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Antimicrobial resistance patterns in *Salmonella enterica* subsp. *enterica* and *Escherichia coli* isolated from bivalve molluscs and marine environment

Federica Giacometti^a, Annalisa Pezzi^b, Giorgio Galletti^b, Marco Tamba^b, Giuseppe Merialdi^b, Silvia Piva^{a*}, Andrea Serraino^a, Silva Rubini^b

^aDepartment of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano dell'Emilia, Bologna, Italy

^bExperimental Institute for Zooprophyllaxis in Lombardy and Emilia Romagna, Via Bianchi 9, 25124 Brescia, Italy

(*) **Corresponding author:** Silvia Piva, Tel.: +39 51 2097321; **Fax.:** +39 51 2097346.

E-mail address: silvia.piva@unibo.it (S. Piva).

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27 ABSTRACT

28 The current study presents data on the antimicrobial resistance (AMR) patterns of 102 *S. enterica*
29 subsp. *enterica* (72 *Salmonella* ser. Typhimurium and 30 monophasic *S. Typhimurium* serovar) and
30 79 *Escherichia coli* (and their phylogenetic group determination) isolates from different species of
31 bivalve molluscs and from the water samples collected from the sub-areas of a mollusc production
32 area near Ferrara (Italy). These areas were classified as Long-line, Lupini, B-Out, B-in, and Sacca.
33 A retrospective evaluation was performed to assess the spatial trends of the resistance patterns of
34 *Salmonella* and *E. coli* and the temporal trend for *Salmonella*; the role of molluscs as AMR
35 indicators and the potential use of *E. coli* as a microorganism indicator of AMR occurrence in a
36 seawater environment were also investigated. Overall, 81% of *Salmonella* spp. and 75% of *E. coli*
37 isolates were resistant to, at least, one antimicrobial agent (AA) and 44% and 38% of the isolates
38 were multidrug resistant (MDR), respectively. The resistance levels of *Salmonella* were influenced
39 by the investigated serovars. Monophasic *S. Typhimurium* serovar showed the highest resistance
40 value with 70% of MDR isolates, in contrast with only 33% in *S. Typhimurium*. In monophasic *S.*
41 *Typhimurium* versus *S. Typhimurium*, twofold resistance levels were observed to streptomycin (97
42 versus 43%), ampicillin (80 versus 40%) and tetracyclines (67 versus 36%). Regarding the temporal
43 trend for *Salmonella*, strains were resistant to, at least, one AA, but this resistance was significantly
44 lower during the first years of this 17-year sampling; however, in parallel MDR isolates, the
45 resistance increased from 23% to a maximum level of 57% during the 2008-2012 period. On
46 assessing the spatial trends, the Sacca area was found to show the lowest number of *Salmonella* spp.
47 and *E. coli* strains resistant to, at least, one AA and MDR. For *E. coli*, the most commonly observed
48 resistance was towards ampicillin (56%), streptomycin (52%), sulphonamides (30%) and ceftiofur
49 (24%). The great majority (65%) of *E. coli* isolates belonged to the commensal phylogroups A and
50 B1, with B1 as the dominant one, whereas most MDR isolates belonged to phylogroup C. Molluscs
51 may be an efficient tool for antimicrobial resistance monitoring, and *E. coli* could be used as a
52 microorganism indicator of the occurrence of antimicrobial resistance in seawater environment.

53 1. Introduction

54 Bivalve molluscs represent an important tool for monitoring antibacterial-resistant *Escherichia coli*
55 and other members of the Enterobacteriaceae family in a coastal environment (Bighiu et al., 2019;
56 Grevskott et al., 2017). Antimicrobial resistance (AMR) is a natural phenomenon, but the misuse
57 and overuse of antibacterial agents in human and veterinary medicine, as well as in agriculture,
58 have increased the release of these substances to the environment, which threatens global public
59 health (Davies & Davies, 2010). Marine environments play an important role in accentuating
60 antimicrobial resistance, as an unknown amount of these drugs ends up either indirectly in the
61 receiving waters, or directly, as a result of intensive fish farming. As a consequence, living
62 organisms could be exposed to a variety of these compounds present in the environment at low
63 concentrations (Chiesa et al., 2018). Besides, coastal areas are subjected to faecal contamination of
64 human and animal waste coming from a variety of sources, including rivers, runoff from
65 agricultural and industrial activities and urban wastewater, resulting in the pollution of marine
66 habitats (Vignaroli et al., 2016; Grevskott et al., 2017). In this context, marine ecosystems are not
67 only an important reservoir for AMR, but also drive its emergence (Al-Sarawi et al., 2018; Taylor,
68 Verner-Jeffreys, & Baker-Austin, 2011; Williams et al., 2016).

69 In the European Union (EU), the microbiological quality of coastal waters (Directive 2006/7/EC),
70 as well as the sanitary control of shellfish produced and sold for human consumption, are
71 consistently monitored by measuring the abundance of faecal indicator bacteria, *E. coli* and
72 enterococci, in waters and the content of *E. coli* in the soft parts, flesh and intravalvular liquid of
73 harvested bivalves for the classification of the mollusc production areas as A, B or C (Regulation
74 EC 853/2004; Regulation EC 2017/265; Regulation EC 2019/265; Regulation EC 2019/267). In
75 addition, the food safety criteria concerning bivalves entering the market states that the absence of
76 *Salmonella* and the enumeration of *E. coli* are requested (Regulation EC 2073/2005; Regulation EC
77 2285/2015; Regulation 229/2019). In the European Union, *Salmonella* is the second most common

