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# *Pheno-genotypic resistance in bacteria from freshwater*

Brazil is a privileged country in terms of its natural resources, and among these, water resources play a significant ecological, economic, strategic, and social role. Despite this high potential, anthropogenic action such as the discharge of untreated effluents into rivers, lakes, and streams have negatively influenced water quality, causing a serious imbalance in the aquatic ecosystem. It is important to note that in Brazil, there are no public policies related to improper disposal of waste in the aquatic environment, and this factor contributes to its pollution. Among the common pollutants, it is possible to highlight antimicrobial drugs, as several studies have pointed out the problem of increasing microbial resistance worldwide, including in various environments, including aquatic ones. Based on this and considering that the bacterial microbiota found in live fish is directly related to the microbiota of the environment, this study aimed to evaluate the diversity and antimicrobial resistance profile of bacteria isolated from fish from the Guandu River, Seropédica-RJ. Fish from the Guandu River were collected and identified. Subsequently, necropsy was performed on these fish, and swab samples were collected from their intestines. Bacterial isolation, biochemical and proteomic identification of isolates, phenotypic detection of antimicrobial resistance, as well as extraction of bacterial DNA and amplification of resistance genes, were performed using these samples. A total of 30 fish were collected and identified, with Pimelodus maculatus, Geophagus brasiliensis, and Oligoplites saliens being the most common species. From the intestinal material of these fish, 32 bacterial strains were isolated, distributed among 11 species. Escherichia coli was the bacterial species with the highest incidence. The bacteria showed resistance to gentamicin, sulfazothrim, ampicillin, cefotaxime, ceftazidime, and amoxicillin with clavulanate. After analysis, none of them showed a phenotypic profile for β-lactamase production, but the genes blaSHV, blaCTX-M, qnrS, and mcr-1 were detected in the bacteria. These results highlight the urgent need to create public policies for constant monitoring of aquatic environments, as there are bacteria resistant to a large number of antimicrobials tested in fish from the Guandu River, as well as the presence of resistance genes, indicating the possibility of resistance dissemination throughout the ecosystem. Thus, the present study demonstrates extreme relevance in the field of One Health, alerting to the importance of observing all environments in an interconnected way.

**Keywords:** Microbiology; Bacterial resistance; Antibiotics; One health.

# *Resistência fenogenotípica em bactérias de água doce*

O Brasil é um país privilegiado com relação aos seus recursos naturais e, entre estes, os recursos hídricos têm relevante papel ecológico, econômico, estratégico e social. Apesar desse elevado potencial, ações antrópicas como lançamento de efluentes não tratados em rios, lagos e córregos, têm influenciado de forma negativa a qualidade das águas, ocasionando um sério desequilíbrio no ecossistema aquático. É importante ressaltar que no Brasil não existem políticas públicas relacionadas ao descarte inapropriado de dejetos no ambiente aquático e esse fator colabora para a sua poluição. Dentre os poluentes comuns, é possível destacar os fármacos antimicrobianos, visto que diversos estudos têm apontado para a problemática do aumento da resistência microbiana em todo o mundo e, em vários ambientes, inclusive o aquático. Baseado nisso e levando em consideração que a microbiota bacteriana encontrada nos peixes vivos está diretamente relacionada à microbiota do ambiente, este trabalho teve como objetivo avaliar a diversidade e o perfil de resistência a antimicrobianos de bactérias isoladas de peixes provenientes do Rio Guandu, Seropédica-RJ. Foram coletados e identificados peixes do Rio Guandu. Logo após, foi realizada a necropsia desses animais e amostras de SWAB foram coletadas de seu intestino. Em seguida, foi realizado o isolamento bacteriano e a identificação bioquímica e proteômica dos isolados. Para formulação do perfil de resistência, foi feita a detecção fenotípica aos antimicrobianos e a nível genômico foi realizada a extração do DNA bacteriano e amplificação de genes de resistência. Um total de 30 peixes foram coletados e identificados, sendo Pimelodus maculatus, Geophagus brasiliensis e Oligoplites saliens as espécies de maior ocorrência. A partir do material intestinal destes peixes, foram isoladas 32 cepas bacterianas, distribuídas em 11 espécies. Escherichia coli foi a espécie bacteriana que apresentou maior incidência. As bactérias apresentaram resistência a gentamicina, sulfazotrim, ampicilina, cefotaxima, ceftazidima e amoxicilina com clavulanato. Após análise, nenhuma obteve perfil fenotípico para produção de β-lactamases, mas foram detectados os genes blaSHV, blaCTX-M, qnrS e mcr-1 nas bactérias. Tais resultados evidenciam a urgência na criação de políticas públicas para um monitoramento constante dos ambientes aquáticos, visto que existem bactérias resistentes a grande parte dos antimicrobianos testados nos peixes do Rio Guandu além, da presença de genes de resistência, indicando a possibilidade de disseminação de resistência em todo ecossistema. Assim, o presente estudo demonstra extrema relevância no âmbito da saúde única, alertando sobre a importância de observar todos os ambientes de forma interligada.

