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Uncovering the Resistome of a Peruvian City through a Metagenomic Analysis of Sewage Samples

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Abstract: Background: Antibiotic resistance is a major public health concern globally. In this study, we aimed to evaluate the abundance and diversity of antibiotic resistance genes (ARGs) in sewage water samples from two hospitals and an adjacent community or urban setting in Huanuco, a Peruvian city located in the highlands. Methods: We collected samples from the community wastewater system and from sewage pipes from the two hospitals in Huanuco. DNA was extracted from 250 mL of sewage water samples (n = 6) and subjected to microbiome profiling using 16S rRNA short amplicon sequencing and shotgun metagenomics. We analyzed the taxonomic and functional content in all samples, including alpha and beta diversity metrics, and searched for ARGs. Results: Our results showed that samples taken from the community wastewater system were compositionally different and harbored greater bacterial taxonomic and functional diversity compared to samples collected from the hospitals' wastewater system. We found a high abundance of bacteria associated with resistance to beta-lactams, macrolides, aminoglycosides, fluoroquinolones, and tetracyclines in all samples. However, there were no significant differences in the abundance or composition of ARGs between the community wastewater samples and those taken from the two hospitals. Conclusions: Our findings suggest that metagenomics analyses in wastewater sewage could be a useful tool for monitoring antibiotic resistance in urban settings. These data could be used to develop local public health policies, particularly in cities or countries with limited resources to establish large-scale One Health projects.

Keywords: antibiotic resistance genes (ARGs); sewages; hospitals; urban settings; microbial diversity

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1. Introduction

The current policy framework of the World Health Organization (WHO) relies on the One Health concept. This notion involves not only human and animal contributions to global health but the role of the environment. The interactions between humans, animals, and the environment can foster the development of antimicrobial resistance (AMR) [1].

AMR represents a global health threat due to several drivers contributing to superbug emergence, including overuse and misuse of antimicrobial drugs, poor infection prevention and control, migration of people or animals, people's proximity to livestock, and environmental factors such as antimicrobial use in agriculture or sanitation infrastructure, among others [2–5].

Murray et al. reported that 4.95 million people worldwide presented AMR-related health problems in 2019 [6], with six pathogens directly associated with AMR deaths:

Escherichia coli, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Neisseria gonorrhoeae*. Furthermore, the daunting AMR projections propose that by 2050, one person will die every three seconds; in other words, ten million people will die annually due to AMR [7].

To address this pressing global health challenge, the WHO established the Global Antimicrobial Resistance and Use Surveillance System (GLASS) in 2015 (https://www.who.int/initiatives/glass (accessed on 1 March 2023)). GLASS is a global strategy that seeks to monitor the evolution of AMR and adopt health policies to tackle the problem. It advocates for a shift from laboratory-based surveillance to a more comprehensive system that integrates epidemiological, clinical, and population-level data.

The importance of AMR surveillance cannot be overstated. Such systems play a crucial role in every nation's efforts to prevent and control infectious diseases. This is especially critical for low- and middle-income countries (LMICs), which often have limited resources and infrastructure to address the problem. By adopting and enhancing the implementation of GLASS, LMICs can strengthen their capacity to address AMR at national, regional, and global levels. This will allow them to gain insights into the spread and impact of AMR and develop evidence-based policies and interventions to combat it effectively [8].

Genome-wide analyses have improved our understanding of AMR, and GLASS recommends the use of whole genome sequencing (WGS) for isolates in cultures [9]. However, the cost and complexity of culturing pathogens can be a significant barrier; thus, metagenomics studies from environmental samples could provide better resolution for the detection of AMR in LMICs. Metagenomic experiments from sewage have been proposed by The Global Sewage Surveillance project consortium to gather and report vast amounts of AMR information with cheaper and easier standardization than clinical-based AMR monitoring [10,11].

Given that LMICs suffer from high rates of infectious diseases [6,7], local and regional AMR monitoring could be established with the sewage approach to assess AMR. Furthermore, Peru represents a country with significant AMR-associated issues in the main and most populated cities [11,12], but with little information for the countryside.

In this study, we present a local genomic assessment of antibiotic resistance genes (ARG) in sewage samples from two hospitals and an urban community pipeline in Huanuco, a Peruvian city over 2000 m above sea level. By using a metagenomic sequencing approach, our study aims to provide insights into the spread and impact of AMR in Huanuco and contribute to the development of effective AMR policies and interventions at the local and regional levels.

