



**Ministério da Educação**  
**Universidade Federal do Rio Grande**  
**Programa de Pós-Graduação em Ciências da Saúde**



**IMPACTO DA CONTAMINAÇÃO AMBIENTAL POR  
CEFALOSPORINAS NO SOLO**

**Livia da Silva Freitas**

Rio Grande/2023



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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de Mestre em Ciências da Saúde.

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## RESUMO

A contaminação ambiental por antibióticos é uma preocupação mundial relevante. Eles são capazes de exercer toxicidade em animais, plantas, microrganismos e até mesmo em humanos através da resistência aos antimicrobianos. Pensando nessa problemática, objetivo deste estudo foi avaliar a influência de cefalosporinas no potencial germinativo de sementes de alface (*Lactuca sativa*), no peso fresco e seco das mudas expostas e no perfil funcional da microbiota do solo, com e sem a presença de minhocas, e da microbiota intestinal das minhocas. Para a avaliação do potencial germinativo, sementes de alface foram expostas a diferentes concentrações de antibióticos por 5 dias. A avaliação do perfil funcional da microbiota do solo e do intestino das minhocas californianas foi feita através da Biolog EcoPlate após solos serem contaminados com diferentes concentrações de ceftriaxona (0, 1 e 10 mg/kg) e incubados durante 14 dias, na presença ou ausência da minhoca *Eisenia andrei*. No teste de germinação das sementes das alfaces, a maior concentração testada (500 mg/L) de antibióticos reduziu significativamente a germinação. Houve uma diminuição do peso seco a uma cefalosporina de primeira geração, e uma influência negativa no peso fresco, principalmente a partir da concentração mais baixa (25 mg/L) para Cefepime. Quanto a microbiota do solo, as concentrações de 1 mg e 10 mg de ceftriaxona não foram capazes de impactar significativamente o perfil fisiológico da microbiota do solo. Além disso, não houve uma relação na mudança da comunidade microbiana com presença das minhocas.

**Palavras chave:** Cefalosporinas; Fitotoxicidade; Microbiota; Solo; *Eisenia andrei*.

## **ABSTRACT**

Environmental contamination by antibiotics is a relevant worldwide concern. They are capable of exert toxicity on animals, plants, microorganisms and even humans through resistance to antimicrobials. Thinking about that, the objective of this study was to evaluate the influence of cephalosporins on the germination potential of lettuce seeds (*Lactuca sativa*), on the fresh and dry weight of exposed seedlings and on the functional profile of the soil microbiota, with and without the presence of earthworms, and the intestinal microbiota of earthworms. To evaluate the germination potential, lettuce seeds were exposed to different concentrations of antibiotics for 5 days. The evaluation of the functional profile of the microbiota of the soil and intestine of Californian earthworms was carried out using the Biolog EcoPlate after soils had been contained with different concentrations of ceftriaxone (0, 1 and 10 mg/kg) and incubated for 14 days, in the presence or absence of the earthworm *Eisenia andrei*. In the germination test of lettuce seeds, the highest concentration (500 mg/L) of antibiotics was significantly the germination. There was a decrease in dry weight for a first-generation cephalosporin, and a negative influence on fresh weight, mainly from the lowest concentration (25 mg/L) for Cefepime. As for the soil microbiota, concentrations of 1 mg and 10 mg of ceftriaxone were not able to significantly impact the physiological profile of the soil microbiota. Furthermore, there was no relationship between the change in the microbial community and the presence of earthworms.

**Keywords:** Cephalosporins; Phytotoxicity; Microbiota; Soil; *Eisenia andrei*.

## INTRODUÇÃO

Os antibióticos são importantes para o tratamento e proteção para saúde humana e animal contra patógenos, mas a poluição de matrizes ambientais por estes compostos é uma ameaça global principalmente devido à resistência aos antimicrobianos (Kumar et al., 2019). Segundo a Organização Mundial de Saúde, o número de mortes relacionadas à resistência a estes compostos pode chegar a 10 milhões de pessoas até 2050 com um custo econômico em torno dos 10 trilhões de dólares anualmente (WHO,2018).

Já se sabe que estes compostos podem ser excretados de forma inalterada, ou ainda na forma de metabólitos, facilitando a entrada nos diferentes compartimentos ambientais, incluindo água e solo (Hu et al., 2010). Dentre as principais fontes de contaminação estão os resíduos do tratamento de animais na agropecuária, resíduos de indústrias farmacêuticas, efluentes domésticos e hospitalares, e ainda o descarte direto e incorreto no meio ambiente. Talvez a principal fonte de contaminação seja através do descarte incorreto, já que é muito comum que esses compostos sejam descartados em água corrente, no vaso sanitário e até mesmo no lixo comum (Vieira 2021).

A falta de tecnologia necessária para eliminar esses compostos, tem contribuído ainda mais para esse cenário. Somado a isso, o Brasil é reconhecido pela baixa cobertura de saneamento básico (Phillipp et al 2022). Mesmo com os avanços relacionados ao saneamento ambiental no Brasil, essa disparidade ainda é objeto de discussões, no âmbito econômico, social e ambiental. Segundo o Sistema Nacional de Informação sobre Saneamento (SNIS), 45% da população não tem acesso a coleta de esgoto, e apenas 50,8% desse esgoto é tratado (SNIS, 2021).

Outras duas fontes de contaminação são o lodo de esgoto e os adubos orgânicos. A primeira é um resíduo sólido proveniente do tratamento biológico de esgoto sanitário, rico em matéria orgânica enquanto a segunda fonte é principalmente proveniente de esterco de porcos e galinhas, que são muito utilizados para acelerar o crescimento e desenvolvimento de plantações (Golet et al., 2013; Li et al., 2021). Por fim, o uso desenfreado desses compostos, inclusive, durante a pandemia da COVID-19, em que houve um crescimento na utilização de antibióticos em alguns casos, de forma equivocada e as vezes sem comprovação de infecção bacteriana, fato que parece contribuir com o uso inadequado, descarte incorreto e conseqüentemente contribuiu para a resistência microbiana a esses compostos (Rawson et al., 2020).

Como mencionado anteriormente, os antibióticos ou seus resíduos podem contaminar o ambiente e, entre os compartimentos ambientais, o solo tem recebido menos atenção que os corpos hídricos. Similarmente, as consequências da exposição a antibióticos para os organismos do solo (edáficos) têm sido menos investigadas, mesmo em organismos chave dos processos ecossistêmicos.

O solo é um compartimento importante que desempenha diversas funções essenciais para o ecossistema, entre elas: a produção de alimentos, ciclagem de nutrientes, regulação e purificação de água, sequestro de carbono e gases estufa, habitat e manutenção da diversidade biológica, decomposição de matéria orgânica, e ainda sendo capaz de infiltrar água e recarregar o lençol freático (Schulte et al., 2014).

Todas essas funções elencam o solo como fundamental para a sociedade, incluindo o contato íntimo dos seres humanos desde a infância. Muito comum, crianças brincarem com o solo, se alimentarem de frutas e verduras cultivadas em solo até mesmo a água que bebemos, um dia passou pelo solo (Blum et al., 2018). Para além da relação com os seres humanos, o solo abriga uma diversidade de espécies de microrganismos que formam a microbiota do solo e estes organismos são importantes para manutenção dos processos ecossistêmicos, tais como a mineralização e ciclagem de nutrientes. A microbiota influencia processos como formação e fertilidade do solo, crescimento de plantas e tolerância ao estresse, renovação de nutrientes, e armazenamento de carbono (Naylor et al., 2022). Além dos microrganismos, outros grupos de organismos desempenham papéis cruciais para manutenção da qualidade do solo, incluindo plantas, minhocas e artrópodes (Vezzani e Mielniczuk 2009). Estes organismos são rotineiramente utilizados para mensurar o impacto da contaminação do solo por diferentes contaminantes (De Araújo et al 2012).

Atividades antrópicas podem ser apontadas como uma das principais interferentes na qualidade do solo, uma vez que a contaminação oriunda de atividades agrícolas, mineração, industrial e até mesmo do descarte incorreto de lixo (Cycón et al., 2019), podem levar a modificações no contexto da diversidade ecológica deste ambiente e, conseqüentemente, impactar diretamente na saúde humana, animal e ambiental.

Entre os grupos de contaminantes, os antibióticos merecem destaque em razão do seu uso extensivo na saúde humana e animal, baixa taxa de metabolização e aporte ambiental contínuo (Thiele-Bruhn 2003). Mesmo com menor número de estudos em relação ao



compartimento aquático, alguns estudos dão pistas sobre comportamento e destino dos antibióticos no solo (Wang e Wang 2015, Zhi et al 2019), bem como a relação com a biota (Cycón et al 2019) e a possível fonte de resistência aos antimicrobianos (Xie et al 2018). Ainda assim, classes específicas de antibióticos (Conde-Cid et al 2020) e alguns tipos e usos de solo têm sido priorizados (Wu et al 2022), revelando a necessidade de expansão dos estudos, sobretudo relacionados aos impactos sobre os organismos do solo.

Os antibióticos fazem parte de um grupo heterogêneo de contaminantes chamados de PPCP's. Os PPCP's (*Pharmaceuticals and personal care products*) – traduzido do inglês: produtos farmacêuticos e de cuidados pessoais – são considerados contaminantes emergentes que possuem uma capacidade de produzir efeitos fisiológicos em humanos mesmo em baixas concentrações. Fazem parte deste grupo os medicamentos como analgésicos, antibióticos, antidepressivos entre outros, e produtos de higiene pessoal como cremes dentais, enxaguantes bucais, cremes faciais, perfumes e etc (Arias, 2019).

Ainda com relação aos antibióticos, durante a Segunda Guerra Mundial os antibióticos foram uma ferramenta fundamental para salvar a vida de milhares de pessoas, que até então, morriam em decorrência de infecções causadas por ferimentos. Desde então, os antibióticos vêm sendo utilizado em larga escala pela medicina humana para combater doenças e infecções e na medicina veterinária para tratar doenças, aumentar a produção e desempenho agroindustrial (Rodriguez-Mozaz et al., 2015).

Uma maneira de conter o uso excessivo dos antibióticos, é otimizar o uso dos antimicrobianos, por isso, a Organização Mundial da Saúde, desenvolveu um sistema de AWaRe (AccessWatchReserve) que traduzindo para o português a classificação seria: Acesso, Monitoramento e Reserva (Figura 1). Esse sistema permite classificar os antibióticos de acordo com seu espectro e o potencial de desenvolver resistência aos antibióticos (WHO, 2021).

A amoxicilina e amoxicilina com ácido clavulânico, por exemplo, são os antibióticos mais utilizados em todo o mundo e estão classificados como “acesso”, sendo a primeira escolha no tratamento de infecções. As cefalosporinas de terceira geração, quinolonas e os carbapenêmicos, são considerados como “monitoramento”, pois tem alto potencial de causar resistência. Na categoria “reserva” estão os antibióticos que devem ser considerados como o último recurso, ou seja, quando os demais tratamento falharam (Sharland et al., 2018).

