Antibiotic resistance of Aeromonas spp. strains isolated from Sparus aurata reared in Italian mariculture farms

Antibiotic resistance of *Aeromonas* ssp strains isolated from *Sparus aurata* reared in Italian mariculture farms

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Abstract

Selective pressure in the aquatic environment of intensive fish farms leads to acquired antibiotic resistance. This study used the broth microdilution method to measure minimum inhibitory concentrations (MICs) of 15 antibiotics against 104 *Aeromonas spp* strains randomly selected among bacteria isolated from *Sparus aurata* reared in six Italian mariculture farms. The antimicrobial agents chosen were representative of those primarily used in aquaculture and human therapy and included oxolinic acid (OXA), ampicillin (AM), amoxicillin (AMX), cephalothin (CF), cloramphenicol (CL), erythromycin (E), florfenicol (FF), flumequine (FM), gentamicin (GM), kanamycin (K), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP). The most prevalent species selected from positive samples was *Aeromonas media* (15 strains). The bacterial strains showed high resistance to SZ, AMX, AM, E, CF, S and TMP antibiotics. Conversely, TE and CL showed MIC_{90} values lower than breakpoints for susceptibility and many isolates were susceptible to OXA, GM, FF, FM, K and OT antibiotics. Almost all *Aeromonas spp* strains showed multiple antibiotic resistance. Epidemiological cut-off values (ECVs) for *Aeromonas spp* were based on the MIC distributions obtained. The results

showed a high frequency of *Aeromonas spp* contamination in *Sparus aurata* reared on the Italian coast and an elevated biodiversity in isolated bacterial strains. *Aeromonas* isolates comprise potentially pathogenic species for humans, often resistant to several antibiotics and able to transfer the genes responsible for antibiotic resistance to microorganisms pathogenic for humans throughout the food chain. The few ECV studies available on many antibiotics against *Aeromonas spp* strains isolated from the aquaculture environment highlight the need for further research in this area, while regular monitoring programmes should be stepped up to check for antibiotic resistance.

Keywords: gilthead seabream; *Aeromonas* spp bacteria; minimum inhibitory concentration; antibiotics; epidemiological cut-off values.

1. Introduction

World fish consumption has been growing in the last thirty years reaching 20 kg per capita in 2014. For the first time, the aquaculture production of fish for human consumption has overtaken the supply of wild-caught fish and is expected to rise to 62% by 2030 (FAO, 2016). Gilthead sea bream (*Sparus aurata*) is a very suitable species for mariculture in the Mediterranean basin and has become one of Europe's main fish species in aquaculture. Greece, Turkey and Spain are the main producers worldwide while Italy is the third main producer in the EU (EC, 2017). Large-scale aquaculture is characterized by the intensive and semi-intensive production systems with high stocking density, which leads to poor hygiene conditions and the emergence of infectious diseases (Diana et al., 2013).

The genus *Aeromonas* comprises a group of bacteria with a ubiquitous distribution in natural habitats, including the aquatic environment (Janda & Abbott, 2010) where species such as *A. hydrophila*, *A. caviae*, *A. salmonicida* and *A. veronii* biovar *sobria* cause disease in marine fish (Radu et al., 2003). *Aeromonas* spp are also important human opportunistic pathogens able to cause

intestinal, blood, skin and soft tissue and trauma-related infections, particularly in young children and the elderly (Janda and Abbott, 2010; Real et al., 1994). *Aeromonas* species have been frequently isolated from fish and other foods (Callister and Agger, 1987; Gobat and Jemmi, 1993). These bacteria are responsible for food spoilage and may serve as vectors for disease transmission to humans (Tsai and Chen, 1996). Infection can also occur after contact with contaminated water or fish (Janda and Abbott, 2010). *Aeromonas* pathogenicity is linked to the production of a number of extracellular hydrolytic enzymes such as lipases and proteases, which aid in bacterial invasion and the establishment of infection (Galindo et al., 2006). Among an array of other virulence factors, the biological activities of cytotoxic enterotoxin (Act) include haemolysis, cytotoxicity, enterotoxicity and lethality (Chopra et al., 1991).

The worldwide expansion of intensive fish farming has increased the use of antibiotics to treat bacterial infections (Díaz-Cruz et al., 2003). In aquaculture, antimicrobials are generally added to the feed or directly to the water to prevent the spread of infectious fish disease (Defoirdt et al., 2011) and in some circumstances to promote fish growth illegally (Serrano, 2005). Regulations governing the use of antibiotics in aquaculture differ widely with little to no enforcement in many of the world's major aquaculture-producing countries (Pruden et al., 2013). The extensive use of antibiotics in aquaculture has in turn resulted in the emergence of antibiotic resistance in both foodborne and opportunistic human pathogens (Marshall and Levy, 2011). The resistance of *Aeromonas* species to diverse groups of antibiotics is a major concern for human health (Figueira et al., 2011) as resistant bacteria can spread from the aquatic environment to humans via the food chain or direct contact (Taylor et al., 2011). In addition, resistance genes can be transferred by mobile genetic elements such as plasmids, phages and transposons (Levy and Marshall, 2004). Janda and Abbott (2010) reviewed the general susceptibility profiles of Aeromonads to various antimicrobial classes, showing resistance to sulfamethoxazole, cephalosporins, penicillins (amoxicillin, ampicillin, ampicillin-sulbactam, ticarcillin, oxacillin and penicillin) and macrolides (clarithromycin). *Aeromonas* species resistant to penicillins and first generation cephalosporins are

