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Impact of urbanization on antimicrobial resistance in soil microbial communities

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Soil is one of the most important reservoirs of antibiotic resistance, global threat that needs to be addressed with the One Health approach. Despite urban parks playing a fundamental role in urban ecosystems, the diffusion, maintenance, and human impact of antibiotic-resistance genes in this substrate are still poorly addressed. To fill in this gap, we adopted a molecular and culturomics approach to study antibiotic resistance in urban parks, accounting for the environmental matrix and the level of urbanization. A higher abundance of efflux-mediated mechanisms in undisturbed environments was observed, while antibiotic alteration or inactivation, and target replacement were more abundant in areas with a higher level of urbanization, also confirmed by significant correlations with anthropogenic features of the environmental matrix. Overall, this study highlights the crucial need to monitor antibiotic resistance in urban parks' soil through a dual molecular and culturomics approach to fully understand and fight antibiotic resistance diffusion.

Antimicrobial resistance (AMR) is the result of natural evolution promoted by the selective pressure that some organisms and environments exert through the synthesis and release of antimicrobial compounds[1]. The abuse and inappropriate use of antimicrobial treatments in the clinic and agronomic environments have intensified AMR, leading to increasing resistance of pathogens to treatments and the ineffectiveness of antibiotics. In 2019 alone, 4.95 million people died due to antimicrobial resistance, and among them, 1.27 million died directly due to the ineffectiveness of the treatments used[2]. According to experts, this number will increase in the coming years, making antibiotic resistance one of the ten threats to global health[1,3,4]. To counteract and reduce antibiotic resistance, the World Health Organization (WHO) provided guidelines on the proper use of antibiotics and established a comprehensive antimicrobial resistance monitoring plan in clinical settings[5,6]. However, no single action will per se provide an adequate solution for AMR. Although monitoring needs to be maintained and expanded in the clinical setting (as not all countries can provide data on antibiotic resistance)[7], it is pivotal to monitor the antimicrobial resistance in other types of environments, adopting the One-health approach[4].The ability to exchange genes between microorganisms through horizontal gene transfer (HGT) and the growing evidence that factors other than antibiotics act as selective agents promote the development and spread of AMR, clearly call for the need for a multiple-sector approach to deal with[4]. Anthropogenic activities, such as the pharmaceutical industry, aquaculture, wastewater treatment plants, personal hygiene products, chemicals, pharmaceuticals, plastics, and heavy metals, play a key role in the environmental spread of antimicrobial resistance[8]. Urban soils are continuously influenced by anthropogenic activities, such as residential, industrial, and transportation practices, which may contribute to shaping the composition of the bacterial community and the soil antibiotic resistance[9]. The contribution of soil in promoting and preserving AMR has been so far confirmed for environments linked to direct human activities, as treatments in agricultural settings. Yet, this investigation was only rarely applied to urban soil, a source potentially favoring the transmission of AMR from the environment to humans[10,11]. Furthermore, most studies have investigated the soil resistome either by looking at antibiotic resistance phenotypes in culturable microorganisms or antibiotic resistance genes alone[9], hence missing the opportunity to explore the actual contribution of natural non-pathogenic microorganisms in AMR diffusion.

Our study investigates antimicrobial resistance in the soil of seven urban parks in Turin (Italy), and two undisturbed native habitats near the same city by applying metagenomics and culturomics approaches. Our data provide a novel and comprehensive picture of the environmental antibiotic resistance reservoir occurring in urban soils.

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Results

Occurrence and distribution of antibiotic resistance genes in urban parks are associated with urbanization

Eleven sites in the Turin province were selected as representative of different environmental contexts (Supplementary Fig. 1 and Supplementary Table 1). According to the percentage of the area covered by urban features (Road Network and built-up areas), sites were classified into different urbanization grade (UG) classes, namely lowest (LowestUG), low (LowUG), middle (MidUG), or highest (HighUG). A metagenomics analysis was performed on soil samples allowing the identification of Antibiotic Resistance Genes (ARGs) and, according to the hierarchical organization of the database[12] the corresponding antimicrobial resistance genetic classes and mechanisms (Supplementary Table 2). The abundance of AMR classes and mechanisms was evenly distributed among the investigated sites and urbanization classes (Wilcoxon-Mann-Whitney test FDR > 0.05, Supplementary Table 2), with 14 out of the 17 identified classes (77.8%) and 5 mechanisms (83.3%) found in every samples' urbanization grade group (Table 1 and Supplementary Fig. 2). The percentage of shared molecular features drastically decreased when delving into the components of classes and mechanisms: only 57.8% of ARGs (63) and 29% of IDs (orthologs ARGs associated with different prokaryotic taxa) were present in every urbanization grade group (Supplementary Fig. 2). Furthermore, IDs showed significant differences among different sites: the LowUG NSO site showed a higher number of IDs compared to the PEL, GRO, and TES sites, with the two latest being characterized by a higher urbanization grade, and the TES site (HighUG) shows more IDs compared to the HighUG PAV site (Wilcoxon-Mann-Whitney test FDR < 0.05) (Table 1 and Supplementary Table 2). Despite this data suggesting a site-specific distribution of IDs and ARGs, parks in undisturbed areas (LowestUG, STU and MAN) showed the highest number of IDs and ARGs which were absent or sporadically occurring in the sites with higher urbanization grades (Fig. 1a, and Supplementary Fig. 2). Samples from different sites also showed significant differences in the composition of IDs, ARGs, classes, and mechanisms, as shown by β-diversity analyses (PCoA and permAnova on Bray-Curtis distances, $P = 0.001$, Supplementary Table 2, Supplementary Fig. 3). Conversely, the same diversification observed among sites was not appreciable among urbanization grade groups (permAnova $P = 1$, Supplementary Table 2). These observations suggest that the distribution of ARGs, classes, and mechanisms associated with antibiotic resistance may be affected by environmental features (as shown by differences in alpha diversity according to urbanization grade) and such differences could be associated with the diversity of ARGs bore by site-specific microbial taxa (highlighted by site-specific clustering in ordination analyses).

