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Temporal dynamics of the soil resistome and microbiome irrigated with treated wastewater containing clarithromycin

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ABSTRACT

Clarithromycin, a common antibiotic found in domestic wastewater, persists even after treatment and can transfer to soils when treated wastewater (TWW) is used for irrigation. This residual antibiotic may exert selection pressure, promoting the spread of antibiotic resistance. While Predicted No Effect Concentrations (PNECs) are used in liquid media to predict resistance risks, PNEC values for soils, especially for clarithromycin, are lacking. Thus, this study aimed to assess clarithromycin's fate and its concentration threshold affecting soil microbial communities and macrolide resistance genes.

The study used a soil microcosm approach with TWW containing clarithromycin at concentrations of 0, 0.01, 0.1, 0.5, and 1 $mg/kg_{dry\ soil}$ over a three-month period. Results showed clarithromycin persisted with limited degradation, likely due to strong adsorption to soil particles. Two transformation products were identified: decladinose-CLA (abiotic degradation) and phosphate-CLA (bacterial phosphotransferase activity). Soil bacterial communities were more influenced by TWW than by clarithromycin itself, as its antimicrobial effect was reduced due to adsorption.

While clarithromycin did not significantly affect the abundance of resistance genes like *intl1*, *mphA*, and *ereA*, concentrations above 0.01 mg/kg increased the *ermB* gene abundance during the first week. The *mefA* gene (macrolide efflux pump) showed a hormetic effect: low doses (<0.1 mg/kg) increased gene abundance, while higher doses (>0.5 mg/kg) inhibited gene transfer or the bacteria carrying it.

This study performed under controlled conditions provided insights into antibiotic resistance dynamics in soils exposed to clarithromycin, highlighting key concentration thresholds influencing resistance spread in soils.

1. Introduction

Macrolides belong to the polyketide class of naturally occurring compounds with macrocyclic lactone ring containing eight or more atoms (Arslan, 2022). This broad class of antibiotics, represents Europe's second most widely consumed category, following beta-lactams (Robertson et al., 2021). They are generally bacteriostatic as they suppress or inhibit bacterial growth rather than killing bacteria completely (Arslan, 2022). Given their clinical importance and persistence in the environment, they are a key focus for risk assessment (Monahan et al., 2023); especially as they are classified as critically important antimicrobials of the highest priority by the World Health Organization (WHO, 2017).

Clarithromycin, a broad-spectrum macrolide antibiotic, has been widely administered in humans (Kümmerer and Henninger, 2003; Zhao et al., 2024) and animals (Wang et al., 2021) medicine. It is typically used to treat bacterial infections such as respiratory tract infections *(Haemophilus influenzae, Moraxella catarrhalis)*, pneumonia (*Streptococcus pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae* and *Chlamydophila* (*Chlamydia*) *pneumonia*), skin infections, Lyme disease, and other diseases (Darkes and Perry, 2003). Its mechanism of action involves inhibiting protein synthesis by binding to the 23S rRNA, a component of the 50S subunit of bacteria. After administration, clarithromycin is partially absorbed and metabolized in the liver and the

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stomach. Due to hepatic metabolism saturation, a part of the oral dose non-metabolized can be recovered as parent compound (22%) in urine (±18%) and feces (±4%) (Davey, 1991) ending up in urban wastewater (Rizzo et al., 2013). Although wastewater treatment plants partially remove antibiotics through adsorption or degradation, the processes are often insufficient to completely eliminate macrolides, including clarithromycin, which can still be detected in treated wastewater (TWW). For example, in constructed wetlands, clarithromycin removal efficiencies ranging from 10% to 50% have been reported (Hijosa-Valsero et al., 2011). This results in clarithromycin concentrations in TWW typically around 10–100 ng/L, although peak concentrations exceeding 1 μg/L have been observed in some cases. (Al Aukidy et al., 2012; Cacace et al., 2019; Castaño-Trias et al., 2024; Li et al., 2023; Monahan et al., 2021, 2023).

With the increasing reuse of TWW for irrigation in many countries as a solution to water scarcity (Ungureanu et al., 2020), macrolide residues, including clarithromycin, are being introduced into soils. The transfer of such residues in soil can exert selection pressure, favouring the dissemination of macrolide-resistant bacteria, particularly when the molecule is persistent, as is the case with clarithromycin. Due to its relatively high adsorption coefficient (Kd = 262–400 L/kg, Manasfi et al., 2021) and low biodegradability, clarithromycin tends to accumulate and persist in soils. Reported half-lives (DT_{50}) for this macrolide range from 9 to over 1000 days, depending on soil type, environmental conditions, physico-chemical properties, and the composition of the soil microbial community (Kodešová et al., 2016; Topp et al., 2016). Only a few studies have reported and/or monitored the presence of clarithromycin residues in soils irrigated with wastewater. In soils from a river basin, often irrigated with reclaimed water from a nearby wastewater treatment plant and amended using sludge, clarithromycin concentrations were measured between 0.1 and 1.3 $\mu g/kg_{dry\ soil}$ (Corada-Fernández et al., 2015). In soils irrigated with untreated wastewater for several years, clarithromycin concentration ranging from 3 to 5.4 $\mu g/kg_{dry\ soil}$ were quantified (Dalkmann et al., 2012). In soil mesocosms containing lettuces and irrigated with TWW spiked with 10 μg/L of clarithromycin (included in a mixture of other pharmaceuticals), concentrations of 219 $\mu g/kg_{dry\ soil}$ were reported after the irrigation cycles, showing the accumulation ability of clarithromycin in soils (Gallego et al., 2021). Using a similar approach, another study reported comparable findings with concentrations of clarithromycin ranging from 10 to 50 $\mu g/kg_{dry\ soil}$ after irrigation. Additionally, the study highlighted contamination of lettuce leaves, demonstrating the molecule's ability to transfer from soil to plants. This transfer represents a potential entry point into the food chain, underscoring the associated risks to human health (Manasfi et al., 2021).

To assess whether environmental concentrations of antibiotics pose a risk for the selection and dissemination of AMR in the environment, the Predicted No Effect Concentration (PNEC) for resistance selection is typically assessed (Le Page et al., 2017; Murray et al., 2021). For clarithromycin, this indicator has been estimated at 0.084 μg/L for cyanobacteria in surface water (Le Page et al., 2017), 0.13 μg/L for freshwater organisms, 1.2 $\mu g/kg_{dry\ sediment}$ for benthic species (Ceriani et al., 2015), 0.25 μg/L for specific micro-organisms (Ceriani et al., 2015), 50 μg/L in raw wastewater complex community (Stanton et al., 2020), and 250 μg/L in sewage influent from wastewater (Murray et al., 2018). Additionally, in soil, it was estimated around 0.1 μg/kg, based on clarithromycin's organic carbon-water partition coefficient (K_{oc}) and an organic carbon fraction (Foc) of 0.016 (Castaño-Trias et al., 2024).

Some bacteria have developed or exhibit resistance to macrolides through various pathways encoded by specific genes: (i) *erm*, which encodes a ribosomal methylase that alters macrolide binding sites on the ribosome, (ii) *mefA*, which encodes a macrolide efflux pump (Darkes and Perry, 2003), (iii) *mph* and *ere*, which encode macrolide phosphotransferase and esterase, respectively, leading to the inactivation of macrolide antimicrobial properties (Golkar et al., 2018; Pawlowski et al., 2018). To date, the dose effect of clarithromycin has been assessed in water (Stanton et al., 2020) and in activated sludge (Zeng et al., 2021) regarding microbial ecotoxicity and the dynamics of antibiotic resistance. In agricultural soils, assays have been carried out on the effect of clarithromycin on the structure and/or function of soil bacterial communities for clarithromycin concentrations above 0.5 $mg/kg_{dry\ soil}$ (Rodríguez-González et al., 2021). This study showed that clarithromycin concentrations higher that 1 $mg/kg_{dry\ soil}$ were toxic for soil bacterial communities. Moreover, toxicity was found to decrease progressively between 1 and 8 days of incubation, with the bacterial communities fully recovering in most soils after 42 days of incubation. In another study monitoring the response of soil bacterial community, resistome and mobilome to a decade of annual exposure to macrolides at 0.1 and 10 $mg/kg_{dry\ soil}$, the study showed that at the highest dose (not environmentally relevant) microbial community structure and resistome/mobilome increased (Brown et al., 2022). However, at 0.1 $mg/kg_{dry\ soil}$, a concentration 1000 higher than the estimated PNEC in soil (Castaño-Trias et al., 2024), no major changes were observed (Brown et al., 2022).

