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AVALIAÇÃO DE ELEMENTOS GENÉTICOS DE RESISTÊNCIA A ANTIBIÓTICOS EM ÁREA SOB INFLUÊNCIA DA FERTIRRIGAÇÃO COM EFLUENTE TRATADO DE UMA GRANJA DE SUINOCULTURA

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1 RESUMO

Uma granja de suinocultura que concede seu efluente tratado para fertirrigação de milho, foi escolhida para monitoramento de genes de resistência a antibióticos. Foram investigados a ocorrência e abundância dos genes de resistência aos antibióticos beta-lactâmicos (*blaTEM*), macrolídeos (*ermB*), quinolonas (*qnrB*), tetraciclinas (*tetA*) e sulfonamidas (*sul1*), além do elemento genético móvel integrase classe 1 (*intI1*) e do gene que estima bactérias totais (*RNAr 16S*), em amostras do efluente tratado, do solo fertirrigado, do solo sem histórico de fertirrigação direta, e de água de um açude, ambos localizados à jusante da área fertirrigada. Foram realizadas quatro campanhas amostrais, com coleta de 1L efluente tratado, cinco amostras de 250 g de cada solo, além de 2L de água do açude. A quantificação dos genes foi realizada por qPCR com os *primers* correspondentes a cada gene. Todos os genes investigados e o elemento genético móvel *int*1 foram detectados em todas as campanhas amostrais dos solos, da água do açude e do efluente tratado. O gene de resistência à sulfonamida foi o mais abundante entre as amostras, com exceção das amostras do açude. Comparativamente, os genes *sul1* e *tetA* foram estatisticamente mais abundantes no solo fertirrigado do que no solo não fertirrigado, em todas as coletas. Isso indica que a utilização do efluente de suinocultura, mesmo que tratado, pode causar a disseminação de resistência a antibióticos para o ambiente.

Keywords: genes de resistência, irrigação, dejetos suínos

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EVALUATION OF GENETIC ELEMENTS FOR ANTIBIOTIC RESISTANCE IN AREA UNDER INFLUENCE OF FERTIRRIGATION WITH TREATED EFFLUENT FROM A PIG FARM

2 ABSTRACT

A swine farm that provides its treated effluent for irrigation of corn was chosen to monitor antibiotic resistance genes. The occurrence and abundance of resistance antibiotics genes to

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beta-lactams (*blaTEM*), macrolides (*ermB*), quinolones (*qnrB*), tetracyclines (*tetA)* and sulfonamides (*sul1*), as well as the mobile genetic element integrase class 1 (*intI1*) and the gene that estimates total bacteria (16S rRNA) were analized. Samples of treated effluent, fertigated soil, soil without a direct fertigation, and water from a pond, both located downstream of the fertigated area, were collected during four sampling campaigns. A total of 1 L of treated effluent, five samples of 250 g of each soil, and 2L of water from the pond were sampled. The quantification of genes was performed by qPCR with primers corresponding to each gene. All investigated genes and the mobile genetic element *int*1 were detected in all soil samples, pond water and treated effluent. The *sul1* gene was the most abundant among the samples, but not at the pond water. Comparatively, the *sul1* and *tetA* gene were statistically more abundant in the fertigated soil than in the non-fertigated soil, in all sampling collections. This indicates that the use of swine farming effluent, even if treated, can cause the spread of antibiotic resistance to the environment.

Keywords: resistance genes, swine manure, soil**.**

3 INTRODUCTION

The reuse of pig farm effluent for irrigation has become a common and widespread activity. Pig confinement areas typically have a high concentration of animals per area, generating a concentrated volume of manure composed of feces, urine, and hair.

Considering that in pig farming management practices, antibiotics are offered to animals, both for therapeutic purposes and as growth promoters (ZEINELDIN, ALDRIDGE; LOWE, 2019), it is speculated that animal waste may contain microorganisms resistant to these drugs and that, with the practice of soil fertigation with this waste (raw or treated), there is, therefore, a risk of disseminating these bacteria, as well as their antibiotic resistance genes (GRAs), in the environment.

Conventional wastewater treatments have not been developed to remove low-concentration contaminants such as antibiotics, GRAs, and resistant bacteria. The removal or reduction of these pollutants depends directly on the type of technology used and the size and composition of the effluent (CHEN; ZHANG, 2013; OSIŃSKA *et al*., 2017).

The ability of bacteria to resist antibiotics depends on the presence of chromosomal or plasmid genes that confer abilities such as inactivation of antibiotics by enzymes or structural alterations, a reduction in drug concentration or prevention of antibiotic accumulation and alteration of the drug receptor (CHEN; CHOPRA; KAYE, 2011; SANTOS, 2014). These GRAs have been used as parameters during the monitoring of water, sewage and livestock waste (ARAÚJO *et al.*, 2020; PEREIRA *et al.*, 2021).

