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Research Article

Microbial Communities Associated with the Intestinal Tract of Grey Mullets from a Mediterranean Aquatic Environment

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Abstract

Introduction: Grey mullets comprise different species that represent the most ubiquitous teleost families in the planet's coastal waters. They are an important proportion of the Mediterranean lagoon's production and have been recently considered cultivated marine fish. This study aimed to explore the intestinal microbial communities of grey mullets to understand their possible ecological role for fish and the aquatic environment.

Methods: Thirty-four wild-caught mullets were sampled from a Mediterranean lagoon during four seasons and the V3-V4 hypervariable regions of 16S rRNA (Illumina MiSeq) of the fish gut were sequenced. Parameters of the aquatic environment were detected: temperature, salinity, DO, PO_4, NH_4, NO_3, NO_2, SiO, DIN, and Chla.

Results: The results indicated a high bacterial diversity (mean Shannon index: 4.74 ± 1.12; Simpson index: 0.93 ± 0.08) and variations among seasons. Sixty prokaryotic phyla were identified and the most abundant ones were: Proteobacteria (mean relative abundance 35.4% ± 17.9), Actinobacteriota (mean relative abundance 16.4% ± 9.9), and Firmicutes (mean relative abundance 10.1% ± 10.9). Bacteria belonging to the *phylum* Chloroflexi were relevant in autumn, Spirochaetota, Verrucomicrobiota, Fusobacteriota, and Cyanobacteria were particularly abundant in winter while Bacteroidota characterized summer fish. A total of 332 prokaryotic families were identified with 26 most abundant ones; Rhodociclaceae (Proteobacteria) were dominant in autumn, Brevinemataceae (Spirocheaetota) and Fusobacteriaceae (Fusobacteriota) were especially present in winter and the Staphylococcaceae (Firmicutes) prevailed in spring.

Conclusion: This study sheds light on the variation in the complex gut microbial community structure of Mediterranean grey mullets and their potential ecological role in protecting fish and preserving the aquatic environment.

Abbreviations

DO: Dissolved Oxygen; PO_4: Reactive Phosphorus; NH_4: Ammonium; NO_3: Nitrate; NO_2: Nitrite; SiO_4: Re-active Silica; DIN: Total Dissolved Inorganic Nitrogen; Chla: Chlorophyll a

Introduction

The interest in the intestinal microbiome of aquatic animals is getting more and more pivotal because its knowledge can contribute to the development of effective strategies for fish rearing in captivity, by manipulating gastrointestinal (GI) microbial communities to promote health and productivity through novel therapeutics and feed additives [1,2]. Moreover, flexibility in the gut microbiome may play a role in biological diversity conservation, enabling fish to colonize new and different aquatic environments [3]. The fish GI tract is a complex ecosystem composed of a dynamic consortium of microorganisms that play critical roles in

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nutrition, energy sources, and host health to reduce or inhibit pathogenic microbes and for the safety of the environment [4]. Fish gut microflora can be divided into two groups: the resident (autochthonous) and the transient (allochthonous) communities [5]. The resident can adhere to and colonize the mucosal surfaces, or occur within the epithelial tissues, while the transient communities are characterized by non-adherent free-living microorganisms although they inhabit microniches, especially during periods of stress [6]. The intestinal microbiota of aquatic animals has higher fluidity than terrestrial animals and changes in various factors such as temperature, salinity, and trophic level [7-10]. The studies on Mediterranean fish species indicated that it is influenced by species, sex, age [11], and physiology other than epigenetic factors (season, feeding regimen, water temperature, and salinity) [12,13]. However, the existence of a core gut microbiota within and between different species independent of diet and geographic location does exist [14]. Thus, the monitoring of bacteria present in healthy wild fish in their natural environment is the first step for use in captivity [15]. Research on intestinal bacteria of a wide range of fish species has mostly reported on the isolation, identification, and evaluation of cultivable bacteria; however, only a small part (< 2%) of the GI microbiota may be cultured and these types of procedures fail to provide information of the microbial community as a whole. Metabarcoding sequencing by various Next-Generation Sequencing (NGS) platforms has emerged as a method to discover new groups of micro-organisms with greater accuracy in environmental systems in spatial and temporal scales [5,10,16]. Illumina MiSeq (Illumina, USA) has been widely used for 16S rRNA gene sequencing of gastrointestinal tract microbiota of freshwater [17] and marine fish [16,18].

Fish belonging to the Mugilidae family, commonly known as the grey mullets group, comprise a great number of species and they are one of the most ubiquitous teleost families in the planet's coastal waters [19]. They have omnivorous and herbivorous feeding habits [20] and have been recently considered a cultivated marine fish that can be fed with "alternative" energy such as insects and cost-effective materials, plants, production dis-cards, etc., contributing to the realization of the goal of sustainability in aquaculture [21]. In this sense, grey mullets represent an interesting resource for aquaculture use although large-scale production faces numerous challenges represented by the ecological carrying capacity of existing sites and environmental impact [22]. In any case, the valorization of fish species less considered for the market is a way to preserve the more valued ones and the biodiversity of the aquatic environment [23].

Among Mediterranean Mugilidae, *Mugil cephalus* (Linnaeus, 1758), *Chelon ramada* (Risso, 1827), *Chelon labrosus* (Risso, 1827), *Chelon saliens* (Risso, 1810) and *Chelon auratus* (Risso, 1810) are some of the most representative fish species in Sardinian lagoons [22,24]. Above all, flathead grey mullet, *M. cephalus*, represents about 50% of world mullet production [19], possesses high economic value, and is appreciated in the food market for its eggs processed to obtain seafood which is known by different names such as Avgo-taracho (Grece), Karasumi (Japan) or Bottarga (Italy), depending on the geographical production area [25,26]. In Italy, grey mullets represent an important proportion of the production of coastal lagoons [27] and their culture is carried out in extensive systems, based on natural cycles and dynamics [28]. Production is based on wild fry availability and it cannot compete with intensive cage culture at sea, but it aims to combine environmental compatibility with economic sustainability. This is an advanced form of coastal lagoon management and represents one of the most interesting examples of coastal lagoon management in the world [29]. According to official sources in 2015, the Italian organic mullet species (*M. cephalus*, *C. aurata*, *C. saliens*, and *C. labrosus*) production is estimated at 80 tonnes (source EUMOFA).

