Journal of Hazardous Materials 478 (2024) 135567

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

The integration of *omics* and cultivation-dependent methods could effectively determine the biological risks associated with the utilization of soil conditioners in agriculture

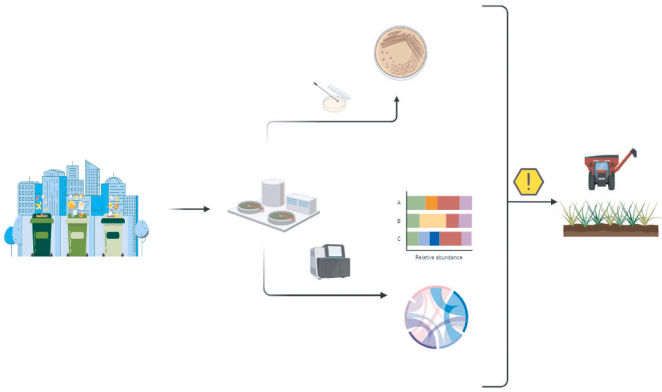
Arianna Peruzzo [a,b], Sara Petrin [a], Aurora Boscolo Anzoletti [a], Marzia Mancin [a], Andrea Di Cesare [c,d], Raffaella Sabatino [c,d], Maria Cristina Lavagnolo [e], Giovanni Beggio [e], Giulia Baggio [a], Patrizia Danesi [a], Lisa Barco [a], Carmen Losasso [a,*]

[a] *Laboratory of Microbial Ecology and Genomics, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro, Italy*
[b] *PhD National Programme in One Health Approaches to Infectious Diseases and Life Science Research, Department of Public Health, Experimental and Forensice Medicine, University of Pavia, Pavia 27100, Italy*
[c] *National Research Council of Italy (CNR) – Water Research Institute (IRSA), Largo Tonolli 50, 28922 Verbania, Italy*
[d] *National Biodiversity Future Center (NBFC), Piazza Marina 61, 90133 Palermo, Italy*
[e] *Department of Civil Environmental and Architectural Engineering, University of Padova, Lungargine Rovetta 8, 35100 Padova, Italy*

HIGHLIGHTS

- Bacterial communities vary significantly based on treatment process.
- Biowaste-derived soil conditioners pose higher health risks.
- Current safety indicators like Salmonella are insufficient.
- Integrated traditional and omic methods are essential for risk assessment.
- Propose using PCR protocols for monitoring soil conditioner safety.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Circular economy
Sludge reuse
Pathobiome
Resistome
Metagenomics

ABSTRACT

In the circular economy, reusing agricultural residues, treated biowaste, and sewage sludges—commonly referred to as soil conditioners—in agriculture is essential for converting waste into valuable resources. However, these materials can also contribute to the spread of antimicrobial-resistant pathogens in treated soils. In this study, we analyzed different soil conditioners categorized into five groups: compost from source-separated biowaste and green waste, agro-industrial digestate, digestate from anaerobic digestion of source-separated biowaste, compost from biowaste digestate, and sludges from wastewater treatment plants. Under Italian law, only the first two categories are approved for agricultural use, despite Regulation 1009/2019/EU allowing the

* Corresponding author.
E-mail address: closasso@izsvenezie.it (C. Losasso).

https://doi.org/10.1016/j.jhazmat.2024.135567
Received 1 June 2024; Received in revised form 15 July 2024; Accepted 16 August 2024
Available online 23 August 2024

use of digestate from anaerobic digestion of source-separated biowaste in CE-marked fertilizers. We examined the bacterial community and associated resistome of each sample using metagenomic approaches. Additionally, we detected and isolated various pathogens to provide a comprehensive understanding of the potential risks associated with sludge application in agriculture. The compost samples exhibited higher bacterial diversity and a greater abundance of potentially pathogenic bacteria compared to other samples, except for wastewater treatment plant sludges, which had the highest frequency of Salmonella isolation and resistome diversity. Our findings suggest integrating omics and cultivation-dependent methods to accurately assess the biological risks of using sludge in agriculture.