However, approaches regarding the effects of the application of treated effluent from the perspective of antibiotic resistance are still scarce and controversial in the literature. The most common studies are on soils irrigated with raw effluent or with the application of animal manure, which present a greater abundance of GRAs after this type of soil treatment than control soils do (HAN *et al*., 2016; HEUER *et al*., 2011; PENG *et al*., 2015). In other studies, no significant increase in the relative abundance of GRAs, such as those that confer resistance to sulfonamides, tetracyclines, and erythromycins, was observed in soils that received treated effluent, even in long-term experiments lasting 6--18 years (BEN *et al.*, 2017; NEGREANU *et al*., 2012). However,

other studies have shown an increase in the relative abundance of sulfonamides, fluoroquinolones, beta-lactams, tetracyclines and mobile genetic element integrase I, among other genes, in soils irrigated with wastewater (CHEN *et al*., 2014; KAMPOURIS *et al*., 2021).

Thus, given that antimicrobial resistance is among the greatest risks to global health (WHO, 2015), the present study aimed to evaluate the occurrence of GRAs in environmental samples (effluent, soil and water) from an area subjected to fertigation with treated effluent from a pig farm.

4 MATERIALS AND METHODS

4.1 Samples and physical–chemical analysis

Four soil sampling campaigns were carried out in the fertigated area (soil 1, code S1), and similarly, a downstream area with no history of direct effluent discharge, called soil 2 (code S2), was selected (CAETANO, 2021). The study soil is characterized as a dystrophic red–yellow latosol, which comprises a soil with low fertility, low phosphorus content, and low amount of water available to plants (ALMEIDA; SANTOS; ZARONI, 2021). In each sampling campaign, approximately 250 g of soil was collected from a 10 cm deep layer at five random points in the study area, as described in a document from the Brazilian Agricultural Research Corporation (FILIZOLA; GOMES; SOUZA, 2006). At the same time, samples of treated effluent from the outlet of the irrigation pipe that is directed to the corn plantation (code E, volume 1 L) and water samples from a reservoir, comprising a composite sample located downstream of the irrigation (code A, volume 2 L), were collected (CAETANO, 2021). Analyses of pH, total organic carbon (TOC), total nitrogen (TN), clay, silt and sand contents were performed, as shown in Table 1. For TOC and TN analyses of the effluent, the samples were filtered through 0.45 μm and 47 mm filtration membranes and diluted 50 times with ultrapure water.

All the samples were preserved for molecular analysis. For this purpose, 10 g of each soil sample was frozen (-20 °C) after two washes in PBS (*phosphate buffer solution*, 140 mM NaCl, 10 mM $Na2HPO4$, 2 mM $KH2PO4$, 3 mM KCl, pH 7.2), followed by centrifugation at 4,500 rpm for 10 minutes. For liquid samples, 300 mL of sample was used, which, after centrifugation and washing of the pellet under the same conditions described above, was frozen at -20 °C.

Table 1. Physicochemical characterization of the soil and effluent samples.

Parameters	Method/ Equipment	Solo 1	Solo 2	Treated effluent
pH	pH meter AZ	6.28	8.02	-
TOC (mg.g $^{-1}$ or mg.L $^{-1}$)	TOC-LCPH/CPN (Shimadzu®)	28.65	17.88	659.30
NT (mg.g $^{-1}$ or mg.L $^{-1}$)		0.31	0.12	2534.50
Clay (%)	NBR NM248.	26.64	13.41	IN
Silt (%)		20.29	14.27	IN
Sand (%)		53.06	72.32	IN

Source: Caetano (2021).
TOC: total organic carbon, NT: total nitrogen; NA: not applicable. Standard deviation for soil 1 (n=20): 0.3, 4.2 and 0.2 (for pH, TOC and NT analyses, respectively). Standard deviation for soil 2 (n=20): 0.5; 7.8; 0.1 (for pH, TOC and NT analyses, respectively).

4.2 DNA extraction and quantification of antibiotic resistance genes, integrases and 16S rRNA

First, 0.25 g of each sample was preserved and subjected to DNA extraction via the DNeasy PowerSoil Kit (QIAGEN®), following the protocol recommended by the manufacturer. The genomic DNA extracted from each sample was subsequently quantified via a spectrophotometer and then subjected to dilutions to normalize all the samples to a concentration of 5 ng/µL.