Earlier studies reported the composition and the predicted functions of the associated gut microbiota on *M. cephalus* of different ages from Northwest Pacific marine environments [30], from Chinese coastal marine areas [31], and on Mediterranean wild thick–lipped grey mullets (*C. labrosus*) [23]. In this work, a metabarcoding study on 34 wild mullets from a Mediterranean transitional aquatic environment has been carried out to analyze, as a first step, the structure of their intestinal bacterial communities during different seasons. These results shed light on the variation in the complex gut microbial community structure of Mediterranean grey mullets and its potential biotechnological role for fish and the aquatic environment.

Materials and methods

Study area and fish samplings

Thirty-four wild-caught mullets, destined for the local food market, were captured by professional fisheries on September 27th, 2018 (autumn), February 10th, 2019 (winter), July 10th, 2019 (summer), and May 18th, 2021 (spring) from Santa Giusta Lagoon (Central west coast of Sardinia (Italy) (coordinates: Lat 39°52'N, Long 8°35'E). The fish were transported inside a refrigerated bag to the Agris Bonassai laboratory within 3-4 hours. Species were determined on a morphological basis, according to identification keys [32,33].

The aquatic area (8.6 km² and 1.0 m mean depth) is a research site of the "Marine Ecosystems of Sardinia" of the Italian Long-Term Ecological Research network (www.lteritalia.it; https://deims.org/6f7581f0-e663-4681-bf9d-4668d6c3f2ba), recognized as a Site of Community Importance for European Union (SCI ITB030037) and is designated by the Sardinian Government as a protected area for animals (INFS code: OR0211) (Figure 1).

Fish measurements and preparation of gut samples

Body and gut weight and total length were measured. The intestine (mean weight 14 ± 6 g) was aseptically removed, diluted (10%, w/v) in saline solution (0.90% NaCl), and homogenized in plastic bags by Stomacher® 400 (FermionX Ltd, Worthing, UK) at room temperature. The whole intestines of fish were collected. The samples for microbiological analyses (homogenates) were made up by mixing the guts of two-three individuals of the same species and immediately stored at

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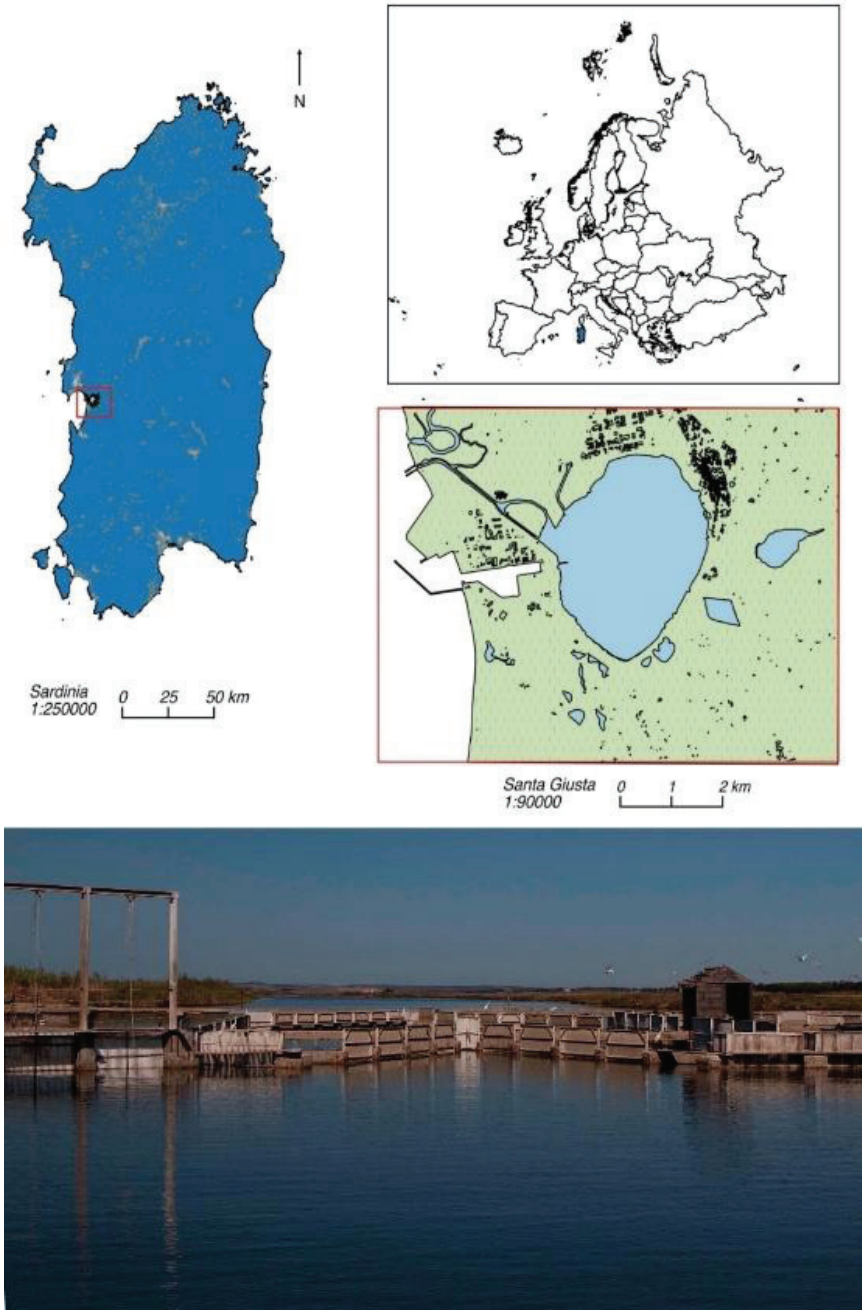

Figure 1: Santa Giusta lagoon: study area.

