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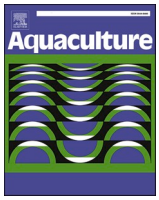

Aquaculture shapes antibiotic resistance in Mediterranean gilthead sea bream farming: From hatcheries to offshore cages

Marco Basili [a,b], Eleni Nikouli [c], Frederik D. Møller [d], Konstantinos A. Kormas [c], Gian Marco Luna [a,e], Veronica Tolosa-Enguís [f], Sonia M. Rodriguez-Ruano [f], Yolanda Sanz [f], Marco Candela [b], Grazia Marina Quero [a,e], Frank M. Aarestrup [d], Pimlapas Leekitcharoenphon [d,*]

[a] Institute for Biological Resources and Marine Biotechnologies, National Research Council (IRBIM-CNR), Largo Fiera della Pesca 2, 60125 Ancona, Italy
[b] Alma Mater Studiorum—University of Bologna, Bologna, Italy
[c] Department of Ichthyology and Aquatic Environment, University of Thessaly, 384 46 Volos, Greece
[d] Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark (DTU), 2800 Kongens Lyngby, Denmark
[e] National Biodiversity Future Center (NBFC), 90133 Palermo, Italy
[f] Microbiome Innovation in Nutrition & Health Research Unit, Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC), Valencia, Spain

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ABSTRACT

Intensive farming in crowded cages increases antibiotic treatments, promoting antimicrobial resistance (AMR) in fish-associated bacteria. Combined with environmental pollution, this accelerates AMR emergence, impacting food safety and ecosystems. To better understand AMR dynamics in aquaculture, this study examined antibiotic resistance genes (ARGs) in farmed gilthead sea bream (*Sparus aurata*) and in environmental samples, including seawater, sediments, and feed. Sampling was conducted across the full production cycle in three Mediterranean countries (Italy, Greece, and Spain) with wild *S. aurata* specimens also collected in Italy and Greece as a comparative baseline. Shotgun metagenomics revealed distinct ARG profiles between farmed fish and their surrounding environment, with notable shifts from land-based phases (eggs to juveniles) to open sea farms (juveniles to adults). Beta-lactam resistance dominated in adult farmed specimens across all sites, underscoring its prevalence in Mediterranean aquaculture. Interestingly, Greek wild fish samples lacked beta-lactamases. This study emphasizes the effectiveness of metagenomics-based approaches for tracking ARGs in fish farming, and underlines its potential to mitigate risks to human health, preserve aquatic ecosystems and make aquaculture more sustainable.

1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the most serious global health challenges of this century (WHO, 2014), with far-reaching implications for human health, food safety and environmental sustainability. The magnitude of this crisis is evident in projections that estimate AMR could cause up to 10 million deaths annually by 2050 (O'Neill, 2016). In 2019, AMR was responsible for an estimated 1.27 million deaths, with the highest burden observed in low- and middle-income countries (Murray et al., 2022). These alarming trends underscore the urgent need for comprehensive surveillance and effective intervention strategies to mitigate the spread of AMR. Antimicrobial resistance is encoded by antibiotic resistance genes (ARGs), which allow microbes to survive and grow in the presence of antibiotics. Water resources play a critical role in the dissemination of ARGs, serving both as vectors and reservoirs for resistant bacteria (Martinez, 2009), facilitating their proliferation and spread through ecological interactions and water movement. Aquatic environments are particularly susceptible to contamination by ARGs due to the persistent influx of pollutants, including pharmaceutical residues and agricultural runoff (Michael et al., 2013). Human activities, such as the discharge of partially treated or untreated wastewater and effluents from livestock farming and aquaculture practices, may further exacerbate this problem by introducing antimicrobials into aquatic ecosystems. Such selective pressure may favor the acquisition and maintainance of resistance and the consequent growth of resistant strains, which can then disperse through the water systems, posing substantial risks to public health and environmental stability (Berendonk et al., 2015; Baquero et al., 2008).

* Corresponding author.
E-mail address: pile@food.dtu.dk (P. Leekitcharoenphon).

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While aquaculture has become an essential industry in meeting the increasing global demand for food from the sea, it also poses environmental challenges, including the use of antibiotics (AB) to treat (and prevent) fish infections, which has been shown to promote the selection of resistant bacteria Ottinger et al., 2016; Topp et al., 2018). Indeed, fish have a limited capacity to metabolize AB, leading to the excretion of unmetabolized active compounds into the surrounding environment via fecal matter (Romero et al., 2012). It is estimated that between 70 and 80 % of the antibiotics administered in aquaculture are eventually released into aquatic systems, significantly contributing to environmental contamination (Burridge et al., 2010). Antimicrobial-resistant bacteria (ARB) generated in aquaculture settings have the potential to contaminate food products, posing risks to human consumers. Moreover, ARBs arising from aquaculture systems are a threat to natural aquatic environments since they may enhance the development or transfer of AMR in wild fish populations and associated food products (D'Costa et al., 2011). Additionally, commercial fish feed has been identified as a critical reservoir for ARGs in mariculture environments, with studies having detected oxytetracycline-resistant isolates in feeds used, for example, in carp farming (Han et al., 2017; Singh et al., 2009).

Despite strict regulation of AB use for veterinary purposes at the EU level, the role of aquaculture in the emergence and spread of AMR, especially in the Mediterranean Sea (EMA, 2023; EFSA et al., 2021), is gaining increased interest as ARGs can be commonly found even in fish farming environments that have never experienced antibiotic treatments (Alduina, 2020). However, our knowledge on AMR dynamics, considering the whole production cycle, is still limited (Reverter et al., 2020; Pepi and Focardi, 2021; Milijasevic et al., 2024; Deng et al., 2025), with most studies published to date in the area referring to AB resistant bacteria isolated from farmed fish and/or farming environment (Chelossi et al., 2003; Zorrilla et al., 2003; Scarano et al., 2018; Di Cesare et al., 2012, 2013) or with focus on only one stage (i.e., mainly adult fish) of the production cycle (Algammal et al., 2025; Salgueiro et al., 2023; Ismail et al., 2024). At the EU level, specific regulations are in place to manage AB veterinary usage, with approved antibacterial agents in Mediterranean aquaculture being mainly represented by oxytetracycline (OTC), oxolinic acid (OA), flumequine (FLU), and potentiated sulphonamides (SA) (Rigos et al., 2021).

In this study, we applied shotgun metagenomics sequencing to analyze AMR throughout the production cycle of Mediterranean gilthead sea bream (*Sparus* [*S.*] *aurata*); one of the main fish species in Mediterranean aquaculture, at three geographic regions; i.e., Italy, Greece, and Spain. Environmental samples, including seawater, sediment, rearing tank surface biofilms, and feed were also analyzed for the AMR content to clarify the possible sources of AMR contamination. The resistance of farmed sea bream populations was also analyzed in comparison to that of wild sea breams to uncover the role of aquaculture in conferring AMR. Our results contribute to understanding the prevalence, distribution, and potential transmission routes of ARGs in aquaculture. This multi-country, multi-matrix metagenomics approach represents a significant advancement in ARGs monitoring across Mediterranean aquaculture. The study emphasizes the effectiveness of metagenomics-based approaches for tracking ARGs in fish farming, with potential usefulness to mitigate risks to human health, preserve aquatic ecosystems and make aquaculture more sustainable (Wright, 2007; Di Cesare et al., 2016; Deng et al., 2025).

