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Bio-composts, soil microbial community, antimicrobial resistance: An *ex-situ* pilot study

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ABSTRACT

The impact of bio-compost on the diversity, composition, and structure of soil microbial communities remains a topic requiring further exploration. Globally, many agricultural soils face degradation, prompting the development of diverse strategies for their restoration. Beneficial soil microorganisms are garnering significant attention due to their crucial role in promoting sustainable agricultural practices, particularly the application of bio-compost. This study presents preliminary findings on the potential effects of a bio-compost on the microbial communities of treated soils, assessing its sustainability in consideration of the possible impact on the spread of antibiotic-resistant bacteria (ARBs) in the view of a One Health approach. Experiments were conducted using an *ex-situ* pilot plant setup, where agricultural soil was treated with two types of bio-compost derived from organic waste. Biomolecular analyses were applied to both untreated and bio-compost-treated soils, enabling an assessment of microbial evolution over time. Early results from a one-year trial revealed that bio-compost treatment fosters bacterial proliferation in the soil, showing consistent growth across the study period. However, species analysis indicated that certain microorganisms introduced to the bio-compost gained dominance, leading to the disappearance of some native species during the experiment. ARBs were searched by cultivation on selective media. Preliminary data indicate the presence of ARBs in the bio-compost, highlighting the importance in performing such analyses when considering an agricultural practice in a One Health view. The data obtained from the experiments on the *ex-situ* plant, suggest that the treatment with the bio-composts does not alter the autochthonous bacteria community in terms of ARBs.

Keywords: One Health; agricultural practice; biobased fertilizer; resistant bacteria; 16SrDNA sequencing.

INTRODUCTION

The intensive and continuous use of chemical fertilizers and pesticides over the past 50 years has had serious repercussions on public health and agricultural productivity worldwide (Ramakrishna et al., 2019). However, these practices have led to many environmental problems, mainly including the decline of soil fertility, reduction of soil biodiversity, increased greenhouse gas emissions, zinc deficiency, accumulation of chemical residues and eutrophication of water bodies (Tilman et al., 2002; Diaz and Rosenberg, 2008). In recent decades, more environmentally friendly approaches, such as the use of bio fertilizers, have gained popularity in the transition to sustainable agroecosystems (Ramakrishna et al., 2019). From an economic perspective, the bio-compost market is growing at a rate of more than 12% per year (Calvo et al., 2014; Owen et al., 2015). The nitrogen-based bio-compost market is expected to grow at a CAGR of 13.25%

by 2020 (Arora, 2018). To date, many strains of PGPB belonging to the genera Acinetobacter, Agrobacterium, Azospirillum, Bacillus, Bradyrhizobium, Burkholderia, Enterobacter, Gluconacetobacter, Pantoea, Pseudomonas, Rhodococcus, and Serratia have been reported to have beneficial effects on plant growth promotion, nutrition, stress reduction, bioremediation, and/or biocontrol of plant pathogen attacks (Sharma et al., 2013; Bargaz et al., 2018).

Within sustainable and holistic agricultural practices, organic farming has emerged as a promising alternative that emphasizes soil fertility restoration and crop quality improvement through nutrient cycling, erosion prevention, as well as sustainable resource use. Organic and conventional farming systems differ in their approaches to soil fertility management and resource use. Organic farming prioritizes the use of natural resources such as compost and manure, avoids the use of synthetic inputs such as fertilizers and pesticides, and diversifies crops to increase resilience, thus promoting soil health and microbial activity (Lori et al., 2017). Although the use of fertilizers from conventional farms is allowed, some regulations must be met (USDA, 2012). In contrast, conventional agriculture relies heavily on the use of synthetic fertilizers and pesticides, with the risk of compromising soil biodiversity and overall ecosystem health (Hartmann et al., 2015).

It is essential to develop the waste management solutions that are economically viable and environmentally friendly, promoting a long-term strategy. In this context, recycling industrial waste and converting it into bio compost could be an environmentally beneficial approach compared to other management systems, contributing to both food security and environmental protection (Villanueva and Wenzel, 2007). Converting industrial by-products into bio compost and using them safely in cropland is an economically viable option, as it reduces the dependence on agro-industrial fertilizers. This method is particularly suitable for promoting regenerative agriculture, improving soil functions and mitigating greenhouse gas emissions in the agricultural and industrial sectors. In addition, it addresses the problem of waste disposal, providing crops with the necessary nutrients and improving ecosystem efficiency (Zaman et al., 2004; Behera et al., 2017; Jamil et al., 2008; Mesman et al., 2007; Kumar et al., 2017; Rangaraj et al., 2007).

In parallel, bio compost production increases the biological energy in soil microbes and promotes beneficial microbial communities, including fungi, bacteria, and actinomycetes, which are key elements in nutrient mineralization processes to achieve higher agricultural productivity. (Jindo et al., 2016; Ros et al., 2006).

The bio fertilizers obtained from organic sources (bio-composts) such as municipal waste, agricultural residues, and manure can be used as a complement to synthetic fertilizers (Chen et al., 2018) as well as promote sustainable agricultural production, sustainability, and soil fertility (Jiang et al., 2020; Rehan et al., 2023).

This approach supports a more sustainable agricultural practice, improving soil fertility and promoting environmentally friendly production (Alburquerque et al., 2012). However, the quality and safety of the bio fertilizers produced, such as digestate derived from anaerobic digestion, must meet stringent standards to limit the risk of contamination by pathogens that could compromise public health (Alfa et al., 2014). The agronomic benefits of digestate have been highlighted in various scientific studies. Literature shows that the digestate derived from agricultural and agro-industrial residues can enrich soil microbial communities and increase their functional diversity. However, further investigations are still needed to fully understand its fertilizing potential and ensure its safe application (Alburquerque et al., 2012).

In the long term, the exclusive use of chemical fertilizers has been shown to reduce soil biodiversity and alter the ecological balance of microbial communities. To address this criticality, the network analysis method has been introduced, useful for investigating the interactions between microbes belonging to different taxa subjected to various fertilization modalities. This tool also allows identifying essential functional microorganisms that directly influence the structures of microbial communities and their potential functions (Ramirez et al., 2010; Sun et al., 2015).

Network analysis is used to investigate the interactions between different microbial taxa subjected to different fertilization treatments (Banerjee et al., 2016). This approach also allows identifying key functional microorganisms that significantly affect the structure and potential functions of the microbial community, thus contributing to a deeper understanding of the

diversity and role of the microbial community (Layeghifard et al., 2017).

It is known that soil is a dynamic ecosystem representing an important reservoir for microbial diversity (Law et al., 2024; Despotovic et al., 2023). Anthropogenic activities, including agricultural practices, may affect soil microbial diversity by introduction of exogenous bacteria some resistant to antimicrobials (ARBs) and harboring antimicrobial resistance genes (ARGs) on genetic elements, like conjugative plasmids, able to mediate their intra- and inter-species horizontal transfer. This topic is largely taken into account by the OneHealth concept, that implies a transdisciplinary approach also focusing on the emerging global problem of the antimicrobial resistance (AMR) (Law et al., 2024). In the context of One Health, natural microbial communities may have an important role in the dissemination of AMR (Despotovic et al., 2023) and agricultural practices should be designed in order to minimize the spread of ARBs and/or ARGs (Sanz et al., 2022).