To better understand the factors driving resistance selection in soils exposed to clarithromycin, we conducted a global microcosm study on soils exposed to a clarithromycin concentration gradient ranging from 0.01 to 1 $mg/kg_{dry\ soil}$. Built on a methodological approach successfully applied with sukfamethoxazole (Della-Negra et al., 2025), the microcosm study aimed to precisely identify the clarithromycin concentration at which resistance selection occurs (anticipated to be between 0.1 and 10 $mg/kg_{dry\ soil}$), explore the relationship between clarithromycin biodegradability, transformation products, physico-chemical properties and resistance selection, and determine the time windows during which the bacterial community, resistance genes, and integrons are most likely to be affected by clarithromycin exposure.

2. Materials and methods

2.1. Sampling

2.1.1. Treated wastewater sampling

Domestic treated wastewater (TWW) came from a wastewater treatment plant located in an agricultural sewershed, at Murviel-les Montpellier (France). The wastewater treatment plant included the usual pre-treatment (screening and grit removal) followed by a biological treatment with constructed wetland (Moulia et al., 2023). This wastewater treatment plant has a nominal capacity of 3000 Inhabitant Equivalent (Ait-Mouheb et al., 2022). The experimental site is used to irrigate a 0.5 ha plot in Murviel-lès-Montpellier using a drip system since 2017, where vineyards, alfalfa and fruit trees are grown. The quality of the TWW meets the criteria for class C reclaimed water (European Parliament, 2020). Small quantities of water were needed for the experiments, so a few liters of TWW were taken on the day the microcosms were started up. In this way, we had a water quality representative of that used to irrigate the agricultural plot from which the soils were taken. The samples were collected at the outlet of the TWW plant with hermetically protected bottles.

2.1.2. Soil samples

Soils were sampled at the experimental platform of Murviel-lès-Montpellier, on an agricultural plot that was irrigated with TWW (treated by constructed wetlands) for 2 years as described in a previous study (Della-Negra et al., 2025). This soil and the crops grown on it are representative of the Mediterranean climate. In recent years, the region has suffered increasingly from drought and is turning to the use of reclaimed wastewater for irrigation (Ait-Mouheb et al., 2018). This matrix is therefore a model of Mediterranean agricultural soil regularly irrigated with TWW.

2.2. Experimental set-up

The experiments were conducted in microcosms as described in (Della-Negra et al., 2025). 25 g of dry soil were added to 250 ml amber glass flasks topped with carded cotton plugs to allow basal respiration. All the flasks were then acclimated at room temperature for 3 days to activate the native soil microorganisms. They were then irrigated in one shot with 20 mL of TWW spiked with clarithromycin (CLA, Sigma, 95%) to obtain final spiked CLA concentrations of 0, 0.01, 0.1, 0.5 or 1 mg/kg $_{dry\ soil}$ (these concentrations do not take into account the residual CLA concentration originally present in the soils which is 0.008 mg/kg $_{dry\ soil}$). The minimum concentration (0.01 mg/kg$_{dry\ soil}$) is close to the environmental concentrations found in the soil after irrigation with TWW (Corada-Fernández et al., 2015; Dalkmann et al., 2012), between 0.1 and 0.5 mg/kg$_{dry\ soil}$, these concentrations can also be found in the soil after sludge spreading (Barreiro et al., 2022) and 1 mg/kg$_{dry\ soil}$ was tested to maximize the potential effects on bacterial communities and enable the detection of transformation products. This concentration range was included in interval that could be detected and quantified by the extraction (QUeChERS) and detection (HRLS-MS-MS) method described here.

Flasks were incubated at 35 °C for 90 days, in a thermo-regulated room. 35 °C was chosen as this temperature maximized bacterial activity (measured by basal respiration) and CLA degradation. From preliminary results, we observed that 1.5–2 months were required to reach a structural equilibrium of soil bacterial community after TWW input. Thus, the experiment was conducted for 90 days (3 months), to encompass the temporal windows of bacterial variability and stability.

Soil moisture content was maintained throughout the experiment to prevent this parameter from influencing the results. A level of 80% of the field capacity was selected as it provides optimal conditions for the efficient diffusion of nutrients and metabolites, while avoiding water saturation that could lead to anoxic conditions. Additionally, this moisture level ensures homogeneous diffusion of antibiotics in the soil, promoting uniform exposure and yielding more reliable and robust results. In this way, soil moisture was adjusted and maintained with milliQ water during the whole experiment (approximately 10 mL were added each week, i.e. around 3 mL were added every two days).

Each condition was tested in triplicate, sacrificial mode was adopted and included 7 sampling times (1, 4, 7, 14, 30, 60 and 90 days after the one-shot irrigation). To assess abiotic transformation of clarithromycin, some flasks containing soil were autoclaved (20 min at 121 °C), incubated for 24 h at room temperature, then autoclaved again to reduce bacterial richness and diversity. These controls were irrigated in one shot with 20 mL of milliQ water spiked with clarithromycin (CLA, Sigma, 95%) to obtain final CLA concentrations of 0, 0.1, or 1 mg/kg $_{dry\ soil}$.

2.3. Molecular biology analyses

2.3.1. DNA extraction and analyses

DNA extraction was performed using the DNeasy® PowerWater® kit (Qiagen) for water and the FastDNA™ SPIN Kit for Soil (MP Biomedical) for soil samples. The kits were used according to the protocol provided by the manufacturer. The quality of the DNA extracted (260/280 ratio), was measured using the Spark® multimode microplate reader.

2.3.2. Quantitative PCR (qPCR)

Amplification reactions were performed using a Bio-Rad CFX Opus 384 real Time PCR system, in reaction mixtures (12 µL) containing 6 µL of Sybr Super Mix (Bio-Rad) and primers at concentrations indicated in SI (Table S1). Total bacterial quantification was performed by qPCR targeting the V3 region from 16S rDNA. Genes related to antibiotic resistance were quantified (macrolide resistance: *ermB*, mobile genetic element: *intl1*, macrolide efflux pump: *mefA*, erythromycin esterase: *ereA* and macrolide phosphatase: *mphA*).

For 16S rDNA, *intl1*, *ermB*, *mefA*, *ereA* and *mphA*, the qPCR programs consisted of initial denaturing at 95 °C (2 min), followed by 40 cycles at 95° (15 s or 10 s for 16S rDNA) for denaturation and Ta (Table S1) (60 s for *intl1* and *ermB*, 30 s for *mefA*, *mphA* and *ereA* and 20 s for 16S rDNA) for annealing and amplification. The fluorescence data was acquired at the annealing temperature, and the final melting curve was constructed with temperature ramping up from 72 °C to 95 °C. For each system, the melting curve was checked, quality criteria of linearity ($R^2 > 0.99$) and PCR-efficiency (between 90 and 110%) were reached and LOD and LOQ were determined (Table S2).

Gene quantification is presented in both relative terms (per 16S rRNA) and absolute terms (per g of dry soil). For absolute quantification, the soil was homogenized by thorough mixing before each sampling, and the moisture content was estimated by comparing the microcosm's mass at time T to its mass at time T0 (dry soil prior to TWW addition). Each soil sample collected for DNA extraction was weighed and adjusted to its dry weight based on the measured moisture content. This process allowed the number of copies of a given gene, estimated in the aqueous solution after DNA extraction, to be accurately related to the amount of soil used for the extraction.

