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Abstract

Sediment samples from three coastal sites - two beach resorts (Beach 1 and Beach 2 sites) and an area lying between an oil refinery and a river estuary (Estuarine site) - were analyzed for antibiotic- and heavy metal (HM)-resistant enterococci.

A total of 123 enterococci, 36 *E. faecium*, 34 *E. casseliflavus,* 33 *E. hirae*, 5 *E. faecalis*, 3 *E. durans*, 3 *E. gallinarum*, and 9 *Enterococcus* spp, were recovered. Strains resistant to erythromycin, tetracycline and quinupristin/dalfopristin (Q/D) were recovered from all sites, whereas multidrug-resistant isolates were recovered only from "Beach 2" (14 %) and "Estuarine" (3.7 %). As regards HM resistance, the strains showed a high frequency (68 %) of cadmium and/or copper resistance and uniform susceptibility to mercury. The prevalence of cadmium-resistant strains was significantly higher among erythromycin-resistant than among erythromycin-susceptible strains. A significant association between cadmium or copper resistance and Q/D resistance was also observed at "Estuarine" site. The levels of the two HMs in sediment from all sites were fairly low, ranging from 0.070 to 0.126 µg/g, for cadmium and from 1.00 to 7.64 µg/g for copper. Mercury was always undetectable. These findings are consistent with reports that low HM concentrations may contribute to co-selection of antibiotic-resistant bacterial strains, including enterococci.

Capsule

In this work is reported a significant association between specific antimicrobial and heavy metal resistances in enterococci from marine uncontaminated sediments.

Keywords

Antibiotic- and heavy metal-resistant enterococci; marine sediment, co-selection.

INTRODUCTION

Enterococci are commensal bacteria of the intestinal tract of animals and humans. Unlike other fecal indicator bacteria (FIB) they can survive prolonged exposure to environmental stressors (low pH, high salt concentrations, wide temperature range) outside their hosts (Byappanahalli et al., 2012). Enterococci are also opportunistic pathogens responsible for severe nosocomial infections; their proneness to acquire mobile genetic elements has given rise to multiresistant strains, which are a cause of grave concern (Gilmore et al., 2014). Antibiotics have long been used in human and animal treatment and prophylaxis as well as to prevent microbial infections in farms (including aquaculture farms), thereby contributing to the spread of antimicrobial resistance. Although their prophylactic use in clinical and farm settings has been declining in the past few years, antibiotic-resistant (AR) strains continue to be recovered in natural environments (Marti et al., 2014; Agga et al., 2015; Berendonk et al., 2015; Singer et al., 2016). AR enterococci found in the marine coastal environment are often closely related to human clinical isolates (Vignaroli et al., 2013; Di Cesare et al., 2014), probably originating from hospital and urban effluents. However, several studies have highlighted a degree of genetic heterogeneity between human and environmental AR enterococci (Castillo-Rojas et al., 2013; Ran et al., 2013; Palmer et al., 2012). In environmental strains, antibiotic resistance may be the result of genetic exchanges with strains of clinical origin which are then followed by a selection process that may be different from the one exercised by antibiotics.

Heavy metals (HMs) are ubiquitous contaminants spread by agricultural and industrial runoff and sewage water (Seiler and Berendonk, 2012; Yu et al., 2017). Lead, chromium, cadmium (Cd), copper (Cu), arsenic, zinc, and mercury (Hg) are those detected most frequently in the environment. Products containing zinc and copper and Cu-supplemented feeds are used in agriculture and farming activities (Loganathan et al., 2008; Fard et al., 2011). Therefore, the beneficial effects of the reduced use of antibiotics in these sectors may be attenuated by the utilization of HM-containing compounds that indirectly select for antibiotic resistance (Baker-Austin et al., 2006; Xu et al., 2017).

