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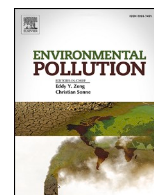

Bacteriological safety and quality of composted products from animal, urban or sewage sludge wastes

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ABSTRACT

This study investigated the presence of culturable bacterial pathogens, and antibiotic resistance and associated genes (quantitative PCR) in commercially available composted products from animal excrements or manure (n = 7), urban wastes (n = 1) or (sewage sludge) (n = 1). Metals quantification and 16S rRNA-based bacterial community composition analyses supported the results to infer potential risks to downstream environments (e.g., soils). *Bacilli* and *Actinomycetes* were the dominant bacterial classes in seven composts, while two were dominated by different classes of *Pseudomonadota* or the class *Bacteroidia*. *Salmonella* spp. was not detected in all composts, meeting recommended quality criteria, while *Escherichia coli* and *Listeria monocytogenes* were only detected in the sewage sludge compost. The antibiotic resistance genes *ermB* and *ermF* were detected in most of the composts, and the antibiotic resistance gene *sul1* and the *intI1* gene (proxy for antibiotic resistance recombination) in all composts in the range of 6–9 log gene copy number/g dry weight. *Listeria* spp. and the gene $bla_{CTX\text{-}M}$ were detected only in chicken/poultry composts suggesting increased risk. All composts, except the urban waste compost, presented at least one metal (zinc, copper, and/or cadmium) above the recommended value. The genes *uidA*, *crAssphage*, *ermB* and $bla_{CTX\text{-}M}$ were negatively correlated with the abundance of total heterotrophs and moisture content, and the genes *intI1* and *sul1* were negatively correlated with the concentration of the metals Cr, Ni and Pb. Overall, the urban waste compost presented the best quality, exhibiting the lowest antibiotic resistance load. These findings alert for the fact that composts may contribute to the dissemination of antibiotic resistance, highlighting the need of regular assessment. It is suggested that multiple factors, including the raw materials, may influence the safety of the final compost, and the knowledge of the variables affecting compost safety need to be thoroughly investigated and understood.

1. Introduction

Composting is an ecofriendly method based on microbial biotransformation, to recycle manure and other organic residues. Composting reduces volume and odour emissions, eliminates pathogens and weed seeds and, above all, releases nutrients for soil amendment, being an excellent organic fertiliser (Keener, 2011; Larney et al., 2006; Onwosi et al., 2020). For this reason, composting is considered a cost-effective and safe process enabling the reuse of nutrients from organic residues. Not only composts of animal origin, but also composts of urban organic wastes (domestic biowaste and sewage from wastewater treatment plants) are increasingly being used as organic fertilisers (Gilbert and Siebert, 2022). In Europe, a goal has been set for 65% of municipal solid waste to be composted or digested (by anaerobic digestion) by 2035 (ECN, 2022; Gilbert and Siebert, 2022). The increase in the production of composts and its consequent availability on the market, may be part of the solution for combating nutrient depletion and erosion observed in agricultural soils (JRC-EC et al., 2016; Scotti et al., 2015). While using composts as fertilisers is part of the soil protection and green deal strategies (JRC-EC et al., 2016), it is increasingly important to assess quality and safety determinants, which monitorisation and control contribute to effective and sustainable uses. A safe compost should not apport to the fertilised soil substances that may contaminate the soil or plants and eventually reach the humans via the food chain. The European and Portuguese legislation define limits for the concentration of metals (mg/kg dry matter: cadmium – 0.7; chromium - 100; copper -

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100; lead – 100; mercury – 0.7; nickel - 50; zinc – 200) and levels of human pathogens (*Escherichia coli* – < 1000 Colony Forming Units (CFU)/g, and *Salmonella* spp. – absence in 25 g) allowed in a final compost to be commercialised as fertiliser (DL103/2015, 2015; EU_2019/1009 and PARLIAMENT, 2019). However, other contaminants, such as antibiotic resistant bacteria or antibiotic resistance genes, whose self-replicative character is recognised in nutrient-rich environments, are not regulated.

The studies addressing the persistence of antibiotic resistance genes in composting products have often reached contradictory results (Ezugworie et al., 2021; Liu et al., 2021). A major explanation may be the high limits of detection of rare genes in soil or complex matrices. It has been shown that it is necessary to have a load as high as 2 log CFU of antibiotic resistant bacteria and 4 log of antibiotic resistance gene copies per gram of dry weight of soil/compost for those to be detectable (Fortunato et al., 2018). Therefore, the occurrence of antibiotic resistance genes in composts may be frequently below the detection capacity, using state-of-the-art methodologies. Moreover, as a man-made process based on natural microbial activity, it is influenced by multiple biotic and abiotic factors, being difficult to predict the fate of some genes or microorganisms. For example, the quality of the final product may be influenced by the combination of variables such as the operating conditions, the matrix and its physic-chemical characteristics (e.g. temperature, pH, moisture content, presence of chemical contaminants such as antibiotics or metals), the structure of the bacterial community, or the nature and potential mobility of the antibiotic resistance genes (Liu et al., 2021).

This study aimed to investigate the occurrence of antibiotic resistance genes and culturable bacterial pathogens in final composts produced from animal manure, urban wastes and sewage sludge, and to infer if their occurrence could be associated with the raw material, the degree of metal contamination, the microbial load or the composition of the bacterial community.

2. Materials and methods

2.1. Composts sampling

The composts investigated (n = 9) in this study were obtained from the retail market (50 kg bags) and stored at ambient temperature. Some were no longer available for sale since they had passed the expiration dates (Table 1). However, we considered that their inclusion in the study would provide an interesting overview of the stability of these products and the type of parameters that may influence the quality of composts. Composts were of animal origin (CA to CG, n = 7), urban wastes (CH, n = 1), and urban sewage sludge (CI, n = 1). All composts were labelled as distinct brands, except CA and CB, which had the same brand and different forms (powder and pellets, respectively). For each compost, a 1 kg composite sample was prepared from 10 fractions of ~100 g, carefully homogenised. Sub-samples were collected from these composite samples for metals quantification (one aliquot of 200 g) and microbiological analyses (three aliquots of 200 g each). The dry weight was determined by drying six pre-weighted compost aliquots of approximately 1 g at 70 °C until constant weight. The compost samples were stored at ambient temperature until further processing for metal and microbiological analyses.