2. Material and methods

2.1. Study area and sampling activities

Samplings were performed in three different countries, farmed samples were collected in Italy (Tyrrhenian Sea), Greece (central Aegean Sea) and Spain (west Mediterranean Sea); in all cases, samples were collected along the production cycle, either in land-based facilities (hatchery, early-stages of the production cycle, i.e. eggs to juveniles, and mainly composed by sterile tank rooms) or in marine open sea cages (breeding of juveniles to commercial-sized adults). The production cycle of gilthead sea bream in intensive aquaculture starts with the collection of fertilized eggs from the mass spawning activity of the broodstock. After hatching (36–48 h), larvae are transferred to rearing tanks and fed with rotifers and microalgae. Artificial feeds are utilised from approximately 40 days post-hatch, and weaning terminates prior to the transfer of larvae to the nursing tanks (juvenile stage). Metamorphosis occurs around days 40–47, followed by weaning at day 60. Juveniles, reaching 1.5–2 g after around 60 days, are then transferred to sea cages until reaching market size (over 350 g) in about 18 months (Table 1). In this study, fish-related samples were grouped in 3 different groups, i.e., "eggs", "early-stage" (i.e., larvae, fry and juveniles) and "adults". Samples of rearing water/seawater, sediments, and feed were also collected from the considered fish farms. Adult wild sea bream individuals were collected in Greece (Ionian Sea) and Italy (Adriatic Sea); no wild specimens were caught from the Spanish Mediterranean coast. Adult fish were collected from the open sea cages from commercial aquaculture establishments and were immediately euthanized with a lethal dose (0.5 g/L) of Tricaine methane-sulfonate (MS222), according to the ethical principles and national legislative context, and placed on ice until arrival at the laboratory. For the early-stage individuals (i.e. eggs, larvae, and fry), DNA was extracted from the entire sample. From each juvenile and adult individual, DNA was extracted from different tissues, i.e.: (1) fish skin (a 2 × 2 cm square from the left side), (2) fish gill (second-gill arch on the left gill), (3) fish gut (tissue and feces or digesta), (4) fish filet (2 × 2 cm square at full depth from the left side). Except for fish feces, all the other tissue samples were rinsed with sterile phosphate buffer solution to remove possible loosely attached microorganisms (Basili et al., 2024). All samples were stored at −20 or −80 °C until further analyses. The list of samples collected and subjected to analysis in this study are reported in Table 1.

2.2. DNA extraction and sequencing

For water samples, one liter of water was filtered onto 0.22 μm cellulose nitrate membrane filters (Sartorius, ø 47 mm) or polycarbonate filters and stored at −20 °C until processing, and the entire filters were used for DNA extraction. For sediment and feed samples three replicates of 0.5–1 g aliquots were used for DNA extraction. DNA from early stages, skin, gill, fillets, feces/digesta, guts, water, sediment, feed, and swabs was extracted using the DNeasy PowerSoil Pro Kit (Qiagen) as previously described in Quero et al. (2023). Extracted DNA samples were quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and sequenced with Illumina NextSeq sequencing. Raw reads were submitted in the European Nucleotide Archive (ENA) under project number PRJEB81273. Individual sample accession numbers can be found in Supplementary Table 1.

2.3. Bioinformatic and statistical analyses of the fish resistome

A total of 440 samples from the three different locations, divided between farmed and wild, were collected for shotgun metagenomic sequencing. After a first DNA quantification step, only 200 samples that showed sufficient DNA for sequencing were processed. All bioinformatics analyses were performed on the Danish National Life Science supercomputer: Computerome2 (https://neic.no/computerome2.0-hpc/). Raw reads were quality checked with FastQC (version 0.11.5, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed with BBduk2 (version 36.49) to remove adapters and low-quality sequences. Paired end and singleton reads were aligned directly to the ResFinder database of acquired ARGs (version 4.0, database update 20,220,825) (Bortolaia et al., 2020) using the k-mer alignment software KMA (v1.2.8) (Clausen et al., 2018) with the CGE preset (−cge), which enforces a minimum relative alignment score of 0.75 and associated scoring penalties that has been tested and found ideal with the small

Table 1
Summary of the collected samples grouped by country and type of samples.

	Farmed Fish			Adults (Days 160–540)	Wild Fish	Environment		
	Early stages (from eggs to juveniles)				Adult	Hatchery	Open Sea	**Total**
	Eggs (Days 0–2)	Larvae (Days 2–40)	Fry-Juveniles (Days 40–160)					
Italy	4 (Eggs)	1 (Larvae)	2 (Juv-Skin) 4 (Juv-Gill)	5 (Skin) 6 (Gill) 28 (Gut)	7 (Skin) 8 (Gill) 37 (Gut)	3 (Tank) 1 (Feed)	3 (Tank) 3 (Seawat) 3 (Sediment)	**115**
	T0 2020/01/26	**T1 2020/01/28**	**T5 2020/06/26**	**T6 – T7 2020/10/14–2021/05/20**		**T2-T3-T4 2020/01/28–2020/02/10 – 2020/03/20**	**T7 2021/05/20**	
Greece		9 (Larvae)	6 (Fry) 1 (Juv-Skin) 2 (Juv-Gill)	4 (Skin) 4 (Gill) 29 (Gut) 2 (Fillet)	3 (Skin) 3 (Gill) 1 (Gut)	5 (Tank)		**69**
		T1 – T2 – T3 2019/12/03 - 2019/12/05 - 2019/12/13	**T4 – T5 2020/01/13 – 2020/03/19**	**T6 – T7 20,200,824 – 20,210,917**		**T2 – T3 – T4 2019/12/05 - 2019/12/13–2020/01/13**		
Spain		4 (Larvae)	4 (Fry)	6 (Gut) 2 (Fillet)				**16**
		T1 – 20,201,213	**T4 – 20,201,209**	**T6- T7 2021/04/30 2022/09/27**				

*Finder databases among the CGE-tools. Paired-end libraries were processed with KMA's default pairing mode ("unite"), and each read was restricted to a single best-matching template (−1 t1). Default KMA quality filters applied (minimum base Phred score 20; minimum depth 1). KMA identity (-ID) and minimum query coverage (−mrc) cutoffs were left at defaults; instead, for summarization we used the KMA's mapstat output and retained only *aligned fragments*. At the gene level, a gene was considered detected if it had ≥1 aligned fragment. Identity and coverage statistics from KMA's res files were recorded but not used as additional filters. No host-DNA removal step was performed prior to ARG detection.

