Biodegradation of the Antibiotic Doxycycline by Bacteria from the Gastrointestinal Tract of Cucurbit Beetles (*Diabrotica speciosa*)

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Antibiotics can contaminate the environment as a result of improper disposal. The induction of doxycycline in the environment could give rise to lineages of the cucurbit beetle *Diabrotica speciosa* containing resistant endosymbiotic microorganisms, giving rise to bacterial strains capable of biodegrading these antibiotic residues. This work determined the relationship between the intestinal microbiota of *D. speciosa* and its ability to biodegrade an antibiotic. Bacterial strains of *Enterobacter cloacae* and *Stenotrophomonas maltophilia* isolated from the gastrointestinal tract of *D. speciosa* were evaluated for the biodegradation of doxycycline. An analytical method for the extraction and quantification of doxycycline by high-efficiency liquid chromatography-ultraviolet detection (HPLC-UV) was developed and applied to monitor the biodegradation rates during bacterial growth for nutrient and nutrient-deficient media, both supplemented with the antibiotic. Degradation kinetics showed a decrease in antibiotic concentration after 96 h in saline medium, with recoveries of 63.1 and 87.7% for *E. cloacae* and *S. maltophilia*, respectively. Two degradation products were then identified and characterized from the liquid chromatography-high resolution mass spectrometry data after bioassays with *E. cloacae*. The gastrointestinal tract of insects such as *D. speciosa* can be a source of useful microbes for biotechnological processes such as the biodegradation of exogenous organic compounds in the environment.

Keywords: doxycycline, biodegradation, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, *Diabrotica speciosa*, chromatographic analyses

Introduction

Antibiotics are chemical products widely used for the treatment and prevention of human diseases, and despite the legal application restrictions, they are also used in the promotion of animal growth and the improvement of their nutritional value. The resistance to these antibiotic groups has gained importance in recent years due to the transmission to other enteric organisms, generating public health concerns. Human and veterinary antibiotic residues have been detected in different matrices, indicating ineffective removal in water and wastewater through conventional treatment methods. Antibiotic residues can be mutagenic, carcinogenic, and teratogenic. They can also reduce reproductive performance, generate allergy to drugs, as well as acute toxicity or intoxication.

Tetracycline antibiotics are well known for their wide range of activities against Gram-positive and negative bacteria, spirochetes, mandatory intracellular bacteria, protozoan parasites, as well as chemotherapeutic compounds. They are widely used in veterinary medicine. However, according to Arsène et al., the excessive use of antibiotics has presented harmful consequences for public health due to their presence, as well as their residues in water, meat, milk, eggs, and animal feed.

On the other hand, microbial degradation proposes a viable option for the treatment of antibiotics and their
residues, with potential to become an important tool in the control of pollutants and environmental improvement. Since microorganisms may encode proteins able to biodegrade specific molecules, including antibiotics, they are of great interest in using them as tools for antibiotic removal from the environment. Therefore, microorganisms able to degrade antibiotics through their metabolic routes into energy, carbon, nitrogen sources, or other nutrients may be applied in the biodegradation or bioremediation of contaminated matrices. Nevertheless, the existence of isolated and pure bacterial strains with the ability to degrade tetracyclines is limited to a small group. Such is the case of the bacterial Bacillus sp. and Shewanella sp. strains which respectively presented a tetracycline degradation efficiency of 98.9 and 97.6% in 100 mg L\(^{-1}\) of seed sludge. Another example is the bacterial consortium of Raoultella sp. and Pandoraea sp. isolated from soils contaminated with tetracycline, subsequently exposed to growth with 35 mg L\(^{-1}\) of the antibiotic, and reporting a degradation of 43.7% in 65 days.

