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METAGENOMA E AVALIAÇÃO DA EXPRESSÃO DE PROTEÍNAS DO INTESTINO DE *POECILLIA RETICULATA* EM RESPOSTA A CONTAMINAÇÃO AMBIENTAL POR ANTIBIÓTICOS.

GABRIELA PUSTIGLIONE MARINSEK

São Vicente
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Tese apresentada ao Instituto de Biociências, Câmpus do Litoral Paulista, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biodiversidade de Ambientes Costeiros.

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Aos 06 dias do mês de junho do ano de 2024, às 14:00 horas, no(a) Salão Nobre do IB/CLP (modo híbrido), realizou-se a defesa de TESE DE DOUTORADO de GABRIELA PUSTIGLIONE MARINSEK, intitulada **Metagenoma e avaliação da expressão de proteínas do intestino de Poecillia reticulata em resposta a contaminação ambiental por antibióticos.**. A Comissão Examinadora foi constituida pelos seguintes membros: Prof. Dr. MARCOS ANTÔNIO DE OLIVEIRA (Orientador(a) - Participação Presencial) do(a) Departamento de Ciências Biológicas e Ambientais / IB/CLP - UNESP, Dra. PALOMA KACHEL GUSSO CHOUERI (Participação Presencial) do(a) UNISANTA / Universidade Santa Cecília, Profa. Dra. MARIA RAQUEL MARÇAL NATALI (Participação Virtual) do(a) Universidade Estadual de Maringá, Prof. Dr. LEANDRO MANTOVANI DE CASTRO (Participação Presencial) do(a) Departamento de Ciências Biológicas e Ambientais / IB/CLP - UNESP, Prof. Dr. ANDERSON FERREIRA DA CUNHA (Participação Virtual) do(a) Departamento de Biotecnologia / UFSCAR. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: Aprovada. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Prof. Dr. MARCOS ANTÔNIO DE OLIVEIRA

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"Devemos acreditar que somos talentosos para algumas coisas, e que essa coisa, a qualquer custo, deve ser alcançada."
(Marie Curie)

RESUMO

A crescente descarga de xenobióticos em ambientes aquáticos tem levantado preocupações significativas devido aos potenciais efeitos adversos que esses compostos podem causar nos ecossistemas. Entre eles, os antibióticos são particularmente preocupantes, pois interrompem inúmeras vias fisiológicas em organismos aquáticos, potencialmente levando a danos celulares, teciduais e em órgãos. O monitoramento da qualidade dos ecossistemas e o estudo dos efeitos desses compostos em organismos geralmente se concentram em órgãos-alvo, como brânquias e fígado, que respondem direta ou indiretamente aos contaminantes. No entanto, a microbiota intestinal, que interage diretamente com contaminantes através da camada mucosa do trato gastrointestinal, também é um alvo crítico nesses processos. Estudos metagenômicos recentes mostraram que a exposição a antibióticos pode causar açterações na composição desta comunidade, impactando em sua interação com o hospedeiro via sistemas nervoso entérico e central. Este estudo teve como objetivo avaliar os efeitos tóxicos da azitromicina em espécies de peixes não-alvo, *Poecilia reticulata*. Avaliamos o impacto em órgãos-alvo ecotoxicológicos, como fígado e brânquias, bem como os efeitos na microbiota intestinal e na homeostase intestinal geral. Nossas descobertas indicam que a azitromicina pode induzir dano histopatológico significativo em órgãos-alvo e prejudicar mecanismos de defesa antioxidante. Mais notavelmente, ela altera a composição da microbiota, levando a problemas de absorção e inflamação no do trato gastrointestinal.
Key-words: Microbiota, mucosa intestinal, biomarcadores, expressão de proteínas, antibióticos.