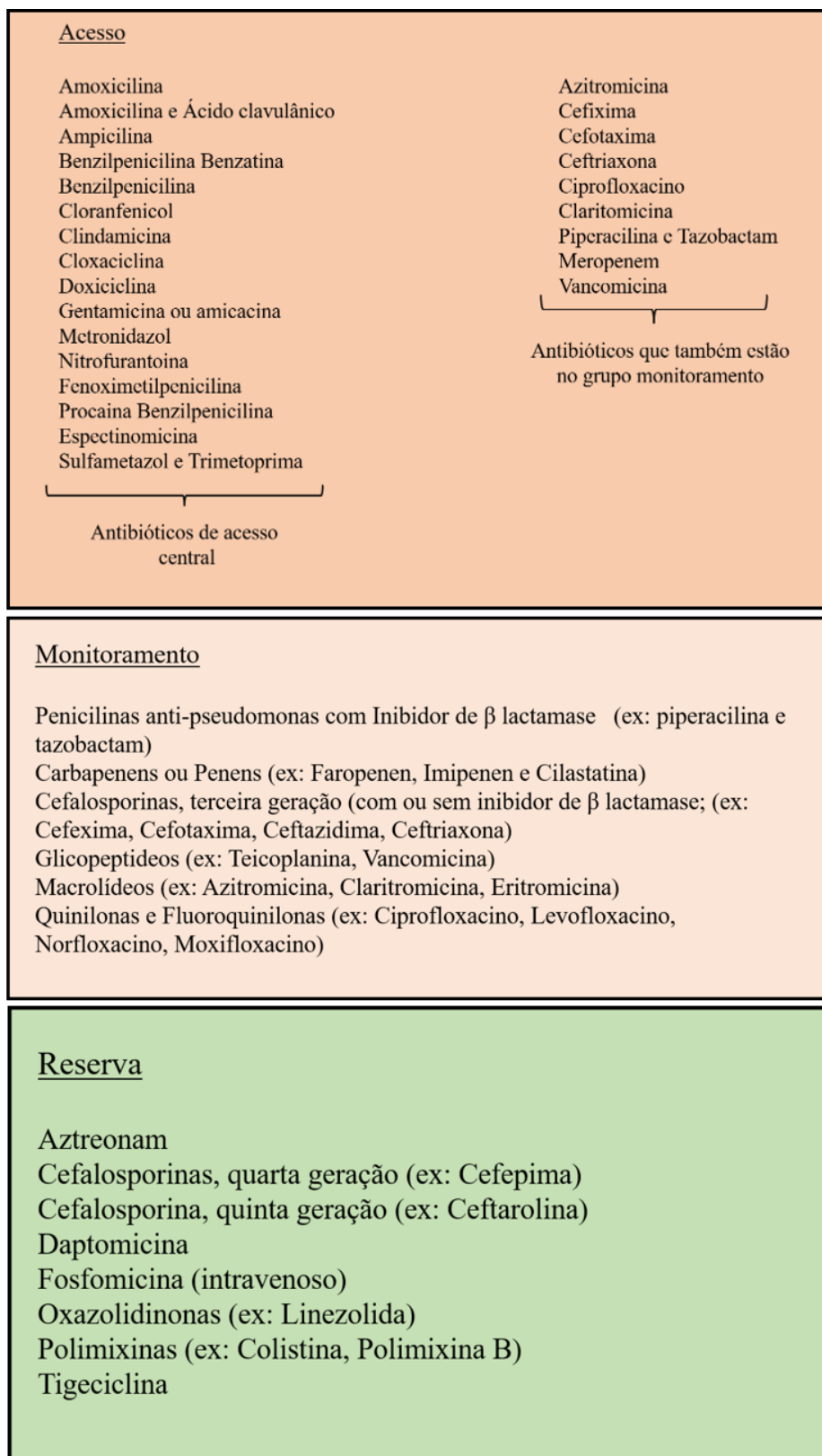


Figura 1: Lista dos Antibióticos classificados nas categorias acesso, monitoramento e reserva para orientar as prescrições. Fonte: Sharland et al., 2018.

As cefalosporinas são antibióticos beta-lactâmicos, assim como os carbapenêmicos, penicilinas e monobactâmicos. O que diferencia os beta-lactâmicos dos demais antibióticos é o anel beta-lactâmico em sua estrutura, que é responsável pela inibição da síntese da parede celular de peptidoglicano bacteriano levando à lise da célula procariótica (mecanismo de ação) (Figura 2). É uma classe muito importante devido sua baixa toxicidade e alta eficácia terapêutica (Saviano & Lourenço, 2018).

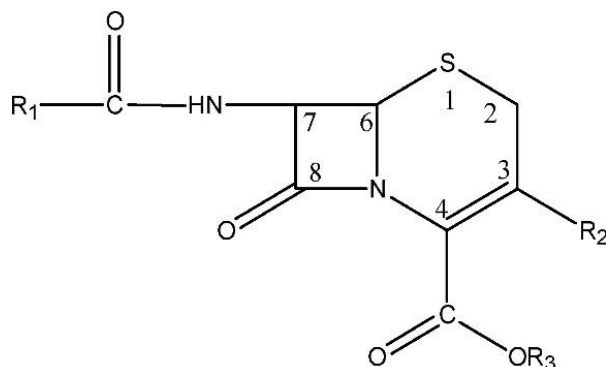


Figura 2: Estrutura dos antibióticos cefalosporínicos

O grupo das cefalosporinas é dividido em cinco gerações: primeira geração: Cefalotina, Cefazolina, Cefadroxila e Cefalexina; segunda geração: Cefoxitina, Cefuroxima e Cefaclor; terceira geração: Ceftriaxona, Cefotaxima e Ceftazidima; quarta geração: Cefepima e Cefpiroma e quinta geração: Ceftaroline e Ceftobiprole.

Levando em consideração sua forte ação bactericida, seu amplo espectro e sua baixa toxicidade, diversos estudos mostraram que as cefalosporinas são mundialmente mais prescritas (Estrada et al., 2012; Newman et al., 2012; Wacharachaisurapol et al., 2021; Moretto et al., 2023). Na pecuária, as cefalosporinas são utilizadas para tratar doenças bacterianas em suínos, bovinos, ovinos e aves (Salmon et al., 1996) e também são as mais prescritas e usadas por veterinários globalmente (Hornish e Katarski, 2002; Yuan et al., 2011). Ainda na medicina veterinária, a mais consumida é a cefalosporina de terceira geração, Ceftiofur, que em 2018, somente na China foram utilizadas mais de 260 toneladas (An et al., 2021).

O presente estudo focou na avaliação dos impactos dos antibióticos da classe das cefalosporinas em organismos do solo, incluindo plantas, minhocas e a microbiota. Estes organismos desempenham papel fundamental na manutenção da qualidade do solo e

como já mencionado são comumente utilizados como indicadores de qualidade do solo, através de bioensaios.

O uso de bioensaios rápidos para detectar alterações causadas por agentes tóxicos no ambiente tem sido frequente em estudos de diagnóstico e monitoramento ambiental. Para estes fins, os bioensaios precisam reunir algumas características, tais como: padronização, baixo custo, *endpoint* bem definido e ser suficientemente sensível para distinguir diferenças entre sítios distintos (Paton et al., 2005).

Bioensaios com plantas têm sido utilizados para avaliação de fitotoxicidade de contaminantes ambientais (Da Silva Júnior et al 2013, Honscha et al 2015). Existem inúmeros protocolos padronizados para avaliação da fitotoxicidade de substâncias químicas (EPA, 1996; OECD, 2003; ABNT, 2009). As espécies recomendadas para a realização dos testes são vegetais usados para o consumo humano (verduras e hortaliças), por exemplo, tomate, pepino, alface, soja, milho, cenoura e cebola.

Diversos estudos avaliaram a fitotoxicidade de antibióticos. Entre estes, Pan & Chu (2016) investigaram a fitotoxicidade de antibióticos veterinários (tetraciclina, sulfametazina, norfloxacina, eritromicina e cloranfenicol) em culturas de alface, tomate, cenoura e pepino, e observaram que, todos os cinco antibióticos avaliados exerceram efeitos tóxicos nas culturas, sendo o alongamento da raiz o mais sensível, seguido pelo alongamento da parte aérea e por último a germinação. Li e colaboradores (2023) também chegaram ao mesmo resultado (inibição do crescimento das mudas e alongamento das raízes) quando avaliaram a fitotoxicidade da oxitetraciclina e a enrofloxacinina em culturas de trigo.

Com relação à microbiota do solo, inúmeros parâmetros têm sido apontados como valiosos para avaliação da qualidade ambiental. Entre eles estão a avaliação da biomassa microbiana, a respiração edáfica, atividade enzimática, biodiversidade microbiana (Zilli et al 2003, De Oliveira Silva et al 2021). Mais recentemente, o perfil funcional microbiano tem sido avaliado pela técnica Biolog EcoPlate. A Biolog EcoPlate se baseia em medir a intensidade de consumo de 31 fontes de carbono, essas fontes são agrupadas em seis categorias de substratos: carboidratos, polímeros, ácidos carboxílicos, aminoácidos, amidas e controle. O resultado permite encontrar um padrão característico da comunidade microbiana nomeado “impressão digital metabólica” (no original, *metabolic fingerprint*) (Gryta et al., 2014). Nosso grupo de pesquisa tem aplicado a técnica para avaliação do

impacto de diferentes contaminantes sobre o perfil funcional microbiano em solos impactados por rejeitos de mineração (Honsha et al 2021) e antimicrobianos (Volção et al 2020, Ramires et al 2021).

Assim como a microbiota, as minhocas, conhecidas como “engenheiras do solo”, também desempenham papéis importantes no solo. Elas têm a capacidade de influenciar e modificar as propriedades do solo, aumentar a atividade microbiana e acelerar a ciclagem dos nutrientes (Wang et al., 2023).

As minhocas também têm sido propostas como invertebrados que podem provocar alterações microbianas importantes no solo, fato esse que tem sido, recentemente, explorado na vermicompostagem em indústrias, que visam promover biodegradação de poluentes orgânicos, tais como os antibióticos e estabilização de poluentes inorgânicos como os metais (Li et al., 2021). Elas também são capazes de reduzir a abundância de genes de resistência a antibióticos no processo de vermicompostagem (Cui et al., 2019).

Embora as minhocas possam indicar e servir como uma biorremediação de solos contaminados (Arora et al., 2021), impactos significativos estão sendo observados na população de minhocas. Parente e colaboradores (2021) avaliaram o impacto das forragens de granjas de frangos (também chamadas de cama de frango) contaminadas com fluoroquinolonas nas populações das minhocas. O estudo demonstrou efeitos de perda de habitat, letalidade, viabilidade celular e perda de biomassa. Nesta mesma linha, Marquéz- Lazaro e colaboradores também observaram que a presença de fluoroquinolonas induziu o dano oxidativo nas proteínas musculares, afetando a integridade e funcionalidade dessas proteínas em minhocas da espécie *Eisenia fetida* (Marquéz-Lazaro et al., 2021).

Pensando nisso, se faz necessário unir todos os componentes do solo (solo - microrganismos - plantas - organismos terrestres) em um único estudo para tentar representar o ecossistema como um todo, frente a contaminação por antibióticos cefalosporínicos.

## **Objetivo geral**

Avaliar o impacto das cefalosporinas no solo.

## **Objetivos específicos**

- Avaliar a ação da ceftriaxona no perfil fisiológico do solo.
- Avaliar o impacto da ceftriaxona como contaminante no solo sob a microbiota intestinal de minhocas californianas
- Avaliar a influência de três gerações de cefalosporinas no potencial germinativo de sementes de alface.
- Avaliar a influência de três gerações de cefalosporinas na massa seca e fresca de mudas de alface.
- Avaliar criticamente as lacunas de conhecimento sobre a temática dos antibióticos no solo.