**Palavras-chave:** Microbiologia; Resistência bacteriana; Antibióticos; Saúde única.

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**INTRODUCTION**

Brazil is a territory rich in natural resources, and among these, water resources play a significant ecological, economic, strategic, and social role (BICUDO et al., 2010). This country is the largest in South America and the fifth largest in the world in terms of territorial extent. With high hydrological potential, it boasts over eleven thousand rivers, streams, and creeks documented by the National Water Agency (RIBATEJO, 2015). Its vastness allows for a wide variety of climates, temperatures, ecosystems, and consequently, suitable environments for the commercial cultivation of many native fish species (SARTORI et al., 2012).

Despite its great potential, anthropogenic actions such as the discharge of untreated effluents into rivers, lakes, and streams have negatively influenced water quality, causing a serious imbalance in the aquatic ecosystem (REIS et al., 2022). It's important to note that in Brazil, there are no public policies related to the improper disposal of industrial waste into aquatic environments, contributing to pollution. Among the common pollutants, antimicrobial drugs can be highlighted, as several studies have pointed out the problem of increasing microbial resistance worldwide, including in various aquatic environments.

Antimicrobial resistance is directly related to the indiscriminate use of these drugs, as well as their improper disposal, prescribed as a prophylactic measure in agriculture, livestock, and even in human and veterinary medicine (HOLMES et al., 2015). This microbial resistance is a naturally conserved evolutionary phenomenon due to microorganisms' contact with antimicrobials, as bacteria, fungi, and viruses have interacted over thousands of years in the environment, which is essential for their existence and propagation (VERDI et al., 2016). However, the improper use of antimicrobial drugs has led to an increase in the spread of resistant bacteria, due to strong pressures on environmental bacterial communities and the dissemination of their resistance genes, as well as selective agents that move between different ecosystems and exert their effects (BARRIOS et al., 2015).

Anthropogenic actions can directly and indirectly influence antimicrobial resistance in river systems. According to Santana (2022), in addition to incorrect disposal, antibiotics enter aquatic environments through excretion in human and animal urine and/or feces, cadavers, manure, hospital sewage, pharmaceutical industry waste, and municipal effluents, as well as from livestock and fish farming industries, which likely constitute the main sources of antibiotic-resistant bacteria and genes being released into the environment. Previous studies have reported that antimicrobials can accumulate in soils and reach surface and groundwater through erosive processes such as leaching and runoff, increasing selective pressure in these environments. Once contaminated, water can be an important avenue for the dissemination of antimicrobial resistance through animal consumption, human consumption, and agricultural crop irrigation.

Once antimicrobials enter the environment, in their original form or as active metabolites, they can be transported and distributed in water, reaching groundwater and potentially causing adverse effects on fish, invertebrates, turtles, marine sediments, among others (ANDERSON et al., 2012). Thus, water not only serves as a means of disseminating antimicrobial-resistant organisms but also as the pathway through which

resistance genes are introduced into the natural bacterial ecosystem, altering the resistome, which represents a diverse set of known and unknown resistance determinants within an ecosystem (HEUER et al., 2011; KOZHEVIN et al., 2013; BLAIR et al., 2014).

It is worth noting that in aquaculture, antibiotics are directly released into surface waters. Fish, the final product of aquaculture, are a significant source of protein consumed in human diets. According to the Food and Agriculture Organization of the United Nations, fish consumption has been growing over the years at higher rates than other meats, such as beef and chicken, which are the most consumed in Brazil (FAO, 2018). Nationally, data from 2015 indicate that freshwater fish production is the main category within Brazilian aquaculture, accounting for 84% of the country's aquaculture production (IBGE, 2016).

The bacterial microbiota found in live fish is directly related to the microbiota of the environment. In other words, the microorganisms present on their body surface, gills, gastrointestinal tract, and muscles are linked to those in their environment (GUZMÁN et al., 2004; MELO, 2015). Currently, various studies emphasize the need to monitor the widespread dissemination of aquatic bacteria carrying antimicrobial resistance genes (GOGRY et al., 2019). Therefore, it is essential to track this dissemination in the aquatic environment as a whole, assessing everything from the water to the present fish, and establishing an epidemiological profile.

The general objective of the article is to evaluate the diversity and antimicrobial resistance profile of bacteria isolated from fish originating from the Guandu River, Seropédica-RJ. The specific objectives are: collect and identify fish from the Guandu River, isolate and identify intestinal bacteria from fish from the Guandu River; evaluate the resistance profile of fish bacteria to antimicrobials, detect the production of broad-spectrum beta-lactamases in isolated bactéria and assess the presence of antimicrobial resistance genes in bacteria from fish originating from the Guandu River.

Although microbial resistance is a natural evolutionary phenomenon, various anthropogenic actions, such as the inappropriate use and disposal of antimicrobials in human and animal medicine, can exacerbate this problem. Once antimicrobials enter the environment, either in their original form or as active metabolites, they can be transported and distributed in water bodies, reaching groundwater and increasing selective pressure in these environments. Once contaminated, water can become an important route for the dissemination of antimicrobial resistance through animal consumption, human water supply, and agricultural irrigation.