79 associated with the production of chromosomally encoded beta-lactamases (Janda and Abbott,
80 2010). Other important resistance determinants to beta-lactam antimicrobials and tetracyclines are
81 *bla* genes and *tet* genes respectively encoded in mobile genetic elements (Agersø et al., 2007; Wu et
82 al., 2011) or integrons, responsible for resistance to tetracyclines, aminoglycosides,
83 chloramphenicol and trimethoprim (Chang et al., 2007; Kadlec et al., 2011). Indeed, acquired
84 antibiotic resistance among fish pathogens could determine serious therapeutic problems in humans
85 following the use of molecules whose class and structure are similar or, in some cases, identical to
86 those used in mariculture (Cabello, 2006). Despite recent efforts by international agencies such as
87 the European Centre for Disease Prevention and Control and the National Antimicrobial Resistance
88 Monitoring System (EFSA, 2014), the role of antibiotic usage in aquaculture in the development
89 and dissemination of antibiotic resistance genes is still poorly understood. The potential risk of
90 transferring such resistance from the aquaculture environment to humans is underestimated
91 (Cabello et al., 2013) so the effectiveness of antibiotics used in fish farming should be carefully
92 monitored.
93 Little information is available on the susceptibility of *Aeromonas* spp isolated from mariculture to
94 antibiotics used in both fish farming and human therapy. Antimicrobial susceptibility is generally
95 tested by measuring the drug's minimum inhibitory concentration (MIC). MIC breakpoints are the
96 MICs at which an organism should be considered susceptible, intermediate or resistant. Breakpoint
97 values are published by organizations such as the European Committee on Antimicrobial
98 Susceptibility Testing (EUCAST) and the American Clinical Laboratory Standard Institute (CLSI),
99 based on pharmacokinetic/pharmacodynamic data and clinical studies. MIC_{50} and MIC_{90} indicate
100 the lowest concentrations of the antimicrobial agent inhibiting visible growth of 50% and 90% of
101 the bacterial population respectively. However, few interpretation criteria for *Aeromonas* spp have
102 been published to date. The only available breakpoints proposed by the CLSI are from clinical
103 isolates adapted from *Enterobacteriaceae*, while no criteria have been established by EUCAST.
104 Epidemiological cut-off values (ECVs) must be set to discriminate wild-type strains (with no

acquired resistance mechanism to the tested antibiotic) from non-wild-type strains (with one or more acquired resistance mechanisms) (Kahlmeter et al., 2003). These cut-off values are the upper limit of the MIC distribution of fully susceptible strains.

The purpose of the present study was to estimate the MICs of *Aeromonas* spp strains isolated from *Sparus aurata* against 15 antimicrobial agents, and to determine the ECVs for *Aeromonas* spp.

2. Materials and methods

2.1 Fish sampling

The study was conducted on gilthead sea bream (*Sparus aurata*) collected from six offshore mariculture farms in three Italian regions (Sardinia, Sicily and Tuscany). All fish farms were characterized by intensive rearing systems in sea cages. Water salinity was ca. 33% and the temperature ranged between 16°C and 22°C. Twenty commercial size (~250 g) *Sparus aurata* specimens were randomly collected at each farm during two different visits conducted four months apart. After collection, fish were slaughtered by immersion in fusing ice, placed in expanded polystyrene boxes and covered with a plastic film then transported to the laboratory under refrigeration and processed within three hours after collection.

2.2 Microbiological analysis

Samples of skin, gills, muscle and intestinal content were aseptically collected from each specimen for microbiological analysis. The initial suspension and decimal dilution for microbiological examination were prepared according to ISO 6887–1:1999. Each matrix was tested for *Aeromonas species* (presence/absence) inoculating 0.1 mL of homogenized PBS (pH 7.4) on plates of Aeromonas Medium Base (Ryan's medium) (Oxoid, Basingstoke, UK) supplemented with ampicillin selective supplement at 5 mg/L. The agar plates were incubated at +30°C for 48 hours. Colonies with typical growth characteristics, opaque dark green with darker centres, were picked and subcultured on brain heart infusion (BHI) agar plates (BHI, Oxoid, Basingstoke, UK). After incubation, isolates were tested as follows: morphology in Gram staining, cytochrome oxidase,

amylase and trealose fermentation. After presumptive genus identification, strains were stored at -80°C for subsequent genetic confirmation and species identification.