ResFinder mapping counts were adjusted for differences in both ARGs gene lengths and bacterial sequence abundances by computing FPKM values for each ARGs associated read (Clausen et al., 2018; Munk et al., 2018). Total antimicrobial resistance per sample was quantified by calculating the total AMR fragments per kilobase per million fragments per sample (FPKM), and then the AMR genes were clustered per antimicrobial class (named 'AMR class') similar to previous published work (Munk et al., 2018).

All statistical analyses were performed in RStudio (2015). Once ARGs data were obtained, alpha- and beta diversity were analyzed using the *vegan* package (Oksanen, 2015). Alpha diversity of AMR data was assessed by calculating richness (i.e., number of different ARGs in a given sample) and Simpson's diversity index (which takes into account the number of genes present as well as their abundance). The occurrence of statistical differences in ARGs alpha diversity was assessed (ANOVA and PERMANOVA) (i) across the three countries, (ii) among the different types of tissue, (iii) between environmental and fish samples, and (iv) between wild and farmed fish, with effect sizes reported as η^2 to indicate the magnitude of group differences. To observe the dissimilarities among samples resistome, non-metric Multidimensional Scaling (nMDS) ordinations were calculated from a Bray–Curtis dissimilarity matrix after square root transformation and Double Wisconsin standardization (R *vegan* function *metaMDS*).

3. Results

A total of 200 samples collected from Italy ($n = 115$), Greece ($n = 69$), and Spain ($n = 16$) were analyzed for this study (Table 1). These samples included early stages of the production cycle (i.e., $n = 28$ including eggs, larvae, fry, and juveniles), adults from open sea cages ($n = 95$), environmental samples from the aquaculture ($n = 17$ including seawater, sediment, and biofilm and n = 1 feed) and wild adult fish ($n = 59$). A total of 16.2 billion paired-end reads were obtained from the 200 samples (average 81.1 million PE reads, range 4.5–215 million sequencing results and statistic information were reported in Supplementary Table 1). A total of 342 AMR gene variants were obtained from the Kmer mapping of all the samples on the ResFinder database; all the variants of each gene were then grouped under the same AMR gene names, obtaining a total of 181 different genes, classified in 19 AMR classes.

Alpha diversity analyses revealed a significantly higher ARGs richness in environmental samples (avg. 16.5 ± 9.11) compared to fish samples (avg. 4.8 ± 2.2), including both early developmental stages and adult phases (ANOVA, $p < 0.001$, $\eta^2 = 0.58$) (Fig. 1). The only notable exception was represented by fish eggs, which exhibited richness values comparable to those of environmental samples (Fig. 1). In contrast, values of Simpson's index did not reveal significant differences between environmental and fish samples, nor were any statistical differences observed among fish samples from different countries or across developmental stages (ANOVA, n.s.). Additionally, a significant difference in both richness and Simpson's diversity was found between wild fish samples from Italy and Greece, with the former displaying higher diversity values in skin and gill samples (Fig. 1) (ANOVA, $p < 0.001$, $\eta^2 = 0.20$). The nMDS revealed a clear separation between the resistomes of environmental samples and fish tissues (Permanova $F = 4.9$, $p < 0.001$) (Fig. 2). However, eggs' resistome clustered more closely with environmental samples than with other fish tissues (Fig. 2). Moreover, no distinct clustering patterns were observed based on country, developmental stage, or, in adult fish, on tissue type; on the other hand, sediment and (sea)water samples exhibited a tendency to cluster together.

Resistomes were further analyzed in order to understand the distribution and level of sharing across the type of samples and environment separately for each country (Fig. 3). To this end, samples were categorized into five groups: (i) environmental samples from hatchery; (ii) early-stage fish samples; (iii) farmed adult fish; (iv) wild adult fish; and (v) environmental samples from open sea aquaculture. In samples collected in Italy, out of 124 ARGs, only eight were detected across all groups, while most of them ($n = 45$) were found exclusively in environmental samples from open sea aquaculture. In early developmental stages, we found that a considerable number of ARGs ($n = 23$) was shared between fish samples from early developmental stages and early-