As a confirmation of this hypothesis, significant differences in the relative abundance of mechanisms of antibiotic resistance across sites and urbanization grades were observed (Fig. 1b and Supplementary Table 3). The antibiotic target alteration was significantly less represented in the LowestUG sites than in more urbanized areas, and those differences were confirmed for the classes associated with this mechanism of resistance (glycopeptide, macrolide, macrolide-lincosamide-streptogramin, mupirocin-like, Fig. 1c) with the only exception of the class multidrug. In fact, this class was significantly higher in LowestUG sites, but its abundance increased with the rise

Site ID	Park	Urbanization Grade	IDs	ARGs	Classes	Mechanisms
MAN	Parco La Mandria	LowestUG	2335.50 +/-239.20	175.50 +/-21.00	20.17 +/-1.17	6.00 +/-0.63
STU	Parco Naturale di Stupinigi	LowestUG	2501.67 +/-74.14	177.00 +/-9.54	21.00 +/-1.00	6.33 +/-0.58
NSO	Parco Sangone	LowUG	2896.80 +/-275.87	242.6 +/-11.01	22.20 +/-0.84	6.60 +/-0.55
NST	Parco Sangone	LowUG	2300.80 +/-178.83	190.80 +/-13.72	20.40 +/-0.55	6.00 +/-0.00
PEL	Parco della Pellerina	LowUG	2196.67 +/-251.90	175 +/-17.53	21.17 +/-0.98	6.00 +/-0.00
ARM	Parco Cavalieri di Vittorio Veneto	MidUG	2288.67 +/-264.96	191.00 +/-23.35	20.17 +/-1.17	6.50 +/-0.55
DOR	Parco Dora	MidUG	2312.50 +/-171.51	196.83 +/-14.96	20.33 +/-0.82	6.67 +/-0.52
VAL	Parco del Valentino	MidUG	2234.67 +/-278.43	174.17 +/-14.73	21.00 +/-1.67	6.00 +/-0.00
GRO	Giardino Nicola Grosa	HighUG	2212.00 +/-133.35	181.83 +/-9.54	21.17 +/-1.60	6.17 +/-0.41
PAV	Parco Arte Vivente	HighUG	2604.33 +/-244.02	198.50 +/-17.67	21.17 +/-0.75	6.33 +/-0.52
TES	Parco della Tesoriera	HighUG	2104.17 +/-154.21	167.50 +/-12.08	20.67 +/-1.75	6.00 +/-0.00

Table 1. Global amount of antibiotic resistance IDs. ARGs, classes, and mechanisms in the sites of study. For each studied site, the average number of AMR genes belonging to different microbial taxa (IDs, ARGs orthologs), Antibiotic Resistance Genes (ARGs), antibiotic resistance classes (Classes), and antibiotic resistance mechanisms (Mechanisms) are reported with the corresponding standard deviation value. Parks are grouped according to the type (undisturbed or urban) and grade of urbanization of the environmental matrix (Urbanization Grade, ranging from the most - "HighUG"- to the least urbanized area - "LowestUG").

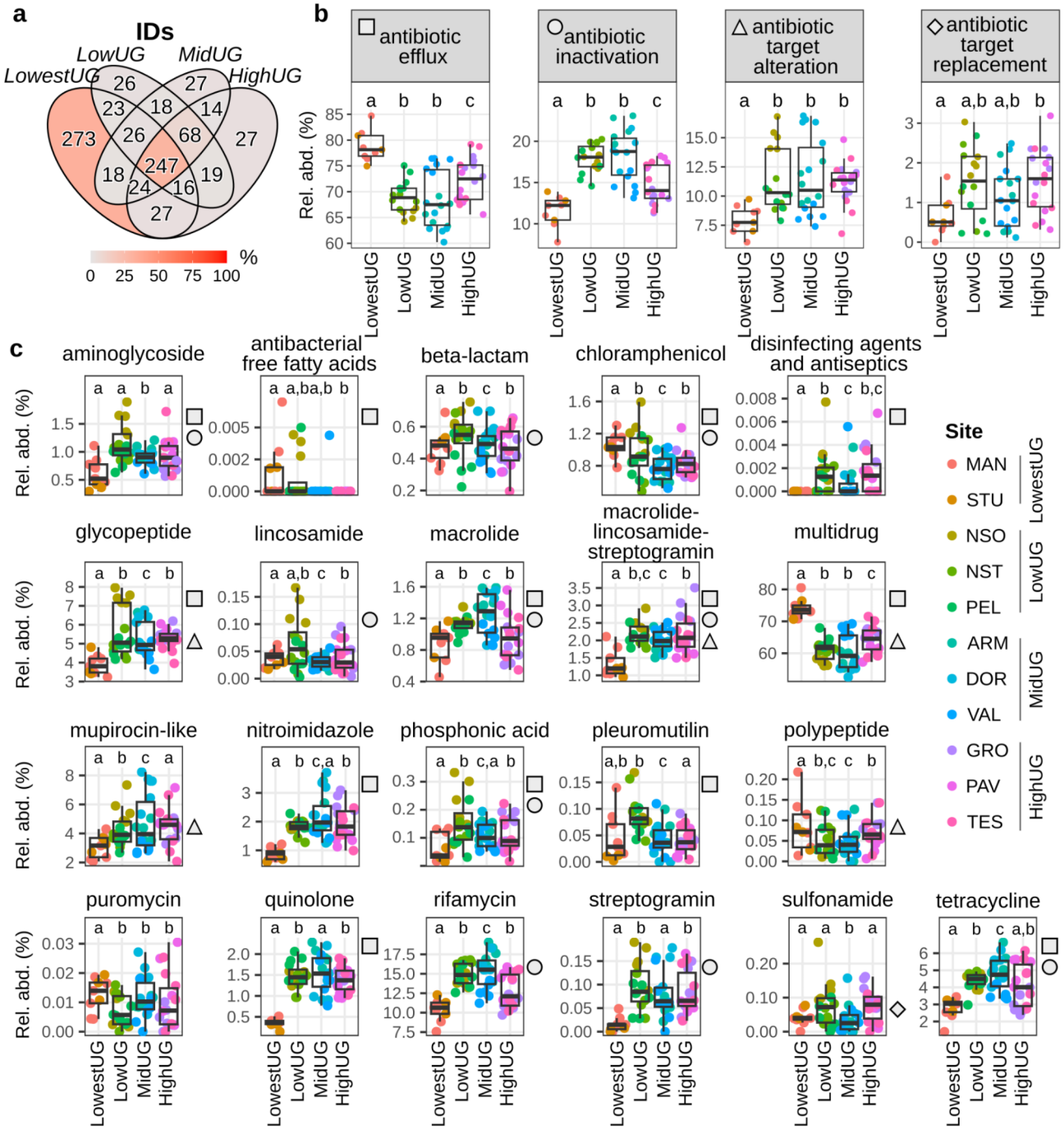

Fig. 1. Distribution of antibiotic resistance genetic features among sites characterized by different degrees of urbanization. (**a**) Venn diagram summarizing the comparison of genes belonging to different microbial taxa (IDs) present in every sample. (**b**) Relative abundance of antibiotic resistance mechanisms evaluated according to the presence of ARGs. (**c**) Relative abundance of ARG classes; symbols on the right of each plot correspond to the mechanism of antibiotic resistance associated with the corresponding ARG class. Letters in the plots indicate statistically supported differences across urbanization grades according to the Wilcoxon-Mann-Whitney test (FDR < 0.05).

