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Situational status of antibiotic resistance of *E. coli* in the Escalerilla WWTP, Arequipa, Peru

Estado situacional de la resistencia antibiótica de *E. coli* en la PTAR Escalerilla, Arequipa, Perú

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KEYWORDS
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ABSTRACT: During the COVID-19 pandemic in Peru, a notable increase in self-medication with antibiotics has been observed, raising concerns about the increase in bacterial resistance. In this context, we evaluated the situation in wastewater treatment plants (WWTPs) using biochemical methods and sensitivity tests, such as the Kirby-Bauer assay. The results of our study show a high sensitivity to antibiotics in most of the strains analyzed, particularly towards β-lactams. However, this sensitivity appears to be influenced by seasonal patterns of medication consumption, supported by studies indicating that its contribution to the total flow is minimal. We analyzed 49 strains of *Escherichia coli*, 27% of which showed no resistance to any antibiotic, while the highest resistance was observed against tetracycline (63%). High levels of resistance to fluoroquinolones, such as ciprofloxacin and levofloxacin, were also recorded. In contrast, amikacin and piperacillin-tazobactam showed minimal resistance, with only one strain resistant to each. Multiresistance, defined as resistance to at least two antibiotics, was identified in 35% of the strains, with two of them showing resistance to 8 and 10 antibiotics, respectively.

RESUMEN: En Perú, durante la pandemia de COVID-19, se ha observado un notable incremento en la automedicación con antibióticos, lo que ha generado preocupación por el aumento de la resistencia bacteriana. En este contexto, evaluamos la situación en las plantas de tratamiento de aguas residuales (PTAR) utilizando métodos bioquímicos y pruebas de sensibilidad, como el ensayo de Kirby-Bauer. Los resultados de nuestro estudio muestran una alta sensibilidad a los antibióticos en la mayoría de las cepas analizadas, especialmente hacia los β-lactámicos. Sin embargo, esta sensibilidad parece estar influenciada por los patrones estacionales de consumo de medicamentos, respaldado por estudios que indican que su contribución al caudal total es mínima. Se analizaron 49 cepas de *Escherichia coli*, de las cuales el 27% no presentó resistencia a ningún antibiótico, mientras que la mayor resistencia se observó frente a la tetraciclina (63%). También se registraron altos niveles de resistencia a fluoroquinolonas,

como ciprofloxacina y levofloxacina. En contraste, la amikacina y la piperacilina-tazobactam presentaron una resistencia mínima, con solo una cepa resistente a cada una. La multirresistencia, definida como la resistencia a al menos dos antibióticos, se identificó en el 35% de las cepas, y dos de ellas mostraron resistencia a 8 y 10 antibióticos, respectivamente.

1. Introduction

Antibiotic resistance has become an imminent global problem. Research has identified resistant bacteria and resistance genes in various settings, including clinical analyses, livestock, and wastewater [1]. Currently, wastewater analysis is emerging as a reliable and complementary epidemiological tool to monitor pharmacological substances like antibiotics, providing real-time data and enabling the identification of geographical variations and temporal trends in their consumption [2]. Wastewater treatment plants (WWTPs) are recognized as major drivers of antibiotic resistance since most of these drugs, both from human and veterinary use, are excreted unmetabolized and reach WWTPs via urine and feces, entering the sewage system [3].

Bioaccumulation of pharmacological substances like antibiotics occurs in WWTPs due to their limited or absent elimination. This constant flow towards receiving water sources can negatively impact human health and ecosystems, promoting the proliferation of resistant bacteria and their genes [4]. Studies have shown that the selection and transfer of antibiotic resistance occur even with exposures to very low concentrations, below the minimum inhibitory concentration, in various environmental settings [5].

The increase in antimicrobial resistance is an imminent threat to public health. Lack of intervention could lead us into a post-antibiotic era, severely limiting the effectiveness of these treatments against common and severe infections. Human migration and globalization have contributed to the spread of resistant bacteria [6]. According to a report from the United Kingdom, it is projected that by 2050, deaths due to drug-resistant infections could surpass 10 million, exceeding even those related to cancer. Global economic losses to antimicrobial resistance could reach $100 trillion [7].