Due to the need of new alternatives for the isolation of microorganisms with biotechnological applications, identification and purification techniques of these pathogens on complex matrices are necessary. A viable alternative to finding new bacteria strains that can perform these biodegradation processes is through the symbiotic relationship between insects and microorganisms in their gastrointestinal tract. Such microorganisms contribute to the detoxification of the insect feed, often caused by persistent organic chemicals from natural or exogenous sources. Some bacterial strains isolated from insect digestive tracts can even produce antimicrobial compounds that contribute to protection against pathogens. An example is the Enterococcus mundtii strain isolated from Spodoptera littoralis, with the ability to secrete the antimicrobial mundticin KS against invading bacteria, while the purified compound can cure larvae infected with Enterococcus faecalis. In relation to the degradation of tetracyclines, there are reports of intestinal bacterial communities of Hermetia illucens (black soldier fly) that were able to degrade up to 82.7% of the antibiotic in concentrations of 2000 mg L\(^{-1}\). The evidence so far implies that the intestinal microorganisms of insects may be helpful in removing antibiotic residues. However, studies on the microbial biodegradability of antibiotics face different obstacles and require analytical strategies with high detection sensitivity and quantification capacity. Among the available techniques, liquid chromatography-mass spectrometry (LC-MS) is the most commonly used technique for the separation and quantification of tetracycline residues in food of animal origin. However, LC-MS has limitations in complex matrices, such as ion suppression, leading to inaccurate and non-reproducible quantifications.

For this work, we selected the symbiotic microorganisms Stenotrophomonas maltophilia and Enterobacter cloacae strains previously isolated from the gastrointestinal tract of Diabrotica speciosa. These microbial strains were isolated and identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF MS) and partial sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene sequencing method along with 17 other bacterial genera including Streptomyces, Empedobacter, Sphingobacterium, Aurantimonas, Rhizobium, Ochrobactrum, Acidovorax, Burkholderia, Delftia, Luteibacter, Klebsiella, Kluyvera, Stenotrophomonas, Serratia, Pseudomonas, Acinetobacter, and Enterobacter. E. cloacae was specifically selected because it is an opportunistic and multi-resistant bacterial strain with redundant regulatory cascades that control its membrane permeability, ensuring bacterial protection and expression of detoxifying enzymes involved in antibiotic degradation. In turn, the S. maltophilia strain was selected because it is associated with a high potential for xenobiotic degradation, even when genomic manipulations or modifications are required to make the microorganisms more effective and efficient in eliminating these persistent compounds. Brooke reviewed and reported the potential and degradation kinetics of S. maltophilia for abamectin (an antiparasitic and acaricide), emamectin, erythromycin, spiramycin, and carbendazim (a fungicide) in contaminated water and soil.

As such, our main objective was to evaluate the viability of antibiotic-degrading bacterial strains isolated from the gastrointestinal tract of Diabrotica speciosa (Germar, 1824) (Coleoptera: Chrysomelidae). A quantitative analytical method was developed using high-efficiency liquid chromatography-ultraviolet detection (HPLC-UV) to verify degradation percentages, and thus evaluate the viability of this strategy. Ultra-high-performance liquid chromatography-time-of-flight mass spectrometry (UHPLC-QTOF MS) was used in order to identify byproducts produced by the bacterial strains Enterobacter cloacae and Stenotrophomonas maltophilia when exposed to doxycycline. In this work, we highlight the importance of searching for novel microbial biomes. We have identified two microbial strains with potential for application in the biodegradation of tetracyclines from the gastrointestinal tract of D. speciosa. Finally, analytical methods were used to monitor biodegradation and validate the microbial potential.
Experimental

Chemical products

Doxycycline (molecular mass (MM) = 444.43 g mol⁻¹, Figure 1) was obtained from Acquativa (São Carlos, Brazil). Nutrients used to prepare the mineral salt-based medium, as well as Yeast Nitrogen Base medium (YNB) were purchased from Sigma-Aldrich (3050 Spruce Street, St. Louis, USA). The LC-MS grade acetonitrile solvent and formic acid were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (50 Spruce Street, St. Louis, USA), respectively. Ultrapure water was obtained by Milli-Q system (18.2 mΩ cm) (Millipore Corporation, Watford, United Kingdom).

Bacterial strains

The pest insects Diabrotica speciosa were collected in Phaseolus vulgaris L. (common beans, cultivate “Bolinha”) crops (21°14’05”S, 48°17’09”W) and reared in a laboratory by Prof Arlindo Leal Boiça-Junior from the Department of Agricultural Sciences at Universidade Estadual de São Paulo (UNESP) in Jaboticabal, Brazil. Microorganisms were isolated from the gastrointestinal tract of D. speciosa according to Perlatti et al. In total, 73 bacterial strains belonging to 17 different genera were isolated. We selected E. cloacae (T8.5N1) and S. maltophila (T9.1N2) in this work due to few studies describing their application potential in the biodegradation of organic molecules such as antibiotics. The study has been registered in SisGen under the code A9A2B26.

