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Tolerance to glyphosate and antibiotic resistance in gram-negative bacteria isolated from soils of different agricultural management systems in Ceará, Brazil

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Abstract - Brazil is among the world's largest consumers of pesticides, with glyphosate (GLY) being the most commercialized herbicide in the country. Studies showed microorganisms suffer selective pressure when exposed to pesticides, developing tolerance to pesticides and resistance to antibiotics (ABs), in a phenomenon known as "cross-resistance". The present work aimed to evaluate the occurrence of glyphosate-tolerance and AB-resistance in bacteria isolated from different agricultural management systems in Ceará State, Brazil. Gram-negative bacteria isolated from agroforestry (S1), conventional farming (S2) and uncultivated (S3) soils were cultured in the presence of 1.6% acid glyphosate. Overall, 58 strains were isolated. Soils S1 and S2 presented several multidrug resistant (MDR) strains, the majority resistant to ampicilin. Although there was a small percentage of strains resistant to ertapenem (33%, soil S1), the fact they were found is concerning, as Carbapenem antibiotics are used to treat clinical cases of MDR bacteria, which are not common outside hospital settings. *Stenotrophomonas maltophilia* (soil S2), resistant to six of the eight ABs tested, was identified by MALDI-TOF mass spectrometry, and was found as one of the most common opportunistic bacteria in ICUs of Ceará hospitals.

Keywords: Multidrug resistant bacteria. *Stenotrophomonas maltophilia*. Agroforestry. Carbapenem. MALDI-TOF/MS bacteria identification.

Tolerância ao glifosato e resistência a antibióticos em bactérias gram negativas isoladas de solos de diferentes sistemas de manejo agrícola no Ceará, Brasil

Resumo - O Brasil está entre os maiores consumidores de pesticidas do mundo e o glifosato (GLI) é um dos herbicidas mais comercializados no país. Estudos mostraram que microrganismos expostos a pesticidas sofrem pressão seletiva, desenvolvendo tolerância a pesticidas e resistência a antibióticos (ABs), fenômeno conhecido como "resistência cruzada". Este trabalho objetivou avaliar a tolerância

ao GLI e resistência a ABs em bactérias isoladas de diferentes sistemas agrícolas do Ceará, Brasil. As bactérias Gram negativas isoladas no sistema agroflorestal (S1), convencional (S2) e solo sem cultivo (S3) foram cultivadas na presença de 1,6% do ácido GLI. Foram isoladas 58 cepas. Os solos S1 e S2 apresentaram várias cepas multirresistentes a drogas (MRD), sendo a maioria resistente à ampicilina. Apesar da detecção de pequena porcentagem de cepas resistentes ao ertapenem (33%, solo S1), a presença delas foi preocupante, visto que as Carbapenemas são usadas para o tratamento de casos clínicos envolvendo bactérias MRD, que não são comuns fora do ambiente hospitalar. *Stenotrophomonas maltophilia* (solo S2) apresentou resistência a 6 dos 8 ABs testados e foi identificada por espectrometria de massas MALDI-TOF, sendo encontrada como uma das espécies oportunistas mais comuns em UTIs de hospitais do Ceará.

Palavras-chave: Bactérias multirresistentes a drogas. *Stenotrophomonas maltophilia*. Sistema agroflorestal. Carbapenemas. Identificação de bactérias por MALDI-TOF/EM.

Tolerancia ao glifosato e resistencia a los antibióticos em bactérias gram negativas aisladas de suelos de diferentes sistemas de producción agrícola em Ceará, Brasil

Resumen - Brasil se encuentra entre los mayores consumidores de pesticidas del mundo, con el glifosato (GLI) como uno de los herbicidas más comercializados en el país. Estudios han demostrado que los microorganismos expuestos a los pesticidas sufren una presión selectiva, desarrollando tolerancia a los pesticidas y resistencia a los antibióticos (ABs), fenómeno conocido como "resistencia cruzada". Este trabajo tuvo como objetivo evaluar la tolerancia al GLI y la resistencia a los ABs en bacterias aisladas de diferentes sistemas agrícolas en Ceará, Brasil. Las bacterias Gram negativas aisladas de la agroforestería (S1), sistema tradicional (S2) y en el suelo sin cultivo (S3) se cultivaron en presencia de 1,6% de ácido GLI. Se aislaron 58 cepas. Los suelos S1 y S2 presentaron varias cepas resistentes a múltiples fármacos (RMF), la mayoría resistentes a ampicilina. Un pequeño porcentaje de cepas fue resistente al ertapenem (33%, S1), pero se consideró alarmante, ya que las carbapenemas se usan para tratar casos clínicos de bacterias RMF prevalentes en entornos hospitalarios. *Stenotrophomonas maltophilia* (suelo S2) mostró resistencia a 6 de los 8 ABs probados y fue identificado por espectrometría de masas MALDI-TOF, encontrándose como una de las especies oportunistas más comunes en las UCIs en los hospitales de Ceará.

