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Insights into structure and functioning of a soil microbial community amended with cattle manure digestate and sulfamethoxazole

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Abstract

Purpose The present work aimed to fill some knowledge gaps on the effects on non-target natural soil microbial communities of the sulfamethoxazole (SMX) antibiotic potentially found in cattle manure digestate. Both soil prokaryotic and fungal community under different conditions were analyzed using molecular ecology methods.

Methods A previous microcosm experiment with a soil amended with a cattle manure digestate (3% dry mass) and spiked with SMX (20 mg/kg soil) was used for this in-depth study. Microbial live cell abundances were assessed by direct epifluorescence microscope methods. The microbial community structures were studied by DNA extraction and amplification using 16S rRNA primers targeting the V3-V4 region (for prokaryotes) and ITS1 (for fungi); Alpha-diversity indices (Chao1, Shannon and Evenness) were also estimated. Moreover, a prediction functional analysis was performed on prokaryotic 16S rRNA amplicon data with PICRUSt2 in order to predict possible variations in some microbial functioning.

Results Adding digestate to soil promoted both microbial abundance and some bacterial groups. The co-presence of SMX initially lowered these positive effects and decreased diversity. However, at day 61, the antibiotic was almost all removed and the microbial abundance and prokaryotic and fungal diversity showed increased values.

Conclusion The antibiotic detrimental effects on prokaryotic cells were transient and some resistant bacteria (e.g., *Sphingomonas*, *Skermanella*), presumably introduced into the soil with the digestate, were able to remove SMX. Moreover, the digestate long-term effect was to favor some fungal groups such as *Basidiomycota*. The presence of the genus *Trichoderma* (*Ascomycota*), able to transform antibiotics, does not exclude also as possible involvement of this fungus in SMX removal.

Keywords Sulphonamide antibiotics · Microbial nitrogen metabolism · Microbial oxidative stress · Prokaryotic and fungal community

1 Introduction

The European Commission has recently promoted the European Green Deal, a series of actions aimed at boosting the efficient use of resources, with a view to achieving the clean, circular economy; restoring biodiversity; and reducing pollution (COM/2021/400 final). For example, a sustainable agriculture using zootechnical waste (e.g., manure, digestate) as organic fertilizers and limiting the application of chemicals meets these objectives. In this regard, the EU Fertilizing Product Regulations (EU 2018, 2019) discipline the use of organic fertilizers by establishing threshold limit concentrations in nutrients (e.g., nitrogen, phosphourous), organic carbon, trace elements, and pathogens. However, both initiatives do not

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consider any potential emerging contaminants which can be found in organic waste. For example, it is generally recognized that by adding cattle manure, antibiotic residues from treating cattle infections can be introduced into soils (Huygens et al. 2021; Wang et al. 2014). In particular, sulphonamides are found in manure at relatively high concentrations (from 0.1 to 91 mg/Kg, Martínez-Carballo et al. 2007; Qian et al. 2016). Cattle manure can be also used for feeding anaerobic digestion plants, enabling its use beyond the carrying capacity of adjacent fields (Shi et al. 2018).

Anaerobic digestion (AD) is a well-established technology with significant growth potential and a key role in the development of a sustainable society (Kougias and Angelidaki 2018), addressing several agricultural sustainability aspects. The AD process anaerobically degraded and stabilize biogenic wastes by microbial communities of the AD plants, reducing emissions of greenhouse gasses to the atmosphere, compared to storage, landfilling, or composting. Biogas is one of the product of this process; it is a sustainable bioenergy with the potential to replace fossil carbon in the generation of electricity, heat, and motor fuel (Kougias and Angelidaki 2018). Digestate is the other important product of AD process. Thanks to its high content in vital plant nutrients and organic carbon, it is widely used as soil fertilizer (Scarlat et al. 2018). Digestate use as fertilizers not only replaces chemicals, but also improves soil structure and quality (Barra Caracciolo et al. 2015; Egene et al. 2021; Möller 2015), increasing also microbial biomass and decreasing pathogens (Gilbert et al. 2020). However, its physico-chemical composition can vary depending on the feed materials and operational conditions of the AD plant (Barampouti et al. 2020). The direct land application of digestate is a good agricultural practice for recycling organic waste, in line with the circular economy principles and sustainable agroecosystems (Chojnacka et al. 2020); responds to growing demand for nutrients in the agricultural sector; and combats organic carbon loss in soils (European Biogas Association 2020).

Although the use of digestates is in line with the green economy, the potential presence of antibiotics in it is not currently considered. Even though new EU soil strategy highlights the importance of soil biodiversity, the knowledge of how antibiotics can affect natural microbial communities, in particular those related to important ecological functions, needs to be better understood (e.g., nitrogen transformation, methanogenesis, sulfate reduction, nutrient cycling, and organic matter degradation). For example, significant concentrations of sulfonamides in the environment could inhibit denitrification and the application of manure to soil containing antibiotics can negatively influence the nitrogen cycle in soil (Grenni et al. 2018; Laverman et al. 2015; Roose-Amsaleg and Laverman 2016; Grenni 2022).

Sulfamethoxazole (SMX) is a sulfonamide antibiotic with a bacteriostatic effect on both Gram-positive and Gram-negative bacteria. It has been used since the 1960s in both livestock and human medicine for the treatment of various deseases such as pneumonia and urinary tract infections (Thiebault 2020) and it is actually one of the most commonly prescribed and consumed sulfonamide antibiotic. The average use is 350 mg/head/day for disease control and growth promotion in cattle (Congilosi and Aga 2021). A high SMX percentage is excreted unchanged (15–25%) or as acetylate (43%) or glucosinate (9–15%) metabolites by treated organisms (Radke et al. 2009). Consequently, its residues and antibiotic resistance genes (ARGs) reach soil and water in different ways and are found as ubiquitous emerging contaminants (García-Galán et al. 2011; Wang et al. 2014). Antibiotics are aimed at killing pathogenic bacteria; however, once in the environment, they can also affect non-target natural microbial communities and alter their structure and functioning, such as the nitrogen cycle (Grenni et al. 2018; Grenni 2022). However, current knowledge of antibiotic biodegradation and effects on natural microbial communities (including fungi) in soil is limited. Moreover, whether anaerobic digestate can also be a significant antibiotic source in soil and its possible effects on both prokariotic and fungi communities still needs to be thoroughly investigated (Bailey et al. 2016; Congilosi and Aga 2021). Recent studies report SMX degradation during an anaerobic digestion process (Mazzurco Miritana et al. 2020; Visca et al. 2022). However, few papers have studied the potential for antibiotics to enter soil if digestate is used as an organic fertilizer (Barra Caracciolo et al. 2020; Congilosi and Aga 2021). For example, Deng et al. (2020) reported SMX residues (from 21.7 to 51.9 µg/L) in a liquid digestate from cow manure.

A recent study investigated the effect of SMX contamination on soil and found a reduction in soil bacterial diversity and change in the composition of the bacterial and fungal community (Cheng et al. 2020). Moreover, our previous microcosm experiment (Rauseo et al. 2019) showed SMX halved in few days in a soil spiked with this antibiotic (20 mg/kg) and amended with anaerobically digested cattle manure. The *intI*1 gene was also found in both the digestate and amended soil, suggesting that the application of cattle manure digestate might be a potential source of antibiotic-resistant bacteria (ARBs) and genes (ARGs) in agroecosystems.

The present work aims to extend our previus work (Rauseo et al. 2019) providing new aspects related to the effects of adding a cattle manure digestate and SMX on a natural soil microbial community in terms of structure and functioning. The present study focuses in particular on direct effects on the prokariotic cells (e.g., decrease in SMX susceptible bacterial genera with some key functioning such as those related to nitrogen cycle).