2. Methods

2.1. Location and Samples

Peru is a middle–high-income country, according to the world bank, based on the Gross National Income (GNI) per capita [13]. The country has a partitioned healthcare system, consisting of public services such as the Ministry of Health (MINSA), police, and armed forces, as well as social insurance and the private sector [14]. The province of Huanuco, located in Peru, has a population of 293,397 according to the 2017 census. Of this population, 71.8% reside in urban areas, while the remaining 28.2% live in rural settings. Huanuco is known to be one of the poorest provinces in Peru, with high illiteracy rates, particularly in the rural areas, where the rate reaches about 20.3%. Moreover, Huanuco experiences significant migration flows, particularly with Lima, the largest Peruvian city [15].

Our study involved collecting sewage water samples from two hospitals in the city of Huanuco, Peru, in 2021. We collected samples from one social insurance health hospital (ESSALUD) and one public healthcare system (MINSA) hospital (Hospital Valdizan: H. Valdizan). These hospitals are the primary healthcare facilities serving approximately 200,000 residents of Huanuco. In addition to these hospital samples, we also collected urban samples from a location far away from the aforementioned wastewater pipes (5 km). The locations of all sampling sites are depicted in Figure 1.

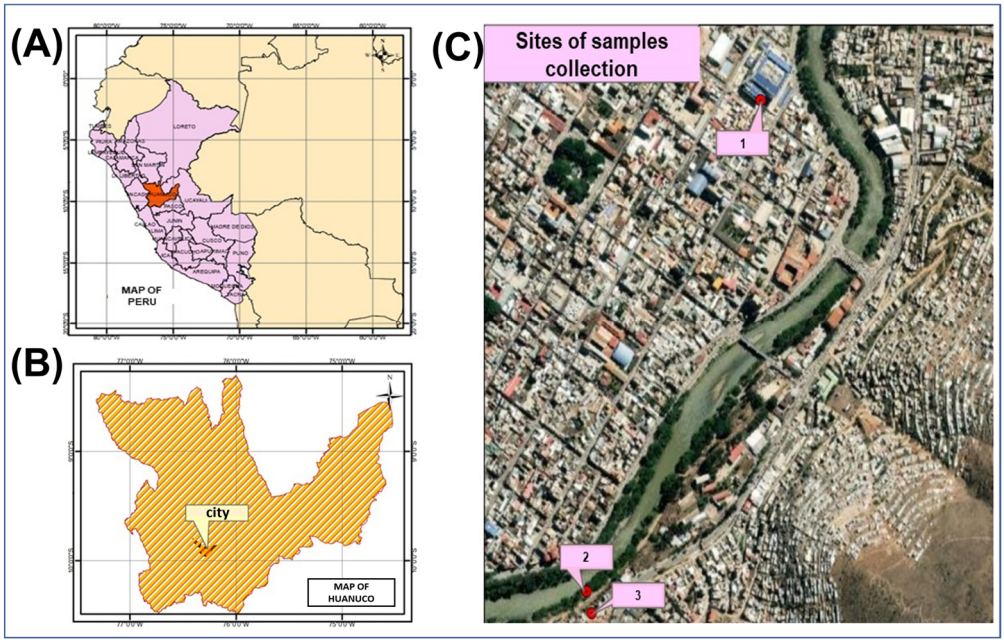


Figure 1. Sampling sites for wastewater samples. The study was conducted in Huanuco, one of the 24 departments, located in the central highlands of Peru, colored in red (**A**). The city where the sewage sites were collected was geographically delimited (**B**). The collection sites for samples included the following: (1) urban pipelines, (2) The H. Valdizan public hospital, and (3) ESSALUD social security hospital (**C**).

One sample of one-liter volume (1 L) each was collected from each sewage on two consecutive days (n = 2/sewage site), controlling for some environmental factors: temperature, osmotic pressure, pH, and oxygen concentration; that is, samples were collected on days with no rain. After collection, the samples were immediately frozen. Then, the samples were thawed for 12 h at approximately 20 °C before processing. After thawing, 250 mL of each sample was sedimented in a centrifuge at 10,000× g for 10 min. The sediment was stored at −20 °C or −80 °C prior to DNA extraction.

2.2. Sample, DNA Extraction, and Sequencing

To extract DNA, the QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, MD, USA) was used following the manufacturer's instructions. DNA quality and quantification of nucleic acids were determined using the QIAxpert System (QIAGEN, USA) and gel electrophoresis.