2.4. Illumina-based 16S rDNA sequencing and sequence data analysis

Sequencing analyses of the DNA extracted from the soil and water were carried out within the Genotoul Lifescience Network Genome and Transcriptome Core Facility platform in Toulouse. All the samples (biological triplicates) were sequenced. PCR amplified the V4-V5 region of 16S rDNA genes with 30 cycles (annealing temperature 65 °C) using the primers 324W (515F - 5′CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMG CCGCGGTA–3′) and 325W (928R - 5′-GGAGTTCAGACGTGTGCTCTT CCGATCTCCCCGYCAATTCMTTTRAGT -3′) (Venkiteshwaran et al., 2016). Amplification was checked by fluorescence with the CFX96TM Touch Real-Time PCR Detection System (Bio-Rad). Sequencing was carried out by the Illumina high-speed sequencer (NGS, Next Generation Sequencing). Adapters were added for multiplexing samples during the second sequencing amplification step. The resulting products were purified and loaded onto the Illumina MiSeq cartridge for sequencing of paired 300 bp reads according to the manufacturer's instructions (v3 chemistry). Sequencing and library preparation tasks were performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). Mothur (version 1.48.0, Schloss et al., 2009) was used to associate forward and reverse sequences and clustering at four different nucleotides over the length of the amplicon. UCHIME was used to identify and remove chimera (Edgar et al., 2011). Sequences that appeared fewer than three times in the entire data set were removed. The remaining sequences were aligned using SILVA SSURef NR99 version 132, Schloss et al. (2009). Taxonomic assignment was done using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) to locate publicly available sequences closely related to the sequences obtained from the samples. Sequences with 97% similarity were sorted into operational taxonomic units (OTUs, Nguyen et al., 2016). The chloroplast sequences from 16S rDNA sequences were removed from the raw data and represented less than 1%. Sequences can be found under the BioProject PRJNA1183871 (from SAMN44729171 to SANM44729360).

2.5. Chemical analyses

2.5.1. Chemicals

Acetonitrile (>99.9%) and methanol (>99.9%) were purchased from Fischer Scientific. EDTA-Mcllavaine solution was prepared from 1.5 g of disodium hydrogen phosphate dihydrate salt (>99%, Merck), 1.3 g of citric acid monohydrate (>99%, Merck) and 0.372 g of EDTA (>99%, Merck), dissolved in 100 mL of Milli-Q water. Clarithromycin (99 %) was purchased from Sigma, d_3-clarithromycin from Caiman Chemical.

2.5.2. General analyses

Analyses of nitrite, nitrate, ammonium and phosphate were performed through the sequential analyzer (Gallery Plus Thermo Fischer, https://doi.org/10.15454/1.557234103446854E12). Liquid samples were directly injected after filtration on 0.45 μm filters. Solid samples were freeze-dried and 3 g of dry soils were then diluted in 9 mL of Milli-Q water, vortexed for 5 min, centrifuged for 5 min at 4000 rpm, filtered on 0.45 μm filters, and injected. In water limit of quantification (LOQ) were: for ammonium = 0.2 mg/L, nitrite = 0.1 mg/L, nitrate = 0.1 mg/L, phosphate = 0.2 mg/L. In soils, LOQ were: for ammonium = 0.6 mg/$kg_{dry\ soil}$, nitrite = 0.3 mg/$kg_{dry\ soil}$, nitrate = 0.3 mg/$kg_{dry\ soil}$, phosphate = 0.6 mg/$kg_{dry\ soil}$.

2.5.3. Clarithromycin extraction from soil samples

Soil samples were freeze-dried and extracted using the QuEChERs extraction method described elsewhere (Manasfi et al., 2021). Briefly, 5g $_{dry\ weight}$ of soil sample was added to a 50 mL-centrifuge tube. 1.5 mL of a d_4-clarithromycin solution in acetone was added at either 3.3 mg/L, 1.7 mg/L, 0.33 mg/L or 0.033 mg/L depending on initial CLA concentration in soils. The tubes were then vortexed and left under a fume hood at room temperature overnight, to allow solvent evaporation and compound interaction with soil matrix. Soil samples were then hydrated with 4 mL of EDTA-Mcllvaine buffer, vortexed, and rested for 1 h 5 mL of acetonitrile were added to the hydrated sample and vortexed. 2.5 g of QuEChERs salt tube (BEKOlut SALT-KIT-AC containing 4 g $MgSO_4$ + 1 g NaCl) was added to the previous mixture that was then hand shaken for 1 min and vortexed for another minute. Finally, the tubes were centrifuged for 10 min at 4000 rpm and 4 °C. 1 mL of the resulting supernatant was evaporated under a gentle nitrogen flow at room temperature until complete dryness. Residues were dissolved in 0.5 mL of a water/MeOH (90/10, v/v) mixture and filtered on 0.22 μm filters before injection.

2.5.4. Clarithromycin quantification and identification of transformation products

Soil QuEChERs extracts were analyzed using a Dionex™ Ultimate 3000 UHPLC (Thermo Scientific™, San Jose, USA) coupled to a high-resolution mass spectrometry Q-Exactive™ Focus Orbitrap mass spectrometer (Thermo Fisher Scientific, Les Ullis, France) equipped with a heated electrospray ionization (HESI) source. Chromatographic separation was performed through a Waters XBridge BEH C_{18} column (150 × 2.1 mm i.d., 2.5 μm particle size). The chromatography assays involved a 10 μL injection volume, a 0.3 mL/min flow rate, and a binary gradient of water +0.1% formic acid (A) and ACN + 0.1% formic acid (B) as follows: 10% B at 0–1 min, 90% B at 10–22 min, 10% B at 27 min, and a stop time at 29 min. Analysis was performed in positive and negative modes in full scan acquisition (70,000 FWHM resolution at m/z 200) and HESI parameters were as follows: 40 arbitrary units (AU) sheath gas; 15 AU auxiliary gas; 300 °C capillary temperature; 200 °C heater temperature, and the electrospray voltage was set at 3.0 kV for the positive and 2.5 kV for the negative ionization mode. The scan range was set between 100 and 1000 m/z with normalized collision energies of 20, 40, and 60 eV. Linearity, accuracy, limits of detection (LODs), limits of quantifications (LOQs), and precision of the method for CLA have been previously validated (Montemurro et al., 2020). Data were treated using Thermo Xcalibur™ 3.1 Software (Thermo Fisher Scientific, CA, USA).

For TPs identification, a literature review of the most common and already identified CLA TPs was constituted and used as a list for their subsequent screening in soil samples (Buchicchio et al., 2016; Calza et al., 2012; Reis et al., 2020; Terzic et al., 2018; Tian et al., 2019; Zeng et al., 2021). Based on this list, their molecular ions $[M + H]^+$ were extracted from the (+)ESI-HRMS chromatograms and $[M - H]^-$ from the (−)ESI-HRMS chromatograms with an isolation width of 5 ppm. If available, structures of some TPs were compared and confirmed with their reference standards.

For the identification of CLA TP, full scan data-dependent MS/MS fragmentation (dd-MS2) was set to acquire TP fragment spectra. Collision energies of 10, 20, 30 and 40 eV were tested.

2.6. Statistical analyses

Statistical analysis and graphical representations were performed using R software (R Core Team, 2022). Graphical representation, PCA and PCoA analysis were performed using ggplot2 (Wickham, 2009) and phyloseq packages (McMurdie and Holmes, 2013). Linear discriminant analysis effect size (LEfSe) was performed using the Microeco package (Liu et al., 2021) to identify biomarkers between CLA concentrations, with an absolute linear discriminant analysis (LDA) score >3.0 and a p value < 0.05 as cut-off values. The results of this analysis are depicted in Fig. 3 (showing taxa significantly impacted by CLA addition) and Table S5 (showing LDA scores, p values and relative abundances and impacted taxa). Significant differences between CLA concentrations in qPCR assays were assessed by performing ANOVA followed by post hoc tests. Significant differences are indicated in the figures by alphabetical letters (distinct letters mean significant differences: p value < 0.05).

3. Results

3.1. Fate of clarithromycin over time

The concentration of clarithromycin in TWW was measured at 2.7 ± 0.1 μg/L, a concentration in the upper range of that found in TWW, but usual for the Murviel plant (Della-Negra et al., 2024; Manasfi et al., 2021). In soils, CLA concentration was estimated at 8.2 ± 2.1 μg/kg $_{dry\ soil}$, the same order of magnitude compared to concentrations found in soils irrigated with untreated wastewater over extended periods (Dalkmann et al., 2012).

Using LC-HRMS analysis and d_3-CLA as an internal standard, we were able to monitor and quantify CLA in soil samples. As shown in Fig. 1a, CLA concentrations were lower after 1 day of incubation compared to the initial concentration, with reductions between 39% and 80%, depending on the conditions. In the control samples (autoclaved soils irrigated with Milli-Q water), concentrations were much higher, around 0.8 mg/kg $_{dry\ soil}$ for the 1 mg/kg condition, and remained unchanged for the 0.1 mg/kg condition. For the rest of the experiment, no significant change in clarithromycin concentration was observed in the 1, 0.5, and 0.1 mg/kg $_{dry\ soil}$ conditions. After 90 days of incubation, clarithromycin was no longer detectable or was below the quantification limit in the 0.01 mg/kg $_{dry\ soil}$ condition (Fig. 1a).