Moreover, the possible effects on fungal community structure were evaluated. For this purpose, the prokaryotic and fungi communities of soil were assessed by 16 s rRNA sequencing using Miseq Illumina. PICRUSt2 was used for predicting functional abundances in prokaryotic community based on 16S rRNA gene amplicon datasets. Moreover microbial abundance (DAPI counts) and viability (live/dead method) were assessed using epifluorescence direct count methods.

2 Materials and methods

2.1 Characteristics of digestate and soil and microcosm set-up

As reported in detail in the previous work (Rauseo et al. 2019), the anaerobic digestate was derived from a digestion plant that produces biogas by using fed-in biomass from a mix of dairy cattle manure (70%) and energy crops (maize silage up to 30%) and waste from the agro-food industries in the area surrounding the digestate plant. The main characteristics of the digestate were total solids (TS) 6.44% w/w; volatile solids (VS): 3.74% w/w; chemical oxygen demand (COD): 1.16 g O_2/g TS; and organic carbon: 5.5 g/L.

The pristine soil (0–20 cm) was taken from an uncultured land (48% clay, 24% silt, 28% sand) located in Montelibretti (Rome, Italy). The organic carbon (OC) and total nitrogen (N), measured by a CHNS analyzer (Carlo Erba NA 1500 series 2 C/H/N/O/S, Milan, Italy), were 1.47% and 0.16%, respectively. The soil reaction was slightly alkaline (pH in H_2O: 7.6) and the electrical conductivity was 0.10 dS/m.

Prior to setting up the microcosms, the soil was air-dried in order to better sieve it (2-mm), and to uniformly add digestate and SMX solution. The digestate/soil ratio was 3% (dry mass) in line with agricultural practices (Christian et al. 2003). SMX was not detected in the pristine soil.

Soil microcosms were performed in accordance with previous works (Barra Caracciolo et al. 2015; Rolando et al. 2021). A total of nine microcosms (three replicates for each condition) were set up. Six microcosms were filled with the digestate-amended soil (600 g) and three of them were spiked with a antibiotic solution (final concentration: 20 mg/kg), using a SMX standard (purity 99%, Sigma-Aldrich) dissolved in ultrapure water (18 MΩ/cm quality). After SMX solution addition, the soil was homogenated using a sterilized glass rod to distribute homogeneously the antibiotic using ultrapure water. The water added to soil was appropriate for reaching 60% of the maximum soil water holding capacity (30% soil moisture), which maintains optimal conditions for activity of aerobic soil microorganisms (Atlas and Bartha 1997). Finally, three microcosms (600 g of pristine soil with no SMX addition) were used as microbiological controls (TQ). In the latter, only ultrapure water was added to reach the same soil moisture.

Each test condition was named as follows:

- D-Soil: soil amended with cattle manure digestate (three replicates).
- D-Soil + SMX: soil amended with cattle manure digestate and spiked with SMX (three replicates).
- TQ: pristine soil with no SMX (three replicates).

The experimental set was kept in an incubation chamber under dark conditions and 20 °C for 2 months. Each microcosm was closed with a sterile cotton plug enveloped in a gauze. Soil moisture was maintained constant (replacing possible daily water loss). At days 1, 7, and 61, aliquots of soil were sampled from each microcosm; in each replicate, SMX concentration and microbial analyses were performed. Each datum was the average of chemical or microbiological analyses obtained from each replicate-microcosm. Finally, digestate alone was also analyzed.

2.2 Chemical analyses: SMX determination

SMX was extracted from the pristine soil (TQ), D-Soil, and D-Soil + SMX samples (about 1 g for each replicate condition) by Pressurized Liquid Extraction (PLE, Thermo Scientific Dionex ASE™ 150), as reported in Rauseo et al. (2019). The operating PLE conditions were a mixture of methanol (VWR, Radnor, PA, USA) and ultrapure water (50:50, v/v) as extraction solvent, pressure: 1500 psi; temperature: 100 °C; static time: 5 min; number of cycles: 3; flush volume: 120%; purge time: 60 s. The final extract was successively purified by Solid Phase Extraction (SPE) as reported in Göbel et al. (2005). The analytical SMX concentration in the purified extracts was obtained using high-performance liquid chromatography (HPLC, Micro Pump Series 200, Perkin Elmer, USA) coupled to a UV detector (UV/Visible Spectrophotometer Detector, mod. LC95, Perkin Elmer, USA) following the method described in Rauseo et al. (2019). The recovery of SMX from soil was in the range of 90–95%. The limit of detection (LOD), calculated in accordance with the IUPAC method (Thompson et al. 2002), was 0.4 µg/kg and the limit of quantification (LOQ) was set at three times of the LOD.

2.3 Microbial live cell abundances

Microbial live cell abundance evaluation was performed in order to detect if SMX presence had an effect on the overall microbial community. Total microbial abundance and cell viability were analyzed using direct epifluorescence microscope methods (without any DNA extraction from soil). The total microbial abundance (N. cells/g soil) was

assessed performing total direct counts using the DAPI dye (4′,6-Diamidino-2-phenylindole dihydrochloride) as the DNA fluorescent intercalant. The DAPI method can detect all microbial cells in a sample regardless of their physiological status and metabolic activity. Formaldehyde-fixed soil samples (1 g for each replicate condition) were processed as reported in detail in Barra Caracciolo et al. (2005, 2015).

The cell viability (% live cells/live + dead) was evaluated in fresh soil samples (1 g each experimental condition replicates). The two dyes used for measuring the ratio of live to dead cells were propidium iodide and SYBR Green II (Sigma-Aldrich, Germany) respectively, as described in detail in previous works (Grenni et al. 2012).

The microbial live cell abundance (N. live cells/g) was obtained by multiplying each total microbial abundance with DAPI count by the corresponding cell viability datum, as reported in other works (Amalfitano et al. 2008).

2.4 Microbial community composition: DNA extraction and sequencing of 16S rDNA and 18S rDNA amplicons

The prokaryotic and fungal community was analyzed from microcosms (three replicates) at each sampling time and experimental condition (including TQ and digestate samples).

The total DNA was extracted from 0.25 g of soil (for each replicate) using the DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA), following the manufacturer's recommendations. A DNA-free sample was also analyzed as the negative control during the whole workflow. The extraction yield and quality of the DNA were assessed using spectrophotometric measurements (Multiskan Sky Microplate Spectrophotometer, Thermo Fisher Scientific, USA). DNA extractions were stored at −20 °C until sequencing.

The DNA extracted was used as the template for sequencing the hypervariable V3-V4 region of 16S rRNA (for *Prokaryotes*) and for the region ITS1 and ITS2 (for fungi) with the MiSeq Illumina. The 341F and 805R primers for *Prokaryotes* and the ITS3tagmix and ITS4ngs primer for fungi were used and are reported in the Supplementary Material, Table S1. The raw sequences were imported and demultiplexed using QIIME2 next-generation microbiome bioinformatics platform v2019.11 (Bolyen et al. 2019) and denoised with the DADA2 plug-in described by Callahan et al. (2016). The primers were removed using the "trim-left-f" (forward) and "trim-left-r" (reverse) primer DADA2 functions (Mazzurco Miritana et al. 2020). These functions remove the sequences from the beginning of a sequence to a specific position. The exact length of the primers was 17 nucleotides for the forward and 21 nucleotides for reverse for the prokaryotic primers and 28 nucleotides for the forward and 21 for reverse for the fungal primers. The amplicon sequencing variants (ASV) obtained were sorted using the Silva 132 database (https://www.arb-silva.de) for *Prokaryotes* and UNITE database (version 8.2) for fungi (Abarenkov et al. 2020) with a naive Bayes classifier trained on the amplified regions with 80% confidence (Bokulich et al. 2018).

