UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus do Litoral Paulista (CLP)

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.

São Vicente
2024

M339m

Marinsek, Gabriela Pustiglione
Metagenoma e avaliação da expressão de proteínas do intestino de Poecillia reticulata em resposta a contaminação ambiental por antibióticos. / Gabriela Pustiglione Marinsek. -- São Vicente, 2024
83 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Instituto de Biociências, São Vicente
Orientador: Marcos Antônio de Oliveira
Coorientadora: Renata de Britto Mari

1. Morfologia. 2. Histologia. 3. Peixes. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Universidade Estadual Paulista (UNESP), Instituto de Biociências, São Vicente. Dados fornecidos pelo autor(a).

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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

AGRADECIMENTOS

Tão desafiador quanto escrever esta tese foi colocar em apenas algumas páginas todos as pessoas que fizeram parte de minha jornada de 12 anos no Campus do Litoral Paulista – UNESP.

Inicio este pequeno texto agradecendo aos meus pais, Silvia e Francisco, que sempre me incentivaram incondicionalmente a percorrer este caminho, muitas vezes ingrato, que é a carreira acadêmica. Obrigada por sempre acreditarem em meu potencial, por me apoiarem em todas as minhas escolhas e por terem investido nos meus estudos. Vocês sempre foram exemplo de retidão, amor e perseverança.

Aos meus irmãos, Bruno e Luiza, que sempre estiveram ao meu lado durante toda minha trajetória, me apoiando e puxando minha orelha quando necessário. Obrigada por sempre acreditarem em mim.

À minha companheira, Mariana. Seu apoio, amor e bondade foram cruciais nesta etapa final. Obrigada pela companhia tantas madrugadas adentro e por não me deixar desistir – principalmente das minhas membranas.

Uma vez dentro da universidade, algumas pessoas foram incentivadores importantes para que eu me mantivesse no caminho da pesquisa.

Primeiramente, agradeço à minha sempre orientadora Prof.ª Dr.ª Renata de Britto Mari. Eu poderia contar sobre a nossa trajetória e sobre os motivos que me fizeram querer continuar, mas vou apenas reforçar algo que sempre te digo: Re, você é um exemplo de professora, educadora, pessoa, mãe e amiga. Obrigada, primeiramente, por ter me apresentado a complexidade do trato gastrointestinal e nosso tão querido sistema nervoso entérico. Além disso, obrigada por sempre acreditar em mim, por me incentivar e me apoiar durante esses 12 anos de convivência.

Agradeço ao meu orientador Prof. Dr. Marcos Antônio de Oliveira, que aceitou de coração aberto me orientar no doutorado, deixando seu laboratório de portas abertas para que eu pudesse desenvolver minha pesquisa. Marcão, obrigada! Eu aprendi muito durante esses anos de convivência, você sempre será um grande exemplo a ser seguido!

À todos os colegas do LABMA e LABIMES, que tornaram essa trajetória mais leve e descontraída. Obrigada pelas boas conversas, pelo apoio durante meus experimentos – e fora deles. Em especial Alexandre e Kainã, obrigada pelos longos anos de trajetória. Todos vocês sempre terão um lugar especial em meu coração!

Em especial, gostaria de agradecer às minhas alunas de IC que são uma parte importantíssima do meu doutorado. Giovanna, Bia, Luana e Manu, obrigada por compartilharem comigo este momento tão importante. Contem comigo sempre!

À Isa, meu anjo particular, obrigada por simplesmente existir em minha vida. Amo você!

Ao programa de Pós-graduação em Biodiversidade de Ambientes Aquáticos (PPG-BAC), em especial à coordenadora do Programa Prof. Dra. Ana Júlia Fernandes e à Fabiana Cerqueira, Supervisora da Seção Técnica de Pós-graduação.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo financiamento de minha pesquisa no país e pela bolsa de doutorado sanduíche (Programa PDSE).

À Prof. Dra. Anna Capaldo e aos colegas da *Università degli Studi di Napoli Federico II*, que me receberam de braços abertos e fizeram eu me sentir em casa. Muito obrigada!

Ao universo, que de alguma maneira me guiou até aqui.

"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).

GENERAL OBJECTIVES

Considering the need to understand the action of azithromycin on non-target aquatic organisms due to their environmental persistence, this work was divided into two chapters presented in the form of scientific articles:

Chapter I: This chapter presents the results obtained through the evaluation of target organs used in ecotoxicological studies, such as liver and gills when exposed to the antibiotic azithromycin for 15 days. We aim at the biochemical and histopathological alterations that the presence of antibiotics can cause in organs widely used in ecotoxicological studies.

Chapter II: This chapter aimed to evaluate the influence of azithromycin on the intestinal microbiota of a non-target fish species (*Poecilia* reticulata) and how changes in the intestinal microbiota can affect intestinal homeostasis and the expression of neurotransmitters important in maintaining digestive tract function.

Assessing azithromycin's ecological toll: unveiling multifaceted impacts on *Poecilia reticulata* through biomarker analysis.

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HIGHLIGHTS

- Azithromycin exposure elevates the hepatosomatic index in *Poecilia reticulata.*
- Altered redox responses in gills indicate oxidative stress induced by azithromycin.
- The exposure led to significant, dose-dependent tissue damage in liver and gills.
- Principal component analysis reveals distinct differences in homeostasis between control and exposed groups.
- Multi-biomarker assessments are essential for accurate environmental health diagnostics.

ABSTRACT

This study investigates the impact of environmentally relevant concentrations of azithromycin on *Poecilia reticulata*, through biomarkers at different structural levels. To this end, the somatic indexes of *P. reticulata* were evaluated, and liver and gill samples were collected and analyzed for biochemical and histopathological alterations. Azithromycin caused significant effects in *P. reticulata*, such as increased hepatosomatic index, altered redox responses, particularly in gills, indicating oxidative stress, and notable tissue damage in the liver and gills in a dose-dependent response manner. Principal Component Analysis highlighted differences between control and exposed groups, demonstrating the azithromycin's influence on organismal homeostasis. This research underscores the importance of understanding azithromycin action in nontarget organisms of aquatic environments.

Key-words: emerging contaminants; antibiotic; fish; liver; gills; histopathology; biochemical biomarkers.

INTRODUCTION

The use of antibiotics has undeniably brought immense human health benefits; however, their widespread consumption for fighting against bacterial infections and for livestock production has also triggered environmental concerns. Upon ingestion, antibiotics undergo metabolism through two distinct pathways (Kapoor et al., 2017). The first pathway involves oxidation, reduction, hydrolysis, and alkylation reactions, while the second pathway involves the formation of more polar and hydrophilic metabolites, namely conjugates of glucuronides and sulfates (Orhan, 2021). These substances are then excreted through urine or feces (Cunningham et al., 2006; Heberer, 2002; Jones et al., 2008) eventually finding their way into aquatic environments *via* wastewater or improper sewage disposal (Gothwal and Shashidhar, 2015).

Since between 40 and 90% (depending on the class of drugs) of the antibiotic dose is excreted in the feces and urine as a parent compound, that is in the active form (Polianciuc et al., 2020), the release of these compounds into aquatic ecosystems raises major concerns. Indeed, due to the not always-complete removal of antibiotics and their metabolites by wastewater treatment plants (WWTPs) (Polianciuc et al., 2020), these substances are traceable in sludge and effluent disposal sites, due to the discharge of personal hygiene and medical products, pharmaceutical industry effluents, and hospital wastes (Rivera-Utrilla et al., 2013), coming to contaminate the environment. Because of their cytotoxic properties, antibiotics and their metabolites contribute to the emergence of antibiotic-resistant bacteria and exert their potential chronic effects on biota, despite their presence in exceptionally low doses (Kümmerer et al., 2014). Indeed, adverse effects have been observed on living organisms, such as damage to DNA molecules, inhibition of DNA synthesis, and disruption of cell replication (Yang et al., 2020).

The current high global consumption of antibiotics, especially azithromycin, and the water solubility of these compounds and/or their metabolites make them pseudo-persistent contaminants in aquatic ecosystems (Huang et al., 2014). In addition, when not used within their validity deadline, antibiotics are often irregularly disposed of, reaching sewage treatment systems and, consequently, surface waters and even drinking water (Carvalho and Santos, 2016). Once in the environment, antibiotics may be absorbed by a range of organisms and spread across the trophic chain, thus posing serious threats to animals and humans (Tamtam et al., 2008).

Although antibiotics were originally developed to target pathogenic bacteria, non-target organisms are inevitably exposed to their residues and by-products (Carvalho and Santos, 2016). Macrolides are generally expected to exhibit low solubility in water, but azithromycin (AZM) is an exception, as it can reach high concentrations in aquatic environments (Rizzo et al., 2013). These macrolide's persistence in the environment is attributed to their effectiveness in treating various diseases; moreover, AZM has been widely used in some countries to combat secondary infections in individuals infected with SARS-CoV-2 (Echeverría-Esnal et al., 2021).

Like other macrolides, AZM is a naturally occurring compound characterized by a 14-, 15-, or 16-membered macrocyclic lactone ring, to which one or more deoxy sugars may be attached (Bakheit et al., 2014). This antibiotic exhibits bacteriostatic properties against both gram-positive and gram-negative bacteria, by binding to the P site on the 50S subunit of the bacterial ribosome. This antibiotic mode of action demonstrates high efficacy in treating airway infections. It functions by inhibiting pathogenic invasion via CD147, an integral membrane protein, thereby disrupting cell membranes. Additionally, AZM acts as a lysosomotropic agent, increasing the pH of endosomes and lysosomes, which impedes viral replication. Furthermore, AZM enhances host type I interferon (IFN) kinetics, interfering with viral and bacterial replication processes (Firth and Prathapan, 2020).

While considered one of the safest drugs for any nation's health system by the World Health Organization (OMS, 2019), concerns about the rising presence of AZM in aquatic environments are growing (Li et al., 2022). Azithromycin concentrations in aquatic ecosystems vary widely, ranging from 62 ng/L to 327 ng/L (Li et al., 2022), with exceptionally high concentrations reported in the United States and Portugal (Fernandes et al., 2020; Jones-Lepp et al., 2012; Massey et al., 2010). In Brazil, urban rivers have recorded AZM concentrations reaching up to approximately 4 µg/L (Böger et al., 2021).

Despite its widespread use in aquaculture, elevated AZM concentrations in aquatic environments can pose significant risks to numerous non-target organisms. Several studies have demonstrated its potential to cause histopathological liver damage, neurotoxicity, oxidative stress, and cardiovascular toxicity (Yan et al., 2019; Shiogiri et al., 2017; Mhadhbi et al., 2022). These findings underscore the necessity for toxicological

studies to provide insights into the compartmentalization of this antibiotic in other non-target organisms during chronic exposure events.

The liver and gills serve as pivotal organs in environmental quality analysis due to their roles as primary routes of exposure to contaminants. The gills are in direct contact with the external environment and thus are particularly susceptible to environmental pollutants. Conversely, the liver plays a crucial role in the organism detoxification processes, encountering contaminants absorbed through the gastrointestinal tract via the enterohepatic circulation. Numerous studies have demonstrated the responsiveness of these organs to environmental stressors, especially in terms of histopathological changes, which often correlate with impaired organ functionality (Marinsek et al., 2024).

This study aims to evaluate the histological and biochemical responsiveness of target organs used in ecotoxicological studies to generate data on the impacts caused by the constant disposal of azithromycin on non-target organisms, specifically fish of the species *Poecilia reticulata*. This study will focus on analyzing the effects of chronic exposure to azithromycin on the gills and liver, organs that play crucial roles in responding to environmental stressors and in the detoxification of contaminants. By investigating histopathological and biochemical changes in these organs, we seek to better understand how azithromycin, a persistent substance in aquatic environments, can affect the health and functionality of aquatic organisms, thereby providing essential information for environmental risk assessment and the formulation of pharmaceutical waste management policies.

MATERIAL AND METHODS

Chemicals

Azithromycin (purity >98%) was purchased from Sigma-Aldrich® (product number: 75199). The stock solution was diluted in dimethylsulfoxide (DMSO, product number: D8418) (50mg/ml) and was stored at -20ºC, and the aliquots for each group were removed from this solution.

Organisms and Experimental Design

The experiment used adult specimens of *Poecilia reticulata* (females) obtained from a local fish producer. Females were chosen because they are bigger than males and provided us with the minimum amount of tissue necessary to fulfill the analysis. The body parameters presented an average length of 2.5 cm and an average weight of 0.3 g. Before the experiment began, the specimens were acclimated to the laboratory conditions for 72 hours in a tank containing 250 L of dechlorinated tap water under a natural photoperiod. After the acclimation period, 25 fish were distributed in each aquarium containing 10 L of water, maintained under constant aeration; physicochemical parameters such as temperature, pH, salinity, and dissolved oxygen were closely monitored and regulated throughout the exposure period.