Both antibiotic and HM residues have been described in marine sediments, which are a potential reservoir of AR strains (Matyar et al., 2008; Vignaroli et al., 2013; Di Cesare et al., 2014). However, HMs are more stable and resistant to degradation than antibiotics. Moreover, in aquatic sediments even low HM levels can contribute to the emergence and spread of AR strains through co-selection of genetic elements encoding both HM and antibiotic resistance (Seiler and Berendonk, 2012; Gullberg et al., 2014; Roosa et al., 2014). Low HM levels in polluted environments can support the maintenance of multiresistance plasmids harboring antibiotic resistance genes as well as genes encoding resistance to biocides and HM. Mechanisms other than co-selection, like cross-resistance, co-regulation, and biofilm induction (Baker-Austin et al., 2006; Yu et al., 2017), may also be involved. Conjugative plasmids have been implicated in the co-transfer of macrolide and copper resistance (Feßler et al., 2017), whereas copper sulfate (used as a feed supplement) appears to be involved in the selection of AR enterococcal populations in farmed animals (Hasman and Aarestrup, 2002). The association of *tcr*(B) and *erm*(B) and their co-transfer by conjugation have been documented by our group in an *E. hirae* isolate from marine sediment collected in the Adriatic Sea in front of the town of Ancona (Marche, IT) (Pasquaroli et al., 2014).

In this study we investigated the frequency of antibiotic and heavy metal resistance in enterococci from coastal marine sediments collected in the area around Ancona at sites variably impacted by human activities. The presence of Cu, Cd and Hg in sediments and the association between antimicrobial and heavy metal resistance were analyzed to evaluate whether metals can contribute to the selection and persistence of antibiotic resistant enterococci.

MATERIALS AND METHODS

Sampling sites and strategy

Sediment samples were collected along a stretch of the Adriatic coast, approximately from latitude 43° 45.300'N, longitude 13° 12.630'E, to latitude 43° 39.0'N, longitude 13° 22.0'E (Fig. S1), as described previously (Di Cesare et al., 2012, 2013). They were obtained at 3 sites: "Beach 1", "Beach

2", and "Estuarine". At "Beach 1", a small but very popular summer resort, samples were collected at a depth of 11.5 m; at "Beach 2", a cove with beaches, restaurants, and three hotels, characterized by intense traffic of commercial and pleasure craft, they were collected at 15 m in; finally, "Estuarine" site (depth, 4.5 m) is off a small town that is close to an oil refinery and a river estuary. Sediment samples (3 replicates) were collected monthly at each site from March 2012 to May 2013. Aliquots of 20 g from each replicate were homogenized and used to isolate enterococci as described previously (Vignaroli et al., 2013). A total number of 45 samples (15 per site) were analyzed.

At each site, the environmental parameters of the water overlying the sediments being sampled were recorded using a conductivity-temperature-depth (CTD) profiler (Model 30 handheld, temperature, salinity and conductivity system, YSI, Yellow Spring, OH, USA).

Chemical analysis of sediments

Trace elements (Cd, Cu, and Hg) were determined in sediment according to validated methods (Benedetti et al., 2014; Etiope et al., 2014). Samples were dried at 60 °C overnight to constant weight and pulverized; 0.5 g was digested in a microwave digestion system (Mars CEM, CEM Corporation, Matthews, NC, USA) using 5 ml nitric acid and 1 ml hydrogen peroxide. Quality assurance and quality control were performed by processing blank samples and standard reference material (SRM NIST 2977, National Institute of Standards and Technology, Gaithersburg, MD, USA). Cadmium and copper were determined by atomic absorption spectrometry (Agilent, Varian SpectrAA 240Z, Agilent Technologies, Santa Clara, CA, United States) using graphite furnace atomization and the Zeeman effect, adding palladium solution (1 g/l, 10 % nitric acid, 5 % citric acid) as a chemical matrix modifier. Where necessary, the standard addition technique was used for resolution of matrix effects. Mercury was detected based on the formation of cold vapor (Agilent, Cetac Quick Trace Mercury Analyzer M6100) following the manufacturer's recommendations. Concentrations were expressed as micrograms per gram of dry weight (d.w.; $\mu g \cdot g^{-1}$). The values obtained with the standard reference material were consistently within the 95 % confidence interval (CI) of certified values.

117 ***Isolation and identification of enterococci***

118 Enterococci were detected by membrane filtration as previously described (Di Cesare et al., 2012;
119 Vignaroli et al., 2013) using Slanetz-Bartley (SB) (Oxoid, Basingstoke, UK) agar plates for
120 selection.

121 Colonies grown on filters were counted and enterococcal abundance was reported as colony-
122 forming units (CFU) / g of sediment. Presumptive enterococci were restreaked on SB agar and
123 identified to the genus level by PCRs targeting a genus-specific 16S rDNA sequence (Di Cesare et
124 al., 2012). Species identification was performed by PCRs targeting species-specific ddl sequences
125 (Di Cesare et al., 2012; Vignaroli et al., 2013). The strains used as positive controls in these PCR
126 assays are listed in Table S1. *E. hirae* PN 1.1, identified during the study, was used as the control
127 strain after sequencing of a ddl amplicon.