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MANUSCRITO 1

**Soil antibiotics: how long will this matrix be neglected?**

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## **Soil antibiotics: how long will this matrix be neglected?**

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## **Abstract**

One of the first things we think of when we talk about antibiotics is curing infectious diseases. But did this great discovery only bring benefits to humanity? The answer is simple and direct: *No*. Over decades we have observed that the indiscriminate use, and sometimes incorrect, together with the lack of concern in the disposal of these compounds, has brought many risks to human health. This problem has already been discussed by public health experts, in the context of antimicrobial resistance, since there is an estimate that, in just over two decades, it will exceed 10 million deaths and cause economic losses in the order of 10 trillion dollars per year. Only more recently have studies directed efforts to investigate the effects of antibiotics in the context of *One Health*, incorporating environmental health into this context. When we look at the distribution and fate studies of antibiotics, it is clear that most studies are focused on the aquatic compartment. Soil, in turn, although it is a direct receptor of antibiotics, especially in veterinary and agricultural areas, has received less attention. This becomes even more complex when trying to assess the effects of antibiotics on organisms and edaphic communities that occupy key ecological niches for ecosystem services. Many questions are open about the ecotoxicological impact of antibiotics on non-target soil organisms and their ecological, economic and social consequences. This is certainly a question that scientists in the field of ecotoxicology must be concerned with answering.

**Keywords:** One health; Edaphic organisms; antimicrobials, Ecotoxicology.

## **Antibiotic use and inappropriate disposal**

Since their discovery, antibiotics have been used in the treatment of various infections and in agro-industrial development. However, its irrational and inappropriate use and incorrect disposal has brought many public health problems worldwide. The most widely addressed problem refers to bacterial resistance to antimicrobials, which, according to estimates by the World Health Organization (WHO), in 2050 will be responsible for more than 10 million deaths, with economic losses in the order of 10 trillion dollars per year (WHO, 2018).

Between 2000 and 2010, there was a 35% increase in the use of antibiotics, in 2015 the increase was 65% and about 76% of this increase occurred in middle- and low-income

countries, such as Brazil (Klein et al., 2017). During the COVID-19 pandemic, a 10% increase in monthly COVID-19 cases was associated with sales of macrolides and other antibiotics (such as cephalosporins and penicillins) in Europe, North America and Africa (Nandi et al., 2023).

The increase in the use of these drugs is directly related to the increase in environmental disposal. In this way, the consumption and excretion pathway allows antibiotics to be disseminated through direct and indirect emissions such as excrements, sewage irrigation and sludge compost and enter urban and agricultural soils (Wang and Wang, 2015). Studies shows that hospitals, industries, lively stock farming, and domestic garbage release contaminated effluents by antibiotics in water and soil (Robles-Jimenez et al., 2021). In addition to these sources, domestic use and its consequent improper disposal has been identified as a source of antibiotics for the environment, since most people throw away medicines from domestic storage (Jafarzadeh et al., 2021).

A consequence of the use and disposal of antibiotics are environmental impacts, which are closely associated with impacts on animal and human health. For this reason, we invite scientists and public health managers to pay special attention to the environment.

### **Antibiotics in the environment**

Antibiotics are recognized for their low toxicity in humans, but the extent of damage to the environment and to living organisms is poorly understood. In the environment, several factors can contribute to the behavior and toxicity of antibiotics in soil, including physicochemical characteristics of antibiotics, presence of other toxic agents, environmental factors and biological receptors in the environment (Zhi et al., 2019).

With regard to degradation, there are some factors that can contribute and accelerate the degradation of antibiotics: adsorption, which will depend on the physical and chemical characteristics of the soil (p.H. value, ion strength and organic matter) and the characteristics of the antibiotics; hydrolysis, which will depend on the p.H. and temperature (for example, amoxicillin is easily degraded in aquatic environments, due to the hydrolysis of beta-lactam ring); photodegradation, where light sensitive antibiotics will be degraded more easily (such as fluoroquinolones), and finally, biodegradation where antibiotics are degraded by microorganisms present in the soil (exactly like the

Proteobacteria and Bacteroidetes phyla that are associated with the degradation of ceftiofur) (Yang et al., 2021).

It is already known that antibiotics can reach and contaminate different environmental compartments (Larsson, 2014; Alduina, 2020). Among the main sources of this contamination are residues from the drug manufacturing industries, hospital and domestic effluent residues and residues from animal treatment in agriculture (Chaturverdi et al., 2021). It is known that antibiotics are not fully metabolized, most of which are eliminated reaching inefficient treatment plants, when considering their low capacity to totally eliminate these compounds (Das et al., 2019; Polianciuc et al., 2020). In places where the coverage of basic sanitation is lower, this problem can be even greater, as this facilitates the entry of these compounds into water bodies, also reaching soil, plants, animals and microorganisms. This problem also extends to humans, as this low sanitation coverage is also related to high morbidity and mortality from infections. This is a reality in developing countries (Twinomucunguzi et al., 2022). But this problem can be even greater, when we think of agriculture or the disposal directly into the environment, where antibiotics do not go through any treatment plant and no metabolization process, that is, they reach the environment directly, which can cause even greater damage to the environment, fauna, flora and human health (Du & Liu, 2021; Jechalke et al., 2014).

A concept that has gained traction in recent years is One Health, which is based on interconnection between human, environmental and animal health. An event that exemplifies this concept well is the COVID-19 pandemic, in which one of the hypotheses is that the virus was transmitted by a wild animal (Mishra & Tripathi, 2021). The environmental imbalance, whether due to deforestation, climate change and even the growth of urban areas, has caused animals to move around seeking more favorable environments and facilitating the contact of wild animals with humans. Defending the balance between man, animal and environment is essential to prevent new pandemics.

Within the context of One Health, the concern of scientists about the dangers of antibiotics is more focused on the problem of resistance (Robinson et al., 2016; Pollock et al., 2020; Eriksen et al., 2021). On the other hand, there is a vast field to be explored regarding the ecotoxicity of antibiotics and their dangers to ecosystem services. There are many studies mentioning aquatic contamination with antibiotics (Carvalho & Santos, 2016;



Kovalakova et al., 2020; Li et al., 2020), a matrix that is important for fish farming, or even studies on the presence of these compounds in organic fertilizer enriched with sewage sludge, used in plantations. But on the other hand, little attention has been paid to soil contamination by antibiotics (Akram et al., 2017; Albero et al., 2018). Furthermore, studies on soil antibiotics have focused on the distribution, fate, biodegradation of antibiotics and the direct impacts on soil microbiota (Cycon et al., 2019; Zhi et al., 2019; Robles-Jiménez et al., 2021). This compartment is vital for food safety and environmental safety and plays an essential role for living organisms, microorganisms and for humans.

### **Soil antibiotics: what is known and the main gaps**

Soil is responsible for providing nutrients necessary for the maintenance of forests, responsible for filtering water, regulating temperature and emission of greenhouse gases. When well managed, it is still able to infiltrate water and recharge the water table.

In addition, it is the most biodiverse habitat on Earth, contains the most diverse microorganisms has also been the original source of most antibiotics used in human and veterinary medicine (Woolhouse et al., 2015). The frequent association between different environmental compartments has facilitated the spread of resistant microorganisms, especially those associated with soil, in particular related to agriculture and waste water originated from human and animal waste (Wang et al., 2021). On the other hand, it returns to humans, via the food chain, drinking water, or the environment. In this sense, it has been considered a critical core of *One Health*, especially due to the constant exposure to various contaminants, arising from the intervention of human activity (Figure 1), which facilitates and contributes to the selection on existing genes, resistant mutants or horizontal gene transfer between soil microbiota, highlighting this niche, therefore, as one of the major environmental reservoirs of antimicrobial resistance (Cycón et al., 2019).

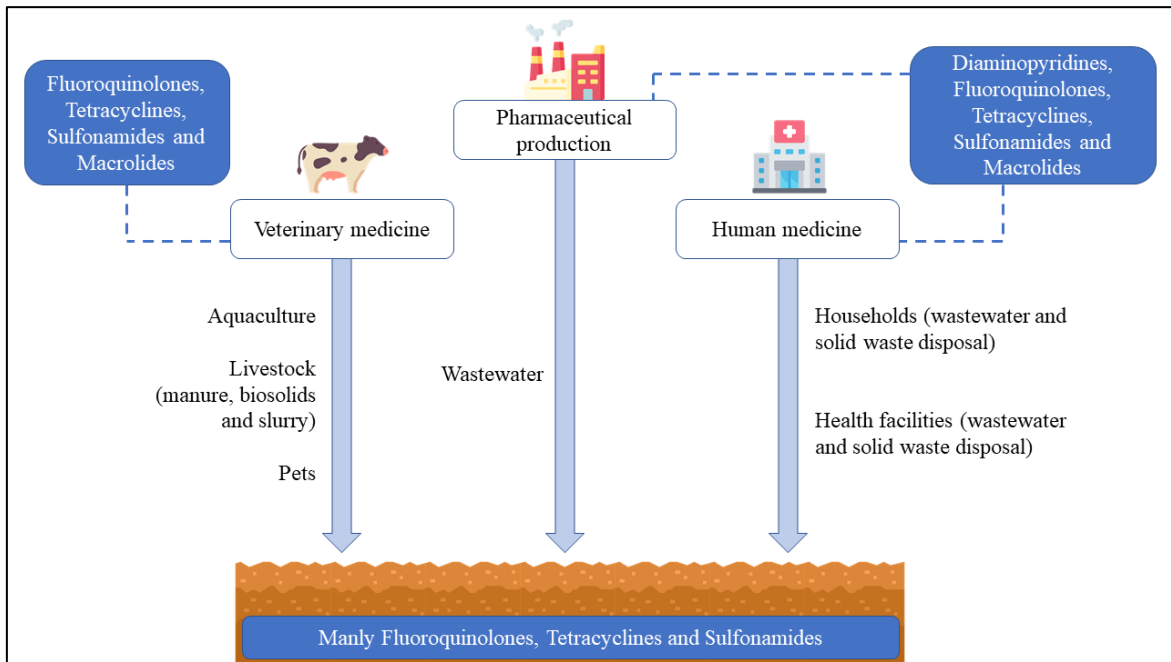


FIGURE 1. Main sources and fate of antibiotics in the soil. Source: Cycón et al., 2019

A recent review on worldwide traceability of antibiotic residues from livestock pointed to only 34 studies of detection of these compounds in soil (Robles-Jiménez et al., 2021). The same study pointed out that the number of compounds analyzed is very low and that research by country is still scarce. In addition, only 42 countries have a system for collecting data on the use of veterinary antibiotics. If, on the one hand, studies on the occurrence of antibiotics in soil are still very low, information on toxic effects is even more limited. The major concern of the studies has been the investigation of the effects of antibiotics on the soil and rhizosphere microbial community and the phytotoxic effects (Du & Liu, 2012; Jechalke et al., 2014; Cycon et al., 2019).

More recent studies have expanded this view of organisms. The study by Volção et al., 2020, investigated the effect of two antimicrobial agents on the microbial community of the gut of the terrestrial isopod *Baloniscus selowii*, while the study by Ramires et al., 2021, used a multispecies system (plants, animals and microbiota) to investigate the impact of the antimicrobial Triclosan on a micro scale. Some studies have been conducted using some classical edaphic organisms (Havelkova et al., 2016; Litskas et al., 2018; Maccari et al., 2022), but most of the information regarding the ecotoxicity of antibiotics is focused on aquatic organisms and systems (Ribeiro et al., 2018; Sodhi & Singh, 2021; Hu et al., 2022) and with a limited number of antibiotics.

One of the main gaps is due to the fact of not knowing the dynamics of these compounds in the soil. It is extremely important to carry out the mapping of possible sources of antibiotics, to know more about the half life of all compounds that are used in humans and animals, what is the real amount that these compounds arrive in the different matrices and mainly to use the One Health perspective so that we can understand the type of impact that can be caused when this compound arrives in the environment. This is exactly what is done with other types of contaminants. Elucidating the dynamics of these compounds, especially in the soil, becomes essential for us to identify and actually mitigate the risks associated with the use and disposal of these compounds.