2.2. Metals quantification

The content of metals (copper, zinc, cadmium, chromium, nickel, lead, and mercury) was determined by atomic absorption spectrometry (AAS), after digestion of 0.2 g (dry weight) of sample with HNO_3 (67–69%, vol/vol) and H_2O_2 (30%, vol/vol). Copper and zinc were determined by flame AAS; cadmium, chromium, nickel and lead with electrothermal AAS; and mercury with cold vapor AAS, as recommended by the equipment producer (PerkinElmer). These analyses were outsourced to Center for Quality and Food Safety (CINATE), an accredited laboratory (ISO/IEC17025:2018, 2018).

Table 1
Composts included in the study, their form, compositions and moisture content.

Sample ID	Form	Composition	Years after production (year of production)[b]	Moisture content (%)[a]
CA	powder	75% animal organic matter (**horse**), 25% plant organic matter	~2 y (2021)	53.0
CB	pellets (4 mm)		~2 y (2021)	10.8
CC	pellets (3 mm)	dried **poultry** manure, animal by-products: Peruvian guano, phosphate guano, feather meal, beet vinasse, kieserite	~5 y (2018)	8.9
CD	pellets (5 mm)	60% **sheep** manure, 20% **horse** manure and the rest from extensively produced **poultry**	~1 y (2022)	34.9
CE	pellets (3 mm)	feather meal and **chicken**-dried manure	~16 y (2007)	14.5
CF	pellets (4 mm)	100% **animal** excrement, manure and effluents	~17 y (2006)	23.6
CG	pellets (3 mm)	organic matter	unknown	14.5
CH	powder	waste from **forest** exploitation and **food waste** (meat/fish, fruit/vegetables, dairy products, bakery, etc.)	<1 y (2023)	20.9
CI	powder	**sludge** from an urban water treatment plant (50%) and calibrated **pine** bark (50%)	<1 y (2023)	59.6

Note: Composts CA and CB were from the same brand, with different forms (powder and pellets).
[a] Determined in this study, based on dry weight determination.
[b] For the composts that did not have information about the year of production (CA, CB, CC, CE), that was estimated based on the expiration date, frequently defined as 1 year after packaging.

2.3. Culture-based microbiological analyses

These analyses included the enumeration of total viable microorganisms at 37 °C and 22 °C, *Bacillus cereus*, *Listeria* spp., *Listeria monocytogenes*, *Escherichia coli*, yeasts and moulds, and the detection of *Salmonella* spp., *Listeria* spp., *Listeria monocytogenes*, and sulfite-reducing *Clostridium* spores.

For microbial enumeration, 25 g from each 200 g aliquot of compost were transferred, in triplicate, into new sterile bags, suspended in 225 mL Buffered Peptone Water (BPW; Biokar diagnostics) and homogenised by hand for 1 min. Appropriate decimal dilutions of each sample were prepared in Ringer's solution (Biokar diagnostics) and plated onto the respective culture media: total viable microorganisms on Plate Count Agar (PCA, Biokar diagnostics), incubated at 37 °C or 22 °C for 72 h (ISO4833-1, 2013); *Bacillus cereus* on Mannitol Egg Yolk Polymyxin Agar (MYP, Oxoid), incubated at 30 °C for 18–24 h (or 48 h, if negative after 24 h) with typical colonies confirmed by the presence of hemolysis (ISO7932, 2004); *Listeria* spp. and *L. monocytogenes* on Agar Listeria Ottavani & Agosti (ALOA, bioMérieux), incubated at 37 °C for 48 h (ISO11290-2, 2017); *Escherichia coli* on Tryptone Bile X-glucuronide agar (TBX, Biokar diagnostics), incubated at 44 °C for 24 h (IPQ4396, 2002); yeasts and moulds on Rose Bengal Agar (Oxoid), incubated at 25 °C for 5 days (ISO3277-1, 1987).

For the detection of bacteria expected to have a low abundance, enrichments were performed in prepared BPW (Biokar diagnostics) bags for *Salmonella* spp. or half-Fraser broth (Merck) for *Listeria* spp. and

L. monocytogenes (25 g, in triplicate, in 225 mL half-Fraser broth, homogenised by hand for 1 min). For the detection of *Salmonella* spp., after pre-enrichment in BPW for 24 h at 37 °C, 0.1 mL was transferred to Rappaport-Vassiliadis Soya Peptone Broth (RVS, bioMérieux), and 1 mL to Mueller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn, bioMérieux), and incubated at 41.5 °C and 37 °C, respectively, for 24 h (ISO6579-1, 2017). For the detection of *L. monocytogenes* and *Listeria* spp., after pre-enrichment in half-Fraser broth for 24 h at 30 °C, 1 mL was transferred to new Fraser broth tubes and incubated at 37 °C for 24 h (ISO11290-1, 2017). After inoculations into appropriate selective culture media, typical colonies were confirmed according to the respective ISO standard protocols. The detection of sulfite-reducing *Clostridium* spores was performed on a base culture medium with added sodium sulfite and ammoniacal iron solutions, incubated at 37 °C for 1–5 days (IPQ2262, 1986).