Microbial growth media

We used a saline mineral salt-based nutrient-poor medium containing 5.12 g Na₂HPO₄, 1.20 g KH₂PO₄, 0.240 g NaCl, 0.400 g NH₄Cl, 0.400 g (NH₄)₂SO₄, 1.023 g of MgSO₄·7H₂O, 0.132 g of CaCl₂, and 1.83 × 10⁻² g FeSO₄·7H₂O in 1000 mL of ultrapure water. The pH was adjusted to 7.0 before autoclaving at 120 °C for 20 min. The saline medium was then supplemented with 50 mg of doxycycline as the only carbon source at a final concentration of 50 mg L⁻¹.

The commercial nutrient culture medium YNB was implemented because it is a nutrient-rich microbial broth composed of amino acids, nitrogen, vitamins, trace elements, and salts. The inclusion of ammonium sulfate in the composition provided a readily available source of nitrogen and amino acids for assimilation by the bacteria. Following the supplier’s instruction, 1000 mL of nutrient medium was prepared by adjusting the medium to pH 7.0 before autoclaving at 120 °C for 20 min. Finally, 50 mg of doxycycline were added to the medium until adjusting to a final concentration of 50 mg L⁻¹.

Biodegradation assays sampling

Enterobacter cloacae and S. maltophila microbial cell suspensions were inoculated (2.63 and 1.09% v/v, respectively) in the saline and YNB nutrient medium with a pH of 7.0 supplemented with 50 µg mL⁻¹ of doxycycline and incubated at 28 °C at 125 rpm in a shaker for 96 h. Bacterial growth and biodegradation was evaluated at 0, 24, 48, 72, and 96 h, while each sample was performed in triplicates (n = 3).

Extraction and detection of doxycycline

For the evaluation of the extraction method, solutions were prepared using doxycycline at concentrations of 5, 50, and 100% of the working concentration and diluted with 5.0 mL of saline medium without bacterial strain. These solutions were slightly agitated with 5.0 mL of ethyl acetate. Subsequently, 20 mL of Na₂EDTA/Mcllvaine buffer solution (pH 4) were added, while the pH was adjusted with a 0.10 mol L⁻¹ NaOH solution. The content was submitted into an ultrasonic bath (Model Soni-top 404A, Soni-tech) for 10 min at 30 °C, and centrifuged (Model 5810, Eppendorf) at 2,000 × g for 10 min at 10 °C. The supernatant was collected and the process repeated twice more. Finally, the collected supernatant was grouped and evaporated. The extracted material was resuspended with methanol and analyzed through the HPLC-UV method. This protocol was developed for evaluating matrix effects and recovery to different doxycycline concentrations.

HPLC-UV and UHPLC-QTOF MS analyses

Quantitative HPLC-UV analyses were performed with an Agilent 1200 Series Liquid Chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a degasser (G1322A), quaternary pump (G1311A), autosampler (G1367B), column oven (G1316A) held at 30 °C, UV-Vis detector (G1316A) set at 350 nm,
and controlled by the EZChrom SI software. A C18 Xtimate™ column (150 × 4.6 mm internal diameter, 5 µm; Welch Materials, Inc., Shanghai, China) was used. The mobile phase was 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B), while the following gradient was programmed as: 0-1 min, 85% B; 1-15 min, 85 for 15% B; 15.1-17 min, 15% B; and 17-20 min, 15% B. The flow rate was 1.0 mL min⁻¹ and the injection volume was 20 µL. The HPLC equipment control, data acquisition and processing were performed by Agilent OpenLAB CDS ChemStation. Products from the degradation process were characterized with an Agilent 6545 QTOF MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with an ESI Jet interface in positive ionization mode with a capillary voltage of 2.5 kV. The desolvation gas flow was 11.0 L min⁻¹ and the gas flow rate was 10.0 L min⁻¹. Collision energies varied from 10 to 35 V. Source temperature was 350 °C, fragmentor at 110 V, skimmer at 45 V, and nozzle voltage at 700 V. Molecular and ions fragments were acquired simultaneously using a mass-to- mass (MS/MS) acquisition mode. The data ranged from 100 to 600 Da. They were processed by the MassHunter Workstation Software.