The amplification and quantification of GRAs were performed via a real-time PCR 7500 (*Applied Biosystems*). Primers containing beta-lactam, erythromycin, sulfonamide, tetracycline, and fluoroquinolone resistance genes (*blaTEM, ermB, sul1, tetA,* and *qnrB genes*, respectively) were used, in addition to the mobile genetic element integrase 1 (*intI*) and the 16S fraction of the ribosomal RNA gene that represents the bacterial domain (16S rRNA) (Table 2). For each reaction, 3.8 µL of ultrapure water, 5.0 µL of PowerUp™ $^{SYBR™}$ Green Master Mix, 0.1 µL of each primer pair (30 µM), and 1.0 µL of DNA sample were added.

Table 2. List of primers

Gene	*Primer*	Sequence	Temp (°C)	Amplicon (pb)
titA	*tetA* fw *tetA* rv	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	54.6	210
south1	*sul1* fw *south1* rv	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	55.8	163
ermB	*ermB* fw *ermB* rv	CGTGCGTCTGACATCTATCTGA CTGTGGTATGGCGGGTAAGTT	56.8	190
qnrB	*qnrB* fw *qnrB* rv	GCGACGTTCAGTGGTTCAG TGTCCAACTTAACGCCTTGTAA	54.7	148
bla TEM	*bla* TEM fw *bla* TEM rv	GCACGAGTGGGTTACATCGA GGTCCTCCGATCGTTGTCAG	48	311
int1	*int1* fw *int1* rv	CCTCCCGCACGATGATC TCCACGCATCGTCAGC	60	280
16S rRNA	1055 fw 1392 rv	ATGGCTGTCGTCAGCT ACGGGCGGTGTGTAC	53	337

Source: Caetano (2021); References: 1- Mao *et al*. (2015); 2 - Tao *et al*. (2014); 3 – Goldstein *et al.* (2001); 4- Ferris, Muyzer and Ward *et al*. (1996)

Absolute gene quantification was obtained via calibration curves with controls for each gene analyzed. These were obtained by cloning each specific gene into a plasmid vector (pGem Easy Vector Kit, Promega), followed by transformation into *Escherichia coli* JM109. Standard curves for each gene in the study were designed, containing six points, with serial 1:10 dilutions of the controls. For each standard curve, the equation was obtained by crossing the log of the concentration of each point (initial concentration of the positive controls) and the *threshold cycle (Ct)* for each point. The Ct corresponds to the point in the qPCR at which reflective fluorescence is detected in each sample, allowing the quantification of the number of DNA copies generated.

Ideal curves were considered when $R^2 > 0.99$, the slope of the line was between -3.1 and -3.6, and an efficiency between 90 and 110% was achieved. Each analysis was performed in triplicate for the curve and samples, and a blank sample without *template was added*. To estimate the quantification of gene copies per µL and per gram of each sample, the Ct was substituted into the equations of each standard curve, with subsequent correction, observing the volume, estimated and actual DNA quantification (after extraction), soil and *pellet masses*, and the solids content of the liquid samples. Thus, the absolute abundance was obtained, represented by the number of gene copies per mL of liquid sample or gram of soil. Relative quantification was determined by the ratio of the absolute abundance (number of gene copies per mL^{-1} or g of sample) of each gene to the quantity of total bacteria (number of 16S rRNA copies per mL^{-1} of sample or g of soil). Further details can be found in Caetano (2021).