128 ***Antibiotic susceptibility testing***

129 All strains identified as enterococci were screened for their susceptibility to ampicillin (AMP),
130 chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), tetracycline (TET),
131 streptomycin (STR), vancomycin (VAN), and quinupristin/dalfopristin (Q/D), all purchased from
132 Sigma-Aldrich (St. Louis, MO, USA). Isolates were streaked on SB agar plates supplemented with
133 the antibiotics at concentrations reported previously (Di Cesare et al., 2012). The minimum
134 inhibitory concentrations (MICs) were determined by broth microdilution (CLSI, 2015) using *E.*
135 *faecalis* ATCC 29212 as the reference strain. Enterococci resistant to at least one agent in 3 or more
136 antimicrobial classes were considered as multidrug resistant (MDR).

137 ***Heavy metal susceptibility testing***

138 The MIC of cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$) and mercuric chloride ($HgCl_2$)
139 (Sigma-Aldrich) was determined by agar dilution as described previously (Pasquaroli et al., 2014).
140 Stock solutions of $CdCl_2$, $CuSO_4$, and $HgCl_2$ were prepared in water (0.5 M; $CdCl_2$ and $CuSO_4$) or
141 PBS (0.33 M; $HgCl_2$,), filter-sterilized, and stored at 4 °C for up to a month. For MIC
142 determination, HM solutions were added to Muller-Hinton agar (Oxoid) to obtain doubling

143 concentrations from 0.19 to 25 mM. pH was adjusted to 7.0 using 1 M NaOH. Overnight cultures in
144 Mueller Hinton broth (Oxoid) were diluted to an optical density (625 nm) of 0.1; then 5 µl was
145 spotted onto HM-supplemented Muller Hinton agar plates. Growth was assessed after incubation at
146 37 °C for 48 h ($CuSO_4$ and $HgCl_2$) or 5 days ($CdCl_2$). The lowest concentration of the metal salt
147 inhibiting bacterial growth was considered as the MIC. *E. faecalis* ATCC 29212 was used as the
148 control strain and the HM resistance breakpoint was set at ≥ 25 mM (Pasquaroli et al., 2014).

149 ***Detection of antibiotic and heavy metal resistance genes***

150 The antibiotic [*blaZ*, *qnr*, *erm*(A), *erm*(B), *mef, tet*(M), *tet*(L), *tet*(O), *ant*(6)-I, *vat*D] and HM
151 (*cadA*, *tcrB merA* and *merB*) resistance genes were detected by PCR as described previously
152 (Pasquaroli et al., 2014; Citterio et al., 2017) using the positive controls and primer pairs reported in
153 Tables S1 and S2, respectively. The STR-resistant strain *E. gallinarum* PN 2.3 (this study) was used
154 as the positive control in PCR assays targeting *ant(6)-I* after amplicon sequencing.

155 ***Data processing***

156 The multiple antibiotic resistance (MAR) index was computed for each strain and sampling site
157 according to Krumperman (1983). MAR values >0.2, when applied to a single strain, indicate
158 multidrug resistant (MDR) strain. When applied to the sampling site, the MAR index was
159 calculated by the formula $a/(b \cdot c)$, where a is the sum of the MAR indicexes of the resistant
160 isolates from the site, b is the number of tested antibiotics, and c is the total number of isolates from
161 the site. MAR values ≥0.2, indicate a site in which there is a high risk of potential contamination by
162 MDR strains.

163 ***Statistical analysis***

164 Odds ratio (OR) and exact 95 % CI were used to determine the association between multiresistance
165 to antibiotics (resistant enterococci with an MAR index ≥ 0.2) and resistance to Cu or Cd. An OR ≤
166 1 indicated negative correlation and OR > 1 positive correlation (Resende et al., 2012).

Differences in the prevalence of AR strains and in HM levels, at each site, were analyzed by the χ^2 test. The significance of the association between resistance to a specific antibiotic and to Cu or Cd resistance was analyzed by Fisher's test and a p value < 0.05 was considered significant.