On the world stage, this issue has been much debated and taken seriously, information campaigns on use, disposal and impacts. But, even with all these information campaigns, several countries, such as Brazil, that despite the creation of the National Action Plan for the Prevention and Control of Antimicrobial Resistance in the Scope of One Health, no specific legislation has yet been implemented to regulate the presence of these compounds in environment. The issue of bacterial resistance to antibiotics still needs to advance a lot across the globe (especially in developing and underdeveloped countries) and it is expected that other impacts (such as ecotoxicological effects) will also be taken into account.

Thinking about this whole context, there is an urgent need to carry out studies with soil and its edaphic components and also the use of more relevant antibiotics according to their demand for use. Although we have few answers on the subject, scientists and public managers need to direct attention to studying the adverse effects that the indiscriminate release of these drugs can cause to the environment.

In addition, it is necessary to intensify campaigns on the use and disposal of antibiotics. An interesting alternative would be to hold educational events in health units, hospitals, neighborhood associations and schools, providing simple and effective information to try to mitigate the impacts of antibiotics on the environment and on human and animal health.

Another point worth mentioning is the investment in agriculture, which through conscious consumption and controlling the use of these compounds makes it possible to have greater profitability and, most important, food security. And finally, investing in technologies so that treatment stations can effectively remove these compounds before the effluents are discharged into the environment.

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MANUSCRITO 2

**Antibiotics in the environment: prescribing risks to non-target organisms**

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## Antibiotics in the environment: prescribing risks to non-target organisms

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**Abstract: Background.** The cephalosporins class are among the most widely used group of antimicrobials worldwide. Antibiotics together with other drugs and personal care products make up a group of emerging contaminants. The effects of exposure to this group of chemical contaminants on non-target organisms are not well understood, as they are still poorly studied. So, this study was to evaluate the phytotoxicity of five cephalosporins in *Lactuca sativa*. **Methods.** Lettuce seeds were exposed to different concentrations of antibiotics (25 to 500 mg/L) for 5 days, in the dark. After this period, the germination percentage, and the wet and dry weights were recorded. **Results.** The highest tested concentration (500 mg/L) inhibited the germination of lettuce seeds ( $p < 0.05$ ), there was a decrease in dry weight when exposed to a first-generation cephalosporin ( $p < 0.05$ ). In addition, there was a significantly negative influence ( $p < 0.05$ ) on the fresh weight, especially in the group that evaluated the exposure of seeds to 25 mg/L of Cefepime. **Conclusion.** We emphasize that there is no record of environmental concentrations of cephalosporins in soil and therefore, we can indicate that it is possible to have

environmental damage resulting from the inappropriate and constant disposal of cephalosporins in the environment.

**Keywords:** Cephalosporins; Antimicrobials; Phytotoxicity; Lettuce.

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## 1. Introduction

Since its discovery, antibiotics have been widely used to treat infectious human and animal diseases and improve agro-industrial performance [1]. The indiscriminate and erroneous use of these compounds in these areas, combined with the low efficacy of the effluent treatment have been pointed out as the main factors related to antimicrobial resistance. This can be attributed to the spread through mobile genetic elements, which facilitate the transfer of these genes between microorganisms [2]. According to estimates by the World Health Organization (WHO), this scenario will culminate in the leadership of antibiotic resistance among the causes of mortality worldwide by 2050 [3]. The number of deaths could reach 10 million and the economic damages even greater, approximately 10 trillion dollars [4].

Once in the environment, these compounds can act on non-target organisms (plants, animals or microorganisms) and cause damage to populations, communities and ecosystems. Antibiotics and other pharmaceuticals and personal care products, also called *Pharmaceutical and Personal Care Products* (PPCP's), are an important group of emerging contaminants that have the ability to produce physiological effects in humans even at low concentrations. This group includes medicines such as analgesics, antibiotics, and antidepressants, among others, and personal hygiene products such as tooth-pastes, mouthwashes, face creams, perfumes, etc [5 - 6]. These compounds have been detected in all environmental compartments, from residuals water, soil, organic fertilizer, sewage sludge, among others, and they are co-responsible for accelerating the spread of antimicrobial resistance and increasing human, animal and ecological risks [7–8].

In Brazil, this scenario is even worse. A recent review, whose objective was to create a global map of bovine antibiotic residues in water and soil, showed that, although Brazil is the largest meat producer and the second largest consumer of antibiotics in the world, there is only one study that mentions the residues of these compounds in the water and

soil [9]. A qualitative study also carried out in Brazil explored the use of antibiotics on a pig farm. This study showed that 67% of farmers produced their feed, and when they found it convenient, they added powdered antibiotics to the feed for pig treatment or prophylactic use. Moreover, 45% of respondents reported that they did not know the differences between human and veterinary antibiotics, and 21% reported that there were no differences between the drugs. In the same study, according to the information provided, it was suggested that pigs were exposed to large amounts of antibiotics for a long period, and yet, almost half of the producers still considered the use of antibiotics indiscriminate [10]. To further aggravate the Brazilian scenario, according to data from the National Health Information System, in 2020, only 55% of the population had access to the sewage network, and only 50.8% of this collected sewage was treated [9,11].

Beta-lactams are a group of antibiotics widely used in community and hospital infections. This group includes carbapenems, monobactams, penicillins, and cephalosporins. Cephalosporins have a broad spectrum of action and have emerged significantly in human, animal, and environmental samples, including hospital wastewater and food products, as well as in animals for human consumption [10–12].

Cephalosporins are an important class of antibiotics, the second most consumed group in Europe [13], and have been growing in another niche, gaining even more visibility for being an option in the treatment of infections and prophylaxis in dental procedures [14]. In Japan, first-generation cephalosporins were the most prescribed by dentists, around 66% from 2015 to 2017 [15].

Although a recent review has shown the toxicity and degradation of cephalosporins in the aquatic environment [14], the dynamic of this class of contaminants for terrestrial organisms is not yet fully understood. Combined with the continuous input of these compounds and the lack of knowledge of the effects of this group of antibiotics on terrestrial organisms, studies point to a longer half-life of some cephalosporins in the terrestrial environment than in the aquatic environment (over 40 days) [16–18].

Among environmental spaces, the soil has been highlighted to be an excellent niche for the growth of numerous microorganisms and probably has the largest and most divergent resistome (set of all antibiotic resistance genes) comprised of bacteria with intrinsic and acquired resistance to antibiotics [19,20]. From an economic point of view, this

compartment has direct and indirect effects on the growth and development of plants, livestock, and food products, in general [21]. Once in the soil, the behavior of these compounds can be diverse and may undergo leaching, be transported to water bodies, or even accumulate in plants or in the soil itself [22].

In this context, managing the potential risk of these substances on non-target organisms of economic interest is essential to broaden the view of the potentially harmful aspects of the inappropriate disposal of antibiotics in the environment and their environmental and economic consequences [23]. Many PPCPs can be absorbed by vegetables during chronic exposure, even at a low level, and consequently, can be transferred along the food production chain [24]. In this sense, mimosa lettuce, the most consumed vegetable in the world and, according to the US Environmental Protection Agency [25], a species indicated for phytotoxicity studies in standardized protocols, was chosen as an experimental model.

Lettuce (*Lactuca sativa* Mill.) is among the most consumed leafy vegetables worldwide and has its culture widely distributed throughout Brazil. This fact is directly related to its wide adaptation to severe climatic conditions, which has favored its prominence among the species of greatest economic and social importance [26]. In addition, to maintain the exponential and qualitative increase of this species, farmers have invested in complementary resources to make seedlings healthy and vigorous, as well as phytosanitary strategies.

Thus, the present study evaluated the influence of three generations of cephalosporins on the germinative potential of lettuce seeds and the fresh and dry weight of the seedlings after five days of exposure, using the acute toxicity test with *L. sativa* seeds.

## **2. Materials and Methods**

### *2.1. Plant Species and Antibiotics Tested*

Hanson lettuce seeds (*Lactuca sativa*) commercially obtained, brand ISLA Ltda., Brazil (pesticide free), were used. The seeds were selected manually, verifying the uniformity of size, weight, and color. Wilted, moldy, stained, discolored, and damaged seeds were excluded. The cephalosporins of the first (Cephalothin and Cefazolin), third (Ceftriaxone and Ceftazidime), and fourth generation (Cefepime) with 100% purity and obtained from Sigma Aldrich were used.

## 2.2. Experimental Design

Phytotoxicity tests were conducted by assessing the acute toxicity of antimicrobials in lettuce seeds in the following concentrations: 25, 50, 100, 250, and 500 mg/L, diluted in mineral water, according to OECD 208 [27]. In addition, mineral water was used as a negative control. The experiments were carried out in three independent replicates, using 25 lettuce seeds in each 9 cm diameter Petri dish containing a paper filter moistened with 3 mL of each corresponding concentration and no addition of pesticides. The Petri dishes containing the seeds and antibiotics remained in a BOD-type germination chamber, at a constant temperature of 25 °C, in the dark, and the standards for phytotoxicity of each test were measured after five days (germination rate, fresh weight, and dry weight of seedlings) [28,29]. According to the Seed Analysis Rules of the Brazilian Ministry of Agriculture, Livestock, and Supply [30], the presence of visible root protrusion was considered a germination criterion. The experiments were carried out in triplicate.

## 2.3. Data Analysis

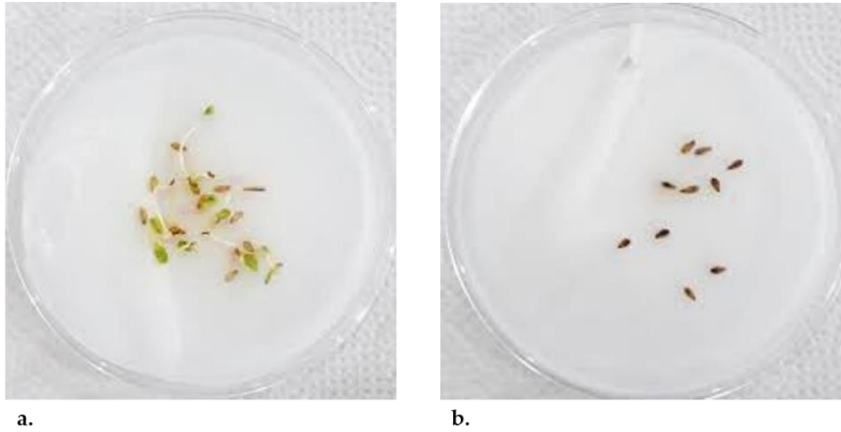
The results were expressed as the mean  $\pm$  standard deviation. To compare the means, analysis of variance (ANOVA) was performed, and when necessary, the a posteriori test (Tukey) was applied for comparison between the groups and the control (5% of statistical significance,  $p < 0.05$ ). GraphPad Prism 4 software was used for data analysis and to build the graphs.