Suspect screening for CLA transformation products (TPs) revealed only two main TPs (Fig. 1b–d). Analysis of the MS^2 spectra (Figs. S1–3, Calza et al., 2012) confirmed the presence of Decladinose-CLA and Phosphate-CLA (Fig. 1d). As both TPs were not quantified, the dynamics of their peak area are only reported here.

Decladinose-CLA, a result of clarithromycin hydrolysis, is a common degradation product observed in several biodegradation studies (Buchicchio et al., 2016; Calza et al., 2012; Reis et al., 2020; Terzic et al., 2018; Tian et al., 2019; Zeng et al., 2021). It is also a known impurity in commercial clarithromycin formulations, typically accounting for 0.2% of the drug by mass, and can constitute up to 16% of clarithromycin by mass in wastewater (Senta et al., 2019). While Decladinose-CLA can be formed through abiotic hydrolysis (Tian et al., 2019), some studies have shown that it can also be produced in hypoxic environments by glycosylase activity (Gonzalez-Gil et al., 2019). In this experiment, Decladinose-CLA was not detected in the commercial CLA reference or TWW samples. However, it was detected in soils at T0, potentially due to previous irrigation. Following the addition of TWW, the peak area of Decladinose-CLA increased rapidly with rising CLA concentrations, then gradually decreased over time (Fig. 1b). The loss of CLA's cladinose moiety occurred in soils as a function of CLA concentration. This phenomenon was also observed in abiotic controls, suggesting that an abiotic transformation was taking place here (Fig. 1b).

In contrast, phosphate-CLA was only detected in soils spiked with

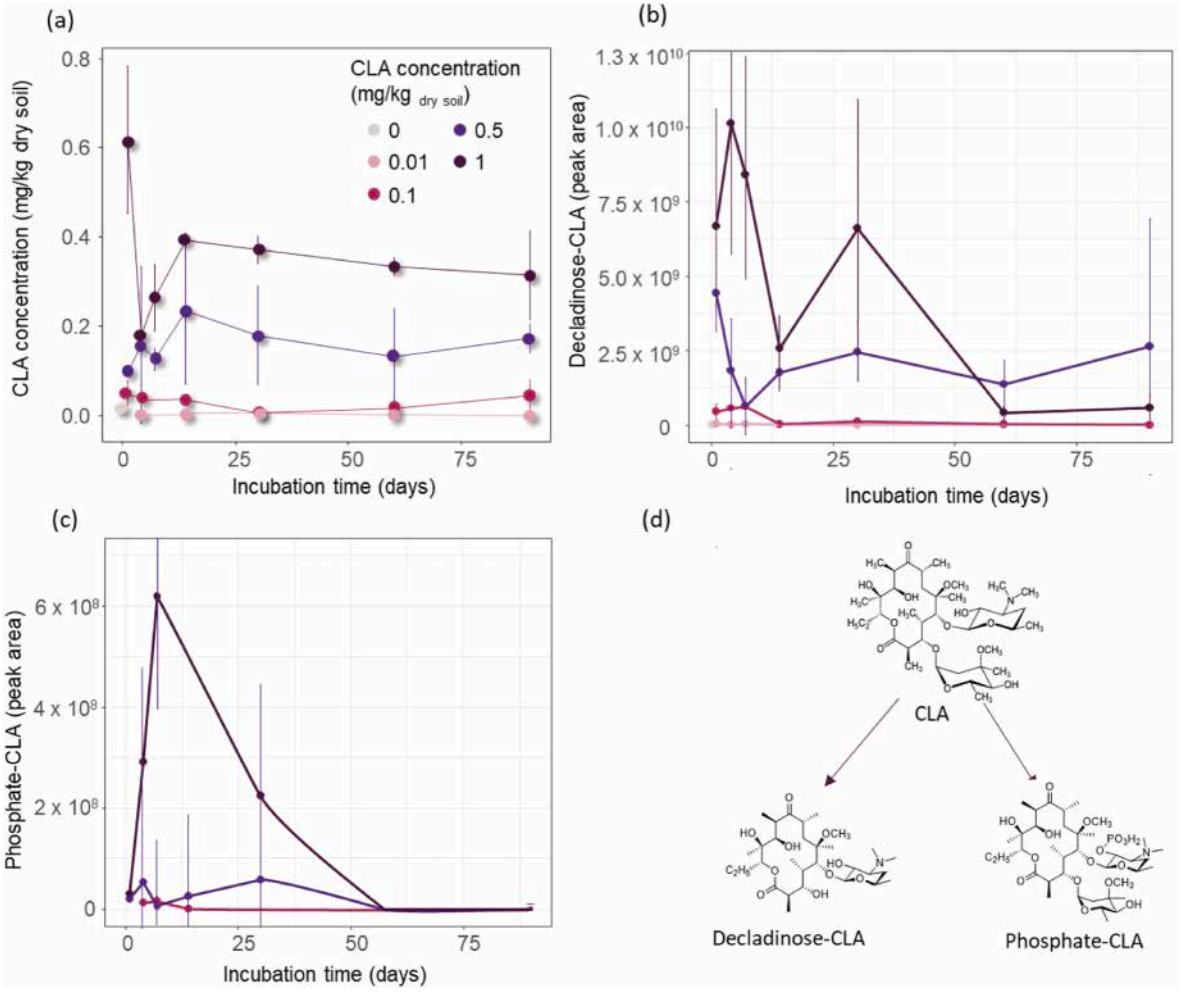

Fig. 1. (a) Temporal dynamics of clarithromycin concentrations in soils exposed to TWW. (b) Temporal dynamics of the peak area of Decladinose-CLA transformation product in soils. (c) Temporal dynamics of the peak area of phosphate-CLA transformation product in soils. At T60 and 90 days, the TP was below the LOD for the conditions. (d) Structures of clarithromycin and its transformation products.

higher concentrations of CLA (>0.1 mg/kg dry soil) and appeared between 1 and 30 days of incubation (Fig. 1c). This TP is typically associated with CLA phosphorylation mediated by macrolide phosphotransferase (Pawlowski et al., 2018), and was not observed in abiotic controls.

3.2. Dynamics of physico-chemical properties in soil

As observed in Table 1, TWW was characterized by moderate concentrations of nitrate (12.9 mg/L) and phosphate (12.8 mg/L) and low concentrations of ammonium and nitrite. This range of concentration is usually observed with this type of TWW treated by constructed wetlands (Ait-Mouheb et al., 2022; Della-Negra et al., 2024; Moulia et al., 2023). The sampled soil was also characterized at the beginning of the experiment and showed the presence of ammonium (2.4 mg/$kg_{dry\ soil}$, Table 1) a quite high concentration of nitrate (13.4 mg/$kg_{dry\ soil}$), indicating a long-term irrigation with TWW (Moulia et al., 2023). Just after TWW input, phosphate concentrations increased in soil microcosms reaching a concentration of 7.1 mg/$kg_{dry\ soil}$ (Table 1), the same order of magnitude of what was brought by 20 mL of TWW to 25g of dry soil. However, nitrate concentration dropped, which could indicate denitrification or microbial immobilization of nitrate for bacterial growth. During the experiment, ammonium concentration remained constant up to 7 days, then decreased from 7 to 30 days; phosphate concentration decreased from 1 to 14 days, nitrate and nitrite concentrations remained constant (Fig. S6, Table 1). Final concentrations can be found in Table 1.

Table 1
Physico-chemical analyses of TWW and soil microcosms before or after TWW input.

Parameter	TWW (mg/L)	Soil at T0 (mg/$kg_{dry\ soil}$)	Soil at T1 day (mg/$kg_{dry\ soil}$)	Soil at T90 days (mg/$kg_{dry\ soil}$)
Ammonium	0.2 ± 0.1	2.4 ± 0.3	2.5 ± 0.2	0.8 ± 0.1
Nitrite	0.2 ± 0.1	1.1 ± 0.5	0.5 ± 0.1	0.4 ± 0.1
Nitrate	12.9 ± 0.5	13.4 ± 1.2	0.3 ± 0.1	2.4 ± 0.1
Phosphate	12.8 ± 2.1	1.1 ± 0.2	7.2 ± 0.7	2.7 ± 0.6

3.3. Dynamics of soil microbial communities irrigated with TWW

As seen in Fig. S4, TWW used in this study was characterized by the presence of *Pseudomonadota* and *Bacteroidota*, representing more than 80% of the bacterial relative abundance; and dominated by *Hylemonella, Pseudomonas, Acinetobacter, Aeromonas, Perlucidibaca, Rheinheimera, Acidovorax* and *Fluviicoccus* for *Pseudomonadota* and *Flavobacterium, Bacteroides, Fluviicola* and *Cloacibacterium* for *Bacteroidota* (Fig. S4). This composition was globally found in previous studies (Della-Negra et al., 2024, 2025) and was characteristic of wastewater treated by constructed wetlands (Moulia et al., 2023).