2.3. 16S rRNA Gene Amplification

To determine bacterial composition, the V4 variable region of the 16S rRNA bacterial gene was amplified using the primers 16S-515F (GTGCCAGCMGCCGCGGTAA) and 16S-806R (GGACTACHVGGGTWTCTAAT). Libraries were constructed using the dual-index approach [16]. Sequencing of pooled libraries was carried out using the Illumina MiSeq platform (San Diego, CA, USA) at the University of Minnesota to generate 2 × 300 bp sequences (~50 K reads/sample). The 16S rRNA sequences were processed using custom-made Perl scripts and the Qiime2 pipeline [17,18]. Briefly, raw sequencing data were processed to remove primers and low-quality end reads (Phred quality score < 30) using cutadapt. These high-quality reads were considered for denoising, merging, chimera removal, and finally generating unique amplicon sequence variants (ASV) using the Dada2 plugin of Qiime2 [17,18]. Representative sequences of each ASV were aligned using MAFFT, and phylogenetic trees, both rooted and unrooted, were constructed using FasTree. Taxonomic assignments of bacterial ASVs were carried out by trained naive Bayes classifiers on reference sequences (clustered at 99% sequence identity) from Green-

genes [19]. For both taxonomic assignments, Qiime2 plugins feature-classifier fit-classifier naïve-Bayes and feature-classifier classifier-sklearn were used. The R statistical software R-4.3.1 was used to calculate alpha and beta diversity metrics as well as indicator species for treatment groups.

2.4. Shotgun Metagenomics Sequencing

From DNA samples, library prep was conducted using the Nextera XT kit (Illumina Inc, San Diego, CA, USA) (quarter reaction), and libraries were sequenced on the NovaSeq sequencing platform to produce 2 × 150 pair-end reads (~15 M reads/sample). Filtering and processing of metagenomic sequence data were performed using the KneadData tool pipeline (https://github.com/biobakery/kneaddata (accessed on 1 March 2023)) to remove low-quality reads, primers, and host contamination. The Bowtie2 index of the GRch37 human reference genome was used as the human reference. Trimmomatic parameters within kneadData were set to 4 base sliding window sizes, only keeping reads with a greater than 20 Phred score. The minimum length of kept samples was also set to at least 90 percent total input read length. Kraken2 was used for taxonomic annotations, using kraken2's built-in database [20]. Taxonomic confidence was set to 0.1. Kraken2's reports were then processed through Braken for abundance analysis with a minimum length of 100 and at least 10 reads to perform re-estimation [21]. Analysis of gene pathway abundance and gene families was performed using Humann3 (http://huttenhower.sph.harvard.edu/humann (accessed on 1 March 2023)). Chocophlan and uniref were used as nucleotide and protein databases, respectively.

2.5. Antibiotic Resistance Analysis

Analysis was first performed using the RGI tool for gene family, drug class, and resistance mechanism abundance using high-quality, clean sequences [22]. DIAMOND was used as the alignment tool for analysis. Secondary analysis of antibiotic resistance was performed using AMRPlus-Plus for annotations of drug resistance mechanisms, resistance type, and functions [23], also leveraging built-in MEGAres databases.

3. Results

The alpha diversity analyses (bacterial richness and Shannon and Simpson indices) based on 16S rRNA analyses indicate that, in the city of Huanuco, the urban sewage samples tended to have higher taxonomic diversity and richness compared to the samples collected from hospitals. The Bray–Curtis index demonstrates differences in beta diversity of bacterial populations between locations, i.e., hospitals and urban dwellings. Approximately 74% of the variance between the bacterial composition at each location was explained in the weighted Bray–Curtis index, respectively, with the H. Valdizan samples showing maximum dissimilarity from the urban and ESSALUD samples (Figures 2 and 3).