2.5 Predictive soil functional analysis of *Prokaryotes*

The PICRUSt2 software tool (https://github.com/picrust/picrust2) made it possible to predict several genes in the functional profiling of the prokaryotic community, using the 16S rRNA gene amplicon datasets (Douglas et al. 2020). In particular, the ASV table of prokaryotic cells generated by DADA2 was the input. The prediction of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KO, https://www.genome.jp/kegg) and Enzyme Commission number relative abundances was performed with hidden-state prediction (Louca and Doebeli 2018) and used to infer pathway abundances (Ye and Doak 2009). Several genes were predicted. In particular, those related to nitrogen cycling (nitrogen fixation, complete nitrification, Supplementary Material, Table S2) and those associated with reduction of oxidative stress (ROS detoxification), such as superoxide dismutases, superoxide reductase, thioredoxin-disulfide reductase, and peroxidases, were also evaluated (Supplementary Material, Tables S3 and S4). The data are reported as EC (Enzyme Commission number) relative abundances (%).

Moreover, the folic acid superpathway based on the MetaCyc pathway database, comprising the dihidroperoidate synthase enzyme, which is the SMX target inside a cell (Sköld 2000), was also verified by the pathway-level inference. The MetaCyc pathway abundances were estimated by regrouping the EC and identified with MinPath of PICRUSt2 (Franzosa et al. 2018; Ye and Doak 2009).

2.6 Statistical analyses

The diversity of both the prokaryotic and fungal community was analyzed using the Evenness and Shannon diversity indices, while the Chao 1 index (Chao et al. 2004) was used as an estimator of potential richness. All the statistical analyses were performed using R (4.0.4 version https://www.r-project.org). Pairwise PERMANOVA based on the Bray–Curtis dissimilarity matrix (Anderson and Willis 2003) was performed using the function *pairwise.perm.manova* from the package *RVAideMemoire* (Hervé 2021) in order to evaluate the significance of ASV changes in the prokaryotic and fungal community composition in the different experimental conditions. The Kruskal–Wallis test (a non-parametric one-way ANOVA using the *kruskal.test* function) together with the *pairwise.wilcox.test* function, as

the post-hoc test (Benavoli et al. 2016), was performed to find any significant differences within the Alpha-diversity indices (Chao1, Shannon, and Evenness), microbial live cell abundance, and prokaryotic (genera and classes) and fungal (phyla and genera) groups, and within predicted functional genes (EC numbers) in the different experimental conditions.

Relative abundances of the prokaryotic and fungal genera most detected were displayed in heatmaps generated by the *pheatmap* function using the *pheatmap* package from R program. In the heatmaps, bacterial or fungal genera and experimental conditions were grouped in accordance with a hierarchical clustering dendrograms, which are shown at the top and on the left side of the heatmaps. Finally, all histograms and stacked bar plots were made with MS Excel 2013.

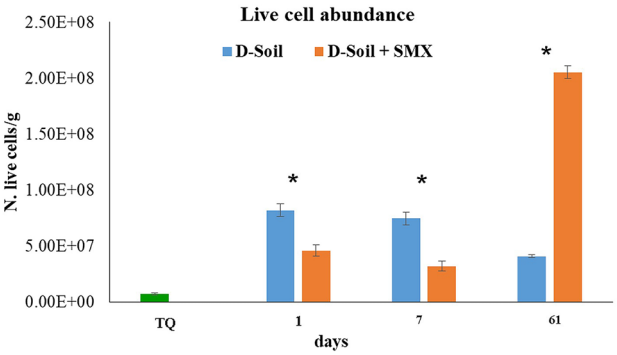


Fig. 1 Microbial live cell abundance (N. live cells/g soil) in the non-treated soil (TQ) and in the soil amended with digestate (D-soil) and with both digestate and the antibiotic sulfamethoxazole (D-soil + SMX). Data are means of 3 independent replicates. The vertical bars represent the standard errors. * refers to significant differences ($p < 0.05$ by Kruskal–Wallis)

3 Results

3.1 Soil chemical analysis

Adding digestate, both organic carbon (OC) and total nitrogen (N) significantly increased from 1.47 to 2.63% and from 0.16 to 0.2%, respectively ($p < 0.01$). Digestate did not have any influence on soil reaction (pH) and electrical conductivity.

SMX in the digestate was negligible (below the limit of detection). The antibiotic spike into the soil decreased rapidly; in fact, its initial concentration (20 mg/kg dry soil) halved after only 7 days, and at the end of the experiment (day 61) only 5% (1 mg/kg) was found.

3.2 Microbial live cell abundances

The microbial live cell abundance (N. live cells/g) in the digestate was $4.4 \times 10^8 \pm 9.7 \times 10^5$. The microbial numbers were significantly ($p < 0.01$) higher at day 1 in both digestate-amended soils (D-Soil and D-Soil + SMX), if compared to TQ (Fig. 1). However, the microbial growth stimulation of digestate at days 1 and 7 was significantly lower ($p < 0.01$) with the antibiotic presence. On the other hand, at day 61, the live cell abundance increased ($p < 0.01$), with a peak in D-Soil + SMX.

3.3 Microbial community diversity and composition

Rarefaction curves (Supplementary Material, Fig. S1) were plotted to compare the number of DNA reads with the identified ASV. These graphs show that the sequencing was able to capture the entire diversity found in both the prokaryotic and fungal community in the different conditions; in fact, a clear asymptote was reached (Gotelli and Colwell 2001).

The Alpha-diversity indices (Chao1, Shannon, and Evenness) for the prokaryotic community are reported in Table 1. All these indices for digestate were much lower ($p < 0.01$) than for soil samples (TQ). As expected, the Chao1 diversity value was significantly higher ($p < 0.05$) in the digestate-treated soil (D-Soil) if compared to the untreated soil (TQ), but this difference was not found in the presence of both digestate and SMX (D-Soil + SMX). The Shannon (H) and Evenness (E) indices were significantly lower ($p < 0.05$) in both digestate-amended soils (D-Soil and D-Soil + SMX) than in TQ, showing an initial effect on some microbial groups of adding digestate and this result was particularly evident in the antibiotic presence. At day 7, a further decrease in both the H and E indices was observed only in D-Soil. At the end of the experiment (day 61), the H and E indexes increased in both D-Soil and D-Soil + SMX and their values were similar in both conditions.

Regarding microbial community composition, *Proteobacteria* (36.4%) and *Bacteroidetes* (33.5%) phyla dominated the digestate microbial community (Supplementary Material, Fig. S2). *Deinococcoccus* (5.9%) together with *Tenericutes* (2.6%), *Firmicutes* (2.4%), *Gemmatimonadetes* (1.5%), and *Verrucomicrobia* (0.5%) was also found. The most abundant genera were *Pseudomonas* (*Gammaproteobacteria*), *Truepera* (*Deinococcoccus*), and *Proteiniphilum* (*Bacteroidetes*), which are bacteria commonly found in mesophilic digestate from dairy manure. Moreover, *Methanosarcina* (*Archaea*), involved in methanogenesis (Fernandez-Bayo et al. 2020), was also found.

The dominant phyla of the TQ soil (Supplementary Material, Fig. S2) were *Actinobacteria* (41.8%) and *Proteobacteria* (36.4%). *Acidobacteria* (7.6%), *Chloroflexi* (7%), and *Planctomycetes* (2.9%) were also detected.