-80 °C until DNA extraction. The pooling of the samples was performed to minimize the individual variations in the fish [34,35]. It was not possible to find mullets of the same species in the local food market during the considered seasons. This was probably due to the migratory behavior of all the wild species that enter and go out from the lagoon and the management of the lagoon (the aquatic area is submitted to different closures to the sea by the fishermen for production purposes) and other uncontrolled factors (the great environmental instability of the lagoon).

Environmental characterization

To explore the seasonal dynamics in the lagoon during the study period, a total of 49 water samples were collected. Water samplings were carried out monthly in autumn 2018, winter and summer 2019, and in May 2021. Temperature, salinity, and Dissolved Oxygen (DO) were measured in situ using a YSI 6600 v2 (YSI Inc., Yellow Springs, USA) multi-parameter probe.

Samples for nutrient analyses were collected at about 30 cm depth. Nutrients were analyzed within a few hours after sampling. Concentrations of inorganic nutrients such as reactive Phosphorus (PO_4), Ammonium (NH_4), Nitrate (NO_3), Nitrite (NO_2), and reactive Silica (SiO_4) were determined on the filtered samples according to Strickland and Parsons, 1972 [36]. Total Dissolved Inorganic Nitrogen (DIN) was calculated as the sum of NH_4, NO_3, and NO_2. Chlorophyll a (Chla) was determined following the SCOR-UNESCO protocol 1997.

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The Dipartimento Specialistico Regionale Idrometeoclimatico (SAR-ARPAS: http://www.sar.sardegna.it/) provided daily data on rainfall (Rain).

Rain data were obtained by summing daily rainfall values to get seasonal accumulations.

DNA extraction, amplification, and sequencing

DNA was extracted from ca. 0.750 g of homogenate (n = 12) obtained by mixing the guts of three individuals of the same species.

All extractions were performed according to the PowerSoil DNA Isolation Kit (Qi-agen®, Hilden, Germany) following the manufacturer's instructions. DNA quality and quantity were assessed by spectrophotometry (NanoDrop®, Wilmington, DE, USA) and fluorometrically (Qubit® Life Technologies, Paisley, UK) to ensure optimal measurement of DNA quantity and purity. The V3-V4 region of the 16S rRNA gene was amplified using universal bacterial primer pair 341F (5'-CCTACGGGNBGCASCAG-3') and 785R (5'-GACTACNVGGGTATCTAATCC-3'). The PCR mixtures, in a final volume of 25 µl, were as follows: 10 ng of template DNA, 0.5 U of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, USA), 1X Phusion HF buffer, 0.5 µM of each primer and 200 µM of each dNTP. PCRs (98 °C for 4 min; 35 cycles of 98 °C for 20 s, 57 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min) were set up in triplicate to smooth possible in-tra-sample variance. PCR products were visualized on 1.5% agarose gels, then amplicon triplicates were pooled and purified using 0.8X volumes of AMPure XP beads (Beckman Coulter, Brea, CA, USA).

The pooled PCR products were indexed and subsequently normalized according to the "16S Metagenomic Sequencing Library Preparation" protocol, with minor adjustments: 1) 0.5 U of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, USA) was used for each reaction and 2) PCR amplicons were purified using 0.7X volumes of AMPure XP beads (Beckman Coulter, Brea, CA, USA). Finally, amplicon libraries were equally pooled and sequenced using the Illumina MiSeq system, in the 2 x 300 bp format (Illumina, San Diego, CA, USA). The 16S amplicon sequences generated for this study can be found in the Sequence Reads Archive (SRA) at NCBI under the accession number PRJNA893889.

Bioinformatic analyses

Raw reads were initially processed with Cutadapt, v. 2.1 to remove primer sequences and reads shorter than 100 bp [37]. All further analyses were conducted in R, v. 4.1 (R CoreTeam, 2019). At first, using DADA2, v. 1.20 [38], forward reads were trimmed at 270 bases and reverse reads at 170 bases, also truncating reads where bases with quality 2 were found and allowing 0 Ns and maximum expected errors equal to 2. At the end of this process, reads shorter than 20bp were discarded. The resulting reads were then denoised and merged to obtain the Amplicon Sequence Variants (ASVs) in the samples. Taxonomy annotation was performed in DADA2 using the SILVA database v. 138 clustered at 99% identity [39]. Subsequent analyses were performed using the phyloseq R package [40] for data handling and further filtering. All ASVs assigned to Eukarya, mitochondria, and chloroplast, or not assigned at the phylum level, were removed; only ASVs with total counts above 10 reads across all the samples were retained for further analyses. Rarefaction curves were produced with a vegan R package. Alpha diversity indexes were computed with phyloseq dedicated functions; beta diversities principal coordinates analysis plots were computed on Bray-Curtis distances matrixes. To describe the different microbial communities, their differences, and similarities, relative abundances of ASVs were computed in all samples. A Venn diagram was drawn to reveal the unique and shared families in the different samples. ASVs were grouped at family levels and analyzed with the Vienna package.

Results

Fish biometry

The morphological traits of the wild mullets are represented by the equation shown in Figure 2.

The graphic shows the relationship between total weight and length of the mullets and the determination coefficient (R^2) indicates a good correlation (r = 0.916) between these biometric variables.

Aquatic environmental characterization

Physical and chemical parameters of the aquatic environment during the period of study are reported in Table 1. Seasonal rain accumulates showed the highest mean values (396.2 mm) in autumn 2018 and the lowest ones (40 mm) in summer 2019. Mean temperature data showed seasonal variations with the highest values recorded in summer 2019 (26.83 ± 2.03 °C) and the lowest ones in winter 2019 (12.59 ± 2.19 °C). Mean salinity data indicated an increase along the seasons from autumn 2018 (20.84 psu ± 7.54) to summer 2019 (35.10 psu ± 2.99). The lowest DO (5.78 mgL-1 ± 0.67) mean values were registered in summer 2019 while the highest (10.03 mgL-1 ± 1.07) ones were in winter. Regarding nutrients, the highest mean values were detected in autumn for all those considered (Table 1). The lowest mean chlorophyll content (0.55 mg m-3 ± 0.26) was detected in spring while and the highest mean value was in autumn (22.42 mg m-3 ± 4.84).