Method performance evaluation

The validation of the method was performed following the instructions of the Brazilian health regulatory agency (ANVISA), observing parameters such as sample stability, method selectivity, retrieval, matrix effect, limits of detection (LOD) and quantification (LOQ), linearity, accuracy, and precision. A stock solution was prepared for doxycycline quantification by accurately dissolving 10.0 ± 0.1 mg of the standard in 10.0 mL of methanol (J.T. Baker, LC-MS degree) in a volumetric balloon flask (Pyrex®). The solution was stored at −5 °C. The prepared stock solution presented a nominal concentration of 1000 µg mL⁻¹. The linear working range was established between 1.00 and 100 µg mL⁻¹.

Working solutions for the construction of an external calibration curve were prepared from the standard solution. The calibration curve solutions were prepared by diluting the standard solution into the following concentrations: 1.00; 2.50; 5.00; 10.0; 20.0; 40.0; 60.0; 80.0, and 100 µg mL⁻¹. All solutions were prepared in triplicates (n = 3). These solutions were used to evaluate the calibration curve, linearity, and linear dynamic interval of the analytical method. The LOD and LOQ were estimated for each analyte using the standard deviation of the intercept and the slope from the regression analysis.

In order to assess the accuracy and precision of the analytical method, three other doxycycline solutions (1.20, 50.0, and 100 µg mL⁻¹) were prepared independently in quintuplicates (n = 5) through the procedure previously described for representation of low, medium, and high-quality control (LQC, MQC, and HQC, respectively). The same quality control solutions were applied to growth media in order to establish the recovery method. All the standards in different doxycycline concentrations were analyzed according to their respective areas in the chromatographic data.

Statistical analyses

A single factor analysis of variance (ANOVA) was performed on linear regression measurement data to evaluate the proposed method and validate the analytical method by using Microsoft® Excel software. Statistical significance was defined in p-values < 0.05, in order to indicate that the model is explained by the proposed regression in a 95% confidence interval.

Results and Discussion

Microbial degradation of persistent organic compounds such as tetracyclines is considered an effective and advantageous approach due to the remarkable catabolic potential of microorganisms, which, after rapid genetic transformation, utilize complex contaminants and convert them into carbon and energy sources. This strategy, often induced by optimized cultivation media, allows the establishment of new metabolic pathways for the degradation of xenobiotics through biodegradation.

Bacterial growth

The saline medium growth curves were similar for bacterial strains, even in the presence of doxycycline during the retardation phase (0 h) (Figure 2). The microorganisms vary in the initial phase due to each strain’s adaptive process to the growth medium, undergoing frequent and reversible phenotypic changes from genetic alterations in the microorganisms. The bacterial strains were in an adaptive process to the new growth medium (delay phase) after 24 h, when there was no increase in cell numbers, but great metabolic activity. The E. cloacae strain shows significant cell growth in saline medium against S. maltophilia in the logarithmic growth phase (24-48 h), even in the presence of doxycycline. For E. cloacae, cell growth follows a division trend with the span characteristic of the strain,
exponentially increasing the number of bacteria during
growth. In its turn, S. maltophilia showed a decrease
in cell content, possibly associated with difficulty in
repairing and replacing damaged subcellular components
during transition to the saline growth medium. Finally,
the E. cloacae strain bacterial cells did not show significant
behavior in the stationary phase (48-72 h), Figure 2. This
is a phase where growth rate stabilizes in the growth
medium. S. maltophilia cells did not show stable growth
in the stationary phase, leading to a phase of cell decline
by consecutively decreasing in small cell fractions more
resistant to the antibiotic until total mortality.