It is worth noting that fish are used daily in human consumption, and if they contain resistant bacteria, they can potentially contaminate the entire food chain. Although bacteria from their microbiota are not commonly associated with human infections, they can serve as reservoirs of resistance genes, which can contribute to the increased transfer of these genes to clinically relevant pathogenic microorganisms.

Therefore, environmental contamination, food contamination, and the occurrence of microbial resistance are reasons for concern, as they not only pose a risk to human health but also contribute to the spread of resistance genes in various environments. Thus, it is of paramount importance to monitor the presence and distribution of bacteria and resistance genes in aquatic environments—a problem that extends

not only to surface waters but also to groundwater, where multidrug-resistant bacteria have also been isolated. Furthermore, bacterial resistance is an inevitable process, and understanding its origins, evolution, and dissemination provides vital information for seeking alternatives to mitigate the effects of these drugs on bacterial populations in aquatic environments and their derivatives.

## MATERIALS AND METHODS

### Study Area

The Guandu River watershed is formed by the Guandu, da Guarda, and Guandu-Mirim rivers. The Guandu River, the focus of this study, spans approximately 80 kilometers and covers eight municipalities in the state of Rio de Janeiro, namely: Piraí, Paracambi, Itaguaí, Seropédica, Japeri, Queimados, Nova Iguaçu, and Rio de Janeiro (Figure 1) (INEA, 2012).


**Figure 1**: Map indicating the extent of the Guandu River.

The primary use of the Guandu River waters is for the treatment of drinking water, and it is treated at the largest water treatment plant (ETA) in the world. Concerning Rio de Janeiro, the Guandu River holds great significance as it, along with other rivers in the watershed, is responsible for supplying water to over 12 million people, constituting around 80% of the inhabitants of the Metropolitan Region. Additionally, it plays an important role for the local population, as there are many fishermen in the region. Despite the population not relying on artisanal fishing as their main nutritional source, it still has a significant impact on factors such as providing a livelihood for part of the riverside population (INEA, 2012).

Near the water treatment plant, the Guandu River receives water from the Abel River, Poços River, and Queimados River, as well as streams from the municipality of Seropédica that are polluted by domestic sewage, industrial effluents, and waste. This is a concerning issue because it contributes to a portion of the population in Rio de Janeiro receiving water contaminated by these pollutants from this source (INEA, 2012).

**Fish Collection**

Fish were collected in May and November 2019 from the Guandu River, located in the municipality of Seropédica, Rio de Janeiro. A total of 30 fish were captured using casting nets and nets and immediately transported alive to the Fish Parasitology Laboratory, located in the Department of Parasitology at the Federal Rural University of Rio de Janeiro, where they were euthanized by spinal cord transection (Figure 2A). It is worth noting that the fish were caught by a local fisherman who sells these fish as a source of income. In the laboratory, a necropsy was performed, during which the gills and visceral cavity were exposed to remove intestinal material (Figure 2B). Additionally, swab samples were collected (Figure 2C) and immersed in 0.9% saline solution, and finally transported to the Veterinary Bacteriology Laboratory, where all microbiological analyses were conducted.

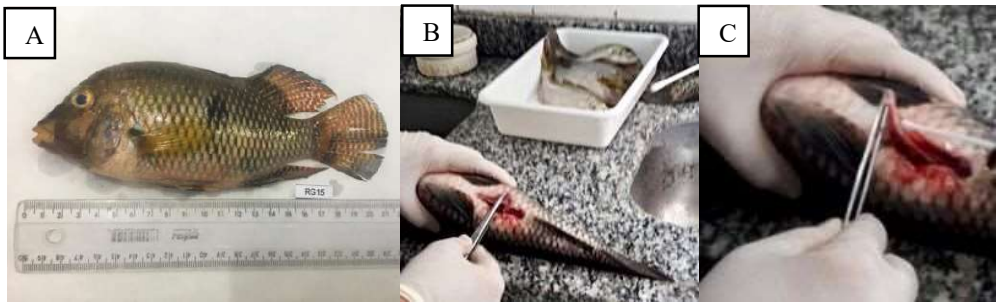


**Figure 2:** Sample Processing. Fish in the necropsy process (A). Removal of intestinal material (B). Swab sample collection (C).

**Isolation and Biochemical Identification**

An aliquot of the saline solution was streaked onto MacConkey agar (HiMedia®) for the isolation of Gram-negative bacteria, and the plates were subsequently incubated at 37°C for 24 hours. After this period, the presence and morphology of colonies were observed (Figure 3), followed by the Gram staining method to confirm Gram-negative bacteria (KONEMAN et al., 2012).

**Figura 3:** Bactérias isoladas no ágar MacConkey.

After Gram staining, the isolates underwent biochemical analyses, including motility in indole medium, citrate utilization, mixed acid and acetoin production, as well as sugar fermentation with gas production (KONEMAN et al., 2012).

