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# Implications of the use of organic fertilizers for antibiotic resistance gene distribution in agricultural soils and fresh food products. A plot-scale study

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## HIGHLIGHTS

- Fertilization increased loads of clinically relevant ARGs.
- Measurable ARG loads in lettuce (*tetM, sul*1) and radish (*sul*1)
- Soil and food ARG loads strongly depended on the type of fertilizer.
- Lowest ARG loads found in minerally-fertilized soils and crops.
- Bacteria from organic fertilizers may increase ARG loads in soils and crops.

## GRAPHICAL ABSTRACT


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## ABSTRACT

The spread of antibiotic resistance genes (ARG) into agricultural soils, products, and foods severely limits the use of organic fertilizers in agriculture. In order to help designing agricultural practices that minimize the spread of ARG, we fertilized, sown, and harvested lettuces and radish plants in experimental land plots for two consecutive agricultural cycles using four types of fertilizers: mineral fertilization, sewage sludge, pig slurry, or composted organic fraction of municipal solid waste. The analysis of the relative abundances of more than 200,000 ASV (Amplicon Sequence Variants) identified a small, but significant overlap (<10%) between soil's and fertilizer microbiomes. Clinically relevant ARG were found in higher loads (up to 100 fold) in fertilized soils than in the initial soil, particularly in those treated with organic fertilizers, and their loads grossly correlated to the amount of antibiotic residues found in the corresponding fertilizer. Similarly, low, but measurable ARG loads were found in lettuce (*tetM, sul*1) and radish (*sul*1), corresponding the lowest values to samples collected from minerally fertilized fields. Comparison of soil samples collected along the total period of the experiment indicated a relatively year-round stability of soil microbiomes in amended soils, whereas ARG loads appeared as unstable and transient. The results indicate that ARG loads in soils and foodstuffs were likely linked to the contribution of bacteria from organic fertilizer to the soil microbiomes, suggesting that an adequate waste management and good pharmacological and veterinarian practices may significantly reduce the presence of these ARGs in agricultural soils and plant products.

## 1. Introduction

The use of organic wastes as agricultural fertilizers, allows a better management of the finite resources we dispose for soil fertilization and food production. Among all the different types of organic wastes that are generated annually, wastewater treatment plants' sludge and animal slurry represent the most abundant ones, making them usual candidates for organic fertilization (Bosch-Serra et al., 2020; Fernández et al., 2009; Pascual et al., 2018; Terrero et al., 2018). The use of sludge from municipal wastewater treatment operations is usually a subject under strict regulatory control (Alvarenga et al., 2015; Murray et al., 2019). There are different types of sludge depending on the level of thermal treatment and drying,

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alkaline stabilization, digestion or composting, which lead to liquid or cake-like sewage biosolids while animal manure is a combination of feces, urine and animal bedding that depending on the level of "turning" can result on solid, semi-solid or liquid manure (slurry) (Qian et al., 2016). The most common treatment used to stabilize the N and improve handling characteristics in this type of organic waste is composting. The product of composting is a material that has a much smaller C:N ratio than the original mixture and may result in a decrease of the concentration of some contaminants including antibiotics (ABs) (Dolliver and Gupta, 2008; Goss et al., 2013). However, there is a potential tendency of increasing of Cu, Zn, K and P concentration during this process (Tejada et al., 2001).

While organic fertilizers can provide macro- and micronutrients to the soil, they may also contain components that can be harmful for animal, plant, and human health (Chen et al., 2018; Urra et al., 2019; Zhou, Zhu et al., 2019). The presence of pathogens represents an obvious threat, but other pollutants, like pharmaceuticals, hormones and ABs may also represent a potential risk. The presence of these last ones, may promote the increase of bacteria resistant to multiple antimicrobial and antibiotic drugs. The acquisition of Antibiotic Resistance (AR) is a natural phenomenon, but the application of organic fertilizers may step up its dissemination and evolution in the soil. Therefore, the potential transmission of AR from amended soils to crops and, ultimately, to consumers is a matter of major concern, particularly for plants that are usually consumed raw (Berendonk et al., 2015; Chen et al., 2016; Freitag et al., 2018; Koch et al., 2017; Yang et al., 2018). Antibiotic Resistance Genes (ARGs) may spread from the organic fertilizer to the soil-plant continuum via endophytes or by adhering to plant surfaces or soil particles, nonetheless few studies have deepened in this topic (Cerqueira et al., 2019b; Marano et al., 2019). Considering those facts and under the actual climate change scenario, there is an urgent need to consider organic sources of nutrients as key factors to a sustainable food production chain. The main question remains in finding the right source that finds balance between the benefits in support of the plant growth and the potential threats and risks (Goss et al., 2013).

The concentration of some antibiotics and other molecules in sludge and slurry may vary depending on the origin and nature of the waste and the different composting processes (Berendsen et al., 2018; Bondarczuk et al., 2016; Ezzariai et al., 2018; Gros et al., 2019; Liu et al., 2015; Qian et al., 2016; Widyasari-Mehta et al., 2016). The potential long-term presence of ABs in the soil under organic fertilization may exert selective pressure over soil microbiomes, leading to changes in its composition (Cerqueira et al., 2019b; Pan and Chu, 2016; Zhang et al., 2014). This usually happens at sub-inhibitory concentrations favoring antibiotic resistant (AR) bacterial strains over sensitive ones, thus turning soil into hotspots for ARGs (Andersson and Hughes, 2011; Cerqueira et al., 2019b).

While it is generally known that organic fertilizers may alter microbiomes and transcriptomes from the receiving soils and, ultimately, from crops, there are only few studies specifically focused in comparing these effects between different types of organic fertilizers in different crops (Buta et al., 2021; Cerqueira et al., 2019a; Muhammad et al., 2020; Radu et al., 2021; Tadić et al., 2021; Xie, Shen et al., 2018). Yet, the development of agricultural practices minimizing the potential risk of their use is a requirement for many world regions in which, like in the Mediterranean region, intensive pork farming co-exists with limiting water availability and strong organic fertilization needs, since soils are almost depleted of organic matter (Noya et al., 2017; Palma-Heredia et al., 2020). In this work, we intend to investigate three key aspects that have been seldomly addressed in an integrated way: 1- The influence of the type of organic fertilizer in the final ARG loads in soils and crops; 2.- The contribution of bacteria from fertilizers to the changes observed in the receiving soils' microbiome and how stable this contribution is; and 3.- The origin of ARGs found in foodstuffs and particularly whether they come from the fertilizer or from the original soil resistomes. To address these questions, we used an integrated setup in which experimental plots located in an agricultural environment (the Llobregat River Delta, South of Barcelona, Spain), using three different organic fertilizers: sewage sludge, piglet slurry and the organic fraction from municipal waste (OFMSW), in addition to a conventional chemical fertilizer (Mineral), and two crops of agronomic interest that are commonly eaten raw, lettuce and radish (*L. sativa* and *R. sativus*). We completed two agricultural cycles, to obtain temporal information, and performed molecular analyses of microbiomes and ARG loads from the different compartments. The microbial population present in the fertilizers and soil samples was studied using high throughput 16S rDNA sequencing techniques at the ASV (Amplicon Sequence Variant) level, which provides an extremely detailed identification of the bacterial and archaea strains present in the samples that allows tracking the transmission of these strains from fertilizers to soils and from soil to plants. In addition, seven ARGs of clinical relevance (*sul*1, *tet*M, *qnr*S1, *mec*A, $bla_{TEM}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{OXA\text{-}58}$) were analyzed using quantitative real-time PCR methods (RT-PCR) in samples of biosolids, soil, and harvest products. The integron class 1 (*intI*1) was added to the analysis as it is considered a marker of anthropogenic pollution and Horizontal Gene Transfer activity (HGT) (Agerso and Sandvang, 2005; Forsberg et al., 2014; Gillings et al., 2015; Marano et al., 2019; Zheng et al., 2020). It has been found closely associated to *sul*1 (Poey et al., 2019) and to several genes encoding extended-spectrum β-lactamases and resistance for tetracycline and quinolones (Agerso and Sandvang, 2005; Chen et al., 2010; Gillings et al., 2008; Quiroga et al., 2013).