78 cause of human gastroenteritis (EFSA & ECDC, 2018). A considerable amount of epidemiological
79 data regarding the presence of *Salmonella* in seafood and its related illnesses is available, and the
80 risks of foodborne diseases associated with *Salmonella* in molluscs are classified as low (Davies &
81 Davies, 2010; NACMCF, 1992). Even if the microorganisms of the genus *Salmonella* are not
82 natural inhabitants of aquatic environments, several *Salmonella* serovars are widely distributed in
83 water (sea, estuarine, river) and in a variety of seafood, with the highest prevalence in molluscs,
84 shrimps, clams, and various fish species (Novoslavskij et al., 2016). *E. coli* is a common inhabitant
85 of the human and animal intestinal tract. *E. coli* may inhabit a host as a harmless symbiont or,
86 depending on the spectrum of encoded virulence factors, it can cause either intestinal or
87 extraintestinal infections (Logue et al., 2017). *E. coli* is a common indicator organism of faecal
88 contamination in aquatic systems, and it is recognized as an important player in the spread of
89 antibiotic resistance (Henriques et al., 2006; Szmolka & Nagy, 2013) due to its plasticity and high
90 aptitude to acquire genetic information through horizontal gene transfer. These characteristics
91 enable *E. coli* to exchange genetic material with other bacterial species (Araújo et al., 2017).

92 In Italy, a continuous microbiological monitoring of compliance with both the shellfish harvesting
93 areas and food safety criteria has been established by the official Veterinary Authorities, as required
94 by the EU regulations. Therefore, a continuous baseline data on the presence of *Salmonella* and *E.*
95 *coli* enumeration in water, shellfish flesh and intravalvular liquid is available. However, this
96 monitoring does not include the isolation and typing of *E. coli* isolates, as well as the AMR
97 evaluation of *Salmonella* and *E. coli*. The current study presents data on the AMR patterns of
98 *Salmonella* isolated with a continuous sampling history, from 2001 to 2017, and of *E. coli*, isolated
99 from 2016 to 2018, in the mollusc production area in the province of Ferrara, Emilia-Romagna
100 Region, Northern Italy. Here, even the distribution of *E. coli* in the phylogenetic groups was
101 investigated. A retrospective evaluation was performed with the following objectives: i) to
102 determine and compare the antimicrobial susceptibility among *Salmonella* serovars and the most
103 prevalent *E. coli* phylogenetic groups identified from bivalve molluscs and sea and brackish water,

104 as well as from different sampling areas; ii) to assess the spatial trends of resistance patterns of
105 *Salmonella* and *E. coli* and the temporal trend for *Salmonella* in the mollusc production area of the
106 province of Ferrara; iii) to investigate the role of molluscs as AMR indicators and to evaluate the
107 potential use of *E. coli* as a microorganism indicator of AMR occurrence in a seawater
108 environment.

109

110 2. Materials and methods

111 2.1 *Salmonella* spp. strain collection

112 In the present study, 102 *S. enterica* subsp. *Enterica* isolates from a strain collection of *Salmonella*
113 isolates were selected: a total of 72 *Salmonella* ser. Typhimurium and 30 monophasic *S.*
114 Typhimurium serovar (4,[5],12:i:-), isolated from 2001 to 2017, were considered. All these strains
115 were isolated during the official monitoring performed by the regional Veterinary Authority and
116 during a shellfish monitoring program using live bivalve molluscs and water samples from the
117 seashore and inland channels of the province of Ferrara, Emilia-Romagna region, Italy. Most of
118 these strains belong to a previous study (Rubini et al., 2018), which involved a total of 237
119 *Salmonella* isolates identified as *S. enterica* subsp. *enterica* (collected from a total of 10,757
120 seawater and molluscs samples from 1997 to 2015), in which 53 different serovars were observed.
121 *S. Typhimurium* was the dominant serovar (26.9%), followed by its monophasic variant 4,[5],12:i:-
122 (11.8%), Derby (6.3%), Newport (5.5%) and Thompson (4.6%). The *Salmonella* isolates considered
123 in this study represent about 50% of isolates and almost all the serovars monophasic *S.*
124 Typhimurium and *S. Typhimurium* collected in the investigated molluscs area. Each isolate refers to
125 a specific area of classification (A or B), a different area of sampling (namely, specific sampling
126 points within the considered area), a different source (Manila clams (*Ruditapes philippinarum*) (n =
127 30 isolates), striped clams (*Chamelea gallina*) (n = 1 isolate), mussels (*Mytilus galloprovincialis*) (n
128 = 3 isolates) and water samples (n = 68 isolates), and a different time of sampling (see Table 1 of
129 supplemental material). All the *Salmonella* strains were isolated in the North Western area of the

130 Adriatic Sea, facing the Southern area of the Po river delta, the major Italian river which, from
131 spring to estuary, flows through the Po Valley (Pianura Padana) for a total of 652 km. The Po
132 Valley is a densely populated area with a high number of large intensive animal farms. The Po
133 river, near its end, in the Adriatic Sea, creates a wide delta with a surface area of 31 km² and an
134 average depth of 1.5 m; its hydrographic network is mostly artificially regulated and, as a
135 consequence, its freshwater flows are partially independent of rainfalls. More than one third of the
136 lagoon surface is exploited for clam farming, with an annual production that has reached a
137 maximum of 87,000 t/year in 2011 (Bison, 2012). This area has been divided into five sub-areas,
138 following the mollusc production areas: i) long-line: the marine class A area used to breed mostly
139 mussels and secondary oysters; ii) Lupini: the coastal marine area, including seawater between 1
140 and 2 nautical miles, that is classified as a class A area in which natural banks of striped clams are
141 present and harvested; iii) B-Out: the narrow sea coastal area and inland waters, classified as a class
142 B area; iv) B-In: class B area that includes the inner channels directly connected to the sea, together
143 with the internal waters; v) Sacca: the class B area included between the Po river and the marine
144 coastline (figure 1). These last three sub-areas are used to breed mussels, Manila clams and oysters.