Although no genera detected in the digestate were found in the TQ soil (except for the ubiquitous *Alphaproteobacteria Devosia*), the addition of digestate alone and together with

Table 1 Alpha-diversity indexes for the prokaryotic community calculated based on ASV (Amplicon Sequences Variant) expressed as means of three independent replicates ± standard errors (*$p < 0.05$, Kruskal–Wallis)

Prokaryota		Reads filtered	Chao1	Shannon (H)	Evenness (E)
Digestate		279,180	299 ± 52	8.92 ± 0.00	0.92 ± 0.001
Day 1	**TQ**	217,586	1786 ± 430	10.62 ± 0.00*	0.97 ± 0.001*
	D-Soil	329,398	1894 ± 210*	9.73 ± 0.70*	0.96 ± 0.003
	D-Soil + SMX	89,080	1732 ± 240	8.41 ± 0.36	0.94 ± 0.000
Day 7	**D-Soil**	116,479	1709 ± 112	6.83 ± 0.38	0.92 ± 0.003
	D Soil + SMX	102,268	1683 ± 139	8.58 ± 0.37	0.95 ± 0.000
Day 61	**D-Soil**	233,367	1893 ± 200	9.77 ± 0.22	0.96 ± 0.003
	D-Soil + SMX	278,845	1763 ± 137	9.89 ± 0.48	0.96 ± 0.002

SMX affected the soil microbial community differently at day 1 (Fig. 2). A significant ($p < 0.05$) decrease in *Acidobacteria* (D-Soil: 3.9% and D-Soil + SMX: 0.8%) and *Actinobacteria* (D-Soil: 24.7% and D-Soil + SMX: 19%) and an increase in *Firmicutes* (D-Soil: 26.3% and D-Soil + SMX: 41.4%) were observed. Moreover, in D-Soil + SMX, the lowest percentages ($p < 0.05$) of *Acidobacteria* (0.8%), *Proteobacteria*, and in particular *Alphaproteobacteria*

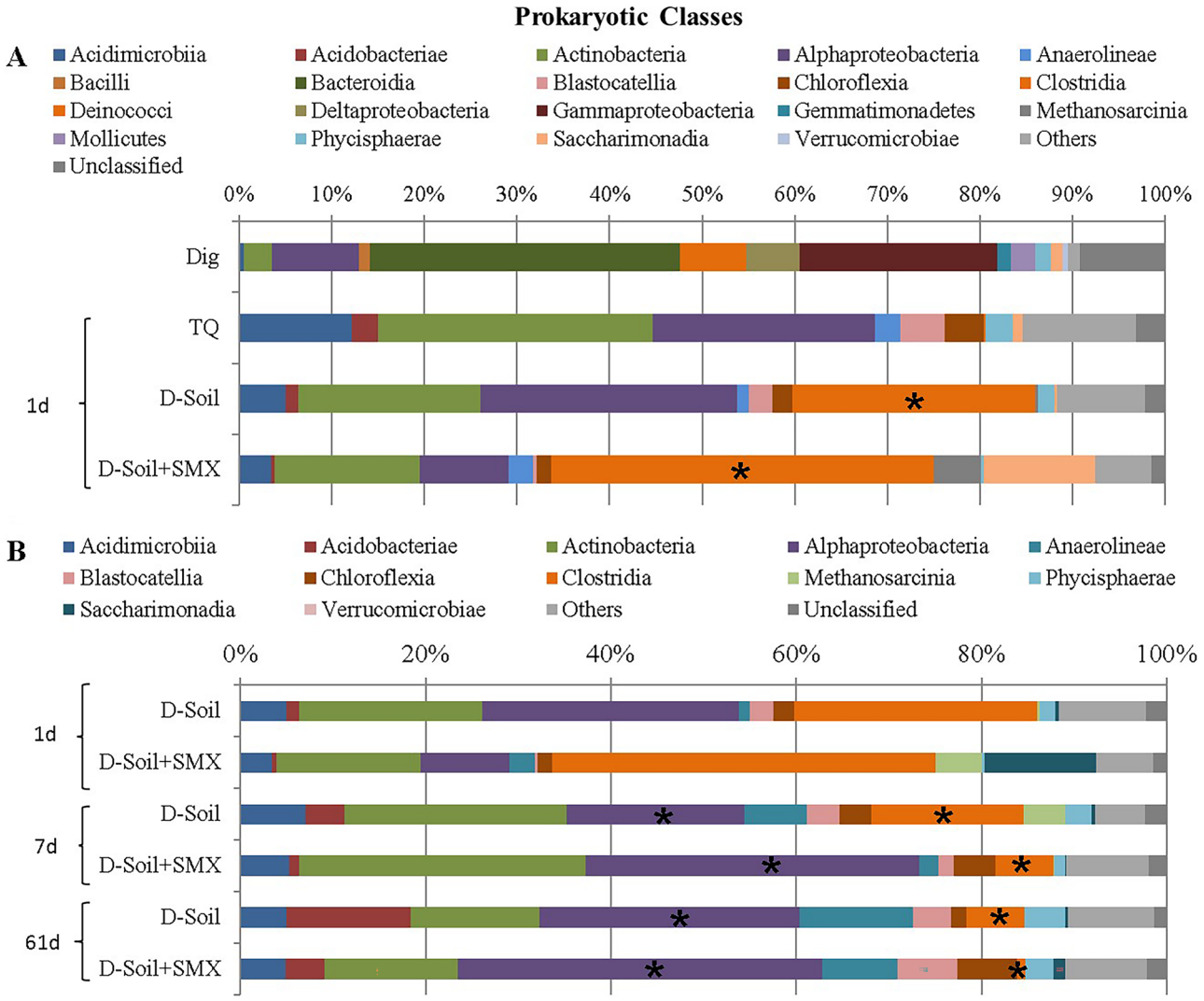


Fig. 2 Prokaryotic relative abundances (%) at class level with an average presence > 1% in each experimental condition and experimental time, **A** Day 1, **B** Days 1, 7, and 61. Data are means of 3 independent replicates

and *Planctomycetes* were found; at the same time, other microbial groups such as *Saccharimonadia* (*Candidatus Saccharibacteria*), *Euryarchaeota*, and *Clostridia* (*Firmicutes*) were detected in higher percentages ($p < 0.05$) with the antibiotic presence than in D-Soil (Fig. 2A).

Although *Alphaproteobacteria* were initially negatively affected where the antibiotic was present (D-Soil + SMX), a subsequent significant increase ($p < 0.05$) in their percentages was observed at days 7 and 61. Moreover, *Saccharimonadia* and *Clostridia* decreased and were significantly lower in D-Soil + SMX than in D-Soil (Fig. 2B).

Figure 3 reports a heatmap of the most abundant prokaryotic genera (top 40 genera, net of "unclassified" one) detected in the overall samples, which covers 49–64% of total identified sequences (ASV). *Sphingomonas* (*Alphaproteobacteria*) was the most abundant genus found in all soil samples; at day 1, this genus was significantly higher ($p < 0.05$) in D-Soil (10%) than TQ soil (3.9%). Adding the antibiotic initially affected *Sphingomonas* (3.9% in D-Soil + SMX1 day); however, at day 7 (corresponding to the SMX DT_{50}), this percentage increased (9%) and, at the end of the experiment, was comparable (5%) in both digestate-amended soils (D-Soil and D-Soil + SMX at day 61). Initial detrimental effects of adding the antibiotic on prokaryotic diversity were also significant ($p < 0.01$) for several *Alphaproteobacteria* genera such as *Bradyrhizobium* (TQ: 1.1%; D-Soil + SMX: 0.2%), *Microvirga* (TQ: 1.15; D-Soil + SMX: 0.8%), and *Dongia* (TQ: 1.1%; D-Soil + SMX: 0.1%), which declined 80%, 27%, and 91%, respectively. In addition, two *Acidobacteria* genera (*Bryobacter* and *Blastocatella*) initially diminished by 86% if compared to TQ. However, all the above genera were found in higher percentages at day 61 (between 1.5 and 2.7%). Other four bacterial genera (KD4_96, FFCH7168, Gitt_GS_136, and TK10, belonging to the *Chloroflexi*) were found to be in D-Soil + SMX 61–94% significantly ($p < 0.01$) lower than TQ. Finally, several *Actinobacteria* genera (IMCC26256, *Aeromicrobium*, *Streptomyces*, *Mycobacterium*, *Iamia*, and *Micromonospora*) were also detected in D-Soil + SMX significantly ($p < 0.01$) lower than TQ.