The addition of TWW to soil microcosms triggered a dynamic shift in

the soil microbial community over time, as shown in Fig. 2b. At the phylum level, *Bacillota, Bacteroidota, Chloroflexi, Actinomycetota*, and *Verrucomicrobia* were the most affected (Fig. 2a). Specifically, shortly after TWW input, an increase in *Flavobacterium* (*Bacteroidota*), Bacillus (*Bacillota*), and certain *Pseudomonadota* (*Microvirga*, *Hylemonella*) was observed (Fig. 2b, Fig. S5). These bacteria are known to include denitrifiers, pollutant degraders, and potential pathogen carriers of multiple antibiotic resistance genes (ARGs)(Bartlett et al., 2022). The residual carbon, nitrogen, and various contaminants in TWW (including pharmaceuticals and heavy metals, Ibekwe et al., 2018) may have been temporarily selected for these genera. Indeed, the dynamics of these taxa was directly correlated with the dynamics of nitrite, phosphate and ammonium (Fig. S7).

Between days 4 and 14/30, there was an increase in *Gracilibacter* and, to a lesser extent, unclassified Family XVIII and *Lutispora* from the Bacillota phylum, along with *Flavisolibacter* (*Bacteroidota*) and *Lacunisphaera* (*Verrucomicrobia*) (Fig. 2b). A similar trend was observed in previous experiments (Della-Negra et al., 2025), attributed to the organic matter and nutrients supplied by TWW (Ibekwe et al., 2018). These denitrifying bacteria are known to be enhanced in soils irrigated with TWW (Della-Negra et al., 2024; Farkas et al., 2023; Guo et al., 2017; Moulia et al., 2023) likely outcompeting the previous ones for nutrients and nitrogen (dynamics correlated with ammonium evolution, Fig. S6).

By the end of the experiment (days 30/60–90), an increase in Anaerolinaceae (*Chloroflexi*), Fimbriimonadaceae (*Armatimonadetes*), and some Betaproteobacteria (*Pseudomonadota*) was observed (Fig. 2b–S5). These bacteria have been identified as settlers in soils irrigated for long-term with TWW (Chand et al., 2023; Della-Negra et al., 2024; Ma et al., 2020).

Overall, it appears that TWW either introduced carbon and nutrients or increased the availability of these components, driving a temporal dynamic in the structure of the soil microbial community, likely driven by the competition effect between the different microbial species (Della-Negra et al., 2024; Frenk et al., 2015; Moulia et al., 2023).

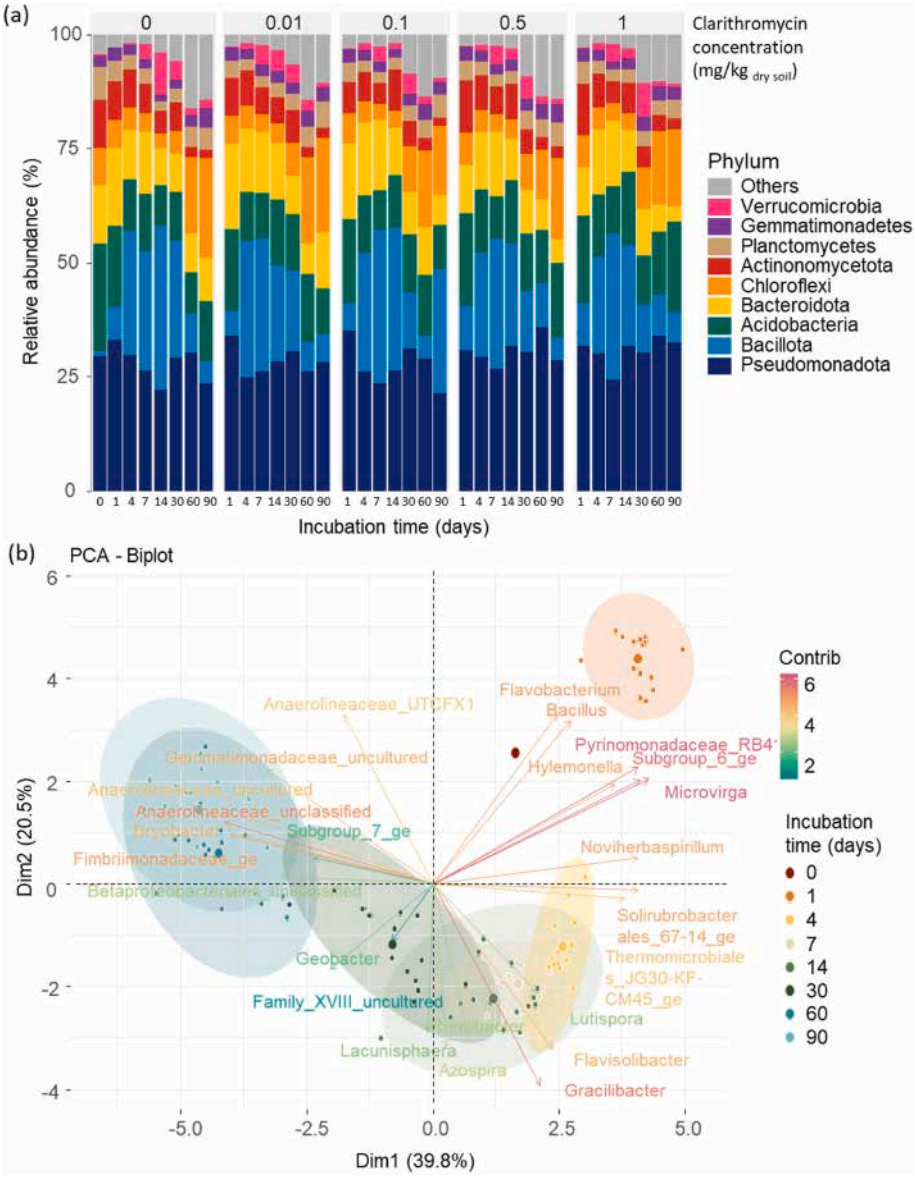

Fig. 2. (a) Bar plot of relative abundance (% reads) of bacterial communities at a phylum level in microcosm soils over time, depending on clarithromycin concentration. (b) Principal component analysis of the bacterial community structures in soil microcosm samples exposed to TWW and clarithromycin at different incubation times and the contribution of the 25 most abundant genera.