2.3 Bacterial identification

Genus identification of isolates was confirmed by PCR (Khan et al., 2009). To avoid over-representation of clones, 16S ribosomal DNA sequencing was conducted on a selection of strains to identify bacterial species. A hierarchical method was used to select up to three strains from each of the following nested criteria: region of collection (three levels), fish farm (two levels), fish specimens (40 levels) and fish matrix (4 levels). For species identification, colonies with morphological and biochemical features of *Aeromonas* spp were grown overnight at 37°C in tryptone soya broth (Oxoid). DNA was extracted using the following protocol: 1mL of broth culture (10^8 CFU/mL) was centrifuged at 12,000 g for five minutes, then the pellet was resuspended in 1mL of phosphate-buffered saline, boiled for five minutes, and centrifuged again (Bottero et al., 2004). The supernatant was stored at -20°C until use. The DNA was quantified using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). All extracted DNA were subjected to sequencing analysis with the MicroSeq 500 16S rDNA bacterial sequencing kit (Thermo Fisher Scientific). 16S rDNA amplicons were purified by Exo-Sap treatment according to the manufacturer's recommendations (USB Europe, Staufen, Germany). Forward and reverse sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Thermo Fisher Scientific). The extended products were purified with DyeEx 2.0 Spin kit (Qiagen, Valencia, CA, USA) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyzer (Thermo Fisher Scientific). The electropherograms were analyzed using Chromas 2.22 software (Technelysium, Epoch Life Science Inc.) and the sequences were submitted to the BLAST similarity search software on the National Center for Biotechnology Information (NCBI) website.

2.4 Antibiotic susceptibility

156 Antibiotic susceptibility was determined for *Aeromonas* strains at the genus level. MICs of 15
157 antibiotics were measured by the broth microdilution method (CLSI, 2011). The antimicrobial
158 agents chosen among those mainly used in aquaculture and human therapy were: oxolinic acid
159 (OXA), ampicillin (AM), amoxicillin (AMX), cephalothin (CF), cloramphenicol (CL),
160 erythromycin (E), florfenicol (FF), flumequine (FM), gentamicin (GM), kanamycin (K),
161 oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim
162 (TMP). To obtain stock solutions, the antibiotic powders (Sigma Aldrich, MI, Italy) were weighed
163 and dissolved in the following solvents (Sigma Aldrich): phosphate buffer, pH 8.0, 0.1 mol/L (AM),
164 phosphate buffer, pH 6.0, 0.1 mol/L (AMX and CF), ethanol 95% (CL and E), methanol 96% (FF),
165 aqueous alkaline solution NaOH 0.1M + ethanol 2:1 (FM), aqueous alkaline solution NaOH 0.1M,
166 pH 10 (OXA), methanol-water 2:1 (OT and TE), aqueous acidic solution HCl 10% (TMP), and
167 deionized water (GM, K, S and SZ). Once dissolved, each stock solution (2,560 µg/mL) was
168 dispensed in 1.5 mL aliquots into polypropylene vials and frozen at - 80 °C until use. Each
169 microtitre plate was prepared with 12 serial twofold dilutions of each antibiotic stock solution
170 (Work Station - Micro Star, Hamilton, Bonaduz GR, Switzerland) with deionized water (phosphate
171 buffer, pH 6.0, 0.1 mol/L, only for AMP and AMX antibiotics). The antibiotic concentrations
172 obtained ranged between 0.06 µg/mL and 128 µg/mL (0.12-256 µg/mL for SZ antibiotic). Strains
173 were subcultured twice in BHI plates before preparation of the inoculum. After overnight
174 incubation at 37 °C, two or more colonies were picked from BHI plates and dissolved in salt
175 solution (0.85% w/v) to obtain 0.5 McFarland turbidity, measured using a portable photometric
176 reader (Densimat, bioMérieux, Lyon, France). Each bacterial suspension was further diluted (1:100)
177 in cation-adjusted Mueller Hinton broth (CAMHB, Oxoid, Basingstoke, UK) supplemented with
178 NaCl (1%) to obtain an inoculum concentration of ca.10^6 cfu/mL. Fifty µL of the final suspension
179 were transferred into microtitre wells (one strain for each row of the microplate) containing 50 µL
180 of each antimicrobial agent. The density of the final inoculum in each well was ca. $5x10^5$ cfu/mL.
181 The reference strain *E. coli* ATCC 25922 was used as quality control. Each microplate was

subsequently incubated under aerobic conditions for 20 hours at 35 °C. The MIC of each antibiotic was compared with breakpoint values to determine resistance (CLSI, 2005, 2007, 2011, 2016; NCCLS, 1998, 1999, 2002). The MIC range and mode, MIC_{50} and MIC_{90} of each antimicrobial agent were also determined. Multiple antibiotic resistance (MAR) among *Aeromonas* spp strains was evaluated applying the MAR index defined as *a/b*, where "*a*" was the number of antimicrobials the isolate was resistant to and "*b*" was the number of antibiotics against which the isolate was tested. According to Krumperman (1985), a MAR index below 0.2 is interpreted as strains originating from animals in which antibiotics are seldom or never used, while a MAR index above 0.2 is interpreted as strains originating from an elevated selective pressure environment where antibiotics are frequently used.