Bio-composts, or microbial inoculants, are formulations containing live microorganisms that promote crop growth (Mahanty et al., 2017), enhance disease resistance of plants, counteract bacterial infections, and reduce soil-borne diseases (Mazzola and Freilich, 2017; Niu et al., 2020). Furthermore, they improve the physical, chemical, and biological characteristics of the soil (Demir, 2020). Despite the clear agronomic and ecological advantages associated with replacing synthetic or organic (animal-derived) fertilizers with bio-composts, little research has focused on the actual effects of bio-composts on soil microbial community especially with regards to the introduction of ARBs and conjugative elements harboring ARGs.

This study reports preliminary data about the potential effect of a bio-compost on microbial community of treated soils and its sustainability regarding the impact on diffusion of antimicrobial resistance more specifically on cultivable ARBs. Experiments were performed on an *ex-situ* pilot plant consisting of an agricultural soil treated with two types of bio compost obtained from organic waste. The two bio composts differ only for the physic form: powder or granular. The pilot plant was monitored along a one-year period for both the microbial community composition by molecular biology approach and the presence of ARBs by cultivable approach.

MATERIALS AND METHODS

Ex situ plant

The research activities were conducted on a pilot plant (Figure 1) consisting of three tanks, containing:

- Tank 1: soil (pb),
- Tank 2: soil + bio compost pellets (pg),
- Tank 3: soil + powdered bio compost (pp).

The pilot plant was set up to analyses the direct interaction between soil and bio compost. Therefore, the same type of soil was used as in an apricot field, and to standardize the experiment, the quantities of bio compost applied in real fields were calculated. On the basis of the full-scale applications, it was estimated that for each plant in the real field was created a cubic furrow with a side of 50 cm.

Knowing that the specific weight of the soil in the real field is equal to 1,500 kg/m^3, and that the volume of the excavation is equal to 0.125 m^3, it was obtained that for each plant, an amount of soil equal to 187.5 kg was taken.

For each plant, therefore, a quantity of bio compost pellets equal to 5 kg was applied premixed to the soil taken and subsequently reintegrated into the excavation, while for each plant, a quantity of bio compost powder equal to 2.5 kg was applied premixed to the soil taken and subsequently reintegrated into the excavation.

Therefore, it was possible to set up the pilot plant by creating the exact proportions of quantities used on a full scale and in particular:

- Tank 1: 37.5 kg of soil (bp),
- Tank 2: 37.5 kg of soil + 1.00 kg of bio compost pellets (pg),
- Tank 3: 37.5 kg of soil + 0.50 kg of bio compost powder (pp).

The material, just like for the real field, was premixed and only subsequently inserted into the tanks. Times of sampling were the following: T0 October 2023, T1 November 2023, T2 December 2023, T3 April 2024, T4 November 2024. Sampling involved taking a sample from each tank. The sample taken at the five points through the use of a core barrel for the collection of the entire soil column was mixed and used for laboratory investigations (Figure 1).

Figure 1. a) Pilot plant, b) sampling of soil in the tank, c) final sampler of soil

Characterization of microbial community

DNA extraction

DNA was extracted from the soil samples using standard bead-beating protocols (NucleoSpin Soil, MACHEREY-NAGEL, Germany) (Figure 2). Quantification of DNA was performed by Qubit 4.0 (Invitrogen, Thermo Fisher Scientific) (Figure 3).

Sequencing

Sequencing process TWO – the Oxford nanopore technologies (ONT) MinION sequencing pipeline has been used. Library preparation involved a one-step PCR process following the Oxford nanopore technologies 16S library preparation protocol (Oxford Nanopore Technologies, n.d.). First, the ~1500 bp 16S V1V9 region was amplified and barcoded using the 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK). The primers used were: 27F: 5'-TTTCTGTTGGTGCTGATATTGC AGRGTTYGATYMTGGCTCAG-3′ and 1492R: 5'-ACTTGCCTGTCGCTCTATCTTC-CGGYTACCTTGTTACGACTT-3'. The following PCR conditions were used: Initial denaturation at 95°C for 1 min, 35 cycles of 95°C for 20 s, 55°C for 30 s, and 65°C for 2 min, followed by a final extension at 65°C for 5 min. Barcoded fragments were then cleaned up using the AMPure XP system (Beckman Coulter Inc., California, USA). The eluted samples were quantified using a Qubit fluorometer (Thermo Fisher Scientific, Massachusetts, USA). As pooling was necessary to sequence all samples in one experiment, all barcoded libraries were pooled together to obtain the final desired ratio of 50–100 femtomoles in 10 µL of 10 mM Tris+HCl buffer (pH 8.0) with 50 mM NaCl. The library was primed using a Rapid Sequencing Adapter (RAP), which contains adapter sequences and motor proteins on these sequences to ligate to the tagged ends. The library was loaded into the MinION flow cell (Oxford Nanopore Technologies, Oxford, UK) (Figure 4), and sequencing undertaken.