Palavras claves - Bacterias resistentes a múltiples fármacos. *Stenotrophomonas maltophilia*. Agroforestería. Carbapenemas. Identificacíon de bacterias por MALDI-TOF/EM.

Introduction

The discovery of antibiotics (ABs) was a gigantic step for modern medicine, which briefly led the scientific community to believe that bacterial infections were under control. Likewise, the foremost development of agrochemicals was one of the pillars of the "Green Revolution", based on intensive agricultural techniques, chemical inputs, and mechanization. According to Food and Agriculture Organization (FAO), this paradigm is at its limit and needs to be more connected with sustainability (Campanhola and Pandey 2019). Worldwide consumption of ABs and agrochemicals has grown uncontrollably, reaching millions of tons per year for use in human disease treatment, farming, livestock, or poultry (Kirchhelle 2018; WHO 2018; Zhang 2018).

Brazil is one of the leading consumers of pesticides with glyphosate (GLY) being the most used herbicide in the country. This is due to the predominance of transgenic glyphosate-resistant soya monoculture in Brazil, amounting to 218 thousand tons sold in 2019 (Pignati et al. 2017; IBAMA 2019). Conversely, agribusiness began in Ceará State after the construction of irrigation canals during the 1980s, mainly in the Apodi Plateau and Ibiapaba Mountains, with emphasis on the cultivation of flowers and fruits for export. Since then, although the State has presented the lowest rate of pesticide commercialization by planted area in the country (VSPEA 2018), cases of occupational cancer, suicides and acute intoxications have increased, as have reports of baby malformation and early puberty attributed to the misuse of pesticides (Carneiro et al. 2015; Ferreira and Viana Jr. 2016; Rigotto and Aguiar 2017). Of the "derisory" amount of 760 tons of pesticides consumed in the State during 2019, 210 tons corresponded to GLY and 255 tons to dichlorophenoxyacetic acid (2,4-D), both from herbicide class (IBAMA 2019).

GLY is a non-selective systemic herbicide belonging to the substituted glycines class with a broad action spectrum that inhibits the enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), a catalyst for the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan, and inhibitor of chlorophyll production, reducing protein synthesis. The shikimate pathway enzymes are essential for many other organisms, including bacteria, being an attractive target for antibiotics. So GLY inhibits the growth of bacteria by cell starvation while it can also induce oxidative stress and differential expression of more than a thousand genes responsible for regulating essential functions such as the central carbon metabolism, chemotaxis, and cell motility, causing a severe effect on the general bacterial physiology (Fei et al. 2013; Hertel et al. 2021). Although the US Environmental Protection Agency considered GLY as a low toxicity agent when compared to its organochlorine and organophosphorus analogues (Baer and Marcel 2014), GLY can be absorbed by soil particles as well as its primary metabolite, the aminomethylphosphonic acid (AMPA), persisting in the environment. Traces of these substances have been detected in human urine samples, which proves their persistance and bioaccumulation, with potential to cause chronic effects (Singh et al. 2020). The increasingly widespread application of GLY generates a selective pressure capable of altering the biological composition of various habitats, including soil, water bodies, plant surfaces and even the animal microbiota. The consequences of long-term exposure to GLY are currently not well understood, leading the World Health Organization to reclassify GLY as a likely carcinogenic to humans (Van Bruggen et al. 2018).