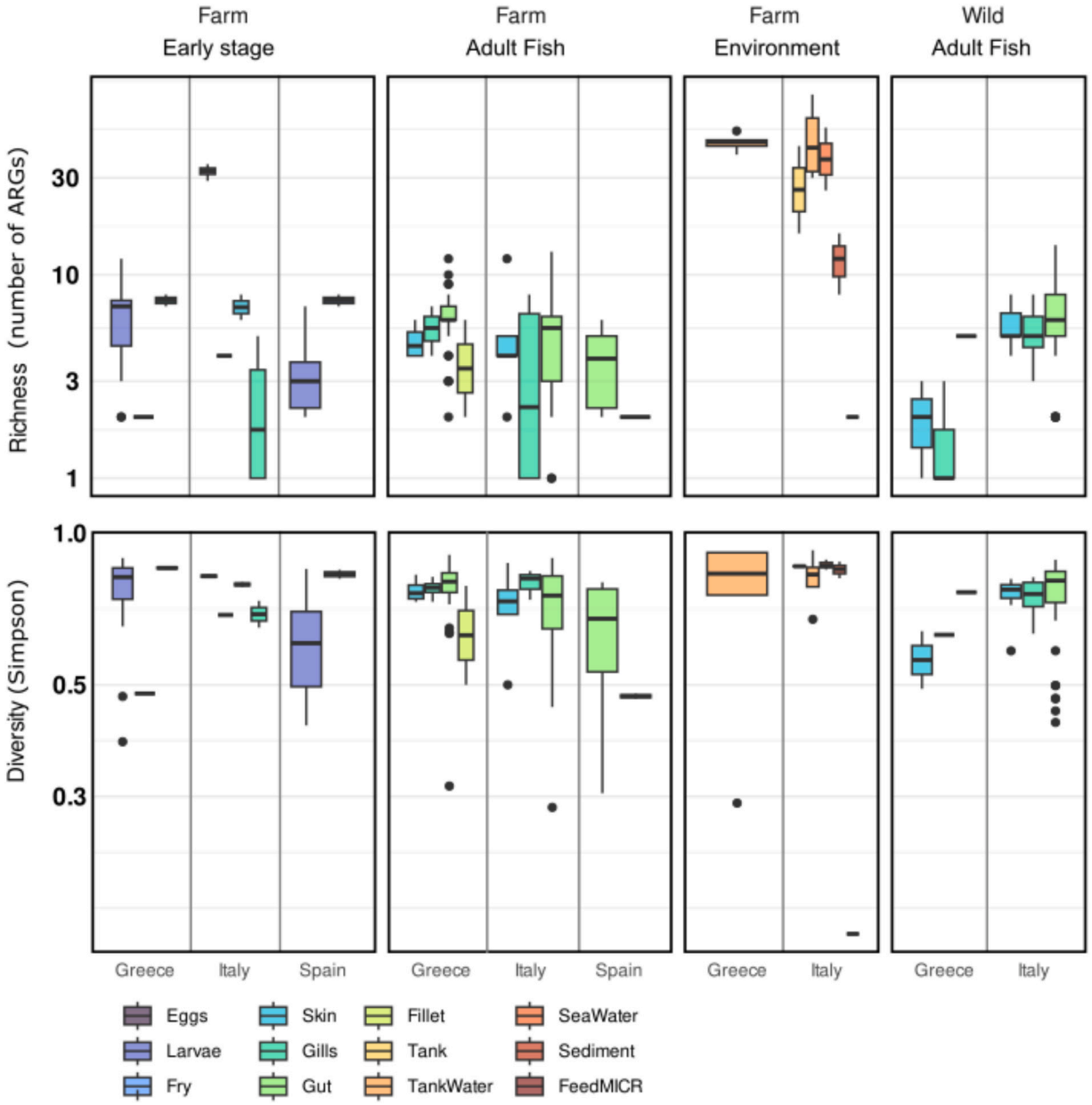

Fig. 1. Alpha diversity of ARGs found in farmed fish along the production chain, in environmental samples and in wild fish. Upper panels report boxplots showing richness values, lower panels report showing Simpson's diversity. Data are grouped by country (Italy, Greece, Spain), life stage (early stages or adult phases) and are coloured according to the type of tissue of sea bream.

stage environmental samples; similarly, wild and farmed adult fish resistomes shared a relatively high number of ARGs ($n = 14$) (Fig. 3). In samples collected in Greece, the majority of ARGs (total of 86) were found exclusively in environmental samples ($n = 42$; only environmental samples from hatchery); however, a considerable number of ARGs ($n = 15$) were shared among early and adult developmental stages of the farmed fish (Fig. 3). Finally, in samples collected in Spain (27 ARGs), 10 ARGs were shared between early stages and adult fish, whereas other ARGs ($n = 10$ and $n = 7$, respectively) appeared to be exclusive of each of those two stages (Fig. 3). Supplementary Table 2 summarizes the detailed list of the ARGs shared between each pair of groups (e.g., early stages vs. adult fish), as well as those shared by samples collected in all countries. Interestingly, genes encoding for resistance to tetracycline (tet) and beta-lactamases (bla) were shared by all countries at all life stages (including adult wild fish), and they were present in all environmental samples analyzed. The early stage-adult comparison revealed a total of five genes shared across Italian, Greek, and Spanish samples, highlighting cross-national similarities.

We further examined the top 10 most abundant ARGs in fish (Fig. 4, upper panel) and environmental samples (Fig. 4, lower panel). Genes coding for the extended spectrum OXA-type beta lactamase represented the most abundant and widespread type of ARG, since they were found both in fish samples (juveniles and adult fish) and seawater samples from open sea cage. However, beta-lactamases were not detected in wild sea bream samples collected in Greece. Overall, wild fish from Greece showed a distinct resistome profile compared to farmed fish collected in the same country, whereas adult farmed and wild sea bream collected in Italy shared a considerable number of ARGs (Supplementary Table 2). In addition to OXA-type beta lactamases-encoding genes, CME-, SPU-, and CARB-type beta-lactamases genes were also present in nearly all fish samples, with no significant differences among countries or tissues (ANOVA n.s.). The analysis of environmental samples showed that each

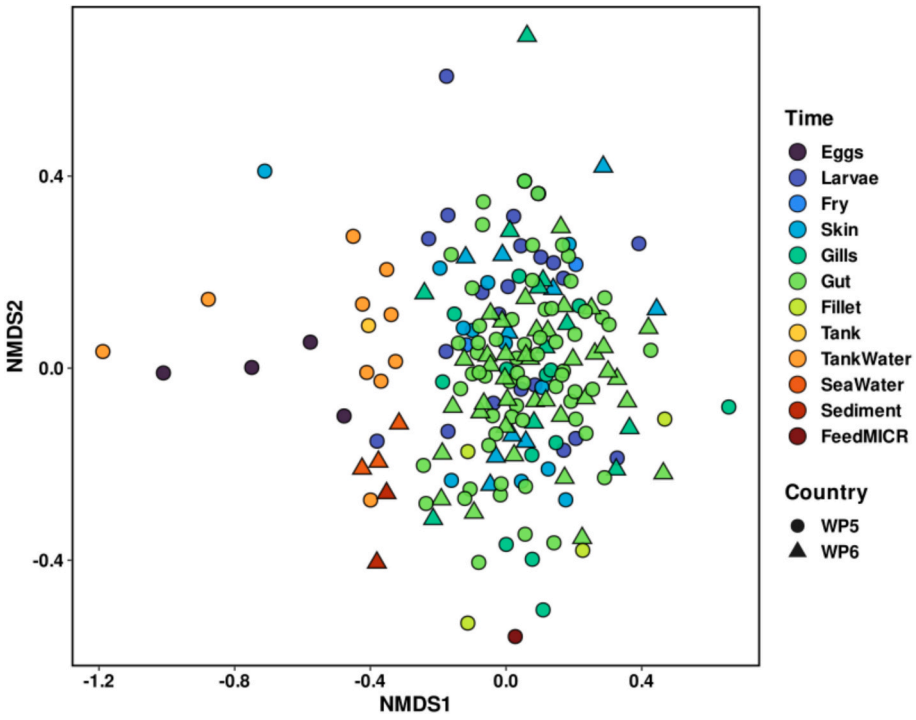

Fig. 2. nMDS plot calculated on a Bray–Curtis dissimilarity matrix of ARGs abundance table of farmed (at the different developmental stages) and wild sea bream samples, as well as of environmental samples. Different colors indicate different developmental stages or the different tissues of adult fish, shapes indicate whether samples are from aquaculture or from wild specimens.

group of samples displayed a different resistome profile, with environmental samples from the early developmental stages of farmed fish being dominated by genes conferring resistance to tetracycline (mainly *tet*(34)), aminoglycosides and sulfonamides. Interestingly, tank water samples from the hatchery shared some of the most abundant ARGs (*sul*2, *aph*(3″), *aph*(6)) with eggs, but they were no longer observed in tissues of later fish developmental stages. Sediment samples exhibited a completely different resistome profile compared to fish and seawater, with seawater samples being dominated by OXA-type beta-lactamase and *tet*(34) genes (Supplementary Table 2). The presence of the 10 most abundant genes was also analyzed in order to observe the ARGs profile change across the cycle (Fig. 5); the results showed a consistent