On the contrary, the two genera *Lachnospiraceae* NK4A136 group (*Firmicutes*, *Clostridium*) and *Saccharimonadales* (*Saccharimonadia*) were initially stimulated by the antibiotic presence and they were the most abundant ones (16 and 12%, respectively) (see D-Soil + SMX 1d in Fig. 3).

However, in soil merely amended with digestate (D-soil 1, day), few genera reductions were observed and several genera, such as *Sphingomonas* (*Alphaproteobacteria*), *Bradyrhizobium* (*Alphaproteobacteria*), and *Microvirga* (*Rhizobiales*), were significantly ($p < 0.05$) higher than those found in TQ. *Lachnospiraceae* NK4A136 bacteria were also found in digestate (D-soil 1, day), but at lower percentages than in D-Soil + SMX and were not present in TQ, suggesting their digestate origin.

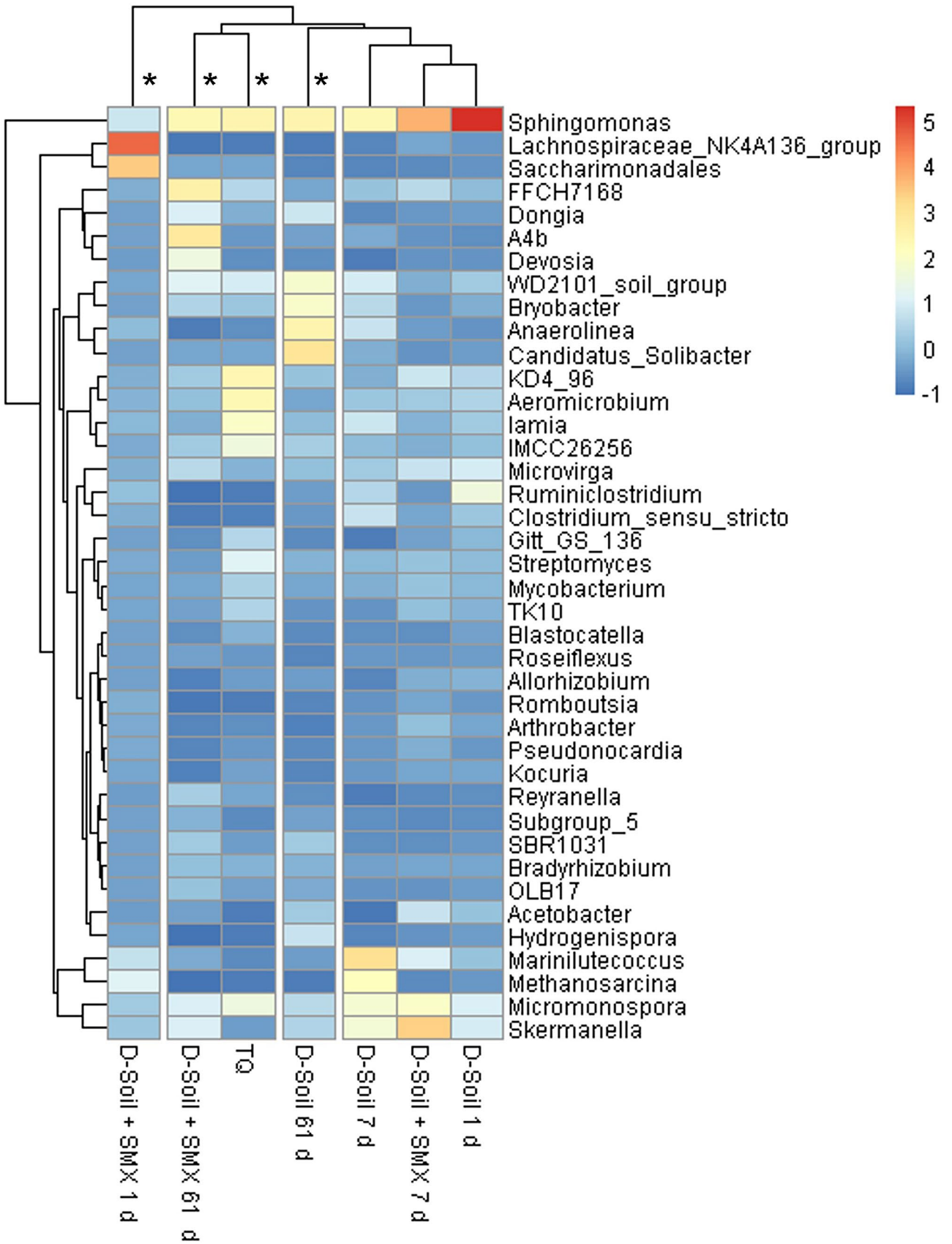
3.4 Predictive soil functional analysis of Prokaryotes

Several functional genes were identified in the prokaryotic community using PICRUSt2. Although it has some limitations (Douglas et al. 2020) and could underestimate functions if compared to shotgun functional profile (Toole et al. 2021), it is one of the main tool used for this purpose and displays the highest precision, compared to other prediction methods (Douglas et al. 2020). The relative abundances of the predicted functional genes related to nitrogen cycle in the pristine soil (TQ) and in the digestate-amended soil (D-Soil and D-Soil + SMX) are shown in Fig. 4A. The three genes related to the nitrogen fixation (all corresponding to the 1.18.6.1 EC number), that is *nifDKH* for Nitrogenase I (Fe-Mo), Nitrogenase II (Fe-Mo), and Nitrogenase (Fe), are reported as a sum, in accordance with Sickerman and Hu (2019). As it is possible to see in Fig. 4A (blue columns), digestate presence had a positive effect ($p < 0.05$) on bacterial species associated with nitrogen fixation at all sampling times (days 1, 7, and 61). However, the antibiotic partially lowered ($p < 0.05$) this positive effect. In fact, the EC number relative abundance (i.e., the value of the EC number analyzed divided by the sum of all EC numbers in the same condition) was higher in D-Soil than in D-Soil + SMX.

Moreover, the digestate also promoted an increase in prokaryotic cells potentially able to perform a complete nitrification (Fig. 4A, orange columns); this effect was significant ($p < 0.05$) at day 7. In a similar way to the nitrogen fixation, SMX partially lowered the positive digestate effect.

Among the predicted functional genes associated with oxidative stress (Fig. 4B), significant differences were found only for superoxide reductase (SOR) and thioredoxin-disulfide reductase (TrxR). In particular, in accordance with the presence of a high SMX concentration at the start of the experiment, SOR (red columns) and TrxR (purple columns) were significantly higher in D-Soil + SMX than D-Soil ($p < 0.05$) at day 1. They subsequently (days 7 and 61) decreased in both amended conditions.

Figure 4C reports the relative abundances of the predicted folic acid pathway of prokaryotic cells (superpathway of tetrahydrofolate biosynthesis). An initial increase in this pathway was observed in the digestate-amended conditions, with significantly ($p < 0.05$) higher percentages with the antibiotic presence. At day 61, no significant differences were found between presence and absence of SMX.


