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Antibiotic resistance in the superficial mucosa microbiota in an Amazonian fish, mapará (*Hypophthalmus* spp.)

Resistência a antibióticos na microbiota do muco superficial em peixe amazônico, mapará (*Hypophthalmus* spp.)

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ABSTRACT

Little is known about environmental variables and pressures that shape this microbiota. The objective of this research was to analyze the susceptibility to antimicrobials among bacteria present in the superficial mucosa microbiota of specimens of the Amazonian fish mapará (*Hypophthalmus* sp.) captured for industrial benefit and in local popular fairs. Surface mucosa samples from 86 mapara specimens were collected using sterile *swabs*. Material was plated using the *pour plate* technique in TSA culture medium and following isolation were made from grown colonies. Isolated bacteria were differentiated according to the structure of the cell wall and subjected to an antibiogram test against representatives of various antibiotic classes. The isolates presented mono and multidrug resistance profiles to the tested antibiotics typical for environments where aquaculture activity is not yet established. Penicillin resistance was the most frequent phenotype for the set of strains. The growth of riverside populations without sewage treatment infrastructure and the presence of heavy metals may have been decisive for the profiles found.

Keywords: teleost, mucosa, microbiome, antimicrobials, resistance.

RESUMO

A comunidade microbiana presente no muco de diferentes espécies de peixes marinhos e de água doce já é estudada, mas pouco se sabe sobre variáveis e pressões ambientais que moldam essa microbiota. O objetivo desta pesquisa foi analisar a susceptibilidade a antimicrobianos entre bactérias presentes na microbiota do muco superficial de espécimes do peixe amazônico mapará (*Hypophthalmus* sp.) capturado para beneficiamento na indústria e em feiras populares locais. Amostras do muco superficial de 86 espécimes de mapará foram coletadas com uso de *swabs* esterilizados. O material foi plaqueado pela técnica de *pour plate* em meio de cultura TSA e feitos isolamentos a partir das colônias crescidas. As bactérias isoladas foram diferenciadas de acordo com a estrutura da parede celular e submetidas a teste de antibiograma contra representantes de várias classes de antibióticos. Os isolados apresentaram perfis de mono e multirresistência aos antibióticos testados, típicos para ambientes em que a atividade de aquicultura ainda não está estabelecida. A resistência a Penicilina foi o fenótipo mais frequente para o conjunto de cepas. O crescimento das populações ribeirinhas sem infraestrutura de tratamento de esgotos e presença de metais pesados podem ter sido determinantes para esses perfis encontrados.

Palavras-chave: Linxacariosis, domestic cat, mite.

1 INTRODUCTION

Environmental microorganisms represent the most relevant reservoir of resistance to antibiotics and other drugs (Fernández et al., 2012). The indiscriminate use of antibiotics by the world population over the past 80 years, combined with anthropogenic activities in the production of food of plant and animal origin, has contributed to the spread of resistance genes mainly among bacteria (Apjok et al., 2019; Usui et al., 2016; Aarestrup, 2015; Haniffa, Viswanathan, Jancy, Poomari, and Manikandan, 2014; Latha and Marhon, 2013; Rakers et al., 2013). Considering that Amazonian cities lack a sanitary system and that the aquatic environment continuously receives human and veterinary antimicrobial residues through domestic and hospital sewage, the emergence and spread of resistance to bacterial antibiotics can be an aggravating factor for public health problems already faced by the population (Apjok et al., 2019; Kang et al., 2017; Bouki, Venieri, and Diamadopoulou, 2013).

Antibiotic resistance studies analyzing environmental microbiota and AGRs (antibiotic resistance genes) may be the most viable alternatives to fully understand the cycle of resistance acquisition by pathogens (Leisner, Jørgensen, and Middelboe, 2016; Forsberg et al., 2014; Martinez, 2012; Bhullar et al., 2012; Davies and Davies, 2010).

Recent studies have revealed the emergence of "superbugs" that carry a number of ARGs that tolerate most antibiotics (Malhotra-Kumar et al., 2016).