**Identification by MALDI-TOF MS**

Following biochemical identification, all isolates underwent proteomic analysis using the Matrix-

Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) technique at the Laboratory for Medical Microbiology Investigation at the Paulo Góes Microbiology Institute of UFRJ.

For sample preparation, strains were cultured on BHI agar at 37°C for 24 hours. Each bacterial culture was transferred to a microplate (96 MSP, Bruker-Billerica, USA), and a lysis solution (70% formic acid, Sigma-Aldrich®) was added to cover the bacterial sediment. Then, 1 µL of matrix solution (alpha-cyano-4-hydroxycinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich®) was used to cover the bacterial extract before processing.

Spectra for each sample were generated using a mass spectrometer (MALDI-TOF LT Microflex Bruker, Bruker®) equipped with a nitrogen 337 nm laser in linear mode controlled by the Flex Control 3.3 program (Bruker®). The spectra were collected in the mass range of 2,000-20,000 m/s and subsequently analyzed using the MALDI Biotyper 2.0 program (Bruker®) with standard settings for bacterial identification.

The MALDI Biotyper 2.0 program compares the spectra of the unknown sample with reference samples in the database and categorizes the results on a scale from zero to three (Table 1), where a higher value indicates a more reliable identification. Identifications with values ≥ 2,000 were considered acceptable.

**Table 1:** Information extracted from the Bruker Daltonik MALDI program describing the meaning of the values in relation to the score obtained in the analyzed sample.

| ***Score*** | **Identificação** | **Símbolo** | **Cor** |
|---|---|---|---|
| 2.300 – 3.000 | Highly probable species identification | ( +++) | Green |
| 2.000 – 2.299 | Secure genus and probable species identification | ( ++ ) | Green |
| 1.700 – 1.999 | Probable genus identification | ( + ) | Yellow |
| 0.000 – 1.699 | Unreliable identification | ( - ) | Red |

**Phenotypic Detection of Antimicrobial Resistance and Double Disc Synergy Test**

Phenotypic detection of resistance and the double disc synergy test for assessing the production of extended-spectrum beta-lactamases (ESBL) were carried out simultaneously on the same plate following the standards established by the Brazilian Committee on Antimicrobial Susceptibility Testing (BRCAST, 2017). An inoculum containing 1.5 x 108 cells/mL, adjusted according to the McFarland scale at a 0.5 dilution, was prepared. Subsequently, a disc diffusion assay was performed by evenly spreading the bacterial suspension (0.1 mL) with a swab in three directions across the surface of Mueller-Hinton agar plates, where antimicrobial discs were deposited. The plates were then incubated for 18 ± 2 hours at 35 ± 1°C.

The antimicrobials used were: Beta-lactams - amoxicillin with clavulanic acid (10/20µg), ampicillin (10µg), aztreonam (10µg), cefepime (10µg), cefotaxime (30µg), cefoxitin (30µg), and ceftazidime (10µg); Fluoroquinolone - ciprofloxacin (5µg); Tetracycline - tetracycline (30µg); Aminoglycoside - gentamicin (10µg); Sulfonamide - sulfamethoxazole + trimethoprim (25µg). Some Beta-lactam discs were strategically placed on the plate for interpretive reading to indicate the production of betalactamases. Therefore, an amoxicillin + clavulanic acid disc was placed in the center of the plate, surrounded by discs of third-generation cephalosporins (cefotaxime and ceftazidime), fourth-generation cephalosporin (cefepime), and aztreonam, all at a distance of 20 mm, center to center, as shown in Figure 4 in red. All other discs were randomly placed on the plate only to detect resistance, as illustrated in blue in Figure 4.

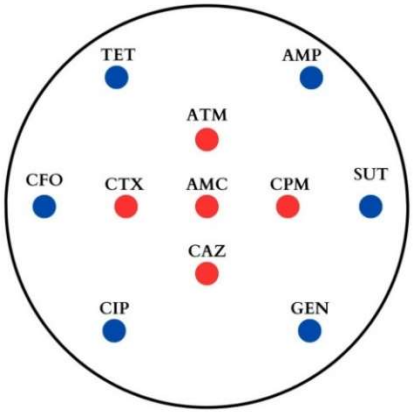


**Figure 4:** Positions of antimicrobial discs on the Mueller-Hinton agar plate for interpretative reading. Legend: AMC – amoxicillin with clavulanic acid, AMP – ampicillin, ATM – aztreonam, CPM – cefepime, CAZ – ceftazidime, CFO – cefoxitin, CTX – cefotaxime, CIP – ciprofloxacin, GEN – gentamicin, SUT – sulfamethoxazole + trimethoprim, TET – tetracycline.

After incubation, the reading was performed by evaluating the halo formed around each antimicrobial disc (Figure 5), i.e., the diameters formed around the disc were measured in millimeters using a ruler. The results were interpreted based on the cutoff points established by the Brazilian Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute (BRCAST, 2022; CLSI, 2020).