This work integrates different approaches to provide a global picture of the effect of organic fertilization in the presence of clinically relevant ARGs in soils and crops, linking their levels to the composition of the corresponding fertilizers in terms of microbiome profiles, ARG loads and antibiotic residue levels. The final goal of this project is to help devising agricultural practices that minimize the spread of ARG from organic fertilizers to agricultural soils, foodstuffs and, eventually, consumers.

## 2. Materials and methods

### 2.1. Experimental site and conditions

The present study was developed along two productive cycles (1st cycle: March 2019-May 2019; 2nd cycle: November 2019-March 2020) at the scientific-technical unit of Polytechnic University of Catalonia (UPC) ("Agrópolis", Viladecans (Barcelona), 41°17′19,1″N, 2°02′43,4″E). Fig. 1 shows weather conditions and the schedule of fertilization, planting, soil sampling and harvesting along the two cycles. The sandy-clay (sand 40%, silt 35.2% and clay 24.8%) soil used in this experiment did not have any history of agriculture prior to this date. Thirty-two experimental plots were cultivated, with half destined for lettuce cropping (*Lactuca sativa* cv "Maravilla", 16 plots of 10 $m^2$) and radishes (*Raphanus sativus* cv "Redondo rojo", 16 plots of 3 $m^2$). Twelve lettuce seedlings were planted in each of three rows spaced at 30 cm and 200 radish seeds per row spaced at 7–8 cm. Each plot received one of the three tested biosolids (sludge, slurry, OFMSW) or a mineral fertilizer ($n = 4$, per treatment) and were randomly distributed in the field. The sludge-based fertilizer consisted in anaerobically-digested sludge from Gavà-Viladecans (Barcelona, Spain) wastewater treatment plant (WWTP), the slurry came from a piglet farm in Osona (Barcelona, Spain) and the OFMSW-derived compost was made of urban waste composted with wood residues for 3–4 months. Main physico-chemical properties of the studied organic fertilizers are shown in Supplementary Table S1. In each cycle, OFMSW was spread along its respective plots, sludge was applied as small moist aggregates and the slurry was applied as a liquid (dry matter content below 5%) using a watering can. All the amendments were incorporated manually to a depth of 5 cm immediately after application. The amount of organic fertilizer added per plot was calculated to ensure the same amount of ammoniacal nitrogen in all treatments (100 kg of N per ha for lettuce and 80 kg of N per ha for radish), corresponding to the average extractions of the crops (Ramos and Pomares, 2010). Specifically, the application rate for OFMSW, sludge and slurry was 4.5 kg/$m^2$, 3 kg/$m^2$ and 4 l/$m^2$ for lettuces and 2.8 kg/$m^2$, 2.3 kg/$m^2$ and 3 l/$m^2$ for radishes, respectively. For the inorganic fertilization group, ammonium nitrate (34% N) was added to provide the same amount of N as in the organic treatments (100 kg N/ha for lettuce and 80 kg N/ha for radish), and superphosphate (43.6% $P_2O_5$) and potassium


**Fig. 1.** Average temperatures, daily accumulated precipitation, organic fertilizers application, sow and harvest dates along the experimental period. Note the different sampling times for lettuce and radish, as well as for the corresponding soil samples, imposed by weather conditions and plant growth.

sulphate (54% $K_2O$) were added to adjust potassium (40 kg $P_2O_5$/ha in lettuce and 30 kg $P_2O_5$/ha in radish) and phosphate levels 185 kg $K_2O$/ha in lettuce and 90 kg $K_2O$/ha in radish) to meet Spanish agronomical guidelines (Ramos and Pomares, 2010). Main physico-chemical properties of the studied soils are shown in Supplementary Table S2.

### 2.2. Biosolids, soil and vegetable sampling, characterization and processing

In each production cycle, organic fertilizers were collected just prior to their application. Moreover, before the experiment and between the first and the second fertilization cycle, soil samples (Initial soil and Soil 2, respectively) were taken at a 10 cm depth with 50 ml sterile polypropylene tubes to characterize the soil along the experimental period, using each tube as a biological replicate. At harvest, eight heads of lettuce and eight handfuls of radishes were randomly sampled per treatment plot respectively and taken refrigerated (4 °C) to the laboratory in Ziploc bags for their processing, treating each head or handful as a biological replicate. Immediately after harvest, 3 × 11.5 cm lettuce soil and radish soil cores were sampled, at a 10 cm depth with 50 ml sterile tubes (Soil 1 for the first fertilization cycle, Soil 3 for the second fertilization cycle). Note that Soil 3 (post harvest) samples were taken at two different time points, as lettuces and radish had different harvesting times at this particular campaign. As the two sets of data ($n = 32$ each) were statistically identical for the analyzed parameters, we opted to treat them as a single set of samples (not shown). Once at the laboratory, excess soil was removed from the plants using a sterile gauze to achieve the visual cleanliness desired by consumers. About 90 g of product per biological replicate were processed in a grinder (Retsch GRINDOMIX GM200). The crushed material was transferred to a beaker along with 50 ml of sterile PBS, mixed thoroughly with a hand blender, and then filtered through a gauze to remove the pulp. This procedure was repeated twice to ensure a proper bacterial rinse. The flow through was then transferred to 50 ml sterile polypropylene tubes through a 100 μm mesh nylon Cell strainer (Corning® Cell Strainer), centrifuged at 4500 rpm for 15 min, and the pellets stored at −20 °C until further bacterial DNA extraction. For further clarification, an scheme of the procedure is shown in Supplementary Fig. S1.

### 2.3. Bacterial DNA extraction and genetic elements quantification

DNA from soil, organic fertilizers, and from pellets from vegetal matrices (250 mg each), was extracted using the DNeasy PowerSoil Kit (Qiagen Laboratories, Inc.), to a final elution volume of 100 μl. The concentration and the quality of the DNA were tested using a NanoDrop Spectrophotometer 8000 (Thermofisher Scientific, Inc). Extracted DNA samples were stored at −20 °C.

Absolute abundance values (copies/g of sample) were calculated for 16S rDNA, *intI*1 and the following ARGs: *sul*1 (Encodes for the enzyme dihydropteroate synthetase, that confers resistance to sulfonamide), *qnr*S1 (a protein from the Pentapeptide Repeat Protein (PRP) family, which contains a tandem of five amino acid repeats, that inhibits the effect of quinolones), *tet*M (a ribosomal protection protein, which confers tetracycline resistance by binding to the ribosome and chasing the drug from its binding site), *mecA* (penicillin-binding protein 2A (PBP2a), which enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis), $bla_{TEM}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{OXA\text{-}58}$, (β-lactamases conferring resistance to beta-lactamic antibiotics such as cephalosporins, monobactams, and carbapenems). The seven selected ARGs confer bacterial resistance against five types of antibiotics widely used both in clinical and veterinary practices. They often associate to mobile genetic elements and they are considered highly relevant in environmental settings (Berendonk et al., 2015). They have been intensively studied in our laboratory for the past four years as robust indicators of the presence of antibiotic resistance genes in the samples (Cerqueira et al., 2019c; Cerqueira et al., 2019b; Cerqueira et al., 2019a). Quantification was performed by real time PCR reactions in a LightCycler 480 II (A F. Hoffmann–La Roche AG, Inc), using the primer sequences listed in Supplementary Table S3. Reactions were conducted in 20 μl volumes on 96-well plates, using primer concentrations of 200 nM for $bla_{TEM}$ and of 300 nM for the rest of genes. Dynamo ColorFlash SYBR Green (Thermo Scientific, Inc.) was used for *mecA*, *tet*M and $bla_{OXA\text{-}58}$ qPCR quantifications; all the other genes were quantified with LightCycler 480 SYBR Green I Master (A F. Hoffmann–La Roche AG, Inc). Amplification protocol was adapted following manufacturers guidelines and different annealing temperatures were used as indicated

in Table S3. All samples were run as technical duplicates along with the standard curve to reduce variability between assays. Plasmids used for the quantification curves were pNORM1 conjugative plasmid (Gat et al., 2017) for *intI*1, *sul*1, *qnr*S1, *tet*M, $bla_{TEM}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{OXA\text{-}58}$ and individual pUC19 plasmids for *mecA*, *tet*M and $bla_{OXA\text{-}58}$ (Laht et al., 2014; Szczepanowski et al., 2009; Tamminen et al., 2011). Quantification limits (LOQ) were established as the minimum amount of plasmid that could be detected without interference from the negative control, the corresponding values are reported in Supplementary Table S3. The quality criteria within the standard curve was a $R^2 > 0.99$, and a slope between −3.1 and −3.4. The accepted efficiency of the reactions ranged from 97% to 100%. Melting curves were obtained to confirm amplification specificity.