2.5 Epidemiological cut-off values

The distribution of MIC values served to determine the ECVs. The statistical determination of ECV values for each antimicrobial agent at genus level was conducted according to Turnidge et al. (2006) using the freely available ECOFFinder Microsoft Excel spreadsheet calculator (https://clsi.org/education/microbiology/ecoffinder/). The spreadsheet is designed to estimate the ECVs based on the observed MIC of the tested bacterial population, i.e. it estimates the MIC value best describing where wild type distribution ends.

3. Results

3.1 Isolation and identification of Aeromonas *spp*

Aeromonas spp were observed in 98 skin samples (30.6%), 154 gills (48.2%) and 40 gut contents (12.5%) whereas the bacteria were never detected in muscle. One hundred and four *Aeromonas* spp strains isolated from *Sparus aurata* were speciated by 16S ribosomal DNA sequencing. Of the total strains, 59 originated from Tuscany, 40 from Sicily and five from Sardinia. The *Aeromonas* strains were isolated from skin (n. 48), gut content (n. 20) and gills (n. 36). Sequencing identified 23 different *Aeromonas* species or species-complex. The most frequently recovered species were

208 *Aeromonas media* (15 strains, 14.4%), *Aeromonas salmonicida/bestiarium/hidrophila/caviae*
209 species-complex (12 strains, 11.5%), *A. molluscorum* (11 strains, 10.6%) and *A. bivalvum* (10
210 strains, 9.6%). Table 1 reports a complete list of *Aeromonas* species and species-complex identified
211 and the relative number of strains. Fig. 1 shows their distribution by region of origin.
212 *3.2 Antimicrobial susceptibility*
213 For some of the selected antimicrobial agents, the reference strain used as quality control for the
214 MIC determination assay indicated the Aeromonas strains in compliance with CLSI
215 recommendations (CLSI, 2005). Over 90% of the speciated *Aeromonas* strains showed
216 susceptibility to CL, FF and GM antibiotics. Table 1 lists the resistance profile for each *Aeromonas*
217 species. All tested *Aeromonas* strains showed resistance to two or more antibiotics. One strain of *A.*
218 *bivalvium* and one strain of the *A. punctata/hydrophila/enteropelogenes* species-complex were
219 resistant to 11 antibiotics while one *A. molluscorum* strain was resistant to 12 antibiotics. Table 2
220 reports the MAR index indicating the multiple antibiotic resistance of *Aeromonas* spp strains by
221 region of origin. Table 3 shows the MIC_{50}, MIC_{90}, mode and range and cut-off of MICs for each
222 tested antibiotic, and the number of sensitive, intermediate and resistant strains with reference to the
223 CLSI breakpoints. The most frequent combination of antibiotic resistance profiles was AM, AMX,
224 CF, E, S, SZ and TMP. For CF and SZ, the MIC_{50} and MIC_{90} values were above the tested range
225 (128 and 256 µg/mL respectively).
226 *3.3 Determination of wild-type strains*
227 The ECVs were computed for 12 out of 15 antimicrobial agents. In addition to CF and SZ, no
228 values were computed for TMP due to a high number of isolates with MIC values greater than the
229 upper limit of the tested dilutions (128 µg/mL).
230 The wild-type strains ranged between 59.6% and 96.2% of the tested strains. Thirty-one strains
231 (29.8%) were wild-type for all antibiotics with a computable ECV. One strain (*A. bivalvium*)
232 resulted wild-type exclusively for E, one strain for GM and K (*A.*
233 *punctata/hydrophila/enteropelogenes* species-complex) and one strain (*A. molluscorum*) for GM, K

234 and S. The remaining 70 strains were wild-type for five up to 11 different antibiotics, yielding 31
235 different combinations of antibiotic wild-type profiles. More than 80% of wild-type strains were
236 resistant to eight antibiotics (CL, E, FF, GM, K, OT, S, TE). Table 3 reports the complete results on
237 the ECVs and percentage of wild-type strains.
238
239 **4. Discussion**
240 The worldwide growth of aquaculture has seen the development of intensive fish farming. This in
241 turn has been associated with an extensive use of antibiotics to treat or prevent bacterial infections.
242 Regulations governing the antimicrobial agents authorized in fish farming differ from country to
243 country. The selective pressure exerted by intensive fish farming has resulted in the emergence of
244 antibiotic-resistant food-borne pathogens, opportunistic pathogens and human commensal flora of
245 food animals (Sorum, 2006; Teuber, 2001; Witte, 2000). The potential transfer of antibiotic
246 resistance from the aquatic environment to humans through direct contact or via the food chain is a
247 serious concern for human health (Marshall and Levy, 2011). Antibiotic resistance monitoring fails
248 to collect extensive information of the classes of antimicrobials used in aquaculture and the efficacy
249 of antibiotics (Cabello et al., 2013).
250 The present study provided useful information on the resistance of *Aeromonas* spp isolated from
251 gilthead sea bream (*Sparus aurata*) reared in Italian fish farms. *Aeromonas* spp were widely
252 distributed in skin, gills and intestinal content of *Sparus aurata* whereas they were never detected in
253 muscle. *Aeromonas* spp can potentially cause human illness by direct contact or through the
254 ingestion of contaminated fish (Janda & Abbott, 2010).
255 Clinical breakpoints are useful to assess the efficacy of antibiotics during treatments, while the
256 determination of ECVs will establish the emergence of antibiotic resistance mechanisms within a
257 bacterial population. Based on these values, the present study documented high resistance rates for
258 β-lactams, erythromycin, sulfadiazine and trimethoprim. The MIC_{90} of ampicillin, amoxicillin and
259 cephalothin (>128 µg/mL) were higher than the reference breakpoints for resistance and the number