ABSTRACT

The increasing discharge of xenobiotics into aquatic environments has raised significant concern due to the potential adverse effects these compounds can have on ecosystems. Among these, antibiotics are particularly worrisome as they disrupt numerous physiological pathways in aquatic organisms, potentially leading to cellular, tissue, and organ damage. Monitoring ecosystem health and studying the effects of these compounds on organisms often focus on target organs like gills and liver, which directly or indirectly respond to the contaminants. However, the intestinal microbiota, which directly interfaces with contaminants through the mucous layer of the gastrointestinal tract, is also a critical target in these processes. Recent metagenomic studies have shown that exposure to antibiotics can cause dysbiosis, impacting not only the microbiota's composition but also its interaction with the enteric and central nervous systems. This study aimed to evaluate the toxic effects of azithromycin on non-target fish species, *Poecilia reticulata*. We assessed the impact on ecotoxicological target organs, such as the liver and gills, as well as the effects on intestinal microbiota and overall intestinal homeostasis. Our findings indicate that azithromycin can induce significant histopathological damage in target organs and impair antioxidant defense mechanisms. More notably, it disrupts the microbiota's composition, leading to absorption issues and inflammation within the gastrointestinal tract.

Key-words: Microbiota, intestinal mucosa, biomarkers, protein expression, antibiotics

Sumário

GENERAL INTRODUCTION

Environmental Contamination by Antibiotics

Antibiotic use has brought numerous health benefits to patients in the fight against infections caused by microorganisms. However, its consumption also causes environmental problems, since after ingestion, its metabolites are eliminated through feces and urine and released into aquatic environments through wastewater or inadequate disposal (Larsson & Flach 2022). In this sense, the greatest concern with the release of these compounds into aquatic environments arises from the chronic effects, since they are present in very low doses and are part of the biological cycle of numerous aquatic organisms (Cheng et al., 2020). They are biologically active compounds that are difficult to degrade and are highly found in effluent dump sites, mainly due to the presence of personal hygiene products, and effluents from pharmaceutical industries and hospitals (Rodriguez-Mozaz et al., 2020). The antibiotic dumping into aquatic environments can harm the resident biota, mostly because they have similar functions as those of their origin, causing side effects on organisms present in the ecosystem.

Antibiotics in general have great potential to cause negative effects on the microbiota, as their cytotoxic effects can damage DNA molecules, inhibiting their synthesis and interrupting cellular replication. After being ingested, they can be metabolized through two distinct pathways, the first being composed of oxidation, reduction, hydrolysis, and alkylation reactions and the second, where conjugates of glucuronides and sulfates are formed and excreted through urine or bile, thus forming metabolites. more polar and hydrophilic (Jones et al., 2008; Cunningham et al., 2006; Heberer 2002, Hoffman, 1984). Therefore, these compounds reach water bodies through inclusion in their conventional form or feces and urine after consumption (Bilal et al., 2020).

The current high consumption of antibiotics, especially azithromycin, around the world and the fact that these compounds and/or their metabolites are soluble in water means that these contaminants become pseudo-persistent in aquatic ecosystems (Harrower et al., 2021). Furthermore, we must pay attention to the fact that, when not used, they are often discarded irregularly, reaching sewage treatment systems and, consequently, surface water and even drinking water (Monahan et al., 2021; Monteiro et

al., 2018), which makes them easily introduced into the trophic chain, thus posing serious threats to animals and humans (Lyu et al., 2023).

Antibiotics were developed to act directly on pathogenic bacteria. However, non-target organisms are exposed to residues and by-products of these drugs, with primary producers and decomposers, essentially important for maintaining ecosystem dynamics, being the main organisms affected, which can lead to serious changes in processes. ecosystems (Lyu et al., 2023). Studies have reported that the presence of antibiotics in water can drastically reduce populations of organic matter-degrading and nitrifying bacteria (Li et al., 2021), which influences nutrient cycling (Oldenkamp et al., 2019). Macrolides are not expected in high concentrations due to their low solubility in water, with some exceptions, such as azithromycin (Rizzo et al., 2013). However, the persistence of these antibiotics in the aquatic environment is due to their high significance in numerous disease treatments.

Bioindicators of Environmental Quality Assessment

To assess the health of an ecosystem constantly exposed to environmental contamination, several chemical techniques have been employed. Such techniques provide data on the availability of a specific contaminant in abiotic compartments, such as water and sediment. However, the organisms' biological responses can only be provided through analyses performed in target organs, such as the liver and gills.