of urbanization levels (Wilcoxon-Mann-Withney FDR < 0.05, Fig. 1c). It has to be considered that the multidrug class is also associated with the antibiotic efflux mechanism, which shows the same trend among urbanization levels (Wilcoxon-Mann-Whitney FDR < 0.05, Fig. 1b and Supplementary Table 3). It is interesting to note that only three out of 13 classes associated with the antibiotic efflux mechanism show a significantly lower abundance in LowestUG samples than other sites, whereas the other classes were less represented in LowestUG sites and showed some grade of differentiation among more urbanized sites (Wilcoxon-Mann-Whitney FDR < 0.05, Supplementary Tables 3 and Fig. 1c). Antibiotic inactivation and antibiotic target alteration were poorly

represented in LowestUG sites and the abundance of ARGs associated with those mechanisms decreased in more urbanized sites with the increase of urbanization (Wilcoxon-Mann-Whitney FDR < 0.05, Supplementary Tables 3 and Fig. 1c). The abundance of ARGs associated with the antibiotic target replacement mechanism increases with the rise of the urbanization grade (Wilcoxon-Mann-Whitney FDR < 0.05, Supplementary Tables 3 and Fig. 1b). If we consider the antibiotic classes associated with this mechanism, only sulfonamide showed significant differences associated with low urbanization levels exhibiting the lowest abundance in the MidUG samples, hence not mirroring the mechanism pattern (Fig. 1c).

All these observations highlight the link between the presence/abundance of AMR molecular features and the urbanization level.

ARGs abundance and diversity are associated with the soil microbial composition

The observation of differences between the type and abundance of ARGs in the studied sites stressed the importance of delving into the composition of the microbial communities. With this aim, the microbial communities were investigated both through molecular information gathered from the metagenomics data and through culturomics approaches. Taxonomic profiling of metagenome libraries revealed that bacterial families Nocardioidaceae (6.7%), Nitrobacteraceae (5.4%), and Solirubrobacteraceae (5.2%) and the fungal genera *Aaosphaeria* (5,5%), *Exophiala* (5.0%), and *Aspergillus* (4.3%) were the most represented taxa across sites (Supplementary Fig. 4a and 5a). Significant differences in the alpha-diversity metrics measured (observed, Shannon, and Simpson alpha diversities, Wilcoxon-Mann-Withney FDR < 0.05, Supplementary Table 4) and community assembly (beta-diversity) of both bacterial and fungal populations were found when comparing samples from different sites (Supplementary Fig. 4b and 5b). Conversely, samples grouped according to the urbanization grade differed from each other only when considering the observed bacterial alpha diversity (Supplementary Table 4) being significantly lower in LowestUG than other sites (Wilcoxon-Mann-Whitney FDR < 0.05, Supplementary Fig. 4c). On the other hand, fungal communities were significantly richer in the LowestUG sites than in other sites, but Shannon's and Simpson's indexes followed the opposite trend (Supplementary Fig. 5b). These observations suggest that, despite being more complex, fungal populations in wilder environments (LowestUG) are more unevenly composed than urban ones. Observed fungal diversity showed positive correlations with both Shannon and Simpson bacterial diversities (Spearman's rho equal to 0.287 and 0.341, respectively). Still, fungal Shannon's and Simpson's indexes were negatively correlated with the same indexes for bacterial populations (Spearman's rho, Shannon fungal vs. bacterial −0.309, Simpson fungal vs. bacterial −0.276, respectively; Fig. 2a). Conversely, the comparison of bacteria and fungi beta diversities revealed a significant association with the sampling site, which overgrew the potential impact of urbanization (Supplementary Table 4 Supplementary Fig. 4c and 5c). However, the linear discriminant analysis effect size (LEfSe) analysis revealed that the urbanization grade was associated with the presence of group-specific bacterial families and genera: Hyphomicrobiales (also known as Rhizobiales) for LowestUG sites, Geodermatophilacae and the *Blastococcus* genus for LowUG sites, Rubrobacteriaceae and the *Rubrobacter* genus for MidUG, and Verrucomicrobiaceae for HighUG sites (Supplementary Fig. 4d). Previous studies showed that urbanization decreases root nodule formation in *Trifolium repens*, potentially supporting the observation of the specificity of Hyphomicrobiales and *Blastococcus*, components of rhizobia, in undisturbed areas[13]. On the other hand, *Rubrobacter* is commonly found in soil from agronomic environments such as olive plantations[14] and Verrucomicrobiaceae in clinical settings[15], hence perfectly matching the identification in samples from parks exposed to increasing urbanization impacts. No fungal taxa were found to be specifically associated with the urbanization grade.

Significant positive correlations were observed between bacterial alpha diversities and antibiotic resistance molecular indicators (IDs, ARGs, classes, and mechanisms) (Wilcoxon-Mann-Whitney FDR < 0.05, Supplementary Tables 4 and Fig. 2a). Conversely, fungal Shannon and Simpson alpha diversities and IDs, ARGs, and mechanisms showed negative correlations (Wilcoxon-Mann-Whitney FDR < 0.05, Supplementary Tables 4 and Fig. 2a), suggesting that unevenly composed fungal populations (corresponding to low Shannon and Simpson indexes) are associated with higher bacterial antibiotic resistance, potentially because of the predominant presence of competitive fungal species. Indeed, among the most abundant fungal species peculiar to LowestUG, the group presenting the lowest Shannon and Simpson indexes, included *Aspergillus wentii* (previously found to produce antibacterial metabolites active against *Pseudomonas aeruginosa*)[16], *Entomortierella parvispora* (associated with Burkholderiaceae-related endosymbiotic bacteria)[17], *Gongronella butleri* (producing 2-Pentenedioic acid derivatives)[18], and *Penicillium* spp. (*P. canescens*, *P. cinerascens*, and *P. lagena*; a genus known to produce antibiotic metabolites)[19](Supplementary Table 4).

Distinct associations were also observable between mechanisms of resistance and microbial taxa: whereas antibiotic target alteration and antibiotic inactivation ARGs were clearly associated with defined bacterial taxa (Fig. 2c and d), antibiotic efflux ARGs showed associations with bacterial species belonging to multiple taxa (Fig. 2b and Supplementary Table 5). Nevertheless, some antibiotic efflux ARGs showed significant positive correlations with relevant bacterial taxa, among which Pseudomonadaceae, Paracoccaceae, Phyllobacteriaceae (highly abundant in HighUG samples), and Nitrobacteriaceae (highly abundant in LowestUG) (Fig. 2b). Conversely, the ARGs associated with antibiotic inactivation and antibiotic target alteration (Fig. 2c and d) showed significant positive and negative associations with specific bacterial taxa. To note, whereas ARGs associated with antibiotic inactivation or antibiotic target alteration showed negative correlations with the genus *Bradyrhizobium*, highly abundant in LowestUG samples, they showed positive correlations with the family Nocardioides, whose species were evenly distributed among LowUG, MidUG, and HighUG (Fig. 2c and d).