of resistant strains ranged between 40.4% and 86.5%. The literature reports resistance rates for these antibiotics as high as 100% (Hatha et al., 2005; Snoussi et al., 2011). The MIC_{50} for ampicillin and amoxicillin was 16 µg/mL, an intermediate value between the reference breakpoints for susceptibility and resistance, while the MIC_{50} for cephalothin was greater than the reference value for resistance (>128 µg/mL). Amoxicillin and ampicillin are susceptible to β-lactamase and to rapid onset antibiotic resistance especially when the antibiotic is repeatedly used in a short time period, typical of intensive fish farming systems. Three different types of β-lactamase have been observed in *Aeromonas* spp (Walsh et al., 1997), but little information is available on the $ECVs_T$ for *Aeromonas* spp and limited to few antibiotics, hampering a comparison with the MIC distribution observed in our microbial population. Despite the high resistance rates observed for β-lactam antibiotics, based on the ECVs computed for amoxicillin and ampicillin, an elevated percentage of strains could be considered wild-type.

Among the quinolone antibiotics, various countries have authorised oxolinic acid (a first generation quinolone) for therapeutic use in aquaculture, while flumequine is the only one of the five fluoroquinolone antibiotics listed in Reg. EC 37/2010 authorised for fish farming. These antibiotics are used in mariculture for the treatment of furunculosis caused by *Aeromonas salmonicida* (Giraud et al., 2004). Oxolinic acid and flumequine showed resistance in 32.7% and 22.1% of the tested *Aeromonas* spp strains respectively. The antibiotic resistance of *Aeromonas* spp in the present study is in agreement with previous investigations conducted in mariculture farms where resistance was between 25% and 50% (Inglis et al., 1991; Snoussi et al., 2011; Cattoir et al., 2008).

The ECV computed in the present study was 0.25 µg/mL for both oxolinic acid and flumequine while the literature reported values of 0.031 µg/mL and 0.06 µg/mL respectively (Baron et al., 2017; Smith and Kronvall, 2015). However, these results are not comparable as the values obtained in our study coincided with the lowest dilution tested.

Due to their broad-spectrum activity, low toxicity and cost, tetracyclines are the most commonly used antibiotics in both human and veterinary medicine. In mariculture, oxytetracycline is

authorized for therapeutic immersion in Europe, while elsewhere (USA and Asian countries) it is also administered with medicated foods. The widespread use of tetracycline has resulted in the dissemination of resistance to many marine bacteria (Furushita et al., 2003) with the number of resistant strains ranging from 7.7% (TE) to 11.5% (OT). These results are comparable with previous studies where *Aeromonas* spp strains showed sensitivity to tetracycline and oxytetracycline (Awan et al., 2009). *Aeromonas* spp strains showed high in vitro sensitivity against both oxytetracycline (80.8%) and tetracycline (85.6%) in the tetracycline class with MIC_{50} values below the reference breakpoint of susceptibility (≤ 1µg/mL) and MIC_{90} of 4 µg/mL and 16 µg/mL for tetracycline and oxytetracycline, respectively. The MIC_{50} and MIC_{90} observed in our study were within the range reported in previous investigations conducted on Aeromonads isolated from freshwater fish (Baron et al., 2017; Čížek et al., 2010). The ECV of *Aeromonas* spp was 2 µg/mL for both tetracycline and oxytetracycline, values greater than those reported by Barone et al. (2017).

Among the Macrolides, erythromycin is the bacteriostatic drug of choice against Gram-positive bacteria. Although it is not approved for aquaculture use in most European countries, the EU has established maximum residue limits (MRLs) (Reg. EC 37/2011). In the present study, erythromycin showed little effectiveness against *Aeromonas* spp. The MIC_{90} of erythromycin was higher than the reference breakpoints (8 µg/mL) with 84.6% of the tested *Aeromonas* strains showing resistance. This high resistance rate and the MIC_{50}, MIC_{90} and ECV for erythromycin are in agreement with other studies (Mejdi et al., 2010; Baron et al., 2017).