Recent studies indicate that microorganisms exposed to pesticides suffer selective pressure with consequent development of resistance to antimicrobial agents and/or tolerance to other xenobiotics. When occorring concomintantly, the phenomenon is called "cross resistance", which has previously been observed in soil bacteria (Rangasamy et al. 2017) and in Gram-negative bacteria cultured in the presence of sub-lethal concentrations of GLY, dicamba and 2,4-D (Kurenbach et al. 2015). Resistance can be intrinsic, namely structural or functional characteristics inherent to a species allow its members to resist the action of certain antibiotics. In addition, bacteria can acquire or develop antibiotic resistance through different mechanisms, including minimizing the intracellular concentrations of the antibiotic through poor penetration or antibiotic efflux; by modifying the antibiotic target structure via *de novo* mutation or post-translational modification; via antibiotic inactivation by hydrolysis; and through horizontal acquisition of antibiotic resistance genes (Blair et

al. 2015). Particularly, bacteria can evolve GLY resistance by reducing GLY sensitivity by increasing EPSP synthase production through overexpressing the coding gene or by gene amplification; by degrading GLY through hydrolases, to access the phosphorous present in the molecule as an energy source; or detoxifying GLY by covalent modifications; by decreasing the uptake or increasing the efflux of the herbicide due to the overproduction of carrier proteins, among other mechanisms (Hertel et al. 2021). On the other hand, tolerance can be a heritable and evolvable trait, and describes bacterial ability to survive transient exposure to a high concentration of the antibiotic without changing their minimum inhibitory concentration. This is often mediated by a change in the physiological state such as slowing down essential cellular process, but inter- and intra-species interactions also play a role and may even reduce the antibiotic concentration in the environment. By evolving under intermittent exposure to antibiotics, bacteria can become resistant (Bottery et al. 2021).

In this work, we intend to investigate if soils submitted to different agricultural management systems show a diverse bacterial profile in respect to the occurrence of tolerance to GLY and AB resistance. To achieve this, Gram-negative bacteria were selectively isolated on GLY from agroforestry, conventional farming, and non-cultivated soils of Eusébio municipality in Ceará State. Bacteria presenting multiple drug resistance (MDR) were identified by MALDI-TOF spectrometry and were associated with reports of opportunistic infections in hospitals in the State of Ceará.

Materials and methods

Soil characteristics and sampling

On the morning of 12 Sept. 2018, during the dry season, agroforestry (S1), conventional (S2) and non-cultivated (S3) soils were collected in two farms located in Eusébio municipality, Ceará State, Brazil. Soils S1 and S3 came from the same farm.

Agroforestry (3°51'29.14"S / 38°25'6.59"O) system had been applied in the farm since 2014, without previous crop history. Soil was sandy, amended with the addition of phosphate rock and manure obtained from poultry feedlots. Irrigation was practiced with water from the property's well. Sampling was carried out randomly, collecting five sub-samples from 0 to 10 cm depth in an area of 20 m^2 containing several crops, e.g., cassava, beans, lettuce and sweet potatoes.

Uncultivated soil (3°51'31.87"S / 38°25'6.16"O) was sandy. The presence of weeds, domestic animals and their excrements prevailed, in addition to being used for the deposit of trees debris and other organic materials from cleaning of the property. Sampling was performed as in S1 soil.

Conventional farming soil, also sandy, was collected (3°50'17.55"S / 38°26'30.60"O) from 0 to 10 cm depth, in five beds of about 10 m2, each containing a different crop, e.g. coriander, chives, okra, cassava, beans and sweet potatoes. Use of hog, livestock, or chicken manure as natural fertilizers as well irrigation using water from a weir excavated near the crops were frequent practices. GLY was being applied on crops during the exploratory visit, as well a fungicide (name not informed) to prevent diseases in beans, as reported directly by the owner.

The sub-samples of each soil were transported to the lab inside plastic bags immediately after being collected. Then, they were mixed, stirred manually and vigorously for about two minutes to obtain a homogenized and representative sample of each soil type.