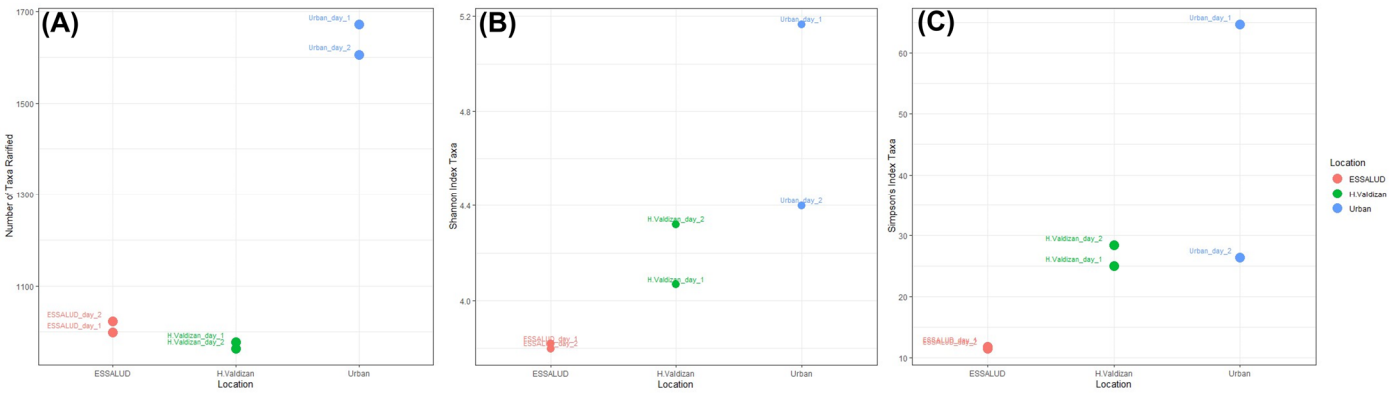


Figure 2. Alpha diversity analyses. (**A**) Number of ASVs rarified according to taxa. (**B**) Shannon Diversity Index and (**C**) Simpson's Diversity Index (ASV: amplicon sequence variants).

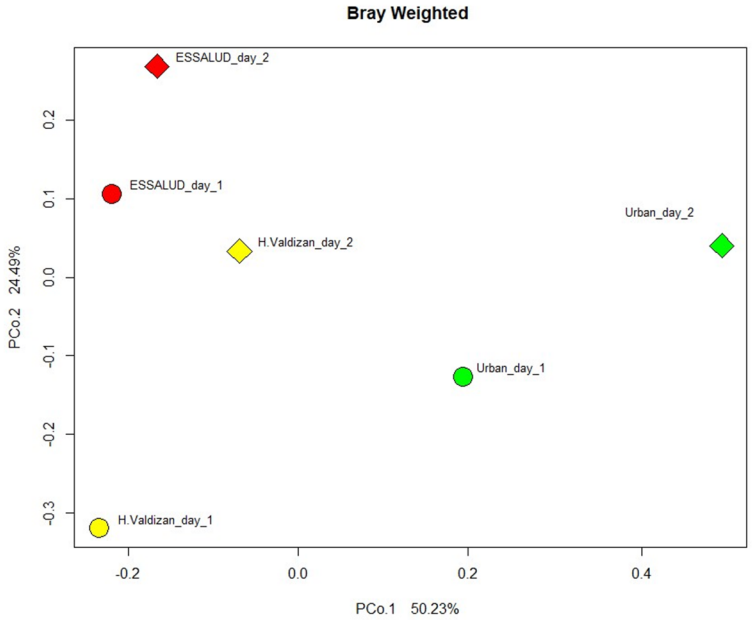


Figure 3. Beta diversity analyses. Principal coordinate analysis (PCoA) of beta diversity (unweighted and weighted Bray distances).

The sewage location influenced the taxonomic diversity between samples. For instance, the ESSALUD hospital shows the highest abundance of *Prevotella copri* among all the wastewater samples. Conversely, the urban biospecimen portrayed the highest abundance of *Aeromonas cavie* compared to those collected from the two hospitals. Even though these proportions were similar between the first and the second sample, some differences in taxa abundance were observed between days for all sites (Figure 4).

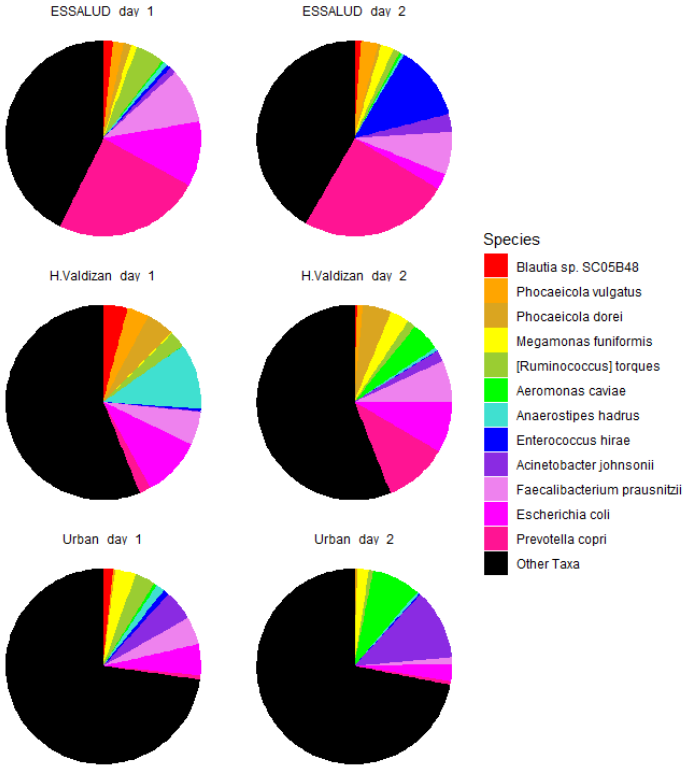


Figure 4. Taxa abundance according to samples and location.