The water from each aquarium was 50% renewed with the total concentration of AZM for each group and the animals were fed once a day. All procedures were performed in compliance with relevant laws and institutional guidelines and the appropriate institutional committee(s) have approved the experiment (CEUA: 01/2020).

The fish were exposed for 15 days to environmentally relevant concentrations of azithromycin, based on the levels reported to Brazil (Böger et al., 2021), divided into five groups: a control group (C0) exposed to water, a solvent control containing the vehicle DMSO (16u/l), and three experimental groups exposed to azithromycin concentrations at 2, 4, and 16 µg/L, respectively. Each group was housed in separate aquariums with a total volume of 10 L, containing 25 individuals (0.8 g per liter) across three replicates per group (total of 12 aquariums). To maintain the integrity of the azithromycin molecule, the water containing the total AZM concentration was 50% renewed every three days (Tong et al., 2011).

After the 15-day exposure, the fish were euthanized using a 0.5 ppm benzocaine solution until opercular movements ceased. Subsequently, all the individuals were measured and dissected under a stereomicroscope, and their liver and gillswere carefully collected. 10 organisms were destined to histopathological technique and the rest to biochemical analysis, which was performed in sample polls of 5 individuals each.

Somatic Biomarkers

The physiological condition of the tested fish, their general well-being and adaptation to the environment were evaluated through the condition factor (CF) and the hepatosomatic index (HSI). The CF is used to investigate the degree of rigidity that the organism finds itself in each environment, which reflects on the animal's well-being (Le Cren, 1951). To determine the condition factor (CF), organisms' total weight and total length were obtained and used in the equation adopted by Vazzoler (1996).

$$CF = Wt/Lt^b$$

Wt is the total weight, Lt is the total length and b is a weight-to-length ratio coefficient.

The hepatosomatic index indicates the weight of the liver to the total body weight, (Al-Ghais, 2013) and is calculated by the equation:

$$HSI = Lw/Tw \times 100$$

Where Lw means liver weight and Tw means the total weight of the organism.

Biochemical biomarkers

The following biomarkers, involved in oxidative stress response, were evaluated: glutathione-S-transferase (GST) and glutathione peroxidase (GPx) enzyme activity, as well as total glutathione (GSH).

Liver and gills were homogenized at 4% w/v in Lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM DTT, 50 mM sucrose, 150 mM KCl, 1 mM PMSF, pH 7.6). The remaining sample was centrifuged at 12,000 g for 20 min at 4 °C, and aliquots of the supernatant were separated for analyses of GST and GPx enzyme activity and total GSH quantification.

The kinetics of GST (Keen et al., 1976), and GPx (Sies et al., 1979) were determined by spectrophotometry at 340 nm and GSH quantification was analyzed at 415 nm. All results were normalized by the total protein concentrations, which were determined spectrophotometrically at 595 nm (Bradford, 1976) using a standard curve of BSA (bovine serum albumin protein) with the following concentrations: 1mg/ml, 0,5mg/ml, 0,25mg/ml, 0,12mg/ml, 0,06mg/ml and blank. Absorbance and fluorescence readings were performed in a microplate reader (Biotek-Synergy ™ HT).

Histopathological index

Liver and gills samples were fixed in 10% formaldehyde, diluted in aqueous solution, and subjected to standard histological procedures. Then, 3 semi-serial sections of 5 µm thickness each were obtained from each animal and stained with hematoxylin-eosin for morphological and histopathological analysis proposed by Bernet et al. (1999). The lesions were identified according to the pre-established importance factor (w) according to the severity of the lesion: The value 1 was applied for lesions with minimal pathological importance, 2 for intermediate lesions, and 3 for lesions with high pathological importance that are usually irreversible.

The anomalies found in the organs evaluated were classified according to a score (a) ranging from 0 to 6 depending on the degree and extent of the lesion, where 0 represents no occurrence and 6 is a very frequent occurrence. Then, the importance factor (w) was multiplied by the score (a), and, thus, we obtained the alteration index. To calculate the total histopathological alteration index (HPI) of the organ, the sum of the importance factor x score (total alteration = $\sum(w * a)$) was performed.

Morphometrical analysis

The morphometric analysis was performed only on gills, from an adaptation of the method proposed by Nero and colleagues (2006). We randomly captured three photomicrographs (100× magnification) of each of the sections available on each slide, totaling 9 photomicrographs/animal. From each captured image, the measurements were performed in each of the secondary lamella that appeared completely in the image, and the obtained measurements were secondary lamella length (SLL), secondary lamella width (SLW), interlamellar distance (ILD), and basal epithelium thickness (BET). For the SSL and ILD measurements, three measurements were taken representing the basal, medial, and apical portions of the lamella, and the results were averaged.

The thickness of the basal epithelium was measured 6 times per image captured, with two measurements taken at the beginning, middle, and end of the sampled epithelium, respectively. From these measurements, an average of the BET per section was taken. The proportion of the secondary lamellae available for gas exchange (PAGE)

was averaged for each filament of an individual and calculated as (Hughes and Perry, 1976):

$$PAGE = 100 \times ((mean\ SLL)/((meanBET+meanSLL)))$$

Statistical Analysis

The data was initially checked for normality, using the Shapiro-Wilk test, while homoscedasticity was evaluated with Levene's test. Statistical differences in mean values for each variable (n=10) were analyzed using analysis of variance (ANOVA), followed by Tukey's test for pairwise comparisons if parametric test assumptions were met. In cases where assumptions were violated, Kruskal-Wallis's test was utilized, followed by Dunn's post-test.

The control (C0) and DMSO groups were compared with a T-Student test and did not show differences. In this sense, the data presented as control is referred to as solvent control only (control).

The Analysis of Similarities (ANOSIM) was employed to assess the impact of various AZM concentrations (0, 2, 4, and 16 µg/L) on biochemical and histological biomarkers. ANOSIM was chosen for its robustness in handling multivariate data and its ability to reveal statistical significance in treatment differences. A total of 9999 permutations were conducted to ensure statistical reliability. Dissimilarity within each concentration group (mean rank within) and between different concentration groups (mean rank between) was calculated to understand treatment variance. The R-value quantified dissimilarity between groups, providing an effect size measure. Statistical significance was determined using a p-value, with a threshold of 0.05 indicating significance.

Calculation of IBRv2

IBRV2 values were calculated according to the method proposed by Sanches and coworkers (2013). Briefly, the individual biomarker data (Xi) were compared with the mean reference value (X0) and a log transformation was performed to reduce the variance.

$$Yi = log\ (Xi/X0)$$

After, the general mean (m) and standard deviation (s) were calculated, and the Yi values were then standardized using the formula:

$Zi = (Yi-m)/s$

To create a zero basal line and to show the variation of a biomarker according to this basal line, the mean of the standardized biomarker response (Zi) and the mean of reference biomarker data (Z0) were used to define a biomarker deviation index (A). A>0 indicates that the biomarker was induced, and A<0 means it was inhibited.

$A = Zi - Z0$

To obtain an integrated multi-biomarker response, IBRv2, the absolute value of A parameters was summed and divided by the number of biomarkers.

RESULTS

Somatic biomarkers

No statistical differences were observed among groups for the conditional factor, even if a discernible decrease in this biomarker was noted in exposed groups. Azithromycin exposure resulted in a notable increase in the hepatosomatic index (HSI), particularly evident in the group exposed to the highest concentration (16 µg/L)-(Figure 1).

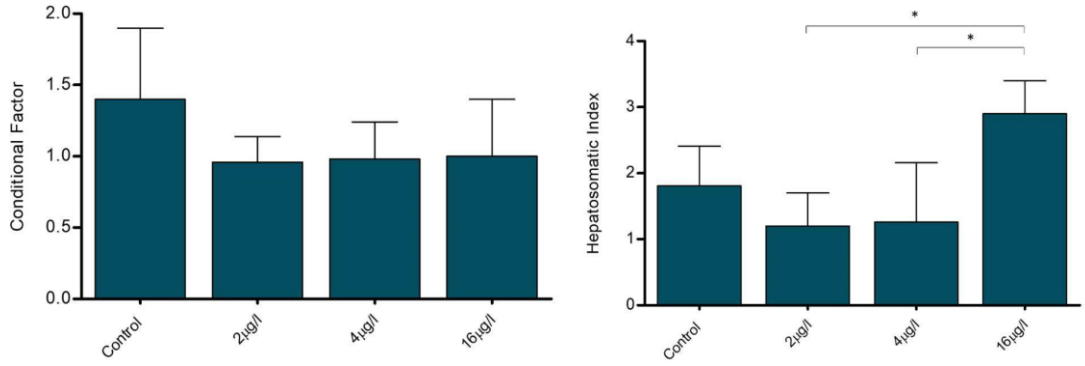

Figure 1. Somatic biomarkers (conditional factor and hepatosomatic index) of fish *Poecilia reticulata* exposed to the antibiotic azithromycin at 0µg/L, 2µg/ L, 4µg/L, and 16µg/ L for 15 days.

Biochemical biomarkers

The exposure to AZM induced alterations in redox responses in both analyzed organs, but the response was more prominent in the gills. It was possible to observe a considerable reduction in GSH content in all groupxs exposed to the antibiotic, and an increase in both GPx and GST activity (Figure 2).

In the liver, GSH content was considerably increased in the group exposed to the higher concentration (16 ug/l). An increase in GPx activity in the animals of this group was also observed, whereas changes in GST activity were not observed (Figure 2).

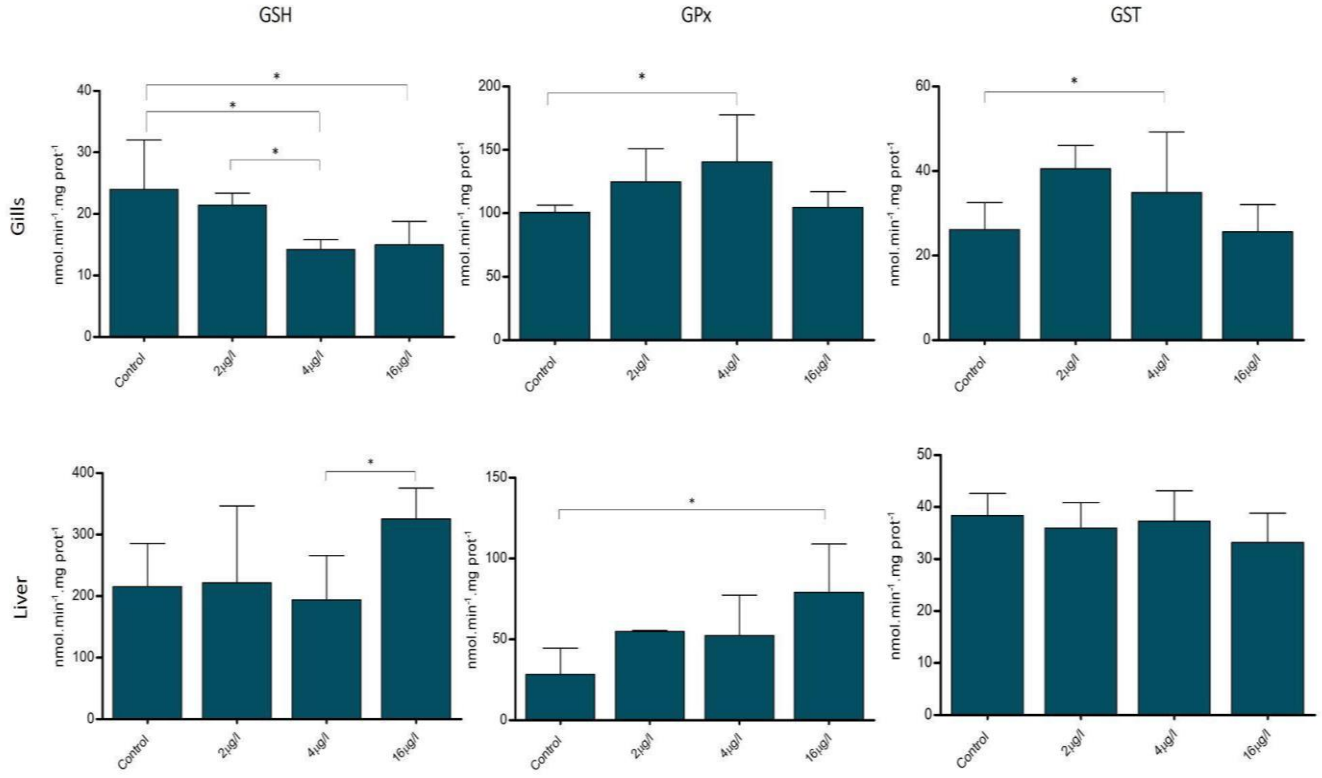

Figure 2. Gills and liver biochemical biomarkers (Total glutathione - GSH, Glutathione peroxidase - GPx, and Glutathione S-transferase - GST) of fish *Poecilia reticulata* exposed to the antibiotic azithromycin at 0µg/L, 2µg/L, 4µg/ L, and 16µg/ L for 15 days.