Isolation of glyphosate-tolerant Gram-negative bacteria

Samples were prepared in triplicate. Approximately 20 g of each homogenized soil sample was weighed into previously sterilized 500 ml Erlenmeyer flasks. 180 ml of sterile extraction solution (Cunha and Batista, 2014) was added to disaggregate the bacteria from soil grains. Briefly, 0.18 g of tetrasodium pyrophosphate (Merck), 0.18 ml Tween 80 (Merck), 1.53 g NaCl (Vetec) were dissolved in 174.6 ml distilled H2O. After sterilization and cooling, a suffice volume of commercial glyphosate solution (Nufarm), pre-filtered through a 0.2 µm pore size nylon syringe filter, was added to yield an extraction solution equivalent to 1.6% glyphosate acid, similar to the recommended agricultural dilution (Defarge et al. 2018), in order to generate a selective shock favoring growing of glyphosate-tolerant bacteria. Glass beads were added to enable the disaggregation process and the flasks were placed in a shaker incubator (TE 420, Tecnal, Brazil) at 150 RPM and 37°C for 30 min. After cooling to room temperature, aliquots of 1 ml were submitted to serial dilution, considering the initial sample as the first dilution at 10-1 concentration. Subsequent dilutions (10^{-2} to 10^{-5}) were carried out in test tubes containing 9 ml of sterile 0.85% NaCl solution. Aliquots of 1 ml from each dilution were pour plated in duplicate in MacConkey Agar (Difco) aiming the isolation of Gram-negative Enterobacteriaceae. Plates were incubated for 48 h at 35°C to provide sufficient time for lactose fermentation. The bacteria were counted in the range of 25 to 250 colonies. Viable cells were expressed in colony-forming units (CFU/g soil) from duplicates mean.

Isolated bacteria were transferred to tubes containing Trypticase Soy Agar medium (Difco) enriched with the equivalent of 0.5% glyphosate acid and stored at 4oC. The purity and morphology of the isolated bacteria were evaluated by Gram stain method modified by Hucker (Levy 2004).

Antibiotic resistance screening

Antibiotic susceptibility of glyphosate-tolerant strains was evaluated by disk diffusion method (Bauer et al. 1966) on Mueller-Hinton agar (Difco), incubated for 24 h at 35ºC. The antibiogram was performed using eight antibiotics from six different classes at the following concentrations per disk: β-lactams ampicillin and ertapenem (Cecon; 10 µg), chloramphenicol (Laborclin; 30 µg), fluoroquinolone ciprofloxacin (Cecon; 5 µg), macrolide erythromycin (Laborclin; 15 µg), tetracycline (Cecon; 30 µg), aminoglycoside streptomycin (Laborclin; 10 µg) and gentamicin (Cecon; 10 µg). E. coli ATCC 25922 strain was used to monitor test performance. The diameter of growth inhibition zone around the disks were measured in millimeters by pachometer. The results were interpreted according to CLSI criteria for Entereobacteriaceae (2017). Isolates were classified according to Magiorakos et al. (2012), with strains resistant to three or more antibiotics of different classes considered multidrug-resistant (MDR). The Neisseria criterion (Andrews 2009) was used for erythromycin since there was no registered test of this antibiotic for Enterobacteriaceae.

Identification of antibiotic multiresistant bacteria by MALDI-TOF/MS

Bacterial isolates were inoculated in duplicate on Mueller-Hinton agar (Difco) and cultivated for 24 h at 35ºC. The direct transfer protocol, referred to as whole-cell measurement,

was followed to obtain mass spectra. Briefly, the bacterial material of each plate was directly transferred to twelve MALDI target spots and dried at room temperature. The sample spots were overlaid with 1 µL of matrix solution (20 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 1% trifluoroacetic acid) and dried at room temperature. MS analysis was performed on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) in linear positive mode. Calibration was performed using the Bacterial Test Standard (Bruker Daltonics, Germany). Twenty four mass spectra (12 spots from each duplicate plate) were obtained for each strain using the Flex Control software version 3.3 according to the standard measurement method for microbial identification, in a mass range of 2-20 kDa. Bacterial identification was performed by BioTyper software version 3.1 (Bruker Daltonics, Germany) using identification score.

Statistical analysis

The analyses were carried out using BioEstat software 5.3. Simpson index indicated diversity and proportion of bacteria: the higher the value, the greater the diversity of species. Shannon-Wiener index accounted for both abundance and evenness (equitability) of the species present, which assumes values between 0 and 1, with 1 being complete evenness (same frequency). The data normality was assessed by Shapiro-Wilk test. Kruskall-Wallis one-way ANOVA determined the diferences of AB profiles between the soils and significance was established by Student-Newman-Keuls test at 95% confidence level.

