SUMMARY OF PhD THESIS

Modulation of antibiotic susceptibility by environmental conditions in *E. coli* strains isolated from turkeys

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Antibiotic resistance is a global problem with significant implications for human and animal health (WHO, 2014). Studies in this field are crucial for understanding and combating this issue. Antibiotic-resistant bacteria can become pan-resistant, posing a major threat to global health.

National surveillance programs have been implemented in many European countries, but harmonization is necessary for data comparison (DE JONG, 2013). Researchers are trying to understand the origin and evolution of antibiotic resistance at the epidemiological, genotypic, genomic, and phenotypic levels (BELLO-LÓPEZ, 2019). Antibiotic resistance mechanisms have evolved and become integral components of basic bacterial physiology, leading to more widespread resistance (JAYARAMAN, 2009).

pH changes can influence antibiotic resistance, but the exact mechanisms are not fully understood (CULEBRAS, 1996). Moreover, bacteria have developed resistance not only to antibiotics but also to heavy metals, and this phenomenon can be influenced by agricultural and medical practices (HOBMAN, 2015; ARGUDÍN, 2018).

By gaining a deeper understanding of the interactions between environmental factors such as pH and heavy metals and the development of bacterial resistance to antibiotics, there is a possibility of developing new approaches to control antibiotic resistance (DE JONG, 2013; BELLO-LÓPEZ, 2019; JAYARAMAN, 2009; CULEBRAS, 1996; HOBMAN, 2015; DAS, 2016; ARGUDÍN, 2018). Studies in this field contribute significantly to the development of effective strategies to combat this global threat to public and animal health.

The doctoral thesis entitled "Modulation of antibiotic susceptibility through environmental conditions in *E. coli* strains isolated from turkeys" is structured into two parts, each containing a series of chapters, designed according to current norms.

Part I "Current State of Knowledge" is divided into 3 chapters and provides a concise description of the information available in the specialized literature regarding the taxonomy and importance of *Escherichia coli* bacteria, antibiotics and antibiotic resistance, as well as their known interactions with environmental factors.

The **first chapter** briefly describes aspects related to the taxonomy, ecology, isolation, identification, pathogenicity, and importance of *Escherichia coli* bacteria, highlighting its variability and versatility as a representative study model.

Chapter two is a review of fundamental aspects related to antibiotics and antibiotic resistance. This chapter presents the mechanisms of action of different classes of antibiotics and summarizes the mechanisms of antibiotic resistance, along with the transmission of antibiotic resistance and the influence of environmental factors on it.

In the **third chapter**, information available in the specialized literature regarding the influence of environmental factors on *E. coli* is presented, with a focus on the impact of pH and the heavy metals cadmium and lead.

Part II presents the personal contribution and is structured into 6 chapters containing the results of the author's own research conducted according to the purpose and objectives of the work, which are presented in **chapter 4**.

Chapter 5 describes the origin of the samples, the isolation and identification protocol of the *E. coli* strains under study, the methodology for evaluating susceptibility to antibiotics, as well as the stages of selecting the antibiotics for study,

selecting the strains for study, and the results of the initial antibiotic susceptibility tests of the strains studied.

In the study, the antibiotic resistance profiles of a total of 48 strains of *Escherichia coli*, isolated from 67 individuals, were analyzed. To include representative strains with and without antibiotic resistance, pathogenic and non-pathogenic strains, from various age categories and different growth systems, the final selection included 19 wild strains (labeled Pr1 to Pr19), along with the reference strain *E. coli* ATCC25922 (labeled Pr20).

To isolate and identify the study strains from the samples collected from turkeys, MacConkey agar was used for isolation. The presumptive colonies of enterobacteria obtained were subsequently transferred to Nutrient Agar and confirmed through oxidase, indole, and beta-glucuronidase tests using TBX agar.

For the initial antibiotic susceptibility evaluation of *Escherichia coli* strains, the disc diffusion method with antimicrobial-impregnated disks (Kirby-Bauer antibiogram) was employed, following the recommendations provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Susceptibility testing considered antibiotics commonly used in veterinary and human medicine to ensure relevance and applicability/impact in current practice. Two sets of antimicrobials were selected: one for veterinary use (T30 oxytetracycline, AX25 amoxicillin, FFC30 florfenicol, SXT25 sulfamethoxazole + trimethoprim, CT10 colistin, ENR5 enrofloxacin) and one for human use (DO30 doxycycline, CTX30 cefotaxime, CTC40 cefotaxime + clavulanic acid, AZM15 azithromycin, CN30 gentamicin, CIP5 ciprofloxacin).