## 3. Results

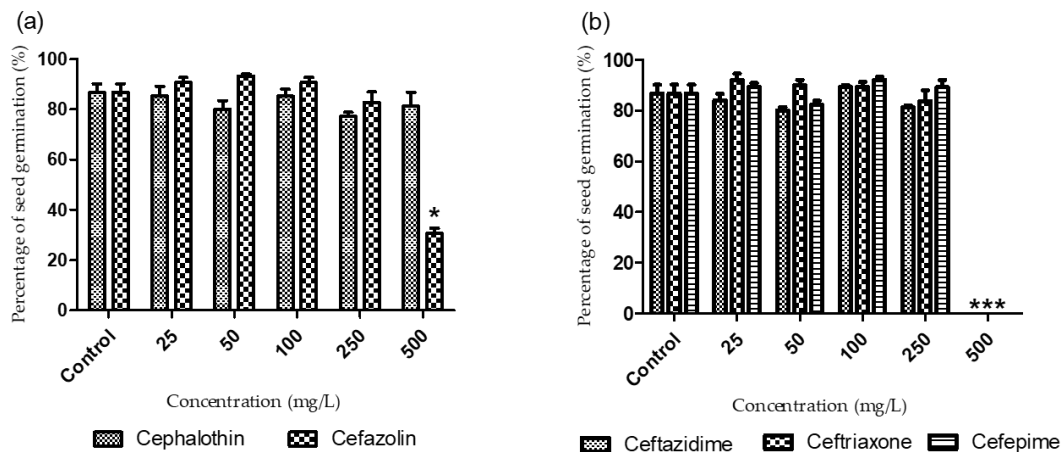
Considering the percentage of seed germination, the highest tested concentration (500 mg/L) of antibiotics significantly reduced germination compared to the negative control, after five days of exposure (Table S1). Also, the 3rd and 4th generation cephalosporins reduced the germination rate to zero at a concentration of 500 mg/L (Figures 1 and 2).

Similar to germination, fresh weight (parameter related to initial seedling growth) was not altered by exposure to Cephalothin in any of the concentrations (Figure 3, Tables S2 and S3). The dry and fresh weights were obtained by weighing the seedling on an

analytical balance, but the dry weight was weighed after drying in an oven at 105 °C for 24 h.



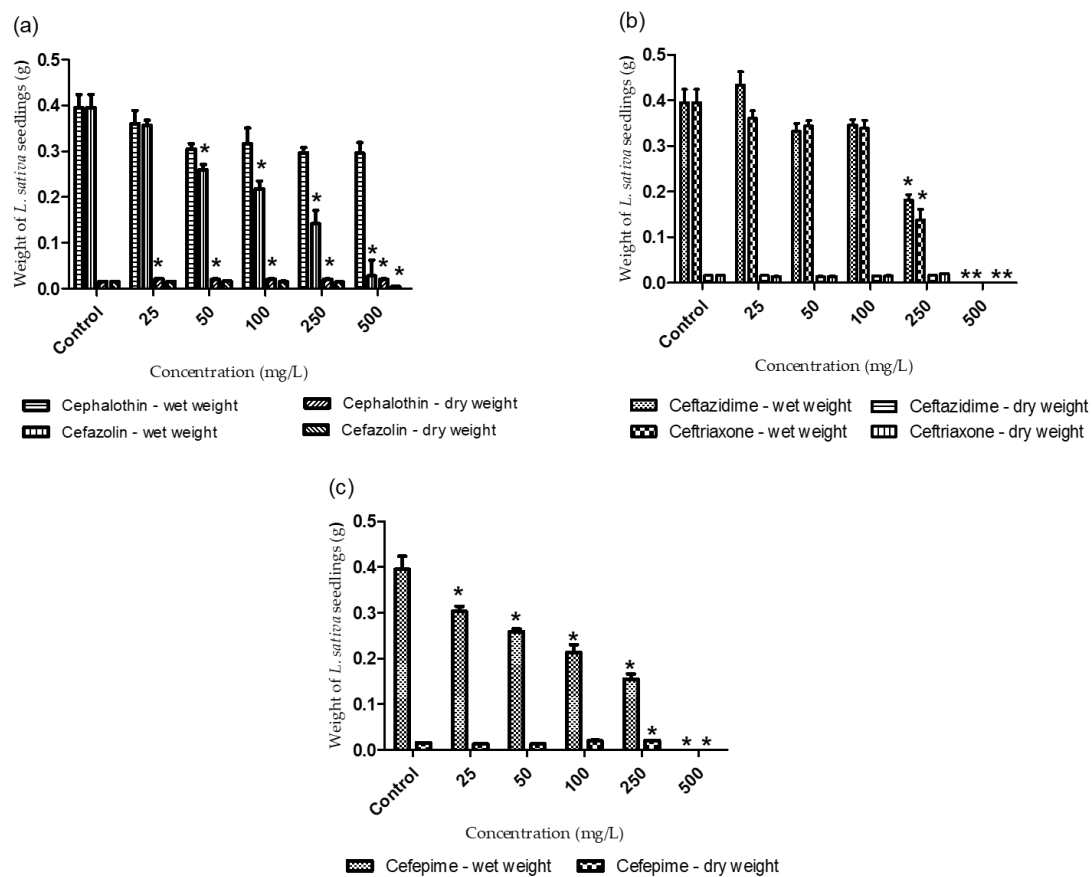
**Figure 1.** Petri dishes containing seeds exposed to different concentrations of cephalosporins, demonstrating (a) *Lactuca sativa* seeds germinated after five days of exposure to 25 mg/L of Ceftazidime and (b) the absence of germination of *L. sativa* seeds exposed to 500 mg/L Ceftazidime.



**Figure 2.** Average germination percentage of *L. sativa* seeds exposed to different cephalosporins. (a) average germination in cephalosporins of the first generation (Cephalothin and Cefazolin), (b) average germination in cephalosporins of the third (Ceftazidime and Ceftriaxone) and fourth generation (Cefepime). \*indicates significance at the  $p < 0.05$  level compared to the control.

On the other hand, for the other antibiotics, especially, of 1st, 3rd and, 4th generation, there was a statistical difference between the concentrations evaluated with regard to fresh weight. The reduction in fresh weight for Cefepime occurred from the lowest concentration tested (25 mg/L) ( $p < 0.05$ ), from the 50 mg/L of Cefazoline ( $p < 0.01$ ) and from the 250 mg/L of Ceftazidime and Ceftriaxone ( $p < 0.001$ ).

The dry weight results are shown in Figure 3. Considering the Cephalothin antibiotic, the dry weight of the seedlings was increased in all concentrations in relation to the negative control ( $p < 0.05$ ). On the other hand, the other antibiotics reduced dry weight by at least one concentration tested compared to the control. The antibiotic Cefepime reduced the dry weight at a concentration of 250 mg/L and for the other antibiotics, only the highest concentration had a significant reduction compared to the control.



**Figure 3.** Wet and dry weight of *L. sativa* seedlings exposed to different cephalosporins. (a) wet and dry weight in cephalosporins of the first generation, (b) wet and dry weight in cephalosporins of the third and (c) wet and dry weight fourth generation. \*indicates significance at the  $p < 0.05$  level compared to the control.



#### 4. Discussion

Despite some studies that point to the low potential phytotoxic of antibiotics [31,32], the present study showed antibiotic toxicity for at least one of the evaluated parameters. High concentrations of cephalosporins significantly influenced seed germination; however, Cephalothin was the only antimicrobial agent evaluated that maintained the stability of the germination process even after exposure to 500 mg/L.

The fact that the highest concentration evaluated prevented the germination of *L. sativa* for most of the cephalosporins evaluated could be related to the degree of adsorption of these compounds since, as recently mentioned in the study by An et al. [33], the increase in the concentration of ceftiofur (the third-generation cephalosporin) reduces the degree of adsorption and the desorption capacity and facilitates the antibiotic reaching the surface and underground environments [34].

Other studies have reported that although there is a tendency for cephalosporins to be stable in an aquatic environment, for example, the rates of hydrolysis and photolysis can vary according to the antibiotic evaluated and act on the ecotoxicity of these compounds [35,36]. Therefore, similar to the findings of our study, cephalexin, a first-generation cephalosporin, such as Cephalothin, tends to exhibit lower acute toxicity against *Vibrio fischeri* than other generations of cephalosporins, which could be associated with a more intense hydrolysis process of these antimicrobials [36].

The germinative process involves different stages, starting with the seed imbibition, when the metabolic activity of the seed is restored, briefly paralyzed due to physiological maturation, followed by the absorption period and culminating with the protrusion of the primary root. Considering that each of these stages is crucial for seedling development and, therefore, the production of viable vegetables, in this study, in addition to root protrusion as a germination indicator, we also evaluated fresh weight (before starting treatment with the antibiotics) and dry weight (after total germination) [37].

The comparison of results based on fresh and dry weight indicated that fresh sprouting biomass is a more sensitive outcome than dry sprouting biomass, similar to other studies that showed the low responsiveness of the germination rate of plants exposed to antimicrobials [28,38,39].

Additionally, it should be noted that the concentrations tested in the present study are high, in the range of mg/L and that antibiotics, in general, are detected in environmental samples in the order of ng or  $\mu\text{g}$  per liter or per kilo [40], including cephalosporins in an aquatic environment [36]. As far as we know, there are no reports of environmental cephalosporin concentrations in soil samples or plants, but several studies point to concentrations of some non-cephalosporin antibiotics in the soil in the order of mg/kg and mg/L [34,41–43]. According to Das et al. [13], the presence of third-generation cephalosporins, such as ceftriaxone, in pharmaceutical effluent has already been reported in the range of 125–175 mg/L. Moreover, Ye et al. [44] and Cyco'n et al. [34] have reported the low biodegradability of these antimicrobials in wastewater, which favors their persistently high concentration.

Even though no studies identified the environmental concentration of these antimicrobials in soil, Pagaling et al. [45] showed that bacteria isolated from the soil after exposure to ceftriaxone show inhibitory concentrations in the order of mg/L. In addition, Qian et al. [46] showed that high concentrations of ceftriaxone (25–50 mg/mL, including those evaluated in this study) contributed to the death of *Zebrafish* embryos, reinforcing the need to investigate possible selective pressure that high concentrations of these antimicrobials may be exerting both directly on non-target organisms such as plants and animals, but also indirectly influencing the microbial composition of this microenvironment.

Recently, Wilkinson et al. [47] evaluated the role of pharmaceutical products in rivers around the world, emphasizing that antibiotics are among the compounds found as a pollutant in this most frequent environment, including concentration ranges of four to five orders of magnitude. Moreover, they point out that this is probably related to the failure of regulatory oversight and inadequate use and sales of these compounds in human and animal health, especially in low- and middle-income countries, where the occurrence and concentrations of antibiotics in the environment are higher [9,10].

Once antibiotics are released on agricultural land, the crops are exposed to them due to their persistence, and the level of exposure depends on the physicochemical properties of the compounds, sorption potential, and environmental conditions. Therefore, as identified in this study, the significant interference of different cephalosporins in the germination process of a vegetable of extreme economic relevance worldwide and nationally,

combined with the ability of antibiotics to affect diverse environments by reducing their biochemical activities and diversity and modifying the microbial community, could impact directly or indirectly agroindustrial losses, in addition to reducing the therapeutic options in human and animal health by the selection of resistant pathogens.

## 5. Conclusions

This study evidenced that cephalosporin antibiotics can cause toxicity in *L. sativa*, although the perceived effects were in high concentrations. The fourth-generation cephalosporins were more toxic, considering the parameters evaluated. Toxicity to plants due to exposure to PPCP's must be monitored, which may indicate a possible risk that these residues move through the food chain. Added to this, the lack of information on environmental concentrations of cephalosporins in soil, despite its widespread use worldwide, alerts us to a potential hazard to non-target organisms. Further research is needed to evaluate the effects of PPCP's on plants in realistic field practice, such as irrigation with treated wastewater containing residues of these products.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pollutants2040029/s1>, Table S1. Average germination percentage of *L. sativa* seeds exposed to different cephalosporins; Table S2. Wet weight of *L. sativa* seedlings exposed to different cephalosporins; Table S3. Dry weight of *L. sativa* seedlings exposed to different cephalosporins.