Metagenomic analyses demonstrated that the most common ARGs detected were TEM beta-lactamase and OXA beta-lactamase in all the samples, followed by the major facilitator superfamily (MFS) antibiotic efflux pump, resistance-nodulation-cell division (RND) antibiotic efflux pump, the GES beta-lactamase, the quinolone resistance protein, and the CRX-M beta-lactamase genes (Figure 5A). We can report that resistance to beta-lactam antibiotics was the most abundant across all samples, followed by cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides (Figure 5B). Notably, the ESSALUD samples exhibited a similar pattern of ARGs to the urban biospecimens, especially in lower amounts of TEM beta lactamase and greater amounts of OXA beta lactamase compared to the H. Valdizan hospital.

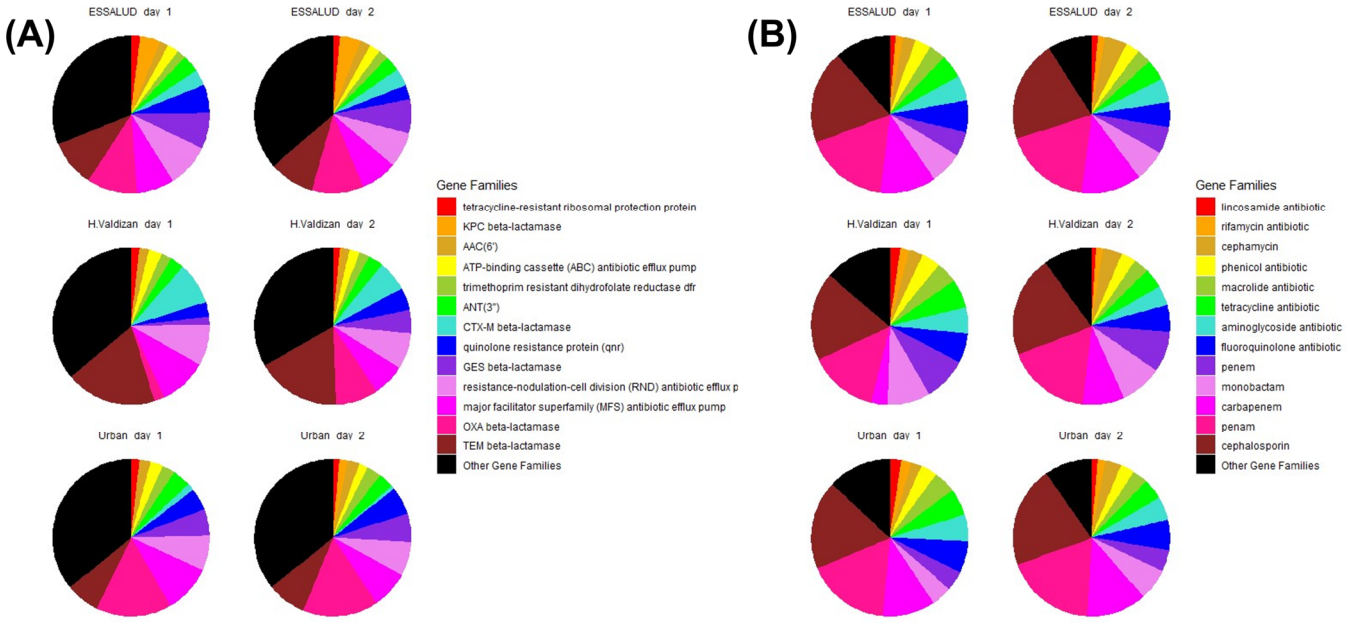
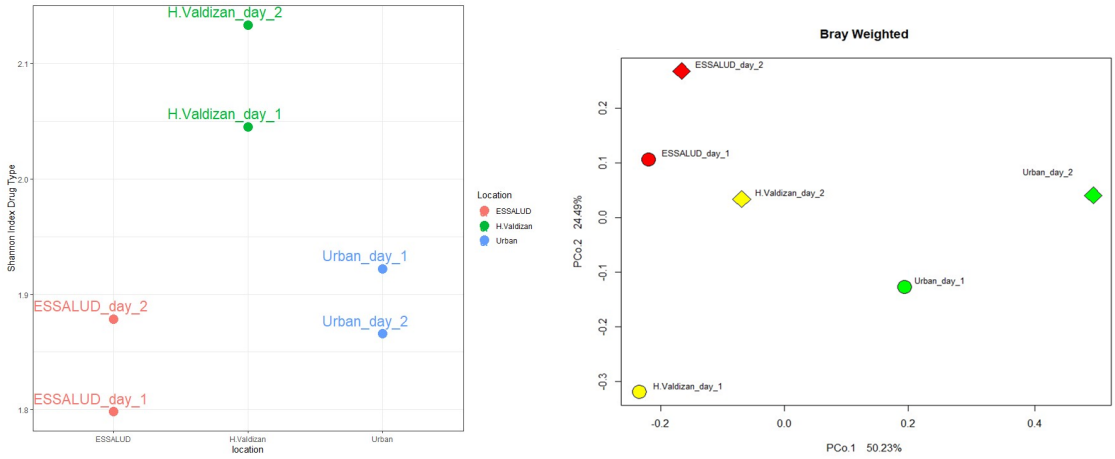