Detection of metals in chicken manure

Dried chicken manure used as organic soil fertilizer on S1 soil (the brand was not informed) was analysed by Inductively Coupled Plasma Optical Emission Spectrometer with axial vision (ICP-OES iCAP 6000, Thermo Scientific). Sample was prepared according to Milestone application note HPR-OF-07 adapted from AOAC official method 999-10 (1999). Briefly, 7 ml of 65% nitric acid and 1 ml of 30% hydrogen peroxide were added to 0.5 g of pulverized sample, which was digested into microwave at 200oC for 45 min. After been cooled at room temperature, sample volume was brought to 50 ml with deionized water.

Results and discussion

Glyphosate-tolerant bacteria count and morphotintoral characterization

Counting of bacteria grown on McConkey agar revealed that the agroforestry management system (S1) displayed the highest number of glyphosate-tolerant bacteria (1.6×10^5 CFU/g) followed by conventional farming (S2) (5.6×10^4 CFU/g) and non-cultivated soil (S3) (1.0×10^2 CFU/g). After counting, bacterial colonies were isolated in TSA medium enriched with GLY. Soils S1 and S2 yielded 30 colonies each, resulting 27 and 26 viable isolates, respectively (Tables 1 and 2). From soil S3, 12 colonies were isolated, resulting only five viable isolates (Table 3).

Prior to obtaining the results, soil S2 was expected to have the highest number of glyphosate-tolerant bacteria among the three studied soils, as continued exposure to pesticides creates an

environment that allows the bacterial population to develop detoxification mechanisms (Jørgensen et al. 2018; Kurenbach et. al. 2018; Rangasamy et al. 2017). However, it was surprising that soil S1 showed so many glyphosate-tolerant bacteria, since its management does not imply the use of pesticides. A possible cause may be the manure used to fertilise the soil. Several Brazilian farms, focused on the production of broiler and pig, add antibiotics in animal feed as growth promoters (AGPs), which may cause strains resistant to antibiotics (Bokma et al. 2014) and a consequent tolerance to GLY. It is also known that several pesticides can permeate soil and sub-soil and contaminate groundwater (Lima et al. 2021; Milhome et al. 2009), which could be a second reason for the presence of tolerant/resistant bacteria, since soil S1 was frequently irrigated with well water. In addition, chicken manure may contain lead due to treated wood chips used as litter material (Crippen et al. 2016) as well zinc and copper used as an alternative to AGPs and as mineral supplements (Gaste et al. 2002; Yazdankhah et al. 2014).

The presence of heavy metals in soil may induce bacteria tolerance to metals through various mechanisms including some used by several bacteria to detoxify themselves from pesticides and ABs (Jarosławiecka and Piotrowska-Seg et al. 2014; Curutiu et al. 2017).

In fact, the ICP-OES analysis of a chicken manure sample used on the farm revealed high levels of lead (439 ppm), copper (256 ppm) and zinc (254 ppm), while the maximum allowable values were 45, 70 and 200 ppm, respectively (MAPA 2011).

Antibiotic susceptibility profile

The antibiograms of the viable bacteria revealed different profiles according to the soil origin. Of the 58 viable strains tested, 93% showed resistance to at least one of the antibiotics tested (Tables 1, 2 and 3). Most of the strains were resistant to ampicillin, chloramphenicol, and erythromycin (Table 4).

In Brazil, beta-lactams, tetracyclines, quinolones and sulfonamides were banned as AGPs since 1999, mainly for meat destined for the international market and to a lesser extent, for the home market. In addition, the use of zinc and copper salts has increased in veterinary use (Bokma et al. 2014), which could explain the high levels of these metals found in the chicken manure and could induce the emergence of the bacterial resistance phenotype observed in soil S1. In addition, 58% of the strains from soil S2 did not show intermediated resistance, but 39% of them were classified as MDR bacteria according to Magiorakos et al. (2012), against 22 and 20% from soils S1 and S3, respectively. Only bacteria resistant to three or more ABs were identified by MALDI-TOF mass spectrometry analysis (Tables 1, 2 and 3).

Susceptibility to streptomycin and gentamicin aminoglycosides as well to the fluoroquinolone ciprofloxacin was prevalent and had similar diversity index and evenness values among the three studied soils (Table 4), perhaps due to the policies restricting the use of these antibiotics in agriculture and veterinary (Bokma et al. 2014; Kirchhelle 2018). Soils S1 and S2 did not display statistically significant differences between their AB susceptibility profile but both did in relation to soil S3 ($p < 0.01$).

