

1 **A phenotypic study of the environmental resistome.**

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38 **Abstract**

39 Since the discovery of antibiotics, the dispersion of resistance genes has increased  
40 exponentially until reaching current state, in which it has become increasingly difficult to  
41 achieve an effective treatment against infectious diseases. The enormous capacity for  
42 genetic exchange between microorganisms is causing resistance genes to be able to reach  
43 all environments, even those where there is no anthropogenic impact or exposure to these  
44 drugs. In this work, a phenotypic study of the resistome has been conducted in a peri-  
45 urban ecosystem (Granada, Spain), in which it has been determined the resistance against  
46 32 antibiotics of 710 bacterial strains isolated from 70 samples from different ecological  
47 niches with different levels of exposure to antibiotics and anthropic action. The results  
48 obtained show a high percentage of resistance in all the subsystems analysed, stating high  
49 multi-resistance profiles. Vancomycin and erythromycin were the antibiotics in which the  
50 highest levels of resistance were observed, while the lowest levels were obtained in  
51 chloramphenicol. Regarding the environments studied, wastewater, farms and food  
52 showed the highest percentages of resistance. It should be noted that in natural soil  
53 samples not exposed to antibiotics or anthropogenic activities, worrying levels of  
54 resistance against practically all groups of antibiotics analysed were detected, although  
55 with quantitatively lower levels than the other ecological niches. All these results lead to  
56 the conclusion of the importance of resistome control and proper management of waste  
57 that may contain antibiotics or resistant bacteria to prevent the wide propagation of the  
58 resistome in the environment.

59

60 **Keywords**

61 Antibiotics

62 Antibiotic resistance

63 Resistome

64 Environmental resistome

65 Phenotypic resistance

66

67 **1. Introduction**

68 Before the discovery of antibiotics at the beginning of the 20<sup>th</sup> century, communicable  
69 diseases were the first cause of morbidity and mortality throughout the entire history of  
70 mankind. Since then, the indiscriminate use of these drugs, both at clinical and veterinary  
71 level, has produced the widespread dissemination of antibiotic resistance (AR)  
72 worldwide. Could this occurrence cause a regression towards the pre-antibiotic era?

73 Antibiotic resistant bacteria (ARB) are an urgent public health problem. Modern medicine  
74 depends on the effectiveness of antimicrobial drugs, however, really high rates of  
75 infections caused by a wide range of resistant microorganisms have been reported all  
76 around the world. Strengthening surveillance of antibiotic consumption and AR is crucial  
77 to improve the scientific basis, evaluate risks and act for their mitigation (Tamhankar and  
78 Stålsby Lundborg, 2019). The threat of the AR can only be tackled if strategies and  
79 interventions at different levels are based on solid data (WHO, 2022).

80 Although the AR mechanisms developed by bacteria are diverse, with the exception of  
81 some constitutive resistance, all of them share a genetic basis susceptible to transmission.  
82 AR are principally mediated by genes found in the environment or in clinically isolated

83 microorganisms, but it has been demonstrated that, in both cases, they are genetically  
84 similar. Actually, AR already existed in nature long before antibiotics were discovered.  
85 Consequently, after this discovery, beyond the study of AR in clinical pathogens, the  
86 concept of resistome was proposed as the set of antibiotic resistance genes (ARGs) from  
87 pathogenic and non-pathogenic bacteria present in a particular environment (Kim and  
88 Cha, 2021).

89 Even if it is possible that microorganisms possess AR naturally or that the AR appears by  
90 spontaneous mutation in the bacteria genome, the most habitual is that AR is acquired by  
91 horizontal gene transfer. This genetic exchange between bacteria can be conducted by  
92 three main mechanisms: transformation or incorporation of free DNA; transduction or  
93 gene exchange through bacteriophages; and conjugation or transfer of mobile genetic  
94 elements (plasmids, integrons, ...) from one bacterium to another. The latter is the main  
95 and most relevant mechanism in the dissemination of ARGs in nature and their  
96 transmission between humans, animals and the environment (Iskandar *et al.*, 2022;  
97 Partridge *et al.*, 2018). For this reason, the problem of the AR cannot exclusively rest with  
98 the study of bacteria of clinical interest (although the high consumption of antibiotics in  
99 the hospital environment exerts a powerful driving force for the development of  
100 resistomes) (Sun *et al.*, 2023), since bacteria proliferate and interact with each other in  
101 the environment, exchanging ARGs which can eventually end in pathogenic bacteria  
102 (Collignon and McEwen, 2019; Despotovic *et al.*, 2023).

103 There is evidence that human activity has greatly contributed to the dispersion of ARGs  
104 throughout the environment, and it is not only due to the massive use of antibiotics in the  
105 treatment of human and animal infectious diseases (Czatkowska *et al.*, 2022). Human  
106 wastes from urban centres, hospitals, agricultural, livestock and aquaculture activities, as  
107 well as residues from wastewater treatment plants and pharmaceutical industries,  
108 contaminate the environment by realising antibiotic residues, ARB and ARGs (Cai *et al.*,  
109 2022). This contamination not only reaches areas close to anthropogenic activities, but is  
110 distributed across natural ecosystems with little or no exposure to antibiotics, such as  
111 forest soils, rivers or oceans (Larsson and Flach, 2022; Scott *et al.*, 2020).

112 The peri-urban environment constitutes an ecosystem where urban and rural  
113 environments interact, in which the transmission and dispersion of ARGs is facilitated.  
114 The administration of antibiotics in the livestock industries, in addition to being a food  
115 safety problem, increases the selection pressure of ARB that are finally released into the  
116 environment, contaminating soils, air and surface waters. Furthermore, in many cases,  
117 solid wastes from these animals are used as fertilizer in the agricultural sector, increasing  
118 the spread of resistances even more (Gbadegesin *et al.*, 2022; Rousham *et al.*, 2018; Xie  
119 *et al.*, 2018).

120 Wastewater treatment plants (WWTPs) are another important source of ARB and ARGs  
121 (Zhang *et al.*, 2022). The treatments currently conducted in WWTPs do not completely  
122 achieve the elimination of microbiological pollution or antibiotic residues, and many of  
123 the effluents and biological sludge treated in these plants are used as irrigation or fertilizer  
124 in agriculture or end up in natural waters (seas, rivers or lakes).

125 On the other hand, the soil microbiota is one of the largest reservoirs of ARGs, since it  
126 constitutes an ecosystem where, naturally, antibiotics-producing microorganisms induce  
127 the appearance and transfer of these genes (Peterson and Kaur, 2018). This fact is  
128 reinforced by the addition of contamination from anthropogenic activities such as  
129 livestock or agriculture. Considering the above, it could be concluded that human,

130 animals and the environment jointly contribute to the increase and propagation of AR  
131 (Tamhankar and Stålsby Lundborg, 2019).