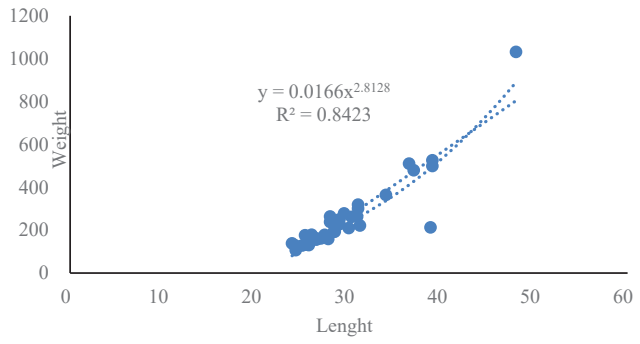

Figure 2: Fish length (cm) and weight (g) of the mullets analyzed in the study.

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16S rDNA sequencing

Diversity analysis: 16S barcode sequencing yielded a total of 4,995,563 row sequences. After the initial steps of filtering, denoising, merging, and chimera removal, a total of 2,618,159 sequences were obtained. From these, 12,045 Amplicon Sequence Variants (ASVs) were identified considering only those that were not assigned to eukaryotic phyla. Considering all samples, ASVs with fewer than 10 sequences were discarded, resulting in a final number of 7817 ASVs. The rarefaction curves showed that the sequencing effort was sufficient to assess the biodiversity of the samples (Supplementary Material. Figure S1). Table 2 shows the number of total counts relative to the ASVs remaining after the different filtration steps, the number of ASVs identified in the microbiota of each fish intestinal sample in each season, and the microbial α-diversity expressed by Shannon and Simpson indexes. No significant difference was found among the α-diversity indexes of samples.

Composition of intestinal microbiota of grey mullets

Bacterial phyla: The 7817 ASVs identified in the dataset were assigned to 60 different phyla (Supplementary Table 1). Figure 3 shows the relative abundances of the phyla identified in the mullets during the various seasons. Phyla with a median abundance of more than 1% or a variance greater than 85% of the entire dataset were considered in the analyses. In this regard, Acidobacteriota, Actinobacteriota, Bacteriodota, Chloroflexi, Cyanobacteria, Desulfobacterota, Firmicutes, Planctomycetota, Proteobacteria and Verrucomicrobiota had a median abundance of more than 1%, while Spirochaetota and Fusobacteriota showed an overall low abundance but high variability among the samples, as they both accounted for more than 40% of the prokaryotic community in MUG-SG06 sample during winter. All the remaining 48 phyla were included in the "Other" group (Figure 3 and Supplementary Table 2).

The gut microbiota of grey mullets was dominated by Proteobacteria (35.4% ± 17.9), followed by Actinobacteriota (16.4% ± 9.9) and Firmicutes (10.1% ± 10.9) which together accounted for 61.9% of the total population identified, and constituted the "core" microbial group. In this "core" microbiome, the Actinobacteriota resulted significantly higher in fish captured in May (spring) (84.68%) with respect to February (winter) (43.82%), July (summer) (37.21%) and September (autumn) (29.8%) ($p < 0.01$) and the Firmicutes were at the highest number in spring, but they were significantly higher in summer with respect to winter and autumn and also in autumn with respect to winter ($p < 0.05$) (Figure 4). Generally, the highest number of bacterial phyla was observed in summer; however, the few phyla that appeared specific to a given season, always showed low abundances, so they should not have a strong effect on the structure and dynamics of the microbial communities in the fish gut (Supplementary Table 1).

To explore the prokaryotic diversity of a single fish intestinal sample, the three phyla with the highest abundance were examined in each of them (Supplementary Table 2). These identified phyla were represented by

Table 1: Mean values ± sd of the environmental parameters in Santa Giusta lagoon during sampling seasons.

	Autumn	Winter	Summer	Spring
Total rains (mm)	396.2a	106.2a	40.0b	69.4b
Temperature (°C)	14.53±4.22a	12.59±2.19a	26.83±2.03b	19.65±1.38c
Salinity (psu)	20.84±7.54a	22.83±3.85a	35.10±2.99b	27.33±2.38ab
DO (mgL^{-1})	9.57±1.85a	10.03±1.07a	5.78±0.67b	7.01±0.73c
DIN (μM)	4.91±0.75a	2.60±1.32b	3.48±1.71ab	3.42±1.57ab
PO_4 (μM)	1.96±1.02a	1.20±0.49b	0.45±0.26c	0.77±0.13bc
SiO_4 (μM)	296.73±38.53a	63.61±71.38b	96.01±52.21b	32.01±8.06b
Chla ($mg\ m^{-3}$)	22.42±4.84a	3.70±2.82b	3.43±2.11b	0.55±0.26c

DO: Dissolved Oxygen; DIN: Dissolved Inorganic Nitrogen; Chla: Chlorophyll a.

Table 2: Number of amplicon sequence variants (ASVs) and microbial α-diversity indexes of mullets' intestinal microbiome.