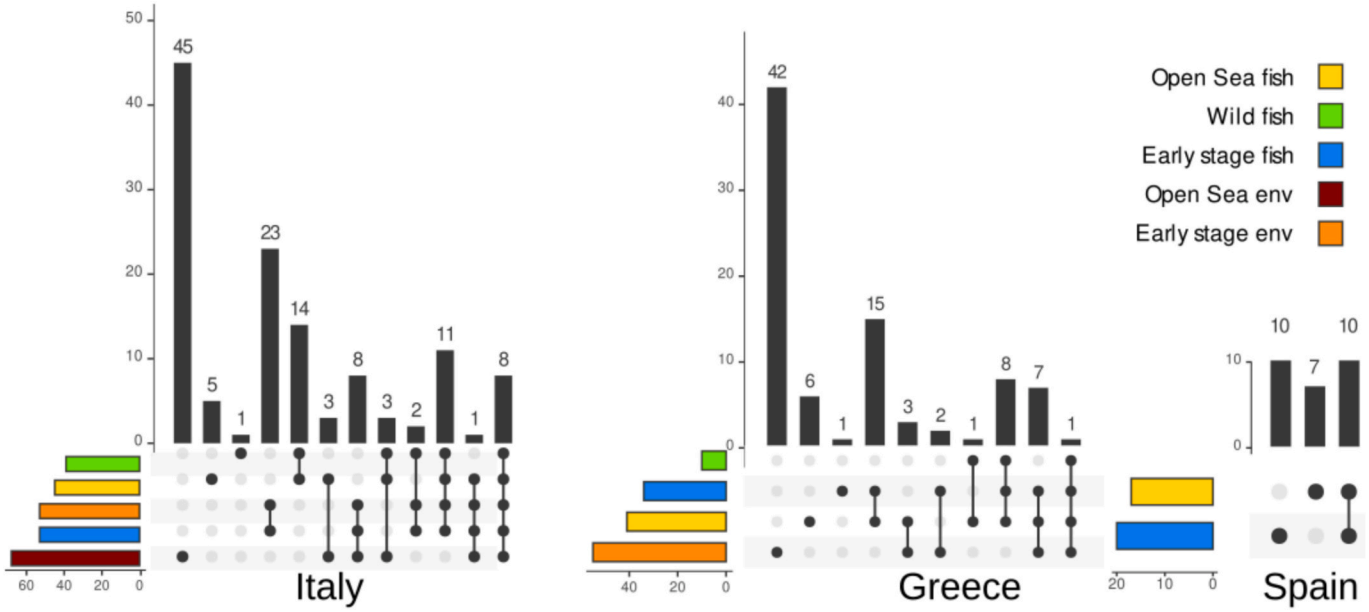

Fig. 3. Upset plots showing the ARGs distribution across the country, hatchery (early stage, blue for the organism and orange for the environment), farmed open sea stages (yellow for the organisms and red for the environment), and wild adults (green); highlighting the sharing of several ARGs between the different groups. The top bar plot indicates how many genes were found in each single group (as shown in the legend) or shared among the groups. For each bar, the corresponding samples were indicated below the top bar plot by the black dots (single dot if the bar identify the unique ARGs of a group; multiple dots connected with the line if the bar indicate the ARGs shared among the indicated groups). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

composition, enlightening the persistence of the resistome profile across the development of the sea bream. In Greek samples, where a complete overview of the growth stages (or life stages) is provided, consistency in the resistome composition was observed from larvae (T2) to juveniles (T4); the same resistome profile was clearly present among the farmed adults (T5-T7), similar to the profile found in the Italian wild samples. However, the wild fish samples collected in Greece lacked the OXA-type of beta-lactamase genes, which caused their resistome profile to be different from the others adult samples.

Considering the content of ARGs in each considered sample at the class level, we observed that resistance to beta-lactams was the most abundant type of AB resistance, representing more than 50 % of the resistome in fish samples (Fig. 6). Resistance to macrolides, lincosamides, and streptogramins (A and B) were also highly represented in fish tissues (Fig. 6), while resistance to tetracyclines, folate pathway antagonists, and aminoglycosides were highly enriched in environmental samples, eggs, and feed (Fig. 6). Overall, the resistome profiles of farmed adult fish samples collected across countries appeared to be similar; on the other hand, we observed a marked difference in resistome composition comparing farmed and wild adult sea bream individuals.

4. Discussion

AMR is a critical and growing global health challenge. Aquaculture has been shown to play a key role in the spread of ARGs in the environment, with particular reference to the water and sediments surrounding fish farming facilities. However, it remains unclear whether aquaculture acts primarily as a source or a sink of AMR: while antibiotics used in aquaculture can introduce resistance into the aquatic environment and food chain (Santos and Ramos, 2018), external inputs such as rivers, urban runoff, and wastewater can also bring AMR contaminants that accumulate in farmed fish (Kampouris et al., 2022). However, AMR