132 The health and environmental problem currently raised by AR, make it necessary for the  
133 continuous investigation and control of the resistome, trying to reduce the selection  
134 pressure of AR as much as possible. Presently, there has been great progress in the  
135 methodology for the detection and study of ARB. Advances in genetic procedures are  
136 providing relevant knowledge of the molecular resistance mechanisms and the  
137 characterization of the genes which produce them (Yadav and Kapley, 2021).

138 The most utilised techniques for the study of ARGs are qPCR and metagenomics, and  
139 each of them has advantages and disadvantages. For instance, soil may contain thousands  
140 of bacterial species of which just a small fraction would be cultivable; in these type of  
141 studies, metagenomics can be useful to provide genetic data on non-cultivable bacteria  
142 (Geelen *et al.*, 2018). As a disadvantage, in metagenomic analyses the genetic sequences  
143 have to be defined in the libraries. For their part, qPCR techniques have the drawback of  
144 requiring the utilisation of a large number of specific primers for each gene. Therefore, it  
145 could be denoted that genetic techniques are highly effective in the ARGs detection, but,  
146 exclusively, those genes which have been previously characterised. Likewise, the  
147 presence of one gene in the bacterial genotype does not always lead to its phenotypic  
148 expression.

149 These reasons justify the relevance and usefulness of phenotypic procedures as a  
150 complement of genotypic studies. Among the advantages found in AR phenotypic  
151 studies, it can be mentioned: a) the ease of conducting the determination of resistances in  
152 minimally equipped microbiology laboratories; b) the economics of phenotypic  
153 procedures; c) the possibility of establishing AR determined by non-previously  
154 characterised genes; d) the quantification of bacteria in which the presence of a gene  
155 actually expresses the resistance effectively; e) the possibility of deducing the potential  
156 presence of the previously described ARGs from resistance phenotypes associated with  
157 those genes.

158 Heterotrophic aerobic bacteria (HAB) form the most salient group in relation to the  
159 appearance and transfer of AR. They include aerobic and facultative anaerobic  
160 heterotrophic bacteria and the majority of the pathogenic bacteria. For this reason, the  
161 most efficient way to conduct a phenotypic study of the resistome is by means of this  
162 group of bacteria.

163 In the present study, a phenotypic study has been performed of the environmental  
164 resistome in a peri-urban area, analysing and quantifying the HAB resistances in different  
165 ecological niches integrated in this ecosystem. The study includes the quantification of  
166 the percentage of the environmental microbiome resistant to antibiotics, the determination  
167 of phenotypic patterns of multi-resistance and the identification of possible genes  
168 potentially involved in these resistances.

169

## 170 **2. Materials and methods**

### 171 *2.1. Sampling*

172 The phenotypic study of the resistome has been accomplished in the peri-urban ecosystem  
173 of the city of Granada (Spain) during 2021. A total of 70 samples have been collected,  
174 originating from different environmental compartments: wastewater (n = 6), urban solid  
175 waste (n = 6), agricultural soil (n = 6), food (n = 12, of which 6 are raw meat and 6 are

176 fruits and vegetables), education centres (n = 12, of which 6 are air and 6 are surfaces),  
177 livestock industries (n = 18, of which 6 are air, 6 are feeders and 6 are solid waste) and  
178 natural soil not affected by anthropogenic activity (n = 10).

179 Solid and liquid samples were collected in sterile polyethylene containers and transported  
180 refrigerated to the laboratory (8-10 °C), being processed within 24 hours. Samples of raw  
181 wastewater and solid waste were obtained in municipal facilities in the peri-urban area.  
182 Soil samples were collected from the surface layer (approximately 10 cm) in different  
183 locations. Food samples were acquired in local establishments from the study area. Air  
184 samples were taken using an inertial volumetric sampler (Air Ideal 3PTM, BioMérieux),  
185 collecting between 500 and 1000 L of air. Finally, RODAC (Replicate Organisms Direct  
186 Agar Contact) contact plates were utilised to sample the surfaces.

## 187 2.2. *Biome extraction*

188 To extract the biome from the solid samples, 25 g of sample were mixed with 100 mL of  
189 peptone water (Panreac). Subsequently, the mixture was introduced into a homogenizer  
190 (Stomacher 400 Circulator) at 150 rpm for 15 minutes. Hence, the biome remained  
191 suspended in the liquid medium.

192 In the air and surface samples, the biome was obtained directly from the culture plates  
193 used in the sampling.

## 194 2.3. *Screening to determine resistant strains*

195 In order to select and quantify resistant HAB contained in the samples, BHI (Brain-Heart  
196 Infusion) agar (Panreac) was utilised in all cultures. 0.1 mL of each homogenized sample  
197 were inoculated on the surface of each plate, one without antibiotic, which will serve as  
198 the total BAH recount (control), and the other 8 plates containing an antibiotic each, at  
199 the appropriate concentration: ampicillin (32 µg /mL), kanamycin (64 µg/mL),  
200 tetracycline (16 µg/mL), ciprofloxacin (4 µg/mL), erythromycin (8 µg/mL), gentamicin  
201 (16 µg/mL), chloramphenicol (32 µg/mL) and vancomycin (16 µg/mL). These antibiotics  
202 were selected for being indicative of the presence of transferable ARGs, using the MIC  
203 (Minimum Inhibitory Concentrations) breakpoints concentrations to consider a bacterium  
204 as resistant (CLSI, 2022; EUCAST, 2022). Regarding air and surface samples, the same  
205 culture medium with and without antibiotic was used, just like the homogenized samples.  
206 All cultures were always conducted in duplicate. The cultured plates were inoculated at  
207 37°C for 48 hours to, successively, perform the recount. By comparing the control plates  
208 (without antibiotic) with the plates cultured with each antibiotic, it was possible to obtain  
209 the percentage of biome resistant to each of the antibiotics utilised.

## 210 2.4. *Phenotypic analysis*

211 From all the culture plates with antibiotic recounted in the screening, a maximum of 16  
212 morphologically different strains were selected, obtaining a total of 710 strains, in which  
213 has been conducted the phenotypic resistance study. To this aim, sensitivity to antibiotics  
214 was performed using the Kirby-Bauer disk diffusion susceptibility test, following  
215 standardized methods (CLSI, 2022; EUCAST, 2022). Each strain was analysed against  
216 32 antibiotics (disk concentration is indicated in brackets): amikacin (30 µg),  
217 spectinomycin (100 µg), streptomycin (10 µg), gentamicin (10 µg), kanamycin (30 µg),  
218 tobramycin (10 µg), trimethoprim-sulfamethoxazole (25 µg), amoxicillin-clavulanate (30  
219 µg), doripenem (10 µg), imipenem (10 µg), meropenem (10 µg), cephalothin (30 µg),  
220 cefazolin (30 µg), cefepime (30 µg), cefotaxime (30 µg), ceftaxidime  
221 (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg),  
222 ciprofloxacin (5 µg), levofloxacin (5 µg), fosfomicin-trometamol (200 µg), teicoplanin

223 (30 µg), vancomycin (30 µg), clindamycin (2 µg), erythromycin (15 µg), aztreonam (30  
224 µg), ampicillin (10 µg), piperacillin (100 µg), ticarcillin (75 µg) and tetracycline (30 µg).