Trimethoprim is mainly used in fish culture and often combined with sulfadiazine in commercial preparations. Because of the potential carcinogenic effect of both antibacterial agents, the EU set MRLs in fish muscle. The present study tested the two antimicrobials independently. Low efficacy was obtained for sulfonamides with resistance rates of 92.3% and 69.2% of strains for sulfadiazine and trimethoprim, respectively. For both sulfadiazine and trimethoprim the MIC_{50} and MIC_{90} were above the reference breakpoints, so the ECVs could not be estimated. These values could not be

311 compared with other studies on *Aeromonas* spp as these antibacterials are generally used in
312 combination.
313 Aminoglycoside antibiotics showed intermediate MIC_{90} values for gentamicin (8 µg/mL) and
314 kanamycin (32 µg/mL), whereas they were above the breakpoint for streptomycin (64 µg/mL) to
315 which 39.4% of strains were resistant. The ECVs for gentamicin and streptomycin were greater than
316 those observed by Baron et al. (2017), while the MIC_{50} for streptomycin was comparable with data
317 obtained by Goñi-Urriza et al. (2000).
318 In the present study, chloramphenicol and florfenicol MIC_{90} were lower than the reference
319 breakpoints. These results were expected for chloramphenicol as it has been banned from use in
320 animal food production since 1994 (EC 1430/94) due to its serious side effects on human health
321 (irreversible aplastic anaemia). While florfenicol is registered for use in aquaculture only in some
322 European countries, resistance to the fenicol category ranged between 2.9 % and 3.8% of the tested
323 strains. The MIC_{50} and ECVs for these two antimicrobials were in agreement with values reported
324 by Baron et al. (2017).
325 Overall, *Aeromonas* spp showed elevated multiple resistance to the antibiotics tested. Most of the
326 strains (82.7%) showed a MAR index between 0.3 and 0.5 (corresponding to resistance to four to
327 eight different antibiotics) while six strains were resistant to nine to 11 antibiotics. One strain, *A.*
328 *punctata*/*hydrophila*/*enteropelogenes*, was resistant to 11 out of 15 antibiotics tested, indicating that
329 the isolates were exposed to high-risk sources of contamination with broad use of antibiotics, as in
330 intensive fish farming. This result is in agreement with previous studies indicating the high
331 antibiotic resistance of *Aeromonas* spp (Dumontet et al., 2000; Nguyen et al., 2014). The antibiotics
332 most frequently associated with multiple resistance were amoxicillin, ampicillin, cephalothin,
333 erythromycin, streptomycin, sulfadiazine and trimethoprim.
334
335 **5. Conclusions**

The present study confirms that selective pressure in the aquatic environment of intensive fish farms leads to acquired antibiotic resistance by *Aeromonas* spp in gilthead sea bream reared in Italy. Compared to clinical breakpoints, measuring epidemiological cut-off values allows a better distinction between wild-type strains and strains which have acquired drug resistance due to selective pressure. The multiple antibiotic resistance of almost all strains raises serious concerns due to the possible transfer via food of antibiotic-resistant bacteria to humans or the acquisition of antibiotic resistance by human pathogens. In the light of these findings, regular monitoring programmes should be stepped up to check for antibiotic resistance in the aquaculture production of fish for human consumption.

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510

Table 1 – Resistance of *Aeromonas* species and species-complex to 15 antimicrobial agents

Species	N (%)	OXA	AMX	AM	CF	CL	E	FM	FF	GM	K	OT	S	SZ	TE	TMP
A. media	15 (14.4%)	8	8	6	13	1	13	5	-	1	3	1	6	13	1	12
A. enteropelogenes	1 (0.9%)	-	1	1	1	-	1	-	-	-	-	-	-	1	-	1
A. bivalvium	10 (9.6%)	2	6	6	10	-	10	1	-	1	1	2	6	10	1	8
A. media/veronii	4 (3.8%)	2	4	4	4	-	4	2	1	-	-	-	-	4	-	4
A. salmonicida/bestiarium/hidrophila/caviae	12 (11.5%)	1	7	8	12	1	11	-	1	-	-	1	7	12	1	9
A. punctata/hydrophila/enteropelogenes	1 (0.9%)	1	1	1	1	1	1	1	-	-	-	1	1	1	-	1
A. salmonicida/bestiarium	8 (7.7%)	-	4	5	8	-	8	-	-	-	-	1	6	8	1	3
A. popoffii	3 (2.9%)	2	1	2	3	-	3	-	-	-	-	-	1	2	-	2
A. molluscorum	11 (10.6%)	7	1	1	4	1	3	6	1	1	2	5	4	11	3	6
A. encheleia	2 (1.9%)	1	1	1	2	-	2	-	-	-	-	-	2	2	-	2
A. hydrophila/salmonicida/bestiarum	4 (3.8%)	1	-	-	4	-	3	-	-	-	-	-	-	4	-	4
A. punctata	6 (5.8%)	1	3	1	6	-	6	1	-	-	-	-	1	4	-	6
A. bivalvium/ popoffii	3 (2.9%)	-	-	-	2	-	3	-	-	-	-	-	-	3	-	1
A. salmonicida/bestiarium/popoffii	2 (1.9%)	1	-	-	1	-	2	1	-	-	-	-	-	2	-	-
A. media/hydro	2 (1.9%)	1	2	2	2	-	2	1	-	-	-	-	-	2	-	2
A. allosacarophila	1(0.9%)	-	-	-	1	-	1	-	-	-	-	-	-	1	-	1