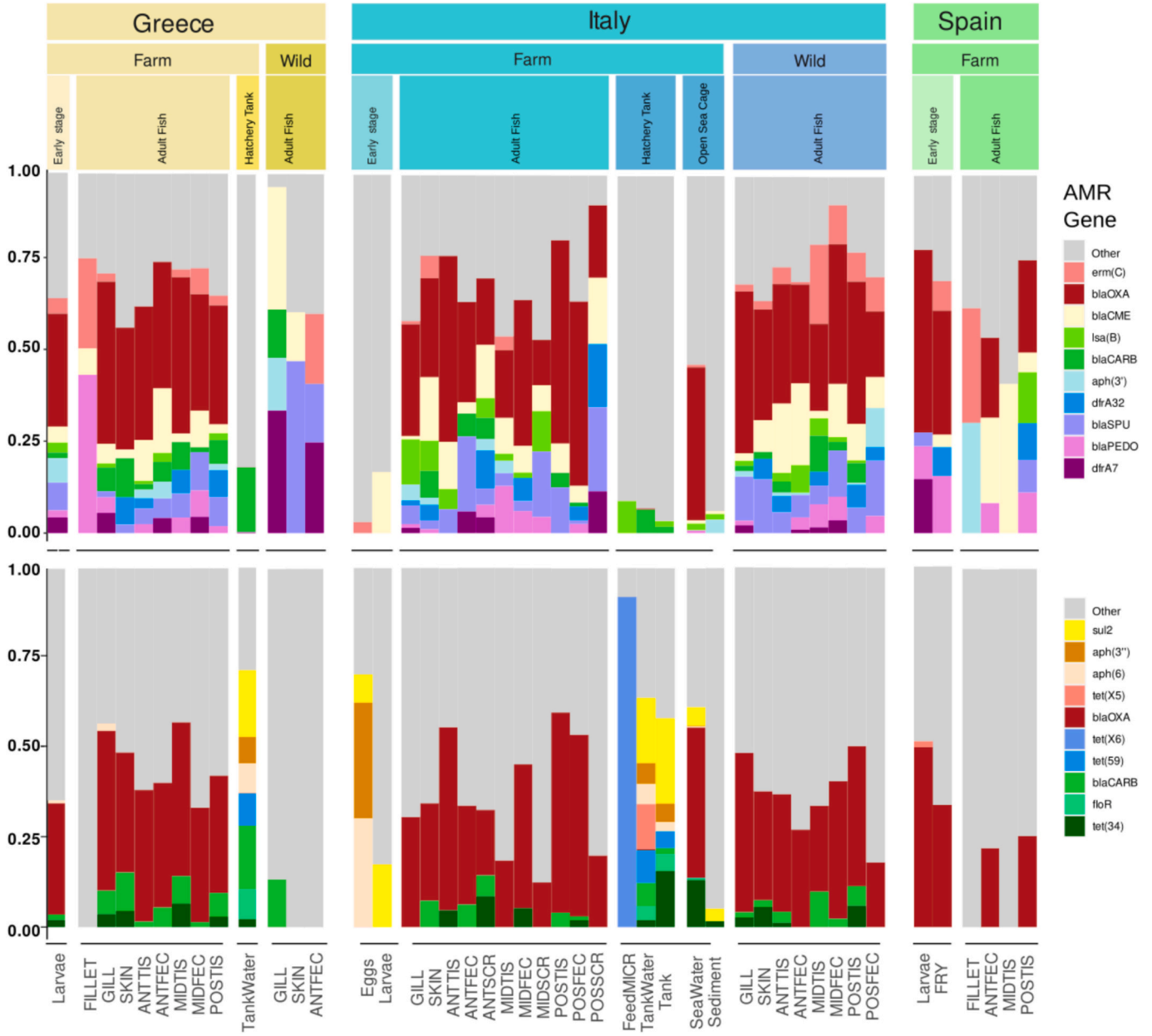

Fig. 4. Barplot showing the relative abundance of ARGs at the gene level in fish samples and environmental samples; samples are separated according to country, sample type (environment and fish), and the origin (farmed or wild). Barplots show only the top 10 most abundant ARGs observed in fish (upper panel) and environmental samples (lower panels).

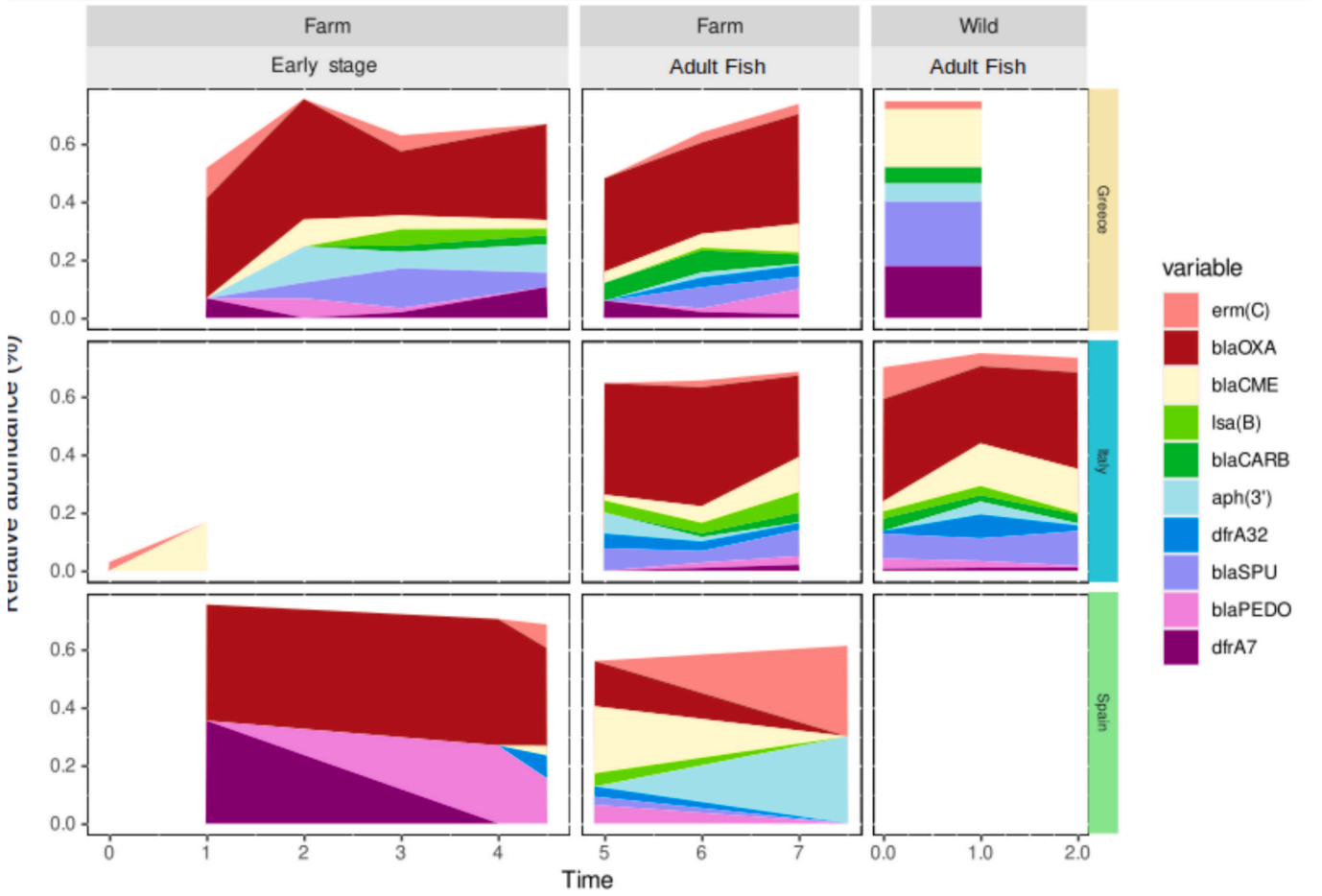

Fig. 5. Relative abundances of resistome profiles at the gene level according to country and time point. Only the top 10 most abundant ARGs observed in fish are reported.