In the human-use set, an integrated system was included to determine if a strain was phenotypically positive for extended-spectrum β-lactamase (ESBL) production. A specific criterion was applied following the EUCAST guideline, stating that the zone of inhibition diameter generated by Cefotaxime + Clavulanic Acid combination must be at least 5mm greater than the zone of inhibition produced by Cefotaxime alone (GISKE, 2017).

In-depth analysis of the initial antibiogram results for the 20 selected *Escherichia coli* strains offered a detailed perspective on their antimicrobial resistance. Multiple resistances were observed for strains Pr1 (CIP5, ENR5, AX25), Pr2 (ENR5), Pr3 (CIP5, ENR5, AX25), Pr4 (CIP5, ENR5, AX25), Pr5 (CIP5, ENR5), Pr6 (CTC40, DO30, T30, ENR5), Pr7 (AX25, ENR5), Pr8 (CIP5, T30, ENR5, AX25), Pr9 (CIP5, DO30, T30, ENR5, AX25), and Pr10 (CTC40). Analysis of the initial antibiotic susceptibility testing results for the first 10 *Escherichia coli* isolates (Pr1-Pr10) revealed a variety of resistance cases. Notably, a high rate of resistance to fluoroquinolones and amoxicillin was observed, indicating that the isolated wild strains had been previously exposed to these substances and adapted by developing general and specific resistance mechanisms. Moreover, strains with multidrug resistance were identified, according to the definition proposed by Magiorakos et al. (2012), referring to "non-susceptibility acquired to at least one antimicrobial agent in three or more antimicrobial categories." Strains Pr6, Pr8, and Pr9 met these criteria and fall into this category, highlighting a high level of resistance to multiple classes of antibiotics. The integrated ESBL detection system did not identify any phenotypically positive strains for extended-spectrum beta-lactamase production.

In the analysis of the initial antibiotic susceptibility testing results for the last 10 *Escherichia coli* strains (Pr11-Pr20), a significant multitude of resistance cases were observed: Pr11 (CIP5, ENR5), Pr12 (AZM15, DO30, CIP5, T30, ENR5, SXT25, AX25), Pr13 (DO30, CIP5, T30, ENR5, SXT25, AX25), Pr14 (without resistance), Pr15 (CTX30, CIP5, T30, AX25, ENR5, FFC30, SXT25), Pr16 (DO30, CIP5, T30, ENR5, FFC30, AX25), Pr17 (CTC40, DO30, CIP5, T30, AX25, ENR5, FFC30, SXT25), Pr18 (DO30, CIP5, T30, ENR5), Pr19 (CTC40, DO30, CIP5, AX25, T30, ENR5), and Pr20 (without resistance).

Particularly, a high rate of resistance to representatives of tetracycline, β-lactam penicillins, fluoroquinolones, and potentiated sulfamides was highlighted. Strains with multidrug resistance were identified, being non-susceptible to 7 out of the 12 tested antibiotics, belonging to four different antibiotic classes. Strains Pr12, Pr13, Pr15, Pr16, Pr17, and Pr19 fall into this category, revealing a high level of resistance to multiple classes of antibiotics. This denotes the presence of complex resistance mechanisms that enable strains to survive and adapt to the pressure exerted by the various antibiotics used in the study.

Overall, the strains considered non-pathogenic (Pr9-Pr19) presented a higher number of antibiotic resistance cases compared to strains isolated from individuals with disease symptoms (Pr1-Pr8). The total percentage of antibiotic resistance cases in strains isolated from diseased contexts accounted for 10.78% of the total, while strains isolated from clinically healthy individuals represented 26.47%, essentially being twice that of those considered pathogenic.

Chapter 6 presents the experiment evaluating the evolution of antibiotic susceptibility under conditions of pH modifications. This chapter includes the conception and description of the experimental protocol as materials and methods, followed by the results and discussions, from which conclusions were drawn for the study of pH influence based on the analysis of the 1440 total tests performed.