*
*
*
*
Sphingomonas
Lachnospiraceae_NK4A136_group
Saccharimonadales
FFCH7168
Dongia
A4b
Devosia
WD2101_soil_group
Bryobacter
Anaerolinea
Candidatus_Solibacter
KD4_96
Aeromicrobium
Iamia
IMCC26256
Microvirga
Ruminiclostridium
Clostridium_sensu_stricto
Gitt_GS_136
Streptomyces
Mycobacterium
TK10
Blastocatella
Roseiflexus
Allorhizobium
Romboutsia
Arthrobacter
Pseudonocardia
Kocuria
Reyranella
Subgroup_5
SBR1031
Bradyrhizobium
OLB17
Acetobacter
Hydrogenispora
Marinilutecoccus
Methanosarcina
Micromonospora
Skermanella
5
4
3
2
1
0
-1
D-Soil + SMX 1 d
D-Soil + SMX 61 d
TQ
D-Soil 61 d
D-Soil 7 d
D-Soil + SMX 7 d
D-Soil 1 d

◂**Fig. 3** Heatmap for prokaryotic relative abundances at genus level in the different conditions (D-Soil + SMX and D-Soil) and sampling times (days 1, 7, and 61). Genera and conditions were grouped in accordance with a hierarchical clustering dendrogram, at the top and the left side of the heatmap. Data are means of 3 independent replicates. * refers to significant differences ($p < 0.05$) by PERMANOVA, based on Bray–Curtis distance

3.5 Fungal DNA sequencing results

The deep DNA sequencing generated 3,538,000 reds and after trim and quality edit, 858,000 reads were retained. The Alpha-diversity indices (Chao1, Shannon and Evenness) for the fungal community are reported in Table 2. The Chao1, Shannon, and Evenness indices of the digestate were significantly lower ($p < 0.01$) than those for all soil samples. At the start of the experiment, adding digestate and, to a lesser extent, digestate plus SMX significantly increased the Chao1 index, but these differences were not found at the end (day 61) of the experiment. At day 7, Chao1 in soil with digestate and antibiotic was the least value found among the amended soils. Regarding H and E, these indexes were similar at the start and at day 7 of the experiment, but at day 61, their values in D-Soil + SMX were significantly higher than D-Soil.

The two main phyla found in all samples (including the digestate) were *Ascomycota* and *Basidiomycota* (Fig. 5). The *Ascomycota* group was the most abundant in the TQ condition (72%) and at days 1 and 7 in both amended and SMX-treated conditions (day 1: 46% in D-Soil and 52% in D-Soil + SMX; day 7: 61% in D-Soil and 51% in D-Soil + SMX). However, at day 61, *Basidiomycota* became the dominant phylum in the amended conditions (D-Soil: 56%; D-Soil + SMX: 41%) and *Ascomycota* decreased (D-Soil: 34%; D-Soil + SMX: 38%).

Based on the current available taxonomic reference database, the thirteen fungal genera identified are reported in a heatmap (Fig. 6). They covered on average 56% of the total fungal sequences identified in all conditions. SMX promoted an initial increase in the *Ascomycetes Urnula*, a genus known as producer of bioactive compounds that can inhibit the growth of other fungi (Poveda 2021). Regarding the other genera, *Trichoderma* (*Ascomycetes*) was initially (days 1 and 7) the most detected genus in all conditions and it was the most abundant genera (14.7%) at day 1 in the antibiotic presence (D-Soil + SMX). The fungal genus *Aspergillus* (*Ascomycota*) was also present (average values of 8.9 ± 1.1%) in both D-Soil + SMX and D-Soil at days 1 and 7. However, at day 61, *Aspergillus* decreased significantly in both conditions, while other *Basidiomycetes* genera, such as *Tomentella* (D-Soil: 26%; D-Soil + SMX: 28%) and *Lacrymaria* (D-Soil: 24%; D-Soil + SMX: 26%), increased (Fig. 6).

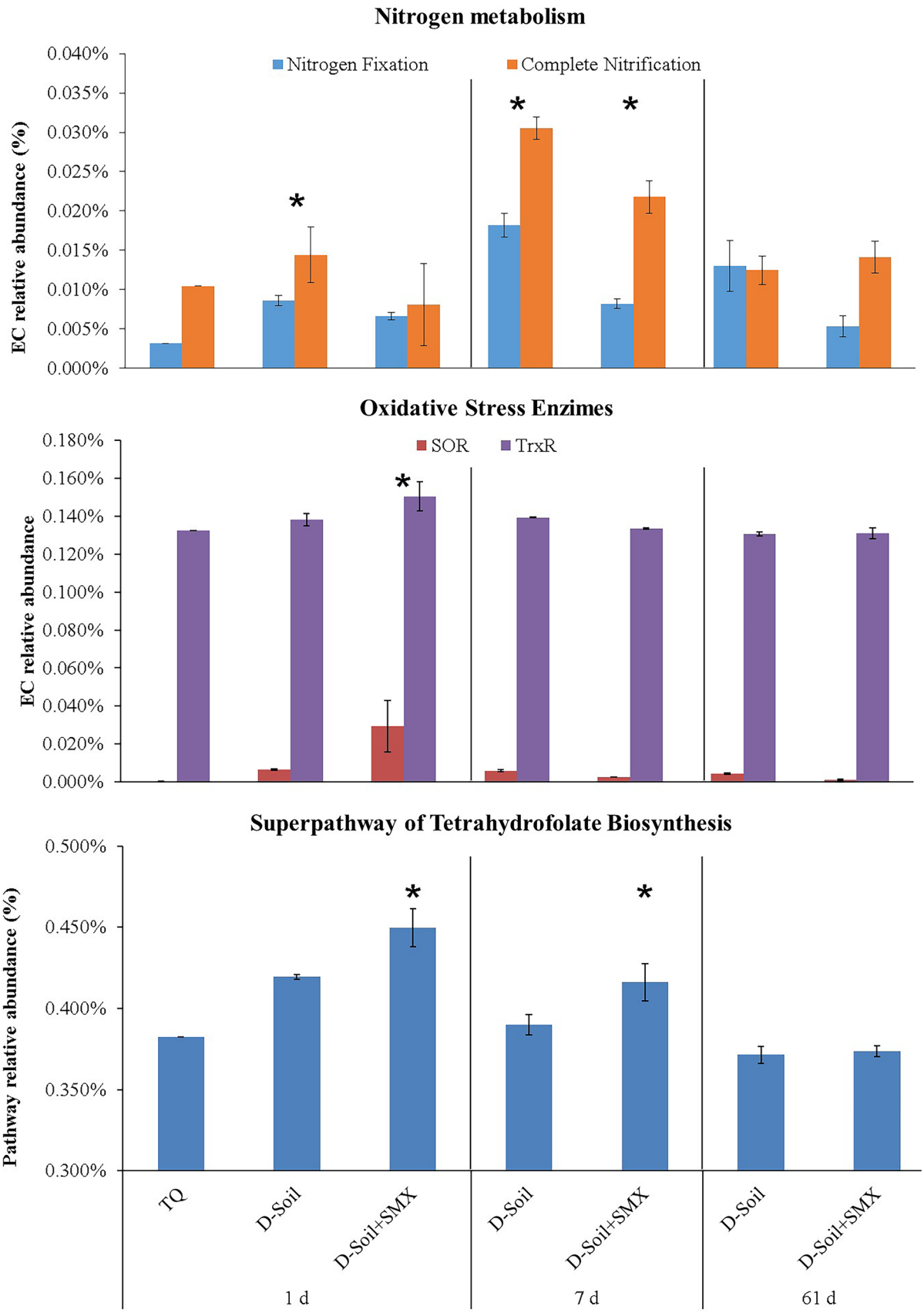
4 Discussion

Adding digestate to soil had a prompt positive effect on the overall microbial live cell abundance. This result was in line with the fact that digestate is a microbial and organic carbon source, as also found in previous works where organic amendments were added to soil (Barra Caracciolo et al. 2015; Di Lenola et al. 2018). Although the microbial abundance increased, this effect was initially lower with the antibiotic presence due to its biocide effect. Interestingly, both the digestate and antibiotic caused a soil microbial community shift and an initial decrease in the bacterial diversity (H and E indices) and an increase in fungal diversity (Chao1) was observed. This phenomenon was presumably due to the mixing of the microbial populations of the digestate with those of the soil, as found in other works (Barra Caracciolo et al. 2015) and on the bactericidal effect of the antibiotic, which favored fungal species. This antibiotic effect was also found by other authors in a soil treated with both sulphonamides and manure (Gutiérrez et al. 2010), but still not reported in the literature for both sulphonamide and digestate addition to soil (Congilosi and Aga 2021).