For the YNB nutrient growth medium, the
log(colony-forming unit (CFU) mL⁻¹) ratio was similar
in relation to the nutrient-deficient growth medium
implemented for E. cloacae cells. On the other hand,
S. maltophilia strains showed a differentiated growth
in relation to the saline medium, even in the presence
doxycline. This is possibly associated with the
low acquired resistance of the antibiotic during the
growth phases, thus causing a low performance in
S. maltophilia cell growth compared to the exponential
growth observed in E. cloacae, as shown in Figure 2. The
growth of E. cloacae cells in the presence and absence
of doxycline in saline and nutrient media did not present
limitations. This indicates that this microorganism
may metabolize the antibiotic used in this study. This
correlates with the tetracyclines, that act by inhibiting
the synthesis of bacterial proteins (bacteriostatics). They
do not eliminate bacterial cells but inhibit their growth,
allowing the antibiotic to be used as a carbon and nitrogen
source, thus becoming an additional factor in the growth
of bacterial cells.

Evaluation of the analytical method

Our initial premise was to evaluate the strains’ ability to
biodegrade doxycline and similar products. As such, we
developed and validated a quantitative analytical method
ensuring that the results were linear and suitable according
to the matrices under investigation. We evaluated different
parameters in the quantitative analytical method in order
to summarize and obtain information about data during
mathematical and statistical analyses. Our approach
allowed us to extract useful information on the variation
doxycline concentrations with regard to possible
matrix effects with accuracy and precision. As such, the
method became a prerequisite for the correct evaluation
and interpretation of the degradation potential of bacterial
cells in the growth media.

The selectivity and specificity of the method were
determined through the detection of doxycline by
retention time (tₚ), with its chromatographic band at
6.78 ± 0.01 min (Figure 3). We also compared the

![Figure 2. Bacterial growth curves in different growth media. (a) E. cloacae in the presence of doxycline, (b) S. maltophilia in the presence of doxycline, (c) E. cloacae in the absence of doxycline, and (d) S. maltophilia in the absence of doxycline. (●) Saline medium, (●) YNB medium.](image-url)
chromatograms after triplicate injections of the sample, consecutively demonstrating a high degree of repeatability. We did not observe chromatographic band overlaps when evaluating the analyte after adding doxycycline in a matrix solution obtained by microbial growth. The quantitative analytical method was linear, ranging from 1.00 to 100 µg mL⁻¹ through the external standard. A determination coefficient (r²) of 0.998 was established between analyte concentrations and their areas in the chromatographic analyses, Figure 3 (Table 1).

The evaluation of the accuracy and precision of the method were determined through doxycycline solutions at low, medium, and high concentrations (1.20, 50.0 and 95.0 µg mL⁻¹) covering the entire linear range through five different samples (n = 5) during three non-consecutive days (n = 3). These analyses showed a mean analyte recovery of 97.4 ± 2.70% and 97.5 ± 1.51% in intra-day and inter-day determination, respectively (Table 2). Precision was assessed by the relative standard deviation (RSD) of intra- and inter-day analyses; the accuracy was determined through average percentage recovery. RSD for repeatability at each concentration level of the standard solutions within a single day (n = 5) and between different days (n = 15) were less than or equal to ≤ 6.9% and ≤ 3.0%, respectively. Accuracy and RSD were 97.5 ± 2.45%, showing a strong agreement between experimental and theoretical values (Table 2).

The LOD, representing the lowest detected concentration of doxycycline, was 0.80 µg mL⁻¹, while the LOQ represents the lowest concentration at which doxycycline can be quantified through the analytical method with acceptable precision and accuracy, was determined to be 2.4 µg mL⁻¹. LOD and LOQ were obtained using the standard deviation of the intercept and slope.

The need for an evaluation of a liquid-liquid extraction method was a prerequisite for measuring the degradation of doxycycline. This would avoid the loss of the analyte in the growth media between steps and enable a sample treatment for HPLC analysis with precision and accuracy. The chosen concentrations enable us to quantify low and

Table 1. Values of the statistical treatments of the linear regression of the doxycycline calibration curve

<table>
<thead>
<tr>
<th>Regression statistics</th>
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<tr>
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<td>r² determination coefficient</td>
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<td>r² adjusted</td>
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<th>Analysis of variance (ANOVA)</th>
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<td>Source of variation    SS     df    MS    F     P-value    F crit</td>
</tr>
<tr>
<td>Between groups         4074379.118 1   4074379.1180  6.9350  0.01807  4.4940</td>
</tr>
<tr>
<td>Within groups          9400123.109 16  587507.6943</td>
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<tr>
<td>Total                  13474502.23 17</td>
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</tbody>
</table>

SS: sum of squares; df: degree of freedom; MS: mean squares; F: F-value.