1. Introduction

The use of treated biosolids (BSO) from municipal wastewater treatment plants (WWTPs) and source-separated biowaste, as soil enhancers in agriculture presents a promising avenue for implementing sustainability policies aimed at reducing the carbon and water footprint of food production systems [1]. However, while this practice offers considerable benefits, concerns regarding potential biological hazards loom large, particularly in the absence of comprehensive monitoring plans within the European Union (EU) [2].

The transfer of chemical and biological contaminants to soil intended for food production raises apprehensions about human health risks through food exposure [3]. Among the spectrum of potential hazards are pathogenic and zoonotic agents including bacteria and parasites, along with antimicrobial resistance genes (ARGs), which can be horizontally acquired by pathogenic microorganisms [4].

Directive 86/278/EEC, designed to regulate the use of biosolids (BSO) in the EU, aims to prevent adverse effects on soil, vegetation, animals, and humans [5]. However, the transposition of this directive into national legislation by Member States has been limited, resulting in a lack of harmonization in implementation criteria. For instance, in Italy, the prevailing reference standard, Dlgs. 99 of 1992, sets the load of *Salmonella* spp. (less than 10^3 MPN/g) as the sole microbiological criterion for BSO conformity, potentially leading to an underestimation of actual biological risks [6].

The presence of pathogens and ARGs in agricultural soils, coupled with their persistence, presents novel epidemiological challenges [7]. These include the creation of new paths for pathogen release into the food chain and the emergence of new microbial variants resistant to antibiotics, facilitated by horizontal gene transfer (HGT) [8]. Indeed, soils serve as reservoirs for antimicrobial-resistant bacteria and ARGs as highlighted by previous literature, demonstrating an increasing diversity and abundance of the antimicrobial resistome (total content of ARGs) over time [9]. Furthermore, previous studies have elucidated the role of practices such as water reuse or sewage sludge application in the spread of ARGs in agricultural soil [10].

Starting from these considerations, this paper endeavors to identify and characterize the biological hazards associated with the use of soil conditioners in agriculture through various approaches:

i) Characterizing the bacterial community, pathobiome (total content of bacterial genera including at least a confirmed pathogenic species, as reported in Bartlett et al., 2022 [11], and antimicrobial resistome using amplicon and shotgun sequencing in various types of BSOs, encompassing both permitted and forbidden for agricultural use.

ii) identifying and isolating potentially pathogenic strains from the various types of BSOs as defined at point i).

iii) Providing a list of bacteria and ARGs as targets to differentiate between BSOs from different sources.

Through these analysis, this study aims to shed light on the potential risks associated with the use of soil conditioners in agriculture and to pave the way for informed decision-making and regulatory measures to ensure food safety and environmental sustainability.

2. Materials and methods

2.1. Sample collection and processing

A total of 140 samples of compost, digestate and sewage sludge were collected from seven commercial and municipal plants located in Northern Italy. The sampling activity was performed in accordance with the European standards on waste sampling to minimize the uncertainty deriving from sampling operations [12]. The samples were collected in two different sampling campaigns.

A total of 33 samples of compost (A) were collected from two treatment plants performing conventional industrial-scale aerobic treatment of separately collected biowaste and green waste. Composting is carried out as a stepwise process beginning with mechanical separation of impurities from biowaste (sieving and metal separation), followed by approximately 30 days of forced aeration and mixing in windrows, which allows the feedstocks to reach temperatures of 50–60 °C. The process is then completed with a further 30 days of maturation in static piles. Samples from agro-industrial digestates (B) were collected downward of two farm-scale digesters equally performing about 25 days of wet (about 10 % w/w of Total Solids) thermophilic (about 55 °C) anaerobic digestion of zootechnical (bovine and suine manures) and agricultural residues (corn silage and straw) for a total of 29 samples. Biowaste digestate (C) was gathered in 22 samples from two public-private multiutility companies operating integrated anaerobic-composting treatment on source-separated biowaste and green waste generated in urban contexts. Both plants generate C by performing approximately 25 days of wet (10 % TS on a weight base), mesophilic (about 40 °C), anaerobic digestion of biowaste, mechanically preselected (*i.e.*, sieving and metal separation) to get rid of impurities, mixed together with shredded green waste. Additionally, 30 samples of compost (D) were collected from the same plants producing C. These samples resulted from the composting of previously chipped green waste mixed with the a solid fraction of compost (C). The composting process was carried out under equivalent conditions used in the production of A samples. Further, 26 samples of treated and untreated sewage sludge (E) were collected from two WWTPs treating urban drainage and household wastewater. In particular, six samples were collected downstream the sludge thickener serving the biological treatment (*i.e.*, nitrification-denitrification) of wastewater. The remaining samples were constituted by the digested sludge undergone about 20 days of mesophilic (about 30 °C), wet (about 4 % TS on a weight base), anaerobic treatment.