The use of fish as indicator organisms of environmental quality in ecotoxicological studies occurs mainly because of the responses that these organisms provide, as they are like large vertebrates' responses. Furthermore, these organisms are a major vehicle for transferring contaminants to humans through consumption. Fish of the species *Poecilia reticulata* belong to the order *Cyprinodontiformes* and the family *Poeciliidae*, being highly tolerant to extreme variations in salinity (euryhaline) and temperature (Gomes Jr and Monteiro, 2007). Due to such characteristics, these organisms are commonly found in coastal basins ranging from the Rio de la Plata, in Argentina, to Venezuela (Lucinda and Costa, 2007). Organisms of this genus have been suggested as key indicators for evaluating the effects of contaminants in the environment (Machado et al., 2013; Mattos et al., 2010), which characterizes them as potential bioindicators.

Previous studies have already addressed, for example, histopathological changes in the liver of animals exposed to iron nanoparticles (Lima Faria et al., 2021), cytogenetic damage triggered by exposure to benzophenone-3 (Santos et al., 2019), as well as changes biochemical processes in these organisms exposed to various toxic compounds such as endocrine disruptors, tannery effluents and organophosphates (Hugget, 2018; Tian et al., 2018; Pereira et al., 2015). Furthermore, *P. reticulata* is an easily manipulated organism and ideal for studies involving bioassays, in addition to having excellent potential for use in public management, such as quality, risk, and environmental monitoring analyses due to its wide geographic distribution, colonization of different habitats and for its presence in environmentally degraded regions.

When using fish as bioindicators, the aim is to evaluate the biological responses of these organisms to various stressors. Such responses are measured using specific biomarkers that vary according to the type of stressor and the response to be assessed. Among the most used biomarkers, the morphophysiological, biochemical, and molecular ones stand out, which establish the relationship between exposure to toxic agents and their effects on the health of organisms mainly using target organs such as the liver and gills (Lomartire et al., 2021).

Exposure of fish to macrolide antibiotics can induce damage to the DNA molecule and generate lipoperoxides through oxidative stress, in addition to causing genotoxic damage through increased nuclear anomalies (Rodrigues et al., 2016; Ji et al., 2012). Antibiotics of this class also can cause cardiac toxicity and alter the activity of antioxidant enzymes such as SOD and EROD (Liu et al., 2017). In addition to causing enzymatic damage, antibiotics' presence can also affect tissue architecture.

Numerous studies have demonstrated that such contaminants can cause histopathological damage that is often irreversible, leading to physio metabolic dysfunctions (Rodrigues et al., 2019). Among the most common pathologies are epithelial desquamation, proliferation and hypertrophy of mucus cells, epithelial hyperplasia, and lamellar fusion. (Rodrigues et al., 2019). In addition, other studies reported the presence of edema, aneurysms, and vascular congestion (Rodrigues et al., 2019). In the liver, exposure to antibiotics induced the formation of pathologies such as hepatocellular degeneration, hepatocyte hypertrophy, increased sinusoidal space, inflammatory

infiltration, and hemorrhage (Rodrigues et al., 2019). In addition, lipid deposition, fibrosis and necrotic spots were also reported (Rodrigues et al., 2019).

The liver and gills are organs widely studied in environmental quality analyses mainly due to their morphophysiological characteristics. The gills are in direct contact with the external environment, being subject to the absorption of xenobiotics available in the water, whereas the liver participates in the detoxification and biotransformation processes, indirectly absorbing contaminants present in the bloodstream or absorbed by the gastrointestinal tract (GIT) (Marinsek et al., 2024).