Correlations were found also between fungal species and ARGs, with genes associated with the antibiotic efflux being positively associated with *Colletotrichum spinosum* (Spearman rho = 0,561) and negatively with *Kalmusia* spp. (rho=-0,533), antibiotic inactivation ARGs negatively correlated with *Tetrapisispora blattae* (rho=-0,539),

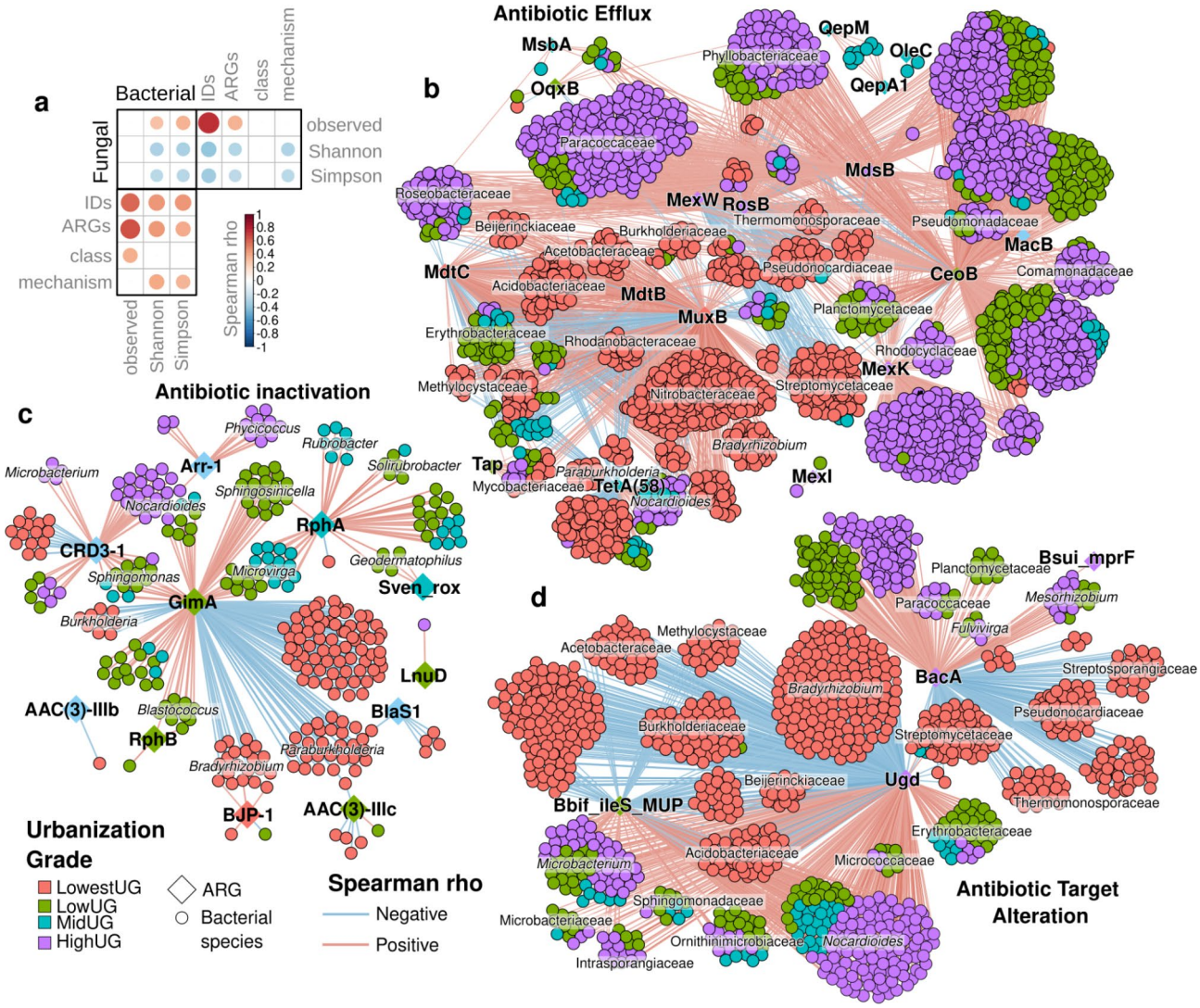

Fig. 2. Microbial composition and association with ARGs. (**a**) Spearman correlations between bacterial and fungal alpha diversities (observed, Shannon, and Simpson) and molecular signatures of antibiotic resistance (IDs, ARGs/genes, classes, and mechanisms). Only significant correlations are shown; point color and size show Spearman's rho. (**b**–**d**) network representations of the significant Spearman correlations between microbial and ARGs relative abundances grouped according to the mechanism of resistance: antibiotic efflux. Networks were drawn with Cytoscape v. 3.10.1[34] (https://cytoscape.org/). (**b**), antibiotic inactivation (**c**), and antibiotic target alteration (**d**). Only significant (FDR < 0.05) correlations with Spearman's rho <-0.75 or > 0.75 are shown; for representation simplification, microbial species have been grouped according to the deepest taxonomic level. Nodes represent microbial species, squares ARGs classes while edges indicate positive (red color) or negative (blue color) correlations according to Spearman's rho. Node colors indicate the urbanization grade bearing the highest relative abundance of the ARG (rhombus) or microbial species (point).

and two different genes responsible for antibiotic target alteration (Erm(37) and Ugd) being negatively (rho=-0,534) and positively (rho = 0,525) associated with *Ceratobasidium* sp. (Supplementary Table 6).