2.2. Detection and isolation of selected pathogenic bacteria and parasites

2.2.1. Shiga toxin-producing E. coli (STEC)

Detection of STEC was performed following the ISO/TS 13136:2012 [13]. Twenty-five grams or millilitres for each sample were collected and homogenised with 225 ml of buffered peptone water (APTS) in a Stomacher bag with filter. The resulting solution (enrichment broth) was then incubated for 18–24 h at 37 °C.

One ml of the enrichment solution for each sample was used for the subsequent DNA extraction as follows: samples were centrifuged at 9000 x g for 5 min and then the supernatant was discarded. The pellet was

washed with 1 ml of phosphate buffer saline (PBS), centrifuged again at 13000 x g for 1 min. The supernatant was discarded and the pellet was resuspended in 200 µl InstaGene Matrix (Bio-Rad) and incubated at 56 °C for 30 min followed by a second incubation at 99 °C for 5 min. Samples were then centrifuged at 12000 x g for 5 min and the supernatants were used to detect *stx* and *eae* genes using the following multiplex real-time PCR (rPCR) protocol. The enrichment broth was conserved at 2–8 °C degree until negative results from the rPCR (see below).

For each sample, the rPCR assay was carried out in 20 µl: 2 µl of DNA, 0.2 µM of each primer (Table 1), 4 µl of QuantiFast Pathogen Master Mix (QIAGEN), and filtered and autoclaved water to the final volume, using a CFX96 Real-Time System (Bio-Rad). The rPCR program was 95 °C for 5 min, 45 cycles of 95 °C for 15 s and 60 °C for 45 s, when florescence was read. Ramp rate in each step was set at 5 °C/s.

In cases when florescence was detected for *stx*1 or *stx*2 gene, microbiological isolation of STEC was attempted by plating the enrichment broth directly onto selective MacConkey agar (MCC) plates, incubated at 37 °C for 18–24 h. Direct plating was performed to ensure the isolation of at least 50 *E. coli* typical colonies. Each presumptive *E. coli* colony was plated in nutrient agar (NA) plates and incubated at 37 °C for 18–24 h. Simultaneously, the presumptive *E. coli* colony was resuspended in 100 µl distilled water for DNA extraction and serogroup determination.

2.2.2. *Salmonella spp.*

Screening of samples positive for *Salmonella* spp. was initially performed by rPCR according to ISO 17604:2015 [14]. Twenty-five grams or millilitres for each sample were collected and homogenised with 225 ml of APTS in a Stomacher bag with filter. The resulting solution (enrichment broth) was then incubated for 18 h at 37 °C. One ml of the enrichment solution for each sample was used for the subsequent DNA extraction with the iQ-Check *Salmonella* II kit (Bio-Rad). The enrichment broth was conserved at 2–8 °C degree until negative results from the rPCR (see below).

Samples were centrifuged at 12000 x g for 5 min and then the supernatant was discarded. Two hundreds µl of Lysis Reagent A were added to resuspend the pellet and then samples were incubated at 95 °C for 10 min. Samples were then centrifuged at 12000 x g for 5 min and the supernatants were used to detect *Salmonella* using the following rPCR protocol.