RESULTS

Enumeration and identification of enterococci from sediment samples

The amount of colonies grown on SB agar varied among sites and sampling times. Altogether, the number of enterococci recovered from March 2012 to May 2013 was low (n=123). Counts were as follows: "Beach 1", 0.93 ± 0.62 CFU/g; "Beach 2", 2.2 ± 1.8 CFU/g; and "Estuarine", 5 ± 6.9 CFU/g. The highest counts were recorded in samples collected from "Estuarine" (6.5x10 ± 1.5 CFU/g) in the warm months (May - September 2012) and from "Beach 2" (1.5x10 ± 0.3 CFU/g) in the cold season (October 2012 - April 2013). *E. faecium* was the predominant species (29.2 %), followed by *E. casseliflavus* (28 %) and *E. hirae* (26.8 %). Whereas these species were recovered from all sites, *E. gallinarum* (2.4 %) was isolated only from site "Beach 2", and *E. faecalis* (4 %), *E. durans* (2.4 %), and additional *Enterococcus* spp. (7.2 %) were retrieved only from "Estuarine" (Fig. 1).

Detection of antibiotic- and heavy metal-resistant enterococci

All enterococcal isolates were tested for their susceptibility to a panel of 8 antibiotics and 3 HMs (Fig. 2 and Table 1). Of the 123 isolates, 39 % (n=48) were AR. Different frequencies were found at each site: "Beach 1", 20 %; "Beach 2", 53.5 % and "Estuarine" , 37.5 %. Strains resistant to ERY, TET, and Q/D were recovered from all sites. TET-resistant isolates were the most frequent, with a significant difference (p < 0.05) only at site "Beach 2". Vancomycin resistance was never detected. The frequency of AR strains was highest at site "Beach 2" and lowest at "Beach 1", where only ERY, TET, and Q/D resistance was recorded (Fig. 2). The highest prevalence of MDR isolates (resistant to three or more antibiotic classes) was observed at site "Beach 2" and the lowest (no MDR strains) at "Beach 1" (Table 1).

192 The highest MAR index, calculated to determine the level of antibiotic resistance of each isolate
193 (Table 1), was 0.62, in strains resistant to 5/8 antibiotics. To assess the possible risk for human
194 health, the index was also computed for each sampling site. Unexpectedly it was highest at "Beach
195 2", albeit it was < 0. 2.
196 As regards HMs, 68 % of strains (84/123) were able to grow in agar plates supplemented with
197 cadmium (57 %) or copper (36.5 %); no mercury-resistant strains were recovered. The MIC
198 results showed that HM-resistant (HMR) strains ranged from 35 to 75 % (Table 1). Resistance to
199 both cadmium and copper was detected in 25 % of isolates, most of which (35.7 %) had been
200 retrieved from "Beach 2".
201 To evaluate the association of HM and antibiotic resistance, the prevalence of strains resistant to
202 cadmium or/and copper among ERY-, TET- and Q/D-resistant or -susceptible enterococci was
203 calculated for each site (Fig. 3 A and B, respectively). Cadmium resistance was more common
204 among AR than among antibiotic-susceptible strains from "Estuarine" and "Beach 2". The
205 association between Cd and ERY resistance was significant ($p < 0.05$) for the isolates from both
206 these sites, whereas the association between Cd and Q/D resistance was significant ($p < 0.05$) only
207 for isolates from "Estuarine" (Fig. 3 A). At "Beach 1" there were no AR isolates, except a single
208 ERY-resistant strain, which was also resistant to Cd. The association between Cd and TET
209 resistance was never significant. As regards copper, a very high significant ($p = 0.004$) association
210 with antibiotic resistance was only that with Q/D-resistant isolates from "Estuarine" (Fig. 3 B).
211 Although the association between a specific antibiotic and Cu or Cd resistance was significant,
212 multidrug resistance (MAR index ≥ 0.2) and Cu or Cd resistance was not significant for isolates
213 from all sites, as shown by the OR data in Table 1. Even at "Estuarine" site where OR>1, the
214 association was not significant, because the OR value (1.06) lies within the 95 % CI of 0.22 - 5.08.