Copy numbers per gene were calculated by extrapolation from the standard curves, and expressed in relation to the processed grams of fresh weight. Prevalence values, understood as the fraction of the bacterial population harboring a given genetic element, was estimated as copies of the genetic element per million 16S rDNA copies (Supplementary Fig. S2 for absolute values). Statistical analysis and plots were performed in the R environment (version 3.6.1; http://www.rproject.org/). Normality and homogeneity of the variances were checked using the Shapiro-Wilk and Levene tests, respectively. Since data followed a normal distribution, an Analysis of Variance (ANOVA) followed by Tukey's B post-hoc correction for multiple tests was performed with the multcomp R package (Hothorn et al., 2016). Significance levels were set at $p \leq 0.05$.

### 2.4. Microbial population analysis by 16S rDNA sequencing

Bacterial communities present in the samples were examined by 16S sequencing analysis. Fertilizers and soil samples from all treatments were sent to Novogene Europe (Cambridge, UK). 16S rRNA genes of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5) were amplified using specific primers barcoding for amplicon generation. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Quality-checked PCR products were mixed at equal density ratios and purified by Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using NEBNext Ultra DNA Library Pre ®Kit for Illumina, following manufacturer's recommendations, and index codes were added. Sequencing was carried out on an Illumina platform and 400 bp paired-end reads were generated (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179685). Total reads per sample and other DNA sequencing quality parameters appear in Supplementary Table S4.

Clean Sequences were analyzed and associated to 201,182 ASVs (Amplicon Sequence Variants) using the DADA2 R package (Callahan et al., 2016). The SILVA database v128, formatted for DADA2, was used to provide taxonomic annotation (Quast et al., 2013). The number of taxa identified and the percentage of taxon coverage (fraction of ASVs annotated to each particular taxonomic level) are shown in Supplementary Table S5. β-diversity analysis were performed with QIIME (Version 1.7.0). The significance of difference among the structure of microbial communities was analyzed by a non-metric multi-dimensional scaling (NMDS) with the Bray–Curtis dissimilarity index (Bray and Curtis, 1957), using the *vegan* R package. Contributions of fertilizers to soil microbiomes were characterized using the FEAST (fast expectation-maximization for microbial source tracking) R package (Shenhav et al., 2019). Correlations between bacteria composition and AB resistance were tested by Spearman's correlations between ASV counts and ARGs and *intI1* abundance. The false positive discovery rate (FDR) correction was set at $p < 0.05$ (Benjamini and Hochberg, 1995), using the *psych* R package (Revelle, 2013).

### 2.5. Antibiotic determination in organic fertilizers

Antibiotic determination in the organic fertilizers used in both campaigns was performed as previously described (Berendsen et al., 2015; Sanz et al., 2021), using 500 mg of sample extracted by ultrasound assisted sonication (35 kHz) in McIlvain-EDTA buffer (pH = 4) and acetonitrile, followed by protein precipitation with lead acetate, centrifugation (3500 *g*) and solid-phase extraction (Strata X-RP, 200 mg / 6 ml, Phenomenex, Torrance CA, USA) clean-up. Chemical species determination was performed by Waters Acquity Ultra-Performance Liquid Chromatography™ System (Milford, MA, USA) coupled with Waters TQ-Detector (Manchester, UK), as described (Tadić et al., 2019). Only aggregated values for sulfonamides (sulfadiazine, sulfamethoxazole, sulfathiazole, sulfapyridine, sulfacetamide, sulfamethazine, sulfamethizole), tetracyclines (chlortetracycline, doxycycline, tetracycline, oxytetracycline) and fluoroquinolones (ciprofloxacin, enrofloxacin, ofloxacin) are presented here. Antibiotic concentrations are expressed in dry or fresh weigh weight basis and are corrected by deuterated surrogate standards (sulfamethoxazole-d4, enrofloxacin-d3, ofloxacin-d3) spiked to the initial sample. A detailed description of antibiotic content in these samples will be published elsewhere (Matamoros et al., in preparation).

## 3. Results

### 3.1. Soil microbiome changes associated to fertilization procedures

Ribosomal 16S rDNA sequencing analysis of soil and amendment samples identified 201,031 amplicon sequence variants (ASV), 83.6% of which were assigned at the level of Family and 59.5% at the level of Genus (Supplementary Table S5). Fig. 2 shows ASV relative compositions of the different samples, averaged by sampling and treatment, and color-labelled at the Phylum level.

The analysis showed a general common microbiome pattern for all soils, with a clear predominance of Proteobacteria, Actinobacteria, Bacteroidetes, and Gemmatimonadota, as well as a significant contribution of Firmicutes in some samples. In contrast, the three types of organic fertilizers showed very different Phyla distributions, both among them and between them and the soil samples. A significant fraction of Archaea appeared in sludge and slurry fertilizers, whereas Firmicutes predominate in OFMSW compost and slurry samples. Finally, Proteobacteria were only predominant in the sludge microbiome, the structure of which was the most similar one to the soil microbiome (Fig. 2). Giving the disparate taxonomic composition between soil and fertilizer samples, subsequent analyses of taxonomic diversity and distribution were restricted to soil samples.

ASV distribution among soil samples varied by both sampling time and the fertilizer used in each plot, with a strong interaction between the two factors (two-way ADONIS, Supplementary Table S6). Sub-setting the data by both factors (sampling and fertilizer used) further confirmed the relevance of both of them (one-way ADONIS, Supplementary Table S6). The analysis showed that mineral fertilization (which in principle did not represent any external addition of bacteria) altered ASV distribution significantly in all samplings, and that the effect of the different treatments was significant both for the pre-amendment Soil 2 and for the post-harvest Soils 1 and 3.

ASV distribution among samples was further analyzed using Non-Metric Dimensional Scaling (NMDS). The analysis only included ASVs representing at least 0.1% of reads in at least one of the soil samples (696 ASVs in total, Fig. 3). Most ASV grouped in the center of the NMDS plot, indicating their relatively uniform presence in most samples (Fig 3A). When the loadings of the different samples were plotted at the same scale (plot B), only the initial, untreated soil samples (red dots), the OFMSW-fertilized samples from Soil 1 and two groups from Soil 3 (OFMSW- and sludge-fertilized samples) appeared separated from the rest, indicating their differential ASV composition. To facilitate further discussion, these four groups have been labelled in Fig. 3B as S0, S1-OFMSW, S3-OFMSW, and S3-Sludge, respectively. Grouping samples from the same treatment resulted in a very poor separation of the groups, except for the initial soil samples (red dots, Fig. 3C). Finally, grouping samples by the sampling time resulted in relatively separated groups, particularly for the initial soil and the second post-harvest sampling (Soil 3, Fig. 3D). Taken together, these results indicate that the time of sampling (pre- versus post-harvest, fertilized versus non-fertilized soils), and not the type of fertilizer applied, appeared as the main driver of the soil microbiome composition.