For each sample, the rPCR assay was carried out in 50 µl: 5 µl of DNA and 45 µl of the amplification mix composed of Reagent B (fluorescent probe) and Reagent C (amplification mix). Volumes for Reagent B and Reagent C were determined following the manufacturer's instructions. A CFX96 Real-Time System (Bio-Rad) was used to perform the rPCR with the following program: 95 °C for 10 min, 45 cycles of 95 °C for 13 s, 58 °C for 5 s and 72 °C for 22 s

Samples were considered positive for *Salmonella* spp. When the threshold cycle (Ct) was greater than 10 in such cases the microbiological identification was attempted from the enrichment broth, according to ISO 6579–1:2017 [15]. An aliquot of 100 µl of the enrichment broth for each sample presumptively positive for *Salmonella* was plated as three distinct droplets onto a modified semisolid Rappaport-Vassiliadis (MSRV) plate and incubated at 41.5 °C for 24 h. At the same time, 1 ml of the enriched broth was added to 10 ml of Muller-Kauffmann tetrathionate novobiocin broth (MKTTn) and incubated at 36 °C for 24 h. After the incubation period, suspected *Salmonella* colonies grown on MSRV were then plated onto brilliant green agar (BGA) and xylose lysine deoxycholate (XLD) agar plates. Ten µl from the MKTTn broth were also plated onto BGA and XLD plates. BGA and XLD plates were incubated at 36 °C for 24 h. Typical *Salmonella* colonies grown onto BGA and XLT plates were transferred onto nutrient agar (NA) plates and incubated at 36 °C for 24 h for subsequent identification and characterization.

Biochemical identification of suspected *Salmonella* colonies was performed by inoculation onto triple sugar iron (TSI) agar, urea-supplemented agar, ONPG semisolid agar, lysin-supplemented broth and tryptone/tryptophan medium (TTM) and incubation at 36 °C for 24 h. *Salmonella* strains are expected to induce a color change in TSI (red slant, yellow bottom with bubbles, and a black halo) and in lysin-supplemented broth. No color changes should be observed in urea-supplemented agar, ONPG semisolid agar, and MTT broth.

Serological identification of isolates identified as *Salmonella* from the biochemical characterization was performed by slide agglutination with *Salmonella* antisera towards somatic (O-) and flagellar (H-) antigens, according to ISO/TR 6579–3:2014 [15]. The serovar names were assigned according to the White-Kauffmann-Le Minor scheme [16,17].

2.2.3. *Listeria monocytogenes*

Screening of samples positive for *Listeria monocytogenes* was initially performed by rPCR according to ISO 17604:2015 [14]. Twenty-five grams or milliliters for each sample were collected and homogenized with 225 ml of *Listeria* special broth (LSB, Bio-Rad) in a Stomacher bag with a filter. The resulting solution (enrichment broth) was then incubated for 25 h at 30 °C. From the enrichment solution, for each sample 1.5 ml was used for DNA extraction with the iQ-Check *Listeria monocytogenes* II kit (Bio-Rad). The enrichment broth was conserved at 2–8 °C degree until negative results from the rPCR (see below).

Samples were centrifuged at 12000 x g for 5 min and then the supernatant was discarded. Two hundred-fifty µl of Lysis Reagent (A+F) were added to resuspend the pellet; samples were vigorously mixed in a cell disruptor for 3 min and then incubated at 95 °C for 15 min. Samples were then centrifuged at 12000 x g for 5 min and the supernatants were used to detect *Listeria monocytogenes* using the following rPCR protocol.

For each sample, the rPCR assay was carried out in 50 µl: 5 µl of DNA and 45 µl of the amplification mix composed of Reagent B (fluorescent probe) and Reagent C (amplification mix). Volumes for Reagent B and Reagent C were determined following the manufacturer's instructions. A CFX96 Real-Time System (Bio-Rad) was used to perform the PCR with the following program: 95 °C for 10 min, 50 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s

Samples were considered positive for *Listeria monocytogenes* when Ct> 10 and in such cases, microbiological identification was attempted from the enrichment broth. An aliquot of 100 µl of the enrichment broth for each sample presumptively positive for *Listeria monocytogenes* was plated onto a RAPID'L. mono selective plate. After an incubation at 37 °C for 24 h, typical *Listeria monocytogenes* colonies were identified.