145

146 2.2. *E. coli* isolation and phylogenetic group determination

147 The isolation of *E. coli* was performed starting from the official samplings of Manila clams (*R.*
148 *philippinarum*) (n = 54), striped clams (*C. gallina*) (n = 3) and mussels (*M. galloprovincialis*) (n =
149 11), collected between 2016 and 2018 in the same aforesaid areas, and analysed for *E. coli*
150 enumeration using the Most Probable Number (MPN) technique specified in ISO 16649-3, and
151 from water samples (n = 11), using a membrane filtering method (APAT, 2003a, 2003b, 2003c).
152 Each sample refers to a different area of classification, namely A and B, a different area of
153 sampling, namely specific sampling points within the considered areas, a different source and a
154 different time of sampling (see Table 2 of supplemental material). The MPN method utilizes
155 Minerals Modified Glutamate Broth (MMGB) as the growth medium, and the material from

positive tubes, i.e., tubes whose colour has changed due to acid production, which was confirmed on Tryptone Bile with X-glucuronide (TBX) agar (Oxoid, Basington, UK); the suspected *E. coli* isolates were streaked onto MacConkey agar (Oxoid, Basington, UK) and incubated aerobically at $37 \pm 1^{\circ}\text{C}$. After 24 hours of incubation, colonies of Gram-negative rods were streaked onto Tryptone Soya Agar (Oxoid, Basington, UK). All the isolates were subjected to a DNA extraction using the REDExtract-N-Amp tissue PCR kit (Sigma, St Louis, MO) and were identified using the PCR procedure described by Clermont and colleagues (Clermont et al., 2008). Overall, 79 *E. coli* strains were considered in this study and were subjected to phylogenetic group determination. *E. coli* isolates were assigned to one of the 7 phylogroups (A, B1, B2, C, D, E or F) based on the PCR amplification of *chuA*, *yjaA*, *arpA* and *trpA* genes and of the TspE4.C2 DNA fragment (Clermont et al., 2013). The reference *E. coli* strain ATCC 25922 and sterile water were used as the quality and negative control samples throughout the procedure, respectively.

2.3 Antibiotic susceptibility testing

All the isolates were tested for their antimicrobial susceptibility to twelve antibiotic agents, according to the agar disk diffusion method described by the Clinical and Laboratory Standard Institute (CLSI, 2016). The antimicrobial panel was chosen considering the importance of antimicrobial classes in the treatment of human and animal infections and the intrinsic resistance of *E. coli* reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antimicrobials tested and the resistant breakpoints used in this study are reported in Table 1. For the breakpoint selection, when available, the epidemiological cut-off values proposed by EUCAST were used as the first choice. The EUCAST clinical breakpoints for Enterobacteriaceae were used as the second choice, and finally, for antimicrobials/bacterial species breakpoints not defined by EUCAST, the CLSI breakpoints for Enterobacteriaceae were chosen. Strains were considered multidrug resistant (MDR) when they showed resistance to three or more antimicrobial classes (Schwarz et al., 2010).

182 2.4 Statistical analysis

183 Descriptive statistics (absolute frequencies and percentages) have been provided regarding the
184 number of isolates: isolates resistant to, at least, one AA and MDR isolates. Findings were
185 presented by area of production, period of sampling, tested antimicrobial agent, *Salmonella*
186 serovars, and phylogenetic group of *E. coli*. The monitoring part was not planned and designed in
187 this study because it followed the real official activities performed by the Veterinary Authority.
188 Given the long period of official monitoring considered in this study, these 17 years of sampling
189 were arbitrarily divided into three different periods, namely, from 2001 to 2007 (17 isolates), from
190 2008 to 2012 (28 isolates) and from 2013 to 2017 (57 isolates), in order to have similar periods with
191 enough data to be compared. Furthermore, a limited number of samplings was performed for Long-
192 line, Lupini and B-out areas (11 for *Salmonella* and 19 for *E. coli* isolates), and therefore, the
193 overall isolates belonging to these areas were merged and, in the aggregate analysis, three definitive
194 areas were considered to assess the spatial resistance differences, namely in B-in, Sacca and the
195 area including Long-line, Lupini and B-out areas. Significant caution should be exerted for data
196 belonging to this merged area.

197 Pearson's chi-squared test and Chi-square test for trend were used to compare the temporal trends of
198 resistance patterns of *Salmonella*, whereas Chi-square test or Fisher's exact test were used to
199 compare the spatial trends for both *Salmonella* spp. and *E. coli* in the mollusc production areas of
200 the province of Ferrara. The significance limit was set at a $p < 0.05$.

201

202 3. Results and Discussion

203 In total, the antibiotic sensitivity tests showed that 19 (18.62%) *Salmonella* and 20 (25.31%) *E. coli*
204 isolates were susceptible to all the antimicrobials tested. Among the 102 *Salmonella* isolates,
205 encompassing the two different serovars, a total of 83 isolates (81.37%) showed resistance to, at
206 least, one antimicrobial agent and 45 isolates (44.12%) were MDR. More specifically, detailing the
207 patterns of resistance associated with the two considered serovars, monophasic *S. Typhimurium*

208 serovar showed the highest rates of resistance, with 96.67% and 70% of the isolates being resistant
 209 to, at least, one antimicrobial agent and having MDR, respectively, whereas 75% and 33.33% of
 210 *Salmonella* ser. Typhimurium isolates were resistant to, at least, one antimicrobial agent and
 211 possessed MDR, respectively. Tables 2 and 3 detail the antimicrobial susceptibility findings of
 212 *Salmonella* isolates of both serovars. The most common resistance in *Salmonella* isolates was
 213 towards streptomycin (58.8%), ampicillin (52%) and tetracyclines (45.1%), but different resistance
 214 levels were observed in the two considered serovars. The monophasic variant and *S. Typhimurium*
 215 showed resistance to streptomycin (97 and 43%, respectively), ampicillin (80 and 40%,
 216 respectively) and tetracyclines (67 and 36%, respectively). A very different behaviour was observed
 217 for other AAs: very high rates of resistance to sulphonamides (60%) were reported for the
 218 monophasic variant, whereas high resistance to carbapenems (23.6%) and chloramphenicol (21%)
 219 was observed in *S. Typhimurium* isolates. Over the years, the trend of resistance to, at least, one
 220 antimicrobial agent, significantly decreased ($p < 0.05$) from 94% to 72% and, in parallel, the
 221 proportions of MDR *Salmonella* isolates increased from 23.53% (4/17) between 2001 – 2007 to
 222 57.14% (16/28) and 43.86% (25/57) in the 2008-2012 and 2013-2017 periods, respectively, even
 223 though no significant differences were observed. More details are presented in Table 4.