Figure 2. NucleoSpin Soil using for the experimental extraction

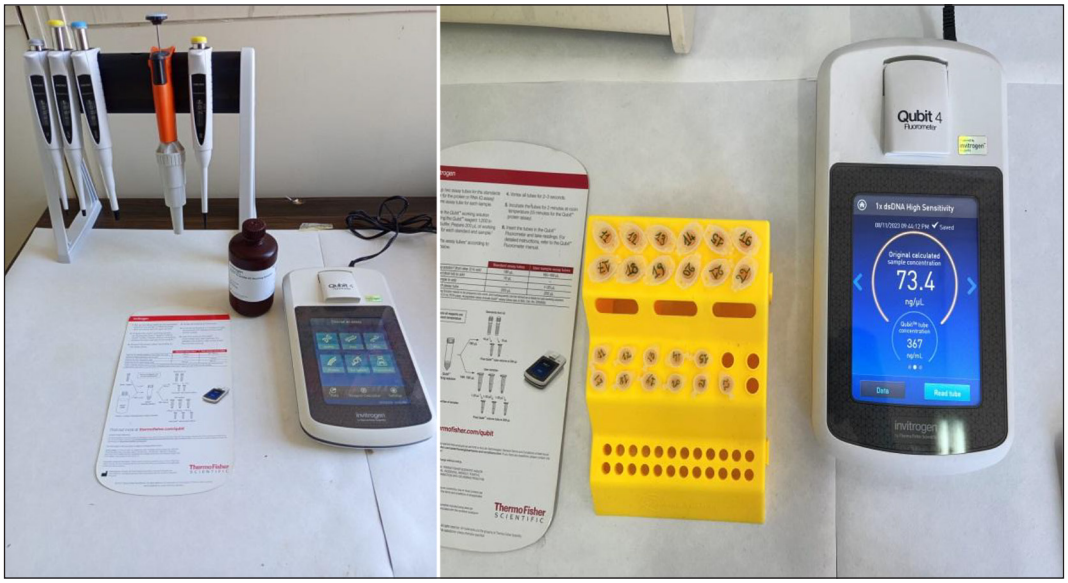

Figure 3. Using Qubit 4.0 and an example of measure of DNA quantification

Bioinformatics analyses

Read1 (forward) and read2 (reverse) reads from the MiSeq for each sample were downloaded from the Illumina Basespace in the fastq.gz format. The DADA2 pipeline was then used in R (version 4.2.1) to recover the Amplicon Sequence Variants (ASVs) from the amplicon data. Dual reads at 300 bp were truncated at 275 bp for forward reads and 250 bp for reverse reads (whilst regarding quality scores). Quality scores above 25 were selected for further analysis. Reads were dereplicated, merged, and chimeras were removed. The Phyloseq package in R was used to construct operational taxonomic unit (OTU) tables. The SILVA 132 train set was used to assign taxonomy. The sequence assignment threshold was 97% as a default setting in DADA2. The OTU table was filtered by abundance below 0.25% to remove spurious OTUs. The "transform_sample_counts" function from the phyloseq

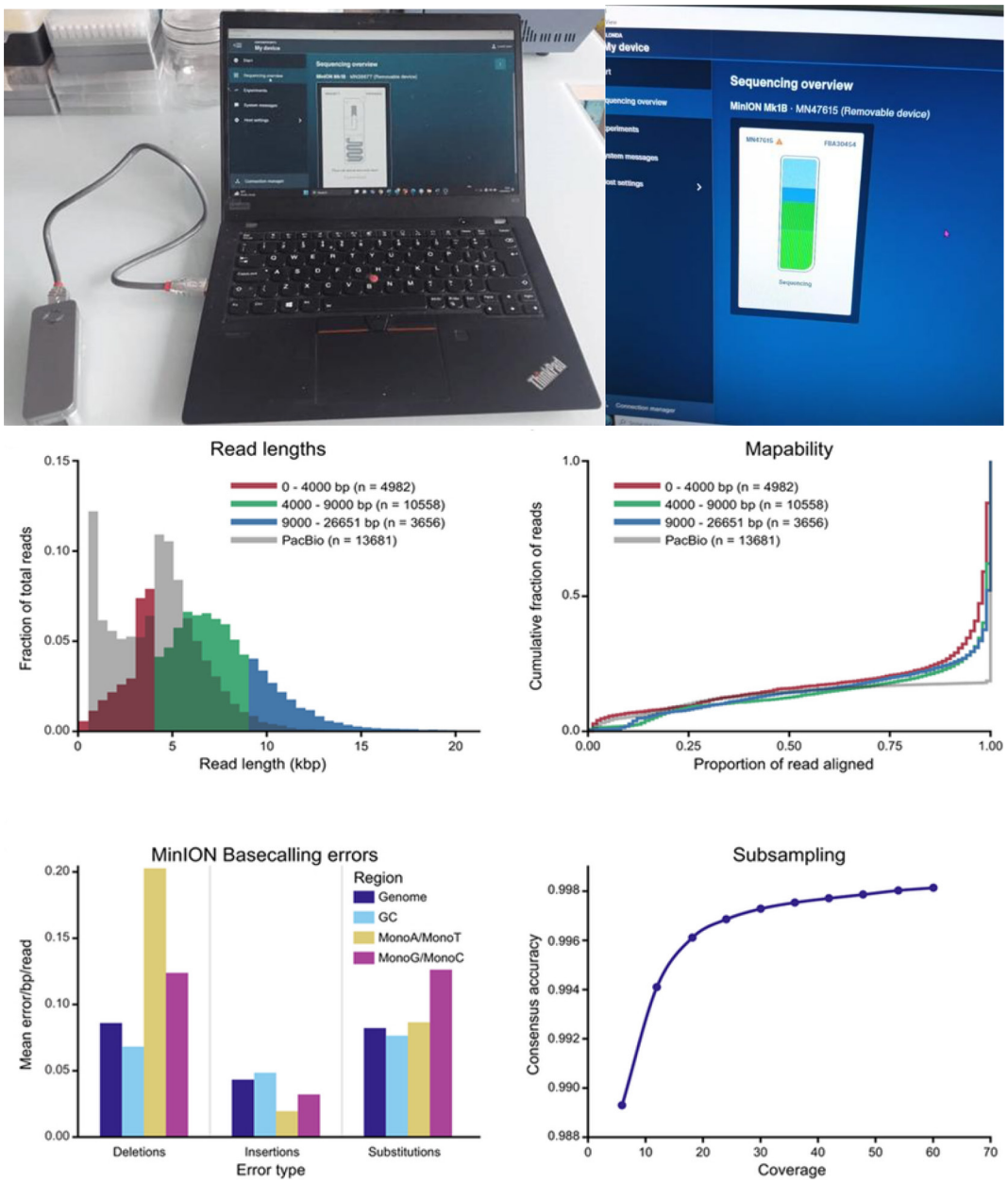

Figure 4. MinION flow cell sequencing a screenshot of the process of sequencing and a typical result of the sequencing

package was used to normalize the data. The "estimate richness" function from phyloseq was used to compute the alpha diversity metrics (effective number of species – exponential of Shannon entropy index). MinKNOW was the operating software for Oxford Nanopore Technologies (ONT), and it was used for data acquisition, real-time analysis, feedback runs, local basecalling, and data streaming. FAST5 (HDF5) and FASTQ files were created by MinKNOW. Raw signal data was stored in FAST5 format and was converted to FASTQ files by Guppy software I. Cakin et al. Journal of Microbiological Methods 220 (2024) 106921 4(v4.0.144). Guppy was the data processing toolkit for ONT devices and was responsible for barcoding/demultiplexing, adapter cutting, and alignment (Werner et al., 2022). The software filtered out the FASTQ files with a quality score of <7. Passed FASTQ files were uploaded to the cloud-based data analysis platform EPI2ME for onward analysis. Reads were then filtered based on quality, and taxonomic classification was made using the Basic Local Alignment Search Tool (BLAST) and entered into the NCBI 16S bacterial database (v2020. 04. 06) with the default parameters of minimum horizontal coverage of 30%

and a minimum accuracy of 77% (Santos et al., 2020). Quality filters, adapter trimming, and the configuration of alignment factors like sequence identity as well as coverage were already defined by default. The GALAXY platform was used to align the ONT MinION reads to the SILVA 132 database for the second taxonomy assignment in addition to the default MinION database. Adapters were removed from filtered FASTQ files by the Porechop tool. Single-end reads were pre-processed by the fastp tool. Qualified quality phred was selected as 9. Reads shorter than 1000 and longer than 2000 were filtered. For taxonomic assignment, Kraken2 (V2.0.8-beta) was used. The confidence interval selected was 0.1, meaning a minimum of 10% of the k-mers should match the database records. Kraken2 reports were visualized in R using the Pavian package.