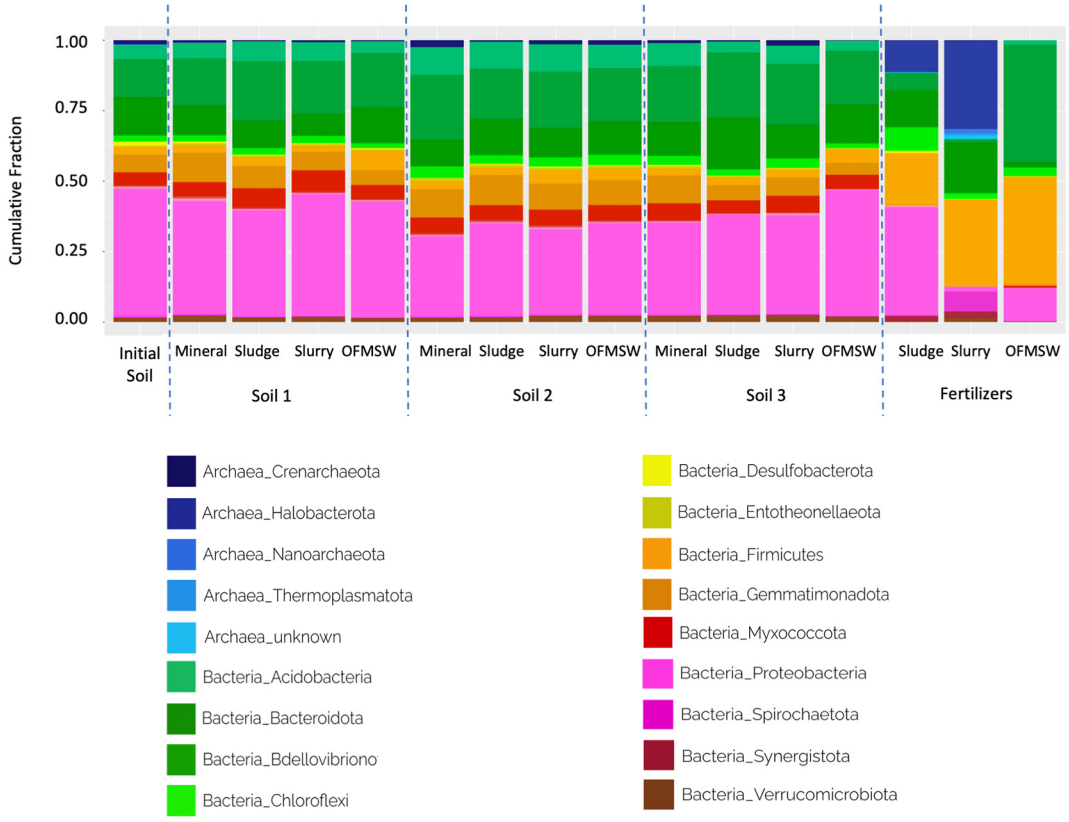


**Fig. 2.** Bacterial and Archaeal Taxon distribution among soil and amendment samples. Sectors correspond to the different Phyla identified in the samples. For sake of simplicity, assignation of the different color codes only includes predominant Phyla.

### *3.2. Contribution of fertilizers to soil microbiomes*

Microbial source-tracking analysis by FEAST showed a very low (below 1%) background level of coincidence between soil and fertilizer microbiomes (Fig. 4), being this figure particularly low for the slurry fertilizer (0.1% or less, Fig. 4). Both sludge and OFMSW fertilization increased the percentage of shared microbiomes to 1–3% in the receiving soils in both campaigns (Fig. 4, pink and red sectors), whereas soils fertilized with slurry only showed a modest 0.2–0.4% of microbiome overlapping with the fertilizer (Fig. 4). These increments appeared to be transient, as pre-amendment Soil 2 values were indistinguishable from those from the initial soil (Fig. 4). These results are consistent with the beta-diversity analysis of soil samples in Fig. 3, as all post-harvest samples shared a similar ASV composition, and that slurry-fertilized samples appeared as less differentiated from the initial soil than sludge- or OFMSW-fertilized ones. These results suggest that at least part of the observed differences in microbiome composition was indeed related to the direct contribution of fertilizer microbiome to the soils.

### *3.3. ARG loads in organic fertilizers, edible crop parts, and soils*

Organic fertilizers showed very high bacterial content, with 16S rDNA levels between $10^{10}$ and $10^{12}$ copies per g of sample (blue squares in Fig. 5A). The highest bacterial load corresponded to the pig slurry in both campaigns, whereas the OFMSW compost showed the lowest bacterial loads, one to two orders of magnitude below the values found for the other two organic fertilizers (Fig. 5A). The levels of *intI*1 and of the targeted ARGs showed a similar trend, with highest values for the slurry fertilizer samples and the lowest ones for OFMSW. The most prevalent genetic elements were *intI*1, *sul*1 and *tet*M, whereas $bla_{\text{TEM}}$ and $bla_{\text{OXA-48}}$ showed high levels in some samples (bars in Fig. 5A). The very high *tet*M loads in the slurry fertilizer was remarkable, although it is consistent with previous studies of ARG loads in different fertilizers derived from swine slurries (Sanz et al., 2021). The distribution of antibiotic concentrations in the different fertilizer samples was consistent with the corresponding ARG profiles, with non-detectable levels in OFMSW compost samples and maximum tetracycline levels in pig slurry samples (Fig. 5B). Note that *sul*1, *tet*M and *qnr*S1 confer resistance against sulfonamides, tetracyclines and fluoroquinolones, respectively.

The initial soil showed very low levels of all targeted genes, as only *intI*1 and *sul*1 were found at levels above quantitation (Fig. 5C, grey boxes). Addition of organic fertilizers significantly increased at least some ARG loads in Soil 1 and Soil3 samples relative to the initial, unamended soil, whereas the effect was only marginal for Soil 2 (pre-amendment samples, Fig. 5C).

Loads of the different genes varied according both the type of fertilizer used and the campaign. The pre-amendment Soil 2 showed essentially no effect of the type of fertilizer on ARG loads, except for $bla_{\text{CTX-M-32}}$ (Fig. 5C, lowercase letters within each graph). In both post-harvest soils (Soils 1 and 3), the lowest ARG levels corresponded to the chemically-fertilized samples in all cases in which significant differences were observed (Fig. 5C). The highest ARG loads corresponded to soils fertilized with slurry for Soil 1 samples, and to all three organic amendments for Soil 3 samples (Fig. 5C). Although making a direct correlation of these data (Fig. 5C) with the ARG loads present in the different organic fertilizers (Fig. 5A) is not straightforward, the overall ARG profiles in post-harvest soils could be related to the profiles of the corresponding fertilizers. The highest ARG loads corresponded to slurry-fertilized soils in both campaigns, consistent with the high levels of antibiotic found in the slurry-based fertilizer (Fig. 5C, orange boxes). In the case of *tet*M, its predominance in the slurry-fertilized soils reflected both the high prevalence of this ARG and the high levels of tetracycline in pig slurry (Fig. 5A–C). Finally, the only soil sample in which the levels of $bla_{\text{OXA-58}}$ were found above quantification limits was the Soil 3 sample fertilized with sludge from the 2nd campaign, coinciding with the high levels of $bla_{\text{OXA-58}}$ found in this particular fertilizer sample (compare Fig. 5A and C).