**Figure 5:** Halos formed after the incubation period of the disc diffusion assay.

For the synergy test, the reading considered a positive result for ESBL production when a "phantom zone" was formed, meaning when there was deformation of the inhibition halo of any cephalosporin disc towards the disc containing clavulanic acid (BRCAST, 2022).

## Genotypic Detection of Antimicrobial Resistance

### Bacterial DNA Extraction

The extraction of bacterial DNA was performed according to the protocol described by Féria et al. (2002). After extraction, the samples were stored at -20°C, and the quantity and quality of the obtained bacterial DNA were assessed using a spectrophotometer (Thermo Scientific). The total DNA integrity was evaluated by agarose gel electrophoresis (0.8%, ADKINS et al., 2007). The gel was visualized under 254 nm UV light, and images were captured using the L-PIX EX photodocumentation (Loccus Biotechnology).

**Amplification of antimicrobial resistance-related genes**

The amplification of the 16S rDNA gene and genes conferring resistance to beta-lactam antimicrobials (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX}$), quinolones (*qnr*S), and colistin (*mcr*-1) was performed using the Polymerase Chain Reaction (PCR) technique, employing the primers described in Table 2. PCR reactions were optimized using the following parameters: 1X Buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.4 µM of each primer, 1 U Taq DNA Polymerase, approximately 20 ng of total DNA, and ultrapure water to reach a total volume of 25 µL.

**Table 2:** Primers and cycling references used in gene amplification by PCR technique.

| Primer | Sequence | Fragment | Amplification condition |
|---|---|---|---|
| 27 F<br>1512 R | AGAGTTTGATCCTGGCTCAG ACGGCTACCTTG TTACGACT | 1500 pb | 94°C 5 min, 30x (94°C 1 min, 58°C 1 min e 72°C 1min), 72°C 10 min |
| *bla*$_{CTX-M}$ F<br>*bla*$_{CTX-M}$ R | AAAAATCACTGCGCCAGTTC<br>CCGTCGGTGACGATTTTAGCC | 862 pb | 94°C 5 min, 40x (94°C 1 min, 55°C 1 min e 72°C 1min), 72°C 5 min |
| *bla*$_{SHV}$ F<br>*bla*$_{SHV}$ R | TTTATCGGCCCTCACTCAAGG<br>GCTGCGGGCCGGATAACG | 931 pb | 94°C 5 min, 40x (94°C 1 min, 55°C 1 min e 72°C 1min), 72°C 5 min |
| *bla*$_{TEM}$ F<br>*bla*$_{TEM}$ R | ATGAGTATTCAACATTTCCGTG<br>TTACCAATG CTTAATCAGTGAG | 861 pb | 94°C 5 min, 40x (94°C 1 min, 55°C 1 min e 72°C 1min), 72°C 5 min |
| *qnr* F<br>*qnr* R | TCAGCAAGAGGATTTCTCA<br>GGCAGCACTATTACTCCCA | 627 pb | 30x (94°C 45s, 48°C 45s e 72°C 45s) |
| *mcr*-1 F<br>*mcr*-1 R | AAAGACGCGGTACAAGCAAC<br>GCTGAACATACACGGCACAG | 213 pb | 94°C 5 min, 30x (94°C 1 min, 55°C 1 min e 72°C 1min), 72°C 10 min |

It is worth noting that the amplification conditions for the 16S rDNA gene (KANE et al., 1993; SUZUKI et al., 1996), *bla*$_{TEM}$ (ESSACK et al., 2001), *bla*$_{SHV}$ (SHAHID et al., 2010), *bla*$_{CTX-M}$ (GESER et al., 2012), *qnr*S (WANG et al., 2004), and *mcr*-1 (LIU et al., 2015) were optimized as described in Table 2.

The PCR reaction products were visualized by electrophoresis on a 1.5% agarose gel containing SYBR Green dye (Invitrogen®). The gel was visualized under ultraviolet light using the L-PIX EX photographic documentation system (Loccus Biotecnologia). Positive controls for each gene and a blank, which contained only the PCR reagents without DNA, were used in all analyses.

**RESULTS AND DISCUSSION**

The collected fish were identified at the species level, including: *Cichla* spp. (3), *Crenicichla* spp. (1), *Geophagus brasiliensis* (5), *Metynnis* spp. (1), *Micropogonias furnieri* (1), *Oligoplites saliens* (5), *Oligosarcus hepsetus* (1), *Oreochromis niloticus* (2), and *Pimelodus maculatus* (11) (Figure 6). These species have been previously reported in the literature in studies from various regions of Brazil.