225 Based on the description of the AR produced by the different genes previously mentioned  
226 (Jian *et al.*, 2021; Leite *et al.*, 2021; Li *et al.*, 2013; Poomchuchit *et al.*, 2021; Zhu *et al.*,  
227 2021; Zhuang *et al.*, 2021; Zurfluh *et al.*, 2020), probable ARGs were estimated on the  
228 basis of the multi-resistance found in the biome, contributing to further information about  
229 the resistome.

### 230 2.5. Statistical analysis

231 The data obtained were entered into the database and processed using IBM SPSS 28  
232 version software. The statistical analysis has basically consisted of a descriptive study,  
233 conducting ANOVA tests and comparison of independent averages, in order to verify  
234 whether the differences between the samples are significant.

235

## 236 3. Results

### 237 3.1. Screening of resistant strains

238 As stated in the methodology, the percentages of resistant HAB to each of the antibiotics  
239 added to the culture medium was obtained in the resistance screening, by comparison of  
240 the recounts with the control plates without antibiotic. In calculating the total average  
241 value of the percentage of strains resistant to every antibiotic, a 12.9% of the HAB present  
242 in the diverse samples have exhibited some resistance to the antibiotics used in the  
243 screening procedure. Taking into consideration each antibiotic, the lowest resistance  
244 percentage is observed in chloramphenicol, 2.7% of resistant strains, and gentamicin,  
245 2.9%; whereas the highest percentage corresponds to erythromycin, 33.4%, followed by  
246 vancomycin, 27%.

247 The frequency of resistant strains in each of the ecological niches of the peri-urban  
248 environment has also been analysed and the differences between the different  
249 environments studied are significant (ANOVA:  $p < 0.001$ ). The highest percentage of  
250 resistant strains has been observed in wastewater samples (32.3% of the strains), and the  
251 lowest percentage, in natural soil samples (3.6%).

252 Foods have been distinguished into fruits/vegetables and meats. The differences between  
253 fruits/vegetables and meats have been analysed using the independent averages  
254 comparison test, observing significant differences in the percentages of strains resistant  
255 to ampicillin, tetracycline, erythromycin, chloramphenicol and vancomycin. The  
256 difference in the total number of resistant strains is also significant. Individually, in  
257 fruits/vegetables the highest percentage of strains manifested resistance to vancomycin,  
258 erythromycin and ampicillin, whereas in meats predominate the strains resistant to  
259 tetracycline.

260 The statistical analysis of the distribution of strains resistant to each antibiotic according  
261 to the origin of the samples, using ANOVA, just indicates significant differences for  
262 ampicillin ( $p = 0.001$ ), erythromycin ( $p < 0.001$ ), chloramphenicol ( $p = 0.004$ ) and  
263 vancomycin ( $p < 0.001$ ). The greatest level of resistant strains has been obtained for the  
264 antibiotics erythromycin and vancomycin in samples from all sources, which coincides  
265 with the global analysis stated above. It can also be observed that wastewater is the  
266 medium with the highest percentage of bacteria resistant to ampicillin, in addition to  
267 erythromycin and vancomycin. On the contrary, farm samples present the greatest  
268 percentage of resistance to chloramphenicol. Both wastewater and solid waste, farms,

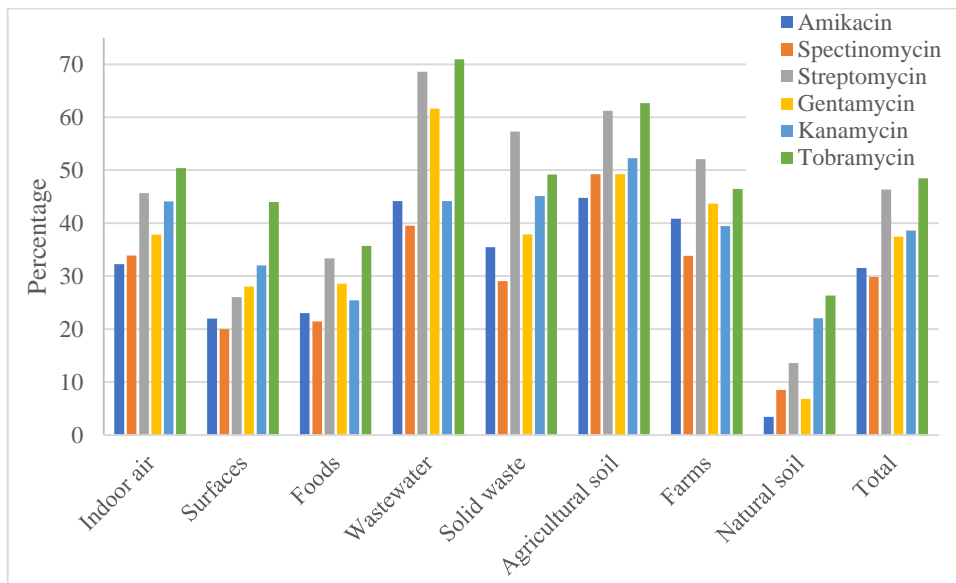
269 indoor air, surfaces and agricultural soil samples, the highest percentage of resistance to  
 270 the antibiotics analysed in the screening has been detected in the presence of  
 271 erythromycin, being the resistance percentages 89.2%, 36%, 35.1%, 30.2%, 25.7% and  
 272 25.1%, respectively; whereas in foods and natural soil samples, the highest frequency of  
 273 resistance was obtained in the presence of vancomycin (48.7% and 7.3%, respectively).

274 *3.2. Resistome analysis.*

275 The resistome analysis was performed on 710 HAB strains isolated from the screening  
 276 test cultures, as indicated in the methodology. The percentage of resistance has been  
 277 analysed for the 32 antibiotics studied, separating them by the different groups of  
 278 antibiotics.