Sample code	Season	Host species	Total counts	Observed ASVs	Shannon	Simpson
		(Fish number)				
MUG-SG01	Autumn	*C. ramada* (3)	26,486	457	5.02	0.98
MUG-SG02	Autumn	C. *ramada* (3)	28,282	487	5.13	0.98
MUG-SG03	Autumn	*M. cephalus* (3)	292,010	705	3.36	0.76
MUG-SG04	Winter	*C. saliens* (2)	96,743	1,211	5.21	0.96
MUG-SG05	Winter	*C. labrosus* (3)	78,504	984	5.45	0.98
MUG-SG06	Winter	*C. labrosus* (3)	342,004	150	1.95	0.76
MUG-SG07	Summer	*M. cephalus* (3)	345,355	1,081	5.99	0.99
MUG-SG08	Summer	*C. ramada* (3)	291,064	1,343	5.93	0.99
MUG-SG09	Summer	*C. labrosus* (2)	127,093	747	5	0.96
MUG-SG10	Spring	*C. auratus* (3)	110,687	514	4.75	0.96
MUG-SG11	Spring	*C. ramada* (3)	311,797	717	4.68	0.95
MUG-SG12	Spring	*C. ramada* (3)	242,564	1,810	4.45	0.9
	Mean ±sd		191,049 ± 124,210	850 ± 458	4.74 ± 1.12	0.93 ± 0.08

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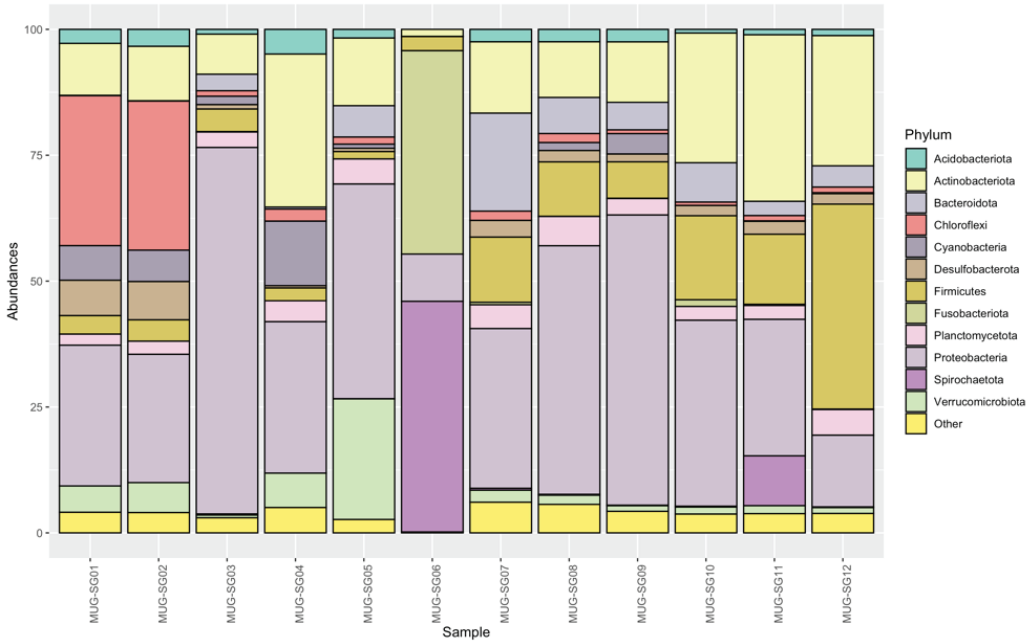

Figure 3: Abundances of the identified phyla. Phyla with a median abundance lower than 1% across the samples are grouped in the "Other" category shown at the bottom of each stacked bar. Abundances in the axis are in percentage.

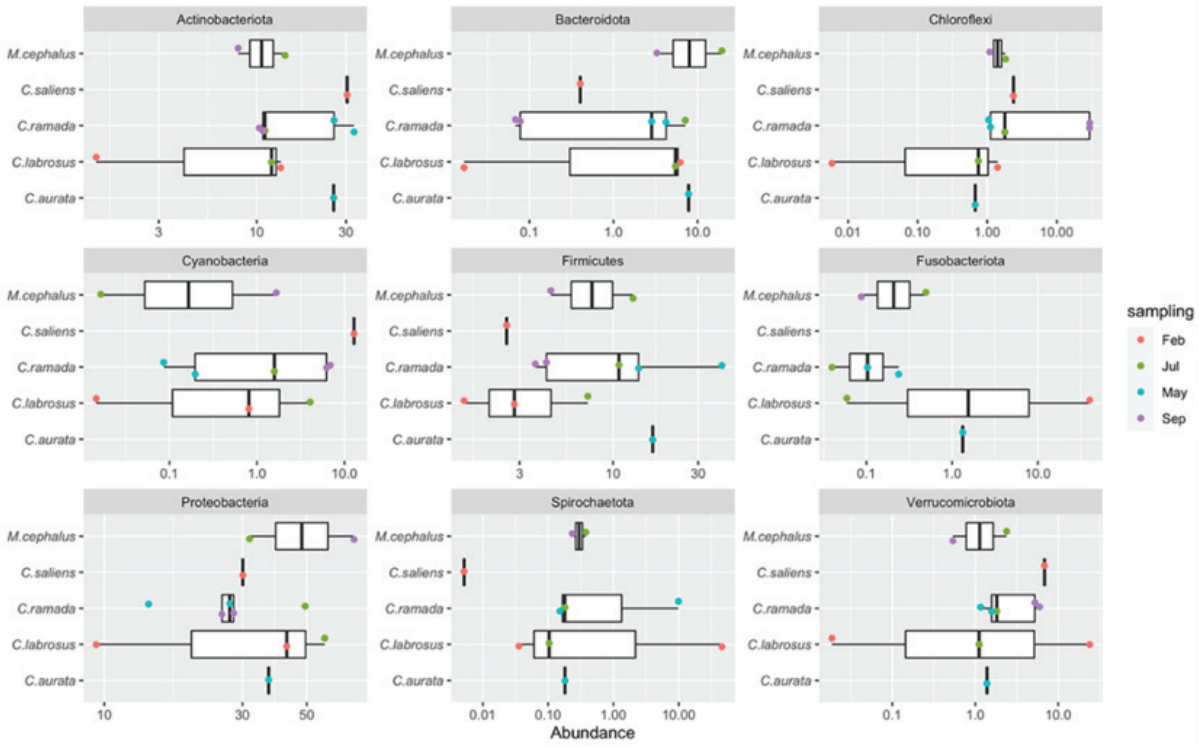

Figure 4: Relative abundances of the most abundant phyla identified in each intestinal microbial community of the mullets during the various seasons. The relative abundances of these nine top phyla are shown across all samples except when zero. Abundances in the X-axis are on a logarithmic scale.