2.2.4. *Campylobacter spp.*

Isolation of *Campylobacter spp.* isolates was performed according to ISO 10272–1:2017 [18]. Ten grams or milliliters for each sample were

Table 1
Primers and probe used in the rPCR for STEC detection.

Primer/probe name	Target genes	Forward sequence	Reverse sequence
VTrt	*stx*1/*stx*2	5′-TTTGTYACTGTSACAGCWGAAGCYTTACG−3′	5′-CCCCAGTTCARWGTRAGRTCMACRTC−3′
EAErt	*eae*	5′-CATTGATCAGGATTTTTCTGGTGATA−3′	5′-CTCATGCGGAAATAGCCGTTA−3′
Probe *stx*1		5′–56-FAM–CTGGATGAT/ZEN/CTCAGTGGGCGTTCTTATGTAA/3IABkFQ/–3′	
Probe *stx*2		5′–5Cy5/TCGTCAGGCACTGTCTGAAACTGCTCC/3IAbRQSp/–3′	
Probe *eae*		5′–/5TEX615/ATAGTCTCGCCAGTATTCGCCACCAATACC/3IAbRQSp/–3′	

homogenized in 90 ml of Preston broth (PR 90) and incubated at 41.5 °C for 24 h in microaerophilic conditions (enrichment broth). After the incubation, the enrichment broth was plated onto modified charcoal cefoperazone deoxycholate agar (CCDA) plates using a 10 µl loop and incubated again at 41.5 °C for 44 h in microaerophilic conditions. Typical or suspected *Campylobacter* colonies were transferred onto Columbia Agar (CA) plates and incubated at 41.5 °C for 24–48 h in microaerophilic conditions. Single *Campylobacter* colonies from CA plates were tested for morphology and motility, ability to grow in aerobic conditions after incubation at 25 °C for 44 h and reactivity to the oxidase test.

2.2.5. Giardia duodenalis and Criptosporidium spp

The detection of Giardia and Cryptosporidium spp. was performed in 1 g of sample fixed using the commercially available immunofluorescence test according to manufacturer instructions (Merifluor® Cryptosporidium/Giardia, Meridian Bioscience, Cincinnati, OH, USA). Quantification of Giardia cysts and Cryptosporidium spp. oocysts was made by counting protozoa elements under the microscope and expressed as a number of (oo-) cysts per 1 g of sample.

2.3. DNA extraction

Total genomic DNA was extracted from the different samples using a commercial column-based kit (QIAamp Fast DNA Stool, QIAGEN), following the manufacturer's instructions with minor modifications. In details, for solid samples, 7–10 g were weighted to be used as starting material together with 10 ml of saline solution that were added in a Stomacher bag and homogenized for 1 min. From the resulting solution, 1.5 ml were collected, centrifuged (1 min, 6000 x g) to collect the pellet that was used for DNA extraction as described below. For liquid samples, 0.5 ml was used as the starting material. After centrifugation (1 min, 6000 x g), the supernatant was discarded and the pellet was weighed. One ml of Inhibitex Buffer was added to the pellet from either liquid or solid samples, and vigorously homogenized using the DistruptorGenie for 1 min. Samples were then incubated for 10 min at 85 °C and then the protocol was followed as described by the manufacturer. DNA was eluted in 100 µl of ATE Buffer and stored at −20 °C until further analyses.

2.4. DNA sequencing

2.4.1. 16 SrRNA gene amplicon sequencing

Each obtained DNA sample was used as a template for amplification of the V3-V4 hypervariable regions of the 16S rRNA gene. The 16 Sr RNA gene library was prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol, following the instructions of the Nextera XT DNA Library Prep kit (Illumina Inc., San Diego, CA, USA). Libraries were checked for both concentration and quality using Qubit dsDNA 1X High Sensitivity and 2200 TapeStation (Agilent), respectively. Samples were equimolarity pooled, and sequencing was performed with an Illumina MiSeq platform using a MiSeq 600V3 cartridge (600 cycles, 2 ×300 bp, paired-end reads). The raw data were deposited on the European Nucleotide Archive (ENA) with Bioproject n. PRJEB76115.