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MANUSCRITO 3

**Assessment of the impact of ceftriaxone on the functional profile of the soil microbiota using the Biolog Ecoplate™**

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## Assessment of the impact of ceftriaxone on the functional profile of the soil microbiota using the Biolog EcoPlate™

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**Abstract: Background:** Antibiotics are important to treat diseases, but they have brought many environmental, economic and health concerns. These compounds can be excreted in an unchanged form or as metabolites and can exert toxicity by contaminating different environmental compartments, including soil. Soil is an important compartment because of the many functions it performs, and because it acts directly on the communities of microorganisms, plants, and animals that constitute it. The functional profile of the soil microbiota is a promising tool to assess soil quality. The objective of this study was, through Biolog EcoPlate, to evaluate the functional profile of the soil microbiota and the gut of Californian earthworms in ceftriaxone contaminated soil. **Methods:** Soils contaminated with different concentrations of ceftriaxone (0, 1 and 10 mg/kg) were incubated for 14 days in the presence or absence of the earthworm *Eisenia andrei*. After exposure, the physiological profile of the soil microbiota and the gut microbiota of the earthworms was evaluated using the Biolog EcoPlate. **Results:** There were no differences in the parameters evaluated between the different concentrations of the antibiotic. The

functional profile of the microbiota of the soils with and without earthworms was similar, but differed from the profile of the intestinal microbiota of the earthworms.

Keywords: Microbiota; Soil; Antibiotics; Ceftriaxone; Ecotoxicity; Earthworms; *Eisenia andrei*.

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## 1. Introduction

Pharmaceutical products are the great landmark of science, saving lives, prolonging life expectancy, curing diseases, improving well-being, and promoting quality of life. Pharmaceutical products consumed by humans and animals include antibiotics, hormones, and anti-inflammatories [1]. Regarding antibiotics, their importance is indisputable. Over the decades, they have been widely used in human medicine to combat diseases and infections, and in veterinary medicine to treat diseases, increase production, and improve agroindustrial performance [2]. However, these compounds have been bringing many environmental, economic, and health-related concerns. In general, about 50 - 60% of these compounds are excreted unchanged in urine, which can contaminate different environmental compartments [3].

Besides environmental contamination, another health and economic concern is the increase in antimicrobial resistance. According to the World Health Organization, the number of deaths related to antimicrobial resistance by 2050 could reach 10 million people and the economic losses around \$10 trillion annually [4].

During the COVID-19 pandemic, there has been an increase in the use of antibiotics, sometimes inappropriately and without evidence of bacterial infection [5]. Cephalosporins, which are widely used, are beta-lactam antibiotics, as are carbapenems, penicillins, and monobactams.

What sets beta-lactams apart from other antibiotics is the beta-lactam ring in their structure, which is responsible for inhibiting bacterial peptidoglycan cell wall synthesis leading to prokaryotic cell lysis [6]. They are an important class of antibiotics due to their low toxicity and high therapeutic efficacy [7]. Given their strong bactericidal action,

broad spectrum, and low toxicity, numerous studies have shown that cephalosporins are the most widely prescribed antibiotics worldwide [8- 9- 10].

In livestock farming, cephalosporins are used to treat bacterial diseases in pigs, cattle, sheep, and poultry [11] and are also the most prescribed and used antibiotics by veterinarians globally [12- 13]. In veterinary medicine, the most consumed cephalosporin is third-generation Ceftriaxone, which in 2018, only in China, more than 260 tons were used [14].

In this sense, considering the increase in the use of antibiotics, it has been widely discussed that once introduced into the environment, antibiotics can cause modifications in the functional, structural and genetic diversity of microbial communities, and may lead to the selection of resistant microorganisms. On the other hand, there is some evidence that some microorganisms could adapt to the action of these compounds and transform them into less toxic products, favoring the recovery of the original microbiota of this environmental compartment [15].

Soil is an important environmental compartment for the ecosystem, since it performs several functions, among them: food production, nutrient cycling, water regulation and purification, carbon and greenhouse gas sequestration, habitat and maintenance of biological diversity, decomposition of organic matter and when well managed it is able to infiltrate water and recharge the water table [16]. In addition, the soil is home to a diversity of species of microorganisms that form the soil microbiota.

Until recently, studies were more focused on the fundamental role of soil in the nitrogen cycle, but recently it has gained even more prominence for being considered one of the main components in global carbon cycling, for example, which has been very closely attributed to the microbial communities that constitute it as an ecological matrix [17].

This has also had repercussions on the complexity of this ecosystem consisting of microorganisms, plants, and a wide fauna, especially, invertebrates that play an important role in maintaining the physicochemical and microbial properties of the soil itself [18]. Furthermore, the abiotic soil environment is widely heterogeneous and influences the functional profile of the soil itself, acting directly or indirectly on the communities of microorganisms, plants, and animals that constitute it [19].

Soil microbial community diversity is one of the ways to assess soil quality and health, since any disturbance, biotic or abiotic affects some basic soil functions, such as nutrient cycling, biomass production, biogeochemical cycling and even soil formation. Techniques such as dehydrogenase, phosphatase and urease are sensitive indicators of microbial response to antibiotic stress in soil [20- 21- 22]. A widely used technique is also the Biolog Ecoplate™ (Biolog, Inc., Hayward, CA, USA), through which it is possible to perform an in-depth analysis of the metabolic profile of microbial communities [23- 24- 25].

In addition to the microbiota, other organisms are important for maintaining soil ecosystem processes. Earthworms, for example, have been proposed as invertebrates that can cause important microbial changes in soil [26- 27]. This condition has been exploited in vermicomposting in industries, which aim to promote biodegradation of organic pollutants such as antibiotics and stabilization of inorganic pollutants such as metals [28- 29- 30].

This set of abiotic and biotic factors directly impacts the biodiversity of environmental matrices, such as soil, allowing soil quality to be measured through the functional diversity of the microbiota, and may even promote the transfer of microorganisms, through cultivated food, to the gut microbiota of animals and humans.

Several authors have proposed that soil microbial diversity is directly proportional to the resistance and resilience of this ecological matrix to environmental disturbances [31- 32], that is, the presence of contaminating compounds such as antimicrobials, may result in changes in soil homeostasis, and consequently, in its ecological functionality [15]. In addition, a contaminated soil will possibly contaminate food which, in turn, could culminate in the contamination of an entire population.

Therefore, it is essential to study the soil matrix to assess the impact that the presence of antibiotics and their residues can cause on the environment and the ecosystem as a whole. Thus, the objective of the present study was to evaluate the impact of ceftriaxone contamination on the functional profile of the soil microbiota in the presence and absence of earthworms of the species *Eisenia andrei*.

## **2. Materials and Methods**

### 2.1. Soil sampling

Soil samples were collected within a protected area on the campus of the Federal University of Rio Grande (FURG), with the following geographic coordinates: 32°04'37.4 "S 52°10'06.9 "W. This area has been used as a control area in studies because of its low degree of contamination [33- 34- 35]. Soil samples were taken using a shovel at a depth of up to 10 to 15 cm and plant residues were removed. The soil was distributed in plastic boxes and dried at room temperature for approximately 4 days. After that, the soil was sieved in a 2 mm diameter mesh and distributed in containers for the experiments (250 grams/recipient).

### 2.2. Test organism and assessed chemical

Californian earthworms *Ensenia andrei* grown in the Laboratory for Pharmacological and Toxicological Testing were used. The compound tested was Ceftriaxone (third generation cephalosporin) 100% pure, obtained from Sigma Aldrich, which was resuspended in sterile distilled water for the experiments. The concentrations used (1 mg/kg and 10 mg/kg) were based on a previous study conducted by Orlewska and colleagues [36].

### 2.3. Experimental design

The experiment was conducted by analyzing two experimental groups: soil contaminated with the antibiotic in the presence and absence of the California earthworms. In the case of the treatments with earthworms, 10 clitellated earthworms were included per container.

Sterile plastic containers with a capacity of 500 mL, with a length of 142 mm, width of 98 mm, and height of 47 mm, received 250 grams of soil prepared as described previously. Afterwards, 50 mL of solution with antibiotic or distilled water (referring to 50% of the field capacity of the soil) were added, in triplicate. The experimental groups were: negative control, 1mg/kg and 10 mg/kg of Ceftriaxone, in the scenario in the presence or absence of earthworms. The experiment was performed in triplicate and the exposure was 14 days, at 25°C, with light/dark photoperiod (12h/12h). During the exposure period, the animals were not fed, the humidity of each replicate was controlled weekly, and there was no need for water replacement.

### 2.4. Microbial Functional Profile

The microbial functional profile was evaluated at the end of 14 days using the EcoPlate system (Biolog, Inc., Hayward, CA, USA), which is a tool used in ecotoxicological assays

[37- 38- 39]. EcoPlate measures the intensity of consumption of 31 carbon sources, which appear to be related to the microorganisms present in the matrix evaluated. These sources are grouped into six substrate categories: carboxylic acids, carbohydrates, polymers, amino acids, starches, and control (well without substrate), as shown in Table 1. The comparison between the groups is done by the amount of substrate consumed.

Table 1. List of substrates evaluated by the functional profile evaluation assay by the EcoPlate system, which uses only water as control. The substrates present in the EcoPlate belong to five groups of compounds that can be oxidized to carbon (carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers).

Substrates	Carbon sources
Polymers	Tween 40
	Tween 80
	$\alpha$ - Cyclodextrin
	Glycogen
Carbohydrates	D - Cellobiose
	$\alpha$ - D - Lactose
	beta - Methyl - D - Glucoside
	D- Xylose
	i - Erythritol
	D - Mannitol
	N- Acety 1- D - Glucosamine
Glucose - 1 - Phosphate	
	D, L- $\alpha$ -glycerol Phosphate



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D - Galactonic acid  $\gamma$ -  
Lactone

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Pyruvic acid methyl ester

D - Glucosaminic acid

D - Galacturonic acid

$\gamma$  - Hydroxybutyric acid

Carboxylic acids

Itaconic acid

$\alpha$ - Ketobutyric acid

D - Malic acid

---

L - Arginine

L - Asparagine

L - Phenylalanine

Amino acid

L- Serine

L- Threonine

Glycyl - L- glutamic acid

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Phenylethylamine

Amines

Putrescine

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Phenolic compounds	2 - Hydroxy benzoic acid
	4 - Hydroxy benzoic acid

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Samples of 5g of soil were collected from each experimental group, and these aliquots were stirred in 45 ml of NaCl solution (0.85%) in a Falcon tube. Soon after, dilution (150x) was performed in saline solution and then 150µL of each suspension was added, in triplicate, in each well of the plate [40].

For the groups in which earthworms were present in the soil, we also analyzed the intestinal contents of these organisms. The earthworms were separated from the soil, washed with 70% alcohol, and dissected. The intestinal lavage from each worm pool present in each group was transferred to Falcon tubes, macerated with three glass beads, in 3 ml of NaCl solution (0.85%) under stirring. Dilution (10x) was performed and then 150µL of each suspension was added in triplicate to each well of the plate.

The plates were incubated in a DBO type incubator in the Laboratory of Pharmacological and Toxicological Assays (LEFT), at 28°C and readings were taken in a spectrophotometer at 590nm (FilterMax F5), in the following times: 48h, 72h, 96h and 120h. The utilization rates of these carbon compounds were quantified through the color change resulting from the transformation of soluble triphenyl tetrazolium chloride present on the plates, which changes to the reduced state: formazan. After each reading, the plates were re-incubated until the end of 120h.

### 2.5. Analysis of EcoPlate data

The Average Well Color Development and the Shannon Diversity Index (H) were obtained from calculations involving the absorbance results.

To calculate the Shannon Diversity Index, we used the following formula:

$$H' = -\sum[(p_i) \times \ln(p_i)]$$

where,  $p_i = n_i/N$ ;  $n_i$  is the number of individuals of species  $i$ ;  $N$  is the total number of individuals. This is a relationship between abundance and richness, expressing the uniformity of abundance values across all species in the sample.