Figure 3. Linearity of the method in the quantitative analysis of doxycycline by HPLC-UV. (a) Calibration curve obtained from standard doxycycline solutions, (b) chromatograms overlay obtained from standard solutions at different concentrations.
Table 2. Accuracy and precision data concerning to the validation doxycycline method by HPLC-UV

<table>
<thead>
<tr>
<th>Concentration / (µg mL⁻¹)</th>
<th>Precision intra-day (RSD) / %</th>
<th>Inter-day (n = 15, RSD) / %</th>
<th>Accuracy (n = 15) / %</th>
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<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td>1.20</td>
<td>98.9 (3.9)</td>
<td>99.6 (6.9)</td>
<td>94.6 (2.8)</td>
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<tr>
<td>50.0</td>
<td>96.8 (1.0)</td>
<td>99.1 (3.1)</td>
<td>99.1 (0.4)</td>
</tr>
<tr>
<td>95.0</td>
<td>97.5 (1.1)</td>
<td>98.5 (1.6)</td>
<td>92.5 (3.7)</td>
</tr>
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</table>

RSD: relative standard deviation.

Table 3. Recovery of doxycycline in growth media

<table>
<thead>
<tr>
<th>Concentration / (µg mL⁻¹)</th>
<th>Saline medium</th>
<th>Nutritional medium</th>
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<tr>
<td></td>
<td>Recovery ± RSD / %</td>
<td>Recovery ± RSD / %</td>
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<tr>
<td>2.50</td>
<td>99.0 ± 0.08</td>
<td>97.9 ± 0.04</td>
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<tr>
<td>25.0</td>
<td>98.4 ± 1.42</td>
<td>98.2 ± 1.31</td>
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<tr>
<td>50.0</td>
<td>99.2 ± 2.49</td>
<td>98.3 ± 1.28</td>
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</table>

RSD: relative standard deviation.

Evaluation of doxycycline biodegradation kinetics

The development of a quantitative analytical method enabled the determination of the antibiotic concentration range at different time intervals with precision and accuracy. Subsequently, the results from the doxycycline recovery evaluation in different bacterial growth media were associated with log(UFC mL⁻¹) values, with no limitations in the growth of E. cloacae cells in the presence of the antibiotic. This indicates that the bacteria metabolize the substance as a source of nutrients. On the other hand, S. maltophilia cells presented limited growth, showing bacterial inhibition in the presence of doxycycline. Apparently, exposure to the antibiotic in the short period of adaptation prevented the immediate response in the antibiotic expulsion pumps, limiting the development of new protection mechanisms for S. maltophilia. Additionally, there is the possibility of insufficient action of the proteins involved in antimicrobial uptake that restricted the resistance mechanism. Nonetheless, after 96 h of exposure, the S. maltophilia cells presented a minimal degradation of 5.02% (94.98% recovered) and 12.30% (87.70% recovered) in the YNB nutrient growth and saline growth media, respectively. The small biodegradation rate obtained by S. maltophilia in the YNB nutrient medium with respect to the saline medium was attributed to the growth medium (YNB nutrient), which is rich in nutrients, releasing the bacterial strain from using the antibiotic as an energy or nutrient source, thus metabolizing the antibiotic in low quantities, as shown in Figure 4. The two media chosen for the study were nutrient-rich and nutrient-poor to assess not only the biodegradation capacity but also the microbial growth capacity. In this case, the nutrient-poor medium (saline) proved to be more effective in biodegrading doxycycline, although it showed a lower cell growth rate (Figure 2). Leng et al. reported some results on the biodegradation of tetracyclines by S. maltophilia showing low biotransformation and significant increases in biomass due to the easily biodegradable substrate; however, they also presented other biotransformation pathways driven by hydrolysis that allowed the reduction of the antibiotic and the identification of six compounds (ca. 168 h).
The *E. cloacae* cells exposed for 96 h presented a degradation of 12.3% (87.7% recovered) and 36.9% (63.1% recovered) in the YNB nutrient and saline growth media, respectively (Figure 4). The versatility of *E. cloacae* is in large part due to the rapid response capacity of regulators that efficiently control membrane permeability, ensuring bacterial protection and the expression of detoxifying enzymes involved in degradation, thus inducing the activation of the metabolic action. *E. cloacae* has become a broad-spectrum bacterium described as an opportunistic and multiresistant bacterial pathogen due to the diffusion of extended-spectrum β-lactamases. The exposure of *E. cloacae* cells to doxycycline as the only food source poses a challenge for bioremediation applications. Nevertheless, taking advantage of acquired bacterial resistance is becoming a strategy for the biodegradation of the antibiotic.