Table 1. Lactose fermenting determination, identification by MALDI/TOF and antibiotic profile of viable Gram negative bacilli isolated from conventional farming soil.

Bacillus	Lactose fermentation	MALDI/TOF identification	Score/ NCBI ID	Matched pattern	Score of matched pattern	AB resistance	AB susceptibility	AB intermediate resistance
C1	+					Amp	All but Amp	NIR
C3	+					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
C5	+					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
C7	+					Amp	All but Amp	NIR
C8	-	*Stenotrophomonas maltophilia* (PX) 23086229 MLD	2.324/ 40324			Amp, Gen, Tet, Ert, Str	Clo, Cip	Ery
C9	-					Ery	All but Ery	NIR
C10	+	*Enterobacter cloacae* ssp *cloacae* DSM 30054T HAM	2.334/ 550	C25 P23	2.873 2.805	Amp, Tet, Ery	Gen, Clo, Ert, Str	Cip
C11	+	*Leclercia adecarboxylata* DSM 5077T DSM_2	1.997/ 83655			Amp, Clo, Tet	Gen, Cip, Ert, Str, Ery	NIR
C13	+					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
C14	+					Amp	Clo, Tet, Cip, Ert	Gen, Ery
C15	-	*Ochrobactrum intermedium* LMG 3301T HAM	1.758/ 94625			Amp, Clo, Str	Gen, Cip, Ert, Ery	Tet
C16	-					Amp, Tet	Gen, Clo,Cip, Ert, Str, Ery	NIR
C17	+	*Klebsiella pneumoniae* ssp *rhinoscleromatis* CCM 5791T CCM	2.276/ 39831	C24	2.741	Amp, Clo, Tet	Gen, Cip, Ert, Ery	Str
C18	-	*Pseudomonas nitroreducens* DSM 14399T HAM	1.727/ 46680			Amp, Str, Ery	Gen, Clo, Tet, Cip	Ert

Bacillus	Lactose fermentation	MALDI/TOF identification	Score/ NCBI ID	Matched pattern	Score of matched pattern	AB resistance	AB susceptibility	AB intermediate resistance
C19	+					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
C20	+					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
C21	-					Amp	All but Amp	NIR
C22	+					Amp, Tet	Gen, Clo, Ert, Str, Ery	Cip
C23	-					Ery	All but Ery	NIR
C24	+	*Klebsiella pneumoniae* ssp *rhinoscleromatis* CCM 5791T CCM	2.364/ 39831	C17	2.740	Amp, Clo, Tet	Gen, Cip, Ert	Str, Ery
C25	-	*Enterobacter cloacae* ssp *cloacae* DSM 30054T HAM	2.336/ 550	C10 P23	2.867 2.741	Amp, Clo, Tet	Gen, Cip, Ert	Str, Ery
C26	-	*Pseudomonas putida* Group 53 PIM	2.030/303	P10 P22	2.713 2.698	Amp, Clo, Ery	Gen, Cip, Str	Tet, Ert
C27	+					Amp, Clo, Tet	Cip, Ert	Gen, Str, Ery
C28	Lac +					Amp, Tet	Gen, Clo, Cip, Ert, Str, Ery	NIR
C29	Lac +					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
C30	Lac +					Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR

Score values: 2.300 to 3.000 = highly probable species identification; 2.000 to 2.299 = secure genus identification, probable species identification**;** 1.7000 to 1.999 = probable genus identification**;** 0.000 a 1.699 = not reliable identification**.**
AB = antibiotic; NR = no AB resistance; NIR = no intermediate resistance; Amp = ampicillin; Gen = gentamycin; Clo = chloramphenicol; Tet = tetracycline; Ert = ertapenem; Str = streptomycin; Ery = erythromycin; Cip = ciprofloxacin.

Table 2. Lactose fermenting determination, identification by MALDI/TOF and antibiotic profile of viable Gram negative bacilli isolated from agroforestry soil.