Histopathological index and morphometry

The exposure to AZM induced changes in tissue architecture in both organs studied; however, the highest histopathological index was observed in the gills, where there was

a dose-dependent response (Figure 3). In the liver, the lower concentrations did not directly affect the histopathological index, but it was possible to observe more drastic pathologies at the higher concentrations (Figure 3).

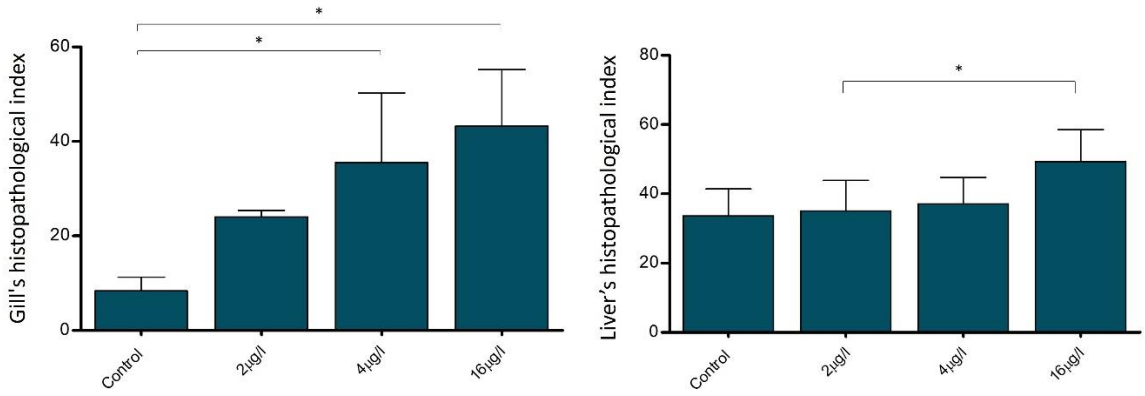

Figure 3. Gills and liver histopathological index (HPI) of fish *Poecilia reticulata* exposed to the antibiotic azithromycin at 0µg/ L, 2µg/ L, 4µg/ L, and 16µg/ L for 15 days.

The most common pathologies observed in the gills were vascular congestion, tissue hypertrophy, and hyperplasia. Some points of lymphocyte infiltration were also observed (Figure 4). In the liver, although the HPI was lower, the pathologies observed were more severe and classified as irreversible. These included intense necrotic and hemorrhagic spots, preceded by hepatocellular degeneration, fibrillar inclusions, and steatosis (Figure 4). Besides histopathological damage, there were no significant differences in gill morphometry and the proportion of the secondary lamellae available for gas exchange (PAGE) (Table 1), indicating that the tested AZM concentrations did not change lamellar morphometry.

Table 1. Gills morphometry and secondary lamellae available for gas exchange (PAGE).

Groups	Secondary lamellar weight	Secondary lamellar length	Inter lamellar distance	Basal epithelium thickness	PAGE (%)
Control	41.7 ± 12.7	9.5 ± 3,6	26.6 ± 6.1	7.5 ± 1,8	84.6
2ug/L	48.0 ± 11.9	9.9 ± 3.9	27.4 ± 11.2	8.0 ± 1.8	85.6
4ug/L	42.8 ± 10.7	11.5 ± 5.4	32.4 ± 8.8	7.6 ± 1.6	84.8
16ug/L	46.4 ± 18.4	10.8 ± 4.3	29.2 ± 9.9	7.8 ± 1.1	84.3

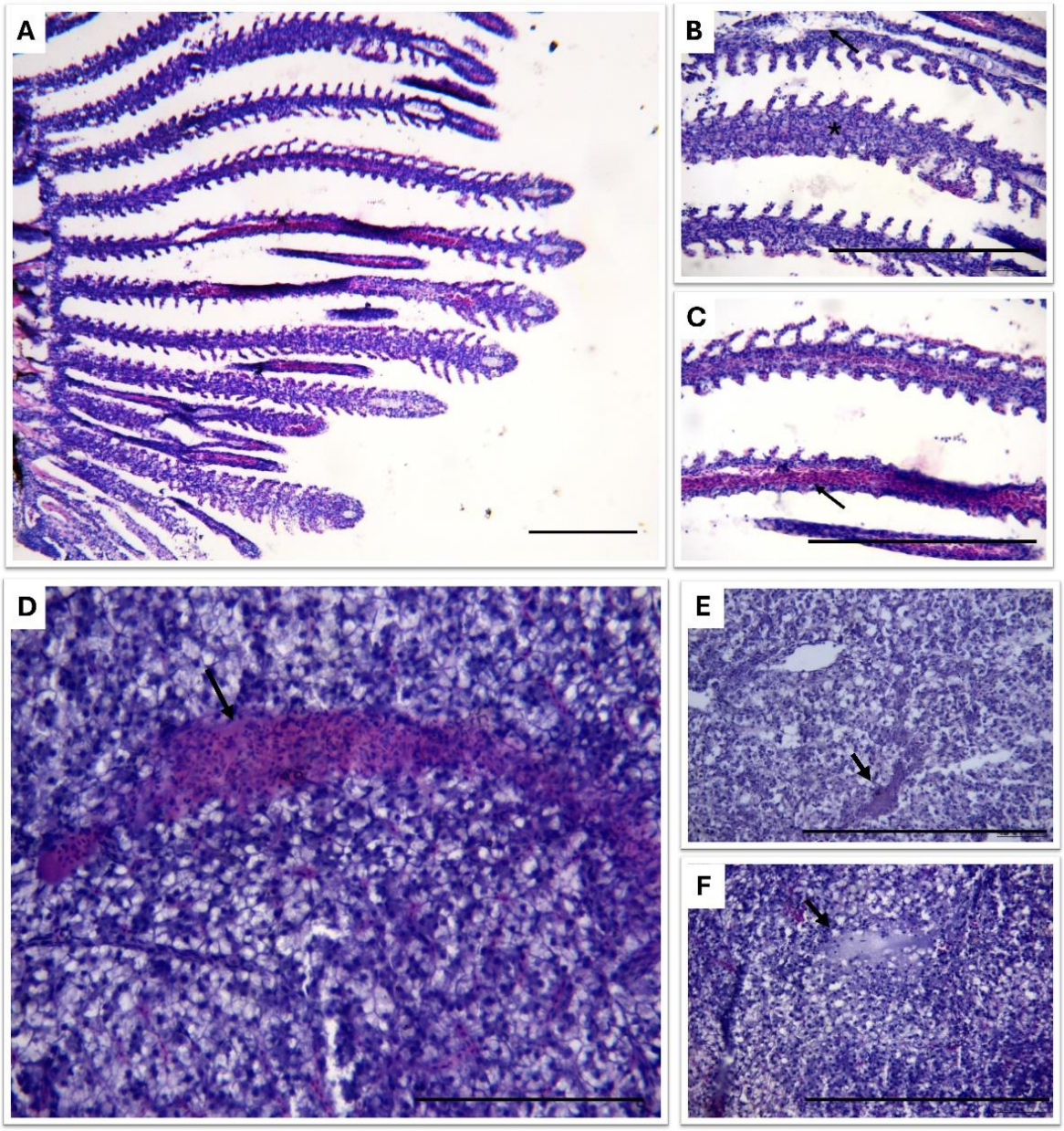

Figure 4. Gills and liver photomicrography of fish *Poecilia reticulata* exposed to the antibiotic azithromycin at 0µg/ L, 2µg/ L, 4µg/ L, and 16µg/ L for 15 days. **A)** gills of exposed fish showing the general architecture of the organ. **B)** It's possible to observe a necrotic spot (arrow) and epithelial hyperplasia and lymphocyte infiltration (*) and **C)** vascular congestion. **D)** It's possible to observe a hemorrhagic spot (arrow) and also steatosis of the exposed group; **E)** fibrillar inclusion (arrow) and **F)** necrotic spot. Stain: Hematoxylin-eosin, bar = 100µm.

Data Integration

The ANOSIM results indicated a statistically significant difference in the effects of AZM at varying concentrations on the studied biomarkers. The analysis revealed a mean rank within groups of 77.8, highlighting the average dissimilarity within each AZM concentration group, and a mean rank between groups of 100.2, suggesting greater dissimilarity between the different treatment groups than within them. The calculated R-value of 0.236 indicates a moderate level of dissimilarity between the groups, implying that different AZM concentrations have distinct impacts on the analyzed biochemical and histological biomarkers. The p-value obtained was 0.0145, indicating that the differences between the groups are statistically significant (Table 2).

IBR2v values

The IBR index was separately calculated for the gills and liver to provide a clearer understanding of each organ's response. In gills, the most prominent biomarker was the histopathological index, which was higher in organisms exposed to 4 and 16 ug/ L, respectively. The liver showed more prominent biochemical alterations, showing a higher alteration in GPx and GST responses also at the higher concentration of azithromycin (16 ug/ L). The histopathological index was also prominent in this group (Figure 5).

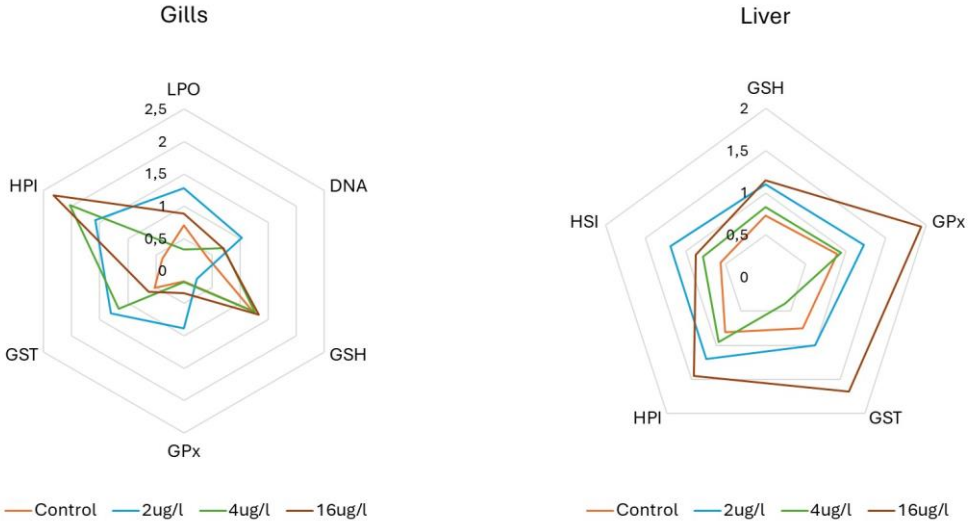

Figure 5. Integrated biomarker response version 2 (IBRv2) for gills and liver of *Poecilia reticulata* exposed to 0µg/ L, 2µg/ L, 4µg/ L, and 16µg/ L azithromycin for 15 days.

DISCUSSION

For exploring azithromycin's (AZM) ecotoxicological impacts on aquatic organisms, our study on *P. reticulata* provides a nuanced understanding that complements and extends findings from previous literature. Our experimental design, which exposed specimens to environmentally relevant AZM concentrations revealed a suite of morphological and biochemical responses, highlighting this antibiotic's subtle yet significant effects on aquatic life.

Our findings indicate an elevated hepatosomatic index (HSI) at the highest tested AZM concentration (16 µg/L), aligning with the broader consensus on its toxicity profile. Notably, AZM has been identified as non-toxic to species such as *Oreochromis niloticus* at concentrations reaching up to 100 mg/L (Shiogiri et al., 2017), who also reported hematological alterations and minor histopathological liver damage following chronic exposure. Our results showed different patterns for *P. reticulata.*

The increase in HSI of exposed organisms may be related to the presence of the antibiotic, as demonstrated in previous studies with different antibiotics such as oxytetracycline (Nakano et al., 2018). The increase in the hepatosomatic index could be related to changes in liver metabolism, which could lead to an increase in the relative weight of the liver in relation to body weight (Kumar et al., 2017). The metabolic stress caused by the chronic presence of the antibiotic may be triggering an increase in hepatic metabolic activity. In addition, studies have reported that the presence of azithromycin can have direct effects on the liver, such as inflammation and fat accumulation (Olayinka and Ore, 2014). The liver of the organisms evaluated in this study showed steatosis as a highly frequent pathology. Another possibility is related to the need to reallocate energy resources to this organ, which could result in an increase in liver weight in relation to body weight.