The Shannon diversity index ( $H$ ) is used to calculate the physiological diversity of bacterial communities. Microbial communities that have the ability to degrade more substrates and/or exhibit similar efficiency in degradation would have higher  $H$  values when compared to the metabolically inactive part of the community that is unable to grow under plate conditions, as pointed out by Muñiz et al [41].

For analysis of the total ability of the microbiota to use different carbon sources, the average well color development ("Average Well Color Development", AWCD) was calculated for all incubation times separately using the equation:

$$AWCD = [\sum (C - R)] / n$$

where,  $C$  represents the absorbance value of the control wells (average of 3 controls),  $R$  is the average absorbance of the response wells (3 wells per carbon substrate), and  $n$  is the number of carbon substrates ( $n = 31$ ). AWCD is an indicator that represents the overall potential metabolic activity of the microbial community, making it an index of total bioactivity for Biolog plates, as described in other studies [42- 43].

Two other indices were also evaluated: NUSE and PUSE. These indices are, respectively, associated with the use of nitrogen- and phosphorus-containing carbon sources. Both are represented by the percentage of the sum of the absorbances from sources containing nitrogen or phosphorus in relation to the sum of the 31 absorbances contained in each microplate replicate. Among the 31 substrate sources allocated to the Biolog Ecoplate™, 10 included nitrogen (8 amino acids, amines and amides), 2 included phosphorus (both present in the carbohydrate group) and the rest of the 19 sources were composed mainly of carbon [44].

## 2.6. Statistical analysis

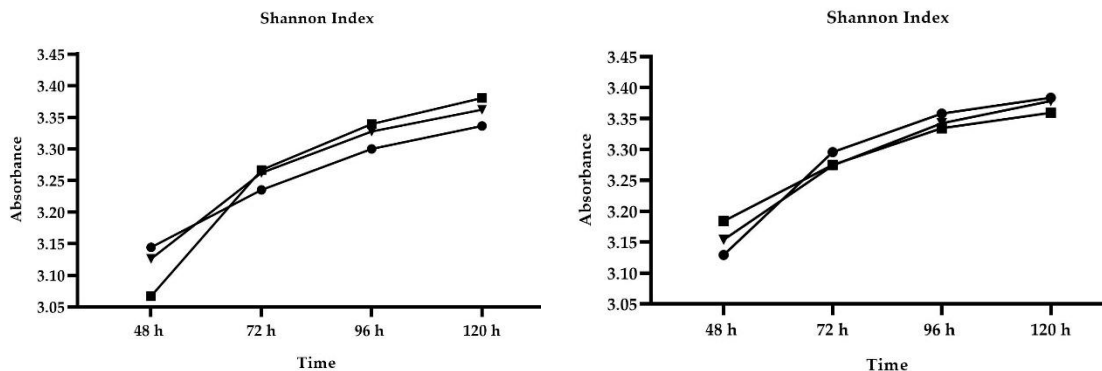
Average Well Color Development, Shannon Index and NUSE and PUSE values were converted into a single measurement (area under the curve) considering incubation times and treatments. Subsequently, a test of variance, two-way ANOVA, was performed in GraphPad Prism version 8 software to evaluate the differences in AWCD, Shannon Index

and NUSE and PUSE values considering antibiotic concentrations and exposure time. All analyses considered a value of  $p < 0.05$ .

### 3. Results

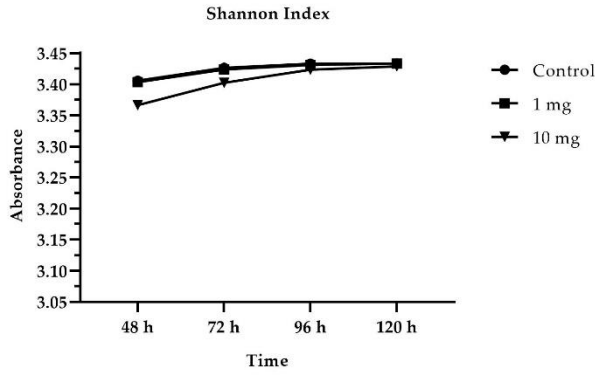
In the treatments where earthworms were present, there was no mortality. Figure 1 shows the behavior of the Shannon diversity index over the four monitored incubation days (48 to 120h) of the Biolog Ecoplate plates of the soil microbiota without and with earthworms (Figures 1a and 1b, respectively) and of the earthworm gut microbiota (Figure 1c). Overall, the behavior of the two treatments with the ceftriaxone antibiotic was similar to the control and this pattern was also found for the other parameters evaluated (data not shown).

Figure 2 shows the mean areas under the curve of the four parameters studied (Shannon diversity index, Average Well Color Development, NUSE and PUSE taking into account the comparison of two factors (ceftriaxone concentration in the soil and source of the microbiota). With the exception of Shannon diversity index, in the other parameters there were no significant differences in the factors evaluated, as well as in the interaction between them. In the case of Shannon diversity index (Figure 2a), the functional profile of the earthworm gut microbiota was statistically different from the soil microbiota in the presence or absence of earthworms.



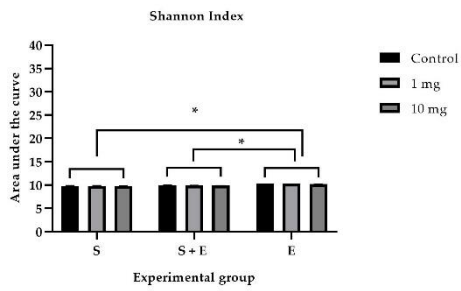
(a)

(b)

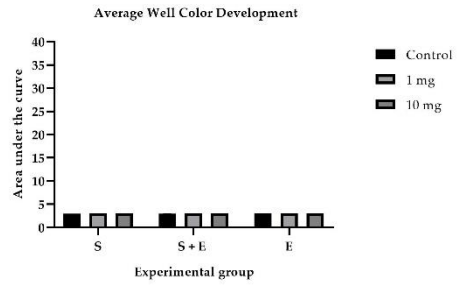


(c)

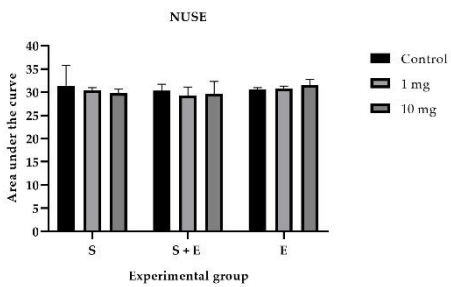
Figure 1. Shannon index of (a) soil, (b) soil with earthworms, and (c) earthworm gut, of the three treatments (control, 1mg, and 10 mg) for all exposures (48h, 72h, 96h, and 120h).



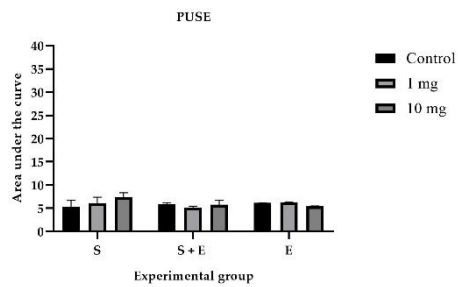
(a)



(b)



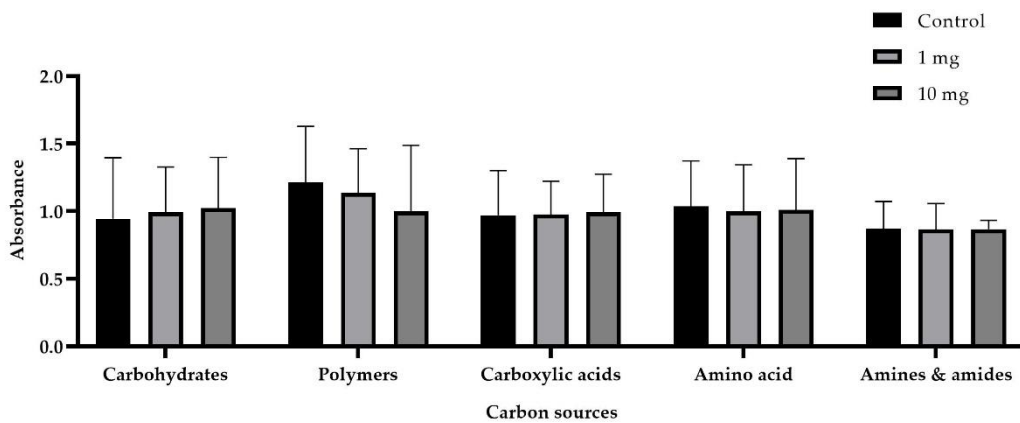
(c)



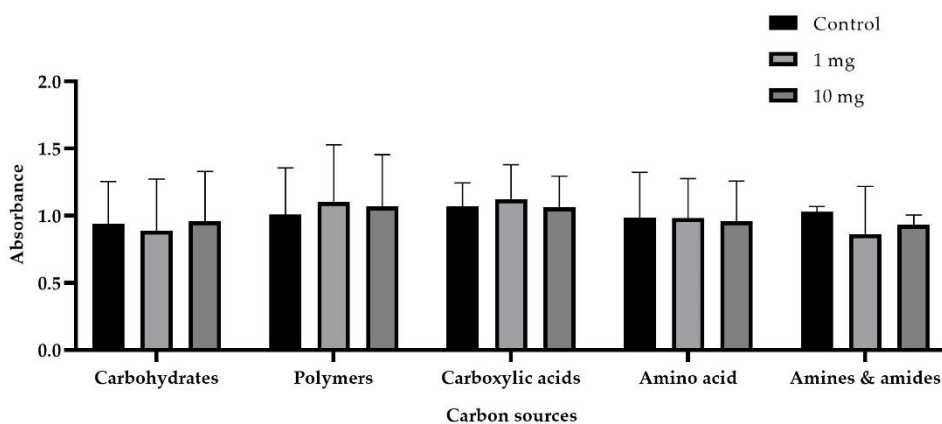
(d)

Figure 2. Results of the two-way ANOVA test of the area under the curve of the parameters (a) Shannon index, (b) Average well color development (c) NUSE and (d) PUSE in the different experimental groups (soil (S), soil with earthworms (S+E) and earthworm intestine (E)). \*p<0,05.

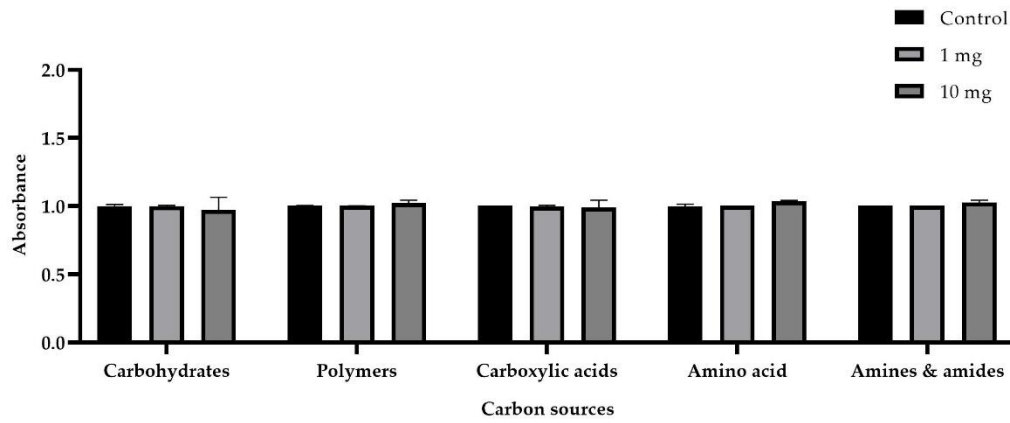
As for the consumption of substrates considering the 5 groups of carbon sources (carbohydrates, carboxylic acids, polymers, amino acids and amines and amides), no statistical difference was observed between the treatments with different concentrations of ceftriaxone, as well as in the source of microbiota (soil with or without earthworms and earthworm gut microbiota) at the time of 120h (Figure 3). On the other hand, Figure 4 presents a heat map with the individual data for each of the 31 carbon sources and a visual comparison shows that the consumption of these sources varies between the treatments with different ceftriaxone concentrations and especially when the source of the microbiota (soil with and without earthworms and earthworm gut microbiota) are compared.