Bacillus	Lactose fermentation	MALDI/TOF identification	Score/ NCBI ID	Matched pattern	Score of matched pattern	AB resistance	AB susceptibility	AB intermediate resistance
P1	-					Amp, Clo	Gen, Cip, Str, Ery	Tet, Ert
P2	+					NR	All	NIR
P4	+					NR	All but Amp	Amp
P5	+					Ery	Gen, Clo, Cip, Ert, Str	Amp, Tet
P6	+	*Serratia ureilytica* DSM 16952T DSM	2.201/ 300181			Amp, Tet, Ery	Gen, Clo, Cip, Ert, Str	NIR
P7	-					Amp	Gen, Cip, Ert, Str, Ery	Clo, Tet
P8	+					Amp, Clo	Gen, Cip, Ert, Str, Ery	Tet
P9	+					Amp	Gen, Cip, Ert, Str	Clo, Tet, Ery
P10	-	*Pseudomonas putida* Group 53 PIM	1.954/303	P22 C26	2.757 2.721	Amp, Clo, Ery	Gen, Cip, Ert, Str	Tet
P11	-					Amp, Clo, Ery	Gen, Tet, Cip, Str	Ert, Ery
P12	-					Amp	Gen, Clo, Tet, Cip, Ert, Str	Ery
P14	-					Amp	Gen, Tet, Cip, Str, Ery	Clo, Ert
P15	+					Amp, Clo	Gen, Cip, Str, Ery	Tet, Ert
P16	-					Amp, Clo	Gen, Tet, Cip, Ert, Str, Ery	NIR
P17	+					Amp, Clo	Gen, Tet, Cip, Str, Ery	Ert
P18	+					NR	All	NIR
P20	-					NR	Amp, Gen, Clo, Tet, Cip, Ert, Str	Ery
P21	+					Amp, Tet	Gen, Clo, Str, Ery	Ert
P22	-	*Pseudomonas putida*_Group 53 PIM	2.112/303	P10 C26	2.748 2.696	Amp, Clo, Ery	Gen, Cip, Ert, Str	Tet

Bacillus	Lactose fermentation	MALDI/TOF identification	Score/ NCBI ID	Matched pattern	Score of matched pattern	AB resistance	AB susceptibility	AB intermediate resistance
P23	-	*Enterobacter cloacae ssp cloacae* DSM 30054T HAM	2.288/550	C10 C25	2.800 2.747	Amp, Tet	Gen, Cip, Ert, Str, Ery	Clo
P24	-					Amp, Clo	Gen, Tet, Cip, Ert, Str	Ery
P25	-					Amp	All but Amp	NIR
P26	-	*Enterobacter cloacae* MB11506_1 CHB	2.099/550	P30 P29 C25 C10 P23	2.855 2.826 2.187 2.180 2.160	Amp, Clo, Ery	Gen, Tet, Cip, Str	Ert
P27	-	*Clostridium beijerinckii* 1011_ DSM 552 BOG	1.358/1520			Amp, Clo, Ert, Str, Ery	Gen, Cip	Tet
P28	-					Ery	Gen, Clo, Cip, Ert, Str	Amp, Tet
P29	-	Enterobacter cloacae MB11506_1 CHB	2.019/550	P26 P30 C25 P23 C10	2.821 2.788 2.194 2.172 2.121	Amp, Ert, Ery	Gen, Clo, Tet, Cip, Str	NIR
P30	-	*Enterobacter cloacae* MB11506_1 CHB	2.024/550	P26 P29 C25 C10 P23	2.850 2.783 2.105 2.092 2.043	Amp, Clo, Ery	Gen, Tet, Cip, Str	Ert

Score values: 2.300 to 3.000 = highly probable species identification; 2.000 to 2.299 = secure genus identification, probable species identification**;** 1.7000 to 1.999 = probable genus identification**;** 0.000 a 1.699 = not reliable identification**.**
AB = antibiotic; NR = no AB resistance; NIR = no intermediate resistance; Amp = ampicillin; Gen = gentamycin; Clo = chloramphenicol; Tet = tetracycline; Ert = ertapenem; Str = streptomycin; Ery = erythromycin; Cip = ciprofloxacin.

Table 3. Lactose fermenting determination and antibiotic profile of viable Gram negative bacilli isolated from uncultivated soil.

Bacillus	Lactose fermentation	AB resistance	AB susceptibility	AB intermediate resistance
S1	-	Amp	Gen, Clo, Tet, Cip, Ert, Str, Ery	NIR
S4	+	Amp, Clo, Ery	Gen, Cip	Tet, Ert, Str
S6	+	Amp, Ery	Gen, Tet, Cip, Ert, Str	Clo
S10	+	Amp, Ery	Gen, Clo, Tet, Cip, Ert, Str	NIR
S11	+	Amp	Gen, Clo, Tet, Cip, Ert, Str, Ery	NIR

AB = antibiotic; NIR = no intermediate resistance; Amp = ampicillin; Gen = gentamycin; Clo = chloramphenicol; Tet = tetracycline; Ert = ertapenem; Str = streptomycin; Ery = erythromycin; Cip = ciprofloxacin.