2.4. DNA-based microbiological analyses

These analyses included the bacterial community metabarcoding based on the sequencing of 16S rRNA gene amplicons and the quantification of genes associated with selected bacterial groups, human faecal contamination and antibiotic resistance. Total DNA was extracted, in triplicate, from 0.25 g (wet weight) of sample, using the DNeasy® PowerSoil® Kit (Qiagen), according to the manufacturer's instructions. DNA concentration was determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The bacterial communities were analysed based on the sequencing of the region V3-V4 of 16S rRNA gene amplicons defined by the primers 341F and 806R. Amplicons were sequenced using an Illumina paired-end platform to generate 250-bp paired-end raw reads that were merged with FLASH (V1.2.11), quality filtered using fastP (Version 0.23.1), and chimeric sequences removed with UCHIME algorithm (Novogene, UK). QIIME2 software was used for the taxonomic annotation of the amplicon sequence variants (ASVs) against the small-subunit (SSU)-rRNA SILVA v.138 database (http://www.arb-silva.de/). The annotation was manually curated considering the LPSN database (https://lpsn.dsmz.de/; February 15, 2024) (Parte et al., 2020). The coverage index (Good, 1953) of the libraries ranged 98.5–100%, confirming an adequate representation of the samples' total diversity (Table S2). The 16S rRNA gene sequences were deposited in the NCBI SRA archive under BioProject number PRJNA1131838.

Quantitative PCR (qPCR), using the SYBR method (StepOnePlus™ Real-Time PCR System, Thermo Fisher Scientific), was used to determine the abundance of total and selected groups of bacteria, and genes, as described in Table S1. Total bacteria and members of the different taxa - phyla *Actinomycetota* and *Bacillota*, classes *Betaproteobacteria* and *Gammaproteobacteria*, were quantified based on the highly conserved or taxa-specific regions of the 16S rRNA gene, respectively. Seven genes associated with human faecal contamination (*uidA*, crAssphage), antibiotic resistance (*sul1*, $bla_{CTX\text{-}M}$, *ermB*, and *ermF*) and genetic recombination (*intI1*) were quantified in the same DNA extracts. Those genes were previously described as good biomarkers for the monitoring of environmental contamination with antibiotic resistance (Teixeira et al., 2023). Gene copy numbers were determined by interpolating the Ct values using standard curves constructed with known concentrations of the target gene. The standard curve methodology was implemented as described in Brankatschk et al. (2012), and the quality criteria for accepting qPCR measurements were the previously described: standard curve efficiency of 90–110%; differences between technical replicates <0.5 cycles in Ct values; observation of a single peak and melting temperature; and absence of increased baseline signal (shoulders) (Rocha et al., 2020).

2.5. Statistical analysis

Bacterial enumeration, genes quantification and metals results were expressed as quantitative units per compost dry weight (Table 1), while bacterial community analysis data was expressed as relative abundance of the number of reads of a *taxon* per total reads number. The comparison of the relative abundance of bacterial taxa among multiple samples was performed using One-way ANOVA with Tukey-Kramer post hoc test, and for two groups comparison the two-sided Welch's *t*-test (confidence of $\geq$99%), using the software STAMP (Parks et al., 2014). The statistical analysis to compare alpha-diversity indexes, and the qPCR quantifications of the different genes was performed using One-way ANOVA followed by Tuckey post hoc test ($p < 0.01$), using the software IBM SPSS (version 28). Analyses aimed at comparing multiple variables were performed using principal component analysis (PCA) and canonical correspondence analysis (CCA) with the software Canoco 5 (Ter Braak and Šmilauer, 2012), and Pearson correlation analysis with GraphPad Prism version 10.2.1 for Windows, with a confidence interval of 99%.

3. Results

3.1. Characterisation of the composts and their quality

The composts analysed in this study (Table 1) varied in the type of raw materials used, specifically, horse organic matter (CA, CB), chicken/poultry manure (CC, CE), a mix of excrements and manure from different animals (CD, CF, CG), urban organic, mainly food, wastes (CH), and sewage sludge from municipal wastewater treatment (CI) (Table 1). The technology applied to produce the compost, in the form of powder (n = 3) or pellets (n = 6), seemed to be the variable most influencing moisture content (ranging from 20.9 to 59.6% or 8.9–34.9%, respectively), although the age might have also affected this parameter. This latter is particularly relevant for sewage sludge compost CI, sampled before packaging (Table 1).

The metals copper and zinc presented the highest values, ranging 50–174 and 158–865 mg/kg dry weight, respectively (Table 2). These values were maximal in composts CF and CI, both with copper and zinc contents above the maximum admissible concentration (Table 2). Mercury was below LOQ or LOD in all composts and lead in two composts (CC and CE), while cadmium, chromium and nickel were quantifiable in all, although in all below the maximum admissible concentrations. In general, composts CI and CF of sewage sludge and animal wastes were those with the highest metal load (Table 2, Fig. S1).

Concerning microbiological quality and according to the regulatory guidelines, *Salmonella* spp. should be absent in 25 g of compost, and *Escherichia coli* should be $<$ 1000 CFU/g of compost (DL103/2015, 2015; EU_2019/1009 and PARLIAMENT, 2019). The only compost that did not comply with these regulatory limits was the sewage sludge compost CI that presented an average level of *E. coli* slightly above the 3

Table 2
Metals content (mg/kg dry weight) of the different composts.

	Cu	Zn	Cd	Cr	Ni	Pb	Hg
Maximum recommended[a]							
Class I	100	200	0.7	100	50	100	0.7
Class II	200	500	1.5	150	100	150	1.5
CA	67	296[b]	0.3	19	7.6	13	<LOD (0.04)
CB	81	270[b]	0.3	11	5.4	9	<LOD (0.04)
CC	50	275[b]	0.9[b]	22	5.4	<LOD (0.20)	<LOD (0.04)
CD	83	406[b]	0.3	8.7	4.4	2	<LOD (0.04)
CE	59	290[b]	0.2	7	2.9	<LOQ (0.67)	<LOD (0.04)
CF	174[b]	865[c]	1.0[b]	25	13	17	<LOD (0.04)
CG	61	201[b]	0.4	20	6.2	6.3	<LOD (0.04)
CH	60	158	0.4	26	10	27	<LOD (0.04)
CI	146[b]	668[c]	0.6	28	38	24	<LOQ (0.13)

Legend: Copper (Cu), Zinc (Zn), Cadmium (Cd), Chromium (Cr), Nickel (Ni), Lead (Pb), and Mercury (Hg). Limit of detection (LOD). Limit of quantification (LOQ).