We established two time intervals (3 and 7 days) for bacterial exposure to different pH levels and selected three distinct pH values (4.5, 6.0, and 8.5) to examine their impact on bacterial resistance. A pre-study was conducted to develop a reproducible and precise protocol for the experiment.

The experimental protocol involved obtaining an acidic culture medium using a commercial acidifier with specific chemical composition. The liquid Lennox LB culture medium was sterilized, and the pH was adjusted to two different values (pH 4.5 and pH 6.0). Additionally, an alkaline culture medium with pH 8.5 was prepared using a NaOH solution. *Escherichia coli* strains were inoculated in the culture media with the three pH values. After incubation for three days, the sediment was transferred to new tubes with the same pH and continued incubation. Isolated colonies were obtained from the initial tubes and tested for antibiotic susceptibility after three days of exposure to the modified pH environment. The selected antibiotics were tested using the same protocol as in the initial susceptibility test. On the seventh day, the isolated colonies obtained from the tubes exposed to pH variations were re-inoculated on MH agar plates for each pH and evaluated for antibiotic susceptibility as before.

Before analyzing the effects of pH modifications on *Escherichia coli* strain antibiotic resistance, blank controls were evaluated. This assessment involved subjecting two strains, Pr20 (*E. coli* ATCC25922) and Pr13, to the same experimental

conditions in duplicate, but without adjusting the pH of the Lennox LB culture medium, and were cultured for 3 and 7 days, respectively. To assess the significance of the differences in results, in the absence of available studies providing a reference point at the current time, we proceeded as follows: we identified the largest absolute difference between the initial antibiogram results and the blank control results. As such, any absolute difference value greater than this reference point is considered significant and attributed to the studied environmental factor. For the study of antibiotic susceptibility evolution under conditions of pH modifications, the reference point was determined to be 3.26 mm.

In the next stage of the study, we conducted tests under different pH conditions for antibiotic resistance, using pH levels of 4.5, 6.0, and 8.5. We recorded and analyzed the results obtained from these tests and compared them to the initial results of the antibiotic susceptibility tests. We analyzed whether there were significant differences in antibiotic resistance depending on the pH used, the tested antibiotic, and the exposure time of *E. coli* strains.

The minimum value was -5.50 mm compared to the initial zone of inhibition diameter, observed in strain Pr16 with the antibiotic azithromycin, pH 4.5, and 3 days of exposure. The maximum value was +6.16 mm compared to the initial zone of inhibition diameter, observed in strain Pr10 with the antibiotic cefotaxime, pH 8.5, and 7 days of exposure.

The summarized interpretation of the obtained results is presented synthetically in tables 1-4 using the notations: Total Number of Differences (ND), Total Number of Significant Differences (NS), Total Percentage of Significant Differences (%S), Maximum (M), Minimum (m), Absolute Reference Value (VR), Percentage of Significant Negative Differences (%-), Percentage of Significant Positive Differences (%+), Total Number of Significant Negative Differences (NS-), Total Number of Significant Positive Differences (NS+).

Table 1. Overview of the study results on pH influence

ND	VR	NS	%S	M	m	%-	%+	NS-	NS+
1440	3,26 mm	105	7,29%	+6,16 mm	-5,50 mm	4,38 %	2,92%	63	42

Table 2. Interpretation of results based on pH

pH 4,5		pH 6,0		pH 8,5	
m -5.50 mm	M +5.69 mm	m -4.61 mm	M +5.43 mm	m -4.55 mm	M +6.16 mm
NS 33	%S 2.29%	NS 41	%S 2.85	NS 31	%S 2.15
%- 1.60%	%+ 0.69%	%- 1.67%	%+ 1.18%	%- 1.11%	%+ 1.04%
NS- 23	NS+ 10	NS- 24	NS+ 17	NS- 16	NS+ 15