Table 4. Diversity indices, equitability and proportion of resistent, susceptible and intermediate bacteria according to the antibiotic tested and soil origin.

		S3 R	S1 R	S2 R	S3 S	S1 S	S2 S	S3 I	S1 I	S2 I
Shannon-Wiener index		0.41	0.61	0.67	0.83	0.85	0.85	0.60	0.66	0.74
Equitability (evenness)		0.45	0.68	0.74	0.92	0.94	0.94	0.67	0.73	0.82
Simpson index		0.57	0.70	0.74	0.85	0.85	0.85	0.75	0.76	0.80
Bacteria proportion (%)	Amp	55.56	43.75	41.38	0.00	2.17	1.15	0.00	10.00	0.00
	Gen	0.00	0.00	1.72	18.52	19.57	17.29	0.00	0.00	11.76
	Clo	11.11	25.00	12.07	11.11	7.97	14.29	25.00	13.33	0.00
	Tet	0.00	6.25	17.24	14.81	10.14	10.53	25.00	33.33	11.76
	Cip	0.00	0.00	0.00	18.52	19.57	18.05	0.00	0.00	11.76
	Ert	0.00	4.17	1.72	14.81	12.32	17.29	25.00	26.67	11.76
	Str	0.00	2.08	5.17	14.81	18.84	14.29	25.00	0.00	23.56
	Ery	33.33	18.75	20.69	7.41	9.42	6.77	0.00	16.67	29.41

S1: agroforestry; S2: conventional farming soil; S3: non-cultivated soil; R: antibiotic resistant; S: antibiotic susceptible; I: antibiotic intermediate resistant; Amp = ampicillin; Gen = gentamycin; Clo = chloramphenicol; Tet = tetracycline; Ert = ertapenem; Str = streptomycin; Ery = erythromycin; Cip = ciprofloxacin

Among the identified bacteria, two species were common to soils S1 and S2. The first, *Pseudomonas putida* (strains P10, P22 and C26), presented the same AB resistance profile, while Enterobacter' strains showed resistance profile much more diversified (Tables 1 and 2), indicating acquisition of AB resistance by different mechanisms. Of the identified MDR bacteria, *Enterobacter* spp., *Serratia* spp., *Klebisiella pneumoniae*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were found among the most frequent species causing laboratory-confirmed primary bloodstream infections (BSIs) in ICUs from hospitals in Ceará State (ANVISA 2020). *S. maltophilia* is a global emerging nosocomial pathogen responsible for cases of bacteremia (BSIs), respiratory and urinary tract infections among others, and associated with high mortality rates. The treatment of diseases caused by this bacterium is difficult due to its intrinsic resistance to several groups of antibiotics including the carbapenems (Brooke 2012).

Enterobacter is an abundant genus in terrestrial and aquatic environments, and is a component of the human intestinal commensal microbiota. *Enterobacter* are also opportunistic pathogens causing endocarditis, septic arthritis, osteomyelitis, skin infections, respiratory and intra-abdominal infections. *E. cloacae* tends to contaminate intravenous devices, surgical equipment and surgical cleaning solutions, and is directly linked to nosocomial outbreaks (Davin-Reglin and Pagès 2015). *E. cloaceae* P29 strain showed resistance to Ert and, consequently, is included in the WHO list of critical priority pathogens for which new AB are urgently needed (WHO 2017).

Conclusion

The obtained results in the present study point to bacteria isolation selected by GLY and co-ocurrence of tolerance to GLY and AB resistance. The frequent exposure of bacteria to pesticides may be favoring the prevalence of bacterial species related to human nosocomial infections in ICUs of hospitals in Ceará State, requiring a meticulous research to relate the origin of the resistant strains in the hospitals and the mechanisms of resistance towards the discovery of new antibiotics.

This work also demonstrated that even sustainable agricultural practices, such as agroforestry, could disseminate resistant bacteria to the environment and cause health problems. Therefore, careful attention is needed to the quality of the natural fertilizers used to enrich the soil.

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