Multi-drug resistant (MDR) *Enterobacteriaceae* and their genes show significant prevalence, possibly linked to intensive animal farming, contributing to their frequency in soil and nearby aquatic ecosystems. It's crucial to maintain constant surveillance of zoonotic bacteria to address this issue [8]. Further research is needed to thoroughly understand the mechanisms of selection, transfer, propagation, and impact of antimicrobial-resistant bacteria, both at environmental and operational levels. Additional studies are essential to develop effective mitigation and control strategies [9].

The COVID-19 pandemic has disproportionately affected low- and middle-income countries (LMICs). Therefore, it is essential to conduct ongoing studies to better understand antimicrobial resistance (AMR) and nosocomial infections in these countries [10].

This study aimed to investigate the prevalence of specific antibiotic resistance in *E. coli* strains at five different points within the Escalerilla Wastewater Treatment Plant (WWTP), from its influent to the final

effluent. The main goal was to identify the antibiotics to which these strains showed higher resistance, using *E. coli* as a common indicator of water quality according to national regulations [11]. Additionally, for the "Tricycle Protocol" developed by the World Health Organization (WHO) under the "One Health" approach [12], the supervision of antibiotic resistance in humans, animals, and the environment was considered. Thus, the aim was to establish a solid informational basis for conducting environmental epidemiological surveillance of wastewater in the city of Arequipa, Peru.

2. Methodology

2.1. Study Location

The Escalerilla WWTP is located at coordinates 219125.74m E, 8188306.97m N, characterized by scarce humidity occasionally reaching up to 50%. High solar radiation leads to an evaporation rate between 4 and 6 times greater than the precipitation rate, resulting in a dry and arid climate with intense sun exposure. Winds range from 6 m/s to 20 m/s, peaking around 1 PM. This treatment plant manages effluents from the Northern Cone of Metropolitan Arequipa, covering the rapidly growing districts of Cerro Colorado and Yura. Furthermore, it has a projected design horizon extending until the year 2036 [13]