(a)

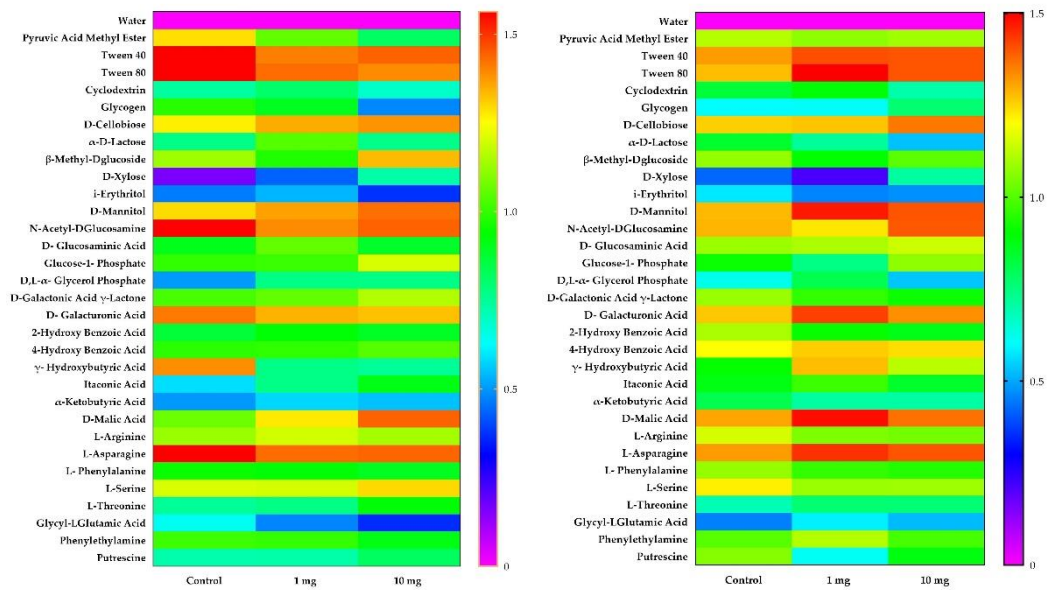


(b)



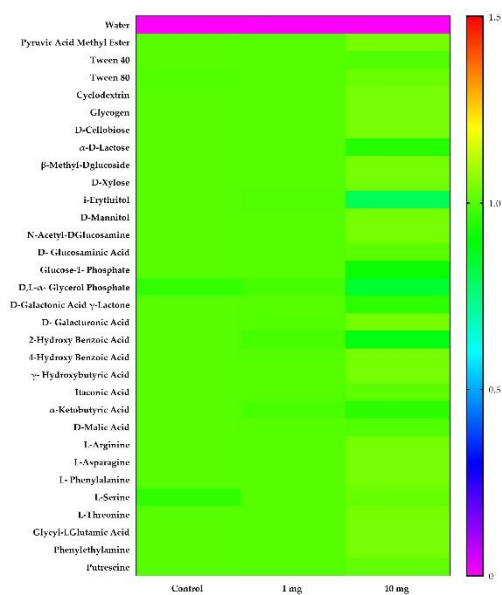
(c)

Figure 3: This figure illustrates the utilization of different substrate types by microbial communities in (a) soil, (b) soil with earthworms, and (c) earthworm guts across three different treatments (control, 1mg/kg, and 10mg/kg) at the 120-hour time point.



(a)

(b)



(c)

Figure 4: Heatmap generated in the Graph Pad Prism program showing the use of the 31 carbon sources in the (a) soil, (b) soil with earthworms, and (c) earthworm gut groups from the three treatments (control, 1mg/kg, and 10 mg/kg) at the 120 h reading.

#### 4. Discussion

There is global concern about issues involving antibiotics: the excessive and inappropriate use, as well as disposal. Although important for disease treatment, they have impacts on various organisms, plants, soil microbiota, and contribute to resistance [4]. Despite all these problems, many countries do not have specific laws regulating their levels in the environment [45]. In general, the data obtained in the present study show low influence of ceftriaxone concentrations in the soil on the functional profile of soil microbiota and intestinal microbiota of worms exposed to contaminated soil. On the other hand, the results show a difference in functional diversity when comparing the intestinal microbiota of worms to the soil microbiota (in the presence or absence of worms).

Some hypotheses can be raised that could explain this absence of alteration in the microbial functional profile. The first one is that the low influence of ceftriaxone on the soil bacterial community may be related to its low availability in a complex compartment such as soil, not exerting its antimicrobial activity under the tested conditions. In fact, studies indicate that ceftriaxone has a high affinity for minerals and this may reduce its



bioavailability [46]. In the case of antibiotics in soil, sorption can affect the bioavailability of these compounds, reducing their antimicrobial activity and limiting their effect in the environment. For instance, ceftriaxone, a broad-spectrum antibiotic, has high affinity for soil minerals, which may reduce its availability for bacteria present in the environment and decrease its impact on the soil microbiota [46].

In addition to sorption mechanisms, a recent study described the impact of different classes of antibiotics on soil microbiota. Ceftriaxone was rapidly degraded in the soil and lost all its antimicrobial activity within 19 hours [47]. Furthermore, the study suggests that unstable antibiotics (such as ceftriaxone) and those with high adsorption rates (such as kanamycin) have smaller or insignificant influences on soil bacterial community compared to other classes of antibiotics [47]. Furthermore, the study points out that unstable antibiotics (ceftriaxone) and antibiotics with high adsorption rate (kanamycin) have smaller or insignificant influences on the soil bacterial community than other classes of antibiotics.

Also, regarding the degradability of the antibiotic, it is possible that ceftriaxone was degraded by bacteria present in the soil. In the study by Mardani and colleagues [48], the genetically modified *Pseudomonas putida* bacterium was able to degrade 69.53% of ceftriaxone from soil. The study points out that Catechol 2,3-dioxygenase producing microorganisms may be able to degrade ceftriaxone effectively in a complex matrix such as soil.

One point to be mentioned is the absence of differences in the functional profile of the soil microbiota in the absence and presence of earthworms. A recent review pointed out that microbial abundance in soils with the presence of epigeic earthworms (*Eisenia andrei*) is variable. Most of the literature shows that they are capable of promoting an increase or decrease in microbial biomass. Less frequent studies describe that the number of microorganisms remains unchanged [49]. In fact, the literature has pointed out that the soil microbiota can be affected by the presence of earthworms and that they can aid in the decontamination and bioremediation of soils contaminated by chemicals, including antibiotics [29]. The study by Pu et al [28] showed that earthworm activity altered the microbial community composition in ciprofloxacin-contaminated soil.

The authors further concluded that the earthworm gut is a critical point for ciprofloxacin removal and may be an option for mitigating antibiotic pollution in soil. However, the

concentration of the antibiotic in the soil is an important factor to take into consideration and it is possible that pronounced effects of ceftriaxone could be seen at higher concentrations. The study by Xia et al [50] reports that low concentrations of antibiotic in soil may not negatively compromise the soil microbial community and that earthworms may even benefit from the presence of these compounds. Another study conducted on polycyclic aromatic hydrocarbon contaminated soils pointed out that earthworm activity changed the structure of the soil microbiota, but did not affect functioning in terms of carbon substrate consumption [51].

Thus, it is possible that structural community changes mediated by chemicals present in the soil may not affect the functional profile of this microbiota, due to a resilience or functional redundancy. The concentration of ceftriaxone in the soil appears to have been unable to affect the pattern of substrate consumption by the earthworm gut microbiota. In fact, there was little similarity in the functional profile of the soil microbiota in the presence of earthworms and the gut contents of these organisms. Therefore, studies such as Wang et al. [23], indicate that the functional diversity of earthworm gut microbiota has a direct relationship with soil microbiota.

Another study conducted with another soil organism also showed a direct relationship between the soil microbiota and the gut of terrestrial isopods. Volcão et al [37] showed that the gut microbiota of the terrestrial isopod *Baloniscus selowii* exposed to soil contaminated with antimicrobial agents (chlorhexidine and triclosan) had a very similar functional diversity to the soil microbiota for the same treatments and the authors justified these findings by considering the contaminated soil microbiota as the main source of microorganisms for the digestive tract of the terrestrial isopod.

The findings of this study point to a resilience of the functional profile and substrate consumption pattern of the soil microbiota and gut of earthworms exposed to the antibiotic ceftriaxone. It is possible that the complexity of the soil environmental matrix coupled with the degradability of the antibiotic may help to counteract possible effects of ceftriaxone on edaphic microorganisms. It is worth noting that the study was conducted only considering the nominal concentration of the antibiotic and there was no monitoring of the degradability of the compound or the pH of the soil over the 14 days of the experiment. It is also possible that the exposure time was not sufficient to cause any significant modification in the physiological profile of the soil.

## 5. Conclusions

The results of our study suggest that even at concentrations as high as 1 mg/kg and 10 mg/kg of ceftriaxone, the physiological profile of soil microbiota remained largely unaffected. While the presence of earthworms is known to be important for maintaining soil quality, our findings revealed no significant correlation between changes in microbial communities and the presence of earthworms. These observations underscore the complex nature of soil microbiota and the need for further research to fully understand the mechanisms underlying these interactions. Future studies should aim to investigate the short-, medium-, and long-term impacts of ceftriaxone and other generations of cephalosporins on soil microbiota, as these investigations are critical in informing the development of effective management strategies to promote biodiversity and maintain the health of the soil and its edaphic components.

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, **L.S.F., F.M.R.S.J.** and **D.F.R.**; methodology, **L.S.F., R.L.B., A.S.B., L.M.V.**; software, **L.S.F.** and **R.L.B.**; formal analysis, **L.S.F., R.L.B.** and **L.M.V.**; investigation, **L.S.F., F.M.R.S.J.** and **D.F.R.**; resources, **L.S.F., F.M.R.S.J.** and **D.F.R.**; data curation, **L.S.F., F.M.R.S.J.** and **D.F.R.**; writing—original draft preparation, **L.S.F., F.M.R.S.J.** and **D.F.R.**; writing—review and editing, **L.S.F., R.L.B., A.S.B., L.M.V., F.M.R.S.J.** and **D.F.R.**; visualization, **L.S.F., F.M.R.S.J.** and **D.F.R.**; supervision, **F.M.R.S.J.** and **D.F.R.**; project administration, **F.M.R.S.J.** and **D.F.R.**; funding acquisition, **F.M.R.S.J.** All authors have read and agreed to the published version of the manuscript.”

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## **CONCLUSÃO GERAL**

A problemática do uso e descarte de antibióticos tem sido recentemente estudada principalmente por sua relação com o aumento da resistência bacteriana a antimicrobianos. No entanto, além deste importante problema, estes compostos podem causar efeitos deletérios em organismos não-alvo, comprometendo os serviços ecossistêmicos e, por conseguinte, a qualidade ambiental. Nosso estudo abordou uma discussão sobre a necessidade de maior número de estudos sobre o impacto dos antimicrobianos na biota, bem como trouxe resultados de experimentos com plantas, animais e microrganismos que apontam o impacto negativo, pelo menos, em altas concentrações ambientais de cefalosporinas.