It is important to notice that the most prominent liver alterations were observed in the group exposed to 16 µg/L, indicating that this concentration has more prominent toxicological effects on the liver. This can be explained by the fact that the lowest concentrations reached this organ in insignificant doses, due to the high metabolic capacity of the organ in question. In fact, at this concentration, the histopathological index was higher, and the liver showed pathologies such as necrosis, hemorrhage, hepatocellular degeneration, fibrillar inclusions, and steatosis. These pathologies can

impair normal liver function, since pathologies such as necrosis are followed by extensive fibrillar inclusions leading to the loss of functional hepatocytes, reducing the synthesis of important proteins such as albumin and coagulation factors, as well as leading to the accumulation of toxins in the body (Krishna, 2017).

In addition to the histopathological changes, biochemical changes were also observed at the same concentration, such as an increase in GSH levels and an increase in glutathione peroxidase (GPx) activity. The increase in GSH may indicate an adaptive response to the oxidative stress generated by the drug since it is one of the main endogenous antioxidants found in cells, playing a crucial role in the body's defense against oxidative damage (Vairetti et al., 2021). Its increase could also be related to the consumption of this tripeptide by antioxidant enzymes like GPx and GST. GPx, in turn, is an antioxidant enzyme that uses GSH as a substrate, neutralizing peroxides such as H_2O_2. The increase in GPx may be associated with a mechanism in the body to metabolize and eliminate peroxides generated during the hepatic AZM metabolization (Vairetti et al., 2021). Azithromycin is metabolized through biotransformation processes such as demethylation, hydroxylation, and n-oxidation, which aim to generate more hydrophilic and polarized metabolites, which are conjugated to glucuronides and sulfates, facilitating their excretion through bile or urine. This metabolism can generate reactive oxygen species such as hydrogen peroxide (Parnham et al., 2014).

Unlike the pattern observed in the liver, where the alterations were frequent at the highest concentration, the gills showed greater alterations mainly at the lower concentrations (2 and 4 µg/L). These alterations can be explained mainly by the different characteristics of the organs. The gills are directly exposed to the contaminants available in the aquatic environment and are more susceptible to the effects of various pollutants (Marinsek et al., 2024), such as AZM, even at lower concentrations. The liver is an organ specialized in metabolizing xenobiotic compounds. It therefore has specific tools to combat the stress generated by the drug, which requires a higher concentration to trigger responses in the biomarkers (Bruslé and Anadon, 2017). This could explain the difference between these two organs' responses.

In the gills, biochemical and histological biomarkers indicated more prominent alterations. The antioxidant responses indicated the activation of mechanisms to combat

oxidative stress, especially at lower concentrations, where it was possible to observe an increase in GPx and GST activity; as previously discussed, GST and GPx use GSH to metabolize available reactive oxygen species. In addition, GST acts in cellular detoxification, playing a crucial role in the conjugation of reduced glutathione with toxic compounds, which facilitates its excretion or neutralization. The increase in the activity of this enzyme indicates that the presence of AZM requires adaptations to deal with the oxidative stress generated by the drug to mitigate the damage to the tissue (Demirci-Cekic et al., 2022).

Histopathological damage to the gills showed a dose-dependent response, which indicates that the higher AZM concentrations, the higher this tissue damage. The most frequently observed pathologies were vascular congestion, tissue hypertrophy, hyperplasia, and inflammatory infiltrates. Although these pathologies are reversible, their chronicity can intensify the inflammatory process, inducing more severe and irreversible pathologies.

Vascular congestion in the gills can actively induce a reduction in gas exchange in the long term, although this data was not observed in PAGE. In addition, the accumulation of blood cells in the vessels can enlarge the production of reactive oxygen species, leading to oxidative stress and compromising gill function. In addition, vascular congestion can negatively affect immune function and compromise the body's electrolyte balance. Problematically, tissue hypertrophy and hyperplasia can lead to obstructed water flow and a reduction in the organ's surface area, which impairs the organ's gas exchange and compromises tissue function.

The integrated biomarker response (IBRv2) indicated, as demonstrated by univariate statistical analysis, that the liver showed more evident responses at the highest concentration, triggering responses in antioxidant defenses (GST and GPX) as well as in histopathological responses. The gills, in turn, responded peculiarly for each group evaluated, with more evident responses in the histopathological index and the amount of GSH in the groups exposed to 4 and 16 µg/L. In this sense, it's azithromycin demonstrates different toxic mechanisms for each organ.

Our study on the exposure of Poecilia reticulata to azithromycin (AZM) enriches the discourse on the potential adverse ecological effects of macrolides, illustrating the

complex interaction between pharmaceutical exposure and the health of non-target organisms. Through the analysis of biochemical and histopathological responses, our results indicate that the liver demonstrates greater resilience, with more evident responses only at higher doses of up to 16 µg/L. In contrast, the gills showed a dose-dependent response, suggesting that even doses below those currently found in the environment can trigger oxidative responses and tissue damage in this organ.

An elevated hepatosomatic index and biochemical changes such as increased levels of GSH and glutathione peroxidase (GPx) activity in the liver indicate an adaptive response to oxidative stress generated by AZM. Additionally, observed liver pathologies such as necrosis, hemorrhage, and steatosis reinforce the toxic potential of AZM at high concentrations. In the gills, histopathological and biochemical alterations were more prominent at lower AZM concentrations, reflecting this organ's direct susceptibility to environmental contaminants. Antioxidant responses, with increased GPx and GST activity, indicate the activation of mechanisms to combat oxidative stress, while pathologies such as vascular congestion and tissue hypertrophy highlight the potential damage caused by chronic exposure.

In conclusion, this study demonstrates that even low doses of azithromycin, currently found in aquatic ecosystems, can trigger significant biochemical responses and tissue damage in non-target organisms. Thus, it is crucial to pay attention to emerging contaminants present in very low doses in aquatic ecosystems, such as azithromycin, as these contaminants can promote changes in populations and communities chronically exposed.

To mitigate the adverse effects of macrolides in aquatic ecosystems, it is essential to implement public policies that include rigorous monitoring of antibiotic levels in water bodies, and establishing limits based on recent ecotoxicological data. Improving the efficiency of sewage treatment plants with advanced technologies to remove antibiotics and their metabolites is crucial. Additionally, promoting awareness campaigns for the proper disposal of medications and implementing specific collection points can prevent environmental contamination. Encouraging the rational use of antibiotics in human and veterinary medicine, restricting over-the-counter sales, and developing educational programs on environmental risks and disposal practices are important measures. Finally,

supporting ongoing research on the ecotoxicological impacts of antibiotics and the development of less harmful alternatives is fundamental to protecting biodiversity and public health.

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Impact of azithromycin on the microbiota and intestinal homeostasis of *Poecilia reticulata*

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HIGHLIGHTS

- Azithromycin alters the intestinal microbiota of *P. reticulata* in a dose-specific manner.
- The exposure to the antibiotic impact's intestinal morphology, leading to inflammation.
- The antibiotic caused a reduction in both nitrergic and vipergic myenteric neuronal density.

ABSTRACT

The intestinal microbiota plays a crucial role in maintaining host homeostasis by modulating and activating the innate and adaptive immune systems, controlling metabolism and nutrient absorption, protecting against pathogenic organisms, and regulating inflammatory processes. Chronic exposure to antibiotics in aquatic environments, due to continuous disposal, can alter the microbial community of non-target organisms, impairing intestinal homeostasis. This study aimed to evaluate whether environmentally relevant concentrations of azithromycin, a widely prescribed broad-spectrum antibiotic, modulate the intestinal microbiota of *Poecilia reticulata*, a non-target fish and whether changes in the intestinal microbiota can affect intestinal homeostasis by reducing absorptive rates, mucosal permeability, enteric nervous system neuron density, and the serotonergic system. Our data indicate that 15-day exposure to the antibiotic resulted in significant changes in the alpha diversity of the microbiota in all evaluated groups (exposed to 2 µg/L, 4 µg/L, and 16 µg/L of azithromycin). These changes were followed by alterations in the goblet cells and intraepithelial lymphocytes density and modifications in the thickness of intestinal layers, significantly reducing the density of VIPergic neurons and increasing the density of nitrergic neurons. A reduction in the expression of the TPH1 enzyme, responsible for serotonin biosynthesis in the gastrointestinal tract (GIT), was also observed. Our results suggest that the chronic presence of azithromycin in aquatic environments can impair the intestinal homeostasis of non-target organisms, affecting nutrition, and potentially disrupting trophic balance in the long term due to its impact on neurotransmitters related to behavioral responses, such as serotonin, which influences predatory and reproductive behaviors.

Keywords: metagenomics, enteric nervous system, histology, western blotting, serotonin.

INTRODUCTION

The gut microbiota consists of a range of microorganisms that relate symbiotically with the host's gastrointestinal tract (GIT) (Andrea et al., 2018; Neish, 2009). The symbiotic relationship works in a beneficial way where the microorganisms benefit the host by extending their metabolic/digestive abilities and compete with pathogenic microorganisms while consuming the nutrients available in their micro-habitat (Kayama et al., 2020). For the relationship between the host and the microbiota to be beneficial, both parties must establish connections that influence and regulate each other in a mutual way (de Weerth, 2017).

The microbiome is essential for maintaining a balance and the health of the GUT environment, supporting beneficial bacteria while protecting the host's intestinal lining and immune system. However, the presence and diversity of bacteria in the intestinal lumen can be a major problem for mucosal enterocytes and the immune system (Kayamana et al., 2020) and to symbiosis occur properly, numerous molecular mechanisms are involved in modulating the intestinal microbiota-mucosa interaction (Katyama et al., 2020). This crosstalk process occurs by host cells' transmembrane or intra-cytoplasmic receptors known as pattern recognition receptors (PRRs) whose main function is to recognize and bind to specific bacterial macromolecules. The specific microbial ligands are called microbial-associated molecular patterns (MAMPs), which include lipopolysaccharides (LPS - produced by gram-negative bacteria), flagellin, peptidoglycans, and peptides (Keogh et al., 2021). Some intestinal diseases are caused by mutations that interfere with the interaction between PRRs and MAMPs, causing severe mucosal inflammation (Qing et al., 2022; Yue et al., 2019).

The activation of PRRs by MAMPs initiates several metabolic pathways such as nuclear transcription factor κβ (NFκβ), MAP kinases, and caspase-dependent signaling cascades (Yoo et al., 2020). These pathway's activation is crucial for the host GIT's normal development. Some studies have demonstrated relevant physiological changes in the intestine, such as increased barrier permeability, altered mucosal immunity, and inflammatory processes in organisms with microbiota dysbiosis, suggesting the fundamental role of these microorganisms for the maintenance and integrity of intestinal barrier functions (Amoroso et al., 2020; Solis et al., 2020).

The complexity of the intestinal barrier stems from several factors that work together to maintain intestinal homeostasis. Among these factors, goblet cells are responsible for numerous functions, mainly associated with intestinal mucosa mechanical defense (Yang and Yu, 2021). The expression of genes related to mucin secretion by goblet cells can be altered depending on the intestinal microbiota (Zhang et al., 2020). In addition, the modulation of intestinal functions through intestinal permeability control), immune functions, motility, nutrient absorption, and enteric nervous system (ENS) activity are also involved with microbiota and its metabolites (Gustafsson & Johansson, 2022; Chakaroun et al., 2020; Fung, 2020; Heiss and Olofsson, 2019).

The enteric nervous system is an intrinsic nervous system in the gastrointestinal tract (GIT) responsible for motility control, secretion, and absorption of nutrients (Furness, 2006). Several studies have shown that changes in microbiota composition can affect the expression of important neurotransmitters for GIT activities, such as nitric oxide (NO) and vasoactive intestinal peptide (VIP) (Bains et al., 2019; Walker et al., 2018). Both neurotransmitters play a crucial role in relaxing intestinal smooth muscle and controlling mucosal functions involved in secretion and absorption (Sarkey & Mawe, 2023). In addition, they are involved in numerous cell signaling pathways, especially in local inflammatory processes. Several studies have reported changes in these neurotransmitter's expression during acute inflammatory events caused by intestinal dysbiosis (Hong et al., 2021).