trends are rarely reported in farmed animals and, even less in farmed fish (Munk et al., 2024). In this study, for the first time for farmed Mediterranean gilthead sea bream (*S. aurata*) production cycle, the presence, dynamics, and contribution of ARGs were assessed both in the farmed fish at the different developmental stages and in the surrounding environment, in order to clarify the possible sources of AMR contamination. This study benefits from a Mediterranean-level perspective, considering that samples from three different countries (Italy, Greece, and Spain) were analyzed using the same methodologies to understand whether common or specific AMR patterns are observable at the basin level for this important aquaculture species. In addition, to further clarify whether and to what extent farming practices contribute to the resistome of farmed sea bream, the content and diversity of ARGs in farmed adult *S. aurata* were compared with those in adult wild specimens from Italy and Greece. Despite the challenges of sampling across three Mediterranean countries, accounting for differences among aquaculture facilities, and dealing with host DNA interference in metagenomic analyses - a methodological bottleneck where the fish microbiome field still needs to make substantial progress -, our study provides one of the first cross-country datasets on fish-associated microbiomes and resistomes in Mediterranean aquaculture. Our results reveal distinct profiles in AMR gene diversity when comparing environmental samples (i.e., tank and seawater, tank surfaces, sediments, feed) and fish samples, with the former exhibiting an overall higher richness, except for fish eggs that resemble the environment to a higher degree. This finding, along with the lack of differences among samples collected from the two countries, suggests that the aquaculture environment - from early production stages in the hatchery to the latest stages in the open sea - may serve as a reservoir of AMR genes and likely as a first source of contamination for the farmed fish. Whether the environmental resistomes arise from wastewater discharge, agricultural runoff as well as from other anthropogenic sources, or whether it is the result of the selective pressure in response to antimicrobial use in fish farming (Cabello et al., 2013; Holmström et al., 2003) remains unclear. However, our results highlight the importance of responsible management of water resources and of sustainable aquaculture practices to control the spread of resistance. Furthermore, the relatively high diversity of the eggs' resistome (as well as its ARG composition comparable to that of tank surface and water) raises concerns about early developmental stage contamination and transmission of resistance along the aquaculture supply chain. Cabello et al. (2013) discussed how antibiotic use in fish farms can lead to the emergence of AMR in eggs, which then contributes to the overall resistance burden in the tank environment, posing risks to both farmed fish and the broader ecosystem. Indeed, the presence of shared ARGs between early developmental stages and adult farmed fish at all sampled locations suggests that resistance may circulate among different stages and is potentially consistent at the Mediterranean basin level (Martinez, 2009; Burridge et al., 2010; Cabello, 2006). However, findings from microbiota profile studies in *S. aurata* and other fish species (Califano et al., 2017; Najafpour et al., 2021; Nikouli et al., 2019) suggest that fish at early stages share a high degree of their microbiome with the surrounding rearing water. This similarity in microbial composition likely explains the observed parallels in the AMR profiles between the eggs and the environmental samples. The most abundant ARGs in this study were represented by resistances to tetracyclines, aminoglycosides, and folate

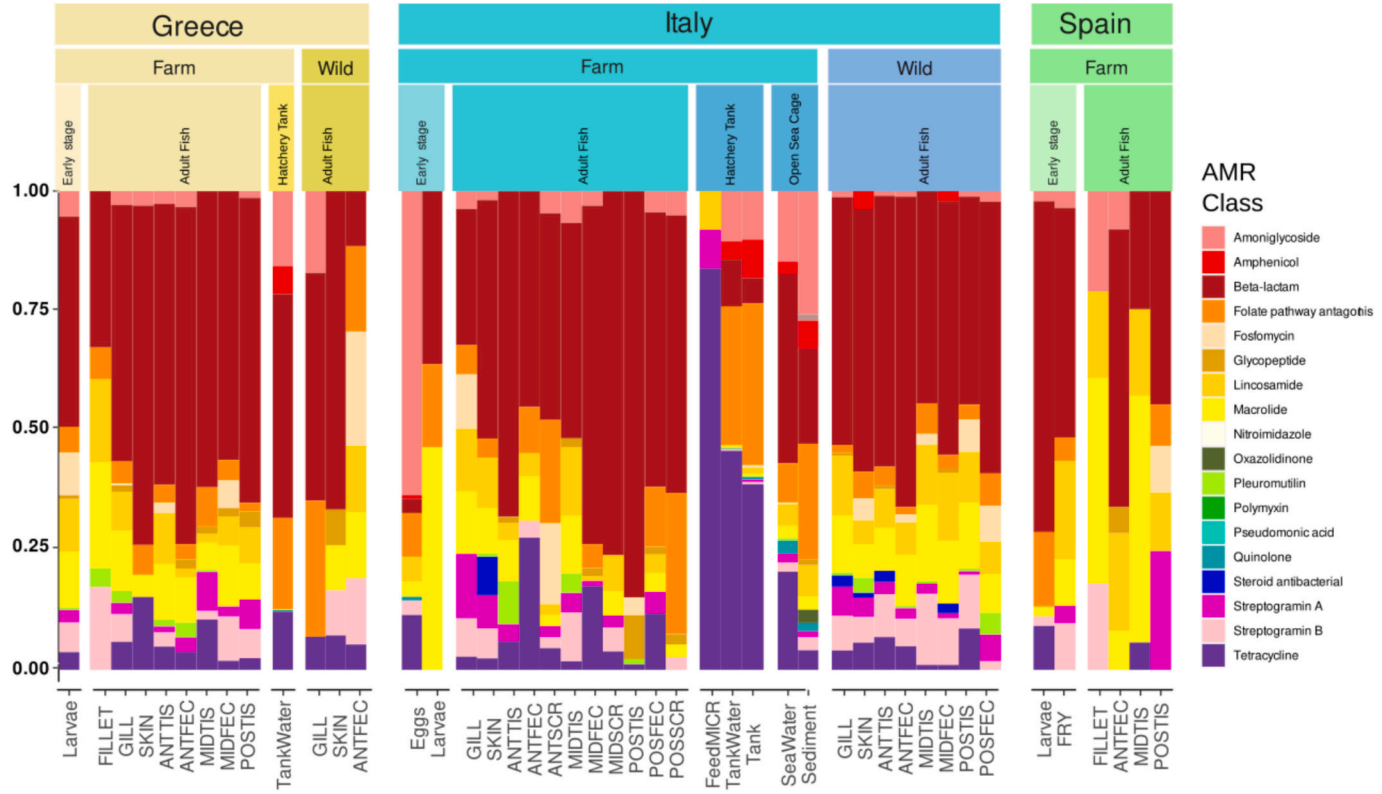

Fig. 6. Barplot showing the relative abundance of ARGs grouped at the class level according to country, sample type (environment vs.fish) and origin (farmed vs. wild) of samples.

pathway antagonists, with resistances to tetracycline and beta lactamases being shared by samples (including wild fish and environmental samples) collected in all countries and across all life stages. Tetracyclines, due to their low cost and broad-spectrum efficacy, are extensively used in aquaculture (Lulijwa et al., 2020; Bondad-Reantaso et al., 2023), leading to the development of resistance genes like *tet*(A), *tet*(C), and *tet*(M), which are frequently detected in fish pathogens across various Mediterranean (Pepi and Focardi, 2021; Fernández Salgueiro et al., 2024; Preena et al., 2020) and Atlantic (Silva et al., 2022) farms, as well as, in general, in aquatic environments (Di Cesare et al., 2013), and contribute to resistance through mechanisms like efflux pumps and ribosomal protection (Ndi and Barton, 2011). Several studies showed that fish farming environments, including sea bream farms, act as a reservoir of transferable tetracycline resistance genes (Di Cesare et al., 2013; Jacobs and Chenia, 2007), corroborating the notion indicating this type of AB resistance as one of the most widespread in marine ecosystems. Sulfonamides, here grouped as folate pathway antagonists, are commonly used in combination with other AB such as trimethoprim and have been also reported to have largely contributed to the spread of resistance genes, e.g. *sul*1 and *sul*2, in the environment as well as in farmed fish (Sousa et al., 2011; Shah et al., 2014). Resistances to these ABs often occur on mobile genetic elements like plasmids, enabling their rapid dissemination across microbial populations in aquaculture systems (Agersø and Petersen, 2007; Marti et al., 2014; Cabello et al., 2016; Watts et al., 2017). This has been shown in diverse aquaculture settings, including, for example red sea bream (*Pagrus major*), where *sul* and *tet* (M) genes occurred in bacterial community in Japanese marine aquaculture environment (Suzuki et al., 2019), or salmon farm in Chile, where *sul*1, *sul*2, class 1 integron-integrase gene *intI*1, *dfr*A1, *dfr*A12, and *dfr*A14 were detected (Domínguez et al., 2019) further emphasizing the relevance of this type of ARGs in aquaculture environments.