Actinobacteriota, Bacteriodota, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteriota, Proteobacteria, Spirochaetota and Verrucomicrobiota. On average, the top three phyla identified in each sample comprised more than 75% of the total abundance and this means that these phyla accounted for most of the microbial community in the gut of each sample. The only exceptions were MUG-SG01, MUG-SG02, and MUG-SG07 in which, however, the most abundant three phyla represented more than 65% of the total abundances.

Considering the gut microbiome during the different periods of study, the ASVs belonging to the Chloroflexi phylum were

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particularly relevant for fish captured in September (autumn) (MUG-SG01 and MUG-SG02), while much lower abundances of this phylum were found in all the other samples captured in the other seasons (Supplementary Table 1 and Figure 4). Remarkably, the *C. labrosus* samples caught in February (winter) (MUG-SG05 and MUG-SG06) showed marked differences with respect to each other. In particular, fewer ASVs were identified in the MUG-SG06 sample, with a large abundance of Spirochaetota (45.8%) and Fusobacteriota (40.4%) as already highlighted, which were at very low abundance or even absent in all the other samples (Supplementary Table 1).

Bacterial families

Looking at deeper taxonomy levels, a total of 332 families were identified. Not all the identified ASVs were assigned to known families and were therefore removed from the analysis. In this regard, 70.1% of the ASVs, comprising 84% of the reads, remained. However, most of the known families had total abundances in all samples below 1%, meaning that they had a low impact on the overall microbial communities. On the other hand, 88 families overpass this threshold (Supplementary Table 3). To have a broad picture of the identified families and their impact on the microbial communities of the mullets' gut, their abundances were further investigated. Figure 5 shows the relative abundances of all the identified families, highlighting those having median abundance in the samples higher than 1% or variance higher than 75% of the entire dataset. All remaining families have been enclosed in the "Other" group, which reaches a large fraction of the overall abundance in some samples. However, some families were found to have a large impact in terms of abundance on the communities, as discussed below.

Sixty microbial families were shared among all fish and represent different amounts of the overall microbial communities ranging from 37.8% to 81.8% with a median value of 54.6% (Supplementary Table 3). Eleven known families were found to be ubiquitous in all fish and seasons. These families were Staphylococcaceae, Comamonadaceae, Beijerinckiaceae, Propionibacteriaceae, Rhodobacteraceae, Rubritaleaceae, Ilumatobacteraceae, DEV007, Halieaceae, Pirellulaceae, and Desulfocapsaceae (Supplementary Table 3). These families had average abundances of less than 5% in all gut microbial communities analyzed. Nevertheless, some trends could be highlighted in certain seasons, such as Staphylococcaceae in May (spring), which reached 35.8% abundance; Rhodobacteraceae and Rubritaleaceae in February (winter), exceeding 20% abundance; Comamonadaceae in July (summer) had an abundance higher than 14%; and Beijerinckiaceae in September (autumn), which had around 10% of the counts (Supplementary Table 3 and Figure 5). In most other cases abundances were lower than 1.9%. A total of twenty-six most abundant bacterial families were found. Looking at the three most abundant families within each intestinal microbial community of the different mullet samples, on average, these three families represented 38.4% of the total abundance, even if they showed very high variability (st. dev: 21.9) (Figure 6). This is because, in half of the samples, the three most abundant families do not reach 30% of the total abundance while in some others they largely exceed 50% (Supplementary Table 4).

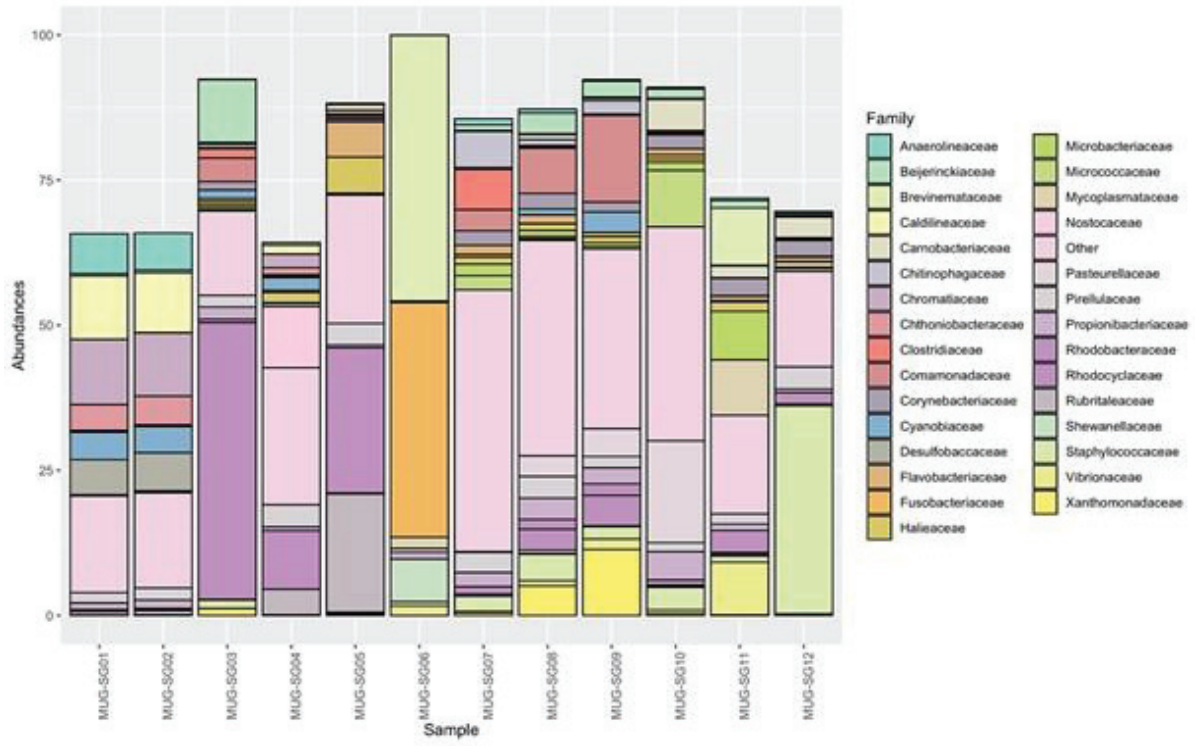

Figure 5: Known families identified in the samples. Families with median abundance below 1% across all samples or showing a variance lower than 75% of the entire dataset are grouped in "Other".