[a] DL103/2015.
[b] Maximum permissible value for class I compost exceeded.
[c] Maximum permissible value for class II compost exceeded.

log-units (3.70 ± 0.48 log CFUs/g dry weight; Fig. 1). This fact might have been due to the short maturation period of this sample (Table 1). The total viable bacteria count ranged from 4 to 9 log CFU/g dry weight, being the highest values observed in the composts with moisture >20.9%, composts CA, CD, CF, CH and CI (Table 1; Fig. 1). The association between the abundance of culturable microorganisms and moisture was expressed as a significant positive correlation (Pearson coefficients of 0.73–0.88, $p < 0.01$; Fig. 2). Total viable microorganisms count at 37 °C and 22 °C were approximately the same in composts CA-CE and CH (up to 0.5 log-units of difference), and significantly lower for total counts at 22 °C in composts CF, CG and CI (0.8–2.2 log-units below the counts at 37 °C) (Fig. 1). The count of total viable microorganisms and moulds were also significantly positively correlated (Pearson coefficients of 0.86–0.87, $p < 0.01$; Figure S1b and Fig. 2). For the samples with the lowest total viable microorganisms count (4–5 log CFU/g dry weight) (CB, CC, CE), moulds were below the detection limit of the enumeration technique (1.0 log CFU/g), or close to this limit as observed for compost CG (1.4 log CFU/g dry weight, in only one of the three replicas) (Fig. 1). For the other samples, mould count ranged between 1.9 and 5.4 log CFU/g dry weight. Yeasts were above the detection limit of the enumeration technique (1.0 log CFU/g) in only four composts, the three powder formulations (CA of horse wastes and CH and CI of urban waste and sewage sludge) and the pellets CB, ranging 1.1 (CB) to 6.30 (CH) log CFU/g dry weight (Fig. 1). Yeast count was not significantly correlated with any other measured variable (Fig. 2). *B. cereus* was quantified in most of the samples, except in CD and CH, in the range 1.5–3.8 log CFU/g dry weight (Fig. 1). *Listeria* spp. were quantified in all the compost of animal origin (CA-CG), in the range 3.5–4.6 log CFU/g dry weight. Although it was not possible to quantify *Listeria* spp. (LOQ = 1.0 log CFU/g) in the composts from urban wastes, the pathogen *L. monocytogenes* was detected in the sludge compost (CI). Sulfite-reducing *Clostridium* endospores were detected in 0.1 g, or less, of all the composts except in CE (Fig. 1).

3.2. Bacterial community

Total bacterial abundance, measured based on the 16S rRNA gene, ranged 8.3–10.5 log gene copy/g dry weight, with compost CA presenting the highest abundance and composts CB and CG the lowest (Fig. S3). These values were not significantly correlated with culturable microbiota enumeration (Fig. 2). The composition of the community represented by these total bacteria was analysed based on the 16S rRNA gene metabarcoding. Horse and chicken/poultry wastes' composts presented the highest Shannon diversity indexes (6.750–9.872), contrasting with sewage sludge compost with the lowest diversity index (4.564–5.016) (Table S2). The phyla *Bacillota* (classes *Bacilli* and *Clostridia*) and *Actinomycetota* (class *Actinomycetes*) were those with the highest relative abundance values in composts CB to CH, observed to range 8.5 (CB) to 10.0 (CC) and 7.9 (CB and CG) to 10.1 (CD) log gene copy/g dry weight of compost, respectively (Fig. S3). The phyla *Pseudomonadota* (classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*) and *Bacteroidota* (class *Bacteroidia*) were those presenting the second highest relative abundance values (Fig. 3). *Beta-* and *Gammaproteobacteria* presented abundance values of 6.6 (CF) to 10.7 (CA) and 6.6 (CB) to 10.5 (CA) log gene copy/g dry weight of compost, respectively (Fig. S3). The most distinct bacterial community compositions were observed in CA and CI, which had the highest moisture content, above 50% (Figs. 3 and 4). Those samples with a higher moisture content also presented a significantly higher ($p < 0.05$) abundance of the classes *Bacteroidia* and *Betaproteobacteria* and a lower ($p < 0.001$) abundance of *Bacilli* and *Actinomycetes*, than all the other composts. Compost form (powder *vs* pellets), more than raw material, is hypothesized to be associated with bacterial community composition (Fig. S2). Composts CA (powder) and CB (pellets), based on the same type of raw material, presented distinct bacterial communities (Fig. 3; Fig. S2b). Compost CA presented a significantly higher relative abundance of *Bacteroidia* and *Pseudomonadota*, while CB had a significantly higher relative abundance of *Bacilli* (Fig. 3). However, the fact that composts of animal origin (CA-CG) presented significantly ($p = 4.22 \times 10^{-6}$) higher relative abundance of members of the class *Clostridia* than composts of urban origin (CH and CI) suggest that the type of raw material may favour the occurrence of some taxa.

3.3. Culture-independent assessment of faecal and antibiotic resistance contamination

The presence of genes *uidA* (encoding the *E. coli* beta-glucuronidase enzyme) and crAssphage (human-associated virus) was examined to assess possible faecal contamination. The gene *uidA* was above the limit of quantification in composts CB, CC, CE and CG, in a range of 5.0–6.8 log gene copy/g dry weight. The crAssphage, specifically associated with human faecal contamination, was quantified in the composts CC,