Isolation of cultivable ARBs and antimicrobial susceptibility assays

Five grams of soils or bio-compost from each composite sample were suspended in 15 ml of 0.9% NaCl sterile solution and mixed thoroughly by shaking overnight (Figure 5). The suspensions were filtered by sterile gauze to remove largest particles and the liquid was recovered in a conical flask and used for detection of bacterial growth. To selectively isolate resistant Gram-negative bacteria form soil, tenfold dilutions of the soil suspensions were spread on a Brilliance solid selective medium (OXOID, Milan, Italy) added with ampicillin (AMP; Cf 100 ug/ml), or Trimethoprim (TMP; Cf 30 µg/ml). For quantitative and qualitative detection of resistant bacteria in bio-compost, tenfold dilutions of the bio-compost suspensions were spread both on the reach medium Nutrient Agar (NA) (OXOID, Milan, Italy) and the Brilliance which were added with AMP (Cf 100 µg/ml) or TMP (Cf 30 µg/ml) (Figure 6). Each soil and bio-compost sample was tested in triplicate.

Single colonies of the selected isolates were transferred to fresh sterile NA medium to obtain pure culture. The selected isolates were characterized for antimicrobial susceptibility by the Kirby-Bauer disk diffusion method (Bauer_1966) with few modifications. Bacterial suspensions were prepared in nutrient broth (OXOID, Milan, Italy) by diluting overnight cultures to achieve OD610nm ranging from 0.1–0.2. Sterile cotton swabs were used to spread bacteria suspensions over NA agar plates. Antimicrobial disks were immediately placed on the medium surface. Zones of growth inhibition around each disk were measured after incubation time of about 48 hours. Absence of inhibition halo was considered as full resistance to the corresponding antimicrobial (Figure 7).

Ten antimicrobials belonging to four different classes were used: beta-lactams (encompassing

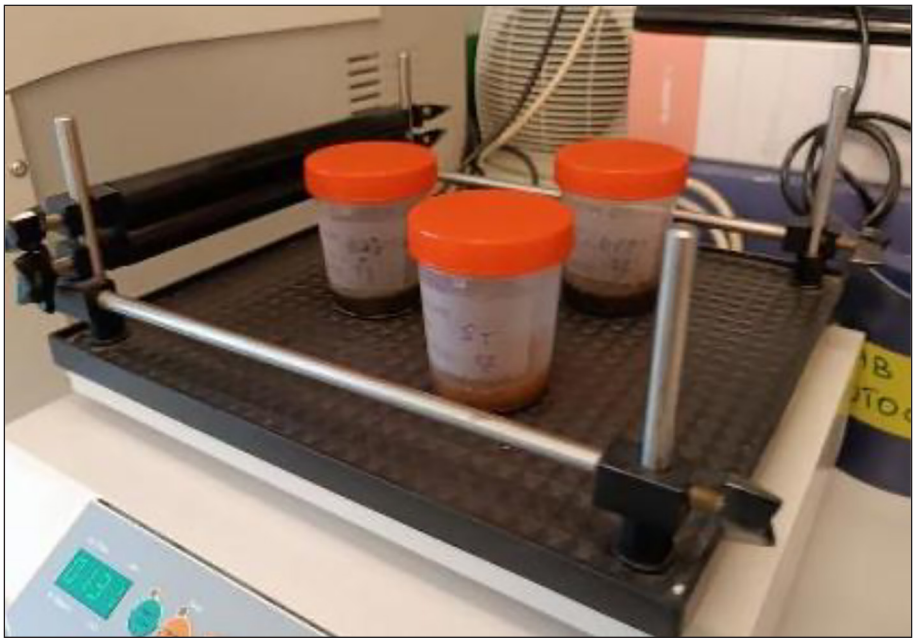

Figure 5. Shake of soil sample suspension soil or bio-compost suspended in 15 ml of 0.9% NaCl sterile solution

the third-generation cephalosporins) AMP (10 ug) and cefotaxime (30 µg) or ceftriaxone (30 µg) (CTX/CRO); aminoglycosides chloramphenicol (CHL; 30 µg), kanamycin (KAN; 30 µg), gentamycin (GEN; 10 µg) and streptomycin (STR; 10 ug); sulfonamide sulfamethoxazole (SUL 25 µg), TMP (5 µg) and the combination sulfamethoxazole/trimethoprim (SXT; 25 µg); tetracycline (TET; 30 µg). Assays were performed in triplicate. *Escherichia coli* ATCC 25922 was used as a quality control strain. In all cases bacterial growth was observed after incubation under aerobic conditions at 25°C.

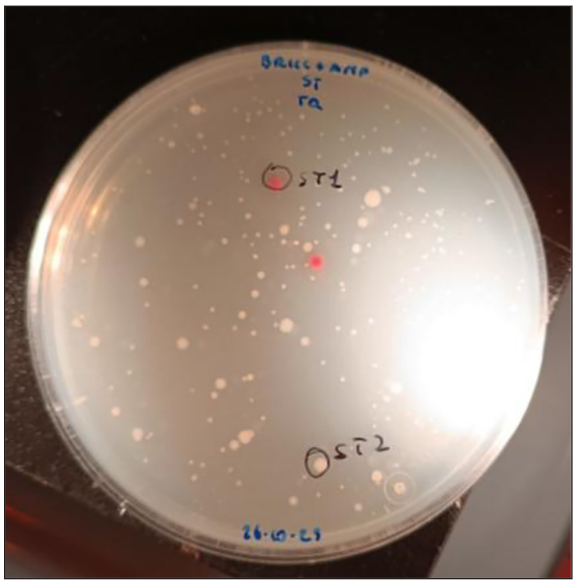

Figure 6. Example of soil sample spread on a Brillance medium added with AMP. Two single colonies has been selected further purification

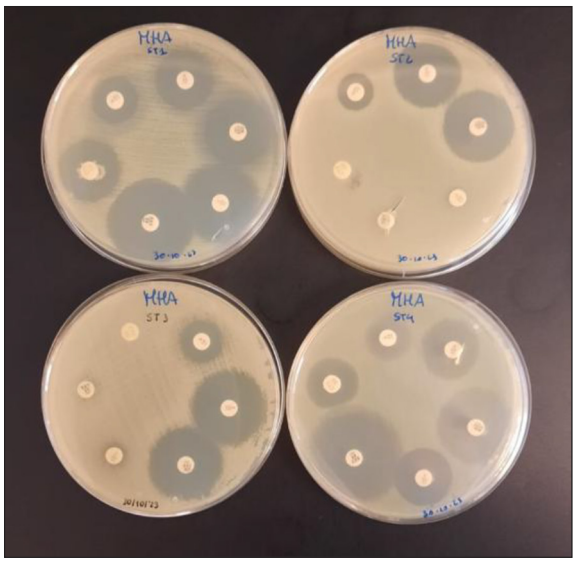

Figure 7. Kirby-Bauer disk diffusion method: example of inhibition halos and full resistance (absence of halo)

RISULTS AND DISCUSSION

Microbial community analyses

To determine the starting point of the soil matrix and the bio compost used, screening analyses were performed on the three samples that are consist in DNA quantification and sequencing. Regarding DNA quantification, it was obtained that the bacterial DNA of the soil was equal to 543,333 ng/kg of soil, the bacterial DNA of the powdered bio compost was equal to 114,253 ng/kg of soil and the bacterial DNA of the bio compost pellets was equal to 164,352 ng/kg of soil. The sequencing of the soil matrix identified a great variability of microorganisms present in which the greater presence of Protobacteria and Actinobacteria was highlighted and in particular the presence of some species in a more abundant manner (Table 1).