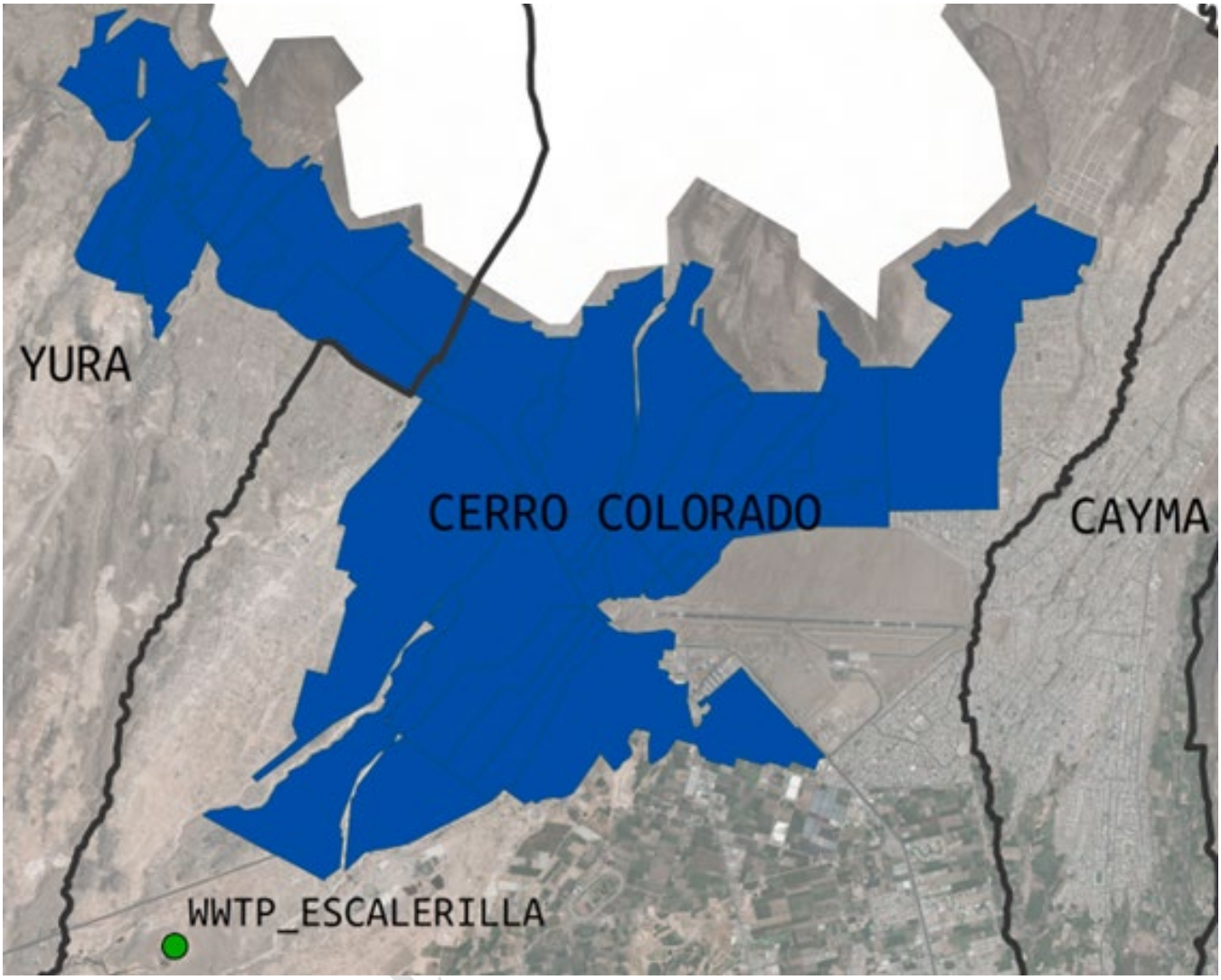

Figure 1. Zones contributing wastewater flow

In **Figure 1**, the districts of Yura, Cerro Colorado, and Cayma are clearly distinguished. The Escalerilla WWTP is marked with a green dot, while the drainage areas, highlighted in blue, represent the zones contributing wastewater flow to this treatment plant. The white areas correspond to uninhabitable zones.

2.2. Hydraulic and Physicochemical Characteristics

The primary collector sewer system directing flows towards the Escalerilla Wastewater Treatment Plant is mainly composed of recently constructed PVC pipes. This system plays a crucial role in treating urban wastewater, covering approximately 9.48% of the city's total area. The treatment method employed is extended aeration, designed to remove between 85% and 95% of contaminants present in the wastewater. The preliminary treatment process includes screening, grit removal, degreasing, as well as fats and oils

removal. On the other hand, secondary treatment occurs through various stages such as the distribution chamber, biological reactor, secondary sedimentation, and external sludge recirculation. Currently, the operation of the biological reactors is alternated, so that one is on standby. In its first phase, the operating reactor supports a maximum mass load of 0.093 kgBOD5/kg SS per day and has a concentration of 4.5 kg/m³ (**Table No. 1**). Each reactor has a total volume of 12.87 m³, with 20% dedicated to anoxia and 80% to aeration [13].

Table No. 1 Flow and Load Variation Over the Years According to Design

Phase	Year	Average Flow (l/s)	Maximum Flow (l/s)	Mass Load kg BOD5/kg SS per day
F1	2013	260	327	0.930
F2	2017	336	423	0.108
F3	2025	390	491	0.126
F4	2036	403	509	0.130

To obtain a representative overview, 5 monitoring points were selected among the main treatment units. These specific points are detailed in **Table No. 2** and were calculated based on an average flow rate of 145 liters per second measured at the treatment plant. The lower treated flow compared to the projected flow is partly due to the ongoing execution of the sanitation projects for "Reservoirs N39 and N31". These projects will complete the missing collectors, allowing their connection to the existing Escalerilla collector.