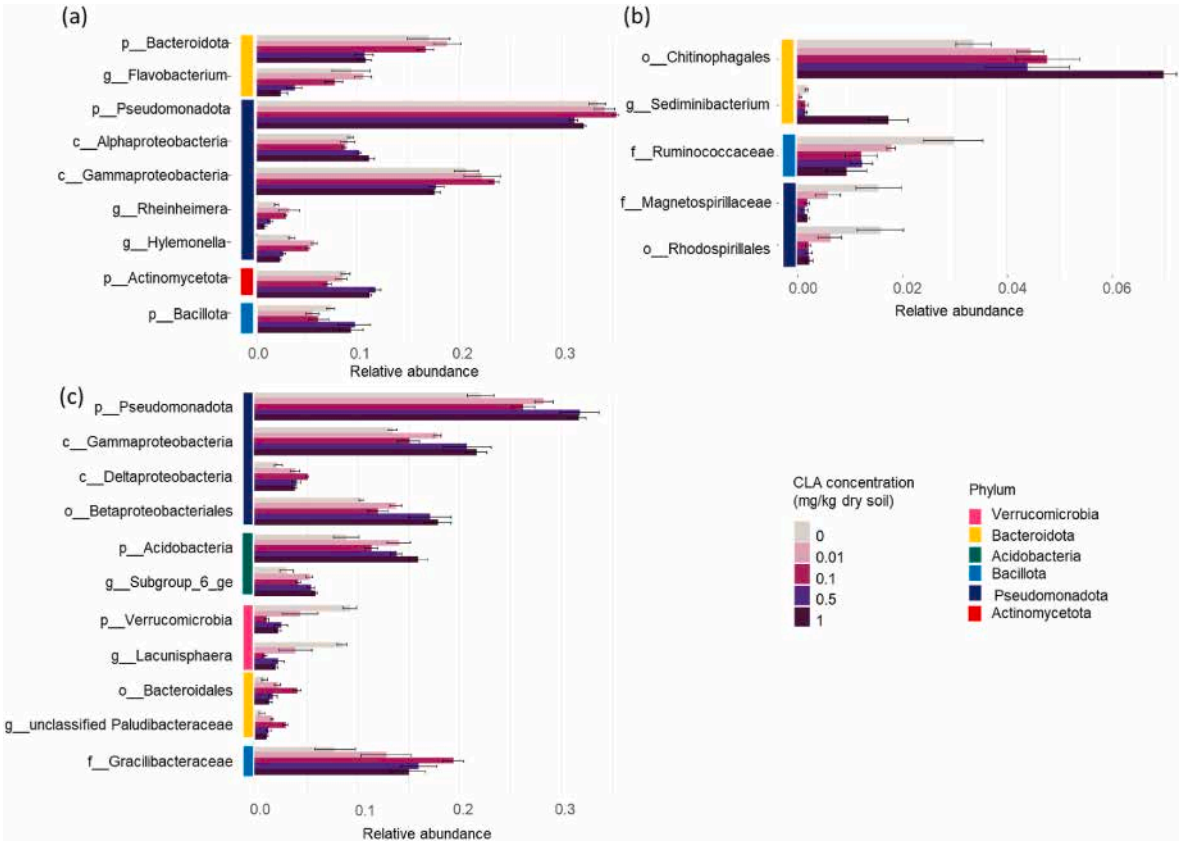

Fig. 3. Biomarkers identified by LEfSe analysis and respective mean relative abundance in soil microcosm samples according to CLA initial concentration at different periods: (a) 1, (b) 30 and (c) 14 days.

3.4. Impact of clarithromycin on soil microbial communities

To assess the impact of clarithromycin on the soil microcosm, a Linear discriminant analysis Effect Size analysis (LEfSe) was performed. LEfSe is commonly used to identify biomarkers of different conditions and was employed here to detect bacterial genera that significantly differed across CLA concentrations (LEfSe combines statistical significance with biological consistency and effect relevance, Segata et al., 2011).

Across the entire experiment (time points grouped by CLA concentration), no significant differences in taxa were found based on clarithromycin levels. However, indicators of CLA concentration were identified at specific time points—1, 14, and 30 days (Fig. 3, Table S4, p_value < 0.05).

One day after TWW input, inhibition of Bacteroidota (*Flavobacterium*) and *Pseudomonadota* (Gammaproteobacteria) was observed at CLA concentrations of 0.1 mg/kg $_{dry\ soil}$, while an inverted U-shaped relationship was seen between CLA concentration and the relative abundance of some taxa. For *Rheinheimera* and *Hylemonella*, maximum relative abundance occurred at 0.01 mg/kg $_{dry\ soil}$. Conversely, for *Actinomycetota*, some Alphaproteobacteria, and *Bacillota*, a U-shaped relationship was observed, with minimum abundance at 0.01–0.1 mg/kg $_{dry\ soil}$.

After 14 days of incubation (Fig. 3), a selection effect was observed on *Pseudomonadota* and *Acidobacteria* phyla, while inhibition was noted for *Verrucomicrobia*. An inverted U-shape profile was also seen for *Bacteroidota* and the Gracilibacteraceae family, with a peak at 0.1 mg/kg $_{dry\ soil}$ CLA.

Finally, by day 30, selection effects were observed for some *Bacteroidota*, and inhibition was identified in Ruminococcaceae and Magnetospillaceae families (Fig. 3).

In this experiment, the addition of clarithromycin did not result in a major shift in the soil microbial community, unlike sulfamethoxazole in a previous study (Della-Negra et al., 2025). However, it did induce specific effects at distinct time windows.

3.5. Impact of clarithromycin on the dynamic of antibiotic resistance genes in soils

In this experiment, several genes were quantified, including 16S rDNA, the methylase gene *ermB*, the erythromycin esterase gene *ereA*, the macrolide phosphorylase gene *mphA*, the macrolide efflux gene *mefA* and the class 1 integron mobile gene *intl1* (Fig. 4). The abundance of the 16S rDNA gene remained relatively stable over time, with no significant differences between CLA concentrations (Fig. 4a). Thus, the total and relative abundances of the selected genes (/16S rDNA, Fig. 4 and Fig. S8) evolved similarly throughout the study.

For *intl1*, *ermB*, *mphA* and *ereA*, an increase in the copy number was observed just after TWW input (from 1.1 log for *ereA* gene to 4.9 log for *ermB* gene, Table S4). Most of these increases exceeded the copy number brought by TWW ($<$0.2 log, Table S5), except for *ermB* gene, where the number of gene copies brought by TWW was similar to the observed increase (4.6 and 4.9 log respectively, Table S5).

The abundance of *intl1* gene then declined over time, with no clear effect of clarithromycin concentration, except at 90 days when copy numbers appeared to increase with higher CLA levels (Fig. 4b). The copy number of the *mphA* gene remained constant between 1 and 7 days, followed by a 0.6-log increase between days 7 and 14, reaching a steady state thereafter. A slight effect of CLA concentration was noted at 60 and 90 days for the 0.1 mg/kg condition, where *mphA* gene abundance was significantly higher than at other concentrations (Fig. 4c).

The abundance of the *ermB* gene decreased throughout the experiment and was no longer quantified after 30 days of incubation. A significant difference was observed between 0 mg/kg $_{dry\ soil}$ and other CLA

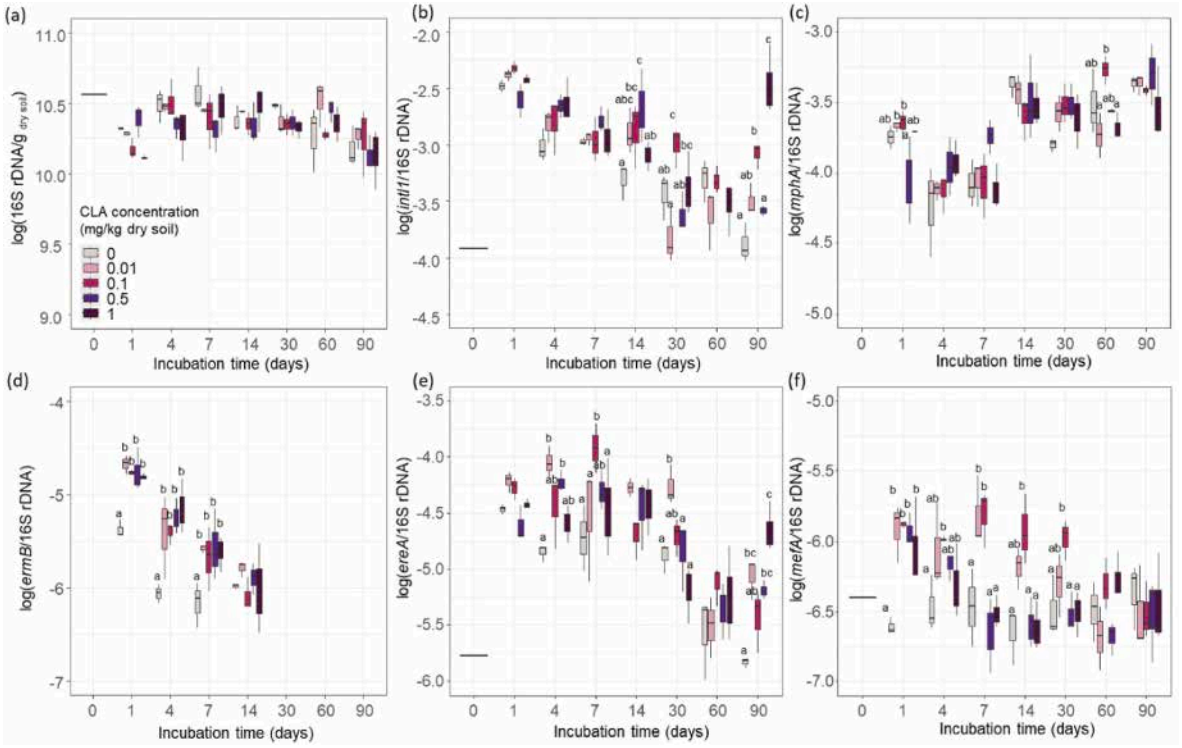

Fig. 4. Evolution of the relative abundance of (a) 16D rDNA/g dry soil, (b) *intl1*/16S rDNA, (c) *mphA*/16S rDNA, (d) *ermB*/16S rDNA (e) *ereA*/16S rDNA, (f) *mefA*/16S rDNA in soil microcosms over time. Letters indicate significant differences between CLA concentrations for each time point (one-way ANOVA). When letters are missing, it means that no significant differences were observed for this time point.

concentrations, whose abundance was on average 0.5 log higher between 1 and 14 days (Fig. 4d). The abundance of *ereA* gene did not evolve a lot between 1 and 30 incubation days, then decreased. Similar to *intl1* gene, *ereA* copy number increased significantly with increasing CLA concentration by day 90 (Fig. 4e, +1.2 log between CLA 0 and CLA 1 mg/kg$_{dry\ soil}$, p value < 0.05).