In the bio compost pellets, sequencing revealed the presence of only abundance of Firmicutes (Table 2). As regards the powdered bio compost, the sequencing highlighted a greater variety of species present in the matrix (Table 3).

The analysis carried out during the monitoring of the three pilot tanks identified, with respect to the soil alone, an initial increase in DNA at T1 (967,500 ng/kg of soil) and a subsequent decrease until T4 reaching a concentration of 375,255 ngDNA/kg of soil. With respect to the bio-compost system an increase in DNA concentration was highlighted in both cases, with a constant trend for the bio compost pellets system reaching a concentration of 1,128,562 ng DNA/kg of soil at T4, while with respect to the bio compost pellets, a slight increase was noted at T1 reaching a concentration of 723,750 ng DNA/kg of soil and a greater increase at T3 reaching a concentration almost equal to the previous bio compost pellets system. At T4, the powdered bio-compost system reaches almost the same concentrations as the bio compost pellet system with a concentration of 1,081,524 ng DNA/kg of soil (Figure 8).

Species analysis during monitoring of the experimental system revealed that, despite the increase in DNA, species diversification exists in the bio compost systems.

Table 1. Percentage of presence of the different species in the soil

Species	% presence	Species	% presence
Streptomyces sp.	18.97%	*Microbacterium sp.*	0.90%
Solirubrobacterales spp.	15.22%	*Phaselicystis sp.*	0.97%
Skermanella sp.	10.33%	*Pseudonocardia sp.*	0.99%
Microvirga sp.	4.76%	*Bryobacter sp.*	0.95%
Microscillaceae uncultured sp.	3.60%	*Agromyces sp.*	0.73%
Acidobacteria Subgroup 6 sp.	2.83%	*Paenibacillus sp.*	0.24%
Blastococcus sp.	2.16%	*Actinomarinales uncultured sp.*	0.12%
Actinophytocola sp.	1.88%	*Lysinibacillus sp.*	0.12%
Gemmatimonadaceae uncultured sp.	1.45%	*Actinomadura sp.*	0.10%
Nocardioides sp.	1.51%	*Thermoactinomyces sp.*	0.03%
Bacillus sp.	1.37%	*Streptosporangiaceae sp.*	0.01%
Steroidobacter sp.	1.03%	*Tumebacillus sp.*	0.01%
Pedomicrobium sp.	1.03%	*Thermoactinomycetaceae uncultured sp.*	0.01%
Chloroflexi sp.	0.97%	*Planifilum sp.*	0.01%
Mesorhizobium sp.	1.00%	Others spp.	26.73%

Table 2. Percentage of presence of the different species in the bio compost pellets

Species	% presence
Paenibacillus sp.	44.01%
Brevibacillus sp.	25.78%
Bacillus sp.	10.85%
Microbacteriaceae uncultured sp.	6.80%
Pseudonocardiaceae Subgroup 6 sp.	1.42%
Planococcaceae uncultured sp.	1.06%
Geobacillus sp.	0.91%
Lysinibacillus sp.	0.78%
Planifilum sp.	0.07%
Phaselicystidaceae uncultured sp.	0.01%
Other spp.	8.31%

The system containing only the soil matrix showed no significant change in species presence, with some species slightly increasing in number while others remained unchanged over time (Figure 9a and Figure 9b)

With regards to the bio compost pellets system, it is noted that there is a notable increase in the presence of *Penibacillus* sp. (final presence percentage equal to 49.19%), *Brevibacillus* sp. (final presence percentage equal to 27.09%), *Bacillus* sp. (final presence percentage equal to 1.56%) and *Agromyces* sp. (final presence percentage equal to 0.46%) (Figure 10a). Of these four species, it was noted that the greatest increase in presence occurred mainly for the species present only in the bio-compost (*Penibacillus* sp. and *Brevibacillus* sp.). Regarding the other identified species, it was found that as many as five species (*Blastococcus* sp., *Streptosporangiaceae uncultured* sp., *Nocardioides* sp., *Pseudonocardia* sp., and *Bryobacter* sp.) present a constant percentage over time; all of these species are present only into the soil (Figure 10b). As many as eleven identified species have a decrease in their percentage presence over time (Figure 10c). Of these, only two species (*Microbacteriaceae* uncultured sp. and *Pseudonocardiaceae* Subgroup 6 sp.) are present only in bio compost pellets, while the remaining nine are present only into the soil. Finally, eleven species present at the beginning of the experiment were absent in the final microbial pool (Figure 10d), of these species three (*Planococcaceae* uncultured bacterium sp., *Geobacillus* sp. and *Phaselicystidaceae* uncultured sp.) were present only into the bio compost pellets, two (*Lysinibacillus* sp. and *Planifilum* sp.) were present both in the bio compost pellets and in the soil and the remaining six were present only into the soil.

As regards the powdered bio-compost, an increase in the percentage presence of twenty-one species was noted (Figure 11a), of which thirteen were initially present only in the powdered bio-compost *Corynebacterium* 1 sp. (final presence percentage equal to 13.05%), *Saccharopolyspora* sp. (final presence percentage equal to 10.49%), *Saccharomonospora* sp. (final presence percentage equal to 10.01%), *Enteractinococcus* sp. (final

Table 3. Percentage of presence of the different species in the powdered bio compost