4.3 Statistical analysis

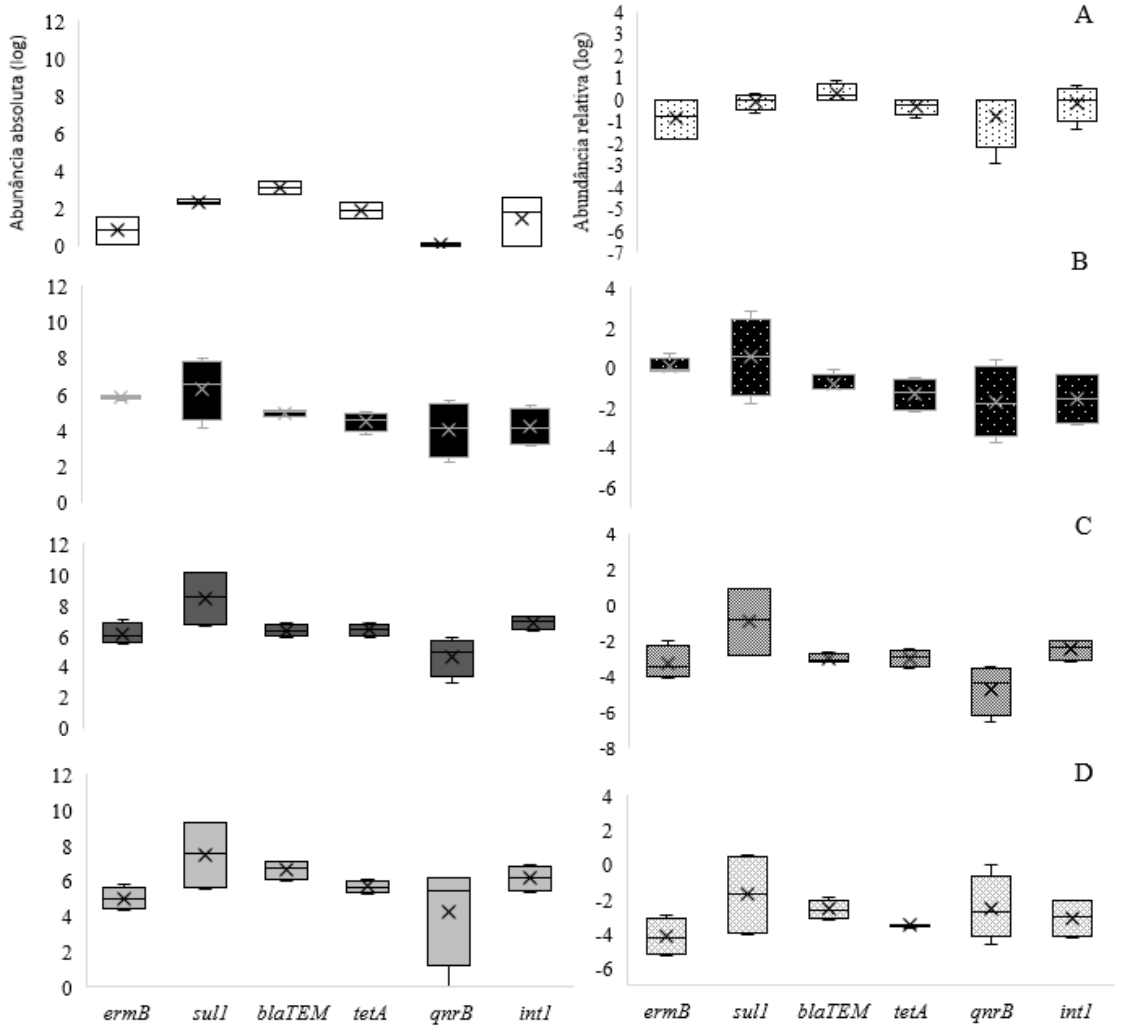
For the statistical analysis, the Shapiro–Wilk test was used to assess data normality, Student's t test was used for parametric data, the Mann–Whitney test was used for nonparametric data, the Wilcoxon test was used for comparisons of soil

physical–chemical data, and Spearman's correlation coefficient was used, in addition to the Kruskal test for statistical analysis of the relative abundance of soil and treated effluent samples.

5 RESULTS AND DISCUSSION

All investigated genes (*ermB, tetA, sul1, int1, qnrB, blaTEM*) and the mobile genetic element *int1* were detected in all the campaigns in S1, S2, A and E (Figures 1 and 2), with the exception of the *qnrB gene*, which was below the detection limit of the analytical method in S2 in collection 1 and in S1 and S2 in 80% of the replicates of collections 3 and 4.

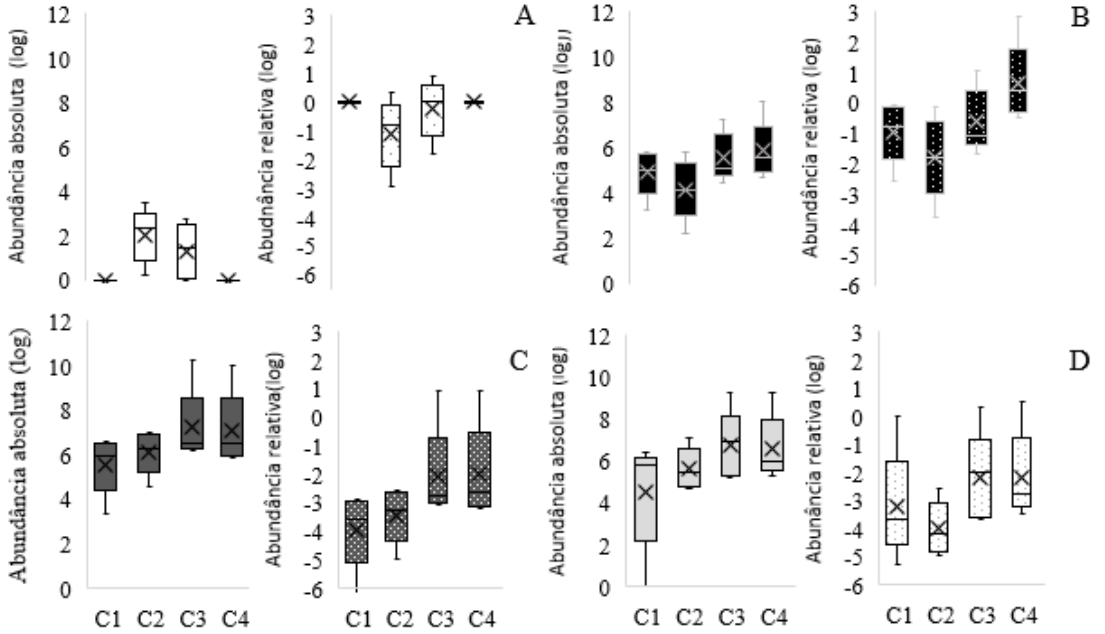
Figure 1. Absolute and relative abundances of each antibiotic resistance gene investigated and the integrase gene in the soil, pond water and treated effluent samples