Table No. 2 Hydraulic Retention Time

No.	Label	Hydraulic Component	Accumulated Retention Time	Unit
P1	A1	Debris Screen - Influent	2.08	min
P2	MR	Distribution Manhole	34.63	min
P3	B1	Bioreactor Point 1	42.32	min
P4	B2	Bioreactor Point 2	1290.51	min
P5	C1	Secondary Clarification	1818.59	min

Table 3 shows the contaminant removal efficiency obtained through monitoring conducted for the National Urban Sanitation Program (PNSU) of Peru at the end of 2020 in an accredited laboratory, using standardized and validated methods. The Wastewater Treatment Plant (WWTP) exhibits the following

removal efficiencies, defined as the difference between the initial and final concentrations, divided by the initial concentration: more than 98% for Settleable Solids, Total Suspended Solids, Oils and Greases, Biochemical Oxygen Demand (BOD5), Chemical Oxygen Demand, Thermotolerant Coliforms, and Total Coliforms. However, the removal efficiency for Total Solids was 68.23%. This difference is due to the WWTP not being specifically designed to remove dissolved solids, as Total Solids are the sum of suspended, settleable, and dissolved solids. The removal of dissolved solids requires other types of processes, such as coagulation-flocculation and/or reverse osmosis.

Table No. 3 Contaminant Removal Efficiency

Parameter	Detection Limit	Unit	Inlet Concentration (P1)	Outlet Concentration (P5)	Removal Efficiency (%)
pH	-	pH units at 25 °C	7.87	7.06	-
Temperature	-	°C	22.6	25.6	-
Dissolved Oxygen	-	mg/L	0.03	3.79	-
Settleable Solids	0.1	mL/L	33.5	<0.1	99.70%
Total Suspended Solids	2.5	mg/L	1038	2.83	99.73%
Total Solids	2.5	mg/L	2902	922	68.23%
Oils and Greases	0.5	mg AyG /L	201	4	98.01%
Biochemical Oxygen Demand	2	mg/L	999	10.2	98.98%
Chemical Oxygen Demand	2.5	mg/L	2424	34.7	98.57%
Thermotolerant Coliforms	1.8	NMP/100 mL	54,000,000	70,000	99.87%
Total Coliforms	1.8	NMP/100 mL	240,000,000	350,000	99.85%

2.3. Sample Collection

Samples were collected between 9:00 a.m. and 9:30 a.m. the last Monday of every month, from November 2022 to February 2023. Each composite sample was obtained by mixing three individual samples. Using a steel sampler, samples were collected and stored in sterile 100 ml plastic bottles. The collection process began at point P5 and sequentially continued to point P1, ensuring the sampler was cleaned between each sample collection. Additionally, in-situ pH data were recorded using ISOLAB TM indicator paper strips, which yielded values ranging between 7 and 7,5. The collected samples were properly packaged, and the cold chain was maintained for transportation to the Microbiology Laboratory of the Faculty of Medicine at the National University of San Agustin, where their analysis would be conducted [14].

It is important to highlight that each year CLSI updates the diameter ranges used to analyze antimicrobial sensitivity and resistance. In this study, in addition to the routinely evaluated antimicrobials, penicillin, amoxicillin, cephalexin, oxacillin, and clindamycin were included, which are not commonly used for *E.*

coli infections. Vancomycin, erythromycin, and azithromycin were also evaluated, although no breakpoints are established for these in the CLSI guidelines. This underscores the importance of establishing specific breakpoints for environmental studies on antimicrobial resistance.

2.4. Microbial Isolation

The determination of *E. coli* was conducted using the Most Probable Number (MPN) technique, as outlined in the 'Standard Methods for the Examination of Water and Wastewater' (APHA) Ed. 2017 [15]. A non-selective enrichment with Buffered Peptone Water was used for the presumptive test, aimed at revitalizing bacteria possibly damaged by storage conditions. To determine the optimal dilution range, 1 ml of the sample was inoculated into a tube containing 9 ml of GranuCult™ Buffered Peptone Water, creating a 10^{-1}dilution. Subsequently, serial dilutions were prepared until achieving a 10^{-3} dilution.