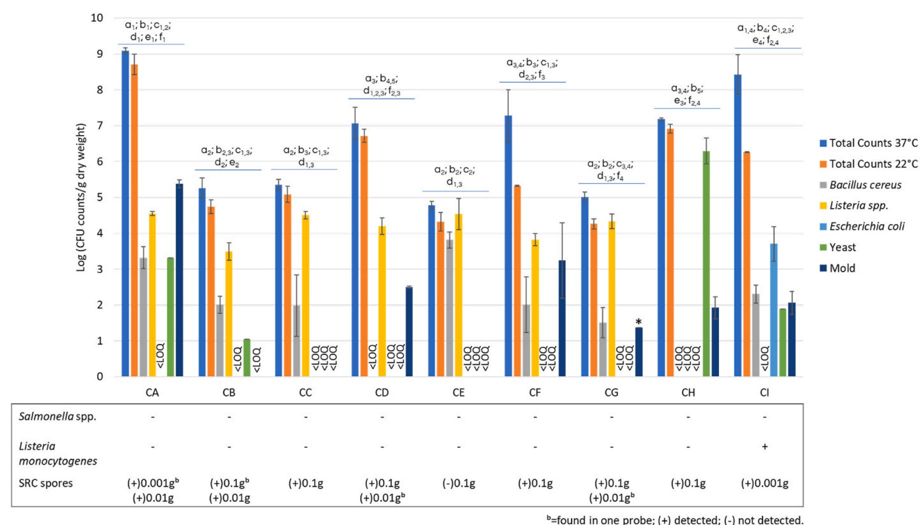

Fig. 1. Results of the microbiological characterisation of the composts. *Salmonella* spp. and *Listeria monocytogenes* were not quantified. Above the bars are indicated the ANOVA post-hoc Tuckey groups ($p < 0.01$) comparing samples for total viable bacteria counts at 37 °C (a), total viable bacteria counts at 22 °C (b), *Bacillus cereus* (c), *Listeria* spp. (d), yeasts (e), and moulds (f).
SRC, sulfite-reducing *Clostridium* spores - presence (+) or absence (−) in 0.1, 0.01 or 0.001 g of sample. (*) moulds were quantified just on one of the three replicas.

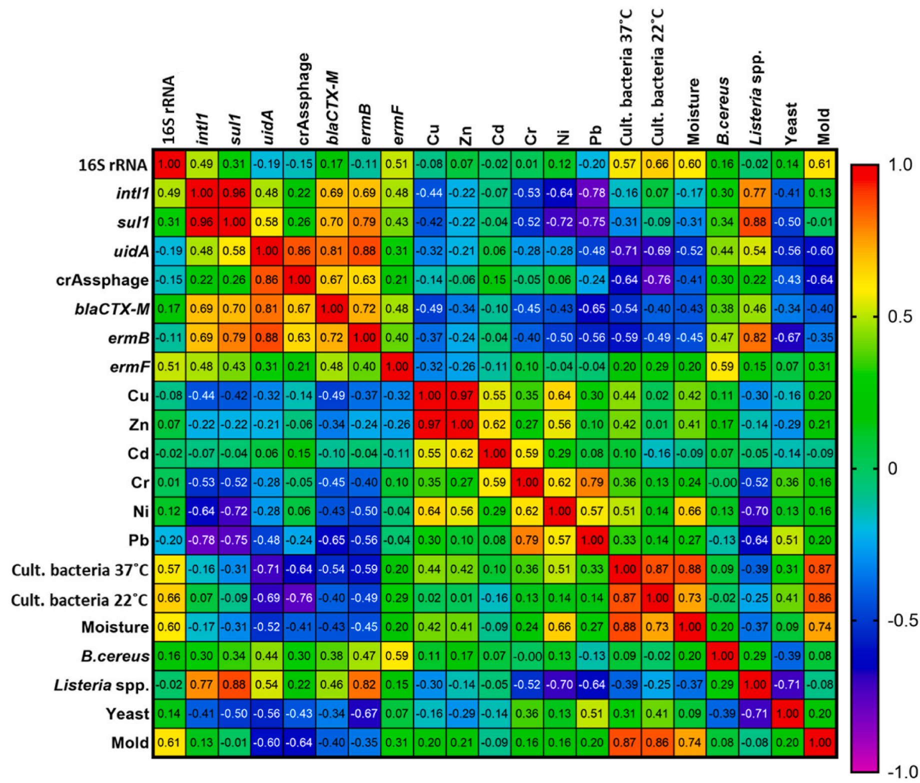

Fig. 2. Pearson correlation analysis for the different parameters analysed: cultivable bacteria, metals and genes. Confidence interval: 99%.

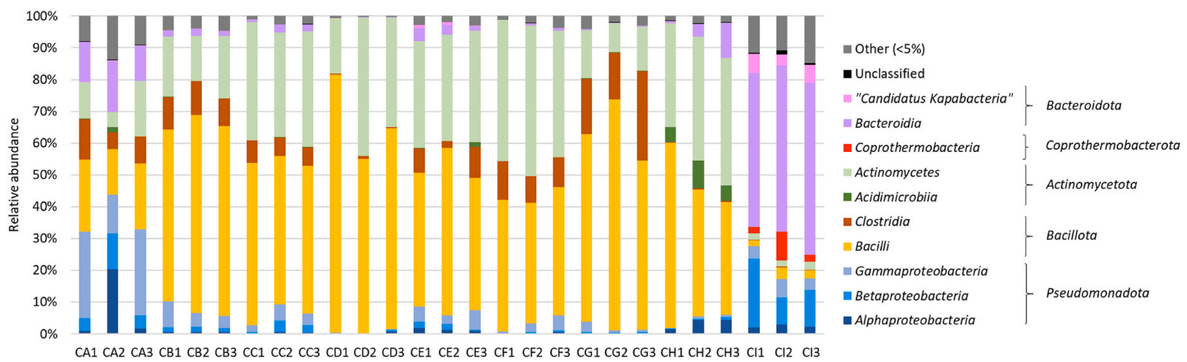

Fig. 3. Bacteria diversity at the class level for the different compost samples.