Source: the author.

A: Number of gene copies/g mL of reservoir water samples; B: number of gene copies/g mL of treated effluent water samples; C: number of gene copies of soil samples 1 and D: soil 2 gene/g sample) (n=20)

Figure 2. Relative and absolute abundance of total antibiotic resistance genes per collection



Source: the author.

A: Gene copy number/g mL of reservoir water samples; B: gene copy number/g mL of treated effluent water samples; C: gene copy number (soil sample 1 and soil sample 2 genes/g sample). Genes: *ermB, sul1, tetA, qnrB, and blaTEM*. C1: collection 1 (Jan 2020), C2: collection 2 (Aug 2020), C3: treated effluent from collection 3 (Mar 2020), C4: treated effluent from collection 4 (Apr 2020). Owing to analytical problems, it was not possible to quantify samples from collections 1 and 4.

5.1 Antibiotic resistance genes in reservoir water

The mean absolute abundance of GRAs in the reservoir water samples ranged from -0.12 to 3.23 log $_{10}$ gene copies.mL $^{-1}$ (Figure 1-A). The reservoir water samples showed a different pattern from the other samples under study, with higher absolute and relative abundances of *blaTEM genes* (3.1 and 0.62 $_{log10,}$ respectively), followed by the following genetic elements for absolute and relative abundance: *blaTEM > sul1 > int1 > tetA > ermB > qnrB.* The relative abundance of GRAs ranged from -3.22 to 0.69 log $_{10}$ copies of GRAs/16S rRNA.

Dong *et al.* (2019) detected lower relative abundances in sediment from an urban lake in China (approximately -2 to -4 log $_{10}$ copies of GRAs/16S rRNA). Arsand *et al.* (2020) reported that the *blaTEM* gene was the most abundant gene in samples from a river in Brazil. In contrast, in some studies with receiving water bodies surrounding pig farms, a predominance of the *sul1 gene was observed* downstream of wastewater discharge (HSU, 2014). The same phenomenon has also been observed in lake samples**,** where the *sul1 gene* was better explained by the proximity of wastewater treatment plants and hospital effluents, ranging from -3 to -1 log $_{10}$ copies of *sul1,* values lower than those found in the present study. (CZEKALSKI *et al.*, 2015).

5.2 Antibiotic resistance genes in treated effluent

The mean absolute abundance of all GRAs in the treated effluent ranged from 4.97 to 7.47 log $_{10}$ gene copies mL $^{-1}$ of treated effluent between collections (Figure 1-B). The *sul1 gene* had the highest abundance in the samples (7.47 log $_{10}$), with the order of *sul1>ermB>qnrB>blaTEM>int1>tetA,*

with a mean absolute abundance higher than the abundance of the RNAr16S gene, which represents the total bacteria present in the samples (5.9 log $_{10}$, ranging from 5.2 to 6.1 log $_{10}$).

There was a variation in relative abundance in the range of -0.17 to 2.23 log $_{10}$ of GRAs/16S rRNA copies, above the detection range reported by Kampouris *et al.* (2021) of approximately -8 to -2 log $_{10}$, who also included in their studies the *int1, sul1*, and *blaTEM* genes in treated wastewater. In another study, tetracycline and sulfonamide resistance genes (*tetM, tetO, tetW, sul1*, and *sul2*) were detected at lower relative abundances than the average of the treated effluent of the present study (approximately -2.0 log $_{10}$) in swine wastewater (BEN *et al.,* 2017).

Negreanu *et al*. (2012) also reported that the relative abundances of the *sul1* and *ermB genes were* apparently lower than those reported in this study (approximately -3 to -2 log $_{10}$ of *sul1* and -4 to -6 log $_{10}$ *of the ermB* gene) in samples of wastewater effluent from a sewage treatment plant.