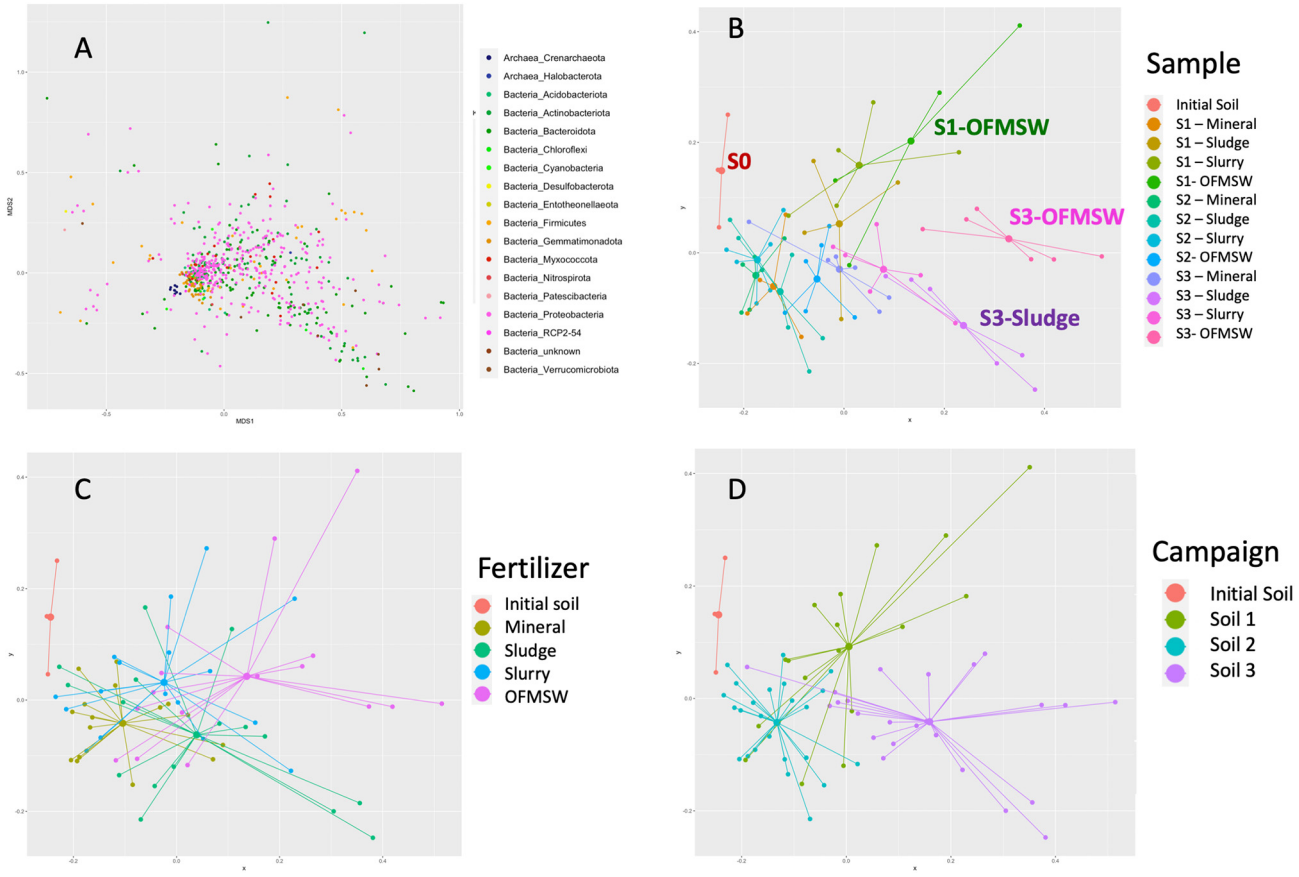


**Fig. 3.** Analysis of ASV distribution among soil samples. A) NMDS (non-metric dimensional scaling) analysis of the distribution of bacterial OTUs. Only OTUs with more than 100 reads in the total sequence effort were consider in the analysis. B–D) Site distribution in the NMDS analysis. Samples were color-coded by sample (B), sampling campaign (C) or type of fertilizer used in the amendment (D). Centroids for each group are indicated as thicker dots.

ARG analysis only detected *intI*1, *sul*1, and *tet*M in lettuce leaves, and only *intI*1 and *sul*1 in radish edible roots (Fig. 5D). ARG loads were in general higher in the first sampling (corresponding to Soil 1 samples) than in the second one (Lettuce1/Radish 1 for Soil 1, Lettuce 2/Radish 2 for Soil 3, Fig. 5D), a trend also observed in soil samples for these particular genes (compare Fig. 5C and D). In fact, the distribution of *intI*1, *sul*1, and *tet*M among lettuce and, in a lesser extent, radish samples roughly reflected the ARG profiles of both soils and fertilizers, with highest levels corresponding to soils and plants fertilized with slurry, and the lowest levels, when significant, to mineral and OFMSW-fertilized ones (compare Fig. 5A, C, and D).

### 3.4. Correlation between ARG loads and bacterial taxa distribution in soils

Correlation analysis identified 717 ASVs whose relative abundance strongly correlated (either positive or negatively) with the prevalence (copies relative to 16S rDNA copies) of *intI*1 or at least one of the analyzed ARGs (Pearson correlation, $p \leq 0.01$, fdr correction, Fig. 6). Hierarchical

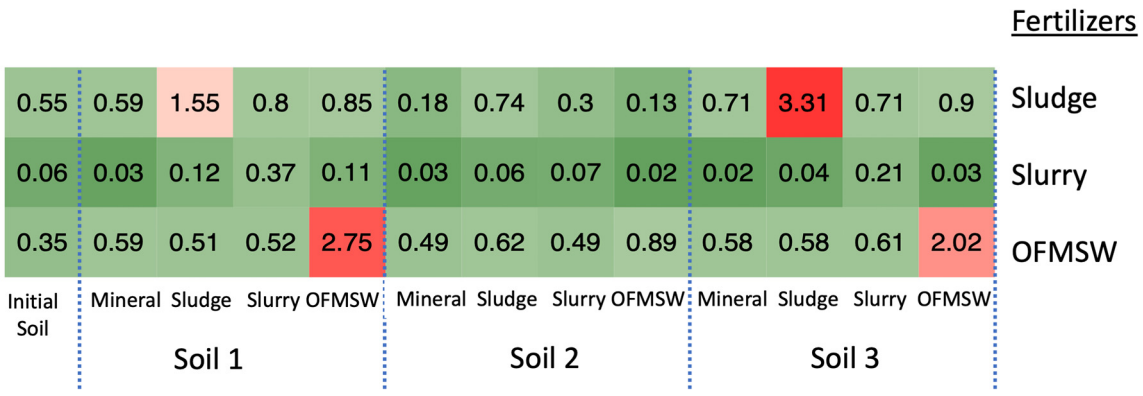


**Fig. 4.** Microbial source-tracking analysis by FEAST. Figures in the graph correspond to the percentage of soil microbiomes (columns) attributable to each fertilizer (rows). Color scale from green to red indicates lower to higher contributions, respectively. Note that the algorithm makes no assumption about the fertilizer used in each soil, so most of the cells show background microbiome coincidence values.

clustering revealed a strong taxonomic dependence of the ARG distribution. Only three Phyla (Actinobacteria, Myxococcota and Proteobacteria), plus a single Bacteroidota Family (Sphingobacteriales) included most of the ASV showing positive correlation with ARGs, whereas Acidobacteria, most Bacteroidota, and Gemmatimonadota showed an equally strong negative correlation with ARG prevalence (Fig. 6, top color bar). In addition to the case of Sphingobacteriales in the Bacteroidota Phylum, other bacterial Families showed specific correlation patterns differentiated from the other groups from the same Phylum, as Pseudomonadales in the Proteobacteria Phylum or Corynebacteriales and Micrococcales in the Actinobacteria Phylum (Fig. 6, labels at the bottom of the heatmap). Clustering analysis showed a very similar distribution for *intI*1 and *sul*1 among the different taxonomic groups (Fig. 6), suggesting a physical linkage between these two genes in a substantial fraction of bacterial cells. A similarly close distribution was observed for *qnr*S1, $bla_{TEM}$, and $bla_{CTX\text{-}M\text{-}32}$, on one side, and for *tet*M and $bla_{OXA\text{-}58}$ on the other (Fig. 6), although in these cases their potential physical linkage is purely hypothetical and would require further analyses.

The relationship between ASV abundance and ARG prevalence can be better analyzed when classifying ASVs according their different distribution among samples. ANOVA analysis identified 1139 ASVs with differential distribution among fertilized soil samples, which could be classified into two clusters by PAM clustering: Cluster A (363 ASVs) and Cluster B (776 ASVs, Fig. 7, left panels). Analysis of the distribution of the components of each of the two clusters revealed a strong dependency on both the sampling campaign and the fertilizer used in each case. Within each campaign, organically-fertilized soils showed higher proportion of ASVs from Cluster A than their chemically-fertilized counterparts, which were correlatively enriched in ASVs from Cluster B. This effect was much stronger in both post-harvest samples (Soil 1 and Soil 3) than in the pre-amendment Soil 2 samples (Fig. 7). The three sample groups with the highest proportion of ASVs from Cluster A corresponded to OFMSW-fertilized Soil 1 and Soil 3 samples and to the sludge-fertilized Soil 3 samples, the same samples identified as more diverse in ASV composition by NDMS (groups labelled as S0, S1-OFMSW, S3-OFMSW, and S3-Sludge in Fig. 3).