Biodegraded products from doxycycline and metabolic pathway

The implementation of a doxycycline quantification method allowed quality control of the biodegradation under environmental conditions. Furthermore, it was possible to observe the formation of new analogous chemical structures that were less complex than the initial compound through the structural transformation of doxycycline by a cellular enzymatic process. The chromatogram of the ions extracted from the doxycycline degradation products is illustrated in Figure 5a. Nevertheless, the biodegraded products may be more harmful than the initial structure. In this case, not only should biodegradation be evaluated, but also the compounds generated by it.

In this work, we established the spectrometric conditions to evaluate degraded doxycycline byproducts using a selected precursor and suitable ions through full scan MS and ion MS/MS products. The identification of the molecular ion peak at mass-to-charge ratio (m/z) 445.1598 [M + H]+ with retention time of 6.695 min in the chromatogram corresponds to doxycycline, Figure 5b. The doxycycline fragmentation pathway describes the peaks at m/z 428.1334 [M + H - NH₃]+ and m/z 410.1225 [M + H - NH₃ - H₂O]+, confirming its presence, Figure 6a. The analysis of the saline medium after *E. cloacae* cells exposure for 96 h described the presence of the product P1, with a peak of protonated molecular ions at m/z 418.1498, describing the fragmentation pathway at m/z 472.1441 [M + H - N(CH₃)₂]+, m/z 390.1533 [M + H - CO₂]+, m/z 372.1441 [M + H - N(CH₃)₂]+ and m/z 279.0641 [M + H - H₂O - 2CO - C₃H₅OH]+, as shown in Figures 5c and 6b. The product P1 corresponds to the deamination of doxycycline and subsequent oxidation by OH. Amide groups may be nitrogen sources for bacterial growth. Some authors have previously described the formation of the P1 product through photocatalytic degradation tests during the elimination of doxycycline in water. In our assays, we evaluated the influence of the photocatalytic action using a control to counteract any influence of light on development, thus establishing the formation of P1 by bacterial doxycycline degradation. Product P2 presented a protonated molecular ion peak of m/z 417.1657 [M + H]+, with a fragmentation pattern of m/z 400.1385 [M + H - NH₃]+, m/z 382.1298 [M + H - H₂O - NH₃]+, and m/z 293.0780 [M + H - N(CH₃)₂ - CO - 2H₂O - NH₃]+, as shown in Figures 5d and 6b (Table 4). This product was generated through decarboxylation with the loss of CO and closure of the doxycycline molecule’s A ring, processes already reported in tetracyclines transformation studies with UV, H₂O₂/UV, and Fe²⁺/UV. The formation of substances analogous to doxycycline in a saline growth medium.

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**Figure 4.** Degradation profiling of bacterial strains after 96 h. (a) Saline growth medium, (b) nutrient growth medium (YNB).
Biodegradation of the Antibiotic Doxycycline by Bacteria from the Gastrointestinal Tract of Cucurbit Beetles (*Diabrotica speciosa*)

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**Populated by *E. cloacae*** indicates structural changes of the antibiotic after 96 h. No doxycycline products were detected in the YNB medium for both bacterial strains, on the other hand. This may be associated with the abundance of nutritional resources for bacterial cells, which would leave the antibiotic as an unattractive food source.