From the intestinal material of these fish, 32 bacterial strains were isolated, belonging to 11 species: *Aeromonas jandaei* (2), *Aeromonas hydrophila* (2), *Aeromonas veronii* (1), *Citrobacter freundii* (7), *Enterobacter* spp. (1), *Escherichia coli* (9), *Klebsiella pneumoniae* (2), *Klebsiella variicola* (1), *Plesiomonas shigelloides* (5), *Pseudomonas* spp. (1), and *Raoultella ornithinolytica* (1). These species have been previously reported in similar studies (COSTA et al., 2008; KIM et al., 2019). The correlation between the isolated

bacteria and the fish species from which they originated is indicated in Figure 7.

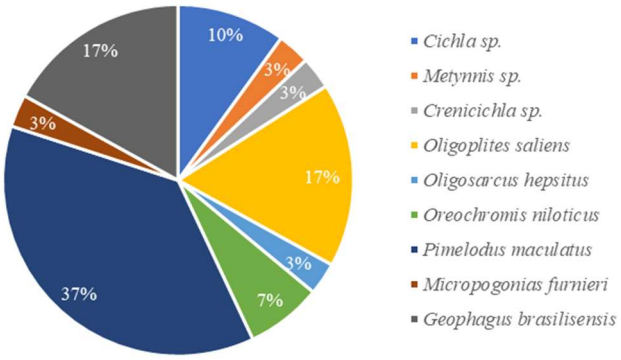


**Figure 6:** Percentage of fish quantity identified by species.

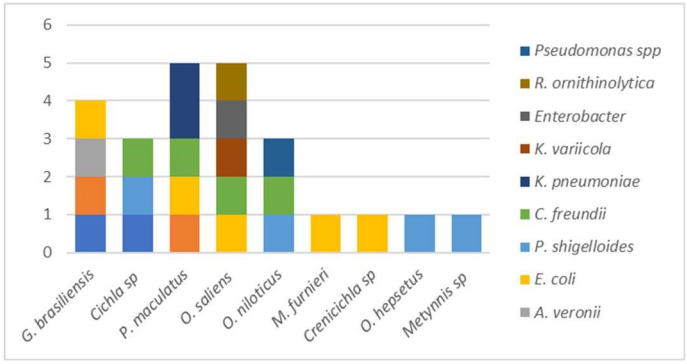


**Figure 7:** Number of bacterial strains isolated by fish species.

Other studies have also analyzed bacterial isolates from fish and detected similar species, such as *Aeromonas* spp., *Plesiomonas* spp., *Citrobacter freundii*, and *Escherichia coli* (COSTA et al., 2008; GUIMARAES et al., 2017; REIS JUNIOR et al., 2019).

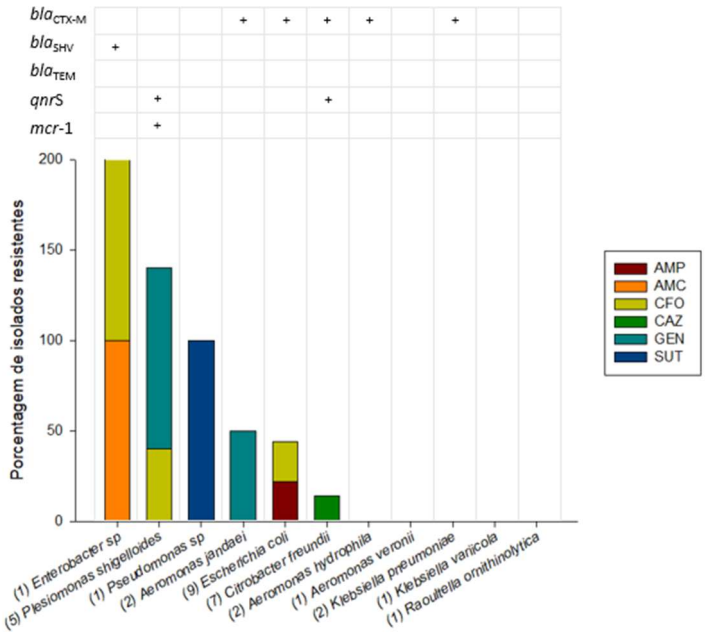


**Figure 8:** Genotypic and phenotypic resistance to antimicrobials of bacterial isolates. AMC – amoxicillin with clavulanic acid, AMP – ampicillin, CAZ – ceftazidime, CFO – cefoxitin, GEN – gentamicin, SUT – sulfamethoxazole-trimethoprim.

In addition to the bacterial diversity from the fish, another important aspect to consider is the potential antimicrobial resistance of the isolates. The bacteria showed resistance to the following

antimicrobials: gentamicin, sulfamethoxazole-trimethoprim, ampicillin, cefotaxime, ceftazidime, and amoxicillin with clavulanic acid (Figure 8). It is important to note that the species *Aeromonas jandaei*, *A. hydrophila*, *A. veronii*, and *Citrobacter freundii* have intrinsic resistance to ampicillin, cefoxitin, and ampicillin with clavulanic acid. *Klebsiella pneumoniae*, *K. variicola*, and *Raoultella ornithinolytica* have intrinsic resistance to ampicillin, and the species *Plesiomonas shigelloides* has intrinsic resistance to ampicillin and amoxicillin with clavulanic acid. Therefore, these antimicrobials were not tested for the mentioned bacterial species.