From this dilution, 1 ml was inoculated into 3 tubes with GranuCult™ Lactose Lauril Sulfate broth with Durham tubes to detect gas production. These tubes were incubated for 48 hours at 37 °C. Tubes showing turbidity and gas production in the Durham tube were subjected to the confirmatory test. For this stage, a selective enrichment was performed using GranuCult™ Brilliant Green Bile Lactose broth (BRILA) at 37°C for 48 hours for total coliforms and GranuCult™ EC broth (*Escherichia coli*) at 44°C for 48 hours for fecal coliforms, both mediums containing Durham tubes. Turbidity and gas production in the Durham tubes were considered as positive results. To confirm the presence of *E. coli*, Kovac's reagent was added to positive EC broth tubes. The appearance of a turquoise ring on the medium surface indicated a positive result for *E. coli*, enabling further selective isolation.

Selective isolation was performed on MacConkey agar GranuCult™, as it allows differentiation of Gram-negative bacteria and the *Enterobacteriaceae* family. Suspected colonies of *E. coli*, identified by their pinkish-red color, underwent biochemical tests using GranuCult™ Lysine Iron Agar (LIA), Triple Sugar Iron (TSI) Agar GranuCult™, and Simmons Citrate Agar GranuCult™ [16]

2.5. Antibiotic Susceptibility Test

The Kirby-Bauer disk diffusion technique was employed to evaluate antibiotic susceptibility (**Table No. 4**). An inoculum of colonies obtained from previous plates was transferred to a tube containing peptone water. Upon reaching a turbidity equivalent to 1.5 x 10-8 CFU/ml (No. 0.5 on the Mac Farlan scale), it was seeded onto the surface of plates with Mueller Hinton NutriSelect® agar. Subsequently, antibiotic disks were placed on these plates, which were then incubated for 24 hours at 37°C. The zones of inhibition surrounding the antibiotics were measured, classifying them as Resistant (R), Susceptible (S), or Intermediate (I), based on standard halo patterns for *E. coli* outlined in the CLSI M100, 2020 Edition, March 2020 - Performance Standards for Antimicrobial Susceptibility Testing [17].

Table No. 4 Antibiotics by groups and sub groups

Groups	Subgroup	Antibiotic
Aminoglycosides	-	Gentamicin (GM)
	-	Amikacin (MK)
Beta-lactams	Beta-lactamase inhibitors	Ampicillin sulbactam (SAM)
	Penicillins	Penicillin (P)
	3GEN cephalosporins	Cefotaxime (CTX)
	Penicillins - aminopenicillins	Amoxicillin (AMX)
	Penicillins - aminopenicillins	Ampicillin (AM)
	Beta-lactamase inhibitors	Amoxicillin + ac. clavulanic (AMC)
	1GEN cephalosporins	Cephalexin (CFL)
	Penicillins - isoxazolylpenicillins	Oxacillin (OX)
	Beta-lactamase inhibitors	Piperacillin – tazobactam (PTZ)
	Cephalosporins – 3GEN	Ceftriazone (CRO)
Cefuroxime	2GEN cephalosporins	Cefuroxime (CXM)
Glycopeptides	-	Vancomycin(VA)
Lincosamides	-	Clindamycin(CC)
Macrolides	14atm	Erythromycin (E)
	15atm	Azithromycin (AZM)
Quinolones	3GEN	Levofloxacin (LEV)
	2GEN	Ciprofloxacin (CIP)
Sulfonamides	-	Trimethoprim sulfamethoxazole (SXT)
Tetracycline	1GEN	Tetracycline (TE)

2.6. Statistical Analysis

The results of this study were obtained through a multivariate analysis of variance (MANOVA) to evaluate significant differences at each analysis point across different monitoring dates. This analysis was performed using IBM SPSS Statistics 7.0.1 IF026 (x64) software.

A total of 49 strains of *Escherichia coli* collected at different points and sampling dates (**Table No. 02**) were analyzed, of which 13 showed no resistance to any antibiotic. Twenty-seven percent of the strains were sensitive to all evaluated antibiotics. The highest resistance was observed against tetracycline, with 63% of the total strains and 86% of the 36 strains that showed resistance to at least one antibiotic. Fluoroquinolones, such as ciprofloxacin and levofloxacin, showed the next highest resistance rates.

The antibiotics with the lowest resistance were amikacin and piperacillin-tazobactam, with only one resistant strain for each. It is important to note that the strain resistant to amikacin was not multidrug-resistant, while the strain resistant to piperacillin-tazobactam showed resistance to four antibiotics.