Microbial isolates confirm the urbanization-driven distribution of ARGs

Since we observed clear links between ARG distribution in urban parks, microbial taxa, and site characteristics, culturomics was undertaken to explore and validate the diversity and distribution of the antibiotic resistance taxa in the sampled sites. A total of 331 bacterial strains (all non-pathogenic) were isolated from 7 representative sites, with a broad representation of bacterial taxonomy (Supplementary Table 7). In most cases, the identified species were isolated from only a few or one of the studied sites (e.g. *Aeromonas*, *Chryseobacterium*, *Deftia*, *Lysobacter*, *Pedobacter*, *Priesta*, *Pseudoxanthomonas*, and *Sphingobacterium*) (Table 2). Several of the genera less frequently isolated, belonging to the *Acidovorax*, *Delftia*, *Ensifer*, *Lysobacter*, *Pedobacter*, *Pseudoxanthomonas*, *Sphingobacterium*, and *Variovorax* were gathered only through selective isolation (in penicillin/streptomycin or geneticin media), suggesting a limited abundance and high antimicrobial resistance of these taxa. The most

	MAN	PEL	ARM	DOR	VAL	GRO	TES	
	LowestUG	LowUG	MidUG	MidUG	MidUG	HighUG	HighUG	Total
Acidovorax[a]	0	1	2	0	0	0	0	3
Aeromonas	0	0	0	0	0	1	0	1
Bacillus	5	0	7	0	3	5	0	20
Chryseobacterium	0	0	0	0	0	1	0	1
Delftia[a]	0	0	0	0	0	1	0	1
Ensifer[a]	0	1	0	0	0	1	0	2
Escherichia	0	0	7	0	0	0	0	7
Lysobacter[a]	0	1	0	0	0	0	0	1
Paenibacillus	0	0	3	0	0	0	1	4
Pedobacter[a]	0	0	1	0	0	0	0	1
Peribacillus	1	0	2	0	0	0	0	3
Priesta	0	0	0	0	1	0	0	1
Pseudomonas	9	4	7	1	1	3	8	33
Pseudoxanthomonas[a]	0	1	0	0	0	0	0	1
Sphingobacterium[a]	0	0	0	0	0	1	0	1
Stenotrophomonas	0	0	2	0	0	0	0	2
Variovorax[a]	0	0	1	0	0	2	0	3
Enterobacteriaceae*	0	0	0	0	3	0	0	3

Table 2. Bacterial genera isolated from the investigated sites. *Identified at a higher taxonomic level; [a]obtained through isolation on a selective medium.

frequently and broadly isolated genera were *Bacillus* (20 isolates from 4 sites) and *Pseudomonas* (33 isolates from 7 sites) (Table 2). To explore the antibiotic susceptibility of the isolated strains, antibiograms were performed with antibiotics clinically relevant and suitable for assessing and verifying the associations between ARG mechanisms and classes. In particular, amoxicillin, cefiderocol, rifamycin, and geneticin were tested to explore the presence of ARGs mediating antibiotic inactivation, with geneticin being also associated with antibiotic efflux; Trimethoprim and Linezolid were chosen as associated with antibiotic target replacement (Supplementary Table 7). The growth inhibition imposed by amoxicillin, cefiderocol, linezolid, and geneticin highlighted significant differences according to the isolation source and corresponding urbanization grade (Fig. 3a). The susceptibility to amoxicillin and linezolid was significantly higher for strains isolated from LowestUG sites compared to other isolates (Wilcoxon-Mann-Whitney FDR < 0.05, Fig. 3a and Supplementary Table 7). This observation was in line with what was observed in metagenomics data, with ARGs associated with antibiotic inactivation (amoxicillin) and antibiotic target replacement (linezolid) being significantly less represented in LowestUG sites (Fig. 1b). The same results were confirmed when comparing the susceptibility of the most frequently found genera (*Pseudomonas* and *Bacillus* spp., Fig. 3b and c), with strains isolated from MidUG sites showing a significantly higher level of resistance to amoxicillin and linezolid compared to LowestUG samples (Supplementary Table 7). Isolates showed a diffused resistance to cefiderocol, with a different trend from what previously observed in metagenomics ARGs data: whereas the genes related to antibiotic inactivation mechanisms or the resistance to beta-lactam antibiotics (both including cefiderocol) were significantly less abundant in LowestUG sites compared to the other (Fig. 1b and **c**), strains isolated from LowestUG sites showed either a higher resistance than other isolates (Fig. 3a) or, in the case of *Pseudomonas* and *Bacillus* isolates, did not differ from other isolates (Fig. 3b and c). The high level of resistance to cefiderocol and the missing differentiation according to urbanization could be ascribed to the fact that cefiderocol has been assigned to the Reserve category of the AWARE classification, because of the still limited information available on microbial resistance to this antibiotic[6,20]. The response of isolates to geneticin highlighted the highest susceptibility in LowUG strains compared to strains isolated from other sites. This observation is in line with the distribution of ARGs associated with the antibiotic efflux mechanism of resistance observed in metagenomics data, but not with the other mechanisms of resistance adopted in response to geneticin (antibiotic inactivation and antibiotic target al.teration, Fig. 1a). The tests on rifamycin, trimethoprim, and penicillin/streptomycin did not show significant differences in strains' response, besides the response to rifamycin, with strains being gradually less resistant to the antibiotic with the increase of the urbanization grade (Supplementary Fig. 6).

The link between antibiotic resistance, environmental features, and microbial populations

To delve further into the potential relationship between the environment and antibiotic resistance features, a capillary investigation was performed to evaluate whether, besides the urbanization grade, additional environmental features could be associated with the selection or promotion of ARGs, possibly through selective pressures exerted on microbial communities. With this aim, data on environmental features (the compositional occupation of different soil use categories within the area surrounding the sampling point, e.g. tree plantations, rivers, roads, and buildings, as described in methods) were gathered from the database Geoportale Piemonte[21]. Redundancy Analysis (RDA) evidenced a strong association between antibiotic resistance mechanisms and