Figure 5. Abundance of resistance gene families (**A**) and drug class resistance (**B**).

Figure 6 illustrates the prevalence of AMR among the collected wastewater samples according to different drug classes. Macrolides, lincosamides, and streptogramins (MLS) were found to be the most abundant, followed by resistance to aminoglycosides and tetracyclines. Alpha and beta diversity analyses of ARGs revealed that the H. Valdizan samples exhibited the highest ARG diversity (as shown in Figure 6A). Notably, the patterns of drug resistance observed in this hospital were different from those seen in the ESSALUD and urban samples, which tended to cluster together (as depicted in Figure 6B). A matter of particular concern was the consistently elevated abundance of oxazolidinone-resistant bacteria observed in the samples from ESSALUD and H. Valdizan in comparison to those from other urban sites. In contrast, the urban sewage samples displayed a higher prevalence of MLS-resistant bacteria compared to their counterparts. Interestingly, a higher abundance of rifampin-resistance genes was detected in the urban wastewater samples than in those collected near the hospitals (as shown in Figure 7). These findings underscore the complex and diverse nature of AMR patterns in different wastewater sources and highlight the urgent need for continued surveillance and monitoring of antibiotic resistance in the environment.



(**A**) (**B**)

Figure 6. Antibiotic resistance according to drug type. Shannon diversity index to depict alpha diversity (similar to Simpsons' metric, not shown) (**A**). Beta diversity shown in the Bray–Curtis index and portrayed in the principal coordinate analysis (PCoA) (**B**).

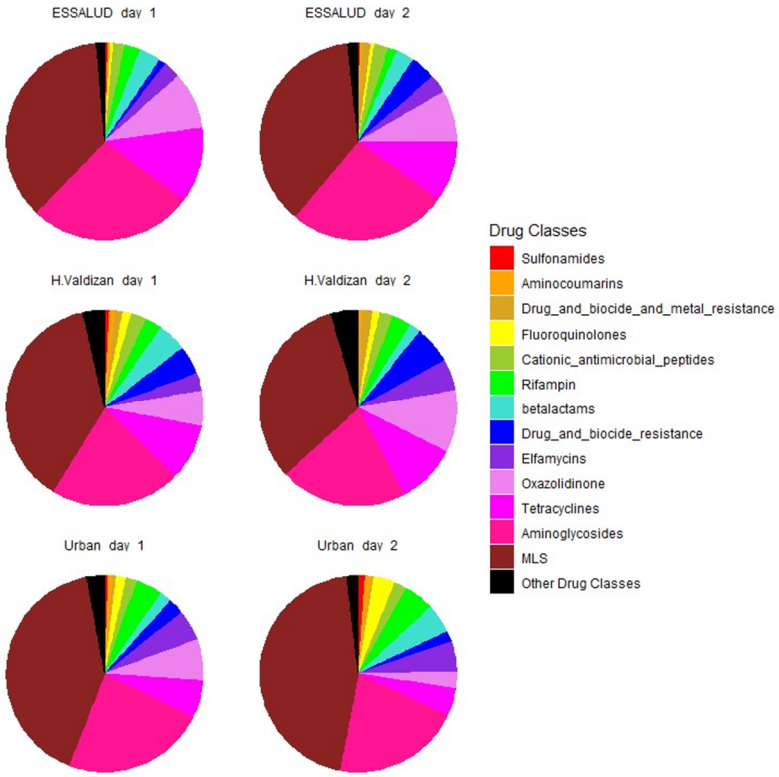


Figure 7. Relative frequencies of bacteria abundance by each type of resistance according to drug type.