Gastrointestinal Tract, Microbiota, and Enteric Nervous System

The gastrointestinal tract (GIT) is innervated by an intrinsic nervous system that controls motility, secretion, and blood flow called the enteric nervous system (ENS) (Furness and Stebbing, 2018). ENS neurons are responsible for controlling intestinal motility and absorption, and their complex relationships perform specific functions in the GIT (Sharkey and Mawe, 2023; Furness and Stebbing, 2018). In this context, neuronal plasticity can be defined as the capability of neurons to adapt to intrinsic or extrinsic changes. Thus, neurons have adaptive properties, altering their biochemical and physiological characteristics temporarily or permanently due to environmental factors (Di Giancamillo et al., 2010). Among the neurotransmitters that control GIT functions, the main ones are acetylcholine, which promotes smooth muscle excitability through muscarinic receptors, and nitric oxide (NO•).

In the peripheral nervous system, NO• is responsible for the primary mediation of non-adrenergic non-cholinergic transmission, promoting the relaxation of the smooth muscles of the gastrointestinal tract (Furness and Stebbing, 2018) and performing important functions such as accommodating the volume of food ingested in the stomach, regulating the tone of the intestinal sphincters and peristaltic movement (Takahashi, 2003). In addition, NO• is involved in kinase pathways and the cyclic AMP transcription factor regulation, pathways that promote cell survival and neuronal protection (Riccio et al., 2006). However, this molecule can become harmful when produced in excess (Pacher et al., 2007), since when a cell is in the pro-oxidant stage, NO• can be involved in oxidation-reduction reactions and form toxic compounds, such as reactive nitrogen

species (RNS), which can cause damage to cellular structures (Pacher et al., 2007). Thus, the proper functioning of the GIT depends on the balance between the neurotransmitters, since morphophysiological changes in this neuronal network, whether in the density of neurons or the alteration of their chemical code, as well as in enteric glial cells, can compromise the functions of the ENS and consequently lead to severe dysfunctions at different levels in the GIT.

Another important neurotransmitter for homeostatic control of the intestine is serotonin (5-HT). This, in turn, acts in the modulation of intestinal motility and platelet function, effectively participating in the hydroelectrolytic regulation of the GIT, modulating thirst and appetite, food intake, energy balance, and behavioral control (Mohammad-Zadeh et al., 2007). Approximately 95% of the body's serotonin is produced in the GIT, either by enterochromaffin cells or serotonergic neurons, and alterations in this neurotransmitter can lead to severe dysfunctions in the homeostasis of organisms (Gershon and Track, 2007). Our studies have shown that environmental contaminants considerably alter the density of nitrergic and cholinergic neurons (Marinsek et al., 2018), affecting neuronal metabolism (Gonçalves et al., 2020). Changes in the plasticity of enteric neurons or even the loss of these neurons can lead to severe gastrointestinal dysfunctions, compromising the health of populations in the medium and long term. (Diamante et al., 2014; Marinsek et al., 2018; Gonçalves et al., 2020). The enteric nervous system, although autonomous, is related to the central nervous system (CNS) through a two-way homeostatic route of communication, which uses hormonal, nervous, and immunological pathways (Mayer, 2011; Mayer et al., 2022). Changes in this pathway can lead to pathological and behavioral consequences and alter responses to stress (Mayer et al., 2022). In addition, changes in the intestinal microbiota resulting from the presence of pollutants can influence the physiological aspects of this pathway, altering the communication between the enteric nervous system and the central nervous system (Jin et al., 2017).

The symbiotic relationship between the GIT and a range of microorganisms such as bacteria, viruses, archaea, and unicellular eukaryotes make up the intestinal microbiota (Neish, 2009). This symbiotic relationship works beneficially: these organisms benefit the host by extending their metabolic/digestive abilities, in addition to competing with

pathogenic microorganisms while consuming the nutrients available in their microhabitat. For the relationship between the host and the microbiota to be beneficial, both parties must establish an association that influences and even regulates each other mutually to ensure homeostasis (Neish, 2009). However, the bacterial presence in the intestinal lumen can be a major problem for mucosal enterocytes and the host's immune system (Neish, 2009). For symbiosis to occur properly, there are molecular mechanisms involved in modulating the microbiota-intestinal mucosa interaction (Marchix et al., 2018). For the crosstalk process to occur between both parties, host cells are equipped with transmembrane or intracytoplasmic receptors known as pattern recognition receptors (PRRs), whose main function is to recognize and bind to specific bacterial macromolecules. Microbial-specific ligands are called microbiota-associated molecular patterns (MAMPs), which include lipopolysaccharides (LPS – produced by gram-negative bacteria), flagellin, peptidoglycans, and formylated peptides (Um et al., 2015). Thus, it has been demonstrated that inflammatory bowel diseases may be related to mutations that interfere with the binding between PRRs and MAMPs (Wells et al., 2011).