The intestinal microbiota also plays a fundamental role in serotonin (5-HT) availability to the central nervous system (CNS). Some studies link changes in microbiota with cognitive problems, learning process, hyperactivity, anxiety, and depression (Simpson et al., 2021; Novotný et al., 2019; Jiang et al., 2018; Cenit et al., 2017), as well as changes in the expression of serotonin receptors (5-HT1A) (Buey et al., 2023). In humans, alterations in microbiota are related to neurodegenerative diseases, depression, schizophrenia, and autism, indicating a strong correlation between the microbiota-intestine-brain axis (Murray et al., 2023; Kushak et al., 2022; Liu et al., 2021).

Serotonin (5-HT) is produced from the essential amino acid tryptophan (Tichard et al., 2009), which can be obtained by digesting protein-based foods and is the precursor of various metabolites such as kynurenine (Gao et al., 2020). Much of tryptophan

metabolism ends up occurring through the physiological kynurenine pathway, mainly through the hepatic enzymes tryptophan-2,3-dioxygenase (TDO), which is mostly induced by the presence of glucocorticoids, and indoleamine-2,3,-dioxygenase (IDO), which in turn responds to inflammatory stimuli (IFN-γ) (Xue et al., 2023).

After being produced, kynurenine can follow two distinct cascades, the first resulting in the formation of kynurenic acid (which is characterized by being highly neuroprotective but can lead to cognitive problems when in high concentrations) and the second resulting in the formation of quinolinic acid, which has neurotoxic effects (Xue et al., 2023). In this sense, it is important to note that the presence of stressful stimuli to the body, such as glucocorticoids and inflammatory processes, can increase TOD and IDO expression, which increase the tryptophan metabolization *via* the kynurenine pathway, reducing the availability of this amino acid for serotonin synthesis, which will cause gastrointestinal alterations as well the increasing the availability of neuroactive metabolites that will impact on the CNS and peripheral nervous system (PNS) (Roth et al., 2021).

Serotonin synthesis and storage, in turn, occurs through tryptophan metabolization, where this molecule is hydrolyzed into 5-hydroxytryptophan by tryptophan hydroxylase-2 enzyme (TPH2) in the central nervous system and TPH1 in peripheral regions such as enterochromaffin cells (Kanova & Kohout, 2021), pancreatic β-cells (Makhmutova et al., 2021), adipocytes (Moon et al., 2022) and osteoclasts (Collet & Coudert, 2021). This molecule is then decarboxylated into 5-hydroxytryptamine (5-HT) by the enzyme L-aromatic acid decarboxylase. Interestingly, only a small portion of 5-HT comes from the CNS, and the vast majority is peripherally synthesized, mainly in the intestine, representing around 95% of the body's 5-HT, regulating motility, secretion, and vasodilation activities (Koopnab et al., 2021).

The intestine synthesis and secretion of 5-HT, mainly by enterochromaffin (EC) cells, is directly influenced by the intestinal microbiota, which is responsible for fermenting food polysaccharides, that are converted into short-chain fatty acids (SCAFs), such as acetate and butyrate, which increase the expression of TPH1 mRNA in EC cells (Reigstad et al., 2015; Jones, 2016). In this way, the synthesis of 5-HT and other neurotransmitters can influence microglial activation and various brain functions (Abdel-

Haq et al., 2019), forming the microbiota-intestine-brain axis (Morais et al., 2021). Sensory information arrives from the gut to the encephalic nucleus, from where afferent limbs branch out to broad regions in the CNS (Sharkey & Mawe, 2023).

The gut microbiota composition, diversity and enzymatic capacity are readily affected by numerous environmental factors, such as the host's lifestyle, diet, and medications such as antibiotics and anti-inflammatory drugs (Song et al., 2020; Claus et al., 2016). In addition, various environmental contaminants can interfere with bacterial growth and induce dysbiosis, which has been associated with intestinal and systemic diseases (Das and Nair, 2019). Therefore, disturbances in microbiota composition induced by chemical agents may constitute an underestimated mechanism by which they interfere with human and environmental health.

The environmental quality of aquatic environments can be harmed by the constant dumping of pharmaceuticals due to their specific effects on biota since they are present in very low doses and can enter the biological cycle of numerous aquatic organisms (Kayode-Afolayan et al., 2022). They are biologically active compounds capable of causing damage to biota, as they can have functions like their origin or even cause side effects in organisms. The high consumption of antibiotics around the world and the fact that these compounds and/or their metabolites are soluble in water make these contaminants pseudo-persistent (Harrower et al., 2022). Furthermore, they are often discarded irregularly, reaching sewage treatment systems and, consequently, surface water and drinking water.

Several effects on the fish GIT microbiome have been reported, such as a reduction in the taxonomic diversity of bacteria and an increase in 90% of bacterial resistance genes when exposed to antibiotics (Yukgehnaish et al., 2020; Carlson et al., 2017). It has been shown that these contaminants can lead to bacterial dysbiosis, which can reduce the diversity of important taxa involved in metabolic capacity and can also promote horizontal gene transfer, leading to microbial drug resistance (Lambert & Schaik, 2022; Mclnnes et al., 2020).

The antibiotics available in the aquatic environment directly target the microbiota, and their absorption by the body affects the microorganisms of the GIT through various signaling pathways, altering the microbiota composition, leading to the development of

diseases in energy metabolism, impairing the absorption of nutrients and the immune defense system, as well as leading to an imbalance in the antioxidant defense system, raising the levels of reactive species and, consequently, leading to oxidative stress (Jin et al., 2015; Zhang et al., 2015).

The presence of antibiotics, specifically azithromycin has been shown to act directly on intestinal motility, which suggests direct action on the enteric nervous system (Delungahawatta et al., 2017) and may be related to mitochondrial toxicity (Jiang et al., 2018). Other studies have reported that antibiotics have specific mechanisms of neurotoxicity, acting directly on neurotransmitters such as NO, VIP, GABA, glutamate, and acetylcholine (Champagne-Jorgensen et al., 2019). Concerning the direct action of antibiotics on nerve cells, studies have shown that azithromycin has pro-apoptotic effects on neuronal cells, also inducing the formation of hydroperoxides and their precursor, mitochondrial superoxide, which leads to oxidative damage to DNA molecules and the cellular membranes (Xiao et al., 2018). The authors also proved that the action of azithromycin on the mitochondria of neuronal cells acts directly to inhibit the enzymatic activity of complexes I and IV of the electron transport chain, gradually reducing mitochondrial respiration and leading to cell death (Xiao et al., 2018).

Considering the ability of azithromycin to alter the microbiota composition and diversity and its effects on the enteric nervous system, this study aims to evaluate the composition of the intestinal microbiota and possible changes in intestinal morphology, in the enteric nervous system composition and the expression of proteins involved in serotonin synthesis in *Poecilia reticulata* exposed to this antibiotic, to understand the chronic effects that this contaminant can cause in non-alvo organisms since this organism is distributed through several aquatic habitats.

MATERIAL AND METHODS

Test Organisms

The experiment was performed on adult specimens of *Poecilia reticulata* (females) obtained from a local pisciculturist and, the body parameters of these fish presented an average length of 2.5cm and an average weight of 0.3g. Before the experiment began, the specimens were acclimated to the laboratory conditions for 72

hours in a box containing 250 liters of water under a natural photoperiod. After the acclimation period, 25 fish were distributed in each aquarium containing 10 liters of water equipped with constant aeration; physicochemical parameters such as temperature, pH, salinity, and dissolved oxygen were closely monitored and regulated throughout the exposure period.

The water from each aquarium was 50% renewed with the total concentration of AZM for each group and the animals were fed once a day. All procedures were performed in compliance with relevant laws and institutional guidelines and the appropriate institutional committee(s) have approved the experiment (CEUA: 01/2020).

Experimental Design

Specimens of *Poecilia reticulata* were subjected to a 15-day exposure to environmentally relevant concentrations of azithromycin. The specimens were separated into four groups: a control group (C0) exposed to water containing the vehicle DMSO and three experimental groups exposed to azithromycin concentrations of 2 µg/L, 4 µg/L, and 16 µg/L, respectively. Each group was housed in separate aquariums with a total volume of 10 liters, containing 25 specimens per liter (0.8 g per liter) across three replicates per group (total of 12 aquariums). To maintain the integrity of the azithromycin molecule, the water containing the total AZM concentration was changed every three days (Tong et al., 2011).

After the 15-day exposure, the specimens were euthanized using a 0.5 ppm benzocaine solution until opercular movements ceased. Subsequently, the specimens were measured and dissected under a stereomicroscope, and the intestine was carefully removed.

Feeding parameters

The experimental procedure was according to Baudou and colleagues (2017). Briefly, the following biological parameters were recorded daily in each aquarium of all groups: Mortality expressed as a percentage of cumulative mortality over time per treatment.

Estimated food intake (I): before each food offer, dead fish were removed from the aquarium and weighed to adjust the feed ratio according to updated biomass. Food was offered every day and any remaining debris was removed by siphoning, filtered, and dried to constant weight at 60 °C.

Food intake, expressed as mg food mg biomass^{-1} day^{-1} , was estimated as the difference of weight between the offered and the remainder amount of food.

Estimated fecal production (F): feces were collected by siphoning before each food offer, filtered, and dried to constant weight at 60 °C. It was expressed as mg feces·mg biomass^{-1} day^{-1}.

Estimated specific assimilation (A) was calculated as I − F.

Bacterial Metabarcoding

Total DNA from the intestinal microbiota was extracted using the QIAmp DNA Stool kit (QIAGEN), following the manufacturer's instructions. For each sample, the gene encoding 16S rRNA was amplified using the forward oligonucleotide (5' *TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***ACTCCTACGGGAGGCAGCA G** -3': the sequence in italics corresponds to the Nextera® transposase adapter sequences A, and the sequence in bold is the widely conserved primer 338F) and the reverse oligonucleotide (5'- *GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***TTACCGCGGCTGCTGGCA C** -3', the sequence in italics corresponds to the Nextera® transposase adapter sequences B and the sequence in bold is primer 239). Subsequently, the Illumina Truseq DNA Sample Preparation v2 kit with a barcode was used to prepare the libraries for each sample. The sequencing was carried out in collaboration with Prof. Dr. Anna Capaldo and Prof. Dr. Ezio Ricca from Università Degli Studi di Napoli Federico II. For metagenomic analysis, data were analyzed using the Illumina 16S Metagenomics software, which performs the taxonomic classification of the v3/v4 region of the 16S rRNA gene through the GreenGenes database.

Western Blotting

For protein expression analyses, primary antibodies for each of the proteins

produced in goats were purchased from ABCAM: TPH1 (Cat Num ab52954), IDO2 (ab131086), and 5-HT (ab273654). The second antibody was anti-goat IgG produced in rabbits and conjugated with horseradish peroxidase (Horseradish peroxidase - HRP) (ab205718). The total protein extract was isolated from GIT samples using RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors. The supernatant was subjected to electrophoresis in 15% SDS-PAGE. After the electrophoretic run, the proteins were transferred to AmershamTM HybondTM 0.45µm PVDF membranes (Cytiva), and the transfer efficiency was evaluated by staining the membranes with Ponceau solution (Ponceau S 0.2%, 3% acetic acid), also used to control the loading of experiments. Then, the membranes were washed with ultrapure water to remove the dye excess, blocked with 5% skimmed milk solution in TBS-T (20mM Tris-HCl pH7.5, 150mM NaCl, 0.1% Tween 20) for 2 hours and subsequently incubated with the polyclonal primary antibody for 2 hours at room temperature. Then, the membrane was washed with TBS-T and incubated with the secondary anti-IgG antibody conjugated to HRP for another 1 hour, at room temperature. At the end of the incubation with the secondary antibodies, the membrane was washed once again with TBS-T, and the detection of proteins conjugated to the antibodies was carried out using the ECLTM Prime Western Blotting kit (Cytiva) and the photodocumentation ChemiDocTM MP Imaging System (Bio-Rad).

Morphometric analysis of the intestine

After fixation in 10% aqueous formaldehyde solution (24 hours), intestine segments were dehydrated in increasing ethanol solutions and cleared in xylene. After inclusion in paraffin, the intestines were subjected to semi-serial transverse sections with 5 µm thickness, totaling 3 sections per slide/animal.

The slides obtained were stained with the Hematoxylin-Eosin (HE) technique for histopathological and morphometric evaluation of the organ extracts, as well as the Periodic Acid-Schiff (PAS)/Hematoxylin technique for goblet cells evaluation and intraepithelial lymphocytes (IELs). After staining, the histological sections were divided into quadrants, from which four fields (0°, 90°, 180° and 270°) were sampled per section, totaling 4 photomicrographs per animal (magnification 1000 ×).

To standardize the measurement of stratigraphic layers, the largest villi that appeared in the quadrant was selected, as well as the layers below it. The density of PAS+ cells and IELs was counted for every 100 enterocytes/quadrant.