Correlation analyses revealed that ASVs from Cluster A showed, in general, a positive correlation with prevalence values for different ARGs (red sectors in the left panels in Fig. 7), whereas most (but not all) ASVs in Cluster B showed a negative correlation (blue sectors in the left panels in Fig. 7). The color bars at the top of each heatmap indicate the different taxa composition of the two clusters: Cluster A was mainly integrated by Actinobacteroidota and Proteobacteria, with minor contributions of Firmicutes and Mixococcota, whereas Cluster B showed a much more complex mixture, including Acidobacteroidota, Actinobacteroidota, Bacteroidota, Gemmatimonadota, and different Alpha and Gamma Proteobacteria (Fig. 7). We conclude that Cluster A included ASVs whose relative abundance in soil depended on the contribution of the fertilizer's microbiome, and that this contribution implicated higher ARG loads. Following the same reasoning, Cluster B included ASVs present in the soil and with a generally low prevalence of the studied ARGs.

## 4. Discussion

Urban solid waste, sewage sludge, and animal manure are among the most commonly used sources of organic amendments in agriculture (Urra et al., 2019). Our comparison between these three sources indicated that the nature of the fertilizer used in each was less important than the process itself, with the result that the final microbiome composition was very similar irrespectively of using mineral, anaerobically digested WWTP sludge, untreated slurry, or composted OFMSW. The results also suggest a relatively stability in the soil microbiome composition, irrespectively from

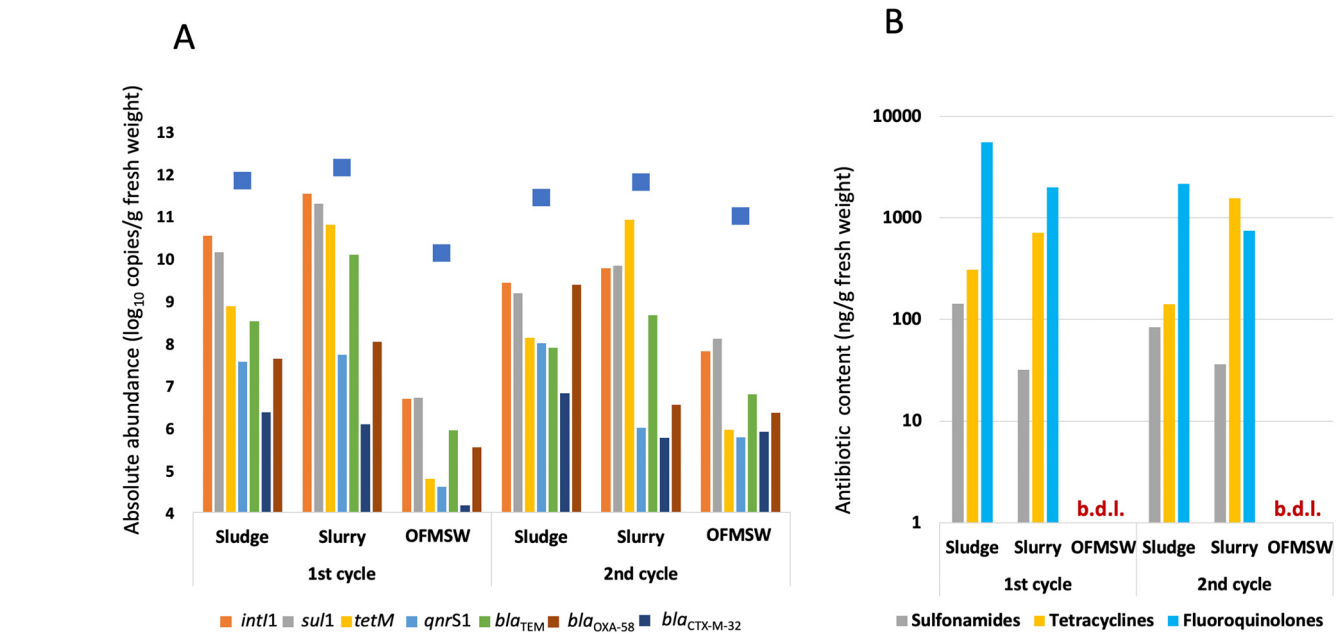


**Fig. 5.** ARG distribution among soil, fertilizers and crop samples. A) Abundance of the targeted genes in fertilizers, expressed as copies per gram of fertilizer (logarithmic transformants). Blue squares indicate copies of 16S rDNA per gram of fertilizer, as indicative of the total bacterial abundance, and represented at the same scale. B) Antibiotic content of the different fertilizers used in this work, expressed in ng/g (fresh weight) of aggregated amounts for each antibiotic family (Sulfonamides, Tetracyclines and Fluoroquinolones). "bdl", below detection levels C) Absolute loads for the different genetic elements in soil samples, expressed as copies per g of soil in logarithmic scale. Thick black lines, boxes and whiskers represent the median, first-to third percentiles and total distribution of samples, respectively. Grey, cyan, brown, orange, and magenta boxes represent the initial soil and soils fertilized with Mineral, Sludge, Slurry, or OFMSW-based fertilizers, respectively. Low-case letters at the top of the graph indicate statistically different distributions (ANOVA + Tukey's B test). Note that ANOVA was performed for each set of soil samples independently (vertical blue dotted lines). "n/s" indicates no statistical difference between genetic element distributions. D) Analysis of the distribution of the different genetic elements in lettuce (top) and radish (bottom). Color-codes and statistical analyses as in Fig. 5A. Only genetic elements detected above LOQ in at least one of the sample groups are included in the graphs.

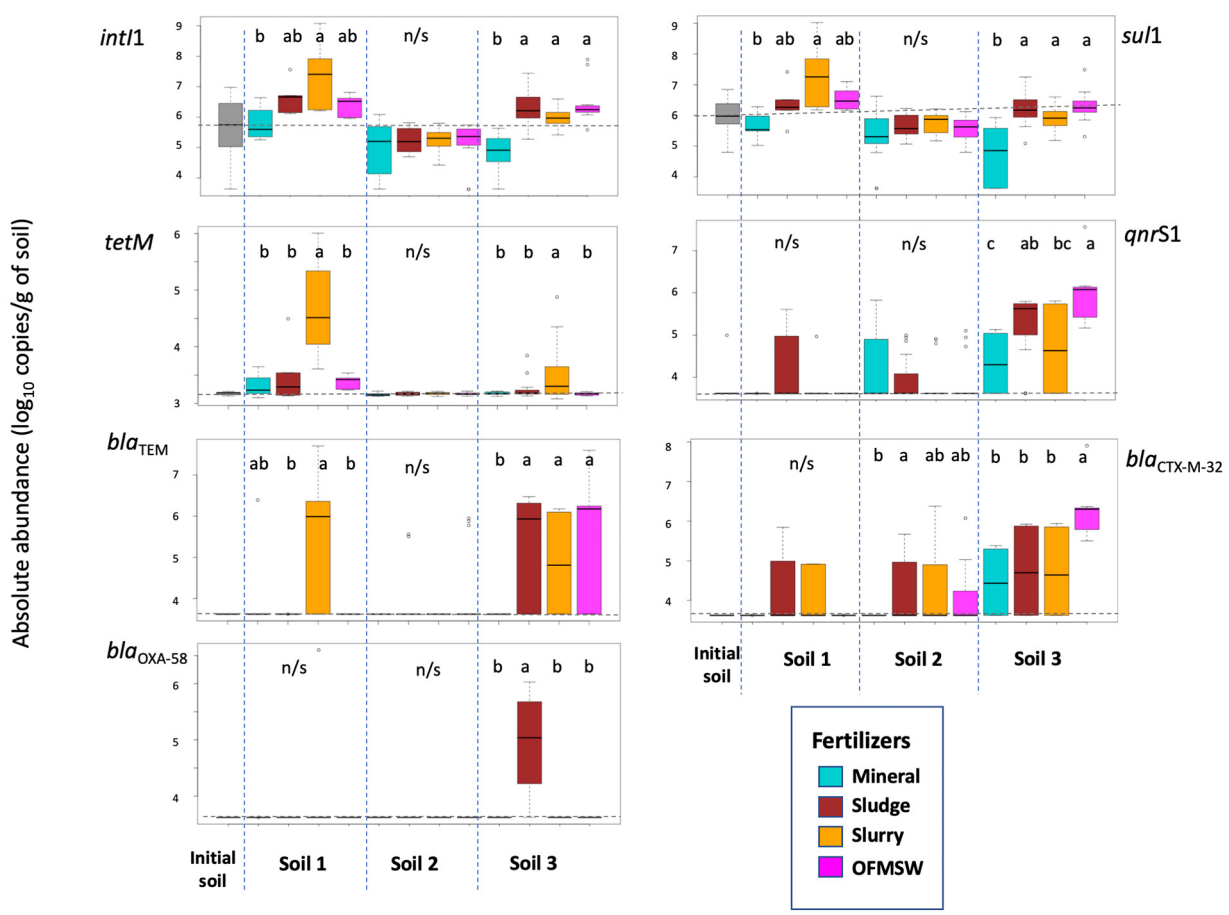


**Fig. 5** (*continued*).

seasonal variations (the whole experiment covered about a year) and from singular weather events, like stormy events or high temperature periods. This notwithstanding, the use of the highly sensitive ASV sequence identification allowed us to identify a small, but significant contribution of fertilizer microbiomes to the receiving soils, indicating a moderate persistence of fertilizer microbiome in soil conditions for relatively long periods of time (months, at least.)