Finally, the abundance of the *mefA* gene remained constant between T0 and T90 days for concentrations of 0 and 1 mg/kg $_{dry\ soil}$. Aside from a slight increase at 1 day, the 0.5 mg/kg $_{dry\ soil}$ CLA concentration followed a similar pattern. However, for the 0.01 and 0.1 mg/kg $_{dry\ soil}$ CLA concentrations, a significant increase in the number of copies of this gene was observed between days 1 and 30 compared to other concentrations (Fig. 4f).

4. Discussion

4.1. Modulation of clarithromycin antimicrobial efficiency by degradation and soil sorption

Despite the limited degradation of clarithromycin in this experiment, two major transformation products (TPs) were observed: decladinose-CLA and phosphate-CLA. Decladinose-CLA appeared rapidly following the introduction of TWW. Given that this TP was absent from both TWW and the commercial formulation of clarithromycin, it is likely that most of this transformation occurred in the soil shortly after the addition of TWW. Abiotic hydrolysis of CLA occurs in water at pH 7, and studies have shown that around 30% of CLA can be transformed after nine days (Frascaroli et al., 2024). With a TWW and a soil close to neutrality (pH 7.1 and 7.5 respectively), CLA transformation may occur in the experiment. Additionally, the nutrients introduced by the TWW may have stimulated bacterial activity, accelerating the degradation of CLA before it reached its adsorption equilibrium, limiting its bioavailability (Frascaroli et al., 2024; Rodríguez-López et al., 2023). During the experiment, the abundance of this TP did not change significantly, which corroborates its probable persistence and its presence in the soil at T0. While this TP has been previously observed in TWW or degradation experiments (Buchicchio et al., 2016; Calza et al., 2012; Tian et al., 2019; Zeng et al., 2021), no studies have yet reported its occurrence in soils exposed to clarithromycin through irrigation with TWW or treated sludge application.

CLA phosphorylation will be discussed in part 4.3 as this transformation is associated with the expression of the *mphA* gene. If it is known that phosphorylation of clarithromycin results in a loss of its antimicrobial activity (Golkar et al., 2018; Pawlowski et al., 2018), declanidose-CLA may also exhibit reduced antimicrobial properties (Ma et al., 2001). Thus, the rapid transformation of a significant portion of CLA could mitigate the selection pressure for antibiotic resistance.

The low degradation of CLA could be explained by its adsorption onto soil particles, limiting its bioavailability. Clarithromycin has an adsorption coefficient estimated between 262 and 400 L/kg (Manasfi et al., 2021), conditioned by (i) electrostatic forces between soil surface charges and those of the molecule, notably associated with the NH $(CH_3)_2$ group of CLA; and (ii) strengthened by van der Waals forces, including hydrogen bonds between CLA and humic acids. CLA adsorption is highly dependent on soil pH and clay content, with approximately 70% of CLA typically adsorbed onto the soil at pH levels between 6 and 7, which aligns with the pH range observed here (Kodešová et al., 2015; Rodríguez-López et al., 2023). In this case, a significant fraction of CLA was likely adsorbed onto the soils. Once adsorbed, clarithromycin may lose its antimicrobial activity. Several studies have demonstrated a negative correlation between the adsorption strength of antibiotics and their antimicrobial activity in soils, with some antibiotics, like colistin, becoming completely inactive (Menz et al., 2018; Subbiah et al., 2011).

Thus, despite its persistence in soil microcosms and more generally in soils, the antimicrobial effect of residual CLA was mitigated or inactivated. It could explain the moderate impact of CLA concentrations on soil microbial communities and the dynamics of antibiotic-resistance genes observed in this study.

4.2. Soil microbial community dynamic after TWW and clarithromycin exposure

The addition of TWW to the soil led to a strong dynamic in the soil microbial community, with distinct changes observed over three time periods: 1 day, 4–30 days, and 60–90 days. These changes were likely driven by competition between species (exogenous and endogenous) for the carbon and nutrients introduced by the TWW (Ibekwe et al., 2018), as well as the selection of bacteria that are resistant or tolerant to the contaminants added to the soil (Fang et al., 2023).

Unlike other antibiotics, such as sulfamethoxazole, tetracyclines, or sulfadiazine (Della-Negra et al., 2025; Rodríguez-González et al., 2021), the introduction of clarithromycin did not result in lasting changes in the structure of the soil microbial community. As seen in previous studies, the toxicity of CLA on soil bacterial communities appears to be time-dependent (Rodríguez-González et al., 2021).

However, there is some consistency in the taxa selected in response to CLA between this study and another that evaluated the dose-response effect of CLA during anaerobic digestion of activated sludge (Zeng et al., 2021). Both studies found a trend in the selection of Gracilibacteraceae (Bacillota), Pseudomonadota, and Chitinophagaceae (Bacteroidota) in the presence of increasing concentrations of CLA. These bacterial groups, which include species known to be multi-resistant to antibiotics, may be better adapted to high CLA concentrations and/or capable of utilizing CLA as a carbon and nitrogen source (Schofield, 2018).

The limited impact of CLA on the structure of the soil bacterial community could be explained by several factors: (i) a greater disturbance caused by the addition of TWW, which contains its own mixture of nutrients and contaminants (Ibekwe et al., 2018), (ii) CLA partial degradation, reducing its efficiency, (iii) CLA adsorption onto soil particles, limiting its bioavailability—one study showed that once tetracycline was adsorbed, no bacterial inhibition was observed in the soil (Subbiah et al., 2011), (iv) and finally, the development of tolerance/resistance by soil bacteria (Fang et al., 2023; Topp et al., 2016). Indeed, CLA was detected in soil samples taken from the agricultural plot, suggesting that the repeated application of TWW containing CLA may have led to an overall adaptation of the soil microbial communities.

4.3. Antimicrobial resistance dissemination in soils exposed to residual concentrations of clarithromycin

Antibiotic resistance genes followed different dynamics according to the gene type.

The *ermB* gene abundance, encoding for the 23S ribosomal RNA methyltransferase, increased just after TWW input. This rise was certainly attributed to the community structure change caused by the introduction of TWW affecting soil physical properties. As observed in Fig. S7, *ermB* gene, nitrite, phosphate and ammonium concentration were directly correlated. Among these bacteria, it is possible that some of them carried genes coding for macrolide resistance. TWW input could also temporary introduced TWW bacteria carrying *ermB* gene into the soil (Gullberg et al., 2011).

Despite the apparent moderate effect on bacterial communities, an increase in the abundance of this gene was observed at concentrations as low as 0.01 mg/kg $_{dry\ soil;}$ suggesting that the Lowest Observed Selection Concentration for CLA in this soil is $<$ 0.01 mg/kg $_{dry\ soil}$ or $<$10 μg/kg $_{dry\ soil}$. No comparable values for soil microbial communities have been reported in the literature. However, predicted no-effect concentrations (PNECs) have been established for complex communities in liquid media (such as wastewater) between 50 and 250 μg/L (Murray et al., 2018; Stanton et al., 2020). Based on a K_{oc} of 23.5 and a Foc of 0.016, PNEC in soils was estimated at 0.1 μg/kg (Castaño-Trias et al., 2024).

In this study, the overall decrease and low abundance of *ermB* gene suggests that bacteria carrying it were outcompeted by other resistant taxa with intrinsic or acquired resistance via alternative mechanisms (Stanton et al., 2020). Here the resistance mechanisms could be activated by the expression of *ereA*, *mphA*, *mefA* genes.