The acute effect (day 1) of the antibiotic was evident with a decrease in some prokaryotic sensitive genera, such as *Bradyrhizobium*, *Microvirga*, *Dongia*, *Bryobacter*, *Blastocatella*, and *Chloroflexi*, and an increase in others (e.g., *Lachnospiraceae* NK4A136 and *Saccharimonadales*) able to resist sulphonamides, as also found by other authors (Sabino et al. 2019; Zhang et al. 2017).

However, the initial detrimental effect of the antibiotic was mitigated in the long term, and at day 61 the microbial abundance and diversity of both prokaryotic cells and fungi were higher in the antibiotic-treated soil (D-Soil + SMX) than in D-Soil. This can be due to SMX decrease (higher than 95%) as found in other works (Müller et al. 2013; Shen et al. 2018). The increase in microbial abundance at the end of the experiment was also probably due to an initial (1 d) SMX selective effect on microbial community. The antibiotic presence probably promoted the growth of resistant/degradative microbial cells, as already found by other authors (Cycoń et al. 2019). In accordance with a reduction in SMX, *Alphaproteobacteria* increased at days 7 and 61. The latter class includes several bacterial genera capable of degrading sulphonamides (Cheng et al. 2020; Reis et al. 2020), such as *Sphingomonas* and *Skermanella*, which increased in D-Soil + SMX. The presence in the soil of bacteria able to degrade SMX was presumably due to adding the digestate, as this antibiotic is frequently present in this organic amendment (Visca et al. 2022).

The predictive soil functional analysis made it possible to highlight the potential effects of adding digestate and antibiotic on the prokaryotic community. In accordance with


Nitrogen metabolism
Nitrogen Fixation
Complete Nitrification
EC relative abundance (%)
0.040%
0.035%
0.030%
0.025%
0.020%
0.015%
0.010%
0.005%
0.000%
*
*
*
Oxidative Stress Enzimes
SOR
TrxR
EC relative abundance
0.180%
0.160%
0.140%
0.120%
0.100%
0.080%
0.060%
0.040%
0.020%
0.000%
*
Superpathway of Tetrahydrofolate Biosynthesis
Pathway relative abundance (%)
0.500%
0.450%
0.400%
0.350%
0.300%
*
*
TQ
D-Soil
D-Soil+SMX
D-Soil
D-Soil+SMX
D-Soil
D-Soil+SMX
1 d
7 d
61 d

◂**Fig. 4** Relative abundances of predicted functional genes and folic pathway of prokaryota. **A** Functional genes involved in nitrogen fixation (blue columns) and complete nitrification (orange columns). **B**. Functional genes involved in oxidative stress. SOR: superoxide reductase (red columns); TrxR: thioredoxin-disulfide reductase (purple columns). **C** Relative abundances of predicted folic acid pathway (superpathway of tetrahydrofolate biosynthesis) of prokaryota. Data are means of 3 independent replicates. * refers to significant differences ($p < 0.05$) by Kruskal–Wallis

an increase in nitrogen content in the amended soil, microbial groups involved in nitrogen metabolism such as fixing atmospheric nitrogen and nitrifying bacteria increased at day 7, as found by other authors (Möller 2015; Safronova et al. 2017). On the other hand, in line with the antibiotic effect, this increase was less evident in D-Soil + SMX than in D-Soil. An inhibition of natural microbial community nitrification due to SMX has been reported in other works (Chen et al. 2021; Grenni et al. 2018).

Reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl ($^{\bullet}OH$) radicals are reactive natural by-products of oxygen during aerobic metabolism. A ROS excess can cause oxidative intracellular damage to DNA, proteins, lipids, and other cellular components (Johnson and Hug 2019) and ultimately cell death. ROS have also been found as a response to lethal stress, such as metal and antibiotic exposure (Schütze and Kothe 2012; Mourenza et al. 2020). However, prokaryotic cells can have detoxification strategies against ROS, such as the protective protein (superoxide dismutases, superoxide reductase, thioredoxin-disulfide reductase, and peroxidases),

Table 2 Alpha-diversity indices for the fungal community calculated on the basis of ASV expressed as means of 3 independent replicates ± standard errors. * refers to significant differences ($p < 0.05$) by Kruskal–Wallis

Fungi		Reads filtered	Chao1	Shannon index (H)	Evenness (E)
Digestate		67,659	50 ± 0	1.32 ± 0.01	0.23 ± 0.01
Day 1	**TQ**	49,216	586 ± 0	6.76 ± 0.01*	0.74 ± 0.01*
	D-Soil	61,458	634 ± 3*	6.32 ± 0.21	0.68 ± 0.02
	D-Soil + SMX	72,991	839 ± 5*	6.52 ± 0.02	0.71 ± 0.01
Day 7	**D-Soil**	67,608	664 ± 63	6.52 ± 0.04	0.72 ± 0.01
	D-Soil + SMX	54,227	437 ± 3*	6.38 ± 0.40	0.73 ± 0.05
Day 61	**D-Soil**	72,066	537 ± 26	4.81 ± 0.21*	0.54 ± 0.01*
	D-Soil + SMX	73,230	548 ± 12	5.84 ± 0.19*	0.65 ± 0.01*