224 Among the 79 *E. coli* considered from 2016 to 2018, a total of 59 isolates (74.68%) showed
 225 resistance to, at least, one antimicrobial agent and 30 isolates (37.97%) were MDR. The most
 226 common types of resistance of *E. coli* isolates were to ampicillin (56%), streptomycin (52%),
 227 gentamycin (35,4%), sulphonamides (30%) and ceftiofur (23%). Regarding the phylogenetic group
 228 determination, most isolates belonged to phylogroup B1 (n = 40; 50.63%), followed by phylogroups
 229 A (n = 11; 13.92%) and C (n = 11; 13.92%), and even to other less frequent phylogroups, D (n = 7;
 230 8.86%), B2 (n = 6; 7.59%) and E (n = 2; 2.53%). Two isolates were untypable and were classified
 231 as “unknown” (2.53%). The highest resistances were observed in phylogroup C, in which 90.91%
 232 and 72.73% of the *E. coli* isolates showed resistance to, at least, one antimicrobial agent and were
 233 MDR, respectively. Phylogroup C showed the highest resistances to almost all the considered

antimicrobial agents (data not shown). Tables 5 and 6 detail the antimicrobial susceptibility findings of *E. coli* isolates, also regarding the distribution of the frequent *E. coli* phylogroups.

No differences were observed regarding the resistance proportions of both *Salmonella* and *E. coli* isolates collected from molluscs and water samples, and from isolates belonging to classes A and B areas (data not shown). However, considering the spatial trends, the *E. coli* and *Salmonella* isolates in the area of Sacca had the lowest resistance for both serovars. Significant differences ($p < 0.05$) among the three different areas were observed, probably mostly regarding the lower percentage in Sacca, for MDR *Salmonella* spp. (20% in Sacca versus 51% in B-in and 73% in Long-line, Lupini B-out) and *S. Typhimurium* strains (9% in Sacca versus 67% in B-in and 39% in Long-line, Lupini B-out), but not for its monophasic variant. No significant differences were found regarding *Salmonella* spp. and *E. coli* strains resistant to, at least, one antimicrobial agent and even MDR among the three different areas. More details are reported in Table 7.

Extremely high resistance levels to, at least, one antimicrobial agent and high multi-resistant levels were observed in *Salmonella* spp. and *E. coli* isolates from molluscs, sea and brackish water in the investigated production area of Ferrara. The *Salmonella* data presented in this study comprise almost half of the overall *Salmonella* isolates of the last 20 years, from molluscs and water collected from the area of Ferrara, that is an important mollusc production area in Italy (Rubini et al., 2018), and therefore, they represent relevant data regarding the occurrence of antimicrobial resistance in *Salmonella* spp. isolates in molluscs. By plotting the amount of resistance of *Salmonella* for each period of the considered 17-year sampling, an evident rise in resistance from the first years of sampling with a doubling of the multi-resistance level in the following years must be mentioned, confirming the global antimicrobial resistance public health concern (ECDC, 2018; EFSA & ECDC, 2019). This increasing resistance is worrisome, particularly since several antimicrobial agents that were considered in this study are empiric or mere antibiotics which are commonly used in cases of serious human and animal infections.

259 Serovars monophasic *S. Typhimurium* and *S. Typhimurium* significantly contribute to the overall
260 numbers of *Salmonella* isolates: in the European framework, they are among the most commonly
261 reported serovars in human cases, as well as in food and animals, with the difference that *S.*
262 *Typhimurium* has been associated with many food and animal sources, whereas monophasic *S.*
263 *Typhimurium* has been mainly associated with pig and broiler sources (EFSA & ECDC, 2018). In
264 molluscs, few data are available in the literature and the positive findings are usually reported as
265 *Salmonella* spp.; however, the complexity of the global epidemiology of *Salmonella* requires
266 improved monitoring data of those serovars which are of the highest epidemiologic importance
267 (Rene et al., 2011).

268 Based on our findings, and in line with literature (EFSA & ECDC, 2019), the resistance levels for
269 *Salmonella* spp. are greatly influenced by the serovars investigated, and therefore, the
270 characterization at the serovar level is indispensable to address the temporal, geographic and source
271 trends. The MDR level was high, overall, but monophasic *S. Typhimurium* had the highest MDR
272 levels (70%) and the resistance level is twice as high for streptomycin, ampicillin, tetracyclines, and
273 sulphonamides (see Table 3); in contrast, the highest proportion of resistance for meropenem and
274 chloramphenicol were reported in *S. Typhimurium* isolates (see Table 3). *S. Typhimurium* and
275 monophasic *S. Typhimurium* were the second and third most common *Salmonella* serovars
276 identified in 2017 from human cases in Europe, respectively, in which the highest proportion of
277 resistance was observed for ampicillin, sulphonamides and tetracyclines, with occurrences of 53, 48
278 and 44% in *S. Typhimurium* and of 87, 87 and 88% in monophasic *S. Typhimurium* (EFSA &
279 ECDC, 2019). These data, even if with clear differences, is in line with our resistance findings,
280 whereas for meropenem, a non-negligible level of resistance was observed in our study, compared
281 to a full susceptibility found in human isolates. From a clinical point of view, fluoroquinolones and
282 third-generation cephalosporins are classified as critically important antimicrobials (CIA) of the
283 highest priority and represent the most important antimicrobial classes for treatment of