215 ***Detection of antibiotic and heavy metal resistance genes***

The antibiotic resistance genes detected in the 48 AR enterococci recovered from the three sites are listed in Table 2. *erm*(B), *tet*(M), and *tet*(L) were the genes detected most frequently in ERY- and TET-resistant strains, whereas *blaZ*, *qnr*, and *vatD* were never detected in strains resistant to AMP, Q/D, or CIP. Overall, 31.25 % of ERY- or TET-resistant strains carried more than one ERY [*erm*(B)-*mef*] or TET [*tet*(M)-*tet*(L); *tet*(M)-*tet*(O)] resistance gene, and most of them (40 %) were *E. hirae*. The most frequent association was *tet*(M)-*tet*(L), recorded in 41.3 % of TET-resistant strains. The cadmium resistance gene *cadA* was recovered in 20 % of cadmium-resistant enterococci, most of which (11/14) came from "Estuarine". Of the 45 copper-resistant strains, 12 carried *tcrB* and none had been isolated in samples from "Beach 2".

Overall, 75 % of AR strains were also resistant to cadmium and/or copper, with 20.5 % of Cd-resistant strains carrying the *cadA* gene and only 1/17 Cu-resistant strains carrying *tcrB*. The co-presence of *cadA* and *tcrB* was never recorded (Table 2).

Seawater environmental parameters and chemical analysis of sediments

Analysis of seawater environmental parameters (temperature, salinity, and conductivity) showed that they were similar at all three sampling sites throughout the study. Temperature ranged from 20.2 to 27.2 °C from May to September 2012 and from 7.5 to 20.8 °C from October 2012 to April 2013. Salinity ranged from 34.8 to 37.1 ppt in the warmer months and from 33.6 to 38.2 ppt in the cold season. Conductivity values were found in a narrow range, the lowest values being recorded in the cold months at "Beach 1" (39.8 µS/cm) and "Estuarine" (42.1 µS/cm) (Table 3).

Chemical analysis of sediment showed cadmium concentrations of 0.07 to 0.13 µg/g of sediment at all sites, whereas mercury was below the limit of detection (< 0.04 µg/g) and showed no significant differences among sampling times or sites. In contrast, different copper concentrations were found in the warm months at the three sites, with average values of 1.07 µg/g at "Estuarine", 2.19 µg/g at "Beach 1", and 4.93 µg/g at "Beach 2". The widest range and highest values (2.73-7.64 µg/g) were

240 seen at "Beach 2" (Table 4). Statistical analysis showed any significant (p>0.05) difference among
241 the three sites.

242 **DISCUSSION**

243 The current regulations envisage a more limited and careful use of the drugs (Costelloe et al., 2010;
244 Ayukekbong et al., 2017), however the emergence and spread of antibiotic resistance in the
245 environment is still cause for grave concern.
246 Nonetheless, additional factors may be involved in the persistence and spread of AR strains,
247 including co-selection of antibiotic and HM resistance genes (Baquero et al., 2008; Oggioni et al.,
248 2013; Baker-Austin et al., 2006; Fard et al., 2011; Pasquaroli et al., 2014). Antibiotics (Manzetti
249 and Ghisi, 2014) and HMs (Roosa *et al*., 2014) released from different sources can both promote
250 selection and dissemination of AR bacteria (Matyar et al., 2008; Seiler and Berendonk, 2012) in
251 different environments. Coastal marine sediments are reservoirs of AR FIB (Di Cesare et al., 2012;
252 Di Cesare et al., 2013; Vignaroli et al., 2013), and sediment resuspension due to wave motion can
253 induce high concentrations of AR FIB in the overlying water, posing a public health risk (Halliday
254 and Gast, 2011; Abia et al., 2016).
255 Antimicrobial compounds and HMs are both capable of exerting a selective pressure; however, HMs
256 are more stable in marine sediment than antibiotics, whose concentration and activity decrease over
257 time through binding to sediment particles and cleavage/modification (Kummerer, 2009); as a result,
258 the pressure exerted by HMs lasts longer (Baker-Austin et al., 2006, Yu et al., 2017).
259 The element concentrations in surface coastal sediments are extremely variable, as a function of
260 biotic and abiotic factors, and it is not easy to establish the thresholds that can be considered as safe.
261 In particular, Cd and Cu concentrations in uncontaminated marine and estuarine sediments fall
262 respectively in the range of 0.01 - 0.6 µg/g (d.w.) and 2 - 70 µg/g (d.w.) throughout the world
263 (Neff, 2002), while these can raise up to about 460 µg/g (d.w.) for Cd and 7500 µg/g (d.w.) for Cu
264 in sediments from heavily contaminated estuaries (Stoffers et al., 1977; Neff, 2002); Hg levels are