279 Resistance to aminoglycosides was detected in a large number of strains (figure 1),  
 280 expressing the highest level of resistance to tobramycin. Globally, 48.5% of the 710  
 281 bacterial strains studied showed resistance to tobramycin, reaching up to 70.9% of strains  
 282 resistant to this antibiotic in wastewater. Streptomycin also presented high levels of  
 283 resistant strains in wastewater (68.6%), agricultural soil (61.2%) and solid waste (57.3%).  
 284 The lowest percentages of resistant strains were observed in natural soil, with a maximum  
 285 value of 26.3% in tobramycin and a minimum value of 3.4% in amikacin.

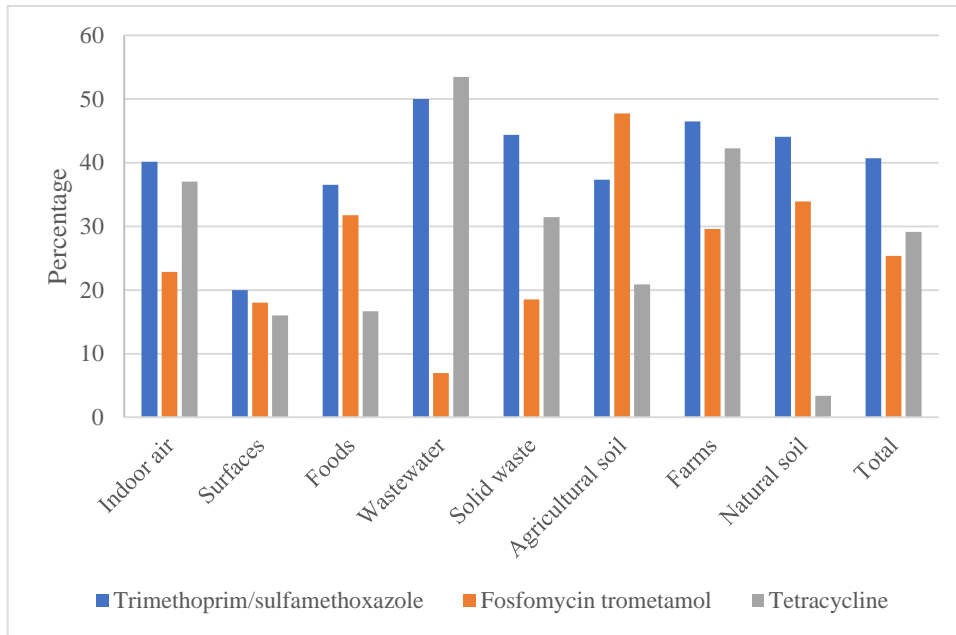
286 **Figure 1.** Resistome analysis. Distribution of resistance to aminoglycosides in HAB  
 287 strains isolated from the screening test cultures.



288 N=710; Chi-square: p<0.001 for all antibiotics.  
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290 Several antibiotics shown in figure 2 also presented a high percentage of isolated ARB.  
 291 Regarding trimethoprim/sulfamethoxazole, 40.7% of the total isolated strains were  
 292 resistant, and it reached a 50% in wastewater and a 44.1% in natural soil. The highest  
 293 percentage of bacteria resistant to fosfomicin/trometamol was found in agricultural soil  
 294 (47.8%) followed by natural soil (33.9%). For tetracycline, few resistant strains were  
 295 found in natural soil (3.4%), nonetheless they were quite abundant in wastewater (53.5%)  
 296 and farms (42.3%).

297 **Figure 2.** Resistome analysis. Distribution of resistance to a miscellaneous group of  
 298 antibiotics in HAB strains isolated from the screening test cultures.

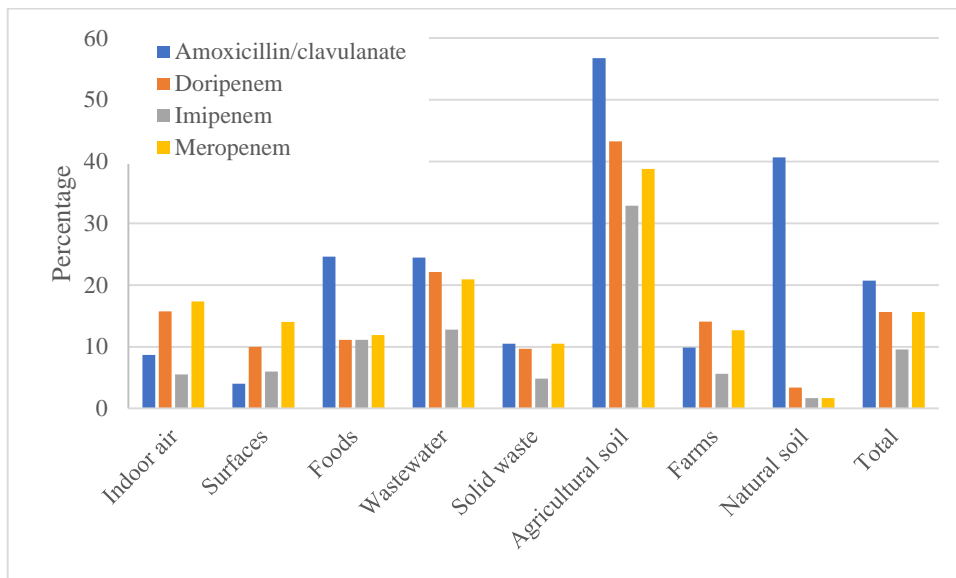


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N=710; Chi-square: trimethoprim/sulfamethoxazole, p=0.034; fosfomycin/trometamol and tetracycline, p<0.001.

302 In relation to the group of beta-lactam and carbapenem antibiotics (figure 3), the highest  
303 number of ARB was found in agricultural soil (amoxicillin/clavulanate, 57.6%;  
304 doripenem, 43.3%; imipenem, 32.8%; and meropenem, 38.8%). The data of 40.7% of  
305 bacteria resistant to amoxicillin/clavulanate in natural soil stood out.

306 **Figure 3.** Resistome analysis. Distribution of resistance to beta-lactams and carbapenem  
307 antibiotics in HAB strains isolated from the screening test cultures.