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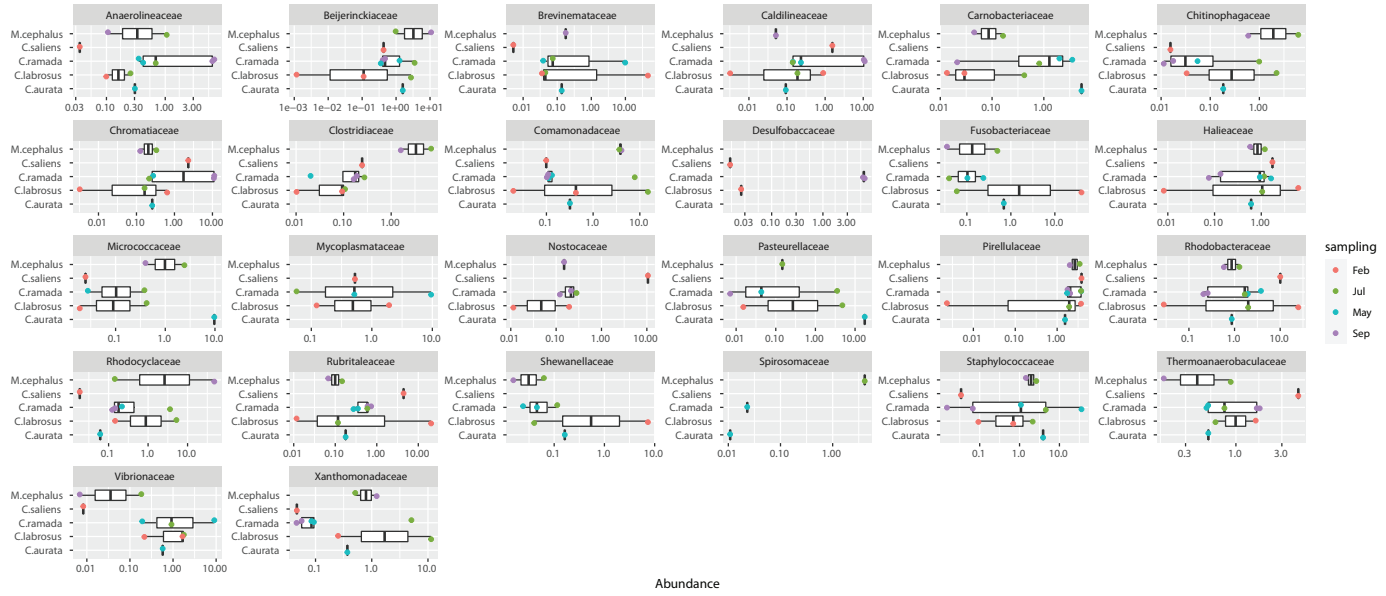

Figure 6: The most abundant families and their relative abundances across the different gray mullets. Zero values are not shown. Abundances in the X-axis are on a logarithmic scale.

The seasonal distribution of intestinal bacterial families shows that Rhodociclaceae (Proteobacteria) (47.7%) was the most abundant group in September (autumn), Brevinemataceae (Spirocheaetota) (45.8%), and Fusobacteriaceae (Fusobacteriota) (40.4%) were dominant in February (winter), and Staphylococcaceae (Firmicutes) (35.8%) prevails in May (spring). On the other hand, the relative abundance of the first three families in the fish caught in July (summer) did not appear particularly high, as only Comamonadaceae and Xanthomonadaceae (both Proteobacteria, respectively 14.92% and 11.39%) exceeded 10% abundance in MUG-SG09 (Supplementary Table 4). The other less abundant families which characterized each season were: Chromatiaceae (Proteobacteria), Caldilineaceae (Chloroflexi), Beijerinckiaceae (Proteobacteria), Anaerolineaceae (Chloroflexi) and Desulfobaccaceae (Desulfobacterota) in September (autumn); Rubritaleaceae (Verrucomicrobiota), Rhodobacteraceae (Proteobacteria), Nostocaceae (Cyanobacteria), Shewanellaceae (Proteobacteria), Halieaceae (Proteobacteria), Thermoanaerobaculaceae (Acidobacteriota) in February (winter); Xanthomonadaceae (Proteobacteria), Clostridiaceae (Firmicutes), Chitinophagaceae (Bacteroidota), Spirosomaceae (Bacteroidota) identified in July (summer), and Pasteurellaceae (Proteobacteria), Brevinemataceae (Spirochaetota), Micrococcaceae (Actinobacteriota), Mycoplasmataceae (Firmicutes), Vibrionaceae (Proteobacteria), Carnobacteriaceae (Firmicutes), Pirellulaceae (Planctomycetota) observed in May (spring) (Supplementary Table 3).