Thus, a complete understanding of the pressures and circumstances that lead to the evolution and spread of antibiotic resistance genes in pathogens is impossible without a detailed examination of the origin and role of resistance genes in the natural environment, being considered the best case of evolution today (Horinouchi et al, 2020; Rogalski et al, 2017; Allen et al., 2010). The analysis of natural resistomes - the set of all antibiotic resistance genes and their precursors in pathogenic and non-pathogenic bacteria - has shown that there are many more resistance genes in nature than those found in pathogens (Martinez, 2012). While, most research has turned to resistance in hospital environments, the critical role of environment, especially the aquatic environment, in the development and dissemination of antibiotic resistance genes (ARG's) has been gaining attention and gathering efforts to understand these dynamics. Antibiotics and ARG's may partially originate from environmental bacteria, and their selection is closely linked to anthropogenic contamination with residual antibiotics (RABs), to which exposure of pathogenic bacteria in the same environment occurs (Gillings, 2013; Baqueiro, Martínez and Cantón, 2008).

In the last decade, many researches have focused on the study of the microbiota present in the mucosa of teleost fish and other aquatic animals in terms of diversity, functionality and microbial ecology, in addition to the established interrelationships and the critical roles for the host (Becker, Longo, Haddad, and Zamudio, 2017; Minniti, Hagen, Porcelatto, and Jørgensen 2017; Sylvain and Derome, 2017; Sylvain et al., 2016).

Fish skin has mucus-producing glands, which is an important substance for the innate defense mechanism and functions as a mechanical and biochemical barrier between fish and the environment. In the mucosa a great diversity of microorganisms is also found, including some pathogenic for man and for fish themselves (Rakers et al., 2013). The constitution of this mucosa varies according to the epithelial surface, the species and the environment in which the animal is inserted (Leonard et al., 2014). Likewise, the surface microbiota of aquatic animals is strongly influenced by the surrounding environment, but it can also be an indicator of human impact.

This research aimed to evaluate the antimicrobial resistance profiles of the superficial mucus microbiota on the skin of the Amazonian fish mapará (*Hypophthalmus* spp.) controlling post-capture manipulation interference and environmental conditions related to time of capture.

2 MATERIAL AND METHODOS

Collection, processing and collection of microbial isolates from the fish's skin superficial mucosa

Samples of superficial mucosa from the skin were collected from 86 specimens of mapará (56 specimens recently arrived to the processing industry and 30 exhibited for sale at a popular fair), from March to June 2015, in the city of Santarém, Pará. The fish were individually packed in sterile transparent plastic bags and transported in an isothermal box to the ICTA / UFOPA Multidisciplinary Teaching Laboratory (Labio) for analysis procedures. Mucus from the mapará skin was collected by smear using a swab soaked in 0.85% saline solution, within a total area of 100 cm². The samples were subjected to serial dilutions up to 10⁻⁵ using 0.85% saline solution as a diluent. 1mL aliquots of the dilutions were inoculated in Triptone Soy Agar (TSA) -BD® medium with duplicates, using the *pour plate* technique and incubated for up to 48 hours at 35° C. After 48 hours of incubation, the standard plate count (CPP) was performed.

Five colonies were selected from the Petri dish cultures and inoculated in tubes containing TSA that were incubated for 24 hours at 35° C. After that period, the grown cultures were analyzed for purity using the Gram staining technique.

Antimicrobial susceptibility test of strains isolated from the superficial mucosa of fish skin

The susceptibility test was performed using the disk-diffusion method (Bauer-Kirby, 1966) on agar with Müller Hinton (MH) -BD® medium against commercial antimicrobial agents (Laborclin®). Resistance cutoff points for Gram positive (8 antibiotics) and Gram negative (7 antibiotics) were used following protocols established by the Clinical and Laboratory Standards Institute - CLSI (2014). The microbial agents tested were: gentamicin (GEN - 30 µg); cephalothin (CFL - 30 µg); chloramphenicol (CLO - 30 µg); erythromycin (ERI - 15 µg); ampicillin (AMP - 10 µg); nitrofurantoin (NFT - 300 µg); penicillin G (PEN - 10 U); nalidixic acid (NAL - 30 µg); sulfametaxazole (SXT - 25 µg); tetracycline (TET - 30 µg).

Colonies of the pure cultures, growing in TSA medium, were transferred to 0.85% saline solution until reaching a suspension comparable to the standard turbidity, corresponding to 0.5 on the McFarland scale (1.5 x 10⁸ CFU / mL). The inoculum was spread evenly with a sterile swab on the surface of the Müller-Hinton (MH) BD® agar,

and the discs impregnated with antibiotics were deposited on the surface of the inoculated culture medium.

The plates were incubated inverted at 35 ° C for 24h. The halo diameter readings of colony growth inhibition were performed with a digital caliper on a millimeter scale (Lorben®).