Species	% presence	Species	% presence
Tumebacillus sp.	23.42%	*Mycobacterium sp.*	0.98%
Corynebacterium 1 sp.	20.41%	*Actinomadura sp.*	0.83%
Saccharopolyspora sp.	12.25%	*Brevibacterium sp.*	0.83%
Streptomyces sp.	7.24%	*Staphylococcus sp.*	0.64%
Saccharomonospora sp.	5.58%	*Thermoactinomycetaceae uncultured sp.*	0.54%
Enteractinococcus sp.	3.14%	*Fodinicurvataceae uncultured sp.*	0.23%
Ambiguous_taxa sp.	2.09%	*Paenibacillus sp.*	0.22%
Atopococcus sp.	2.04%	*Bhargavaea sp.*	0.18%
Thermobifida sp.	2.03%	*Novibacillus sp.*	0.17%
Atopostipes sp.	1.91%	*Defluviitoga sp.*	0.17%
Nocardioides sp.	1.69%	*Methylococcaceae uncultured sp.*	0.15%
Bacillus sp.	1.63%	*Kroppenstedtia sp.*	0.15%
Planifilum sp.	1.51%	*Caldicoprobacter sp.*	0.14%
Steroidobacter sp.	1.47%	*Thermocrispum sp.*	0.13%
Microbacterium sp.	1.41%	*Jeotgalicoccus sp.*	0.13%
Salipaludibacillus sp.	1.30%	Other spp.	4.70%

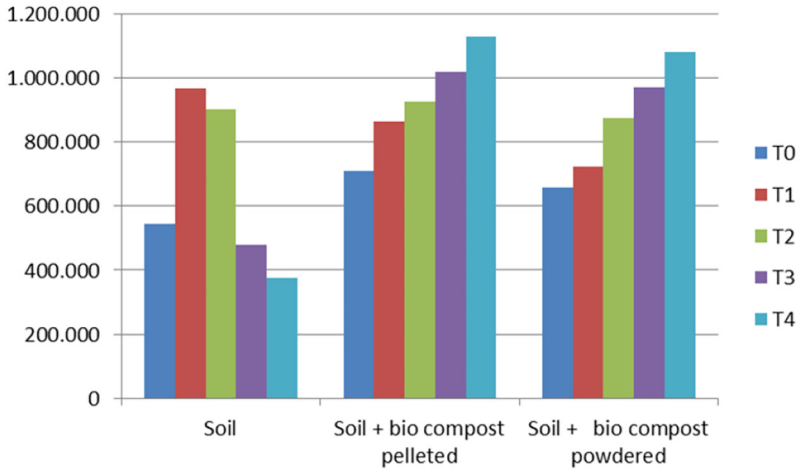

Figure 8. DNA trend in the plant during monitoring activity

presence percentage equal to 2.45%), *Ambiguous_taxa* sp. (final presence percentage equal to 1.96%), *Atopococcus* sp. (final presence percentage equal to 1.91%), *Atopostipes* sp. (final presence percentage equal to 2.16%), six species were initially present both in the soil and in the powdered bio-compost *Tumebacillus* sp. (final presence percentage equal to 17.61%), *Streptomyces* sp. (final presence percentage equal to 8.43%), *Nocardioides* sp. (final presence percentage equal to 0.74%), *Planifilum* sp. (final presence percentage equal to 1.56%), *Thermoactinomycetaceae* uncultured sp. (final presence percentage equal to 0.59%) and *Thermoactinomyces* sp. (final presence percentage equal to 0.62%, and two species were initially present only in the soil *Mesorhizobium* sp. (final presence percentage equal to 1.65%) and Actinomarinales uncultured bacterium sp. (final presence percentage equal to 0.64%). Six species maintained a constant percentage presence over time (Figure 11b). Of these, only the species *Jeotgalicoccus* sp. was initially present in the bio-compost powder, while the other five (*Skermanella* sp., *Microvirga* sp., *Acidobacteria* Subgroup 6 sp., *Pedomicrobium* sp., and *Gemmatimonadaceae* uncultured sp.) were initially present only in

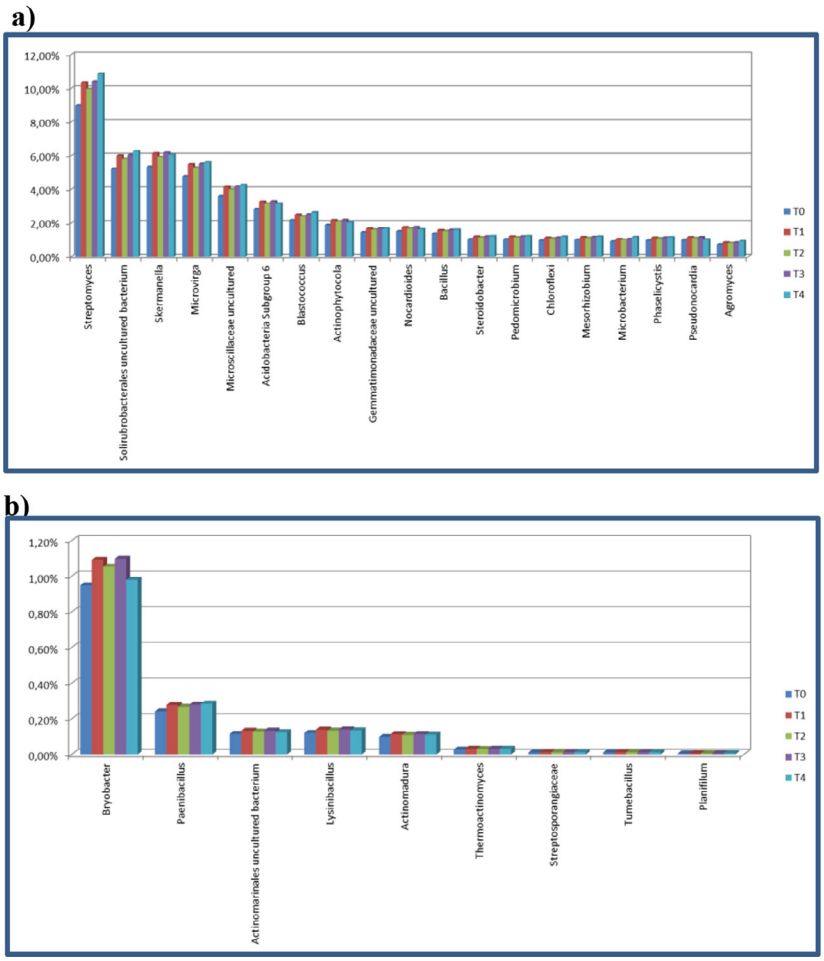

Figure 9. Trend of the main species in the soil: a) species that increase their presence; b) species that present a constant concentration over time