Table 3. Interpretation of results based on the antibiotic

AZM15	CN30	CTX30	CTC40	DO30	CIP5	T30	AX25	FFC30	SXT25	CT10	ENR5
M	M	M	M	M	M	M	M	M	M	M	M
+5.69	+4.08	+6.16	+4.46	+4.07	+6.05	+4.32	+4.20	+4.73	+3.53	+2.57	+3.97
m	m	m	m	m	m	m	m	m	m	m	m
-5.50	-5.07	-4.60	-4.61	-3.47	-4.16	-3.39	-4.55	-4.40	-4.25	-2.66	-4.40
%S	%S	%S	%S	%S	%S	%S	%S	%S	%S	%S	%S
1.88	0.49	0.97	0.69	0.28	0.69	0.35	0.63	0.63	0.35	0.00	0.35
NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
27	7	14	10	4	10	5	9	9	5	0	5
%+	%+	%+	%+	%+	%+	%+	%+	%+	%+	%+	%+
0.90	0.21	0.21	0.21	0.07	0.21	0.28	0.21	0.21	0.21	0.00	0.21
%-	%-	%-	%-	%-	%-	%-	%-	%-	%-	%-	%-
0.97	0.28	0.76	0.49	0.21	0.49	0.07	0.42	0.42	0.14	0.00	0.14
NS+	NS+	NS+	NS+	NS+	NS+	NS+	NS+	NS+	NS+	NS+	NS+
13	3	3	3	1	3	4	3	3	3	0	3
NS-	NS-	NS-	NS-	NS-	NS-	NS-	NS-	NS-	NS-	NS-	NS-
14	4	11	7	3	7	1	6	6	2	0	2

Table 4. Interpretation of results based on exposure time

3 days				7 days			
%-	%+	m	M	%-	%+	m	M
1.88	1.39	-5.50	+4.46	2.50	1.53	-5.07	+6.16
NS-	NS +	NS	%S	NS -	NS +	NS	%S
27	20	47	3.26	36	22	58	4.03

The conclusions for the study results are as follows:

Data analysis was performed using appropriate statistical methods to assess the significance and correlations between the analyzed variables: the pH used, the tested antibiotic, and the exposure time of *E. coli* strains.

In total, 1440 tests (inhibition zones) were conducted for all strains, antibiotics, pH levels, and exposure times.

Exposing *E. coli* to different pH values demonstrated an overall trend of reducing susceptibility to the evaluated antibiotics rather than increasing it. Lower pH values appeared to increase resistance, while higher pH values increased sensitivity. These changes also depend on the specific antibiotic tested.

The exposure time to pH had a crucial role, with the longest interval resulting in the highest number of changes in sensitivity/resistance.

The number of variations in resistance/sensitivity to antibiotics among all strains and tested antibiotics is also influenced by pH. Acidic conditions predominantly induced reductions, with the greatest number of significant decrease occurring at moderate acidity. The most drastic reduction was induced by the lowest pH value, while the highest pH showed the most significant increase in susceptibility. However, the number of variations in either positive or negative directions is approximately equal for the alcaline pH.

Analyzing the results based on the tested antibiotics revealed that azithromycin recorded the most significant differences. Other antibiotics such as gentamicin, cefotaxime, cefotaxime with clavulanic acid, doxycycline, oxytetracycline, amoxicillin,

florfenicol, sulfamethoxazole with trimethoprim, colistin, enrofloxacin, and ciprofloxacin also exhibited significant differences in the response of *E. coli* strains to different exposure conditions.

In conclusion, the study demonstrated that the pH used, the tested antibiotic, and the exposure time are important factors influencing the susceptibility of *E. coli* strains to antibiotics. A detailed analysis of these factors can aid in understanding and appropriately addressing antibiotic resistance, potentially supporting the development of innovative strategies to combat bacterial resistance at a global level.

Following the same pattern as in the previous chapter, **Chapter 7** was structured, presenting the second experiment evaluating the evolution of antibiotic susceptibility in the presence of heavy metals, the conclusions of which were drawn after analyzing a total of 1920 tests.

The experimental protocol was broadly designed following a similar structure to the previous study with pH variations. However, this time, the investigated environmental factor was the presence of heavy metals cadmium and lead. Concentrations of 5 µg/L and 50 µg/L of heavy metals were selected. This choice was motivated by several important considerations. The selected concentrations align with values typically found in certain areas, such as the Danube Delta, where heavy metals are present in water and sediment. Additionally, these chosen concentrations comply with legislatively regulated reference values for surface water, according to ORDIN 161/2006. Moreover, the selection of these concentrations is justified as they do not significantly influence the pH value since the solution in which heavy metals are suspended contains nitric acid. The experiment's implementation followed the protocol previously described in the study of the influence of pH on antibiotic resistance. The *E. coli* strains (Pr1-Pr20) were exposed to the environmental factors (cadmium and lead at concentrations of 5 and 50 µg/L) for 3 and 7 days, respectively, and antibiotic susceptibility was evaluated following the steps described in the previous study.