284 salmonellosis. Our findings showed moderate resistances to third-generation cephalosporins, but
285 which were still higher than data reported in humans, pigs, calves or cattle (EFSA & ECDC, 2019).
286 *E. coli* is an important foodborne pathogen. In addition, it is used worldwide as an indicator of
287 faecal contamination and as a hygiene indicator regarding sanitary quality or unsanitary conditions
288 (Metz, Sheehan, & Feng, 2020). In antimicrobial resistance, *E. coli* is also considered as a sensor of
289 the situation at each moment, and it has emerged as a major player in resistance: *E. coli* is typically
290 chosen as the representative indicator of antimicrobial resistance in Gram-negative bacteria, and its
291 monitoring in a specific population provides valuable data on the resistance occurring in that
292 population (EFSA & ECDC, 2019). Regarding the data on antimicrobial resistance in *E. coli*
293 isolated from molluscs and seafood or marine environments, available in the literature, comparisons
294 are arduous due to the differences in antimicrobial agents and breakpoints used; in most studies, the
295 most common resistances were observed for tetracycline, trimethoprim-sulfamethoxazole,
296 ampicillin and streptomycin, with lower prevalence values than those found here (Changkaew et al.,
297 2014; Grevskott et al., 2017; Ryu et al., 2012; Van et al., 2008; Vignaroli et al., 2015; Vignaroli et
298 al., 2016; Wang et al., 2011) or with values in line with ours (Al-Sarawi et al., 2018; Bighiu et al.,
299 2019). The prevalence of MDR strains in our study is higher than in most of the aforementioned
300 studies, but is still in line with those of other studies (Bighiu et al., 2019; Boss, Overesch, &
301 Baumgartner, 2016; Changkaew et al., 2014; Kumaran et al., 2010; Van et al., 2008). In addition, in
302 the European Union, the monitoring of antimicrobial resistance using the indicator *E. coli* in food-
303 producing animals (pigs and calves) and in their food products, has been mandatory since 2014: in
304 both animal species, tetracycline resistance was the most common trait, followed by resistance to
305 sulfamethoxazole and ampicillin (EFSA & ECDC, 2019).
306 *E. coli* is a common commensal inhabitant of the gastrointestinal tract, but it is also a common
307 cause of severe infections in humans, being the most frequent cause of bloodstream infections and
308 urinary tract infections among the Gram-negative bacteria. *E. coli* is involved in infections of both
309 community and healthcare origin, as well as being associated with intra-abdominal infections and

310 causing neonatal infections, such as early and late neonatal sepsis (ECDC, 2018; Wang et al.,
311 2011). *E. coli* is among the infectious agents whose antimicrobial resistance has reached an
312 extremely worrisome situation (WHO, 2014). According to the last report of the European Center
313 for Disease Control (ECDC) (ECDC, 2018) on antimicrobial resistance obtained through the
314 European Antimicrobial Resistance Surveillance Network database, that included only data from
315 invasive isolates (blood and cerebrospinal fluid), in Italy, in 2017, the prevalence of *E. coli* resistant
316 to aminopenicillins (ampicillin or amoxicillin), fluoroquinolones and to third-generation
317 cephalosporins was 67% (EU mean value, 58%), 45% (EU mean value, 26%) and 29.5% (EU mean
318 value, 15%), respectively. These data roughly reflect our findings regarding the resistance to
319 ampicillin (56%) and to third-generation cephalosporins (24%), except for enrofloxacin (5%) (see
320 Table 5). By contrast, while carbapenem resistance remains rare in Europe, and in Italy (0.3%)
321 (ECDC, 2018), in our study, worrisome percentages (above 10%) of this type of resistance were
322 observed. Carbapenems are among the most frequently prescribed antibiotics for the treatment of
323 bacterial infections in humans, even if their utility is being threatened by the worldwide rise of
324 carbapenem-resistant Enterobacteriaceae. Although the use of carbapenems is prohibited in food-
325 producing animals and restricted to pets in most European countries, these findings illustrate the
326 continuous spread of these highly resistant bacteria, accompanied by emerging public health
327 problems (Roschanski et al., 2017). In our study, resistance to ceftiofur was the fifth most common
328 resistance type. Ceftiofur is used in human and veterinary medicine and it has an activity against
329 both Gram positive and negative bacteria; however, third-generation cephalosporins have been
330 categorized as CIA and substances of the highest priority, while 3rd and 4th-generation
331 cephalosporins, as well as fluoroquinolones and polymyxins, are included in category 2, according
332 to the European Medicines Agency. Thus, those veterinary antimicrobials represent a higher
333 estimated risk for public health than other classes of antimicrobials. Our findings confirm that
334 bivalve molluscs are an efficient tool for identify antimicrobial resistance, being able to detect
335 antimicrobials used in the past and still used in food-producing animals and human beings, as well

336 as a useful opportunity to drive emergent threats to humans in terms of both antimicrobial resistance
337 and clinical aspects.