The abundance of *intl1* gene, (a proxy for anthropogenic pollution and horizontal transfer of ARGs,Gillings et al., 2015) and *ereA* gene (encoding for erythromycin esterase) increased significantly following TWW addition. Such increase after TWW addition is common and certainly attributed to the growth of bacteria carrying these genes and stimulated by the nutrient load brought by TWW. Just as *ermB* gene, *intl1* was well correlated with ammonium dynamics (Fig. S7).

In this study, these two genes exhibited similar trends, with parallel dynamics and changes in abundance at 90 days as a function of CLA concentration. This correlation, also observed in another study (Lu et al., 2021), may be explained by the presence of these genes in the same gene cassette (Partridge et al., 2018). Although the transformation product associated with *ereA* expression was not observed in this study, the significant increase of this gene at 90 days with increasing CLA concentration suggests an activation of this mechanism, or a horizontal gene transfer of this gene among the bacterial population. While community structure seemed stable between 60 and 90 days, a slight increase of nitrite was observed at the end of the experiment (Table 1, Fig. S6). This change might reflect shifts in microbial community functions, disturbed by desorption of clarithromycin, or other factors.

Although phosphate-CLA was detected for high CLA concentrations (0.5 and 1 mg/kg $_{dry\ soil}$), no clear variation of *mphA* gene abundance was detected in this study. However, as the transformation product was detected, the activation of *mphA* gene was certainly triggered by high CLA concentrations, thus modulating macrolide phosphatase activity (Fang et al., 2023). Interestingly, while *mphA* gene abundance increased after 14 incubation days, no corresponding increase in metabolic activity was detected, suggesting that these changes were related to shifts in the microbial community favoring bacteria carrying the *mphA* gene.

Unlike the other genes, the abundance of the *mefA* gene encoding the macrolide efflux pumps increased for concentrations [0.01–0.1 mg/kg $_{dry\ soil}$] and then decreased for concentrations of 0.5 and 1 mg/kg $_{dry\ soil}$. This pattern suggests a hormetic effect, where low CLA doses stimulate and high CLA doses inhibit. This effect has already been observed with sulfonamides and *E. coli*, with such low optimal concentrations promoted bacterial growth, mutation frequency and conjugative transfer (Sun et al., 2019). Depending on antibiotic concentration, they showed that antibiotics could have different target proteins in an organism inducing stimulatory and inhibitory effects. In another publication studying the effect of a macrolide cocktail on soils, the authors reported that the ***mexL*** and ***mexP*** efflux genes, which confer macrolide resistance in *Pseudomonas* spp., were more abundant in soils exposed to 0.1 mg/kg$_{dry\ soil}$ compared to controls. Interestingly, this increase was not observed at a dose of 10 mg/kg$_{dry\ soil}$ (Brown et al., 2022). For our study, these findings seem to confirm that resistance selection (via efflux pumps) may be driven by the presence of clarithromycin at 0.01–0.1 mg/kg$_{dry\ soil}$. The dynamics of *mefA* gene could also explain the similar abundance patterns of specific bacterial taxa such as *Rheinheimera*, *Hylemonella*, some *Bacteroidota*, and the *Gracilibacteraceae* family.

Finally, the gradient of CLA concentration did not show a clear positive correlation with the abundance of targeted macrolide resistance genes. Long-term irrigation tends to enhance sequestration of cationic or uncharged molecules (Dalkmann et al., 2014), as this is the case with macrolide. In soils, it seems that CLA adsorption onto soil particles, coupled with the adaptation of the soil microbial community, increased tolerance to the antibiotic. As observed in several studies, high concentrations of CLA (10 mg/kg$_{dry\ soil}$) were required to observe an increase in the abundance of resistance genes (Brown et al., 2022; Lau et al., 2020). It is possible that the complexity of soil microbial community structure raises the tolerance threshold to CLA, as previously observed in untreated wastewater (Stanton et al., 2020). Finally, in soils, the physico-chemical properties and the endogenous soil microbial community appear to be the main factors driving changes in the resistance genes quantified here.

4.4. Implications for environmental risk assessment of antimicrobials

Residual concentrations of antibiotics in soils, resulting from repeated irrigation with TWW, may accumulate over time and lead to the selection or emergence of resistant bacteria in soils (Christou et al., 2017; Gullberg et al., 2011; Marano et al., 2021). Although these concentrations are significantly lower than therapeutic levels, studies—particularly in aquatic environments—have shown that even low concentrations can be sufficient to enrich antimicrobial-resistant bacteria (Murray et al., 2021). In the agricultural soils context, this risk is particularly concerning as antibiotic resistance could potentially transfer to plants, directly entering the food chain (Xiao et al., 2023). For the case of clarithromycin, some residues were observed to be transferred from soil irrigated with TWW to lettuce leaf, directly entering to the food-chain (Manasfi et al., 2021). If concentrations are expected to be lower in plants than in soils, the different context (endogenous microbial communities, physico-chemical properties) could lead to a higher selection pressure and lower minimal concentration of resistance selection. Antibiotic resistant genes (either free or carried by antibiotic resistant bacteria), could also be transferred from soil to plants as already observed in some studies (Leiva et al., 2021). Once again, these different transfers are modulated by the physico-chemical properties and the endophyte bacteria present in plants that could act as ecological barriers, thus mitigating antibiotic resistance dissemination (Marano et al., 2019).

Several methodologies have been developed and are continuously being refined to assess the environmental risk of antimicrobials, accounting for their selective and persistent potential as well as their environmental impact (Ceriani et al., 2015; Le Page et al., 2017; Murray et al., 2021). One approach involves the establishment of concentration thresholds such as predicted no-effect concentrations (PNECs) (Bengtsson-Palme and Larsson, 2016; Murray et al., 2021).

In this study, using a controlled microcosm model, we applied a concentration gradient of a single molecule to investigate its fate, its impact on soil microbial communities, and the dynamics of certain resistance genes. Comparisons with a similar study on sulfamethoxazole highlight how the nature of the antibiotic (its adsorption capacity and mechanism of action) influences key concentration thresholds for the spread of resistance in soils (below 0.01 mg/kg $_{dry\ soil}$ for this antibiotic, above 0.01–0.1 mg/kg $_{dry\ soil}$ for sulfamethoxazole) (Della-Negra et al., 2025).

This fundamental research provides valuable insights into the factors and mechanisms controlling the spread of antibiotic resistance in soils. To simulate more realistic conditions, future experiments could vary factors such as soil type, water quality, weather conditions, and the way water is supplied (repeated and/or intermittent input). This will help enrich PNEC indicators, which are currently mostly estimated and not measured in complex and solid media.

5. Conclusions

Through this study, which aimed to assess the fate and impact of clarithromycin introduced via soil irrigation with TWW, we were able to identify that.

(i) Clarithromycin remained largely persistent, with limited degradation. Its biodegradation was likely inhibited by the strong adsorption of the molecule onto soil particles. The two major TPs identified were decladinose-CLA, likely of abiotic origin, and phosphate-CLA, produced by phosphotransferase activity.

(ii) The dynamics of the soil bacterial communities appeared to be more perennially affected by the addition of TWW than by the presence of clarithromycin, whose antimicrobial activity was likely reduced due to its adsorption onto soil particles.

(iii) Although the overall abundance of the *ermB* gene remained low throughout the experiment, the addition of clarithromycin at concentrations as low as 0.01 mg/kg $_{dry\ soil}$ led to an increase in *ermB* gene levels in the soil. Conversely, the dynamics of the *mefA* gene exhibited a hormetic pattern, where low clarithromycin doses (<0.1 mg/kg $_{dry\ soil}$) increased the gene's abundance for up to two weeks, while higher doses (>0.5 mg/kg $_{dry\ soil}$) inhibited its transfer or the bacteria carrying this gene.

Further studies should be considered to extend the panel of antibiotics studied and the environmental physico-chemical conditions. Particular attention should also be paid to the exact proportion of the molecule adsorbed and the role adsorption plays in the mechanisms discussed above.

CRediT authorship contribution statement

Oriane Della-Negra: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Marilia Camotti Bastos:** Methodology, Investigation. **Valérie Bru-Adan:** Validation, Supervision. **Gaëlle Santa-Catalina:** Validation, Supervision. **Nassim Ait-Mouheb:** Supervision. **Serge Chiron:** Writing – review & editing, Supervision. **Dominique Patureau:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.120954.

Data availability

Data will be made available on request.

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