Six bacteria exhibited resistance to gentamicin, including all *Plesiomonas shigelloides* strains (5) and one *Aeromonas jandaei*, representing a total resistance rate of 18.75%. For the antimicrobial cefotaxime, 16.66% of the bacterial isolates showed resistance, including two *Escherichia coli* strains, one *Enterobacter* spp., and two *Plesiomonas shigelloides*. Ampicillin resistance was detected only in *Escherichia coli* strains, totaling 6.25%. Regarding the antimicrobials ceftazidime, sulfamethoxazole-trimethoprim, and amoxicillin with clavulanic acid, only one resistant strain was detected for each, which were *Citrobacter freundii*, *Pseudomonas* spp., and *Enterobacter* spp., respectively.

All bacterial strains showed sensitivity to the following antimicrobials: aztreonam, cefepime, cefotaxime, ciprofloxacin, and tetracycline. Therefore, in case of an infectious process caused by these bacteria, these drugs would be considered ideal for a higher chance of therapeutic success, as they would have an effect on the bacteria, leading to their death or growth inhibition.

None of the evaluated bacterial isolates showed a multidrug-resistant profile, meaning resistance to three or more classes of antimicrobials. Regarding the production of broad-spectrum beta-lactamases, none of the bacteria exhibited a characteristic phenotypic profile. However, the presence of resistance genes related to beta-lactamases was detected in genotypic tests.

The genes *bla*$_{SHV}$ and *bla*$_{CTX-M}$, as well as *qnr*S and *mcr*-1, were detected in the bacterial isolates (Figure 8). The only gene not detected in any of the isolates was *bla*$_{TEM}$, which confers resistance to beta-lactams. The *bla*$_{SHV}$ gene was only detected in the species *Enterobacter* spp. Regarding the *qnr*S gene, two isolates (6.25%) tested positive, these being *Plesiomonas shigelloides* and *Citrobacter freundii*.

The *mcr*-1 gene, which confers resistance to colistin, was detected in one isolate of *Plesiomonas shigelloides*. This finding is reported for the first time in this study, emphasizing the need for monitoring bacterial species from fish, as they have the ability to disseminate resistance genes in the environment and potentially generate opportunistic infections with therapeutic failure in both fish and humans.

**DISCUSSION**

From the species found in the Guandu River, we can highlight *Pimelodus maculatus*, which were found in the highest proportion in this study (37%), are reported to be distributed across various South American countries and are found in rivers ranging from the Amazon basin to the southernmost part of Brazil. There are records of this species in the Barbosa Lagoon and the Jacaré-Pepira River, both located in the state of São Paulo, and it has also been reported in the Guandu River, Rio de Janeiro (CASTRO et al., 2018; NEGRELLI

et al., 2021; VIEIRA et al., 2015). According to Negrelli et al. (2021), this fish species is considered opportunistic, has an omnivorous feeding habit, and adapts to a varied diet, consuming algae, insects, crustaceans, or mollusks (NEGRELLI et al., 2021).

The species *Geophagus brasiliensis* typically inhabit freshwater environments; however, fish of this species have been reported to tolerate high salinity levels in natural settings, and high relative abundance of these fish in such conditions has been reported in the southernmost part of Brazil (QUINTELA et al., 2019). On the other hand, fish of the genus *Cichla* spp., commonly known as "tucunarés," are endemic to South America. In Brazil, their natural distribution is limited to the Amazon region and the Tocantins-Araguaia river basin, which is situated in the ecotone between the Cerrado and Amazon biomes (KULLANDER et al., 2006; D'AVILLA et al., 2021).

It's worth noting that *Oreochromis niloticus*, *Cichla* spp., and *Metynnis* spp. are species commonly associated with environmental disturbances, which favor their introduction and establishment in different environments (D'AVILLA et al., 2021). This factor could potentially alter the fauna of the Guandu River. Therefore, further studies related to fish diversity in this river and monitoring of factors such as pollution, overfishing, introduced species, and aquaculture are necessary, as the Guandu River is responsible for supplying water to the Metropolitan region of the state of Rio de Janeiro.

Moreover, considering the high commercial potential of some of these fish, the possibility of inappropriate disposal of antimicrobials in this river becomes a concern, as it can lead to water contamination and, consequently, affect the animals present. The primary detrimental effect of this issue is the alteration of the fish's intestinal microbiota since antimicrobials can exert strong selective pressure, promoting the resistance of opportunistic bacteria and thereby contributing to the occurrence of infections caused by opportunistic bacteria (CASTAÑEDA et al., 2018).

According to the bacterial species found in the samples, it is possible to highlight species of the *Aeromonas* spp. genus are of significant relevance, as they are often associated with fish spoilage due to their ability to produce endotoxins, and they are also important for public health, as they have pathogenic potential for humans (SANTOS et al., 2019).