The mean relative abundance of the *sul1 gene* in the treated effluent was approximately 2.23 log $_{10}$ *sul1*/*16S rRNA* gene copies (ranging from -1.81 to 2.82 log 10 between collections), whereas the *ermB* gene ranged from -0.23 to 0.68 log $_{10}$ between the collection relative abundance means. A positive relative abundance may indicate the possibility of more than one gene per bacterium or that some of the resistance genes persist in the environment even after the bacteria die and could be free in the environment as genetic fragments.

5.3 Antibiotic resistance genes in soil samples

The mean absolute abundance of GRAs in S1 ranged from 6.06 to 9.85 gene copies g $^{-1}$ of sample. The genes with the highest absolute abundance in the S1 soil samples (n = 20) were *sul1* and *int1*, with total means of 9.8 and 7.0 log $_{10}$ number of gene copies/g sample, respectively, following the order *sul1* > *int1* > *ermB* > *tetA* > *blaTEM* > *qnrB*.

The S1 samples presented a variation in relative abundance in the range of -3.32--0.47 log $_{10}$ of GRAs/16S rRNA copies (Figure 1-C). The *sul1 gene* was the most abundant gene, with an average value of 0.47 log $_{10}$, which was greater than that reported in some studies involving fertigated soil, such as Negreanu *et al.* (2012), which reported values of approximately -6 to -3 log $_{10}$ and relative abundances reported by Wang *et al.* (2014) and Kampouris *et al.* (2021), *with* approximately -3 log $_{10}$ of *sul1*/16 s rRNA.

The *qnrB* gene was the least abundant gene in the S1 samples, with an average of 6.0 log10. Importantly, among the 20 (twenty) soil samples collected, only 11 were above the detection limit for the *qnrB gene* (collection 1: 4.0x10 ^{2}, collections 3 and 4: 8.0x10 ^{5}). In the S2 samples, 13 (thirteen) samples had quantification of the *qnrB* gene below the detection limit of the reaction.

The genes *sul1* and *tetA* were significantly more abundant ($p<0.05$) in S1 than in S2 when the statistical analysis was performed with all replicates of all collections. When each collection was analyzed separately, a different pattern was observed for the genes *tetA* and *ermB*, in which the relative abundance of the gene *ermB* was greater ($p<0.05$) in the fertigated soil in collections 1, 2 and 3 and that of *tetA* was greater only in collections 3 and 4, in addition to the gene *int1,* which was greater ($p<0.05$) in collections 1, 2 and 4.

The abundance of the *sul1 gene* was significantly greater in the fertigated soil than in the nonfertigated soil at all collection times. Thus, fertigation may contribute to the increase in the *ermB, tetA*, *sul1*, and *int1 genes* in the soil.

Despite not receiving directly irrigated treated effluent, all the studied

genes were also detected in the S2 samples (Figure 1-D). The mean absolute abundance of GRAs in S2 ranged from 5.53 to 8.95 gene copies/g of soil sample. The *sul1 gene* was also the most abundant gene among the investigated genes, with a mean absolute and relative abundance of 7.33 log $_{10}$, followed by *blaTEM* and *int1 (*6.45 and 6.07 log $_{10}$), with the gene *ranking being sul1 > blaTEM, int1 > qnrB > tetA > ermB.*

The relative quantification of GRAs in S2 ranged from -0.43 to -4.06 GRAs/16S rRNA copies. The relative abundance of the *sul1 gene* (0.25 log $_{10)}$ was considerably greater than that reported in other studies in the literature, such as those by Negreanu *et al.* (2012), Ben *et al.* (2017) and Kampouris *et al.* (2021), who reported relative abundances of approximately -4 to -8 log $_{10}$ in nonirrigated soils with treated effluent.

The considerable abundance of the same genes detected in S1 in the soil samples from S2 is due to their proximity to the manure biodigester chamber. An area of direct leakage of raw manure into the soil was observed near this equipment. Therefore, it is possible that due to rainfall, GRAs from raw effluent washing flowed from this area to Soil 2, contributing to the increase in GRAs in the soil.

Importantly, significant GRAs have also been quantified in soils considered untouched by anthropogenic activity, such as Antarctic soils and unexplored Tibetan soils (WANG *et al.,* 2016, LI *et al.,* 2020). The relative abundance in pristine Tibetan soils ranged from -1.3 to -0.55 log $_{10}$ GRA copies/16 rRNA gene copies, which was within the range reported in the present study for S1 and S2. The GRAs found in the aforementioned Tibetan soil confer resistance to less common antibiotics, such as glycopeptides and rifampicin. In environments with direct human impact, including Tibetan soil under anthropogenic influence, there is a tendency for GRAs, such as fosmidomycin, as well as aminoglycoside and bacitracin, to be more common in the literature, with an emphasis on macrolide-lincosamide-streptogramin, fluoroquinolone and tetracycline (LI *et al*., 2020).