338 Given the worldwide phylogenetic analyses, *E. coli* showed an apparent clonal population structure
339 and a clear phylogenetic signal that appropriately reflect the relationship between strains (Tenaillon
340 et al., 2010). Different phylogenetic groups play distinct ecological roles and, therefore, the
341 classification of *E. coli* in the phylogenetic groups is important to understand its pathogenesis and
342 interaction with hosts. Hitherto, a robust phylogeny, was built and 8 phylogenetic groups were
343 identified (A, B1, B2, C, D, E, F, and the *Escherichia* cryptic clade I). The *E. coli* strains
344 responsible for extra-intestinal infections were far more likely to be members of phylogroups B2 or
345 D, rather than of A or B1, that usually lack a distinct virulence profile and are classified as
346 commensal or diarrheagenic strains (Clermont et al., 2013). In general, strains belonging to
347 different phylogenetic groups show different phenotypic and genotypic traits (Gordon & Cowling,
348 2003); differences in the phylogroup distribution are mainly ascribable to geographical (location
349 and climate) and host (diet, gut morphology, body mass) factors, explaining variable animals and
350 human phylogroup identification in several studies in the literature. The phylogroups that are
351 considered to contain highly virulent extra-intestinal *E. coli* strains are the B2 phylogroup, whose
352 microorganisms are a major cause of bacteraemia and neonatal sepsis (Cole et al., 2019; Vila et al.,
353 2016) and, to a lower extent, the D phylogroup, albeit it was detected as the most common in
354 urinary tract infections in humans (Farajzadeh Sheikh, 2019). Particular *E. coli* clones, more
355 frequently belonging to the phylogroup B2, are more prone than others to cause bacteraemia, but
356 they cannot be considered as having a deterministic causation relationship with extra-intestinal
357 diseases (Vila et al., 2016); the B2 group exhibits the highest diversity at both the nucleotide and
358 the gene content level, supporting its early emergence in the species lineage and suggesting that it
359 has a subspecies status. Considering the findings obtained in our study using Clermont typing, the
360 great majority (65%) of *E. coli* isolates belong to the commensal phylogroups A and B1 (these
361 groups appear as sister groups), but phylogroup B1 strains dominated with a prevalence of 51%,

362 both in general and in each of the three considered sampling areas. These data are in agreement
363 with several studies reporting a higher prevalence of A and B1 phylogroups in molluscs, with A
364 being the dominant one (Luna et al., 2010; Vignaroli et al., 2016; Vignaroli et al., 2012) in surface
365 water, in several animals (Johnson et al., 2017; Tomazi et al., 2018) and in water and vegetables
366 (Araújo et al., 2017). Unlike the studies performed by Vignaroli in *Chamelea gallina* clam in
367 Marche Region, Italy, and in marine sediments in the Adriatic Sea, in which the prevalence of MDR
368 strains was higher in phylogroup A, in our study, most MDR isolates belonged to phylogroup C,
369 followed by B2. Phylogroup C was the only one resistant to chloramphenicol and trimethoprim-
370 sulfamethoxazole, and for which the highest resistance values were reported for ampicillin,
371 streptomycin, sulphonamides, and tetracycline (data not shown). In addition, it should be noted that,
372 whereas isolates belonging to A and B1 phylogroups were observed to be MDR, depending on the
373 considered area of sampling, isolates belonging to phylogroup C were MDR, in spite of the area
374 considered. It has been observed that strains of these phylogroups vary in their phenotypic and
375 genotypic characteristics, ecological niche, lifestyle, and propensity to cause disease (Tenailon et
376 al., 2010); therefore, these findings confirm that waters receive contamination from a variety of
377 sources. In this context, there is a growing body of evidence suggesting that *E. coli* can not only
378 survive for extended periods of time, but also proliferate in several different environments, such as
379 water, soil, algae, plants, and manure, also in the absence of faecal inputs, thus supporting its use as
380 a water quality indicator (Nanayakkara, O'Brien, & Gordon, 2019) and as an indicator organism of
381 faecal contamination (Vignaroli et al., 2015). In addition, *E. coli* isolates that are responsible for
382 elevated counts (blooms) in freshwater reservoirs carry a capsule originating from *Klebsiella* spp.;
383 overall, about 7% of *E. coli* strains have acquired these capsules, which were observed to be non-
384 random distributed and restricted to A, B1 and C phylogroups (Nanayakkara, O'Brien, & Gordon,
385 2019). Given the limited number of isolates used in this study, and also the absence of any isolate
386 characterization, authors could not resolve these doubts. However, the diversity in both the

387 distribution of phylogroups among the different areas and the antimicrobial resistance patterns
388 remains.

389 Regarding the spatial trends of resistance, the area of Sacca was the one with the lowest number of
390 *Salmonella* spp. and *E. coli* isolates resistant to, at least, one antimicrobial agent and MDR;
391 conversely, the B-in area was the area with the highest number of *Salmonella* spp. and *E. coli*
392 strains resistant to, at least, one antimicrobial agent and MDR. However, whereas for *Salmonella*
393 spp., isolates collected from the Sacca area were susceptible to 6 out of the 12 antimicrobials tested
394 and showed the lowest levels of resistance, for *E. coli*, resistance was observed for all the
395 antimicrobials, even if with the lowest levels of resistance observed. In contrast, the B-in area was
396 the area with the highest number of *Salmonella* spp. And *E. coli* strains resistant to, at least, one
397 antimicrobial agent and MDR, as well as having the highest levels of resistance to the antimicrobial
398 agents. See Tables 2 and 5 for more details. Even if neither the area nor the environment could be
399 considered truly pristine, owing to the transfer of antibiotics and resistance genes via the wind,
400 tides, bird migration and other environmental elements (Barkovskii et al., 2012), the B-in area is
401 directly subjected to anthropogenic activities and it is exposed to industrial, municipal, agricultural
402 and zootechnical impacts, whereas the Sacca area is certainly less anthropogenically impacted than
403 the others. The aforesaid occurrence frequencies of antimicrobial resistance, as well as the
404 distributions of resistant *E. coli* isolates among the phylogroups, identify the B-in area as the main
405 source of antimicrobial resistance. *E. coli* strains that can presumably cause human extraintestinal
406 infections are not prominent within the *E. coli* population of the aquatic environment, but, at the
407 same time, our findings allow us to speculate on the fact that molluscs, and therefore, water
408 environment sites, cannot be considered risk-free. Obviously, further studies should be performed to
409 understand and verify the ecology of allochthonous and indigenous bacteria, as well as the
410 pathogens in aquatic environments.