265 also widely variable, although the mercury concentration in uncontaminated marine sediments is
266 generally lower than 0.2 µg/g (d.w.) (Neff, 2002).
267 Additional environmental parameters besides toxic compounds, especially temperature, salinity and
268 conductivity, can influence the structure of the microbial community in coastal sediments and FIB
269 survival (Byappanahalli et al., 2012; Gilmore et al., 2014; Tinta et al., 2015).
270 In this study, three coastal sites in the central Adriatic Sea - two beach resorts and an area close to
271 an oil refinery and a river estuary - were investigated to gain insights into the prevalence of AR and
272 HMR enterococci in marine coastal sediments. A total number of 123 enterococcal strains of
273 different species were recovered. Their different abundance and distribution are likely to be
274 influenced by factors other than temperature, salinity and conductivity, such as pollution and
275 sewage discharge. For instance, the peak abundance found at "Estuarine" site could be related to the
276 nearby river estuary. Since the river flows through farmed fields, its waters are probably
277 contaminated by human and animal waste. This may also explain the high enterococcal species
278 diversity found at this site.
279 The MAR index was consistently < 0.2 at all sites, posing a low public health risk. Accordingly, the
280 levels of cadmium, copper and mercury in samples were fairly low and very similar to those
281 described in other sampling areas in the central Adriatic Sea (Benedetti et al., 2014; Etiope et al.,
282 2014). Nevertheless, isolates resistant to cadmium (57 %), copper (36.5 %), or both (25 %) were
283 recovered from all sites. These findings are in line with reports describing the emergence of resistant
284 strains also at low concentrations of toxic compounds (Roosa et al., 2014). HM resistance was mainly
285 detected in strains from sites "Beach 2" and "Estuarine", with a marked prevalence of cadmium-
286 resistant over copper-resistant isolates. The cadmium and copper resistance found at "Estuarine" may
287 be related to the nearby river estuary and to the probable use by the farms found along the river of
288 copper-supplemented feeds and fertilizers, which usually contain several contaminants (e.g. Cd and
289 fluorine) (Loganathan et al., 2008; Fard et al., 2011). The highly significant (p=0.004) association
290 between Q/D and copper resistance in enterococci from site "Estuarine" supports this hypothesis,

291 also considering that streptogramins are frequently used in animal farming. Since copper
292 concentrations were similar at all the three sites, the significance of the Cu and Q/D resistance
293 association is likely dependent by the higher abundance and different origin of strains collected at
294 this site. The least contaminated site was "Beach 1", where the abundance of enterococci was lowest,
295 no MDR strains were recovered, and a single strain (*E. hirae* S3.1) was co-resistant to cadmium
296 and ERY. These findings are in line with the characteristics of this town (a sea resort); moreover,
297 the location of the sampling site above the estuary of the River Misa involves that the sampling area
298 does not receive the direct impact of human/animal sewage.
299 Unexpectedly, 53.5 % of enterococci from site "Beach 2", a sea resort in Conero Park, were resistant
300 to at least one antibiotic, and 14 % of them were MDR. The finding may be explained with the heavy
301 traffic of commercial and pleasure craft, including ferryboats to Croatia and Greece, that
302 characterizes this area and with sea currents, which may have contributed to accumulate bacteria
303 from distinct sources. Moreover, more than half of the isolates recovered from this site were AR and
304 HMR, the most frequent associations being Cd-TET, Cd-ERY, and Cu-TET. These data, which
305 support the association of HM and antimicrobial resistance genes (Baker-Austin et al., 2006; Yu et
306 al., 2017), suggest that conditions favoring strain persistence are found at this site. The association of
307 HM and antimicrobial resistance can be due to carriage of the relevant resistance genes on the same
308 genetic element (Pasquaroli et al., 2014). In particular, Gullberg *et al*. (2014) have demonstrated that
309 the presence in the environment of very low amounts of single antibiotics or/and toxic compounds
310 can select for a large plasmid coding for resistance to aminoglycosides, beta-lactams, tetracycline,
311 macrolides, trimethoprim, sulfonamide, silver, copper, and arsenic. An efflux system mediating the
312 export of copper/cadmium and TET may also be involved (Hayashi et al., 2000). Carriage of *erm*(B)
313 and *tcr*B by the same conjugative *E. faecium* plasmid has already been described (Hasman and
314 Aarestrup, 2002) as has the co-transfer of *erm*(B) and *tcr*B from a strain of *E. hirae* recovered from
315 marine sediment to a human strain of *E. faecalis* (Pasquaroli et al., 2014).