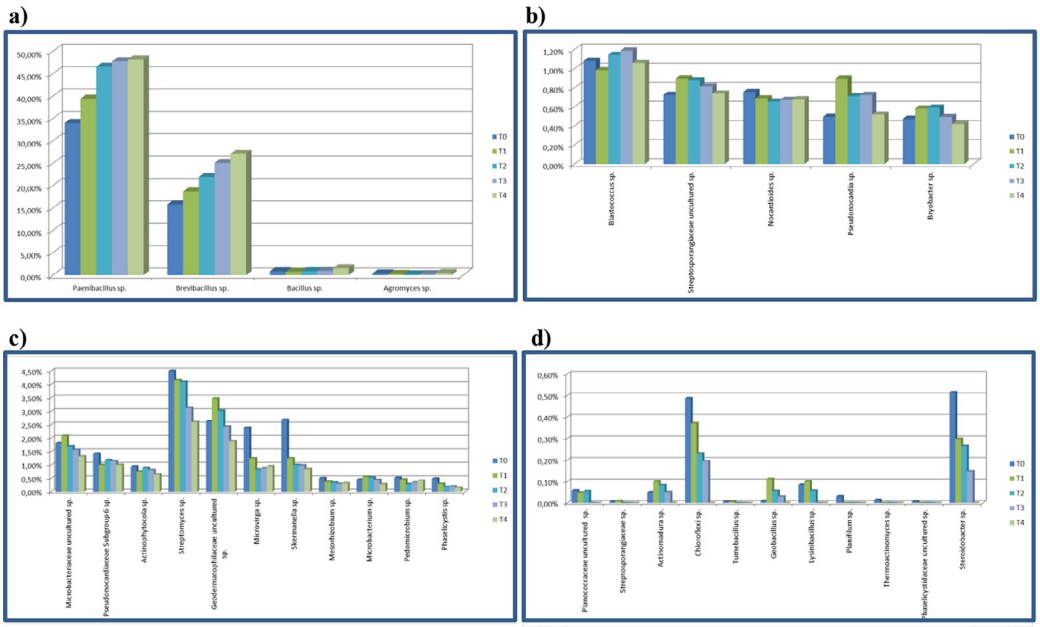

Figure 10. Trend of the main species in the soil+ bio compost pellets:
a) species that increase their presence; b) species that present a constant concentration over time
c) species that decrease their presence; d) species that are absent in the final microbial pool

the soil. During the experiment, it was evident that ten species showed a decrease in their presence in the final microbial pool (Figure 11c). Of these species, three were present only in the bio-compost powder (*Thermobifida* sp., *Brevibacterium* sp., and *Paenibacillus* sp.), one was initially present in both the soil and the bio-compost powder (*Microbacterium* sp.), and six species were initially present only in the soil. Finally, fourteen species present at the beginning of the experiment were absent from the final microbiological pool (Figure 11d). Of these, at the beginning of the experiment, six species were present only in the bio-compost powder (*Salipaludibacillus* sp., *Bhargavaea* sp., *Novibacillus* sp. and *Defluviitoga* sp.), two were present in both the soil and the bio-compost powder (*Bacillus* sp. and *Steroidobacter* sp.) and five were present only in the soil.

Characterization of cultivable ARBs from soil and bio-compost

Two lots (lot 1 and lot 2) of the granular bio-composts have been tested (in triplicate) for the presence of bacteria cultivable on the media Brilliance or Nutrient Agar and resistant to AMP or TMP. No growth has been observed on Brilliance medium added with AMP or TMP indicating absence of Gram-negative bacteria resistant to those antibiotics and cultivable on this medium. Similarly, no growth it has been detected on Nutrient Agar added with AMP suggesting absence of bacteria resistant to AMP. On the contrary, bacteria colonies have been observed on Nutrient Agar added with TMP, indicating presence of cultivable bacteria resistant to this antibiotic. TMPr bacteria have been detected in number ranging from at least 9,00E+03 to about 9,00E+04 CFU/g in lot 1 and from at least 4,00E+03 to about 3,00E+05 CFU/g in lot 2. Ten TMPr isolates were selected based on colony morphology and were characterized for their susceptibility to the 7 additional antimicrobials AMP, CHL, GEN, KAN, STR, SUL and TET by the disk diffusion method. Most of the isolates were susceptible to 6 out the 7 antimicrobials tested. Indeed, all of them showed resistance to SUL, while 2 isolates were resistant also to AMP and 1 isolate shows resistance to STR while sensitive to AMP. The resistance patterns with inhibition haloes are listed in Table 4.

To the best of authors' knowledge, these are first data on the presence of cultivable ARBs in a bio-compost. Although analyses on more than 2 lots should be performed to statistically confirm the regular presence of TMPr bacteria in the bio-compost, this study highlights the importance of such monitoring in view of the One Health approach evaluating sustainability of a bio-compost in agricultural practices. In a

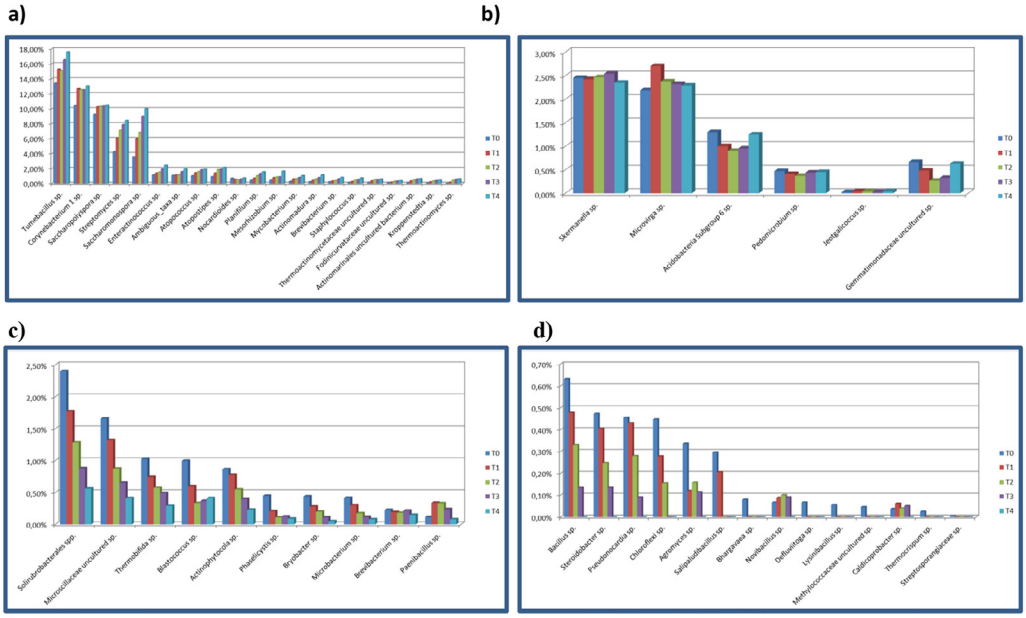

Figure 11. Trend of the main species in the soil+powered bio-compost: a) species that increase their presence; b) species that present a constant concentration over time; c) species that decrease their presence; d) species that are absent in the final microbial pool

Table 4. Susceptibility patterns of the selected TMPr isolates from bio-compost

Isolate	Lot	Inhibition halo* (mm)						
		AMP	KAN	CHL	SUL	GEN	STR	TET
1	1	50	35	35	0	40	40	45
2	1	30	23	25	0	25	26	33
3	1	34	35	25	0	30	21	22
4	1	12	24	30	0	23	26	30
5	1	12	24	29	0	24	27	26
6	2	0	40	30	0	25	40	30
7	2	0	24	29	0	21	25	32
8	2	34	25	25	0	24	30	29
9	2	34	28	30	0	30	30	35
10	2	35	30	25	0	25	0	19

Note: AMP: ampicillin; CHL: chloramphenicol; GEN: gentamycin; KAN: kanamycin; STR: streptomycin; SUL: sulphamethoxazole; TET: tetracycline

*: 0 mm indicates full resistance; haloes >10 mm indicate not full resistance or susceptibility

future perspective, it could be interesting to plan analyses on bio-compost by integrating data on cultivable ARBs and detection of ARGs eventually associated to mobile genetic elements such as plasmids.