5.4 Comparison of the abundance of resistance genes among the soil, treated effluent and reservoir water samples

The mean abundance of total GRAs in this study was represented by the sum of the means of the genes *blaTEM*, *ermB, qnrB, sul1* and *tetA* of the treated effluent, soil and reservoir water samples. The total abundance of GRAs in the treated effluent was 4.24x10 ^{6} (6.62 log $_{10}$ ± 7x10 ^{6} copies of GRAs). In Soil 1, the average abundance of all samples (n = 20) was approximately 3.10x10 ^{10} (10.5 log $_{10}$) ± 3.18x10 ^{10}. For Soil 2, it was 4.5x10 ^{9} (9.6 log $_{10}$) ± 4.5x10 ^{9,} and for Reservoir 2, it was 4.18x10 ^{3} (3.62 log $_{10}$) ± 3.$^{59x10\ 3}$.

The decrease in relative values compared with absolute values of the studied samples suggests a decrease in the proportion of resistance genes in relation to bacteria in the environment, possibly due to the decrease in copies of the resistance gene in relation to the number of copies of 16S rRNA. With respect to the relative data, it was possible to observe a decay of GRAs of up to 1 log $_{10}$ copies between Soil 1 and Soil 2; however, there was no significant difference ($p \leq 0.05$) in the relative data of the GRAs when comparing the two soil samples, which were very similar.

Although it is not possible to compare the relative abundance of the treated effluent to that of the reservoir water, due to the small sample size, a lower quantification of GRAs in the reservoir environment than in the other environments is noted in the first analysis; this environment is a possible sink for GRAs (GAO *et al.*, 2018; ZHANG *et al.*, 2021).

When comparing the relative abundance of the treated effluent and the soil that received this effluent, a difference of up

to 3 log $_{10}$ of GRAs was noted, reflecting a greater significant quantification ($p \leq 0.05$) regarding the genes *ermB*, *blaTEM* and *tetA* in the effluent than in soil 1 (with a decay of 3.06, 3.15 and 2.31 log $_{10}$ of relative abundance, respectively). These results may lead to the hypothesis that there is a decrease in the amount of GRAs after the release of treated effluent into the soil, possibly caused by changes in environmental conditions, differences in the microbiome, and temperature, among other factors, and that direct fertigation does not appear to have influenced the quantification of total GRAs in soil 1, since a similar amount was found in soil 2.

The *sul1* gene was detected at greater abundance in samples S1, S2, and E (averages of 9.81 and 8.95 log $_{10}$ *sul1* gene copies/g of soil and 7.47 log $_{10}$ gene/mL of sample, respectively), and no significant increase or decrease ($p \leq 0.05$) in the relative abundance of this gene was detected among the samples. However, a positive correlation has been found between the *sul1 gene* and the intensity of wastewater irrigation (KAMPOURIS *et al*., 2021), and the same gene was also the most abundant gene in the samples investigated by Ben *et al*. (2017);

Notably, the *sul1 gene* has also been found in high abundance in pristine soils (PARTRIDGE *et al.*, 2009; WANG, 2014). This fact may suggest that the *sul1 gene* is persistent in the environment.

The *blaTEM* gene was detected (mean of 0.31 log, n = 2) but was present at lower abundances in the soil and treated effluent samples (-2.88, -2.59, -0.57 soils 1 and soil 2 and treated effluent, respectively). Despite the smaller data sample (n = 2), the highest relative abundances of the *blaTEM* (enzymatic mechanism of resistance) and *tetA* (efflux pump) genes and the *int1 integrase occurred in the reservoir. The ermB* (mechanism of antibiotic target shifting) and *qnrB (enzymatic mechanism of resistance)* genes were more abundant in the treated effluent samples, and the *sul1 gene* (mechanism of antibiotic target shifting) was more abundant in the soil samples.

5.5 Correlations between antibiotic resistance genes and the integrase 1 gene in soil samples

In the S1 samples, there was a moderate correlation of the 16S rRNA gene with the *blaTEM gene* (0.54), in addition to the strong correlation of the *int1 integrase* with the *B and sul1 genes.* and *tetA* (0.71, 0.88, and 0.74, respectively). The correlation between the blaTEM gene and total bacteria may be associated with the natural soil resistome. Kampouris *et al*. (2021) confirmed that the blaTEM and blaCTX *genes* are *stable* members *of* the *natural* soil resistome since they are not affected by fertigation.