Similarly, the prevalence of *bla*OXA-type genes in fish samples, particularly in adult fish (farmed and, only for Italy, wild individuals), raises concerns about the transmission of beta-lactam resistance within aquaculture systems (Preena et al., 2020). Interestingly, the lack of occurrence of this gene in wild fish samples collected in Greece, suggests that contamination of beta-lactamases is widespread along the coasts of Italy, but localized only in fish farm environments in Greece. This differences, may reflect localized exposure in aquaculture settings, whereas broader environmental contamination from anthropogenic sources (e.g., proximity to urban and agricultural discharge) could contribute to the patterns observed in Italy. Moreover, this type of resistance is of particular interest in the fish farming context, considering that several *Vibrio* (*V.*) species (including *V. cholerae*, *V. vulnificus*, and *V. aestuarianus*) and *Aeromonas* species are known aquaculture-related pathogens harboring beta-lactam resistance genes (Wang and Levin, 2006; Alderman and Hastings, 1998; Vega-Sánchez et al., 2014). Although our dataset did not allow to directly link ARGs to specific taxa, these findings support indications from prior studies reporting Proteobacteria and Bacteroidetes as the dominant phyla carrying ARGs in aquatic systems (Di Cesare et al., 2013; Romero et al., 2012; Rigos et al., 2021). Together with *sul*1, *sul*2, and *tet*(A) resistance genes, extended spectrum beta lactamases (ESBLs) have also been identified in fecal samples of wild sea bream (Sousa et al., 2011), confirming our results and confirming previous knowledge on AB resistance in aquaculture settings. The overall similar AMR profiles observed comparing wild and farmed adult sea bream samples further underlines the role of the aquatic environment as a source of AMR. However, the distinct AMR profile observed in wild adult sea bream collected in Greece, particularly characterized by the lack of OXA-type beta-lactamase resistance, could be attributed to differences in local antibiotic usage practices and environmental factors, highlighting the impact of regional management practices on AMR development. The persistence of these resistance genes throughout the fish production cycle suggests that aquaculture practices may serve as amplification reservoirs for clinically relevant ARGs that could transfer to human pathogens, as intensive antimicrobial

use in aquaculture creates reservoirs of drug-resistant bacteria and transferable resistance genes (Cabello, 2006), thereby compromising the efficacy of last-resort carbapenem therapies used for severe human infections.

The differences in antimicrobial resistance (AMR) profiles between environmental samples and fish tissues suggest that, while fish acquire resistance genes through environmental exposure, the specific AMR patterns observed in fish tissues point to a selective process potentially driven by their unique microbial communities. Moreover, the similar profile observed in the tank water along the productive cycle and the change occurred in the open sea environment indicates that aquaculture environments are complex ecosystems where AMR genes can be exchanged and dispersed through various compartments in both the hatchery and the open sea area, with the open sea affected also by several external inputs (Reverter et al., 2020). Environmental sources, such as water contaminated with antibiotics, play a critical role in introducing resistance genes into aquatic systems, where fish are exposed to resistant bacteria and ARGs (Martinez, 2009). However, studies indicate that fish microbial communities, particularly in the gut, exhibit selective pressures that shape specific resistance profiles, distinct from those found in the broader environment (Romero et al., 2012; Rigos et al., 2021; Rico et al., 2013). This selectivity, confirmed by the consistent profile observed across the various stages, could result from factors like host-specific immune responses (Ferri et al., 2022) and microbial interactions within the fish microbiome, which preferentially favor certain ARGs. Consequently, the AMR profiles in fish reflect both environmental influences and the fish's internal microbial dynamics, creating a unique resistance pattern that differs from the surrounding ecosystem. Further studies are needed to determine the possible role of aquaculture-associated AMR for food safety or impact on recreational water, including the possible emergence of novel ARG variants.

5. Conclusions

This study offers a comprehensive view of AMR occurrence, dynamics, and sources in Mediterranean gilthead sea bream farming across three countries. From hatcheries to offshore farms, our findings show that aquaculture environments can act as reservoirs for ARGs, influenced by environmental factors. The widespread detection of ARGs, especially those for beta-lactams, tetracyclines, and sulfonamides, highlights the need for targeted interventions. Antibiotic use is not the sole driver of AMR — anthropogenic factors like wastewater and agricultural runoff also contribute. Shared ARGs between wild and farmed fish suggest resistance can persist through the production cycle, emphasizing the need for sustainable practices and water management. With aquaculture being vital to global seafood supply, balancing productivity with ethical antimicrobial use is key. Reducing AMR impacts through probiotics, vaccines and biosecurity should be prioritized for a sustainable industry.

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CRediT authorship contribution statement

Marco Basili: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eleni Nikouli:** Writing – review & editing, Investigation, Conceptualization. **Frederik D. Møller:** Writing – review & editing, Formal analysis, Data curation. **Konstantinos A. Kormas:** Writing – review & editing, Resources, Investigation, Conceptualization. **Gian Marco Luna:** Writing – review & editing, Resources, Conceptualization. **Veronica Tolosa-Enguís:** Writing – review & editing, Resources, Investigation. **Sonia M. Rodriguez-Ruano:** Writing – review & editing, Resources, Investigation. **Yolanda Sanz:** Writing – review & editing, Resources, Investigation. **Marco Candela:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Grazia Marina Quero:** Writing – review & editing, Investigation, Conceptualization. **Frank M. Aarestrup:** Writing – review & editing, Resources, Methodology. **Pimlapas Leekitcharoenphon:** Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization.

Ethics statement

The Authors declare that all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected.

Declaration of competing interest

The Authors declare no conflicts of interest.

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Data availability statementThe data that support the findings of this study are openly available in the SRA (https://www.ncbi.nlm.nih.gov/sra) and ENA (https://www.ebi.ac.uk/ena/browser/home) public repositories. All accession numbers are reported in Supplementary Table 1.

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