River level, hydrological period and precipitation

To know the hydrological period of Tapajós River, the river level was calculated to classify the months between the hydrological periods - flood, flood-peak, ebb and drought - the monthly amplitude, average monthly elevation, and the frequency of maximum and minimum elevation for each month were used. Tide elevation data was obtained from the National Water Resources Information System (SNIRH) of the National Water Agency (<http://www.snirh.gov.br/hidroweb/serieshistoricas>).

Precipitation data were obtained from the meteorological station located in the city of Belterra, a Brazilian municipality in the state of Pará, belonging to the Mesoregion of western Para. It is located in northern Brazil, at 02°38'11 "south and 54°56'14" west, about 52 km from the municipality of Santarem and 1245 km from the capital Belem, with the regular weather station code OMM: 82246 for long-term monitoring by INMET (National Institute of Meteorology).

Statistical analyses

Antimicrobial resistant bacteria counts (mono or multiresistance) were obtained. A Pearson correlation test ("r") was performed to verify the effect of precipitation and river level on Gram-positive and Gram-negative resistant bacteria, where the resistant bacteria count was considered a dependent variable (x) and, precipitation and river level the independent variable (y).

3 RESULTS AND DISCUSSION

- Antimicrobial resistance profiles in bacterial isolates from the superficial mucosa of the mapará (*Hypophthalmus spp.*) skin

Susceptibility profiles to antimicrobials were detected in 165 strains (88 Gram-positive and 77 Gram-negative) isolated from mucus in the superficial skin of mapará against 7 classes of antibiotics (Table 1).

Table 1 - Profiles of antimicrobial resistance of the strains obtained from the superficial mucus of the mapará skin (*Hypophthalmus spp.*) according to cell wall characteristic.

Gram-positive			Gram-negative		
Resistance profiles	No. Isolates per site		Resistance profiles	No. Isolates per site	
	Industry	Fair		Industry	Fair
NAL+SXT+TET+PEN+NIT+ERI+CFL	4	0	CFL+NIT+AMP+SXT+TET	4	4
TET+PEN+NIT+ERI+CFL	1	0	CFL+NIT+AMP+TET	3	6
NAL+SXT+TET+PEN+ERI	2	0	NIT+AMP+SXT+TET	1	3
NAL+SXT+TET+PEN	0	1	CFL+AMP+TET	1	2
SXT+TET+PEN+ERI	0	1	CFL+AMP+SXT	1	4
SXT+TET+PEN	2	0	CFL+NIT+AMP	2	1
NAL+PEN+CFL	1	0	NAL+PEN+CFL	1	0
SXT+PEN+CFL	8	1	NIT+AMP+TET	1	0
TET+PEN+NIT	1	0	SXT+TET	1	2
TET+PEN+CFL	1	0	AMP+SXT	0	1
PEN+CFL	11	6	CFL+AMP	3	7
SXT+PEN	2	1	CFL	2	5
SXT+CFL	0	1	AMP	8	5
PEN	17	19	TET	1	0
Without resistance	7	1	NIT	1	1
Total by site of origin	57	31	Without resistance	3	3
			Total by site of origin	33	44

AMP - Ampiciline; CFL - Cephalothin; CLO - Chloramphenicol; GEN - Gentamicin; ERI- Erythromycin; NAL - Nalidixic acid; NIT - Nitrofurantoin; PEN - Penicillin; SXT - Sulfamethoxazole; ETT - Tetracycline; R - Resistant; I - Intermediate; S - Sensitive.

The two antimicrobials with resistance profiles with higher resistance, PEN and AMP, belong to the same class of drugs and are beta-lactam, acting as enzymatic inhibitors of the transpeptidation process during peptidoglycan synthesis (Guimarães, Momesso, and Pupo, 2010). Penicillin is an antibiotic of the penicillin class and the most important mechanisms of resistance to this drug in Gram-positive bacteria are mutations and / or reduction or alterations in the expression of penicillin-binding proteins (PBPs) (Lobanovska and Pilla, 2017; Beceiro, Tomás, and Bou, 2013).

Among the Gram-positive bacterial strains isolated from the processing industry samples, 88% showed resistance to at least one of the antibiotics, of these, the greatest resistance was for penicillin (34%), 22% for penicillin + cephalothin and 16% for sulfamethoxazole + penicillin + cephalothin. A total of 66% of strains were resistant to more than one antibiotic and 8% were resistant to all tested antibiotics.