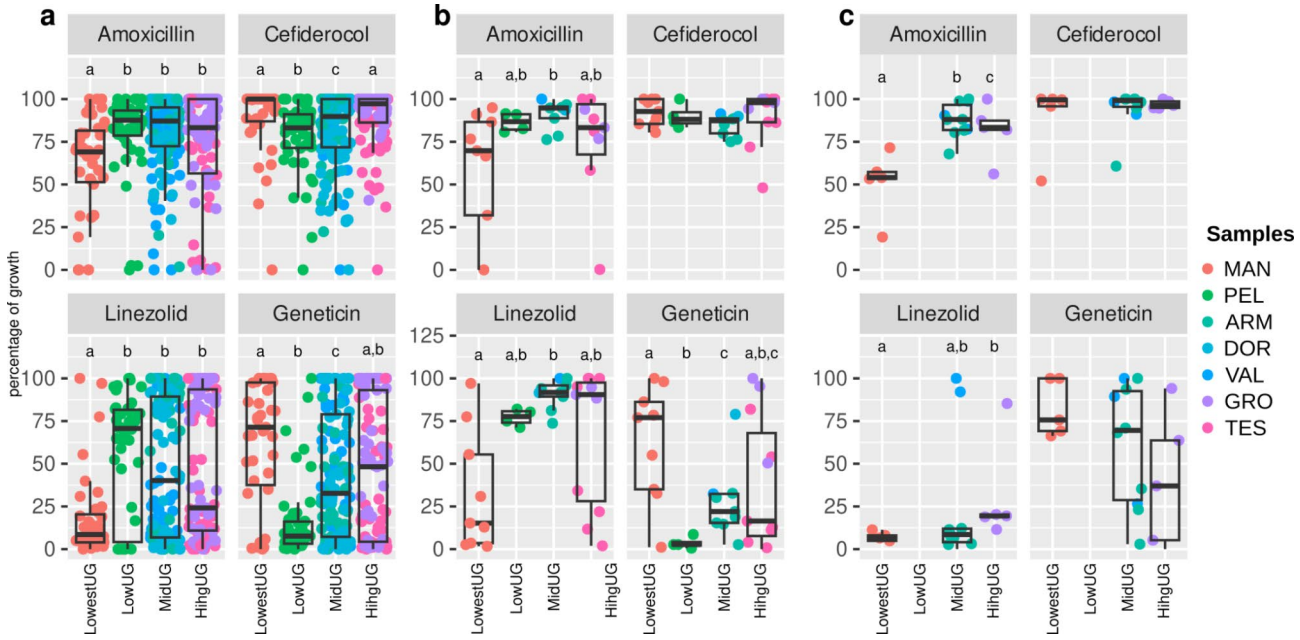

Fig. 3. Susceptibility of bacterial isolates to amoxicillin, cefiderocol, linezolid, and geneticin. Susceptibility of all the strains (**a**), of *Pseudomonas* spp. (**b**), and *Bacillus* (**c**) strains isolated from the sites under investigation. Letters indicate statistically different groups of strains according to the susceptibility to the tested antibiotic (Wilcoxon-Mann-Whitney FDR < 0.05).

environmental features, with 87.59% of the ARG mechanisms abundance variance explained (Supplementary Tables 8 and Fig. 4a). In particular, the antibiotic efflux mechanism showed an association with ecological patches characterized by the occurrence of deciduous leafy tree species including *Quercus*, *Alnus*, Fraxinus, exotic *Quercus*, and other features linked to woodland areas, whereas the antibiotic inactivation and antibiotic target al.teration mechanisms showed relationships with urban-related features, such as densely built-up urban areas, road network tunnels, and parks/cultivated gardens (Supplementary Tables 8 and Fig. 4a). The link between urbanized areas and the antibiotic inactivation and antibiotic target al.teration mechanisms was also confirmed through stepwise regression analysis: sparsely built-up areas, riverbanks fine mobile sediments, railway network, parks/cultivated gardens and railway networks showed significant positive contributions in the model explaining the relative abundance of ARGs associated with those mechanisms (Fig. 4b). On the other hand, the model of the antibiotic efflux mechanism included *Abies/Picea* woodlands, scrublands-recent abandonment (with positive coefficients) and road network, road network bridge viaduct overpass, parks/cultivated gardens, and densely built-up urban areas (with negative coefficients), confirming the association of this resistance mechanism with unmanaged environments (Fig. 4b). Bacterial and fungal family abundances were associated with characteristics of the environmental matrix (Fig. 4c-d), also confirming the association between the composition of microbial communities and antibiotic resistance molecular features (Fig. 2). Acidobacteriaceae and Nitrobacteriaceae and ARGs related to antibiotic efflux, significantly correlated with each other (Fig. 2b), were found to be associated with the same environmental matrix features (e.g. *Quercus/Alnus/Fraxinus* and exotic *Quercus*) (Fig. 4c). Nocardioidaceae showed a clear pattern of association with urbanization levels and provided an interesting insight into the impact of this stressor on antibiotic resistance: besides being more abundantly present in HighUG samples (Fig. 2) and positively associated with densely built-up urban areas, road network tunnels, and riverbanks fine mobile sediments (Fig. 4c), their abundance was positively correlated with antibiotic inactivation and antibiotic target al.terations ARGs and negatively with antibiotic efflux (Fig. 2c-d). The distribution of fungal families showed a moderate association with the environmental matrix (constrained variance = 74.97%, Fig. 4d), as previously observed for the correlation of ARGs abundances and fungal species (Supplementary Table 6). Nevertheless, relationships were observed: between the abundance of Russulaceae and *Quercus/Alnus/Fraxinus*, forest in European mesotrophic soil, and exotic *Quercus* (all associated with antibiotic efflux, Fig. 2a); between Aspergillaceae and railway network and road network tunnels; and Nectriaceae with densely built-up urban areas, sparsely built-up urban areas (Fig. 4d).

Discussion

Our study clearly shows the dramatic need to adopt an approach that considers all environments to which humans are directly or indirectly connected when aiming at mitigating the global threat of antimicrobial resistance.

Metagenomic analysis revealed a higher abundance of antibiotic resistance genes in the soil of urban parks in Turin, Italy, with a culturomics approach confirming this result. Considering resistance mechanisms and urbanization grade, we observed that efflux pumps are the most abundant resistance genes. Conversely, rising