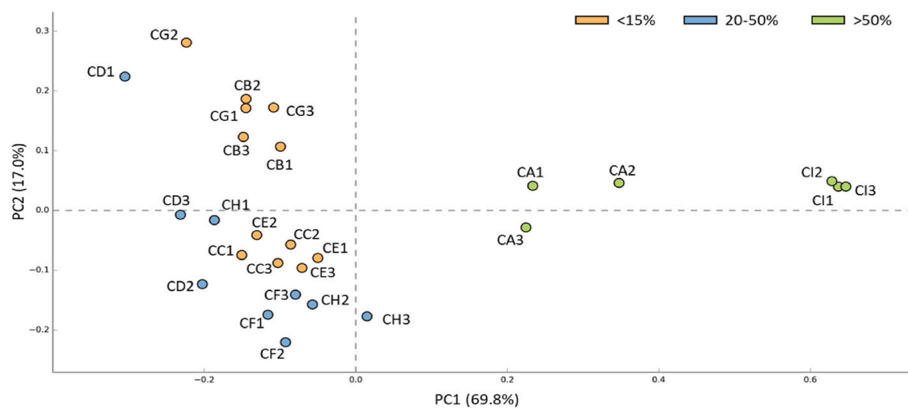

Fig. 4. Samples distribution based on a Principal Components Analysis (PCA) regarding the taxonomic annotation at the Class level. Samples are highlighted according to their moisture content (%).

CE, CG and CI, in an abundance ranging 4.6–6.8 log gene copy/g dry weight (Fig. 5). Both indicators, although not coinciding exactly in the same samples, were observed in composts produced from animal wastes and sewage sludge. The abundance of the two genes, *uidA* and crAssphage, was significantly correlated (Pearson coefficient of 0.86, Fig. 2). Class 1 integron integrase gene (*intI1*) is considered an indicator of the potential of the bacterial communities to mobilise genes by genetic recombination. This gene and the gene *sul1* (sulfonamides resistance), which are frequently genetically linked, were quantified in all samples in abundance values ranging 5.8–9.0 and 6.0–9.9 log gene copy/g dry weight, respectively. Accordingly, the quantification of both genes was significantly correlated (Pearson coefficient of 0.96; Fig. 2). As for faecal contamination, were the composts produced from urban wastes (food waste or sewage sludge) CH and CI that presented the lowest loads of these genes (Fig. 5). The genes *ermB* and *ermF*, associated with macrolide, lincosamide, and streptogramin B (MLSB) resistance phenotypes, were quantified in seven or eight of the composts, respectively, in abundance values up to 9.5 log gene copy/g dry weight (Fig. 5). The gene *ermB* was quantified in all the composts produced with animal wastes but not in the urban waste composts, while the gene *ermF* was not quantified only in compost CD. The abundance of MLSB associated genes was not significantly correlated. However, *ermB*, but not *ermF*, was positively correlated with the abundance of the genes *uidA* and *sul1* (Pearson coefficients of 0.88 and 0.79, $p < 0.01$, Fig. 2). The antibiotic resistance gene observed with the lowest frequency was $bla_{CTX\text{-}M}$, encoding a beta-lactamase. It was quantified only in composts CC and CE, produced from chicken/poultry wastes, in the range of 5.3–6.7 log gene copy/g dry weight (Fig. 5). Its occurrence was significantly correlated with the abundance of the faecal indicator *uidA* (Pearson coefficient of 0.81, Fig. 2). The genes *uidA*, *crAssphage*, *ermB* and $bla_{CTX\text{-}M}$ were negatively correlated with the total viable bacteria counts and moisture content (Pearson coefficients of −0.40 to −0.76, Fig. 2), while the genes *intI1* and *sul1* were negatively correlated with the concentration of the metals Cr, Ni and Pb (Pearson coefficients of −0.52 to −0.78, Fig. 2).

4. Discussion

Composting is a valuable process to give a second life to organic wastes, totally aligned with a circular economy perspective, promoting waste reuse and organic soil fertilisation. This study investigated the occurrence of faecal contamination, antibiotic resistance genes and human bacterial pathogens in final composts produced from animal manure, urban wastes and sewage sludge, and aimed to infer if the raw material, the degree of metal contamination, the microbial load or bacterial community composition might be associated with the presence of those contaminants. The results were also expected to inform about the occurrence of potential microbiological risks for the recipient environment (e.g. soil or crops).

Contrary to most of the organic compounds, metals tend to persist after composting (Chimuka et al., 2009; Dumontet et al., 2001; Ko et al., 2008). For that reason, they are among the parameters that should be monitored (DL103/2015, 2015; EU_2019/1009 and PARLIAMENT, 2019). The observed concentrations of metals were within values commonly observed for composts from sewage sludge or animal origin (Amir et al., 2005; Chimuka et al., 2009; Ko et al., 2008) and were mostly below the maximum admissible concentrations. The compost CI (1:1 sewage sludge and pine sawdust) and the compost CF (non-discriminated animal wastes), were the composts with the highest metals' concentration, and the only ones with zinc above the maximum admissible concentration (Table 2; Fig. S1a). High concentrations of Cu and Zn in composts from animal origin may be not surprising because these metals are frequently used as additives in animal feeds, and they are not totally absorbed by animal bodies but excreted in animal manures (Ko et al., 2008). Also, according to literature, sewage sludge and sawdust are among the major sources of metals in composts due to the enrichment during the wastewater treatment process and the high capacity of lignocellulosic materials to adsorb metals (Chimuka et al., 2009; Dias et al., 2023). Of note is also the fact that compost CI was analysed immediately after production, and it was probably not totally stable, which could result in an overestimation of the metals concentration that tends to decrease along the composting due to immobilisation and stabilisation resultant from processes such as changes in the pH and microbial activity (Amir et al., 2005; Chimuka et al., 2009; Singh