Among Gram-negative isolates, 92% showed resistance to at least one of the antibiotics used for the test and none showed resistance to gentamicin. Among the resistance profiles identified, the most frequent was against ampicillin (18%), followed

by cephalothin + ampicillin (14%) and cephalothin + nitrofurantoin + ampicillin + tetracycline with 13% of resistance. Ampicillin, an antibiotic of the β -lactam class, was among the tested antibiotics to which the isolates were more resistant, both in the samples of the processing industry and in the fair. This drug was present in 9 out of 11 profiles of multiple resistance. In the strains isolated from the fair samples, the most frequent resistance profiles were for cephalothin + ampicillin (17%), cephalothin + nitrofurantoin + ampicillin + tetracycline (15%) and, cephalothin and ampicillin 12% each.

Analyzing the broad-spectrum antimicrobial used in both groups, resistance to CFL was the most frequently detected (48.5%). Cephalothin is a cephalosporin also in the group of beta-lactams. It is possible to affirm that the main resistance mechanism presented among the members of this bacterial community is the production of beta-lactamase enzymes that cleave the beta-lactam ring.

Despite the relative majority of bacteria showing resistance and multi-resistance profiles registered among the isolates from the fair's fish, the greatest diversity of resistance profiles was detected among the bacterial isolates from fish in the processing industry. Tihamiyu et al. (2015) provided more evidence on the occurrence of multidrug-resistant strains in nature, which represents a potential threat to human health.

There are several routes for transferring resistance genes or either resistant bacteria to antimicrobials that can directly and indirectly establish themselves in humans and animals. The consumption of contaminated food or derived products, or direct contact with colonized / infected animals or with biofluids (blood, urine, feces, saliva, semen, etc.) of these animals is considered as potential routes (Le Roux and Blokesch, 2018; Marshall and Levy, 2011).

Resistance genes can be found both in the aquatic environment and in organisms directly linked to it. A study of commercial marine fish caught off the Digha coast (India) suggests that they may play a role as a carrier / reservoir of antibiotic-resistant bacteria, representing a risk to the health of fish consumers (Ghosh and Mandal, 2010). Also, in uncultivated freshwater fish, high percentages of antibiotic resistance were found in the superficial mucosa microbiota, indicating that these profiles are not directly linked to aquaculture but to environmental pressures from anthropogenic activities in general (Ozaktas, Taskin, and Gozen, 2012). Further research has found similarity in the number of antibiotic resistance genes in sediments from aquaculture and natural aquatic environments (Buschmann et al., 2012). High levels of antibiotic resistance have been found in bacteria in a lake in China, posing risk to the health of human and animal

populations that consumed this water (Yin et al., 2013). Gao et al. (2012) isolated *Bacillus* spp. of waters influenced by aquaculture in Tianjin (China), which showed resistance to tetracycline (57.1%) and sulfamethoxazole (63.3%).

Bacillus species, isolated from the intestine of *Labeo rohita* (carp family), showed resistance to β -lactam antibiotics such as ampicillin, cephalixin and penicillin, to Gram-positive specific antibiotic like erythromycin and to broad spectrum antibiotics such as chloramphenicol and tetracycline and gentamicin aminoglycoside (Giri, Sukumaran, and Dangi, 2012). Gram-positive strains present in the superficial skin mucosa of the mapara tested in this research showed profiles of susceptibility to the action of chloramphenicol.

Gram-negative bacteria from the skin mucosa of the gray stingray (*Potamotrygon motoro*) showed a resistance profile to three groups of antibiotics where ampicillin was the most expressive, followed by amoxicillin / clavulanic acid and cephalothin (Domingos et al., 2011). The authors also concluded that in the event of an accident with a streak, mucosa along with bacteria with a multi-resistance profile and in the surrounding water aggravate the injury, making treatment difficult.

Strains of *Aeromonas* spp. isolated from aquarium water and the scales of ornamental fish were resistant to amoxicillin (95%), carbenicillin (96%) and ampicillin (94%) (Dias, Mota, Martinez-Murcia, and Saavedra, 2012). This same bacterial genus isolated from carp (*Carassius gibelio*) in China showed resistance to penicillin, but also showed a sensitivity profile to two other classes of antibiotics (Sun et al., 2016).

Drugs such as chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole can be used to treat salmonellosis. However, resistance to these drugs has increased significantly in recent years among known isolated strains (Meng et al., 2011; Wannaprasat, Padungtod, and Chuanchuen, 2011; Lee and Kim, 2011). In the search for effective treatment for human and animal diseases, fluoroquinolones have been recommended and third-generation cephalosporins are the drugs most likely to be used when there is resistance to fluoroquinolone (Van et al., 2012).