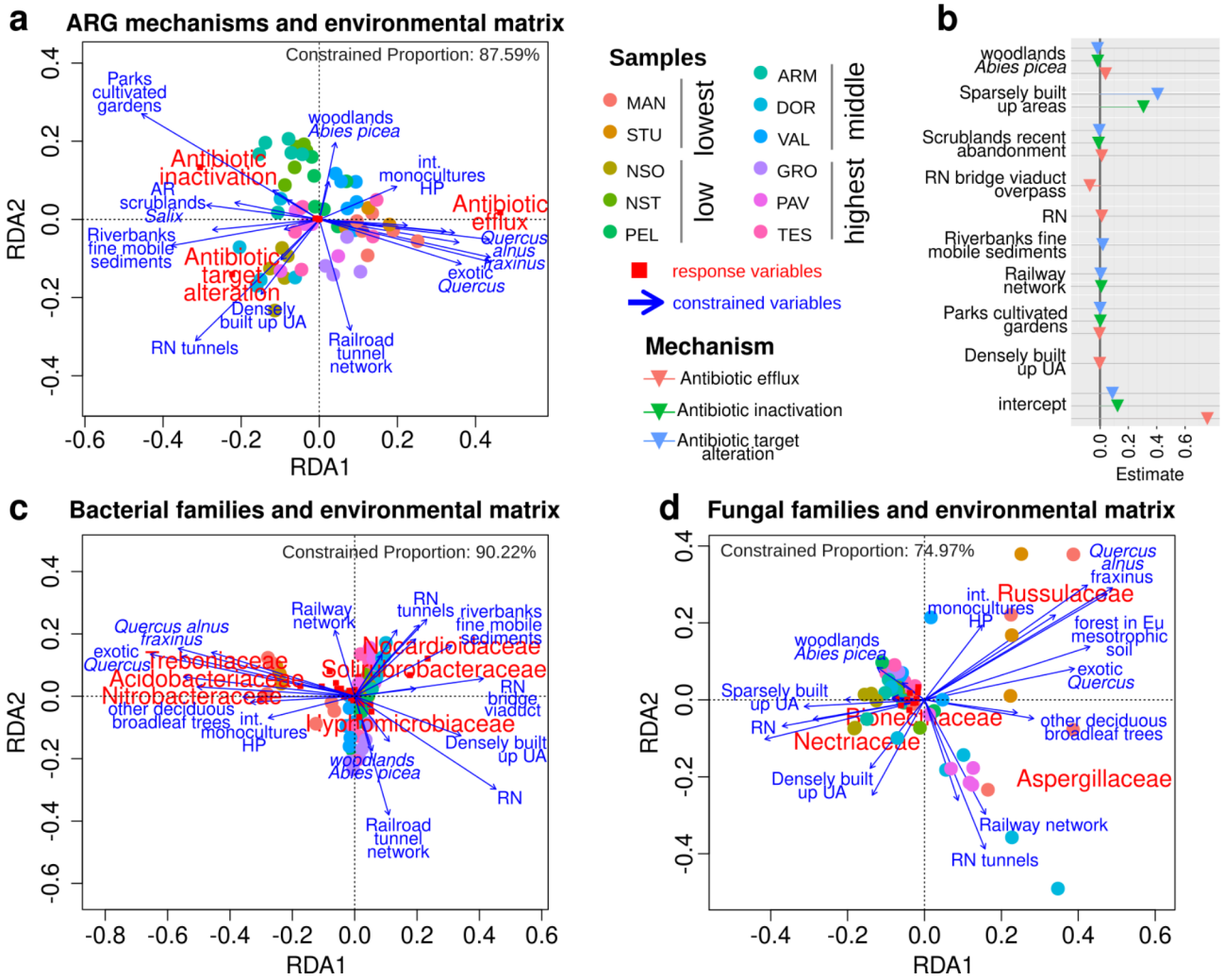

Fig. 4. Associations between environmental matrix and soil characteristics and the ARGs and microbial populations. (**a**) Redundancy analysis (RDA) representation of ARGs mechanisms and environmental matrix features. (**b**) Coefficients estimated through stepwise regression analysis modeling the ARGs mechanism relative abundance in the function of the environmental matrix characteristics of the sites under investigation. (**c**) Redundancy analysis (RDA) representation of bacterial families and environmental matrix features. (**d**) Redundancy analysis (RDA) representation of fungal families and environmental matrix features.

urbanization is associated with a significant increase in resistance genes related to antibiotic inactivation, target al.teration, and target replacement. Urbanization appears to play a role in this differentiation, either directly through anthropogenic factors such as pollutants or indirectly by modifying the environment (e.g. habitat fragmentation) and thus acting on the composition of the soil microbiota. In fact, we observed that in sites where the anthropogenic pressure is lower, or even absent, there is a greater abundance of fungi and a predominance of some antibiotic-producing species, suggesting their role in the enrichment of efflux pump-related genes and confirming the renewed competition between the two kingdoms in soil[22]. On the other hand, our data indicate a higher abundance of bacteria and an even distribution of fungal taxa in sites with higher urbanization, suggesting a role of human activities as selective stressors. This association is also supported by biomarker discovery analysis LEfSe, which showed an association between specific bacterial taxa and the level of urbanization.

Culturomics provided a broader view, validated the data obtained through metagenomics, and provided additional information otherwise unavailable through sequencing. Similarly to what was observed with metagenomics, bacterial strains isolated from more urbanized sites showed higher resistance to amoxicillin and linezolid (antibiotic inactivation and target al.teration mechanism, respectively) compared to those isolated from less urbanized ones. Considering the observed differential distribution of antibiotic inactivation and target al.teration mechanisms, the results of the antibiograms with cefiderocol, an antibiotic counteracted by these two mechanisms, show a warning signal: despite the ARGs classes and mechanisms related to this molecule being more abundant in urbanized sites, the strains isolated from undisturbed locations were highly resistant. This discrepancy could be ascribed to the fact that the exact mechanism of the antibiotic is fully understood yet and so are the antibiotic-resistance genes[23].

Overall, the study highlights the crucial need to monitor antibiotic resistance in urban soil by adopting a dual approach (molecular and culturomics), which is essential for assessing and validating the occurrence of antibiotic resistance. It is important to gain insight into the factors that may contribute to the development and spread of antibiotic resistance in urban environments. This knowledge is fundamental for a better understanding of how cities should become to reduce those factors that could contribute to the development and spread of antibiotic resistance.

Methods

Site selection, sampling, and soil properties analyses

Ten parks in the province of Turin were selected for soil sampling (Supplementary Table 1). The selection was performed to include, according to the local ecological network map provided by the Geoportale Piemonte database[21] and analyzed with QGIS v3.30.1[24], the broadest environmental diversity. QGIS, an open-source software released under the GNU General Public License, was also used to generate maps of the study site. Environmental features within a 500-meter circular buffer around the sampling points were gathered and transformed into percentages. Densely built-up areas as well as the presence of a road within the buffer were used to analyze urbanization around the sampling sites. According to the urbanization gradient, each park was categorized as LowestUG (urbanization features covering < 25% of the analyzed area), LowUG (urbanization between 25% and 50%), MidUG (urbanization between 50% and 75%), and HighUG (urbanization between 75% and 100%). Two sites were selected as being located in not-urbanized and undisturbed areas (MAN and STU), the other sites were located within Turin municipal boundaries. Top soil samples (0–10 cm depth) were collected between October and November 2022 with metal soil corers in grassy areas and near trees, for a total volume of 195 cm^3 of soil for each sampling area. A 50 ml sterile tube was filled and stored for DNA extraction and microbial isolation, while the remaining soil was collected and used for physicochemical analysis. Samples for microbial isolation and physicochemical analyses were temporarily stored at 8 °C and rapidly processed; isolation was performed for samples from 7 out of 10 sites. Samples for DNA extraction were stored at -80 °C and processed within one month from the sampling date.

Soil DNA extraction and sequencing

DNA was extracted from 200 to 250 mg of frozen soil material with the DNeasy PowerSoil Pro kit (QIAGEN, HIlden, Germany) following manufacturer procedures. The quantity and purity (260/280 and 260/230 ratios) of the DNA were checked using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) and metagenome sequencing was performed at IGATech company (Udine, Italy). Briefly, shotgun metagenomic libraries were constructed using the Celero™ DNA-Seq kit (NuGEN, San Carlos, CA) following the manufacturer's instructions. Both input and final library were quantified by a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA) and quality tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent Technologies, Santa Clara, CA). Libraries were then prepared and sequenced on a NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) in paired-end mode with a read length of 150 bp and a minimum target throughput of 80 M reads/sample. Base calling, demultiplexing, and adapter masking were performed by the company with Illumina BCL Convert v3.9.3.

