vibrio* species have also been described as probiotics [63]. In this sense, the presence of *Vibrio* species in the fish gut has been related to the production of hydrolytic enzymes, assisting in the breakdown of dietary components [18]. *Vibrio* strains have been found to produce amylase and lipase [64], cellulose [65], and chitinase [66] among others. Functionality analyses showed the presence of genes related to the *Vibrio choleare* pathogenic cycle. These genes have been associated with the virulence of the *Vibrio* genus [67,68]; however Deng et al. [69] observed a decrease in *V. choleare* pathogenic cycle in crabs affected by the Baimang disease comparing microbiota in healthy crabs.

Prediction of differential microbiome metabolic potential using PICRUSt suggested that the majority of microbial functions were associated with carbohydrate, amino acid, and energy metabolism, membrane transport, and cell replication and repair, which is consistent with the important roles of nutrient digestion, metabolism, and immune functions in the fish gut [31,70–72]. In our study, a high level of the predicted functional pathways which were part of amino acid metabolism could be linked to the feeding habits of mullets, which are based largely on benthic organisms and zooplanktonic organisms [9]. The higher level of gene pathways responsible for carbohydrate metabolism may also play an important role in bacterial nutrition. It is known that the activity of proteases is higher in piscivorous fish compared with herbivorous fish, while the activity of enzymes metabolizing carbohydrates contrariwise is lower in piscivorous and higher in herbivorous species of fish [73]. In this regard, Pujante et al. [74], found a noticeable increase in the protease activity levels in *C. labrosus* as a consequence of the changes in fish-feeding habits. These modifications might reflect a possible compensation mechanism (i.e., by increasing enzyme secretion) that ensures efficient digestive processes, as has been described in other herbivorous fish species such as carp or tilapia [75,76]. *Pseudomonas* sp. and *Mycoplasma* sp., which are present in the *C. labrosus* gut, are protease- and amylase-producing bacteria [77,78] that can participate in amino acid and carbohydrate metabolism. Besides, the high energy metabolism pathway suggested that the intestinal microbiota could supply energy to the host to digest and metabolize food [43,78]. Membrane transport and cell replication and repair are essential biological processes and are widely spread in bacteria [79,80]; thus, it is reasonable that microbial genes involved in these functions were found in high abundance in *C. labrosus* gut.

KEGG 3 level showed statistically higher levels of the following predictive functions in the anterior section of the gut: (i) inositol phosphate, related to intracellular metabolism regulatory pathways and signal transduction [81]; (ii) phenylalanine metabolism, related to normal growth and metabolic processes [82]; (iii) biosynthesis of glycosphingolipid, related to the modulation of signal transduction pathways as well as in cell adhesion/recognition processes and cell proliferation/differentiation [83,84]; (iv) biosynthesis of siderophore, related to pathogenic control [85]. Contrarily, the biosynthesis of pantothenate and CoA, related to intermediary metabolism [86], was significantly higher in the posterior section. In this sense, previous studies have documented that predictive functions of microbial communities differed along the gut of different fish species [87,88].

The gut microbiome has an important role not only in dietary nutrient digestion but also in xenobiotics metabolism and degradation [19,89]. The influence of xenobiotics on fish microbiota composition has already been documented. Exposure to dioxin-type polychlorinated biphenyls (PCB) results in disrupted gut microbiota and host metabolism as well as intestinal inflammation [90,91]. In fathead minnow (*Pimephales promelas*), the exposure to benzo[a]pyrene resulted in shifts in microbial composition and enrichment in hydrocarbon-degrading taxa [92]. In this sense, *C. labrosus* showed statistically higher predictive functions in the anterior section of the gut related to xenobiotic degradation, such as styrene and fluorobenzoate. This enrichment in those degradation pathways of aromatic compounds suggests environmental pollution. As described above, fish specimens were caught in the La Caleta harbor, so the origin of these aromatic compounds could be attributed to the incomplete combustion and/or leak of fuels (i.e., gasoline, diesel) [93].

However, the small size of the harbor, and the constant turnover of fish within it, may diminish the effect of contamination on the fish. Thus, in order to determine the influence of the environment on the microbiota of *C. labrosus*, further research is needed, obtaining samples of captured fish from less anthropized areas.

5. Conclusions

In conclusion, *C. labrosus* do not seem to show a clear differentiation of their microbiota depending on the area of the intestine where it is located. On the other hand, this first study shows a diverse microbial community with the capacity to digest organic matter (providing nutrients to the fish), control pathogens (by producing antimicrobial substances) and degrade toxic substances, revealing that the function of the microbiome could play an important role in the nutrition and health of these fish. However, further research is needed to determine the variability of the microbiota of these fish depending on the environment they inhabit and the type of food they feed on.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7040209/s1, Figure S1: Rarefaction curves for each fish and section; Table S1: Functional KEGG pathways for each fish and section.

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