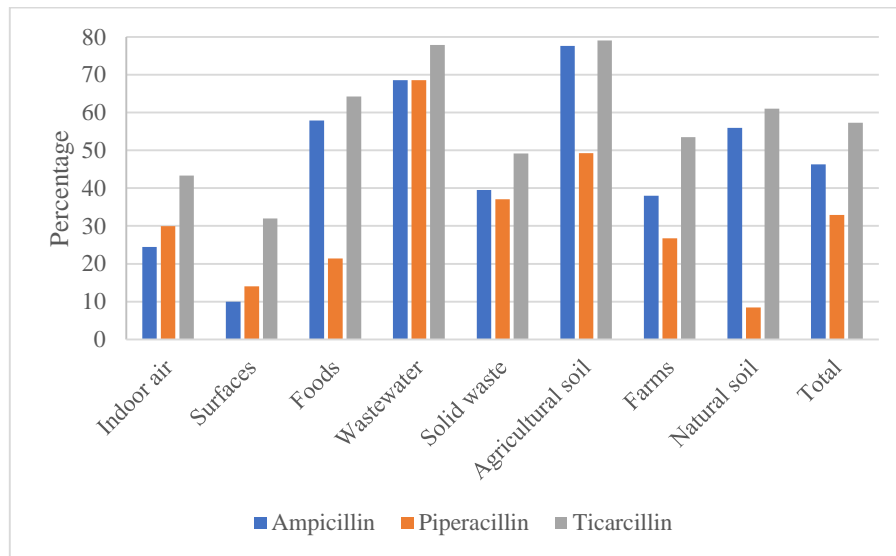


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N=710; Chi-square: p<0.001 for all antibiotics.

310 Resistance to the penicillin group was highly abundant (figure 4). The maximum values  
311 were observed for ampicillin (77.6%) and ticarcillin (79.1%) in agricultural soil, and for  
312 piperacillin (68.6%) in wastewater. In this group of antibiotics was also remarkable the  
313 large number of resistances observed in natural soil samples, in which values of 55.9%  
314 for ampicillin and 57.3% for ticarcillin were obtained.

315 **Figure 4.** Resistome analysis. Distribution of resistance to penicillin antibiotics in HAB  
 316 strains isolated from the screening test cultures.

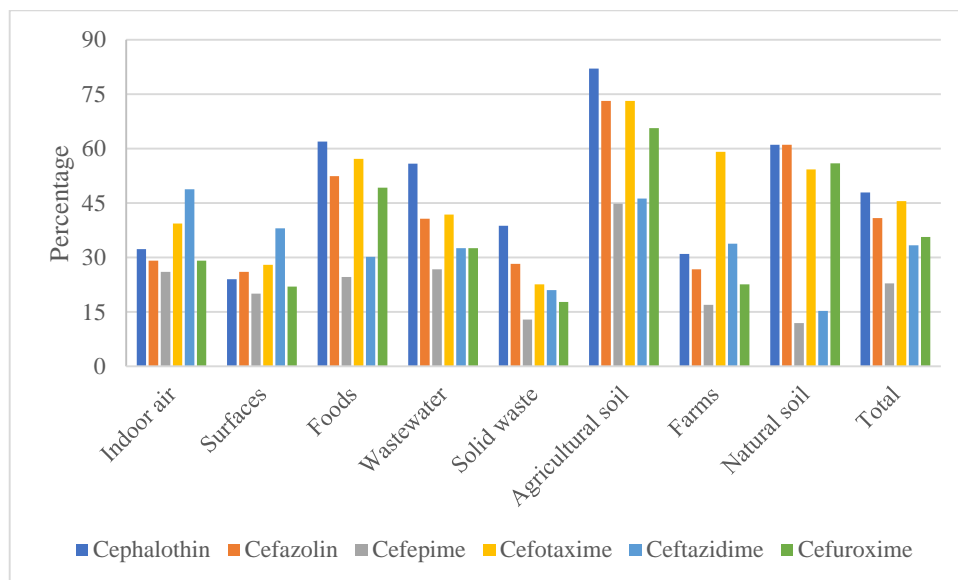


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N=710; Chi-square:  $p < 0.001$  for all antibiotics.

319 Resistance to the cephalosporin group also exhibited high values in all environmental  
 320 compartments (figure 5). Amongst the most outstanding data, it could be noted that the  
 321 highest values of ARB were observed in the agricultural soil, in which 82.1% of the  
 322 strains expressed resistance to cephalothin and 73.1% to cefazolin and cefotaxime.

323 **Figure 5.** Resistome analysis. Distribution of resistance to cephalosporin antibiotics in HAB  
 324 strains isolated from the screening test cultures.

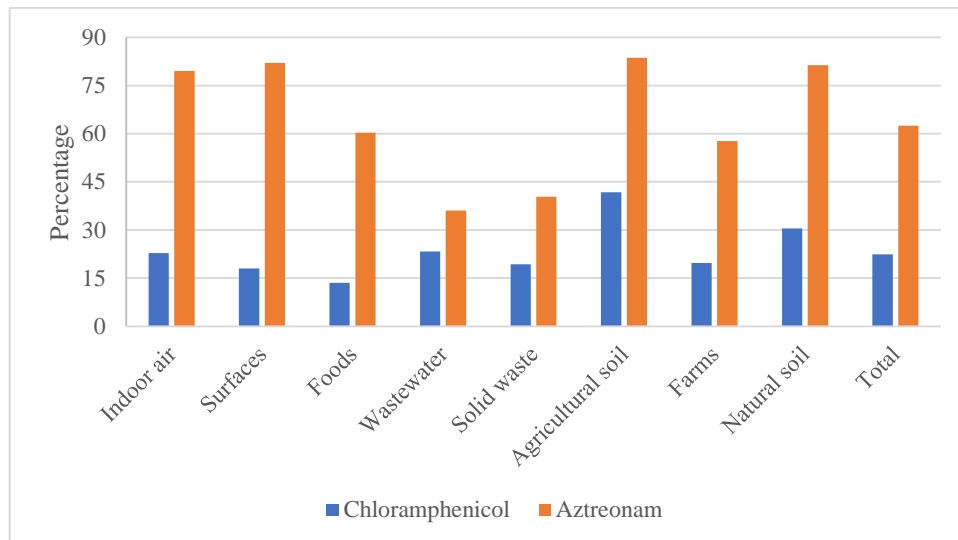


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N=710; Chi-square:  $p < 0.001$  for all antibiotics.

327 With respect to monobactams, aztreonam showed high levels of resistance with a wide  
 328 distribution, exceeding 70% in indoor air, surfaces, agricultural soil and natural soil  
 329 (figure 6). In the phenicol group, chloramphenicol exhibited lower levels of resistance,  
 330 with a 41.8% being the highest value in agricultural soil.

331 **Figure 6.** Resistome analysis. Distribution of resistance to phenicol and monobactam  
 332 antibiotics in HAB strains isolated from the screening test cultures.