Before evaluating the impact of heavy metals on antibiotic resistance in *Escherichia coli* strains, control tests were performed using two strains in duplicate, Pr13 and Pr20 (*E. coli* ATCC25922). These strains were subjected to the same experimental conditions, except that the Lennox LB culture medium was supplemented only with the excipients of the heavy metal suspension, without containing the metals themselves. Subsequently, these strains were cultivated for 3 and 7 days.

In the absence of available studies providing a reference point to evaluate the significance of differences between results, the following approach was adopted: the largest absolute difference between the initial antibiotic susceptibility results and those of the blank controls was identified. This difference was considered the reference point for further interpretation of the differences generated by exposure to heavy metals. Thus, any absolute value of the difference greater than this reference point was considered significant and attributed to the investigated environmental factor.

For the study of the evolution of antibiotic susceptibility in the presence of heavy metals, the reference point was determined to be 3.43 mm.

In the next stage of the study, tests were conducted under different conditions of heavy metal presence to assess resistance to antibiotics, using cadmium and lead at concentrations of 5 and 50 µg/L. The results obtained from these tests were recorded and analyzed, then compared with the initial results of antibiotic susceptibility tests. The results were analyzed based on the metal used, its concentration, the tested antibiotic, and the exposure time. Variations and trends in antibiotic resistance were observed and recorded based on these criteria.

Table 8. Overview of the results from the study on the influence of heavy metals

ND	VR	NS	%S	M	m	%-	%+	NS-	NS+
1920	3.43mm	146	7.60%	+6.55 mm	-6.12 mm	0.52 %	7.08 %	10	136

Table 9. Interpretation of results based on the metal

Cadmium				Lead			
NS 62	%S 3.23	M +6.55	m -6.12	NS 84	%S 4.38	M +6.40	m -4.08
NS + 56	%+ 2.92	NS - 6	%- 0.31	NS + 80	%+ 4.17	NS - 4	%- 0.21

Table 10. Interpretation of results based on concentration

5 µg/L				50 µg/L			
NS 70	%S 3.65	M +6.40	m -4.77	NS 76	%S 3.96	M +6.55	m -6.12
NS + 67	%+ 3.49	NS - 3	%- 0.16	NS + 69	%+ 3.59	NS - 7	%- 0.36

Table 11. Interpretation of results based on exposure time

3 days				7 days			
%- 0.16	%+ 3.18	m -6.12	M +5.98	%- 0.36	%+ 3.91	m -5.83	M +6.55
NS- 3	NS + 61	NS 64	%S 3.33	NS - 7	NS + 75	NS 82	%S 4.07

Our results revealed the impact of heavy metals (cadmium and lead) at different concentrations on the susceptibility of *E. coli* strains to antibiotics, depending on the exposure time.

Following all the conducted tests, it was observed that heavy metals, regardless of type, concentration, or exposure time, increase the susceptibility to all tested antibiotics. The differences between the two metals favor lead, as it shows a greater number of values indicating increased sensitivity.

The concentration of heavy metals applied has a less pronounced influence on sensitivity, as both concentrations led to a remarkable increase in sensitivity.

Regarding the tested antibiotics, significant differences in susceptibility of *E. coli* strains to antibiotics were observed. Cefotaxime in combination with clavulanic acid generated the most significant differences, followed by oxytetracycline, amoxicillin, and sulfamethoxazole combined with trimethoprim.

The exposure time to heavy metals is directly correlated with the obtained effect, as sensitivity increased after 7 days of exposure.

In conclusion, the results highlight how heavy metals influence the susceptibility of *E. coli* strains to antibiotics, depending on the concentration and exposure time. These observations contribute to a better understanding of the complex interaction between environmental factors and bacterial susceptibility to antibiotic treatment.

The general conclusions drawn from the study "Modulation of antibiotic susceptibility by environmental conditions in *E. coli* strains isolated from turkeys" are formulated and presented in **Chapter 8**.

The comparative analysis of the results obtained in the pH and heavy metal studies revealed significant differences in the susceptibility of *Escherichia coli* strains to antibiotics depending on the investigated environmental factor.