Discussion

In this study next-generation sequencing technologies (NGS) and bioinformatics analysis allowed us to gain a greater knowledge of the microbial communities (both the resident and the transient) associated with the gut of Mediterranean wild grey mullets in response to a variety of environmental aquatic factors. The study revealed the structure of a complex ecosystem is, highly influenced by the aquatic environment during different seasons. The fish specimens analyzed represent the typical edible wild fauna from Sardinian coastal lagoons and other Mediterranean transitional aquatic environments [27]. Research attention has been focused on the aquaculture of *M. cephalus*, which represents a traditionally harvested and consumed fish in various European countries such as Italy, Spain, and France, especially appreciated in Tunisia, Egypt, and Taiwan, and a suitable species for feeding populations in developing countries [20,26]. Moreover, the culture of grey mullets Mugilidae species is considered a priority within the current strategies of sustainable European aquaculture [21]. To the authors' knowledge, various studies were made on the gut microbiota of cultured [41] and wild mullets [23,31] and the present study has provided more information on the biodiversity of mullets intestinal microflora and their biotechnological potential. Previous studies on Mediterranean grey mullets have shown some interesting biotechnological traits of intestinal cultivable bacteria as a source of bioactive compounds with immunological and bioremediation functions [42].

The outlined microbiome confirms that the acquisition and maintenance of the gut microbiota is a very complex process, which is dictated by both environmental factors and host physiological pressures [43,44]. Indeed, each captured grey mullet of this study is characterized by a specific breeding period that determines the migratory behavior that depends on spawning, endocrine mechanisms, photoperiod, temperature, and feeding activity [45]. This work has revealed the presence in the mullets of dominant main phyla: Gram-negative (Proteobacteria) and Gram-positive (Actinobacteriota and Firmicutes) which accounted for 61.9% of the total prokaryotic population identified across all the intestinal samples and

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constitute the "core" microbial community in the mullets' gut. This suggests the potential role of these core taxonomic groups for vital functions in the nutrition and/or the immunity of the fish. From an ecological point of view, it was interesting to observe a seasonal influence on the gut microbial composition with a dominance, in spring, of Actinobacteria and Firmicutes which were also detected in the intestinal microflora of other marine and freshwater fish [14,46-48].

The present study detected the occurrence of the *phylum* Chloroflexi, which was particularly abundant in the *C. ramada* individuals caught in autumn (both samples were above 29.5%). Other papers reported the presence of Chloroflexi in the microbial communities of *M. cephalus* gut [30] and in a wide range of aerobic and anaerobic habitats including sediments, hot springs, and methanogenic reactors, where these bacteria are supposed to have a role in sludge stabilization and breakdown [49]. These authors reported that bacteria of Chloroflexi *phylum*, commonly isolated from sludge matrices, have a role in bioremediation processes, being able to degrade complex polymeric organic compounds to low molecular weight substrates (sludge granulation). Moreover, Liang, et al. [50] described Chloroflexi as a component of bacterial communities from petroleum reservoirs and its involvement in toluene degradation. In particular, members of the Chloroflexi *phylum* belonging to the Anaerolineaceae family found at an abundance of more than 6% in the mullets collected in autumn, are described in the literature as methanogenic bacteria (hydrocarbon degrading), frequently encountered in the presence of petroleum. The presence of bacteria able to degrade toxic substances as aromatic compounds (styrene and fluorobenzoate) on *Chelon labrosus* was also pointed out using PICRUSt functional analysis [23], although their role is still unclear and most of this group of microorganisms remains uncultured, and understudied [51].

In this study, the most abundant bacterial family was represented by Rhodocyclaceae (47.7%) (beta-Proteobacteria) as observed by Le and Wang [30] in the gut of *M. cephalus* from the Taiwan Strait. Interestingly, the Rhodocyclaceae species were described by different authors for producing bioactive metabolites and, in particular, being able to transform perchlorate into harmless chloride [52-54]. In this regard, Guarino, et al. [54] reported about the genera Azospira and Dechloromonas of the Rhodocyclaceae family, able to transform perchlorate into harmless chloride, which is widely distributed in different environments such as soil and groundwater. Nowadays, perchlorate (ClO_{4^-}) is a ubiquitous ion released into the environment by anthropogenic activity although significant quantities of perchlorate are naturally formed in the atmosphere, especially during thunderstorms [53,55,56]. The main effect on human beings is its action on the thyroid gland by inhibiting iodide uptake and synthesis of thyroid-stimulating hormone, with serious impairments of growth, metabolism, and reproduction. Another dominant bacterial family identified in the mullets is the Brevinemataceae (Spirocheaetota). This microbial group was found to be very abundant in winter (45.8%) as also reported by Le and Wang [30] for the gut of *M.cephalus* and by García-Márquez, et al. [23] for *C. labrosus* individuals. Members of the Brevinemataceae family are described by other authors as producing butyrate [57] which may have an intestinal barrier function and support mucosal immunity [58]. Throughout this study, another family represented by Staphylococcaceae (Firmicutes) was detected significantly in the gut of fish sampled in spring (35.8%). Different papers described several biotechnological activities of Staphylococcaceae [59] and its capacity for degrading hydrocarbon [50]. Generally, the Firmicutes *phylum* is regarded as beneficial bacteria to the host since it comprises the group of lactic acid bacteria that are highly studied for their probiotic properties [60,61]. In this regard, the Lactobacillales order was identified with an abundance of more than 1% in all fish species caught in spring and summer, seasons with environmental conditions more suitable for this type of bacteria.

The present work is a first study via 16S rRNA metabarcoding technology on the intestinal communities of different species of Mediterranean grey mullets from a Sardinian aquatic environment; as already remarked, it was impossible to find mullets of the same species in the local food market during the different seasons of study, probably due to the different migratory behavior of these wild species and the management of the lagoon. Other metagenetic studies will be carried out considering the single fish species along different seasons and from other Mediterranean aquatic environments to have a broader view of the ecology of the bacterial communities associated with the intestinal tract of wild grey mullets.

Conclusion

The findings of the present work provide interesting insights into the diversity and biotechnological potential of the symbiotic intestinal communities hosted by Mediterranean grey mullets.

The results add new important insights into the intestinal microbial ecology of these fish as alternative candidate species and for rational use in aquaculture, following the EU policy for innovative technology and environmentally and commercially sustainable for a rational use.

Future research could be driven to the mullet aquaculture for preserving their intestinal microbiome which is an added value to protect fish and to preserve the aquatic habitat, a prerequisite of a sustainable aquaculture.

(Supplementary-Tables)

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