2.4.2. Shotgun sequencing

A subset of 54 samples belonging to A (N = 9), B (N = 15), C (N = 10), D (N = 6) and E (N = 14) matrix respectively, were underpinned to shotgun sequencing. The subset of samples was obtained by pooling together DNA samples with similar microbial communities. Libraries were prepared, using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA). The average library size was assessed with a 2200 TapeStation system (Agilent). Samples were equimolarity pooled and diluted until 1.3 pM. Sequencing was carried out on the Illumina NextSeq 550 platform, employing a 2 × 150 bp paired-end read sequencing chemistry. The raw data were deposited on the European Nucleotide Archive (ENA) with Bioproject n. PRJEB76115.

2.5. Bioinformatic analysis

2.5.1. Bacterial community annotation

The raw data obtained by 16S rRNA gene amplicon sequencing were processed in RStudio [15] using the *dada2*package [19]. The sequences were first filtered and trimmed utilizing a truncation based on the quality profiles of the reads. The filtered sequences were then dereplicated to combine all identical sequencing reads into unique sequences. After quality check, a total of 117 over 140 samples were used for the analyses, belonging to A (N = 26), B (N = 28), C (N = 19), D (N = 18), and E (N = 26) matrices respectively. Then, the forward and the reverse reads were merged to obtain the full denoised sequences. To increase the accuracy of the results, chimeras were removed. SILVA ribosomal database [20] was used to assign taxonomy to the identified Amplicon Sequence Variants (ASVs). The pathobiome was investigated by filtering of ASV table to keep only the ASVs belonging to potentially pathogenic genera as described in Bartlett et al., 2022 [11]. Briefly,

a list of 267 potentially pathogenic bacterial genera, retrieved from Bartlett et al., 2022 [11], was used to filter the ASV table, retaining only the ASVs whose taxonomic assignment matched the genera present in this list. This approach defined a subset of the global microbial community here defined as *pathobiome*.

2.5.2. Antimicrobial resistome annotation

The shotgun sequencing raw data were processed using TrimGalore version (0.6.10) [https://github.com/FelixKrueger/TrimGalore/tree/master], using a Phred score cutoff of 20 and keeping only sequences longer than 100 bp.

The resistome was extracted from the trimmed reads using RGI (6.0.3) [https://github.com/arpcard/rgi/tree/master], using the RGI-bwt module, with the CARD database as a reference, with default setting and matching to Protein Homolog Models.

The extracted resistome for each sample was then normalized with the 16S rRNA gene copy number obtained with ARGs OAP (3.2.3) [https://github.com/xinehc/args_oap/tree/master], using the method suggested in Yin et al.(2023). [21].

2.6. Statistical analysis

Statistical analysis and graphical representation were performed using R version 4.2.3 software [16] P-value < 0.05 was considered significant.

2.6.1. Prevalence estimation of selected pathogens

The prevalence and exact binomial confidence intervals (95 % level) of the isolated pathogens were estimated by considering both the overall data and the data specific to each type of sludge.

The potential significance of differences in prevalence among types of sludge was assessed using the one-tailed Fisher's exact probability test. In case of significance, a pairwise comparison, using the FDR (false discovery rate) method to adjust the p-value, was conduct. The packages *binom* [22] and *rstatix* [23] were applied.

2.6.2. Comparison of diversity indexes on microbiome, pathobiome and resistome

Statistical tests were performed on three datasets: the whole bacterial community (n = 117), the pathobiome (n = 117), and the resistome (n = 54). The analysis was conducted by *phyloseq* package [24]. For alpha-diversity measures (Shannon index), the count-tables, derived from the microbiome, pathobiome and resistome data, were normalized using Geometric Mean of Pairwise Rations method (*GMPR* package) [25]. The non-parametric Kruskal-Wallis test was used to compare

alpha-diversity. If significant, the pairwise comparison Wilcoxon-Mann-Whitney test was performed by adjusting the p-value (p adj-value) for multiple comparisons using the FDR method. Beta-diversity was evaluated with Bray-Curtis distance and visualized by a Principal Coordinate analysis (PCoA) plot. PERMANOVA test was used to compare beta-diversity parameters among groups.