The high level of resistance to multiple antibiotics is a common phenotype among bacteria from the skin mucosa microbiota of freshwater fish in places with no history of aquaculture activity (Ozaktas, Taskin, and Gozen, 2012). In our study, high index of multiple resistance was observed among the group of isolates, Gram-negative and positive (Table 1).

It is known that the presence of multidrug resistance in environmental bacteria may be related to the presence of heavy metals in the environment (Dixit et al., 2015).

Heavy metals occur naturally in the environment resulting from pedogenetic processes and arising from anthropogenic actions. The most significant natural sources are erosion and volcanic activity, while anthropogenic sources depend on human activities such as mining, use of pesticides and phosphate fertilizers as well as biosolids (i.e.: animal fertilizers, compost and municipal sewage sludge), among others. (Sabiha-Javied et al., 2009; Modaihsh, Al-Swailem, and Mahjoub, 2004). The imbalance in the ecosystem caused by the presence of metals can pose risks to human, animal and plant health (Beltran-Pedrerros et al., 2011; D'amore, Al-Abed, Scheckel, and Ryan, 2005). High levels of mercury (Hg) were found in different areas of the Amazon, with and without a history of gold mining, with the floodplains being identified as Hg methylation areas (Beltran-Pedrerros et al., 2011).

Wang et al. (2015) explained the high percentages of bacterial resistance to drugs of different types, considering the contamination of water bodies by antimicrobials and heavy metals, given that many of the resistance genes are encoded in the same mobile genetic structure as these elements, in a phenomenon called cross-resistance.

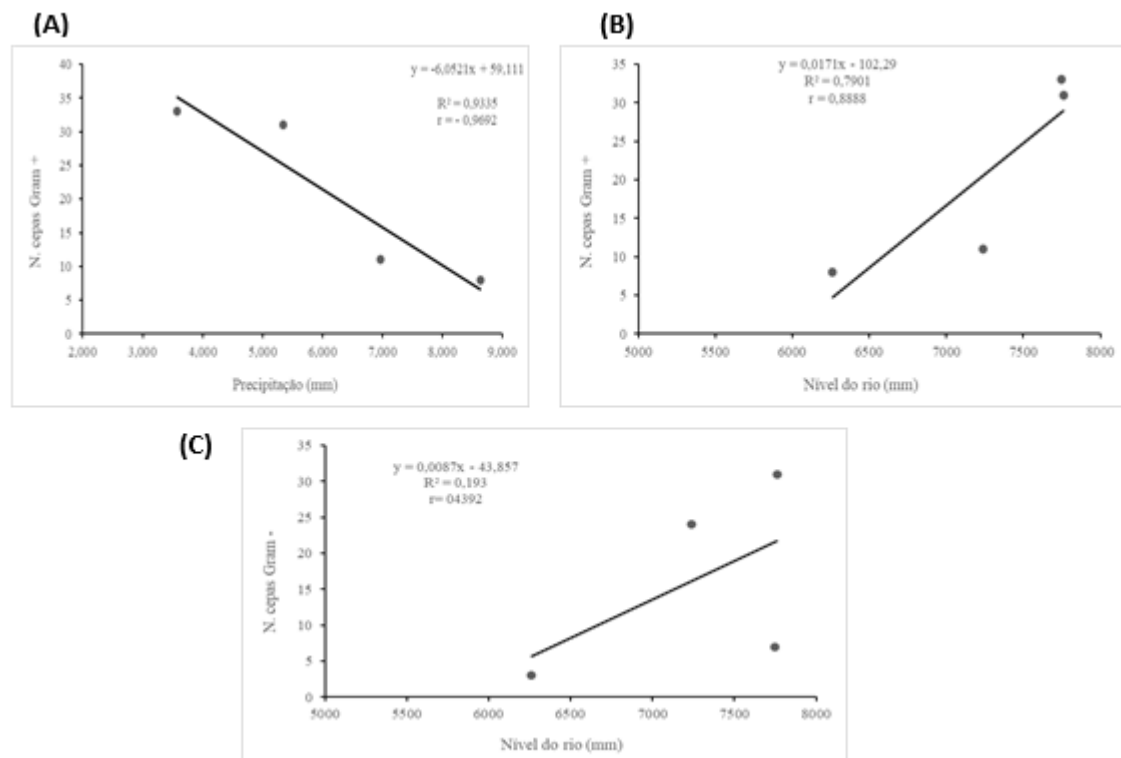
The spread of bacterial resistance to multiple antibiotics is a worldwide problem and its understanding involves broader environmental and social issues. The different patterns of antibiotic resistance of bacterial isolates may be the result of the difference in selective pressures. Our findings showed the presence of antibiotic-resistant strains in fish samples in this region of the country, indicating the potential risk to public health, but is also an indication of the effect of anthropogenic activities on the resistome of aquatic environments and organisms.