4. Discussion

Peru has been facing the dual consequences of the epidemiological transition in recent decades, with prevailing infectious diseases and increasing rates of non-communicable conditions [24]. In our study, we have demonstrated high levels of ARG related to beta-lactams, monobactams, macrolides, and even modern antibiotics such as oxazolidinone or last-resort antibiotics (i.e., carbapenems), especially in samples from the social insurance hospital (ESSALUD) in the city of Huanuco. This is concerning as it suggests that even in remote areas, AMR is becoming increasingly prevalent.

Peru, like other countries in the Americas, depicts antibacterial consumption in its population based on beta-lactams, macrolides, lincosamides, and streptogramins, as well as quinolones. Our study results suggest that these consumption patterns may be associated with the prevalence of antibiotic resistance genes (ARGs) in Peru, with beta-lactams being the most commonly detected ARGs, followed by high rates of MLS and quinolone resistance.

Our analysis revealed differences in beta-lactamase resistance patterns between healthcare settings, with lower TEM and higher OXA prevalence in urban and ESSALUD settings compared to H. Valdizan. This discrepancy could be attributed to TEM's frequent presence in bacteria such as *E. coli* and *K. pneumoniae*, common in treatments at H. Valdizan (Figure 5A). Conversely, OXA could be found in bacterium requiring more intensive treatments, often administered in ESSALUD and private clinics throughout urban areas. Moreover, antibiotic prescribing, inhabitants' consumption habits, and infection control practices may vary between healthcare settings, contributing to the observed differences in resistance patterns.

Therefore, our observations seem relevant due to the high proportions of infectious diseases spread throughout the Peruvian territory, with some iconic examples due to microbes such as *Mycobacterium tuberculosis* or *Helicobacter pylori*. In fact, Peru is considered an endemic tuberculosis (TB) country with high rates of antibiotic resistance [25]. This has been corroborated by our study, where we observed small but important rates of rifampin ARG-related resistance, with considerable rates of fluoroquinolone and carbapenem ARG genes associated with treatment resistance. This is paramount due to the use of these drugs for the treatment of first-line TB-resistant strains [26].

Nevertheless, further studies should explore and confirm our findings, as other authors suggest TB strains with low fluoroquinolone resistance rates [27]. Thus, to avoid any kind of bias, the sewage ARG investigation seems to gather enough information before starting a drug schema in a specific community and could even contribute to suggesting further WGS in TB patients to establish which isolates could have higher resistance susceptibility [28].

On the other hand, *Helicobacter pylori* infections could have resistant strains to standard and second-line therapies. For instance, a recent meta-analysis demonstrated high rates of resistance in patients (6–25%) and on strain assessment (51–63%) [29]. The endemic condition of diseases associated with this bacterium in Peru and the poor sanitary conditions, among other factors, could be promoting the repeated use of antibacterial for *H. pylori* treatment. Despite these alarming resistance rates, Villavicencio et al. could only find seven studies for their conclusions, giving support to an epidemiological sewage approach such as the one we conducted for better treatment assessment and before drug selection in a community.

Besides the irrational antibiotic consumption or overuse in LIMCs such as Peru [7], a special mention must be made of the recent global pandemics. Therefore, resistance to macrolides or fluoquinolones found in our study could be due to the irrational use of azithromycin in the first year of the COVID-19 pandemic [30] or other endemic infectious conditions requiring this sort of treatment (e.g., *Helicobacter pylori* [31], sexually transmitted diseases [32,33]).

The results of our project slightly differ from a similar one performed in two sewages of two Hospitals of the Peruvian main city, Lima. In this study, the authors suggested that all the bacteria they found were multidrug resistant with high rates of ARG associated with extended-spectrum beta-lactamases (ESBL, bla TEM) and carbapenemase genes (ESBL, bla KPC, and bla IMP) [18]. Nevertheless, these authors only studied bacteria containing beta-lactamase (bla)-related ARG.