Moreover, for the pathobiome and the resistome, a differential abundance analysis was conducted *via* the Kruskal-Wallis test. In particular, for the pathobiome, the total abundance of potentially pathogenic genera and the abundance of the single food pathogens were tested; for the resistome, the total ARG abundance and the abundances at the resistance class level were analyzed. If the results of this test had statistical consistency, a set of pairwise tests using the Wilcoxon-Mann-Whitney test was performed.

2.6.3. Variables reduction and classification on microbiome, pahtobiome, and resistome

Bacterial genera, potentially pathogenic bacteria, and ARGs datasets were analyzed to reduce the data complexity in terms of variable numbers and to identify the most important futures to discriminate among the different types of sludge.

In order to reduce the data complexity, a multinomial linear regression model, with LASSO (Least Absolute Shrinkage and Selection Operator) penalty of maximum likelihood, was applied.

The selection of genera and genes was obtained by using the optimal lambda regularization parameter identified as lambda min. Lambda min is the value of lambda (λ) that gives the minimum mean cross-validated error and tends to preserve a greater number of variables.

The random forest (RF) technique was applied considering the genera and genes selected to verify if it is possible to discriminate among the various types of sludge. Out-of-bag (OOB) error was used to measure the predictive ability of the RF model. Multidimensional scaling (MDS) plot of RF results was used to highlight the similarities among the sludge. The importance variables' graph, ordered according to the mean decrease accuracy, was used to show the most relevant genera and genes in the classification of the sludge. The packages *glmnet* [26], *randomForest* [27], and *ggplot2* [28] were applied.

2.6.4. Network analysis

The co-occurrence between ARGs and their possible bacterial hosts was investigated through a network correlation analysis. In particular, for the whole bacterial community, only 54 samples matching the resistome dataset were analyzed. Moreover, ARGs with a total normalized abundance in all samples ≥ 0.003 and bacterial genera with a total normalized abundance in all samples ≥ 1000 were selected. Applying these thresholds, at least 20 % of genes/genera from both datasets was retained. A matrix of pairwise correlations for each type of sludge was obtained by Spearman rank correlation [29], using the *psych* package [30], considering genetic elements significantly correlated for rho> 0.8 and $p < 0.01$. The co-occurrence network, based on such significantly correlated genetic elements, was characterized and visualized using Gephi software v0.10.1 [31]. In the network, a modularity index > 0.4 identified a modular structure of its components (as defined in Newman, 2006).[32].

3. Results

3.1. Identification of selected pathogenic bacteria and parasites

Salmonella was successfully detected and isolated in 27 out of 140 samples. Details on the *Salmonella* serovars identified are reported in Table 1**S**. *Listeria monocytogenes* was isolated from 10 samples as detailed in Table 2S. *Campylobacter spp.* was isolated from 3 samples (**Table 3S**). Cysts (from 2 to 6) of *Giardia duodenalis* were detected in 6 out of 140 samples. All specimens were Cryptosporidium negative. Despite the detection of STEC in a few samples, evidenced by the positivity for *stx* and *eae* genes (**Table 4S**), attempts to microbiologically isolate the strain were unsuccessful. The prevalence and exact binomial confidence intervals (95 % level) of the selected pathogens were reported in Table 2 and **Table 8S**.

The chi-square test indicated that there was not a significant difference among the prevalence of the different sludges for *Listeria monocytogenes* ($p = 0.0816$), and *Campylobacter* spp. ($p = 0.604$), whereas there were significant differences for *Salmonella* spp. ($p < 0.001$) and *Giardia duodenalis* ($p < 0.001$). The pairwise comparison indicated that the prevalence of *Salmonella* spp in E was significantly higher than in A ($p = 0.0066$) and B ($p = 0.0018$). Whereas, the prevalence of *Giardia duodenalis* in E was significantly higher than in B ($p = 0.0397$), and D ($p = 0.0397$).

3.2. Bacterial community composition

As evidenced by Fig. 1A, the alpha-diversity of the bacterial community, as measured by the Shannon index, highlighted differences among the various sludges. Significant differences were found between B and D ($p < 0.001$), with matrix D exhibiting the highest median Shannon index. Statistically significant variances are also observed between A and D, as well as between B and E ($p < 0.05$).