A. tasmaniensis/ hydro/ punctata	2(1.9%)	1	2	-	2	-	2	1	-	-	-	1	1	2	1	2
A. media/ punctata	5 (4.8%)	4	3	2	3	-	5	3	-	1	2	-	2	3	-	4
A. encheleia/ molluscorum	3 (2.9%)	-	2	2	3	-	3	-	-	-	-	-	1	2	-	1
A. salmonicida/ sobria/popoffii	4 (3.8%)	-	-	-	4	-	3	-	-	-	-	-	1	4	-	2
A. salmonicida	3 (2.9%)	-	-	-	3	-	1	-	-	-	-	-	-	3	-	1
A. salmonicida/sobria	1 (0.9%)	-	-	-	1	-	1	-	-	-	-	-	1	1	-	-
A. molluscorum /eucrenophila	1 (0.9%)	1	-	-	-	-	-	1	-	-	-	-	1	1	-	-
Total	104 (100%)	34	46	42	90	4	88	23	3	4	8	12	41	96	8	72

oxolinic acid (OXA), amoxicillin (AMX), ampicillin (AM), cephalothin (CF), cloramphenicol (CL), erythromycin (E), flumequine (FM), florfenicol (FF), gentamicin (GM), kanamycin (K), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP).

Table 2. Multiple antibiotic resistance (MAR) index of *Aeromonas* spp strains isolated from gilthead sea bream reared in 3 Italian regions.

MAR index	Region			Total
	Sardinia	Sicily	Tuscany	
0.1	-	*n* = 1	*n* = 2	*n* = 3
0.2	-	-	*n* = 13	*n* = 13
0.3	-	*n* = 14	*n* = 31	*n* = 45
0.4	*n* = 3	*n* = 7	*n* = 4	*n* = 14
0.5	*n* = 1	*n* = 14	*n* = 7	*n* = 22
0.6	-	*n* = 1	*n* = 1	*n* = 2
0.7	*n* = 1	*n* = 2	*n* = 1	*n* = 4
0.8	-	*n* = 1	-	*n* = 1
0.9	-	-	-	-

Table 3. MIC (µg/mL) and antimicrobial susceptibility of *Aeromonas* spp strains isolated from *Sparus aurata*. *=M45-P (CLSI, 2005); a=M100-S26 (CLSI, 2016); b= M42/49 (CLSI, 2011); c=M31A2 (NCCLS, 2002); d=M31A (NCCLS, 1998).