Strains that showed resistance to at least two antibiotics were considered multidrug-resistant; it was found that 35% of the strains were multidrug-resistant. Additionally, a strain resistant to 10 antibiotics was identified at point P3 and another resistant to 8 antibiotics at point P5, both isolated on the same date during the first month of sampling. A strain resistant to 6 antibiotics was also found at point P1, corresponding to a sampling conducted during the rainy season (February).

The proportion of resistant strains at point P5 was lower compared to other points, representing 50% (5 out of 10) of the strains. At the other points, the proportions were 73% at P4 (8 out of 11), 70% at P3 (7 out of 10), 71% at P2 (5 out of 7), and 100% at P1 (11 out of 11). During the rainy season months, no resistant strains were detected in the final phase of the wastewater treatment plant (WWTP). This could be because the rainiest months coincide with the highest temperatures, which implies a lower consumption of antibiotics due to a reduction in respiratory infections. Additionally, the location of the Escalerilla WWTP means that the flow received from rain is not significant, as even during the rainy season, it does not reach the plant. This could indicate that exposure to lower concentrations of antibiotics, along with competition among different bacteria present in the sludge, reduces the population of antibiotic-resistant bacteria.

Table No. 5 Percentage of Antimicrobial Resistance Level by *E. coli* according to the sampling point

Percentage of Microbial Resistance by Sampling Point	P1				P2				P3				P4				P5			
	R	I	S	N1	R	I	S	N2	R	I	S	N3	R	I	S	N4	R	I	S	N5
Tetracycline	90,9	0,0	9,1	11	57,1	14,3	28,6	7	60,0	10,0	30,0	10	72,7	0,0	27,3	11	30,0	0,0	60,0	10
Ciprofloxacin	0,0	27,3	72,7	11	14,3	14,3	71,4	7	30,0	10,0	60,0	10	45,5	18,2	36,4	11	30,0	0,0	70,0	10
Gentamicin	0,0	36,4	63,6	11	0,0	14,3	85,7	7	30,0	0,0	70,0	10	9,1	27,3	63,6	11	10,0	10,0	80,0	10
Amikacin	0,0	9,1	90,9	11	14,3	14,3	71,4	7	0,0	0,0	100,0	10	0,0	18,2	81,8	11	0,0	30,0	70,0	10
Ampicillin-sulbactam	9,1	0,0	90,9	11	0,0	14,3	85,7	7	10,0	10,0	80,0	10	9,1	0,0	90,9	11	10,0	20,0	70,0	10
Trimethoprim Sulfamethoxazole	0,0	10,0	90,0	10	0,0	0,0	100,0	7	10,0	10,0	80,0	10	0,0	9,1	90,9	11	10,0	0,0	90,0	10
Cefotaxime	20,0	0,0	80,0	10	0,0	0,0	100,0	7	20,0	0,0	80,0	10	0,0	0,0	100,0	11	20,0	0,0	80,0	10
Cefuroxime	10,0	0,0	90,0	10	0,0	0,0	100,0	7	10,0	20,0	70,0	10	9,1	9,1	81,8	11	30,0	0,0	60,0	10
Levofloxacin	20,0	20,0	60,0	10	14,3	14,3	71,4	7	10,0	30,0	60,0	10	36,4	18,2	45,5	11	40,0	0,0	60,0	10
Ampicillin	18,2	0,0	81,8	11	14,3	0,0	85,7	7	10,0	20,0	70,0	10	0,0	9,1	90,9	11	40,0	0,0	60,0	10
Amoxicillin + clavulanic acid	9,1	9,1	81,8	11	14,3	14,3	71,4	7	10,0	10,0	80,0	10	45,5	9,1	45,5	11	0,0	0,0	90,0	10
Piperacillin-tazobactam	0,0	0,0	100,0	11	0,0	0,0	100,0	7	0,0	0,0	100,0	10	9,1	9,1	81,8	11	0,0	0,0	100,0	10
Ceftriaxone	9,1	9,1	81,8	11	0,0	0,0	100,0	7	30,0	10,0	60,0	10	0,0	0,0	100,0	11	10,0	10,0	80,0	10

Note: The percentages were calculated between the types of resistance (R=resistance, I = intermediate and S=sensitive) per monitoring point.