411

412 4. Conclusion

413 In conclusion, bivalve molluscs are confirmed to be an efficient tool to detect antimicrobial
414 resistance. This study presents data on the antimicrobial resistance in *Salmonella* and *E. coli* strains
415 isolated from molluscs and water and showed high multi-resistant levels, as well as spatial and
416 temporal trends of resistance; however, for trend analysis of resistance, further studies are needed.
417 The overlap of the antimicrobial resistance data collected in this study with the previously reported
418 data of isolates from food animals and human beings suggests that testing the isolates from water
419 and molluscs could be a useful tool to monitor the evolution of AMR of some bacterial species. In
420 this context, routine antimicrobial susceptibility testing could be included as a parameter to be
421 investigated by official laboratories using isolates collected from different species of bivalve
422 molluscs, sea and brackish water by the official Veterinary Authorities during the official
423 monitoring of molluscs. Starting from the strength of the European microbiological monitoring of
424 molluscs and their production areas, the implementation of this step could further allow the
425 development of an official network at the national and/or European level, which could be able to
426 optimize the data referring to activities already planned and which could be performed by official
427 veterinary authorities. This monitoring could be useful to raise a constant and ongoing awareness of
428 antimicrobial resistance in the environment.

429

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433

434

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638

Highlights

- Bivalve molluscs are confirmed as efficient tool for antimicrobial resistance monitoring
- High multi-resistant levels were observed in *Salmonella* spp. and *E. coli* from molluscs and water
- Resistance levels for *Salmonella* are influenced by the investigated serovars
- Spatial trends of MDR *Salmonella* strains are observed

Figure 1

Figure 1. Scheme and classification of the five sub-areas in which bivalve molluscs and water samples were collected in the Ferrara area, Italy, from 2001 to 2018.

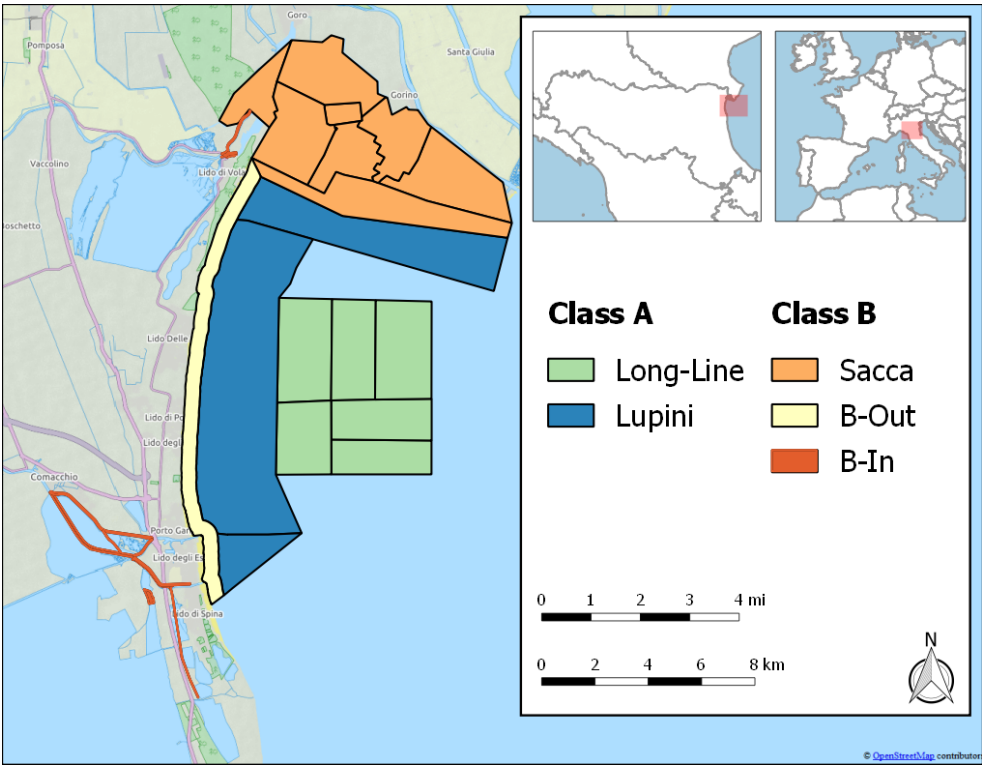


Table 1. List of tested antimicrobials and resistant breakpoints used.

Antimicrobials	Abbreviations	Disk content (µg)	Resistance breakpoint (zone diameter mm)	
			<i>Salmonella enterica</i> subsp. <i>enterica</i>	<i>Escherichia coli</i>
Ampicillin	AMP	10	≤17	≤13
Ceftazidime	CEFTZ	10	≤19	≤21
Ceftiofur	CEFT	30	≤17	≤17
Chloramphenicol	CHL	30	≤18	≤16
Enrofloxacin	ENR	5	≤16	≤16
Gentamicin	GENT	10	≤15	≤15
Meropenem	MER	10	≤26	≤24
Nalidixic Acid	NA	30	≤13	≤13
Streptomycin	ST	10	≤11	≤11
Sulfisoxazole	SULFAX	250	n.p.	≤12
Sulphonamides	SULFA	250	≤12	n.p.
Tetracycline	TETRA	30	≤16	≤11
Trimethoprim-sulfamethoxazole	T-SULFA	25	≤10	≤15

n.p. not performed; EUCAST breakpoints were chosen for all antimicrobials agents except for ceftiofur, enrofloxacin, nalidixc acid, streptpmycin for both *Salmonella enterica* subsp. *enterica* and *Escherichia coli*, as well as for sulfonamides for *Salmonella enterica* subsp. *enterica* and sulfisoxazole and tetracycline for *Escherichia coli*, for which CLSI breakpoints were used.

Table 2. Results of susceptibility testing to antimicrobial agents of *Salmonella* spp. isolates collected in different areas of mollusk production in the province of Ferrara.