Our data shows that ARG loads were generally higher in amended soils than in the initial sample, and that both ARG total loads and ARG profiles depended on the fertilized used and on the time of sampling. Pre-amended soil samples, either from the initial soil or from those taken immediately before starting the second agricultural cycle, showed relatively low ARG loads, and, in the case of Soil 2, essentially no influence from the type of fertilizer used in the previous campaign. Therefore, we concluded that, whereas soil microbiomes stayed relatively stable all through the year-round survey in amended soils, ARG loads in soils appeared as unstable and transient. ARG loads seemed to depend on a combination of the persistence of the fertilizer effect and the input of ARG-harboring bacteria from the different amendments. Soils amended with mineral fertilizer showed higher ARG loads than the original soil in some cases, probably indicating proliferation of ARG-harboring soil bacteria as a consequence of the process of amending itself, as previously reported (Nõlvak et al., 2016; Sun et al., 2019). However, this effect can probably explain only a minor fraction of the total increase in ARG loads, as their profiles varied according the type of amendment and the harvest cycle. In almost all cases, soils amended with inorganic fertilizer (and therefore, with no ARG added in the process) showed the minimal ARG loads within a given sampling campaign.

ARG loads in soils appeared linked to the relative abundance of a small group of bacterial taxa, particularly Actinobacteriota, and Delta- and Gamma-Proteobacteria. These groups were also linked to higher ARG loads in fertilizers based on swine slurries and on their digestates (Sanz et al., 2021). In addition, different Gammaproteobacteria families (Xanthomonadales, Pseudomonadales, Enterobacteriales) appeared linked to a high ARG prevalence in commercial agricultural soils, plants, and fruits (Cerqueira et al., 2019c; Fogler et al., 2019). As most of these bacterial groups have representatives in both swine and human gut, as well as in soil microbiomes, they may represent significant vectors for the spread of antibiotic resistance from animal farms to human populations via fresh food products

ARG loads in crops were relatively low in all cases, particularly in radish samples, although both *intI*1 and *sul*1 in lettuces and radish, and *tet*M in lettuces showed significantly high loads when pig slurry was used as fertilizer, particularly in the first harvest. The differences in ARG profiles between the two species may be related to the particular ARGs we are analyzing in this work, which were chosen according their clinical relevance and the availability of robust analytical protocols to quantify the corresponding antibiotic families in the fertilizers. Previously published shot-gun analyses of resistomes from these two species revealed similar relative ARG loads, but showing different profiles (Fogler et al., 2019). However, the technique used for this particular study did not allow a precise absolute quantitation of ARG loads (sequences per gram of tissue) in the samples. In general terms, we observed a consistent pattern in ARG loads in the three compartments analyzed (fertilizers, soils and crops), in which *intI*1, *sul*1, and *tet*M reached their maximum loads in slurry or slurry-fertilized samples, and particularly in the first campaign. This suggests that pig slurry-based amendments have a strong potential for influencing ARG loads of soils and crops, consistently with previous data on manure fertilization (Sanz et al., 2021; Xie, Yuan et al., 2018; Zhou, Qiao et al., 2019), and despite the limited capacity of colonization of soils shown by the slurry microbiome. Our data is also consistent with previous results that ARG in soils can translocate

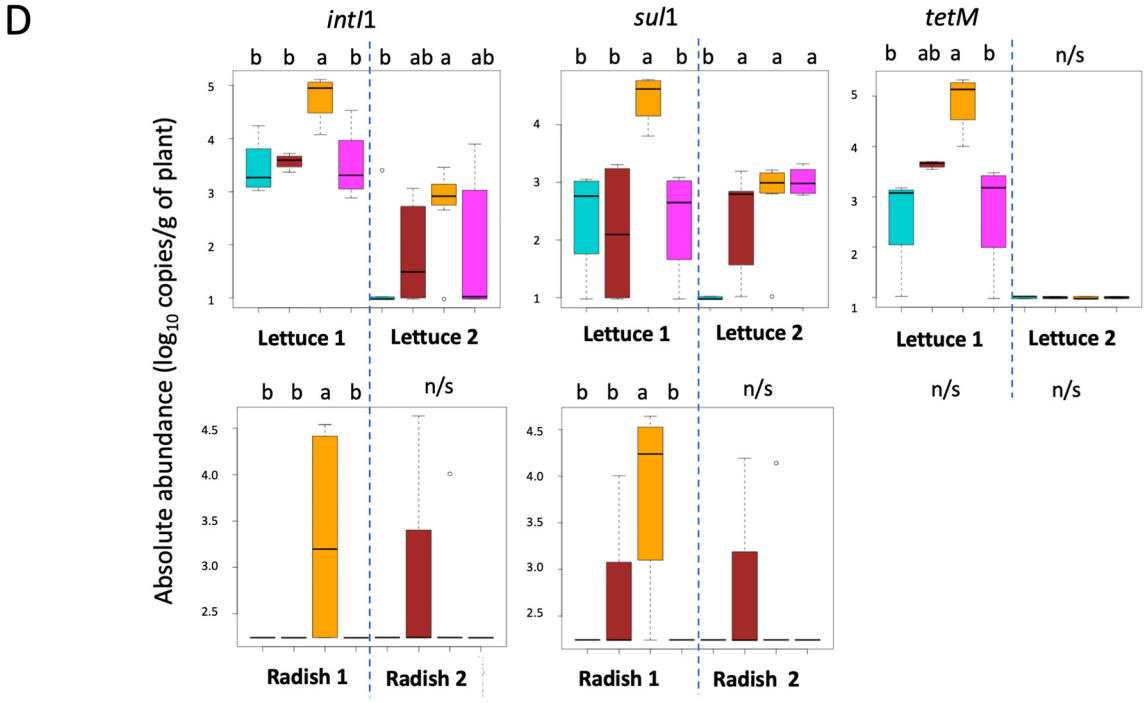


**Fig. 5** (*continued*).

to edible plant parts, and that lettuces are particularly prone to present comparatively high levels of both ARGs, antibiotics, and antibiotic transformation products (Cerqueira et al., 2019c; Christou et al., 2018; Domínguez et al., 2014; Gao et al., 2020; Margenat et al., 2017; Tadić et al., 2020).

There are many indications that soil resistomes are the main driving factors of ARG loads in food products, and that their composition may be affected by agricultural practices, particularly by the application of organic fertilizers (Cerqueira et al., 2019c; Muurinen et al., 2017; Piña et al., 2020). The results presented here are consistent with this interpretation, despite the relative minor fraction of fertilizers' ASVs found in the receiving soils' microbiomes. Furthermore, soil and food ARG loads appeared to be highly dependent on the fertilizer used in each case and on its ARG loads. Fertilizer's ASV contribution to soil microbiomes and ARG levels in soils showed strong and comparable temporal variations, suggesting a link between the two parameters. We propose that fertilizers, rather than the original soil microbiomes, were the main source of clinically-relevant ARGs found in foods. This implicates that a strict control on the ARG loads in the initial waste used to produce the fertilizer will be essential for the minimization of the risk of their spreading to soils and crops (Muurinen et al., 2017). We hope our results will be useful for developing more efficient and safer agricultural management frameworks to regulate the type, dose, and timing of application of the different organic fertilizers, and as a support for a general implementation of protocols limiting the use of antibiotics in pharmaceutical and veterinary practices (Muurinen et al., 2017; Sneeringer and Clancy, 2020).