316 In conclusion, the significant association between specific antimicrobial and heavy metal resistances,
317 found in enterococci from marine sediment, suggests that this environment is suitable for the
318 persistence of resistant bacteria. The factors responsible are largely unclear, considering that resistant
319 strains of non-human species and human multi-resistant strains were recovered from a sea resort
320 characterized by a limited range of human activities. Notably, the higher frequency of metal resistance
321 found among strains resistant to specific antibiotics does not exclude a contribution of heavy metals,
322 even at low concentrations, to the selection of AR enterococci.

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445 **Figure Legends**

446 **Figure 1.** Prevalence of the different enterococcal species at the three sampling sites (Beach 1,
447 Beach 2, Estuarine).

448 **Figure 2.** Prevalence of enterococci resistant to the eight antibiotics found at the three sampling
449 sites (Beach 1, Beach 2, Estuarine). * $p < 0.05$.
450 AMP, ampicillin; CHL, chloramphenicol, CIP, ciprofloxacin; ERY, erythromycin; TET,
451 tetracycline; STR, streptomycin; VAN, vancomycin; Q/D, quinupristin/dalfopristin.

452 **Figure 3.** Prevalence of cadmium- (A) and copper-resistant (B) enterococci among antibiotic-
453 resistant (black bar) and antibiotic-susceptible (white bar) isolates from sites "Beach 2" and
454 "Estuarine". Resistant isolates from "Beach 1" were too few to be reported in a diagram.
455 *the association between HM and antibiotic resistance was barely significant (p=0.05-0.07);
456 **the association between HM and antibiotic resistance was significant (p=0.004).
457 ERY, erythromycin; TET, tetracycline; Q/D, quinupristin/dalfopristin.

458 **Figure S1**. Sampling sites (Google Earth software)

Table 1. Frequency of antibiotic- and heavy metal-resistant enterococci and correlation (Odds ratio) between multidrug (MAR ≥ 0.2) and heavy metal resistance

Site	No. of isolate	Frequency (%) of AR[a] isolates	Frequency (%) of MDR[b] isolates	MAR index range of AR isolates	MAR[c] index of site	Frequency (%) of isolates resistant to			Odds ratio[d] (95 % confidence interval)		
						Cd	Cu	Hg	Cd	Cu	Hg
Beach 1	15	20	0	0	0.003	40	40	0	-	-	-
Beach 2	28	53.5	14.2	0.37-0.62	0.017	75	39.2	0	-	0.25 (0.02-3.06)	-
Estuarine	80	37.5	3.75	0.37	0.008	53.7	35	0	0.62 (0.32-3.08)	1.06 (0.22-5.08)	-

[a]antibiotic resistant, [b]multidrug resistant, [c]multiple antibiotic resistance, [d]OR=1: absence of correlation, OR > 1: positive correlation, OR < 1: negative correlation.

Table 2. Antibiotic/heavy metal resistance phenotype and genotype of AR isolates from the three sampling sites