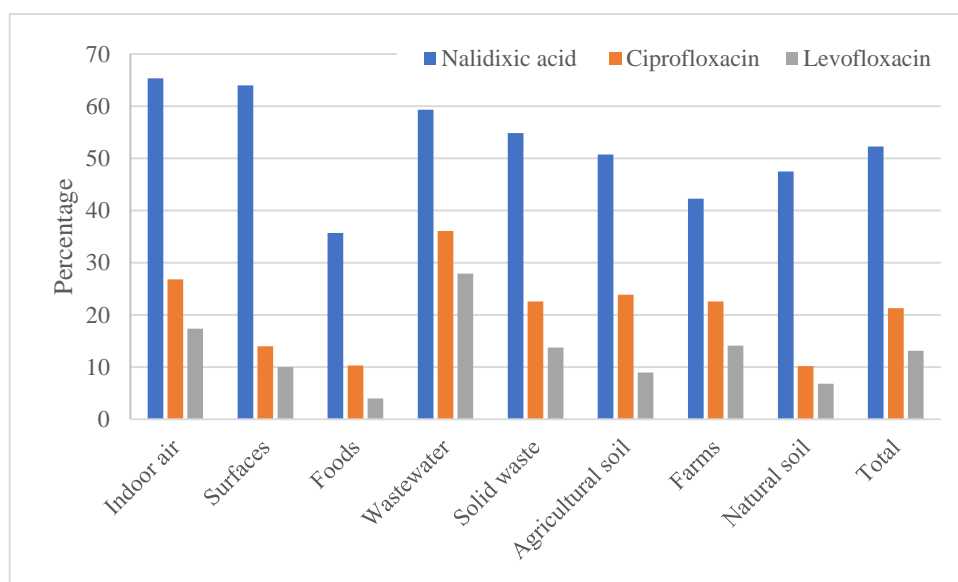
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N=710; Chi-square:  $p < 0.001$  for all antibiotics.

335 In the antibiotics of the fluoroquinolone group (figure 7), the high level of bacteria  
336 resistant to nalidixic acid stood out in all the environmental compartments analysed.  
337 Considering the total of the strains studied in all media, the results revealed that 52.3%  
338 were resistant to this antibiotic. In indoor air and surfaces, resistance to nalidixic acid  
339 exceeded 60% and, in natural soil, 47.5% was observed. For the rest of the  
340 fluoroquinolones included in the study, the resistance levels were lower, although in  
341 wastewater 36.1% showed to be resistant to ciprofloxacin and 27.9% to levofloxacin.

342 **Figure 7.** Resistome analysis. Distribution of resistance to fluoroquinolone antibiotics in  
343 HAB strains isolated from the screening test cultures.

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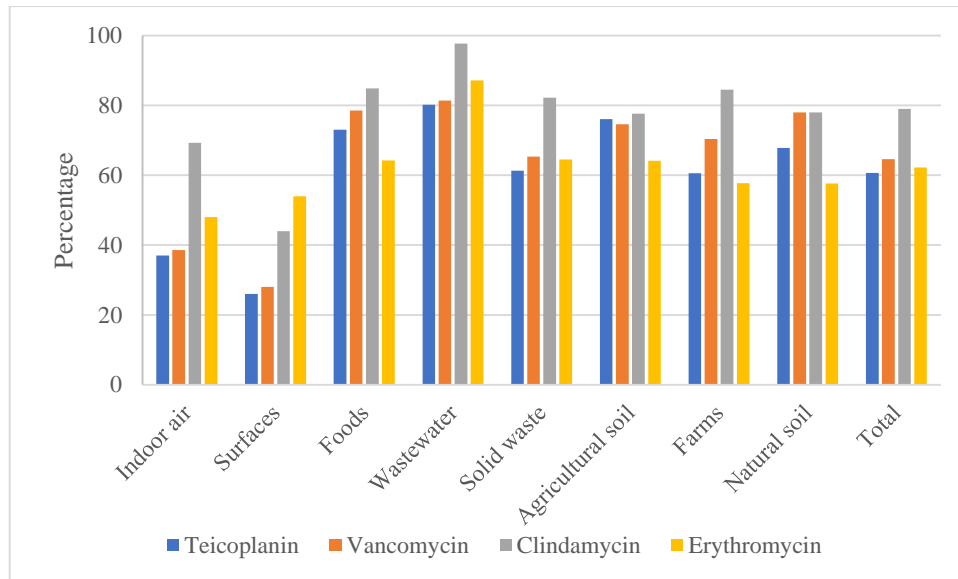
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N=710; Chi-square:  $p < 0.001$  for all antibiotics.

347 In the group of glycopeptide antibiotics (figure 8), 80.2% resistance to teicoplanin and  
348 81.4% to vancomycin was achieved in wastewater. The resistance levels reached for these  
349 antibiotics in food and natural soil were also particularly high. With regard to macrolide  
350 antibiotics, 97.7% of the strains isolated in wastewater were resistant to clindamycin, as  
351 well as 84.9% in foods. The value in natural soil was quite remarkable, 78% of the strains

352 were resistant to this antibiotic. Respecting erythromycin should be noted an 87.2% of  
 353 resistance in wastewater.

354 **Figure 8.** Resistome analysis. Distribution of resistance to glycopeptides and macrolides  
 355 in HAB strains isolated from the screening test cultures.



356

357 N=710; Chi-square: p<0.001 for all antibiotics.

358 Based on the phenotypic resistance profile, the ARGs present in the isolated strains can  
 359 be deduced with a high probability. The ARGs considered, which could cause resistance  
 360 to the groups of antibiotics analysed, were: *bla* (penicillins,  $\beta$ -lactams, cephalosporins,  
 361 carbapenems and monobactams), *qnr/oqx* (quinolones and fluoroquinolones), *aac/ant*  
 362 (aminoglycosides), *str* (streptomycin), *sul/dfr* (sulfonamides), *flo* (chloramphenicol), *fos*  
 363 (fosfomicin), *van* (glycopeptides), *erm* (macrolides) and *tet* (tetracyclines).

364 Through this procedure, the enormous presence of the *bla* gene has been deduced, which  
 365 could be present in 100% of the strains isolated from agricultural soil and in 90.1% of all  
 366 bacteria isolated in all the environmental compartments analysed (table 1). *van* and *erm*  
 367 genes would also be highly frequent, with maximum percentage values in wastewater.  
 368 *aac* and *ant* genes, which encode the aminoglycoside acetyltransferase and  
 369 aminoglycoside nucleotidyltransferase inactivating enzymes respectively, would be more  
 370 abundant in wastewater (83.7%), solid waste (77.4%) and agricultural soil (77.6%) media.  
 371 In natural soil the most frequent probable genes would be *bla* (88.1%), *van* (78%) and  
 372 *erm* (79.7%).

373 **Table 1.** Percentage of probable strains bearing ARGs according to the deduction of the  
 374 phenotypic profile.