Furthermore, AMR could be arising in hospitalized patients due to excessive use of common and last-resort antibiotics, as has been reported by Resurrección-Delgado et al. in a national Peruvian hospital, where authors found that 40% of patients should not have been indicated antibacterial treatment, whereas 90% of inpatients started treatments only by the clinician's empirical choice [34]. This could explain why we detected a variety

of ARG related to even last-resort drugs (i.e., carbapenems), especially in the social insurance hospital (ESSALUD), which has more access to a broader list of treatments than the public institution (MINSA). This resistome could not only affect inpatients, but also healthcare workers if they do not have appropriate sanitary facilities or adequate biosafety, transmitting ARG from hospitals to the community [35].

Our study findings reinforce the GLASS stewardship recommendations for local monitoring of AMR. Contrary to previous studies by the Global Sewage Surveillance project consortium that evaluated ARGs in sewage samples from coastal cities in Peru [11,12], our study sheds light on the AMR landscape in poor, informal urban and rural settings. Specifically, we investigated the resistome of a city located in the Andean region, highlighting the prevalence of aminoglycoside, fluoroquinolone, and macrolide-related ARGs, which have also been shown to be present in Lima and Piura by Munk et al. [12].

One of the strengths of our study is that we assessed ARGs in the sewers of a local settlement, following expert recommendations [10]. This approach is the first of its kind in a province of Peru and can aid in assessing the wastewater system in hospitals. Furthermore, we controlled several factors to have samples with representative information related to AMR, despite the high variability of sewage samples from one day to another due to environmental and human factors. However, we acknowledge that our sample size was limited, and we could only perform this analysis once a year in two separate consecutive days.

In addition, we were not able to introduce metagenomics in tap water to assess the resistome directly consumable by humans or animals in this city. It seems crucial to complement our strategy to other methodologies due to a recent report suggesting insufficient effectiveness of Peruvian municipal wastewater treatment plants, where authors quantified moderate concentrations of antibiotics such as clarithromycin, trimethoprim, ciprofloxacin, sulfamethoxazole, and azithromycin [36]. Furthermore, Huanuco represents a city with high poverty rates and an informal urban environment, where several factors, such as crowded houses, farms, and domestic animals, and soil bacteria, amongst others, contribute to AMR [37]. This adds complexity to the assessment of the microbiome of communities with high migration flux from Huanuco to Lima and other places, as well as any commercial products traveling between these cities. This information could have contributed to a better understanding of how ARG flux contributes to specific migrant-AMR causes from one place to another, as a recent publication suggests that migration plays a crucial role in AMR distribution [38].

For an extensive assessment of ARG in the whole community, it is highly recommended to expand the sample size and carry out samplings at various intervals throughout the year, considering factors such as soil, weather, farming and domestic animal activities, wastewater treatment strategies, and drug availability in the city, amongst others. For this reason, a larger project with a One Health perspective is suggested, with local and regional adaptations in Peru.

5. Conclusions

In conclusion, our study highlights the high prevalence of antibiotic resistance genes (ARGs) in a countryside city in Peru, particularly in the samples collected from the social insurance hospital (ESSALUD). Our findings suggest that the high consumption of antibiotics such as beta-lactams, macrolides, and quinolones in the Peruvian population may be associated with the prevalence of ARGs in the environment. These findings highlight the widespread distribution of common ARGs and the concerning level of antibiotic resistance in the collected wastewater samples, which may have implications for public health and the environment. Furthermore, our study underscores the urgent need for more comprehensive monitoring and surveillance of AMR in Peru, including in poor, informal, urban, and rural settings.

Our study also emphasizes the importance of taking a One Health approach to combatting AMR, which recognizes the interconnectedness of human, animal, and environmental

health. Addressing AMR requires a multifaceted strategy that includes reducing the inappropriate use of antibiotics in human and animal health, improving infection prevention and control measures, promoting vaccination, and developing new antimicrobial agents. Additionally, efforts should be made to improve sanitation infrastructure and hygiene practices in both healthcare facilities and communities, as poor sanitary conditions can contribute to the spread of AMR.

Author Contributions: L.J.-V., N.P.-R., V.C.R.-L. and C.M.-J. conducted the sampling and microbiology experiments. J.A.P., L.J.-V., S.D., A.G. and H.G. conducted the analyses. J.A.P., L.J.-V., N.P.-R., V.C.R.-L., C.M.-J., S.A.-A., M.U., D.P.-L., R.C.-C., S.D., A.G. and H.G. wrote and reviewed the manuscript. H.G. conceived and supervised the research. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Our study was approved by the Ethics in Research Committee of the Universidad de Huanuco.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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