Histochemistry of NADPH-diaphorase neurons

To detect non-adrenergic – non-cholinergic (NADPH – dp) neurons, the intestinal segments were subjected to the action of the enzyme NADPH-diaphorase through the use of an artificial electron acceptor, NBT (Nitro Blue Tetrazolium). After highlighting, the intestines were fixed, sectioned along the longitudinal axis at the mesenteric margin and microdissected under a stereomicroscope, to remove the mucosal and submucosal tunics and preserve the muscular and serous tunics. Then, the samples were dehydrated in increasing alcohol solutions, cleared in xylene and mounted on a slide under a coverslip with Permount® synthetic resin.

Neuronal density (mm^2) was obtained with an optical microscope coupled to the Motic Image® image capture system. For each membrane preparation, 60 random fields were captured, sampling all regions (mesenteric, intermediate and anti-mesenteric) of the intestine.

Immunohistochemistry of Myenteric Neurons

The collected intestines were washed with 0.1M PBS buffer (pH 7.4) to remove residues and tied at their ends, filled, and distended with 4% paraformaldehyde for 2 hours at 4°C. After this period, the segments were washed in 0.1M PBS (pH 7.4) and stored in this same solution plus 0.04% sodium azide, to be micro-dissected. The total preparations of the muscular coat, obtained by removing the mucosa and submucosa through dissection under a stereomicroscope with trans-illumination, were used for the immunohistochemical technique to evaluate the vipergic neuronal population.

To this end, total intestine preparations were washed twice in 0.1M PBS (pH 7.4) with 0.5% Triton X-100 for 10 minutes, and blocked for 1h in a solution containing 0.1M PBS (pH 7.4), 0.5% Triton X-100, 2% BSA and 10% goat serum, to avoid non-specific binding. Subsequently, the tissues were incubated for 48 hours in a solution containing 0.1M PBS (pH 7.4) with 0.5% Triton X-100, 2% BSA and 2% goat serum and the primary

antibody (Anti-VIP antibody [EPR23288-43]). After incubation, the membranes were washed three times in 0.1M PBS (pH 7.4) with 0.5% Triton X-100 and incubated with the respective secondary antibody (Alexa fluor 488) for two hours at room temperature. After further washing with PBS to remove excess antibodies, the membranes were mounted between slide and coverslip with glycerol and analyzed under a fluorescence microscope.

Neuronal density (mm²) was obtained with an optical microscope coupled to the Motic Image® image capture system and transferred to the computer. For each membrane preparation, 40 random fields were captured, sampling all regions (mesenteric, intermediate, and anti-mesenteric) of the intestine.

Statistical analysis

The data were initially subjected to the Shapiro-Wilk test to verify normality and Levene's test to evaluate homoscedasticity. Statistical differences were tested for the average values of each variable using the ANOVA test, followed by the Tukey test when the assumptions for the use of parametric tests were followed. The significance level was 5% and the results were expressed as mean ± standard error of the mean.

RESULTS

Physicochemical parameters

The physicochemical parameters were monitored throughout the experiment, and are available in Table 1 below:

Table 1. Physicochemical parameters measured on the first and last day of fish exposure.

Groups	Initial		Final	
	Temperature	pH	Temperature	pH
Control	25.1 ºC	7.1	25.5 ºC	7.1
2ug/L	25.3 ºC	7.3	25.9 ºC	7.2
4ug/L	25.6 ºC	7.2	26.0 ºC	7.2

16ug/L	24.9 ºC	7.4	26.9 ºC	7.2

Feeding parameters

Data relating to cumulative mortality and dietary parameters are described in the table below. None of the parameters showed statistically significant differences, but was possible to observe a reduction in fecal production in the experimental groups, as well as a reduction in specific assimilation for the groups exposed to 2µg/L and 16µg/L.

Table 2. Cumulative mortality and average dietary parameters were obtained for *Poecilia reticulata* specimens exposed to the antibiotic azithromycin for 15 days.

Parameters	Control	2µg/L	4µg/L	16µg/L
Body size (cm)	2.4 ± 0.2	2.47 ± 0.24	2.46 ± 0.23	2.49 ± 0.25
Body weight (g)	0.69 ± 0.1	0.50 ± 0.2	0.60 ± 0.2	0.50 ± 0.18
Futons' Conditional Factor	1.5	0.9	1.0	1.0
Cumulative mortality (%)	20%	32%	24%	20%
Food Intake (mg food mg biomass^{-1} day^{-1})	0.065 ± 0.04	0.055 ± 0.022	0.060 ± 0.02	0.059 ± 0.025
Estimated Fecal Production (mg feces·mg biomass^{-1} day^{-1})	0.053 ± 0.04	0.039 ± 0.024	0.039 ± 0.03	0.036 ± 0.03
Estimated Specific Assimilation	0.044 ± 0.02	0.029 ± 0.018	0.050 ± 0.029	0.038 ± 0.022

Data expressed as mean ± standard deviation.

Microbiota Composition

Metabarcoding analyses provided information about the diversity and composition of the microbiological communities that make up the total niches of intestinal lumen and mucosa.

The alpha and beta diversity analyses indicated significant differences between the control and antibiotic-exposed groups ($p<0.05$, figure 1). Interestingly, the group that showed the higher diversity was the group exposed to 4µg/L, followed by the group exposed to 16µg/L. The beta diversity analysis demonstrated significant differences between the groups, mainly regarding the diversity of the groups exposed to 2µg/L and 4ug/L (Figure 1D).

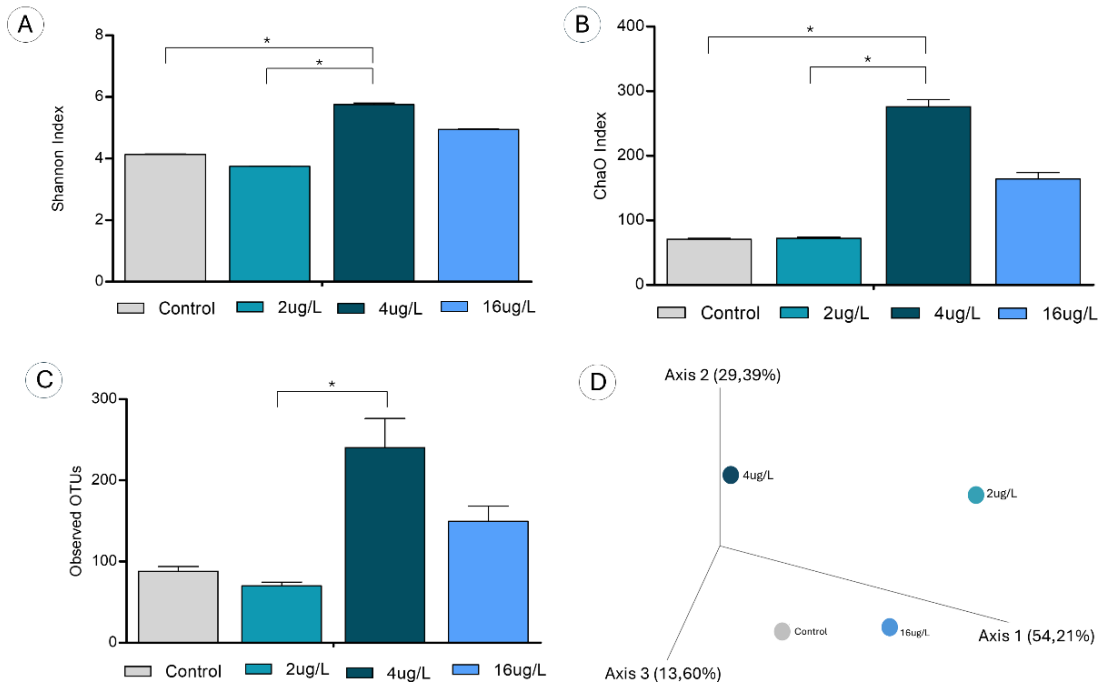

Figure 1. Alpha diversity of *Pocilia reticulata* gut microbiota (A) Shannon index, (B) ChaO Index and (C) Number of Operational Taxonomic Units (OTUs) observed. (D) Beta diversity: principal coordinate analysis (PCoA) of bacterial community structure based on Buray-Curtis distances.

Relative abundance data at the phylum and genus level are highlighted in Figure 2. The microbiological community of the gastrointestinal tract of *Poecilia reticulata* is mainly composed of organisms belonging to the phylum Firmicutes (54.54%), followed by Fusobacteria (22.47%), Proteobacteria (8.20%), Actinobacteria (7.32%) and Bacteroidetes (5.44%). The other phyla observed had a relative abundance below 1%. In all experimental groups, it was possible to observe a reduction in the relative abundance

of the Firmicutes phylum, which occurred mainly in the groups exposed to 2µg/L and 4µg/L, with a reduction of 24.64% and 19.84%, respectively ($p < 0.01$). A significant reduction can also be observed in the Actinobacteria phylum between the groups exposed to 4µg/L and 16µg/L, with a reduction of 1.92% and 3.17%, respectively ($p<0.01$).

Interestingly, the phylum Fusobacteria presented a high increased when exposed to low doses of the antibiotic (2µg/L), with a significant increase of 24% ($p<0.01$). In this same phylum, it was possible to observe that higher doses of the antibiotic caused a significant reduction ($p<0.05$) of 8.91% and 5.81% in the groups exposed to 4µg/L and 16µg/L, respectively. On the other hand, the Proteobacteria and Bacteroides phyla increased in relative abundance after exposure to the antibiotic, especially in the groups that presented higher doses. Proteobacteria increased by approximately 23.8% in the group exposed to 4ug/L ($p<0.01$), while the Bacteoridetes phylum increased by 3.06%, 7.3% and 8.58% among the experimental groups.

The intestinal microbiological diversity of *P. reticulata* also changed at the genus level, where it was possible to observe that the main genera that constitute the microbiota, such as Erysipelotrichaceae and Cetobacterium, suffered a reduction in abundance in the presence of the drug, with an excess observed at a concentration of 2µg /L where it was possible to observe a 24% increase in the abundance of organisms of the genus Cetobacterium ($p<0.01$).

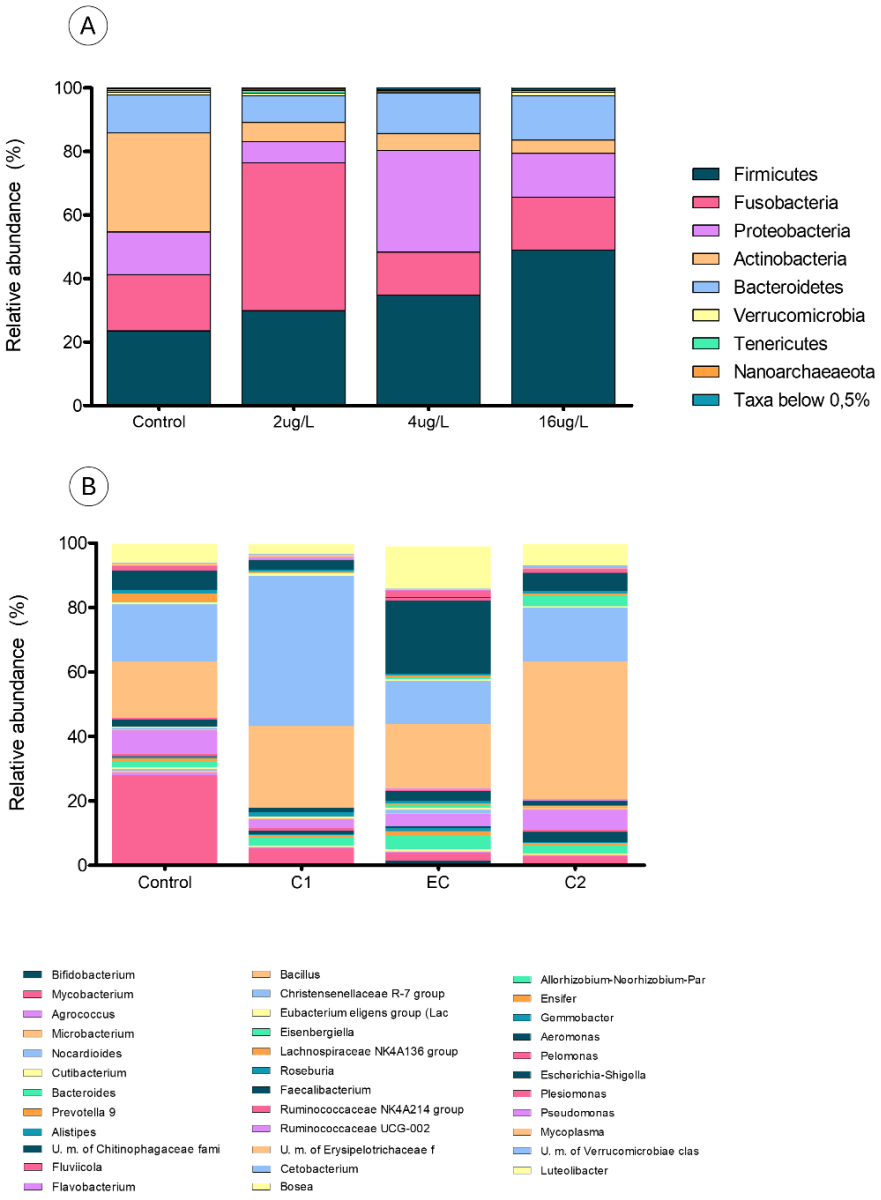

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Figure 2. Microbiota structure before and after treatment with azithromycin. Changes in the relative abundance of phyla (A) and genera (B) that make up the intestinal microbiota of *Poecilia reticulata.*

Western Blotting

The IDO2, TPH1 and 5-HT receptor expression was determined using Western blotting. Figure 3 shows the bands obtained for each antibody using a load control anti β actin. Interestingly, it's possible to notice a reduction of protein expression in all groups exposed to azithromycin at the higher concentration (16µg/L). TPH1 expression was significantly reduced in all antibiotic-exposed groups ($p<0,05$).