The lack of species barriers for the transmission of resistance genes reinforces the need for research efforts in this area. An ecological approach is imperative to understand and mitigate the impacts that the transfer of resistance genes or resistant bacteria can have on the human population (Nelson, Moore, and Rao, 2019; Founou et al., 2016; Tihamiyu et al., 2015; Verraes et al., 2013).

The search for patterns of antimicrobial resistance in bacteria from aquatic organisms intended for food, or not, should consider the effects and pressures of the aquatic environment on these results. The occurrence of rainfall can be an aggravating factor for exposure to bacteria related to sewage in water bodies, as well as the hydrological periods characterized by floods and droughts also have the capacity to influence the dynamics of exposure to bacterial or pollutant groups that can cause co-selection of resistance among native bacteria.

We analyzed resistance frequency among bacterial isolates on the skin surface of fish considering the environmental variables of rainfall and hydrological cycle of the Tapajós River. Among Gram positive isolates, there was a negative correlation with the occurrence of rainfall and no relationship with Gram negative compared to this same variable. As for the level of the river that expresses the hydrological cycle of the river, a positive correlation was found for both Gram-positive and Gram-negative (Figure 1).

Figure 1: Relationship between the number of Gram-positive (A and B) and Gram-negative (C) strains resistant to antimicrobials with precipitation and the Tapajós river level (Santarem, Para, Brazil).



The seasonal and abundant rainfall in the Andean region causes an annual, regular, monomodal, and wide fluctuation in the water level of the Amazon River (Ramalho et al., 2009). The overflow of the main channel of the river and the flooding of a large extension of the Amazon Forest is a consequence of the flood pulse and the flat relief of the Amazonian depression. The flood pulse is decisive for most ecological processes in the floodplain, causing its periodic transformation from terrestrial to aquatic environments.

Di Cesare, Eckert, Rogora, and Corno (2017) evaluated the precipitation capacity over the abundance of five different ARGs of different types of antimicrobials, in bacteria isolated from the Toce river (Italy), and concluded a strong correlation between the

absolute abundances of the ARGs were strongly correlated with each other during the rain, highlighted genes for tetracyclines, sulfonamides and macrolides.

Likewise, the occurrence of ampicillin-resistant bacteria in the water in Puthia Lagoon (Bangladesh) associated with poultry and fish breeding, showed a greater number of bacteria resistant to antimicrobial in the rainy season (Neela et al., 2015).

Historically, humans lived around rivers and, consequently, cities, agriculture and industries developed close to these aquatic environments. Rivers have become one of the most vital sources of water supply and resources for human populations. A reality especially in the Amazon and in general in the northern region of Brazil. However, this Brazilian region has the lowest rate of basic sanitation coverage (<http://www.tratabrasil.org.br>), with its rivers being the final destination of most sewage and effluents resulting from human activity.

Posada-Perlaza et al. (2019) found that the water resistome of the Bogotá river changes along its course due to the presence of human agglomerations confirming anthropogenic activities as important factors in the dissemination of antibiotic resistance genes in the microbiota.

4 CONCLUSIONS

The detection of multiresistance profiles in this study among bacterial isolates, including forbidden antibiotics, is indicative of the selective pressure resulting from anthropogenic sources on the banks of the Tapajós River, or may also be related to cross-resistance events from the presence of heavy metals characteristic of the waters of this river, which determine antimicrobial resistance mainly during the rainy season.

An understanding of the determinants of resistance present in the superficial mucosa microbiome of mapará, and other fish species of the Amazon, may provide information not only on frequencies of resistance to antibiotics, but can also help in the construction of a scenario that reflects the indiscriminate use of antibiotics in the region. Future research should focus on the search for information about the resistance patterns between the bacterial strains and the understanding of the natural and anthropogenic phenomena involved in this panorama, considering the unique characteristics of the Amazon region.

This is one of the first studies focusing on the study of the resistance profile in samples isolated from the Amazon environment, evidently that more efforts are needed to deepen research of this nature.

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