	<i>Bla</i>	<i>qnr/oqx</i>	<i>aac/ant</i>	<i>str</i>	<i>sul/dfr</i>	<i>flo</i>	<i>fos</i>	<i>van</i>	<i>erm</i>	<i>Tet</i>
Indoor air	92,13	66,14	70,87	45,67	40,16	22,83	22,83	40,94	81,10	37,01
Surfaces	95,92	65,31	48,98	26,53	20,41	18,37	18,37	28,57	83,67	16,33
Foods	93,65	36,51	50,00	33,33	36,51	13,49	31,75	80,95	86,51	16,67
Wastewater	87,21	59,30	83,72	68,60	50,00	23,26	6,98	81,40	98,84	53,49
Solid waste	79,03	55,65	77,42	57,26	44,35	19,35	18,55	67,74	85,48	31,45
Agricultural soil	100,00	53,73	77,61	61,19	35,82	41,79	47,76	76,12	86,57	20,90
Farms	91,55	46,48	69,01	52,11	46,48	19,72	29,58	69,01	88,73	42,25

Natural soil	88,14	47,46	32,20	13,56	44,07	30,51	33,90	77,97	79,66	3,39
Total	90,13	53,46	65,59	46,40	40,62	22,43	25,39	66,01	86,32	29,20
Chi-square	<0,001	<0,001	<0,001	<0,001	0,04	0,001	<0,001	<0,001	0,016	<0,001

375 The large majority of bacteria would not present a single resistance gene. Of the 710  
376 bacteria isolated, just 3 bear a single gene: *aac/ant*, *van* and *bla*, respectively. The rest of  
377 the strains contain a diverse number of ARGs in their genotype combined in a different  
378 way to form the resistome. The combination *bla van erm* was the most frequent, being  
379 present in 7.2% of the strains. Other abundant genetic combinations were *bla aac/ant str*  
380 *van erm* (3.9%), *bla aac/ant str sul/dfr van erm* (3.1%) or *bla qnr/oqx aac/ant str sul/dfr*  
381 *flo van erm tet* (2.7%).

382

#### 383 4. Discussion.

384 The genetic and functional diversity of the resistome is highly wide and reflects billions  
385 of years of evolution of microorganisms in close contact with toxic molecules from really  
386 different backgrounds, most of them produced by themselves for their own defence.  
387 Antibiotics and resistance mechanisms have been present in the environment for millions  
388 of years and, hence, long before of being discovered by humanity. Their usage has  
389 enabled endless medical therapies, unthinkable in times when infection control was  
390 absent. However, the massive overuse of antibiotics has occasioned a selection pressure  
391 of the resistance mechanisms. Resistome represents a relevant impediment for using  
392 habitual antibiotics in the treatment of infectious diseases. Therefore, resistance is a real  
393 threat in modern medicine. The lesson from antibiotics history is that all of them are  
394 subject of resistance and the genetic diversity and the propagation mechanisms are  
395 incredibly broad. The enormous pool of resistance elements in environmental bacteria,  
396 accumulated during millennia, provide a huge reservoir of new resistance elements which  
397 can be captured and mobilized in pathogenic agents. The resistance thus, returns  
398 inevitable, demanding the continuous discovering of new drug candidates to maintain the  
399 infection control (Hobson *et al.*, 2021).

400 Antibiotic resistance is expressed in a phenotype that can be detected by growth inhibition  
401 in trials performed by disk diffusion in agar, and is interpreted as a resistant or sensitive  
402 microorganism. The disk diffusion susceptibility method is a simple and well-  
403 standardized method that allow for the study of resistomes and, currently, it is still used  
404 (Hartinger *et al.*, 2021; Papajová *et al.*, 2022). Compared to phenotypic studies, modern  
405 genomic techniques overcome some limitations in comparison with methods based on  
406 bacterial culture (Schmieder and Edwards, 2012). Nevertheless, metagenomic has  
407 constraints: not all genes identified are efficiently expressed in every host, and not all the  
408 genes are found in metagenomic libraries (Nowrotek *et al.*, 2019).

409 In fact, evaluation studies about the translation of genomic data to the phenotypic  
410 resistance profile are not sufficiently reliable at present. Currently, prediction of  
411 phenotypes using genetic sequencing techniques is premature and lacks reliability  
412 compared to traditional bacterial culture methods (Lee *et al.*, 2021). Due to these kind of  
413 limitations, phenotypic studies with microorganisms' isolation should not be dismissed  
414 (Navarro *et al.*, 2011). Some authors even denote that the resistome study using bacterial  
415 culture and the AR determination by disk diffusion or agar dilution continue being the  
416 gold standard tool for every microbiologist (Selvarajan *et al.*, 2023). Phenotypic detection  
417 methods are generally easy to execute, interpret and implement in the laboratory routine  
418 and they also provide relevant information.

419 This investigation has been performed in an urban and peri-urban environment,  
420 comparable to other ecosystems with similar characteristics. In order to accomplish this,  
421 different ecological niches or compartments were distinguished: indoor air, surfaces,  
422 foods, wastewater, solid waste, agricultural soil, farms and natural soil. It must be  
423 considered that these compartments are not closed, but rather there is an exchange of  
424 resistance mechanisms between them that, overall, it defines the resistome from a  
425 particular ecosystem. The study has been conducted on HAB because it is the most  
426 representative group and has the greatest exchange capacity, since anaerobic bacteria  
427 have a further limitation. In the work undertaken, the aim has been to know two different  
428 aspects of the resistome. Firstly, to quantify the percentage of resistant HAB in relation  
429 to the total HAB found in the studied ecosystem, considering the several compartments.  
430 For that purpose, a screening test have been used. Secondly, we have studied and  
431 quantified the phenotype of resistance to a great number of antibiotics, performing an  
432 antibiogram, in a large number of bacterial strains selected at random from the screening  
433 cultures. From this data, according to the resistance profile, it has been defined the  
434 potential presence of ARGs.

435 Culture in media with antibiotics are utilised as a screening test, but it has been made  
436 quantitatively. The results show a microbiome with a high percentage of resistances,  
437 especially to erythromycin and vancomycin. In the quantification of AR by  
438 compartments, it may be observed that all of them present a salient percentage of ARB.  
439 The highest percentage of resistant microbiome is appreciated in wastewater, followed by  
440 farms, foods and solid waste, media which have habitually presence of antibiotics and the  
441 proliferation of microorganisms favors the exchange of genetic material (Anthony *et al.*,  
442 2020; Uluseker *et al.*, 2021). Actually, wastewater and the wastewater treatment plants  
443 are considered critical points for the proliferation of ARB and the exchange of ARGs  
444 (Selvarajan *et al.*, 2023; Shen *et al.*, 2023).