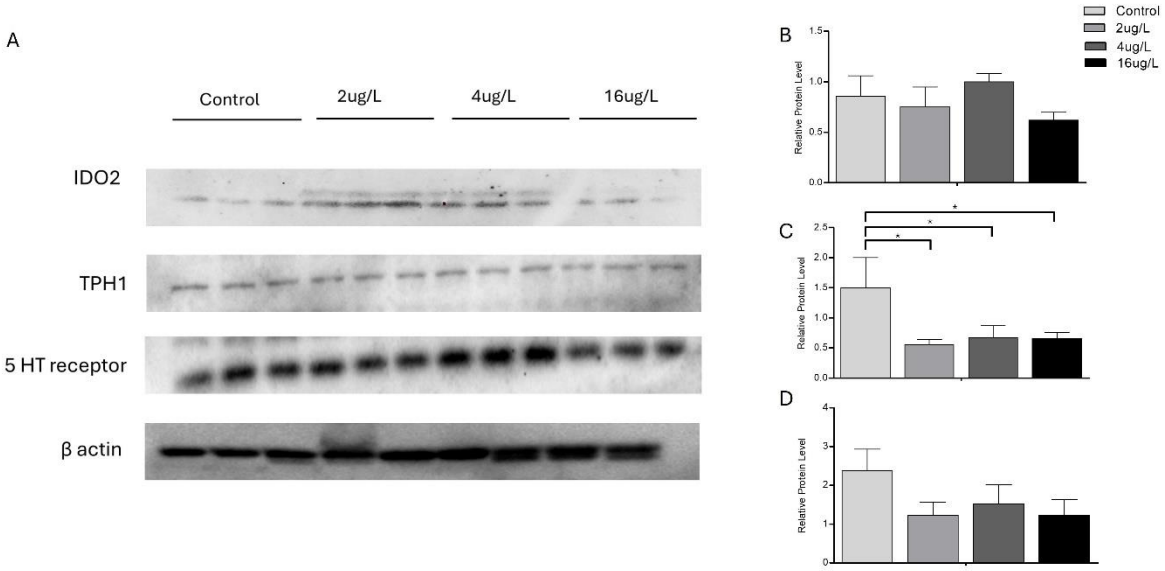

Figure 3. Western blot analysis of IDO2, TPH1, and 5HT receptor in *Poecilia reticulata* intestine from control and exposed to different concentrations of azithromycin groups. The graphs on the right show quantification of the intensity of bands calibrated to the intensity of the β-actin. (A) IDO2, (B) TPH1, and (C) 5-HT receptor. (*) indicate significant differences between groups ($p < 0.05$ one-way ANOVA).

Intestinal morphometry

The morphometric results indicated significant changes between the control group and the experimental groups. Regarding the measurement of the total intestinal wall, it was possible to observe a reduction in the experimental groups, which was significant only in the group exposed to 2µgL. This reduction may be a reflection of the shortening of the villi and submucosa, which are also significant for this group. Regarding the intestinal submucosa, it was also possible to observe a significant reduction in the groups exposed to other concentrations of azithromycin. Interestingly, the muscle layer

responded only at the highest antibiotic concentration (Figure 4).

The results observed in the intestinal mucosa indicated a significant increase in the density of goblet cells concerning the number of enterocytes in all experimental groups. In the groups exposed to 2µg/L and 4µg/L, it was also possible to observe a significant increase in intraepithelial lymphocytes (IELs) (Figure 4).

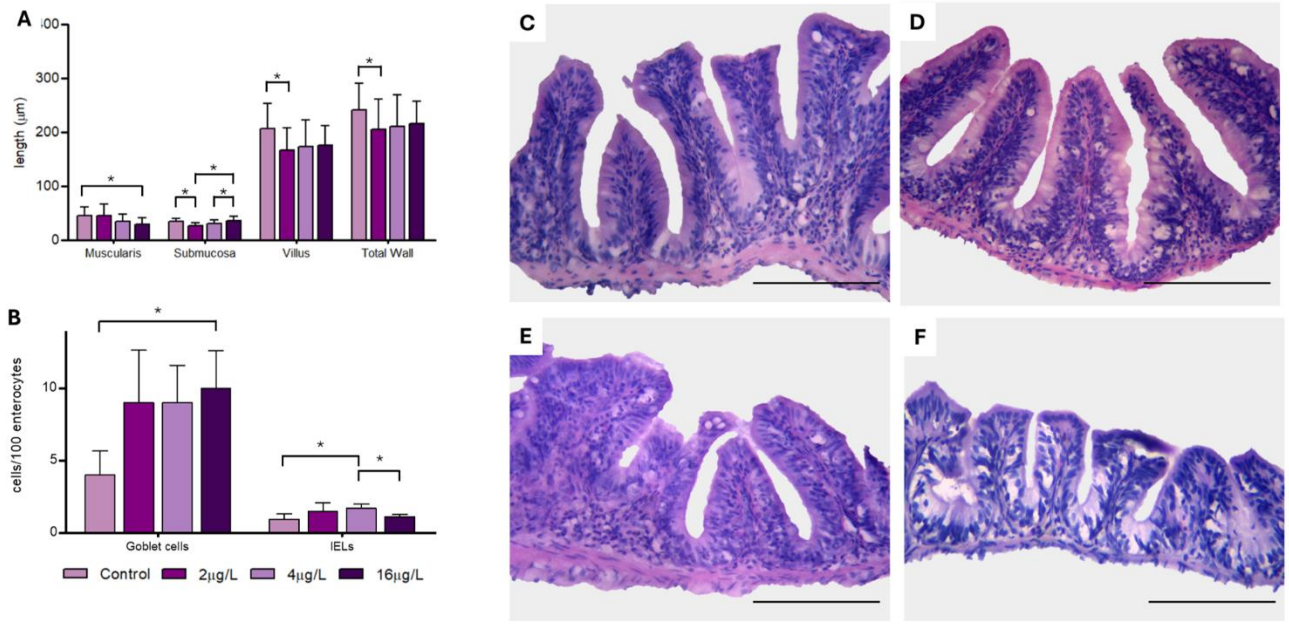

Figure 4. Intestinal morphometry data indicates the thickness of the muscular layers, submucosa, villi, and total intestine wall (A) and the relationship between goblet cells/enterocytes and intraepithelial lymphocytes/enterocytes (B). Statistically significant differences are indicated by an asterisk (*) ($p<0.05$, one-way ANOVA). Besides, photomicrography exemplifying the difference in the thickness of all stratigraphic layers (C) – control group, (D) group exposed to 2ug/L, (E) group exposed to 4ug/L and (F) group exposed to 16ug /L of azithromycin. Staining = hematoxylin-eosin; bar = 100um.

Neuronal Density

Density of Non-adrenergic – non-cholinergic (NADPH-dp) Neurons

Morphologically, the myenteric plexus of *Poecilia reticulata* presented a network-like organization containing primary, secondary, and tertiary nerve fibers. The neurons were concentrated in the primary beams, which communicate with each other

through the secondary and tertiary beams. We found no evidence of the presence of ganglia and the morphology followed a pattern in all groups studied.

Regarding neuronal density, there was a significant increase in neurons evidenced by the NADPH-dp technique in the group exposed to the lowest concentration of azithromycin (2µg/L) and to the highest concentration of the antibiotic (16µg/L) concerning the control group (figure 5).

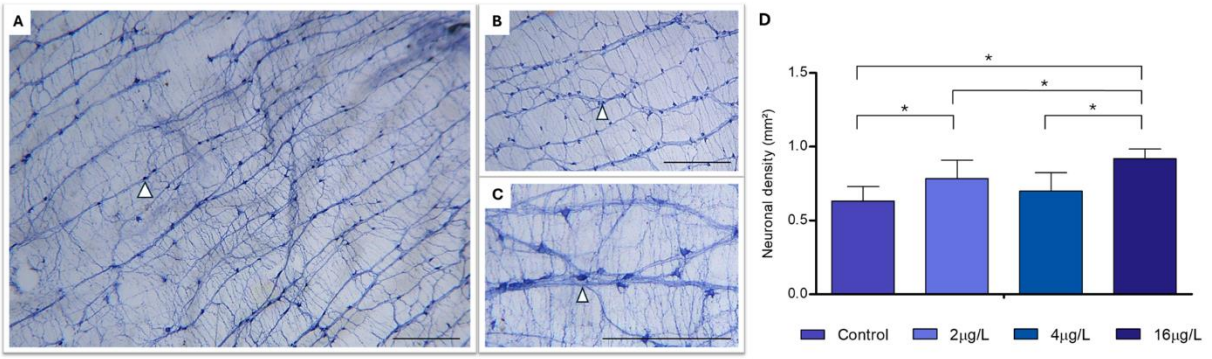

Figure 5. A photomicrography of the myenteric plexus indicates a difference in neuron density between the experimental (A) and control (B and C) of *Poecilia reticulata* exposed to different concentrations of azithromycin. Technique: NADPH- diaphorase. Bar = 100 µm. (D) a graphic representation of NADPH-dp neuronal density. Statistically significant differences are indicated by an asterisk (*) ($p<0.05$, one-way ANOVA).

Evidence of Vipergic Neurons

The quantification of vipergic neurons indicated a significant reduction in the experimental groups compared to the control group (Figure 6).

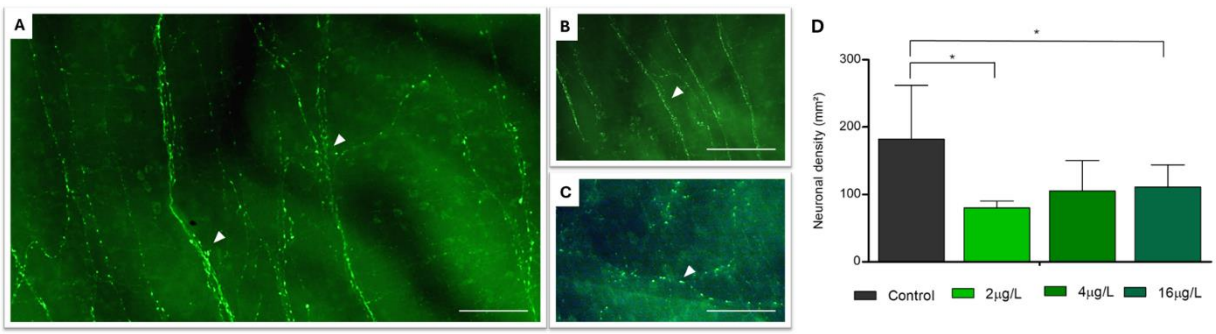

Figure 6. Vipergic neuronal density evidenced by immunofluorescence technique. On the left, a photomicrograph indicating the organization of the plexus in a network containing primary and secondary fibers and a reduction in the density of neurons. (A) control group, (B) and (C) experimental groups. On the right (D) graphic representation of the density of vipergic neurons in the control groups and those exposed to different concentrations of azithromycin. Bar = 100µm.

DISCUSSION

Our results demonstrated that the most abundant phyla found in *Poecilia reticulata*'s gut are Firmicutes, followed by Fusobacteria, Proteobacteria, and Actinobacteria. These phyla are reported by several authors to be the most abundant in fish since these organisms are dominantly colonized by aerobic, facultative anaerobic, and obligate bacteria (Llewellyn et al., 2014), which can vary in genus according to the organism habitat (Austin, 2006). It was also possible to notice that the presence of azithromycin changed considerably microbiotas composition. Several studies have used metagenomic analysis to understand contaminants effects on fish microbiota composition (Yu et al., 2021; Zhang et al., 2021; Wang et al., 2020), which have shown that numerous factors can modulate and alter the composition of this microbiological community.