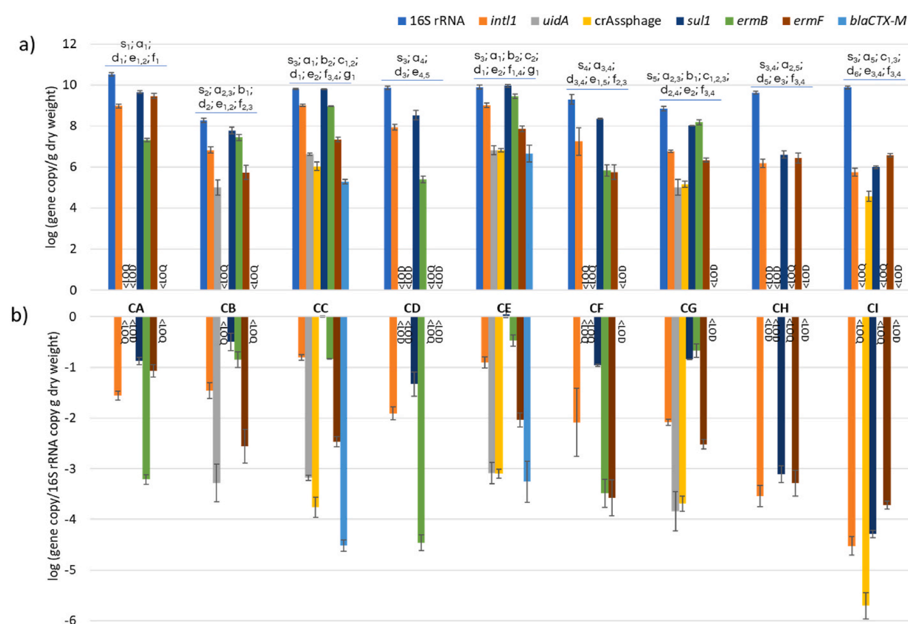

Fig. 5. Quantification of the abundance (a) and relative abundance (b) for the genes 16S rRNA, *intI1*, *uidA*, *crAssphage*, *sul1*, *ermB*, *ermF*, and $bla_{CTX\text{-}M}$ in the different composts.
Above the bars are indicated the ANOVA post-hoc Tuckey groups ($p < 0.01$) comparing samples for 16S rRNA (s), *intI1* (a), *uidA* (b), *crAssphage* (c), *sul1* (d), *ermB* (e), *ermF* (f), and $bla_{CTX\text{-}M}$ (g) genes.

and Kalamdhad, 2012). Notwithstanding, the concentration of the metals Ni, Zn and Pb, higher in CI compost than in most of the others, was significantly correlated with the bacterial community composition, particularly with *Betaproteobacteria* and *Bacteroidia*, also with higher relative abundance in this compost than in others (Figure S4, Fig. 3). The low maturity of the compost CI was also reflected in the microbiological analysis, which revealed the presence of *L. monocytogenes*, numbers of *E. coli* above the recommended limit of 3 log CFU/g dry weight of compost (Fig. 1), and a significantly higher prevalence of *Bacteroidota* and reduced prevalence of *Bacillota* and *Actinomycetota* (Fig. 3). Meng et al. (2019) have previously shown that for dairy manure composting, this shift in the bacterial communities from a higher prevalence of *Pseudomonadota* and *Bacteroidota* at the beginning of the composting process, replaced by *Bacillota* and *Actinomycetota* in the mature compost.

As observed in other studies (Meng et al., 2019; Wang et al., 2020), the moisture content was among the parameters that mostly affected the abundance of cultivable bacteria (Fig. 1 and Fig. S4) and also the bacterial community composition (Fig. 4). Indirectly, the compost form also seemed to have influenced the moisture content (Fig. S2b), probably due to the processes used to produce the pellets. The comparison of composts CA and CB produced from the same type of raw material and with different forms, evidenced that effect - the powder version (CA) presented a moisture content of 53% while the pellet version (CB) had five times less moisture, 10.8% (Table 1). The compost moisture content was observed to have a higher contribution for the bacterial community composition than the raw materials used for the compost production (Fig. 4 vs Fig. S2). No evidence was found that the age of the compost could influence the bacterial community structure. Composts produced more than 15 years ago (CE and CF; Table 1) presented bacterial communities comparable to the other composts produced more recently (Figs. 2 and 3 and Fig. S2).

Bacillota and *Actinomycetota* were the most abundant phyla, except for the composts CA and CI, for which *Pseudomonadota* were also among the most abundant phyla (Fig. 3; Fig. S3). The poor maturation of CI and the high moisture (>50%) of both composts (CA and CI) may explain the distinct bacterial community and also justify that some variables, such as metals concentration or exogenous bacteria (e.g. harboring the gene *ermF*), are observed to be significantly associated with distinct classes of *Pseudomonadota*, that in these composts ranged 9.5–10.7 log copies/g dry weight, about >2 log units more than in other composts (Fig. S3; Fig. S4). Although members of the phyla *Bacillota*, *Actinomycetota* and *Pseudomonadota* were expected to be the most abundant in compost, the reduction of *Pseudomonadota* abundance in a mature compost has been consistently reported (Meng et al., 2019; Wang et al., 2020; Xu et al., 2022). In this study, we argue that it may be moisture content, and not only the compost maturity, that drives the high relative abundance values for *Pseudomonadota*. Short-read amplicon sequencing analysis has some limitations, particularly the low resolution at lower taxonomic ranks, such as the genera or species. That was observed for our data, with percentages of unclassified reads at the genus and species level of 3.3–38.3% and 52.0–95.5%, respectively (data not shown). For this reason, when looking for pathogens, usually identified at the species level, the use of culture dependent methods is more accurate compared to the 16 S rRNA gene short amplicon sequencing. In fact, it is widely demonstrated that culture- and DNA-based methods target different populations (Vaz-Moreira et al., 2011). For example, the pathogen *L. monocytogenes* was not detected using the metabarcoding approach, although they were detected based on cultivation methods (Fig. 1).