In S2, positive correlations were also observed between *int1 and the sul1* gene and *ermB* (0.79 - strong and 0.60 - moderate, respectively), in addition to strong and negative correlations between *int1* and *tetA* (-0.72). In addition, negative and weak correlations were observed between the *blaTEM gene and the int1* integrase in both soil samples (-0.48 in soil 1 and -0.59, $p \leq 0.05$).

int1 integrase corresponds to a potential indicator of horizontal gene transfer of *ermB* and *sul1* (Wang, 2014), it is likely that this mobile element is involved in the dissemination of these genes in the two soil areas sampled. Khan *et al.* (2013) also reported high levels of *sul1* and *int1*, indicating a strong correlation with antimicrobial-contaminated environments. Through multiphase analysis, Kampouris *et al.* (2021) reported that *sul1* and *int1 genes* (in addition to the *qnrS* and *bla* OXA-58 genes) were increased by irrigation with treated effluent.

A previous study using pristine soil samples from Antarctica also revealed 177 genes that confer resistance to natural antimicrobials. However, no mobile genetic

elements, such as integrons, transposons, or recombinases, were found. Resistance appears to have occurred through vertical transfer (from generation to generation of bacteria) rather than horizontal transfer (VAN GOETHEM *et al*., 2018).

Importantly, in a recent study of untouched pristine soils in Tibet, a region of the Asian Plateau, EGM was found, in which the *int1 integron* was the most abundant, suggesting that such elements are not exclusive to anthropized environments (LI *et al.*, 2020).

In conclusion, the presence of synthetic antibiotic resistance genes, such as those that confer resistance to sulfonamides (*sul1*) and fluoroquinolones (*qnrB*), as well as the presence of the mobile genetic element *int1* may indicate the occurrence of horizontal transfer of these genes between soil bacteria, since bacteria can replicate the gene and transfer it to other bacteria in the environment as well as pathogenic bacteria, leading to a public health problem. The correlations of the reservoir samples could not be analyzed because of the small number of samples (n=2), and no correlations were detected between genes in the treated effluent samples (n=4).

5.6 Correlations between genetic resistance elements and TOC, NT, pH and particle size distribution parameters in soil samples

No correlations were detected between the resistance genetic elements (EGR) and the parameters TOC, NT, and pH (n=20, $p<0.05$) among the soil samples in soil 1, which may indicate that the amount of carbon, nitrogen, and pH variation are not related to the GRAs investigated in the fertigated soil. In soil S2, the pH of the S2 samples was correlated with all the genes studied (n=20), highlighting the strong positive correlation with the *ermB, sul1, and int1* genes. (0.72, 0.75 and 0.72); in addition, a moderate correlation was also observed between the 16S rRNA gene and TOC, indicating a possible relationship between total bacteria and natural soil organic matter 2. Thus, the *ermB, sul1* and *int1* genes appear to be more related to the most alkaline soil pH without direct fertigation.

Soil 1, which contains a greater clay content, would theoretically be more conducive to interactions with bacteria than would Soil 2, which tends to have a greater bacterial quantity. However, the averages of the two soils in relation to total bacteria and the sum of GRAs were very close in both samples (soil 1: 9.3 ± 0.17 and 8.6 ± 1.53 and soil 2: 9.23 ± 0.40 and 8.06 ± 1.21 $\log_{10}$ of the number of 16S rRNA gene copies and GRA copies/g of soil), leading to the conclusion that soil particle size does not appear to have interfered with the quantification of bacteria and GRAs. With respect to the treated effluent, no correlations were found between the genes and pH, TOC, or TN.

In summary, it was not possible to perceive a correlation pattern between the organic matter content in the treated effluent and in soil 1 that received this treated effluent in the collections carried out, and despite the probable contribution of soil acidity through fertigation and the contribution of the treated effluent to the increase in total organic carbon and nitrogen, these parameters in the first analysis do not appear to be directly involved in the quantification of GRAs through fertigation in the soil.

qnrB, blaTEM) and the mobile genetic element *int1* were detected in all the samples collected from the soil, treated effluent, and reservoir water. The *sul1 gene,* related to sulfonamide resistance, was the most abundant gene among the samples, with the

6 CONCLUSION

All the investigated antibiotic resistance genes (*ermB, tetA, sul1, int1,*

exception of the reservoir samples, where the *blaTEM gene* (resistant to beta-lactams) was more abundant. The *sul1* and *tetA genes* were significantly more abundant in the directly fertigated soil than in the soil without direct fertigation. Since these genes were present in the treated effluent, it is possible that fertigation contributes to the increase in sulfonamide and tetracycline resistance genes in this environment. The concomitant presence of the integrase gene in the soil samples, as well as the strong correlation observed between this gene and the erythromycin and sulfonamine resistance genes, suggest that horizontal gene transfer is involved in the dissemination of GRAs in the soil.

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