Interestingly, NGS sequencing showed that the relative abundance of the bacterial phyla behaved in a particular way for each analyzed group. In the group exposed to 2ug/L, it was possible to observe an increase in bacteria belonging to the phylum Fusobacteria. These bacteria are obligate anaerobic gram-negative bacteria that naturally make up the microbiological diversity of the gastrointestinal tract (GIT). However, they are considered opportunistic, and their exacerbated increase is related to the occurrence of colorectal cancer in humans (Hashemi et al., 2019). These bacteria can attach themselves to hypoxic sites, usually related to tumor growth, replicating more intensely, and triggering inflammatory processes in the GIT, such as the activation of pro-inflammatory markers, infiltration of myeloid cells such as dendritic cells, macrophages, and granulocytes (Kostic et al., 2013).

Exposure to azithromycin environmental concentration (4µg/L) led to an increase, only at this concentration, in the relative abundance of bacteria belonging to the

Proteobacteria phylum. Although it is one of the main components of the microbiological community of the fish gut, this phylum increase is characterized as a marker for dysbiosis and it's a potential target for some disease diagnoses (Shin et al., 2015). It is suggested that an increase in the abundance of these bacteria is also related to an increase in inflammatory processes in the intestinal niche, especially in terms of deregulation of innate immune response (Shin et al., 2015). In this study, the genus *Aeromonas*, which belongs to the Proteobacteria phylum, was responsible for the considerable increase in the relative abundance of this phylum in the organisms exposed to the environmental concentration of the antibiotic. Bacteria belonging to this genus are recognized worldwide as emerging pathogens (Rizzatti et al., 2017) since this genus contains strains with virulent properties capable of producing enterotoxins and cytotoxins (Mukundan et al., 2018). Some strains belonging to this genus are responsible for causing severe diseases such as gastroenteritis, characterized by accentuating inflammatory intestinal processes (Parker et al., 2011). We therefore highlight that the presence of azithromycin may be favoring the emergence of pathogenic bacteria even at lower doses.

The highest concentration of the antibiotic (16µg/L) led to an exacerbated increase in the Firmicutes phylum, which occurred due to an increase in bacteria from the Erysipelotrichaceae genus. These organisms are rarely mentioned in scientific investigations, although they have relevant immunological effects and have the potential to flourish after treatment with broad-spectrum antibiotics such as azithromycin (Zhao et al., 2013; Palm et al., 2014; Dinh et al., 2015). In addition, an increased relative abundance of these bacteria has also been reported in patients with colon-rectal cancer (Chen et al., 2012) and their abundance is related to increased expression of tumor necrosis factors of the TNF family (Schaubeck et al., 2015).

The increase in this genus of bacteria is mainly related to metabolic alterations, especially in the lipid profile of rats treated with hypercaloric diets (Fleissner et al., 2010). Interestingly, this genus of bacteria is positively related to an increase in fat in the liver (Spencer et al., 2011), data that corroborates with the liver profile of fish exposed to this same concentration. In this group, there was an increase in the hepatosomatic index and a high level of hepatic steatosis (see article II of this document).

Morphological biomarkers responded similarly at all treatment concentrations. In

the mucosa, it was possible to observe a considerable increase in epithelial lymphocytes (IELs) at concentrations of 2µg/L and 4µg/L. These immune system cells play a fundamental role in GIT immunity since they can recognize and respond to a range of antigens (Yang et al., 2008); in addition to being able to migrate to the mucosa by recruitment mechanisms provided by chemokines. We suggest that the IELs increase can be related to the increase of immunogenic bacteria phyla, as shown above. These cells are fundamental in the body's defense by acting in the lysis of pathogenic microorganisms (Olivares-Villagomez & Van Kaer, 2018), which reinforces our hypothesis that the presence of the antibiotic in lower doses favors the multiplication of opportunistic bacteria, causing inflammation in the intestinal mucosa.

Another alteration observed in the intestinal mucosa is related to an increase in PAS+ goblet cells. Goblet cells are known to provide a physical barrier between the mucosal enterocytes and the intestinal lumen, as well as playing an important role in inducing the adaptive immune response by responding to the luminal content and delivering the luminal antigens to lamina proprias' mononuclear phagocytes (Knoop et al., 2020; Kulkarni et al., 2022) by a process that's called trans-epithelial antigen delivery. Some studies have shown that stressful environments, such as the indiscriminate proliferation of bacteria in the microbiota, can induce an increase in the endoplasmic reticulum activity of these cells, increasing mucus production (Johansson & Hansson, 2022). The increase in mucus by goblet cells can be a determining factor in the absorption of nutrients by the gastrointestinal tract (GIT), impairing the body's nutrition in long term.

Our data also indicated morphometric changes in the stratigraphic layers of the intestine in all exposed groups, with a reduction in the size of the villi and submucosa in the groups exposed to the lowest concentrations of azithromycin (2 and 4µg/L) and a reduction in the muscle layer in the group exposed to the 16µg/L concentration. Several studies have shown that the reduction in the histological layers of the GIT is related to an attempt to reduce the epithelial contact surface against contaminants and luminal stressors (Marinsek et al., 2018; 2022; Oliveira et al., 2024). Interestingly, studies that reported an increase in the phylum Fusobacteria also observed a reduction in the size of enterocytes and the striated border, which reflects a reduction in the intestinal abortive rate due to a decrease in cellular metabolism (Huang et al., 2021). These findings reinforce our data

suggesting a reduction in the absorptive rate observed in organisms exposed to azithromycin, especially in the group exposed to 2µg/L where reduced values were also observed in the estimate of fecal production.

The morphological changes observed may be related to alterations in the density of myenteric plexus neurons that express neurotransmitters important for maintaining intestinal homeostasis. It has been shown that vasoactive intestinal peptide (VIP) plays an important role in epithelial modulation and regulation through fucosylation process, and its reduction may impair mucosal homeostasis (Lei et al., 2022). The reduction in villus size may be related to changes in the turnover process, which regulates cell differentiation and extrusion rates, reducing the contact surface between the lumen and the epithelium (Cheat et al., 2015). Additionally, this neurotransmitter plays an important role in regulating the host's immune response through the production of pro-inflammatory cytokines (IL-5, IL-9, and IL-13), in this sense, its reduction can actively interfere with immune responses, making the individual more susceptible to pathogenic organisms (Sharkey & Mawe, 2023) in addition to acting as a regulator in the differentiation of mucosal cells into goblet cells (Schwerdtfeger & Tobet, 2020). As mentioned, the increase in intraepithelial lymphocytes and goblet cells in the mucosa can intensify inflammatory and absorptive processes in the GIT of organisms exposed to azithromycin, reducing the availability of nutrients.

The increase in the density of non-adrenergic-non-cholinergic neurons, which express nitric oxide, has been observed in several studies by our group that report stress in the intestinal lumen promoted by the presence of environmental contaminants (Marinsek et al., 2018; 2022). Nitric oxide (NO) is a molecule involved in various physiological functions and plays an important role in intestinal motility. In addition, some studies have reported that nitric oxide plays a key role in mediating inflammatory intestinal processes, with its expression increased to limit the proliferation of pathogenic bacteria (Sharma et al., 2007); during inflammatory events, it is estimated that the release of NO by the epithelium and neurons is 100 times higher than homeostasis levels (Idrizaj et al., 2021).

Studies indicate that the release of nitric oxide in the GIT can also have an impact on the modulation of microbiological diversity; in fact, an increase in nitric oxide in the

GIT triggered an increase in bacteria of the genus *Sterobacteriaceae* (Proteobacteria) and *Lactobacilaceae* (Firmicutes) in rats, a finding also observed in our study in animals exposed to 4µg/L and 16µg/L. Although it plays an important role in the body's inflammatory processes, the increase in neuronal nitric oxide can also impair the absorption of nutrients by enterocytes. This is because nitric oxide is the main inhibitory neurotransmitter present in the GIT and, together with acetylcholine, promotes peristaltic balance through the contraction and relaxation of smooth muscle (Furness, 2023).

Serotonin (5-HT) is a molecule that acts in various pathways in the body and comes from tryptophan, an essential amino acid. Only 1 to 2% of the tryptophan present in the intestinal lumen is metabolized by the host into serotonin, while the vast majority is used in the kynurenine pathway, where it is converted by indoleamine-2,3-dioxygenase (IDO) into different bioactive substances, such as kynurenic, picolinic and quinolinic acid (Gao et al., 2018; Yokoyama et al., 1979). These other tryptophan derivatives interact with the aryl hydrocarbon receptor, a ligand-activated cytosolic transcription factor that encodes genes that modulate immune responses, maintain epithelial barrier function, and prevent intestinal inflammation (Lamas et al., 2018).

Interestingly, the gut microbiota also indirectly affects tryptophan availability by activating host genes that encode enzymes involved in tryptophan catabolism via the kynurenine pathway (Waclawikova & Aidy, 2018; Desbonnet et al., 2008). For example, certain bacterial strains, such as *Bifidobacterium infantis*, have been shown to increase plasma levels of tryptophan when administered to mice by reducing the activity of IDO in the kynurenine pathway. As a result, more available tryptophan can be used by the host for the synthesis of 5-HT (Desbonnet et al., 2008). Our study showed that despite IDO level maintenance in animals exposed to azithromycin it is possible to notice a significant reduction in TPH1 expression. TPH is a rate-limiting enzyme that catalyzes the conversion of L-tryptophan into 5-hydroxytryptophan (5-HTP) (Yabut et al., 2019). Secondly, there is a rapid conversion of 5-HTP into 5-HT by the enzyme L-aromatic amino acid decarboxylase (Yabut et al., 2019). In this sense, the reduction in TPH1 expression indicates a reduction in the metabolization of tryptophan into 5-HT.

Besides the 5-HT receptor levels did not show significant differences statistically, it's possible to observe a reduction in antibiotic-exposed groups. It is important to note

that serotonin plays an important role in the neurotransmission of nerve impulses and cell signaling, as well as in regulating behaviors related to mood regulation and anxiety. In the GIT, serotonin plays a crucial role in regulating the ENS, activating immune responses, and maintaining epithelial integrity (Mittal et al., 2017; Wan et al., 2020). Reductions in 5-HT availability can trigger catastrophic consequences for the GIT, increasing intestinal permeability and triggering inflammatory processes.

As mentioned, the serotonergic system is highly important in controlling and modulating the plasticity of various behaviors, especially in the context of intra- and interspecific social interactions, nutritional status, and immunomodulation (Winber and Nilsson, 1993). In addition, the presence of serotonin has also been linked to the control of agonistic behaviors, stress responses, endocrine functions, and, interestingly, sexual change in hermaphroditic organisms (Larson et al., 2003). In fish, studies have shown that serotonin metabolism is hyperactivated in stressful situations, such as exposure to predation (Winberg et al., 1993), hierarchical dominance, and agonistic behavior (Winberg et al., 1991, 1992), as well as being related to reproductive behavior by stimulating the release of gonadotropin-releasing hormone (GnRH) by the hypothalamus (Sentilkumaran et al., 2001), gonadotropin by the pituitary gland (Khan and Thomas, 1994), and gonad maturation and sexual behavior (Prasad et al., 2015). In this sense, alterations in the serotoninergic system caused by the contamination of water bodies by antibiotics can directly affect behavioral responses which can interfere at various trophic levels.

In this sense, our data provided a broad view of how contamination of aquatic environments by azithromycin can modulate the intestinal microbiota, leading to various morphofunctional alterations in the GIT. These alterations focus on a reduction in absorptive rates through an increase in goblet cells and a reduction in stratigraphic layers, an increase in intraepithelial lymphocytes, and changes in the density of non-adrenergic - non-cholinergic and viperergic neurons. In addition, azithromycin was able to alter the serotoninergic system of fish, reducing the expression of the enzyme tryptophan hydroxylase (TPH1).

Our results demonstrated that azithromycin promoted severe alterations in *Poecilia's reticulata* gastrointestinal tract, mostly reducing absorptive capability and

causing microbiotas alterations that lead to inflammation in the TGI. Our results are pioneers in that they analyze the alterations in the intestinal microbiota and its relationship with intestinal histopathology, neuron density of the enteric nervous system and serotonin production. We aim to highlight that the inappropriate and constant discarding of drugs in aquatic environments can affect the absorptive homeostasis of non-alvo organisms, potentially also prejudicing behaviors that are involved in serotonergic pathways.

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