The products with smaller monoisotopic mass and complexity than the antibiotic correspond to substances with a nucleus of four rings, one aromatic with a hydroxyl substitution of polar characteristics, known as phenol, two saturated rings made up of oxygen substitutions (carboxyls and hydroxyl), and aliphatic chains made up of amino groups to the rings. The final products were identified with lesser structural complexity than doxycycline, providing an alteration in the chemical and physical features of the antibiotic, thus changing its assimilation into the environment.52

**Conclusions**

A HPLC-UV method was successfully developed and applied for direct quantification of doxycycline in YNB nutrient and saline media after biodegradation assays. The analytical method showed selectivity, precision, and accuracy necessary for antibiotic quantification. The biodegraded percentage allowed the identification of two degraded products by means of UHPLC-QTOF MS spectrometric techniques, allowing us to establish a relationship between the biotransformation of doxycycline to the analogous products generated after the biological tests. The doxycycline biodegradation capacity of the *E. cloacae* bacterial strain showed better potential than *S. maltophilia*, reducing more than 38.05% of the antibiotic in a saline growth medium after 96 h. Both bacteria evaluated were able to grow in the high antibiotic concentration media used in the study, especially in YNB medium. This work demonstrated the ability of some microorganisms to adapt and respond to the presence of an antibiotic. The results make the microbial strains evaluated as candidates for the development of bioremediation systems. Furthermore, the results confirm the hypothesis that we can find microorganisms with biotechnological potential in the most diverse biomes, or microbiomes, such as the gastrointestinal tract of an insect.

**Acknowledgments**

The authors are grateful for the financial support...
Table 4. Standard and degradation products determined by UHPLC-ESI-QTOF mass spectra

<table>
<thead>
<tr>
<th>Product</th>
<th>$t_r$ / min</th>
<th>$m/z$</th>
<th>Assignment</th>
<th>Mass error$^a$ / ppm</th>
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<tr>
<td>DOX</td>
<td>6.695</td>
<td>445.1598</td>
<td>[M + H]$^+$</td>
<td>1.79</td>
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<tr>
<td></td>
<td></td>
<td>428.1334</td>
<td>[M + H – NH$_3$]$^+$</td>
<td></td>
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<td></td>
<td></td>
<td>410.1225</td>
<td>[M + H – H$_2$O – NH$_3$]$^+$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>321.0745</td>
<td>[M + H – 2CO – C$_4$H$_5$OH]$^+$</td>
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</tr>
<tr>
<td>P1</td>
<td>7.438</td>
<td>418.1498</td>
<td>[M + H]$^+$</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td></td>
<td>390.1533</td>
<td>[M + H – CO$_2$]$^+$</td>
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<tr>
<td></td>
<td></td>
<td>372.1441</td>
<td>[M + H – N(CH$_3$)$_2$]$^+$</td>
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<tr>
<td></td>
<td></td>
<td>354.1316</td>
<td>[M + H – C$_3$H$_5$OH]$^+$</td>
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<tr>
<td></td>
<td></td>
<td>323.0556</td>
<td>[M + H – CO – C$_3$H$_5$OH]$^+$</td>
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<td>305.0438</td>
<td>[M + H – N(CH$_3$)$_2$ – C$_3$H$_5$OH]$^+$</td>
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<td>295.0588</td>
<td>[M + H – 2CO – C$_3$H$_5$OH]$^+$</td>
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<td>279.0641</td>
<td>[M + H – H$_2$O – 2CO – C$_3$H$_5$OH]$^+$</td>
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<tr>
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<td>417.1656</td>
<td>[M + H]$^+$</td>
<td>0.239</td>
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<td>400.1385</td>
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<td>382.1298</td>
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<td>293.0780</td>
<td>[M + H – N(CH$_3$)$_2$ – CO – 2H$_2$O – NH$_3$]$^+$</td>
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</table>

$^a$The mass error in ppm was calculated based on the theoretical monoisotopic masses and the observed values. DOX: doxycycline.
received from the Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (grant numbers 406537/2021-6, 429404/2018-2, 307800/2021-0, 465357/2014-8); the Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP (grant numbers 2018/00340-0, 2018/21201-8, 2014/50918-7, 2021/11878-3); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Finance Code 001) and the Research Center of the Universidad de Córdoba (CIUC) for the support granted.

Author Contributions

KSSM and LGCD designed the experiments, acquired, analyzed, interpreted the data, and drafted the manuscript; OICM and MRF conceived the study, analyzed, interpreted the data, and critically read and revised the manuscript with financial support; MRF analyzed, interpreted the data, drafted the manuscript, and provided the financial support; MFGFS participated in the data discussion, and with financial support. All authors contributed to the article and approved the submitted version.

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J. Braz. Chem. Soc. 2024, 000, 00, e-20230183