In the MANOVA statistical analysis, no significant differences were found between the sampling points. However, multivariate tests revealed a significant difference, suggesting that the date on which the samples are taken influences the inhibition halos.

3. Results

In Peru, the self-medication rate with antibiotics before the Covid-19 pandemic stood at 27.6%. During the pandemic, these values increased to 39.2% for antibiotics/anti-inflammatories and specifically to 21.6% for antibiotics. A reduction in the average age of self-medicators was observed, decreasing from 46.5 years before the pandemic to 40.87 years during it [18]. This was mainly attributed to the dispensing of drugs without a prescription, guidance from pharmacists, occasional use of medications, and acquisition without proper consideration of their implications [19].

These factors suggest a probable increase in antimicrobial resistance in WWTPs, especially due to the high rate of self-medication and hospital discharges. The latter contribute to the alteration of the microbiota present in effluents, generating resistance that is affected by the persistence and solubility of the medications [20].

This could be due to the fact that, although hospitals would be expected to be the largest source of discharge in WWTPs, since they host a wide variety of multi-resistant bacteria, they do not represent a significant percentage of the flow to the plants, as confirmed by studies such as that of Verburg et al. which suggests that hospital discharges are 10% compared to other discharges [3]. Rahman et al., in their wastewater monitoring, identified carbapenem-resistant *Enterobacteriaceae* (CRE), vancomycin-resistant *Enterococci* (VRE), and Methicillin-resistant *Staphylococcus aureus* (MRSA) in areas with 5.2 million residents, but evidenced that hospitals were not a predominant source of resistance [21].

This suggests that the major contribution of resistant bacteria may come from other discharges, such as urban, domestic, or livestock wastewater. In urban environments, WWTPs present considerable diversity [22]. The sources of contamination in these facilities include fluoroquinolones, trimethoprim, aminoglycosides, sulfonamides, beta-lactams, tetracyclines, cephalosporins, and carbapenems in extracellular (exDNA) and intracellular (inDNA) DNA [23], [24]. Resistance to broad-spectrum antibiotics such as aminoglycosides, which inhibit protein synthesis in gram-negative infections resistant to β-lactams and other first-line antibiotics [25], can propagate antimicrobial resistance in aquatic environments through horizontal gene transfer, posing a risk for the selection of resistant bacteria in treated wastewater [23].

Our study demonstrated higher sensitivity to antibiotics, mostly belonging to the β-lactam group (**Table No. 5**). This trend could be linked to the rapid hydrolysis of these antibiotics in bodies of water, which could limit their ability to effectively promote resistance [26]. However, it was observed that *E. coli* showed higher resistance to Oxacillin, Penicillin, and Tetracycline. In the case of the latter, various studies have shown a constant prevalence of resistance, as *E. coli* harbors at least 40 tetracycline resistance genes. These genes encode enzymatic inactivation, ribosomal protection, or efflux pumps such as the tetA pump, known for its role in tetracycline resistance in *Enterobacteriaceae* [27].

According to our analysis, no significant differences were found between the sampling points. However, a significant variation ($p<0.05$) was observed in the mean values of each sampling point across the different monitoring dates. Likewise, there wasn't a pronounced prevalence of resistance during the analyzed months. These results align with several studies, demonstrating that the relationship between antibiotic concentration, Wastewater Treatment Plant (WWTP) treatments, and antibiotic resistance is not always strictly positive or negative. This suggests that, while WWTPs might stress bacteria, they aren't always a hotspot for resistance gene proliferation. However, they may increase the relative abundance of mobile resistance genes, such as plasmids [28]. Despite the reduction in *E. coli* population density achieved by some WWTPs, as found in our study (**Table No. 6**), they don't ensure the effective elimination of bacterial DNA, leaving residual fractions in the environment attached to particles, degraded, or absorbed through natural transformation into competent cells [24].