We checked libraries quality using FastQC v0.12.1 and MultiQC v.1.12 and further quality filtered and trimmed residual adapters with BBDuk (within BBTools v39.01 (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/), with the following parameters: 'ktrim = r k = 21 mink = 11 hdist = 1 tpe tbo qtrim = rl maq = 15 minlen = 100 ftm = 5' and the built-in Illumina adapter sequences database. Raw metagenome libraries have been submitted to the NCBI Sequence Read Archive (SRA) under BioProject accession No. PRJNA1158962.

Identification of antibiotic resistance genes

The software ARGs_OAP was used to assess the presence and abundance of antibiotic-resistance genes[25] on the quality filtered and adapter-trimmed reads. Default parameters for both Stage 1 (E-value cutoff for GreenGenes = 1e-10; E-value cutoff for Essential Single Copy Marker Genes = 3; identity cutoff in percentage for Essential Single Copy Marker Genes = 45) and Stage 2 (E-value cut off for target sequence 1e-7; Identity cutoff in percentage for target sequence = 80; query cover cutoff in percentage for target sequences = 75; aligned length cutoff in amino acid for target sequences = 25). The NCRD95 (non-redundant antibiotic resistance genes) database was used to evaluate the presence of antibiotic resistance genes[12] and quantify them according to IDs, ARGs, broad classes, and mechanisms of resistance. The following analyses were carried out on data normalized according to the 16 S rRNA marker gene[25].

Taxonomic classification of metagenomic libraries

Filtered metagenomic reads were classified using kaiju v1.9.2[26] using the default "greedy" mode and the "nr_euk" database (updated with the kaiju-makedb software on April 2 2023) as reference Taxonomical classification of each sample were then obtained using the 'kaiju2table' command, and results imported into R/RStudio session[27] and merged to obtain the final count tables.

Bacteria isolation, identification, and storage

Soil aliquots dedicated to culturomics approaches were stored as described above; 1 g of soil was suspended in 5 ml of sterile distilled water and serial dilutions were performed to plate 100 µL of 10^{-2} to 10^{-4} dilutions onto a non-selective Luria-Bertani (LB) medium, a LB medium supplemented with penicillin/streptomycin (10,000 U/ml and 10 mg/l, respectively) and McConkey agar supplemented with geneticin (32 µg/ml). After 48 hours of incubation at 32°C, bacterial colonies with different shapes, sizes, and colors were further isolated by subculture on the same media of isolation and then stored in a sterile 40% (w/v) glycerol solution at − 80°C.

Identification was performed through Sanger sequencing of the V3-V4 region of the 16S rRNA gene. Briefly, universal primers (Bact8F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and Bact1510, 5′-CGGTTACCTTGTTACG ACTT-3′) were used to amplify the entire 16S rDNA gene. PCRs were performed with GoTaq DNA polymerase (PROMEGA). Following an initial denaturation step at 95°C for 2 min, th\e samples were amplified for 34 cycles (95°C for 30 s, 53.6°C for 30 s, and 72°C for 90 s) plus a final extension at 72°C for 10 min. Sanger sequencing of the V3 and V4 region was performed with the primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3' Sequences were compared against the GenBank database using the standard nucleotide BLAST approach (Supplementary Table 7). Sanger sequences of the 16 S V3 and V4 regions were deposited in GeneBank (PQ256666-PQ256751).

Antibiograms

Isolates were tested for resistance to amoxicillin (8 mg/L), trimethoprim (4 mg/L), cefiderocol (1 mg/L), linezolid (4 mg/L), penicillin/streptomycin (10,000 U/ml and 10 mg/l, respectively), geneticin (32 µg/ml) and rifamycin (4 mg/L). Antibiotics were chosen according to AWARE classification (including amoxicillin and trimethoprim for the access group, cefiderocol and linezolid for the reserve group, and rifamycin for the watch group) and the mechanism adopted by microorganisms for resistance. The EUCAST MICs were used to select the antibiotic concentrations to be tested; considering that the isolated and identified microorganisms did not belong to the species included in the EUCAST, the highest clinical breakpoint indicated for each antibiotic in the EUCAST guidelines (EUCAST clinical breakpoint v 14.0, 1 Jan 2024) was used[28]. Antibiograms were performed in liquid media, and the optical density at 600 nm was measured after 48 h. The growth percentage was calculated by subtracting the mean OD of negative controls (medium with antibiotics) from the ODs of the treated samples (the isolate inoculated in the medium with antibiotics) and the positive control (the isolate inoculated in the medium); the resulting values were used in the following formula: (OD treated/OD control)*100.

Statistical analyses

Statistical analyses were performed in the R statistical environment[27]. Alpha- (observed, Shannon, and Simpson indexes) and beta- (Bray-Curtis) diversities analysis were evaluated with the 'estimate_richness' and 'distance' functions of the phyloseq v1.46.0 R package[29], NMDS coordinates were obtained on Bray-Curtis distances with the ordinate phyloseq R package and then plotted with ggplot2 v3.5.1[30]. permANOVA was performed with the 'adonis' function of the vegan v2.6.6.1 R package[31] to evaluate the grouping of samples according to the Bray-Curtis distances. Wilcoxon-Mann-Withney tests were performed with the pairwise.wilcox.test using FDR as p.adjust.method option[27]. Spearman correlation analysis was performed with the rcorr function of the Hmisc v5.1.3 R package[32] and plotted with the corrplot function[33]. Networks were drawn with Cytoscape v3.10.1[34] on Spearman's correlations rho with an absolute value higher than 0.75 and fdr < 0.05. LEfSe analysis was performed with the 'run_lefse' function of the microbiomeMarker v1.8.0 R package[35].

Data availability

Sanger sequences of the 16 S V3 and V4 regions were deposited in GeneBank (PQ256666-PQ256751). Raw metagenome libraries have been submitted to the NCBI Sequence Read Archive (SRA) under BioProject accession No. PRJNA1158962.

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Conceptualization: M.C., L.L, I.S.; Data curation: D.B., M.C., S.M., I.S; Formal analysis: D.B., M.C., S.M., I.S; Investigation: D.B., M.C., V.F., L.L., S.M., I.S, C.V.; Methodology: D.B., M.C., L.L., S.M., I.S.; Visualization: D.B., I.S.; Writing—original draft: D.B., I.S.; Writing -review and editing: D.B., M.C., V.F., L.L., S.M., I.S, C.V.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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