*Escherichia coli* was the most frequently detected species among the analyzed samples, all of which were derived from the intestines of fish from extractive fishing areas, intended for local consumption. The presence of this microorganism should raise concerns about the hygienic-sanitary aspect of fish, as thermotolerant coliforms like *E. coli* are considered indicators of microbiological contamination in food (DOI et al., 2015; VASCONCELOS, 2019). However, Resolution RDC No. 12, dated January 2, 2001, from the National Health Surveillance Agency (ANVISA), stipulates a maximum limit only for the presence of coagulase-positive *Staphylococcus* in fresh fish, which is up to 103 CFU/g, and the absence of *Salmonella* sp. in 25g (ANVISA, 2001). It is worth noting that in Brazil, infections and/or intoxications transmitted by contaminated water or food can become a significant public health issue (SOUSA, 2006).

The fact that none of the evaluated bacterial isolates showed a multidrug-resistant profile is of utmost importance because multidrug-resistant isolates pose a serious risk to public health by further

reducing therapeutic options in an infectious process (MAGIORAKOS et al., 2012; MIRYALA et al., 2021). In the United States and Europe, it is estimated that around 25,000 people die each year due to infections caused by multidrug-resistant microorganisms, while in China, this number can reach 100,000 (GLASS, 2017; O'NEILL et al., 2016). In addition to mortality, the impact on the cost to the healthcare system associated with reduced productive capacity can reach 1.5 billion euros per year worldwide (ANVISA, 2021).

It is important to emphasize that although most of the cataloged species may not have significant clinical relevance at the moment and did not exhibit multidrug resistance, various articles demonstrate the need for monitoring them in different environments, as they have profiles of emerging pathogens and are responsible for the dissemination of resistance genes throughout the aquatic environment.

For example, bacteria of the *Aeromonas* spp. genus, despite being typical in cases of septicemia in tropical fish (PELLIN et al., 2023), are currently considered emerging enteric pathogens associated with infections in the hepatobiliary system, respiratory tract, soft tissues, and septicemia in humans. In 2004, a diarrhea outbreak occurred in Brazil, with 2,170 cases, and bacteria of the *Aeromonas* spp. genus were isolated from 119 out of 145 analyzed samples. In 2012, in Brazil, bacteria of this genus were reported in 2.7% of diarrheal samples, with *A. caviae* being the most frequently obtained species, followed by *A. hydrophila* (PREDIGER et al., 2012). Furthermore, there have been reports of food poisoning outbreaks caused by the consumption of meat contaminated with A. hydrophila in the Asian kingdom of Bhutan (BALLEZA et al., 2018; TSHETEN et al., 2016).

Latin America has the highest prevalence of ESBL-producing strains, with countries such as Brazil, Cuba, Ecuador, and Venezuela being among them. In these countries, the *Klebsiella* genus is the most abundant in ESBL production reports, while in Guatemala, Mexico, and Peru, *Escherichia coli* is commonly reported (NOGUEIRA et al., 2015). As found in the present study, the most common type of ESBL is CTX-M, although other isolates have TEM and SHV types. This correlates with the results presented, as the $bla_{\text{CTX-M}}$ gene was detected in the highest proportion, being found in five bacterial isolates.

The $bla_{\text{SHV}}$ gene was only detected in the species *Enterobacter* spp. This species is associated with various types of infections, including nosocomial, urinary tract, respiratory, osteomyelitis, endocarditis, and soft tissue infections (MEDEIROS et al., 2023). Another relevant factor is that *Enterobacter* spp. can be found in both terrestrial and aquatic environments and has been isolated from foods such as cow's milk and meat (MEDEIROS et al., 2023).

Regarding the *qnr*S gene, two isolates (6.25%) tested positive, these being *Plesiomonas shigelloides* and *Citrobacter freundii*. The species *C. freundii* is a normal bacillus in the gastrointestinal tract microbiota and can mainly cause urinary tract infections (DARBY et al., 2014; PLAKKAL et al., 2013). In Chile, a case of neonatal sepsis caused by *Citrobacter freundii* was reported for the first time, originating from a cystic lymphatic malformation, demonstrating its increasing pathogenic potential (MARTIRANO et al., 2022).

Assessing the incidence of antimicrobial resistance in bacteria from fish used for human consumption is of utmost importance, as it can facilitate the transfer of resistance determinants to commensal and pathogenic bacteria of humans and animals, posing a concerning factor for public health. Therefore, the

importance of further studies regarding the dissemination of resistant bacteria involving aquatic environments and animals in a One Health approach is reinforced, as they are interconnected directly or indirectly with other animals, different environments, and humans.

## CONCLUSIONS

*Pimelodus maculatus*, *Geophagus brasiliensis*, and *Oligoplites saliens* are the fish species with the highest occurrence in the present study. Among these fish, *Escherichia coli* was the bacterial species with the highest incidence.

Some of the bacterial isolates exhibited antimicrobial resistance, and the genes $bla_{SHV}$, $bla_{CTX\text{-}M}$, *qnr*S, and *mcr*-1 were detected in the bacteria from the fish.

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