Area	No. of isolates	No. of resistant isolates (%)												R at least one AA	MDR
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA		
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFA	T-SULFA		
B-in	61	37(60.7)	-	7(11.5)	13(21.3)	2(3.8)	1(1.6)	2(1.9)	41(67.2)	11(18)	28(45.9)	20(32.8)	2(3.3)	53(86.9)	31(50.8)
Long-line, Lupini, B-Out	11	8(72.7)	-	-	1(9)	-	-	-	9(81.8)	5(45.4)	8(72.7)	4(36.4)	1(9)	9(81.8)	8(72.7)
Sacca	30	8(26.7)	-	2(6.7)	6(20)	-	-	-	10(33.3)	-	10(33.3)	4(13.3)	-	21(70)	6(20)
Total	102	53(52)	-	9(8.8)	20(19.6)	2(1.9)	1(0.9)	2(1.9)	60(58.8)	16(15.7)	46(45.1)	28(27.4)	3(2.9)	83(81.4)	45(44.1)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 3. Results of susceptibility testing to antimicrobial agents of *Salmonella* ser. Typhimurium and monophasic *S. Typhimurium* isolates

Serovar	No. of isolates	No. of resistant isolates (%)												R at least one AA	MDR
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA		
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFA	T-SULFA		
<i>S.</i> Typhimurium	72	29(40.3)	-	7(9.7)	17(23.6)	1(1.4)	1(1.4)	2(2.8)	31(43.1)	15(20.8)	26(36.1)	10(13.9)	3(4.2)	54(75)	24(33.3)
monophasic <i>S.</i> Typhimurium	30	24(80)	-	2(6.7)	3(10)	1(3.33)	-	-	29(96.7)	1(3.3)	20(66.7)	18(60)	-	29(96.7)	21(70)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 4. Temporal trends of resistance to antimicrobial agents of *Salmonella* spp. isolates collected in different years of mollusk production in the province of Ferrara.

Period	No. of isolates	No. of resistant isolates (%)												R at least one AA	MDR
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA		
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFA	T-SULFA		
2001-2007	17	4(23.5)	-	-	10(58.8)	-	-	-	91(52.9)	4(23.5)	4(23.5)	4(23.5)	2(3.3)	16(94.1)	4(23.5)
2008-2012	28	18(64.2)	-	2(7.1)	7(25)	-	1(1.6)	-	22(78.6)	6(28.6)	13(46.4)	8(28.6)	-	26(92.9)	16(57.1)
2013-2017	57	31(54.4)	-	7(12.8)	3(5.3)	2(3.8)	-	2(1.9)	29(50.9)	6(28.1)	29(50.9)	16(28.1)	1(9)	41(71.9)	25(43.9)
Total	102	53(52)	-	9(8.8)	20(19.6)	2(1.9)	1(0.9)	2(1.9)	60(58.8)	16(15.7)	46(45.1)	28(27.4)	3(2.9)	83(81.4)	45(44.1)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 5. Results of susceptibility testing to antimicrobial agents of *E. coli* isolates collected in different areas of mollusk production in the province of Ferrara.

Area	No. of isolates	No. of resistant isolates (%)												R at least one AA	MDR
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA		
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFAX	T-SULFA		
B-in	27	17(63)	-	9(33.3)	4(14.8)	2(14.3)	2(7.4)	12(44.4)	18(66.7)	-	2(7.4)	13(48.1)	-	23(85.1%)	15(55.6%)
Long-line, Lupini, B-Out	19	8(42.1)	-	2(10.5)	1(5.2)	3(15.8)	-	7(36.8)	12(63.2)	-	3(15.8)	5(26.3)	-	14(73.7%)	6 (31.6%)
Sacca	33	19(57.6)	1(3)	7(21.2)	4(12.1)	3(9.1)	2(6.1)	9(27.3)	11(33.3)	1(3)	2(6.1)	6(18.2)	2(6.1)	22(66.7%)	9(27.3%)
Total	79	44(55.7)	1(1.3)	18(22.8)	9(11.4)	8(10.1)	4(5.06)	28(35.4)	41(51.9)	1(1.3)	7(8.9)	24(30.4)	2(2.5)	59(74.7)	30(38)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 6. Results of susceptibility testing to antimicrobial agents in relation to the inclusion of *E. coli* isolates in more and less frequent phylogenetic groups and the sampling area in the mollusk production area in the province of Ferrara; findings were expressed as number of resistant isolates and their percentage value (%).

Area	Phylogenetic group							
	A		B1		C		B2, D, E and unknown	
	R at least one AA	MDR	R at least one AA	MDR	R at least one AA	MDR	R at least one AA	MDR
B-in	7(100)	5(71.4)	9(81.8)	7(63.6)	4(100)	2(50)	3(60)	1(20)
Long-line, Lupini, B-Out	1(100)	-	6(75)	2(25)	2(66.7)	2(66.7)	5(71.4)	2(28.6)
Sacca	3(33)	-	13(61.9)	3(14.3)	4(100)	4(100)	4(80)	2(40)
Total	11(81.8)	5(45.4)	28(70)	12(30)	10(90.91)	8(72.7)	12(70.6)	5(29.4)

R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains

Table 7. Spatial trends of resistance to at least one antimicrobial agent and multi-resistant strains expressed as number of resistant isolates and their percentage value (%).

Area	<i>Salmonella</i> ser. Typhimurium		monophasic <i>S. Typhimurium</i> serovar		<i>Salmonella</i> spp.		<i>Escherichia coli</i>	
	R to at least one	MDR	R to at least one	MDR	R to at least one	MDR	R to at least one	MDR
	AA		AA		AA		AA	
B-In	33(80.5%)	16(39%)*	20(100%)	15(75%)	53(86.9%)	31(50.8%)*	23(85.1%)	15(55.6%)
Long-line, Lupini, B-Out	7(77.8%)	6(66.7%)*	2(100%)	2(100%)	9(81.8%)	8(72.7%)*	14 (73.7%)	6 (31.6%)
Sacca	14(63.6%)	2(9.1%)*	7(87.5%)	4(50%)	21(70%)	6(20%)*	22(66.7%)	9(27.3%)

R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains. Number of isolates in columns bearing * are significantly different (p< 0.05).