In the pH study, it was observed that altering the pH of the Lennox LB culture medium had a significant impact on the antibiotic resistance of *E. coli* strains. The pH of the medium influenced the bacterial susceptibility to antibiotic treatment, with variations observed depending on the tested antibiotic, exposure time, and pH used.

At pH 4.5, the most pronounced changes in the negative direction (decreased susceptibility) were recorded, while at pH 8.5, positive changes (increased antibiotic susceptibility) were observed. At pH 6.0, the most significant changes were predominantly negative, but with a lower intensity than at pH 4.5 and 8.5.

Regarding the tested antibiotics, azithromycin showed the highest variation in inhibition zones, with significant changes in both positive and negative directions. The aminoglycoside gentamicin and the cephalosporin cefotaxime also generated significant differences in antibiotic susceptibility. In contrast, colistin did not show significant changes, indicating stability in susceptibility to this antibiotic under the tested pH conditions.

The antibiotics cefotaxime + clavulanic acid, oxytetracycline, amoxicillin, and sulfamethoxazole combined with trimethoprim presented the most significant differences in antibiotic susceptibility in the presence of heavy metals.

The duration of exposure to different pH levels also had an impact on antibiotic resistance. Exposure for 7 days resulted in the most significant changes, with notable increases in susceptibility, while the greatest decreases were observed at the 3-day exposure time.

In the study of heavy metals, the presence of cadmium and lead in the culture medium led to significant modifications in antibiotic susceptibility. These changes were influenced by the concentration of heavy metals and the exposure time.

The concentration of 50 μg/L caused the most significant differences, with a predominance of increases in the inhibition zone diameter. However, even at a concentration of 5 μg/L, significant changes in antibiotic susceptibility were observed.

Comparing the two studies, it can be observed that both pH and heavy metals have a significant impact on the susceptibility of *E. coli* strains to antibiotics. The obtained results demonstrate that environmental factors can influence how bacteria respond to antibiotic treatment, underscoring the importance of considering environmental factors in antibiotic resistance research.

In conclusion, this study has provided new insights into how pH and the presence of heavy metals affect the antibiotic susceptibility of *Escherichia coli* strains. The findings contribute to a better understanding of the complex interaction between environmental factors and antibiotic resistance and have practical applications in

managing issues related to the antibiotic crisis in the medical field and agri-food industry.

Chapter 9 emphasizes the originality and innovative contributions of the thesis:

It is a study that fills the gap of lacking similar studies or models in investigating the influence of pH and the presence of heavy metals in the living environment of enterobacteria for assessing antibiotic resistance dynamics.

It is a comprehensive study that scientifically investigates and compares the antibiotic resistance/susceptibility of pathogenic versus non-pathogenic *E. coli* in turkeys raised in an industrial versus household setting, of different age categories, and exposed to different prevention protocols.

The practical approach to antibiotic resistance/susceptibility in a rent species, which also serves as a food source, covers the identification and information regarding a wide range of potential sources of environmental and human contamination with plasmids encoding antibiotic resistance.

The experimental approach and the results obtained in the study allow the investigation of the impact of *E. coli*'s living environment on the development and spread of bacterial resistance in diverse contexts and categories of microbial populations.

The chosen influencing factors to be investigated, pH of the environment, and the presence of heavy metals, are important not only for *E. coli*, as the results can be extrapolated to other *Enterobacteriaceae*, but also for the host organisms, supporting the development of adapted and suitable measures for the three sides of the epidemiological triangle: infectious agent, susceptible host, and surrounding environment.

The study's model is innovative, tailored to experimental needs with standardized interpretation boundaries for both pH variations and heavy metal intervention, depending on their nature and experimental dose. Creating a specific reference point for the subsequent interpretation of differences generated by exposure to pH or heavy metals facilitates the processing of an impressive volume of data.

The detailed analysis of these factors can aid in understanding and addressing antibiotic resistance correctly, having the potential to support the development of innovative strategies for combating bacterial resistance at a global level.

The thesis is the first study that simultaneously provides information on the relevance of pH and heavy metals in conditioning the antibiotic resistance/susceptibility of *E. coli*, prioritizing and demonstrating the more pronounced impact of heavy metals, depending on their concentration and exposure duration.