Site	AR isolate	Resistance phenotype	Resistance genotype
Beach 1	*E. hirae* 3.1	ERY, Cd	*erm*(B), *cadA*
	E. casseliflavus 5.1	Q/D	-
	E. hirae 8.6	TET	*tet*(M), *tet*(L)
Beach 2	*E. faecium* 2.1	TET, Cd, Cu	*tet*(M)
	E. gallinarum 2.3	AMP, ERY, TET, STR, Q/D, Cd	*tet*(M), *tet*(L), *erm*(B), *ant(6)*
	E. gallinarum 2.4	AMP, ERY, TET, STR, Q/D, Cd	*tet*(M), *tet*(L), *erm*(B), *ant(6)*
	E. gallinarum 2.5	AMP, ERY, TET, STR, Q/D, Cd, Cu	*tet*(M), *tet*(L), *erm*(B), *ant(6)*
	E. faecium 3.1	ERY, TET, Cd	*erm*(B), *tet*(M), *cadA*
	E. hirae 8.4	TET, STR, Cd	*tet*(M), *tet*(L)
	E. faecium 8.6	TET, Cd, Cu	*tet*(M)
	E. faecium 8.7	ERY, Cd	-
	E. faecium 8.10	TET, Cd	*tet*(M)
	E. casseliflavus 8.11	TET, Cd	*tet*(M)
	E. faecium 8.14	ERY, Cd, Cu	-
	E. casseliflavus 8.16	TET	*tet*(M)
	E. faecium 8.20	TET, Cd, Cu	*tet*(M), *tet*(L)
	E. faecium 8.22	ERY, Cd	-
	E. faecium 8.23	CIP, ERY, TET, Cd	*tet*(M), *tet*(O)
Estuarine	*E. durans* 1.4	ERY, Cd	*erm*(B)
	E. hirae 2.3	TET, Cd	*tet*(M), *tet*(O)
	E. faecium 2.7	TET, Cd	*tet*(M), *tet*(L)
	E. durans 2.13	TET, Cd	*tet*(M)
	E. faecium 2.15	TET, Cd	*tet*(M)
	E. hirae 2.16*	ERY, Cd, Cu	*erm(A), erm*(B), *mef, tcrB*
	Enterococcus spp. 7.1	CIP	-
	E. casseliflavus 7.2	CIP	-
	Enterococcus spp. 7.3	CIP, ERY, TET, Cd	*erm*(B), *tet*(M)
	E. casseliflavus 7.6	CIP	-
	E. faecalis 7.9	ERY, TET, Q/D, Cd	*erm*(B), *tet*(M), *cadA*
	E. casseliflavus 7.12	ERY, TET	*erm*(B), *tet*(M), *tet*(L)
	E. casseliflavus 7.13	CIP	-
	E. faecium 7.17	ERY, Cd	-
	E. faecium 7.20	TET, Cu	*tet*(M)

E. casseliflavus 7.21	CIP, TET, Cu	-
E. faecalis 7.22	ERY, TET, Q/D, Cd, Cu	*erm*(B), *tet*(M), *tet*(L), *cadA*
E. casseliflavus 7.25	CIP	-
E. casseliflavus 7.26	TET, Cd, Cu	*tet*(M)
E. faecium 7.34	ERY, Cd	-
E. casseliflavus 7.40	TET	*tet*(M)
E. hirae 7.41	TET, STR	*tet*(M), *tet*(L)
E. hirae 7.43	ERY, TET, STR	*erm*(B), *tet*(M), *tet*(L)
E. casseliflavus 7.44	Q/D, Cd, Cu	-
E. faecium 7.45	Q/D, Cd, Cu	-
Enterococcus spp. 7.47	Q/D, Cd, Cu	*cadA*
E. casseliflavus 7.48	CHL, ERY, TET, Cd, Cu	*mef, tet*(M), *tet*(L)
E. hirae 7.49	Q/D, Cd, Cu	-
Enterococcus spp. 7.54	Q/D, Cd, Cu	*cadA*
E. faecalis 8.5	ERY, TET, Cd, Cu	*erm*(B), *tet*(L), *cadA*

***Previously described by Pasquaroli et al., 2014**

Table 3. Range of temperature, salinity and conductivity values recorded in the water overlying the sediments being sampled at the three sites during the study

Sampling period	Temperature (°C)			Salinity (ppt)			Conductivity (µS/cm)		
	Beach 1	Beach 2	Estuarine	Beach 1	Beach 2	Estuarine	Beach 1	Beach 2	Estuarine
May 2012-September 2012	20.2-26.6	20.2-26.1	20.3-27.2	35.6-36.2	35.4-37.1	34.8-36.9	49.4-55.5	50.9-55.1	50.1-56.4
October 2012-April 2013	7.5-20.4	8.2-20.8	7.5-19.9	35.4-37.2	36.3-38.0	33.6-38.2	39.8-51.0	43.4-51.7	42.1-50.8

Table 4. Range of heavy metal concentration values detected at the three sampling sites during the study

Heavy metal*	Concentration (µg/g)					
	May 2012 – September 2012			October 2012 – April 2013		
	Beach 1	Beach 2	Estuarine	Beach 1	Beach 2	Estuarine
Cd	0.080 - 0.090	0.085 - 0.126	0.072 - 0.095	0.070 - 0.089	0.080 - 0.087	0.075 - 0.087
Cu	2.16 - 2.46	2.73 - 7.64	1.00 - 1.28	2.64 - 3.16	2.91 - 6.32	1.05 - 1.71

*Hg concentrations were consistently < 0.04 µg/g at all sites