In all the compost analysed it was possible to detect indicator bacteria, being *Listeria* spp., *B. cereus*, and *Clostridium* spores the most common (Fig. 1). *Listeria* spp. were quantifiable only in the composts produced from animal wastes (CA-CG), probably an echo of the common occurrence in livestock manure (Biswas et al., 2018). As observed in the present study, other authors reported low levels of *Listeria* spp. in composts from food wastes (Brinton Jr et al., 2009). However, it is difficult to find associations between the compost raw materials and the presence of pathogens once their survival and persistence in the final compost depends not only on the initial matrix (C:N ratio), but also on the conditions of composting process, for example, the maximum temperature reached, pH, oxygen, moisture content, metabolic by-products and/or enzymes produced by indigenous microbiota, or predation by native microorganisms (Awasthi et al., 2019; Erickson et al., 2014; Lepesteur, 2022).

In addition to human pathogens, it is also of concern that composts may be a vehicle for disseminating antibiotic resistance genes to agricultural soils. The risk associated with the presence of some indicator genes, as those examined in this study, is that their presence anticipates the presence of other antibiotic resistance genes, possibly of higher clinical relevance, despite the lower abundance. The genes examined in this study and others that are indicated by these, may hint an increased risk of contamination of the amended soils and possibly of the cultivated crops, with the possibility of entering the human food chain. In that scenario, the genes need to persist in the soil after the compost application, which has already been demonstrated by some studies. For example, the gene *ermB* was reported to persist for at least five years post-application of swine manure compost (Scott et al., 2018), and also in crops cultivated in soils that were amended with compost (Pu et al., 2019). In this study, most of the antibiotic resistance biomarker genes were negatively correlated with the metals (Fig. 2), not evidencing a possible co-selection effect due to the metal contamination. However, previous publications have suggested that the co-selection of metals and antibiotic resistance is a likely event when the conditions are favorable (Ezugworie et al., 2021; Wei et al., 2020). Another interesting result was the negative correlations of the genes *uidA*, *crAssphage*, *ermB* and $bla_{CTX\text{-}M}$ with the total cultivable bacteria counts (Pearson coefficients of −0.40 to −0.76, Fig. 2). The composts with the highest cultivable bacteria abundance (CA, CD, CF, CH, CI) were samples for which those genes were mostly below the limit of quantification (Figs. 1 and 5). This may be the result of bacteria competition and antagonism. In an habitat rich in organic matter, as is compost, fast-growing bacteria with reduced or no fitness cost associated to antibiotic resistance may become more competitive (Letten et al., 2021), and the negative correlation observed in this study confirms this hypothesis.

The seven genes examined in this study (*intI1*, *uidA*, crAssphage, *sul1*, *ermB*, *ermF*, and $bla_{CTX\text{-}M}$) were previously described as good biomarkers for antibiotic resistance contamination (Teixeira et al., 2023). Although the quantification of genes by quantitative PCR is limited to approximately 4 log gene copies per gram of soil/compost dry weight, due to limitations inherent to the DNA extraction and quantification methodologies (Fortunato et al., 2018), it was possible to quantify all the biomarker genes (Fig. 5). Contrary to the observed for the bacterial diversity that seemed to be more correlated with the moisture content than with the raw materials, the presence of genes associated with antibiotic resistance was more common in composts from chicken/poultry manure (CC and CE) for which all the targeted genes were quantified (Fig. 5). Those were also the only composts for which was detected the $bla_{CTX\text{-}M}$ gene, considered as a gene of high risk, widespread on mobile genetic elements and reported to cause problems in hospitals (Zhang et al., 2021). The gene $bla_{CTX\text{-}M}$ was previously reported in compost and described as positively correlated with several multidrug efflux pump genes (Mao et al., 2021). The genes *intI1* and *sul1*, associated with the mobile genetic elements class 1 integrons, were quantified in all the composts, revealing the potential of the compost bacterial communities to transfer genetic information to other bacteria. The other two antibiotic resistance genes, *ermB* and *ermF*, were quantified in most of the composts of animal origin, but in the urban waste composts, it was only quantified the *ermF* that was positively correlated with the presence of *Pseudomonadota* (Fig. 5 and Fig. S4). The four genes (*intI1*, *sul1*, *ermB* and *ermF*) were already described in livestock manure compost (Ezugworie et al., 2021; Liu et al., 2021; Qian et al., 2016; Youngquist et al., 2016), and the abundance of the gene *ermB* was described as positively correlated with the abundance of the tetracycline resistance

genes (*tetM*, *tetQ*, *tetS*) and the beta-lactamase bla_{TEM} in poultry compost (Esperón et al., 2020) or with the occurrence of transposons in manure biocompost (Pu et al., 2019). These results reveal that composts of animal origin may represent an added risk for antibiotic resistance dissemination in the receptor environments.

5. Conclusions

Moisture content was observed to be strongly associated with the abundance of cultivable bacteria, which was negatively correlated with the abundance of acquired antibiotic resistance genes. Moreover, moisture content was associated with the predominance of members of the phylum *Pseudomonadota* in the bacterial community.

The concentration of metals, in general below the maximum admissible values, was observed to be negatively correlated with antibiotic resistance indicators and positively associated with *Betaproteobacteria*, *Bacteroidia, Coprothermobacteria* and the *Candidatus Kapabacteria*.

The raw materials used for composting were suggested to represent the major driver for the occurrence of acquired antibiotic resistance genes. Composts produced from poultry/chicken manure had higher loads of antibiotic resistance contaminants, representing a higher risk for receptor environments.

However, all composts, irrespective of the origin (animal, urban wastes, and sewage sludge) were observed to be potential sources of antibiotic resistance genes. Based on the results of this study, it is recommended that loads of contaminants in raw materials are examined to produce safe composts. The pre-treatment of raw materials or their combination with other sources of organic matter may be optimized in order to reduce the load of contaminants in the final product.

CRediT authorship contribution statement

Ivone Vaz-Moreira: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Angelo D'Arnese:** Writing – original draft, Methodology, Investigation, Formal analysis. **Maurice Knoll:** Methodology, Investigation, Formal analysis. **A. Margarida Teixeira:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Joana Bastos Barbosa:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Paula Teixeira:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Célia M. Manaia:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125329.

Data availability

Most of the data is available in the manuscript or in public databases. Any missing data will be made available on request.

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