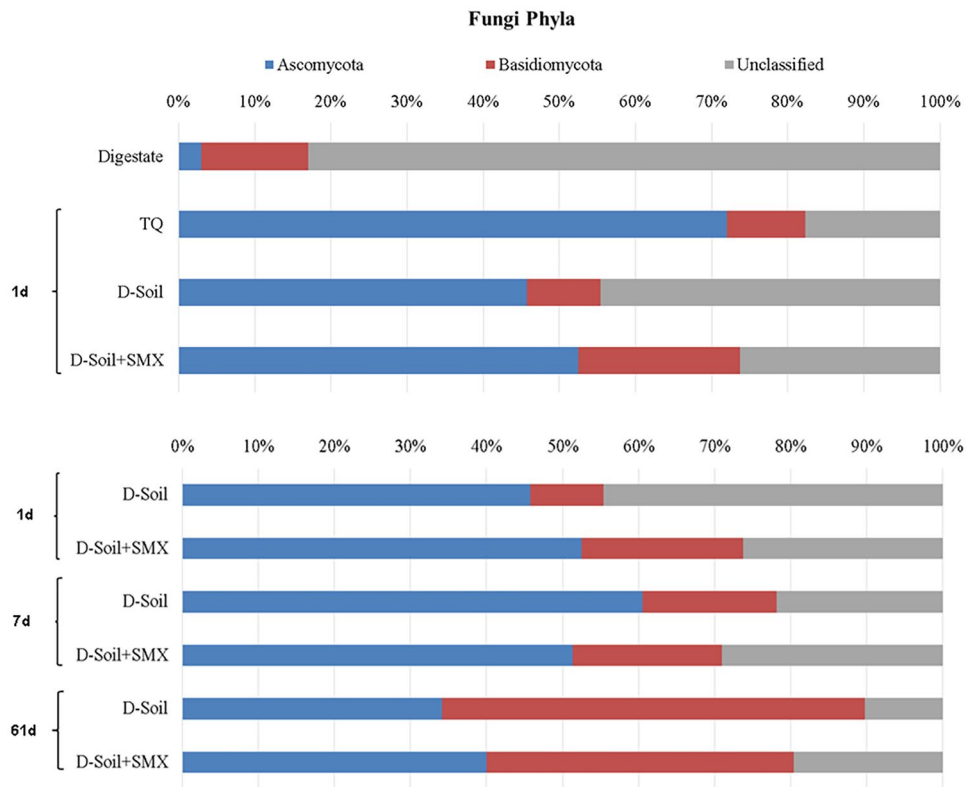


Fig. 5 Relative abundances (%) of fungal phyla in the different conditions (D-Soil + SMX and D-Soil) and sampling times (days 1, 7, and 61). Data are means of 3 independent replicates. * refers to significant differences ($p < 0.05$) by Kruskal–Wallis

and they can be considered a mechanism for resisting antibiotics (Schütze and Kothe 2012). As mentioned, a significantly higher percentage of superoxide reductase (SOR) was found in D-Soil + SMX at day 1. However, in line with SMX decrease from day 7, the predicted SOR gene involved in a protective role against oxidative stress decreased sharply.

In a similar way, the predicted TrxR, which together with Trx is a oxidoreductase system with antioxidant and redox regulatory roles (Zeller and Klug 2006), was at its highest percentage at day 1 in D-Soil + SMX and decreased significantly from day 7, in accordance with the antibiotic decrease.

Finally, the predicted folic acid pathway was also influenced by the antibiotic being present in accordance with the SMX antibiotic target inside bacterial cells. All prokaryotic cells require reduced folate cofactors for biosynthesis of a diverse range of cellular components. Tetrahydrofolate serves as a donor of one-carbon units in a variety of biosynthetic processes, including the formation of methionine, purines, and thymine. In most microorganisms, folates must be synthesized through the folic acid pathway. The presence of this pathway in many pathogenic *Bacteria* and its absence in mammals have made the folic acid pathway an attractive antimicrobial drug target, in particular for sulphonamides, including SMX (Bermingham and Derrick 2002). However, sulfamethoxazole antibiotic-resistant bacteria are able to restore this pathway; in fact, they have alternative genes (*sul*1, *sul*2, *sul*3) encoding variants of dihydropteroate synthase (DHPS) enzymes, which are resistant to the antibiotic effect. Consequently, the initial increase in predicted folic acid pathways (sulfonamide antibiotic target) observed in this work can be ascribed to a prompt selection of resistant bacteria when adding the antibiotic. The latter result is supported by our previous work (Rauseo et al. 2019), in which the same digestate-amended soil and SMX addition showed an immediate increase in the *intI*1 gene. The *intI*1 gene is recognized to be an effective proxy for antibiotic resistance and it is related to *sul*1 (Gillings et al. 2015). Moreover, since in the digestate used in this study SMX was not detected but about 6 mg/kg of one SMX metabolite was found (N4-acetylsulfamethoxazole; Rauseo et al. 2019), the addition of digestate probably added antibiotic-resistant bacteria to soil.

This work has also analyzed the fungal community since they can coexist in soil with prokaryotic cells in both competitive (e.g., nutrient and space accesibility) and synergic interactions. Consequently, since fungi contribute with *Bacteria* and *Archaea* to several regulating ecosystem services, it is crucial to have on overall picture of the possible direct and indirect effects of an antibiotic on overall soil microbial community (Chu et al. 2020). Really, SMX did not affect significantly the structure of the fungal microbial community. However, a possible involvement of some fungal genus such as *Trichoderma* in SMX removal cannot be excluded. In fact, *Trichoderma* was found to be the most abundant genus and its SMX transformation might occur through co-metabolism (Yang et al. 2020), as found in other works where this antibiotic was shown to be removed up to 71% in 7 days (Piyaviriyakul et al. 2021).

Interestingly, when adding digestate, a long-term increase in *Basidiomycota* (day 61) was observed and this might be ascribable to the simultaneous decrease in *Firmicutes* and *Actinobacteria*. In accordance with these results, some authors have reported that *Basidiomycota* are in competition with Gram-positive bacteria (Alves et al. 2012); consequently, they can increase when there is a Gram-positive decrease.

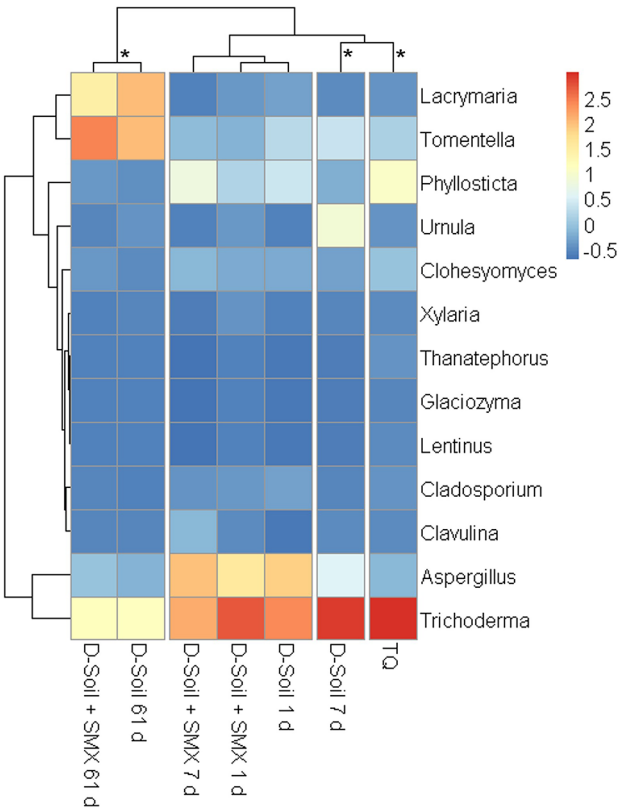


Fig. 6 Heatmap for fungal relative abundances at genus level with an average presence > 1% in different conditions and sampling times. The genera and the conditions were grouped according to a hierarchical clustering dendrogram, which is shown at the top and on the left side of the heatmaps. Data are means of 3 independent replicates. * refers to significant differences ($p < 0.05$) by PERMANOVA, based on Bray–Curtis distance

5 Conclusions

The overall results show how adding digestate, which contained allochthonous prokaryotic cells, fungi, and organic matter, induced a shift in the soil microbial community. Moreover, the overall microbial abundance and bacterial species involved in nitrogen metabolism also increased. These positive effects initially decreased with the antibiotic presence, which negatively affected some microbial groups. However, the detrimental effects of the antibiotic on some prokaryotic cells were transient thanks to the occurrence of resistant bacteria, which were presumably introduced into the soil with the digestate and were able to remove it.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s11368-022-03222-y.

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Author contribution A. Barra Caracciolo, P. Grenni, and L. Patrolecco: research conceptualization and study design; A. Barra Caracciolo: funding acquisition; J. Rauseo, T. Pescatore, A. Barra Caracciolo, P. Grenni, and L. Patrolecco: experimental set-up and chemical and microbiological analyses; G.L. Garbini: data elaboration; P. Grenni and G.L. Garbini: writing of the first draft; A. Barra Caracciolo and P. Grenni: critical manuscript revision. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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