Table No. 6 *E. coli* MPN values from the five sampling points of the Escalerilla WWTP

	MNP per sampling day at the WWTP Escalerilla				
	P1	**P2**	**P3**	**P4**	**P5**
28-November	1500	1600	1200	900	700
26-December	5300	>110000	>110000	24000	1500
30 January	15000	9300	1900	2400	600
27-February	900		>110000	5300	300

Other studies have reported a higher prevalence of antibiotic-resistant bacteria in facilities with more invasive treatments, such as WWTPs utilizing a modified activated sludge process, followed by a laminar settler for nutrient removal, and a final UV disinfection step at an estimated dose of 16 mJ/cm2 [29]. Our results do not support their findings, which could be due to the fact that the monitoring point 'P5' is located before the disinfection process. Antibiotic resistance prevalence is also influenced by how these treatments might alter the abundance of elements carrying resistance genes, such as integrons or plasmids [28].

Despite the development of microbial isolation and molecular methodologies more sensitive than the biochemical methods used in this study, no substantial difference in results has been evidenced between the two approaches in different studies; instead, similar studies have shown comparable percentages when using conventional methods akin to those employed here [30]. Additionally, it has been demonstrated that methods like qPCR have limitations in detecting some bacteria that are identified by culture-based methods, which can incur higher economic costs if multiple targets need detection [31]. Hence, it's crucial to pay attention to these conventional methods, especially in countries with fewer resources where modern methods might not be as accessible.

All of this underscores the importance of epidemiological surveillance of wastewater, integrating technical and business information from Sanitation Service Providing Companies. It's considered crucial to characterize antimicrobial resistance in specific population groups through surveillance at WWTPs, health centers, schools, and strategic sampling points. These data will effectively monitor antibiotic consumption characteristics in the population, aiming to prevent resistance issues in the future.

4. Conclusions

The study did not find significant differences between the monitoring points through the MANOVA analysis. However, a significant variation in average values was observed over the monitoring dates, suggesting that seasonal changes influence the resistance of the collected strains. This highlights the importance of conducting seasonal monitoring to better understand the dynamics of antimicrobial resistance (AMR) and how environmental conditions impact its spread, a central aspect of the environmental monitoring in the Tricycle protocol.

The *E. coli* strains showed higher resistance to tetracycline (63%), followed by fluoroquinolones, emphasizing the need for specific monitoring of these antibiotics. Additionally, 35% of the isolated strains were multidrug-resistant, with some resistant to up to 10 antibiotics. These antibiotics, identified with high resistance rates, require strict public health surveillance to anticipate possible hospital complications. It is essential to exercise greater caution when prescribing these agents to vulnerable patients, ensuring prior identification of the pathogen and its antimicrobial sensitivity.

Although advanced molecular techniques exist, culture-based methods remain effective and cost-efficient for AMR surveillance, especially in resource-limited settings. The Tricycle protocol promotes their use, demonstrating that conventional methods can provide valuable data for monitoring antimicrobial resistance.

A biannual antibiotic resistance surveillance is proposed, with reviews during warmer (summer) and colder (winter) periods. It is also crucial to consider natural phenomena such as El Niño and the impact of global warming in these analyses. This should be reflected in public policies to improve wastewater management and evaluate technologies to reduce the load of resistant bacteria released into the environment.

Finally, it is recommended to conduct additional studies incorporating advanced microbial identification methods, such as proteomics or genomics, in conjunction with biochemical methods, aiming to establish correlations between environmental factors such as temperature, hydraulic retention time, and cell retention time.

Declaration of competing interest

We declare that we have no significant competing interests including financial or non-financial, professional, or personal interests interfering with the full and objective presentation of the work described in this manuscript.

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Author contributions

- Isaac Yanqui Morales: Conceptualization, Funding Acquisition, Methodology, Project Administration, Writing – Original Draft Preparation, Writing.
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- Danitza DelCha: Methodology, Writing – Original Draft Preparation.
- Renzo Aguirre: Formal Analysis, Investigation, Data Curation.

Data availability statement

The data used in this study can be viewed at the following link https://drive.google.com/drive/folders/1xk1nItLtmU2Ie5SAmhzdRaQ4sr68R9R5?usp=sharing

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