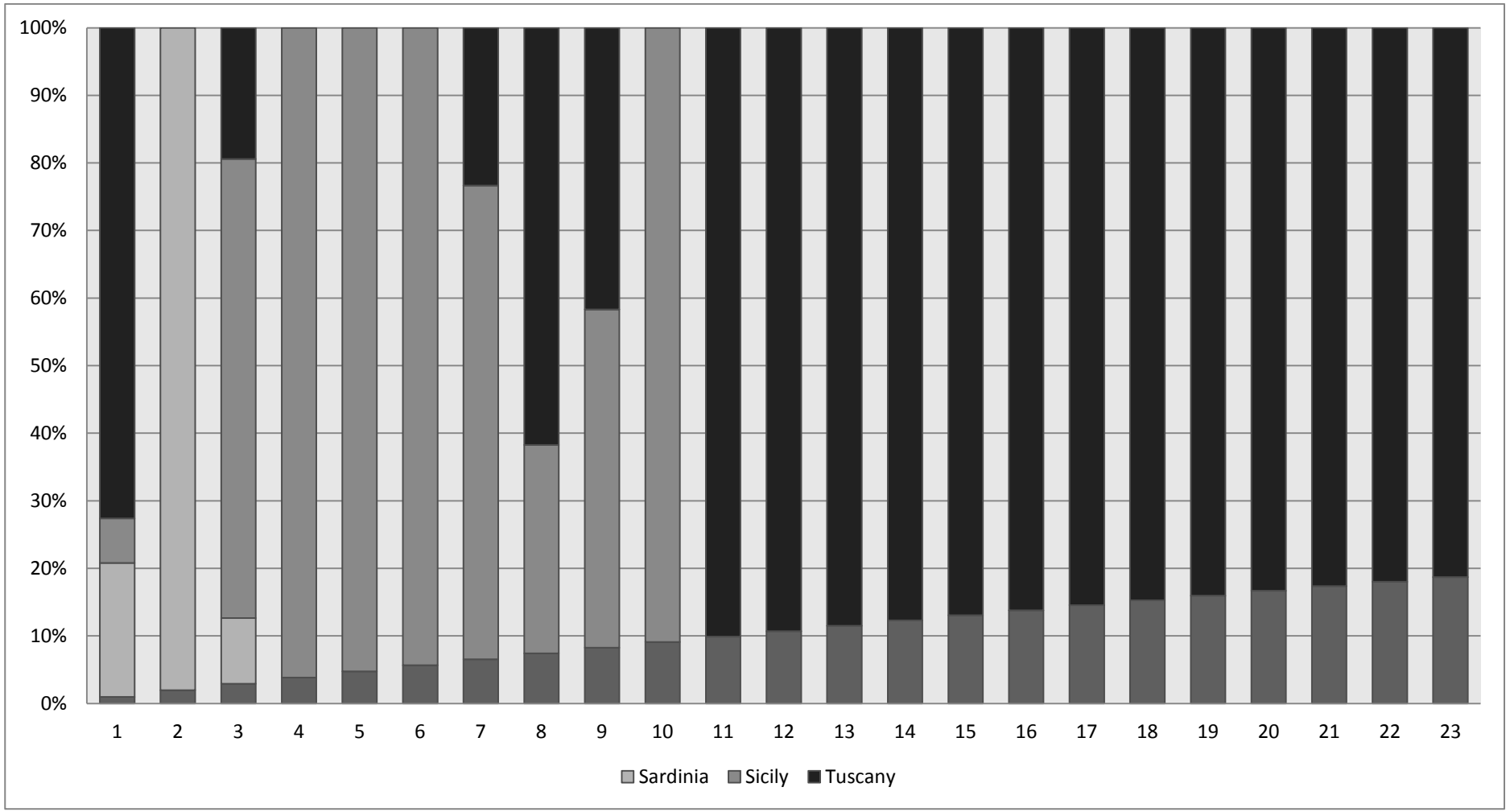
Antibiotic	Breakpoints	MIC_{50}	MIC_{90}	Moda	Range	S (%)	I (%)	R (%)	ECV	WT strains (%)
OXA	≤0.12-≥1[b]	0.06	16	0.06	0.06-≥128	65 (62.5)	5 (4.8)	34 (32.7)	0.25	69 (66.3)
AMX	≤8-≥32*	16	≥128	8	0.06-≥128	44 (42.3)	14 (13.5)	46 (44.2)	32	66 (63.5)
AM	≤8-≥32*	16	≥128	16-≥128	0.06-≥128	42 (40.4)	20 (19.2)	42 (40.4)	64	77 (74.0)
CF	≤8-≥32*	≥128	≥128	≥128	0.06-≥128	14 (13.5)	-	90 (86.5)	ND	ND
CL	≤8-≥32*	0.5	4	0.5	0.25-64	98 (94.8)	2 (1.9)	4 (3.8)	2	91 (87.5)
E	≤0.5-≥8[c]	16	64	8	0.06-≥128	9 (8.7)	7 (6.7)	88 (84.6)	32	93 (89.4)
FM	≤2-≥4[c]	0.12	16	0.06	0.06-≥128	81 (77.9)	-	23 (22.1)	0.25	62 (59.6)
FF	≤4-≥8[b]	1	4	0.5	0.06-64	101 (97.1)	-	3 (2.9)	2	93 (89.4)
GM	≤4-≥16*	2	8	2	0.12-32	92 (88.5)	8 (7.7)	4 (3.8)	8	100 (96.2)
K	≤16-≥64[a]	8	32	16-32	0.5-≥128	89 (85.6)	7 (6.7)	8 (7.7)	32	96 (92.3)
OT	≤1-≥8[b]	0.5	16	0.5	0.12-≥128	84 (80.8)	8 (7.7)	12 (11.5)	2	84 (80.8)
S	≤6-≥25[d]	16	64	16	1-≥128	9 (8.7)	54 (51.9)	41 (39.4.)	64	90 (86.5)
SZ	≤38-≥76[a]	≥256	≥256	≥256	0.12-≥256	8 (7.7)	-	96 (92.3)	ND	ND
TE	≤1-≥8[b]	0.5	4	0.5	0.12-≥128	89 (85.6)	7 (6.7)	8 (7.7)	2	85 (81.8)
TMP	≤8-≥16[a]	32	64	64	0.12-≥128	32 (30.8)	-	72 (69.2)	ND	ND

MIC (Minimum Inhibitory Concentrations), S (susceptible strains), I (intermediate strains), R (resistant strains), ECV (epidemiological cut-off value) (µg/mL), WT strains (wild-type strains), oxolinic acid (OXA), amoxicillin (AMX), ampicillin (AM), cephalothin (CF), cloramphenicol (CL),

erythromycin (E), flumequine (FM), florfenicol (FF), gentamicin (GM), kanamycin (K), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP). N.D.= not determined.

Figure

Figure 1. Percentages of *Aeromonas* species - species complex isolated from 6 mariculture farms in 3 Italian regions.



1=*A. media*; 2=*A. enteropelogenes*; 3=*A. bivalvium*; 4=*A. media/veronii*; 5=*A. salmonicida/bestiarium/hidrophila/caviae*; 6=*A. punctata/hydrophila/enteropelogenes*; 7=*A. salmonicida/bestiarium*; 8=*A. popoffii*; 9=*A. molluscorum*; 10=*A. encheleia*; 11=*A. hydrophila/salmonicida/bestiarum*; 12=*A. punctata*; 13=*A. bivalvium/popoffii*; 14=*A. salmonicida/bestiarium/popoffii*; 15= *A. media/hydro*; 16=*A. allosacarophila*; 17=*A. tasmaniensis/hydro/punctata*; 18= *A. media/punctata*; 19=*A. encheleia/molluscorum*; 20=*A. salmonicida/sobria/popoffii*; 21=*A. salmonicida*; 22=*A. salmonicida/sobria*; 23=*A. molluscorum /eucrenophila*.