The activation of PRRs by MAMPs initiates several metabolic pathways such as nuclear transcription factor κβ (NFκβ), MAP kinases, and caspase-dependent signaling cascades (Man et al., 2018). The activation of such pathways is crucial for the normal development of the GIT of organisms. Some studies with germ-free mice have demonstrated highly relevant morphological, biochemical, and physiological alterations suggesting the fundamental role of the microbiota in the development, maintenance, and integrity of the intestinal barrier functions (Neu, 2007). Such changes are related to motility and modulation of enzymatic activity (Conroy and Walker, 2009), defects in the development of lymphoid tissue associated with the GIT, antibody production, reduction in the number of Peyer's patches and the thickness of the lamina propria, in addition to altering the development of intraepithelial lymphocytes (Zoualli, 2021), which indicates that the microbiota directly influences the maturation and execution of several immune functions that benefit the host.

The complexity of the intestinal barrier comes from several factors that, together, work to maintain intestinal homeostasis. Among these factors, goblet cells are responsible for numerous functions, mainly associated with the mechanical defense of the intestinal

mucosa (Sharma and Tepas, 2010). The expression of genes related to the secretion of mucins produced by goblet cells can also be altered depending on the intestinal microbiota (Sharma and Tepas, 2010). Another important characteristic that makes the barrier effective is proteins involved in cell adhesion, such as tight junctions (TJ), adhesion junctions (AJ), desmosomes, and GAP junctions. Among all the complexes, TJs represent most epithelial junctions, among which the lateral association between occludins and claudins represents the largest gatekeeper of the intestinal barrier (Allam et al., 2020). These junctions have been extensively studied, and some studies have shown that changes in the microbiota tend to disorganize cell junctions. Enteric pathogens tend to alter the distribution of occludins, thus increasing cell permeability (Di Vincenzo et al., 2024). Intestinal changes attributed to the imbalance of the microbiological community may be related to human diseases such as irritable bowel syndrome and severe intestinal dysfunctions (Mayer et al., 2022; Mayer, 2011). Furthermore, such changes can compromise the central nervous system and appear to be involved in genetic diseases such as autism (Mayer et al., 2022; Cryan and Dinan, 2012), Parkinson's disease (Hirayama and Ohno, 2021), Alzheimer's disease (Garcez et al., 2019), and behavioral changes (Dinan and Cryan, 2017).

Additionally, the GIT microbiota is also involved in the control of infectious diseases (Sekirov and Finlay, 2009; Sekirov et al., 2010), since commensal bacteria efficiently compete with pathogenic organisms for nutrients, in addition to stimulating the production of mucus, antimicrobial factors, and signaling molecules that inhibit the growth of pathogens (Hasan and Yang, 2019). In addition to these factors, the modulation of intestinal functions through the control of intestinal permeability (Di Vincenzo et al., 2024;), immune functions (Wang et al., 2019), motility (Zheng et al., 2022), sensitivity (Valdez-Moralez et al., 2014), nutrient absorption (Hadidi et al., 2021) and the activity of the enteric nervous system (ENS) (Sharkey and Mawe, 2023).

The host presents numerous immune and physiological adaptations that allow the interaction between the microbiota and the intestinal barrier to be beneficial, adaptations that prevent the host from encountering pathogenic organisms and developing uncontrollable inflammation. Thus, the intestinal barrier is considered the first line of host defense through tools such as microbial recognition, production of antimicrobial peptides,

mucins, and secretion of antibodies (Zong et al., 2020), and its integrity is controlled by endogenous and exogenous factors to the organism, such as expression of inflammatory cytokines, chemical compounds, and drugs (Maynard et al., 2012). Therefore, changes in barrier functions can trigger persistent immune activations leading to numerous intestinal diseases.