Soils of the Pilot Plant (not treated, with bio compost pellets and with bio compost powered) were monitored for the presence of Gram-negative bacteria cultivable on Brilliance medium and resistant to AMP and/or TMP. Composite soil samples were collected from October 2023 to November 2024 at five different times (T0 October 2023, T1 November 2023, T2 December 2023, T3 April 2024, T4 November 2024) and analyzed in triplicate for quantitative analysis. Preliminary data, showed in Tables 5 and 6, make evident no significant change in concentrations of bacteria populations resistant to AMP or TMP among the 3 soils along the sampling times, suggesting that the bio-compost does not impact the Gram-negative resistant bacteria of the soil microbial community. However, further analyses are necessary to statistically confirm this hypothesis.

Table 5. Concentrations of populations resistant to AMP

Sampling time	CFU/g		
	Soil	Soil + bio compost pellets	Soil + bio compost powered
T0	7.00E+02	7.00E+02	7.00E+02
T1	6.08E+03	3.00E+06	3.06E+03
T2	1.10E+03	6.00E+03	3.06E+03
T3	7.50E+05	2.58E+05	1.30E+05
T4	5.30E+05	1.80E+04	1.60E+05

Table 6. Concentrations of populations resistant to TMP

Sampling time	CFU/g		
	Soil	Soil + bio compost pellets	Soil + bio compost powered
T0	1.24E+03	1.24E+03	1.24E+03
T1	1.80E+03	1.70E+06	2.90E+06
T2	1.40E+03	1.10E+05	1.00E+04
T3	9.00E+05	3.60E+05	4.6E+05
T4	1.20E+06	2.80E+04	2.40E+05

Table 7. Number of isolates from pilot plant selected for disk diffusion assay

Collection time	Soil	Soil + bio ompost pellets	Soil + bio compost powered
T0	15	0	0
T1	11	11	9
T2	15	12	13
T3	12	6	15
T4	6	6	7

Table 8. Distribution of antimicrobial resistance patterns among the tested soil isolates

Resistance pattern*										Number of isolates			TOT number of isolates
										Soil	Soil + bio compost pellets	Soil + bio compost powered	
AMP		STR	KAN	CHL	SXT			CTX/CRO		1			1
AMP			KAN	CHL	SXT			CTX/CRO		3		1	4
AMP				CHL	SXT			CTX/CRO		9	7	15	31
AMP				CHL				CTX/CRO	TMP		3	1	4
AMP					SXT			CTX/CRO		7	2	1	10
AMP				CHL	SXT					4	6	5	15
AMP		STR			SXT					1			1
AMP	GEN	STR								3			3
AMP		STR				SUL				2			2
AMP								CTX/CRO	TMP		3	2	5
AMP						SUL				2		2	4
AMP					SXT					6	2	4	12
AMP									TMP	5	2		7
AMP								CTX/CRO		4	1	2	7
					SXT		TET			1			1
				CHL	SXT							1	1
AMP										6	6	2	14
							TET			1	1		2
					SXT					1	2	5	8
		STR								1			1
									TMP	2		3	5
TOT number of isolates										59	35	44	138

Note: AMP – ampicillin; CTX – cefotaxime; CRO – ceftriaxone; CHL – chloramphenicol; GEN – gentamycin; KAN – kanamycin; STR – streptomycin; SUL – sulphamethoxazole; TET – tetracycline; TMP – trimethoprim
*: patterns are grouped based on the number of resistances as follow: 6, 5, 4, 3, 2, 1 from top to bottom

A total of 138 isolates were randomly selected among those grown on AMP and/or TMP (59 from not treated soil, 35 from soil with bio compost pellets, 44 from soil with bio compost powered) among the collection times (15 at T0, 31 at T1, 40 at T2, 33 at T3 and 19 at T4) and characterized for the antimicrobial susceptibility pattern to 10 commonly used antimicrobials. The number of isolates from each soil type selected at each collection time is listed in Table 7.

Twenty-one different resistance patterns were detected (Table 8), characterized by resistance to one antimicrobial (5 patterns), resistance to 2 antimicrobials (6 patterns) and from 3 to 6 antimicrobials (10 patterns). Only one combination of 6 and 5 antimicrobials (AMP, STR, KAN, CHL, SXT, CTX/CRO and AMP, KAN, CHL, SXT, CTX/CRO) has been detected. Regarding the other pattern categories, different combinations of antimicrobials have been observed, as shown in table 8. Percentages of

isolates showing resistance from 1 to 4 antimicrobials were almost similar (about 22%, 23%, 26% and 25,3% of isolates with resistance to from 1 to 4 antimicrobials, respectively). Only 1 isolate (0.7%) showed the unique pattern with 6 antimicrobial resistances and 4 isolates (2.8%) showed 5 antimicrobial patterns.

Treated and untreated soils seem characterized by specific patterns of resistant bacteria. However, further experiments performed on new pilot plant for a longer time should be performed. Regarding resistance to a specific antimicrobial – that to AMP is present in 15 out 21 patterns and that to SXT in 10 out 21. These preliminary data suggest that the treatment with the bio-compost does not increase the resistant Gram-negative soil populations in one year monitoring. Additionally, resistances to AMP and SXT seem to be the most common among the Gram-negative soil populations tested, as expected in consideration of the experimental plan based on the use of selective media added with AMP and TMP. Finally, the multi-resistant patterns (to 5 or 6 antimicrobials) are rare in the tested populations.

CONCLUSIONS

This study highlighted a direct correlation between soil type and the activity of bio compost applied in two different forms, pelleted and powdered. Initial results from a one-year trial showed that bio compost application promotes bacterial growth in the soil, with a steady increase over time, reaching 59.48% for soil treated with bio compost pellets and 64.47% for soil treated with bio compost powered. Regarding the growth of the present species, it is noted that most of the species introduced by the bio compost tend to predominate in growth, replacing most of the native species, with some disappearing during the trial. However, this trend does not appear to involve the spread of ABRs. Data are undoubtedly preliminary albeit they further highlight the need to assess, in the light of the OneHealth viewpoint, the potential impact of bio-composts in agriculture practices and more generally in the environment in order to minimize the spread of ARBs and/or ARGs.

The data presented here have currently been developed in a closed system free of any plants that would influence the microbiological status.

Future studies will include the use of the bio compost on different soils and a subsequent study in which the interaction with the possible presence of plants should also be analyzed.

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Founding

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