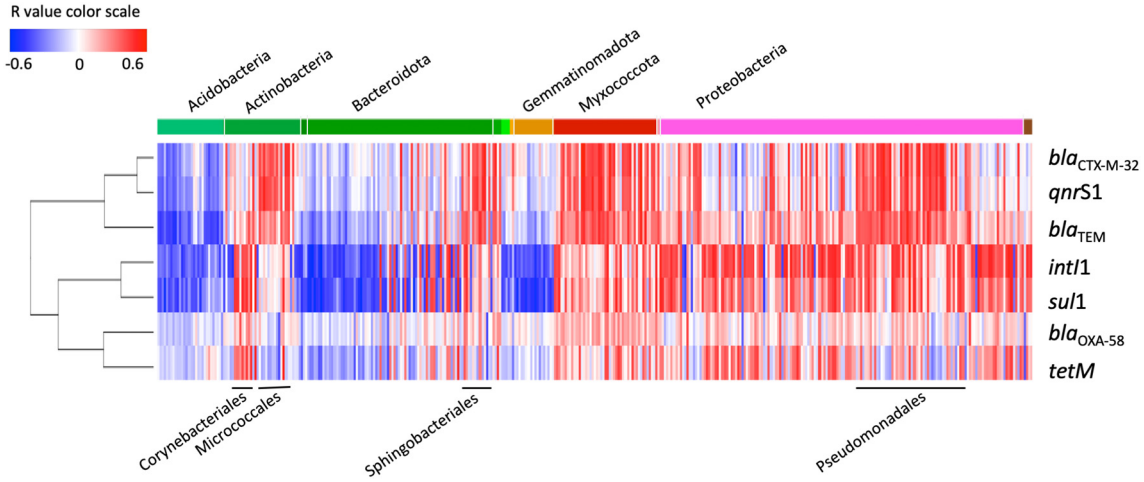


**Fig. 6.** Correlation analysis (Pearson) between ASV abundance and genetic element prevalence (copies per 16S rDNA copy) among soil samples. Initial soil samples were excluded from the analysis. Only AVS with significative correlation with at least one genetic element are represented. Red and blue sectors correspond to positive and negative correlations, respectively (color scale on top). ASVs are ordered taxonomically, as indicated by the color bar at the top of the heatmap, which follows the same color-code as in Fig. 2. Names of some relevant Phyla are indicated at the top; names of some bacterial families are also indicated at the bottom of the graph. Genetic elements were grouped by hierarchical clustering.

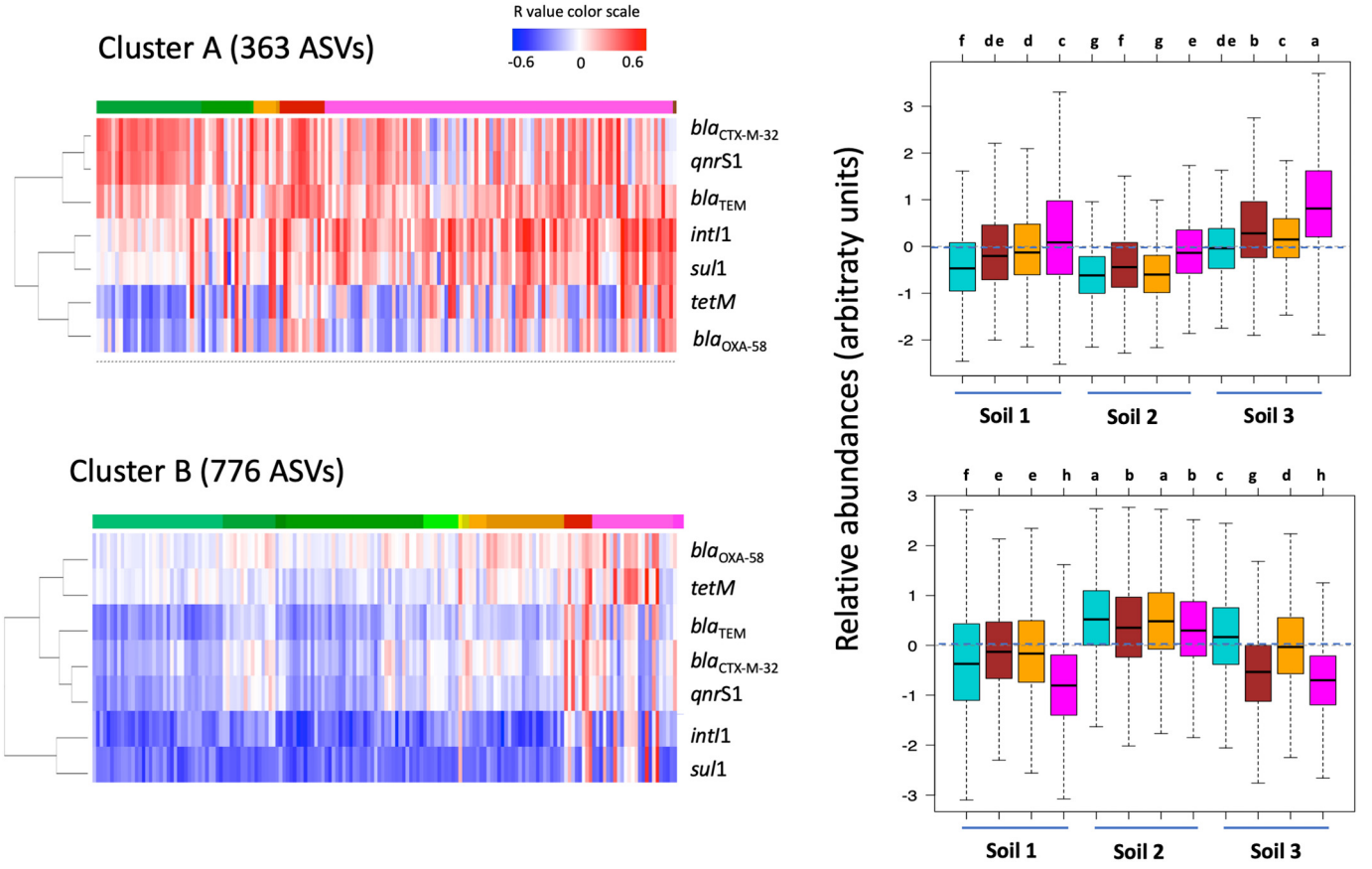


**Fig. 7.** PAM clustering analysis of ASV distribution among soil samples (initial soil samples excluded). The correlation maps on the left show the correlations of the different ASVs in each cluster with the different genetic elements. As in Fig. 6, ASVs were grouped taxonomically (color bar on the top, same code as in Fig. 2). Positive and negative correlations are indicated by red and blue sectors, as in Fig. 6. Boxplots on the right indicate distribution of ASVs included in each cluster (normalized values) in each sample set. Boxes are colored as in Fig. 5. Low-case letters at the top of the graph indicate statistically different distributions (ANOVA + Tukey's B test), using the whole dataset.

## CRediT authorship contribution statement

**Claudia Sanz:** Investigation, Methodology, Formal analysis, Validation, Writing - original draft. **Marta Casado:** Investigation, Methodology. **Laia Navarro-Martin:** Supervision, Validation. **Núria Cañameras:** Conceptualization, Investigation, Resources. **Núria Carazo:** Conceptualization, Investigation, Resources. **Victor Matamoros:** Conceptualization, Investigation, Resources, Project administration, Writing - review & editing, Funding acquisition. **Josep Maria Bayona:** Conceptualization, Project administration, Writing - review & editing, Funding acquisition. **Benjamin Piña:** Conceptualization, Supervision, Formal analysis, Software, Visualization, Project administration, Writing - review & editing, Funding acquisition.

## 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.scitotenv.2021.151973.

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