Antibiotics and their influence on the intestine and the composition and metabolic activity of the intestinal microbiota

The microorganisms that make up the GIT microbiota have been known for decades to be involved in the biotransformation of xenobiotic composts in humans, and it has been suggested that the potential of the gastrointestinal microbiota to metabolize xenobiotics is too large in terms of the capacity of the body (Koppel et al., 2017).

The composition, diversity and enzymatic capacity of the intestinal microbiota are quickly affected by various environmental factors, such as host lifestyle, diet, and medications such as antibiotics and anti-inflammatories (Claus et al., 2016; Rodríguez et al., 2013). Furthermore, various chemical substances found in the environment are only capable of interfering with bacterial growth or inducing dysbiosis (imbalance in the composition of the microbiota) in vitro and in vivo and have been associated with a series of intestinal and systemic diseases (Bäckhed et al., 2012). Therefore, disturbances in the composition of the microbiota induced by chemical products may constitute an underestimated mechanism by which they interfere with human and environmental health.

Especially when it comes to antibiotics, various effects on the microbiome of the TGI of people have been reported as a reduction in the taxonomic diversity of bacteria and an increase in bacterial resistance genes by more than 90% (Carlson et al., 2017). Furthermore, it has been demonstrated that antibiotics can lead to bacterial dysbiosis, reducing the diversity of important taxa involved in metabolic capacity and reducing resistance to microbiome colonization (Lange et al., 2016). Dysbiosis can also promote horizontal gene transfer and microbial resistance to drugs (Stecher et al., 2013). A bibliographic review by Ferrer et al. (2014) confirmed that among the various antibiotics prescribed for humans, 68 lead to alterations in the composition of the microbial communities of the intestine. Notwithstanding this group of antibiotics in the intestinal

microbiota, it was confirmed that fluoroquinolones are responsible for alterations in 32 groups of bacteria in humans (Chen et al., 2014, Kim et al., 2012). Most of the pollutants available in the aquatic environment do not have the microbiota directly, its absorption by the organism affects the TGI micro-organisms through various signaling pathways, altering the composition of the microbiota, and leading to the development of things in the energy metabolism, prejudging the absorption of nutrients in the immune defense system, in addition to causing an imbalance in the antioxidant defense system, raising the levels of relative species and, consequently, raising the organ to oxidative stress (Jin et al., 2015; Zhang et al. al., 2015).

The exact means that the microbiota communicates with the host are not known. It is known that crosstalk between the microbiota and the host occurs through various mechanisms that involve immune modulations, endocrine signals, and metabolic alterations (Lyte, 2014; Rook et al., 2014). These communication pathways pass through the enteric nervous system (ENS), responsible for transmitting this information to the central nervous system through the vagus nerve (Bravo et al., 2012). Thus, alterations in the enteric nervous system may impair communication between the GIT and the central nervous system, causing considerable damage to the host. There is evidence that alterations in the microbiota caused by antibiotics can directly affect ENS since changes induced by antibiotics do not occur in germ-free organisms or animals with resistant microbiota. to antibiotics (Benakis et al., 2016; Bercik et al., 2011). Antibiotics also demonstrate an influence on intestinal motility, which suggests a direct reaction in the enteric nervous system (Delungahawatta et al., 2017) and may be related to mitochondrial toxicity (Jiang et al., 2019). Many studies have reported that antibiotics have specific mechanisms of neurotoxicity, directly affecting neurotransmitters such as GABA, glutamate, and acetylcholine (Champagne-Jorgensen et al., 2019). Furthermore, the authors confirmed that the addition of azithromycin to the mitochondria of neuronal cells directly inhibits the enzymatic activity of complexes I and IV of the electron transport chain, gradually reducing mitochondrial respiration and leading to cellular death (Xiao et al., 2018).

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