445 The high presence of ARGs to beta-lactams and tetracycline in farms coincide with the  
446 published results corresponding to soils that have been fertilized with manure of bovine  
447 and swine origin, which indicate that the presence of these genes are the response to the  
448 selection pressure of the great tetracycline consumption in livestock (Chen *et al.*, 2019;  
449 Osorio *et al.*, 2023; Qi *et al.*, 2023; Xiong *et al.*, 2018). In foods, we have distinguished  
450 between fruits/vegetables and meats. Comparing both subgroups, it may be noted that, in  
451 meats, the highest frequency of resistant strains also corresponds to tetracycline, since, as  
452 it has been mentioned above, it is an antibiotic widely used in livestock; in contrast, the  
453 highest resistance level in fruits/vegetables is to vancomycin, possibly due to the  
454 existence of a greater frequency of gram-negative bacteria.

455 Based on the phenotype of the samples analysed, the ARGs which presence is the most  
456 probable have been estimated. *bla*, *aac/ant*, *van* and *erm* have been the most frequent  
457 genes found in wastewater, which habitually appear in other resistome studies in  
458 wastewater (Rodríguez *et al.*, 2021). The presence of antibiotics, metals and disinfectants  
459 in this medium could act as a selection pressure for the selection and transfer of ARGs  
460 (Kim and Cha, 2021).

461 It should be noted that the majority of the research studies on resistome are limited to  
462 wastewater and water consumption systems, natural soil, agricultural soil and foods.  
463 However, there are recent evidences about AR in the airborne microbiome (Gwenzi *et al.*,  
464 2022). The presence of ARGs is superior in aerosols from indoor environments  
465 compared to outdoor aerosols (probably due to a greater air ventilation); nevertheless, the  
466 composition of the AR is variable. Thus, it has been described a higher abundance of  
467 resistance to beta-lactams and tetracycline in indoor aerosols, while in outdoor

468 environments, although the resistance to tetracycline is also abundant, it predominates the  
469 resistance to aminoglycosides (Zhao *et al.*, 2021). In an investigation accomplished at the  
470 University of Agriculture of Krakow (Poland), it was found that, of the staphylococci  
471 isolated in air, more than a 50% were resistant to erythromycin and about the 33% to  
472 tetracycline (Wolny-Kołodka *et al.*, 2019). Additionally, in another investigation related  
473 to airborne bioaerosols in university facilities in Ibadan (Nigeria), resistance to  
474 tetracycline was detected in the 75% of the strains (Giwa and Adeniyi, 2017). In the  
475 various studies previously conducted in the resistome, a widely variety of genes have  
476 been included, such as *bla*, *erm*, *van*, *tet* and *aac* (Banchón *et al.*, 2021; Gao *et al.*, 2023;  
477 Gwenzi *et al.*, 2022). In our indoor air screening samples, an 8.9% of the resistant strains  
478 were resistant to all antibiotics, and a 30.2% to erythromycin. In the isolated bacteria, the  
479 greatest frequencies of resistance were obtained for aztreonam (79.5%) and clindamycin  
480 (69.3%), being the most frequent resistance genes *bla* (92.1%), *erm* (81.1%) and *aac/ant*  
481 (70.9%).

482 It is relevant to note that natural soil is an ecosystem selected for not having antibiotics  
483 or human action and which only receives rainwater. However, in this environment, a 3.6%  
484 of strains resistant to some of the antibiotics used in the screening test was already  
485 detected, increasing to an 8.9% in the agricultural soil, confirming the presence of ARGs  
486 in natural ecosystems. A study in soils reveals the abundant presence of *erm* genes along  
487 with PKS genes involved in the synthesis of different antibiotic substances, including  
488 erythromycin, but no significant differences were found in *erm* and PKS genes between  
489 soils with or without anthropogenic influence (Yi *et al.*, 2019). Nonetheless, numerous  
490 investigations have verified that the use of antibiotics exerts a selection pressure for  
491 resistance and, despite ARGs to beta-lactam, fluoroquinolone, macrolide and tetracycline  
492 have been found in non-anthropogenically impacted areas, these genes are very much  
493 abundant in areas with anthropogenic impact (Centurion *et al.*, 2022). Even then, many  
494 studies have demonstrated that natural soil is the origin of the ARGs and it is considered  
495 as a salient reservoir of resistome, both intrinsic and gene-acquired resistance. These  
496 genes can be selected and mobilized, emerging ubiquitously, and with special  
497 significance in clinical environments (Kim and Cha, 2021).

498 Analysing our resistome results as a whole, it can be generalized that some ecosystem  
499 compartments constitute the principal reservoir of AR: wastewater, solid waste and  
500 agricultural soil. Previous studies have reported the relevance of the soil microbiome  
501 under the influence of anthropogenic activities on the ecosystem resistome. Organic  
502 fertilizer and treated or untreated wastewaters destined to crop irrigation have been  
503 identified as human and animal waste products capable of influencing the diversity and  
504 prevalence of ARGs in agricultural soils (Kim and Cha, 2021).

505 Our results exhibit that *bla*, *aac/ant*, *van* and *erm* genes appear more frequently in the  
506 total strains, coinciding with the high consumption of beta-lactams antibiotics and  
507 aminoglycosides for a long period, and more recently, with the consumption of  
508 glycopeptides (vancomycin) and macrolides (erythromycin). Moreover, these same genes  
509 are widely distributed in every environmental compartment and, especially, in  
510 wastewater, agricultural soil and solid waste.

511 Finally, it is necessary to emphasize that bacteria very frequently harbour more than one  
512 resistant gene, just as it has been observed in our results: this is the case of the *bla van*  
513 *erm* genotype, the most frequent found, or genotypes as extensive as *bla qnr/oqx aac/ant*  
514 *str sul/dfr flo van erm tet*. This fact is significant since the use of one single antibiotic is  
515 capable of selecting a multigenic resistance strain that lead to the resistance to numerous  
516 antibiotics.

517

## 518 **5. Conclusions.**

519 By the realization of this phenotypic study, it has been possible to verify that the urban  
520 and peri-urban ecosystem exhibit a very extensive resistome, less abundant in the natural  
521 environment, and which it increases until reaching the maximum value in those ecological  
522 niches where exists an abundance of microorganisms and a selection pressure for  
523 antibiotics. The conducted study has allowed to confirm that ARGs are not isolated in  
524 different bacteria within the ecosystem; they rather bear a large genetic load of multiple  
525 ARGs, which confer them multi-resistance, causing one single antibiotic to be able to  
526 select the resistance to other antibiotics of diverse nature, and being transmitted between  
527 different microorganisms from several ecological niches, spreading widely throughout  
528 the environment.

529 The solution to this problem is certainly complicated, but it is clear that it must include a  
530 reduction of the multi-resistant microbiome in the ecological niches of the greatest  
531 anthropic intervention, an urgent rationalization of the antibiotics utilisation and  
532 especially in the greater control of antibiotic residues and ARB released into the  
533 environment.

534

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539

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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