The analysis of beta-diversity has revealed a distinct partitioning among compost matrices (A and D), digestate matrices (B and C), and the treated and untreated, and non-compliant agricultural sludge matrix (E), with samples clustering into three different clusters (Fig. 2A). This partitioning is reflected in the taxonomic composition (Fig. 3A). Indeed, A and D, derived from compost, exhibited higher abundance in Bacilli, Alphaproteobacteria, Actinobacteria, and Deinococci, while B and C, from digestate, showed greater abundance in taxa belonging to the class of Bacteroidia, Gammaproteobacteria, Limnochordia, and Clostridia. For E, approximately 50 % of the bacterial community comprised taxa belonging to the class of Bacteroidia and Gammaproteobacteria.

Regarding unique and shared bacterial genera, we can observe that the matrix showing the highest number of exclusive bacterial genera was E. In this case, the unique genera accounted for 50 % of the bacteria present in the microbial community of this type of sludge. The matrix with the lowest number was matrix D (8 %). For the other types of sludges, the unique genera were: 18 % for A, 12 % for B, and 14 % for C (Supplementary Fig. S1A).

Considering the bacterial genera, the regression model with LASSO penalty selected 67 genera (Table S1 supplementary material). This selection was obtained considering a lambda (λ) value of 0.002. The five groups of sludge were distinguishable in the RF MDS plot (Fig. 4A left panel, OOB=2 %). From this figure, B and C were more similar to each other, as for A and D, while E was different from all the others. The most relevant bacterial genera for the sludge's classification were shown in Fig. 4A (right panel) and the first five were *Dethiobacter*, *Gracilibacillus*, *Oceanobacillus*, *Aliivibrio,* and *Lautropia*.

3.3. Pathobiota composition

After filtering the obtained ASV table for the presence of the 267 potentially pathogenic bacterial genera identified by Barlett et al., 2022 [11], we retained only 8588 ASVs. The prevalence of potentially pathogenic genera (PPG) varied from approximately 10 % to 20 %, with differences stemming from the sludge of origin (Fig. 3B). Specifically, the prevalence of PPG in A was significantly higher than that found in all other types of sludge, while the prevalence of PPG in B was significantly lower than in the other sludges ($p < 0.005$). The prevalence of PPG in D was significantly higher than in matrices B, C, and E ($p < 0.005$); the prevalence of PPG in C is significantly higher than in B ($p < 0.005$), and the prevalence of PPG in E is significantly higher than in B and C ($p < 0.005$).

Regarding the diversity of the pathobiota, the Shannon index varied significantly depending on the type of sludge. Particularly, the greatest

Table 2
Prevalence and confidence interval (CI_{95}) of each pathogen, overall and by type of analyzed substrate (A-E). CI_{95} is indicated between square brackets.

	Prevalence [and CI_{95}]					
Pathogens N pos/N tot	Overall (140)	A (33)	B (29)	C (22)	D (30)	E (26)
Salmonella spp. 19/140	13.57 % [8.37; 20.38]	3.03 % [0.08; 15.76]	0 % [0; 11.94]*	22.73 % [7.82; 45.37]	10.00 % [2.11; 26.53]	38.46 % [20.23; 59.43]
Listeria monocytogenes 10/140	7.14 % [3.48; 12.74]	6.06 % [0.74; 20.22]	3.45 % [0.09;17.76]	18.18 % [5.19; 40.28]	0 % [0; 11.57]*	11.53 % [2.44; 30.15]
Campylobacter spp 3/140	2.14 % [0.44; 6.13]	0 % [0; 10.58]*	0 % [0; 11.94]*	4.54 % [0.11; 22.84]	3.33 % [0.08; 17.21]	0 % [0; 13.23]*
Giardia duodenalis 6/140	4.29 % [1.59; 9.09]	0 % [0; 10.58]*	0 % [0; 11.94]*	0 % [0; 15.43]*	0 % [0; 11.57]*	23.07 % [8.97; 43.65]

(*) one-sided, 97.5 % confidence interval

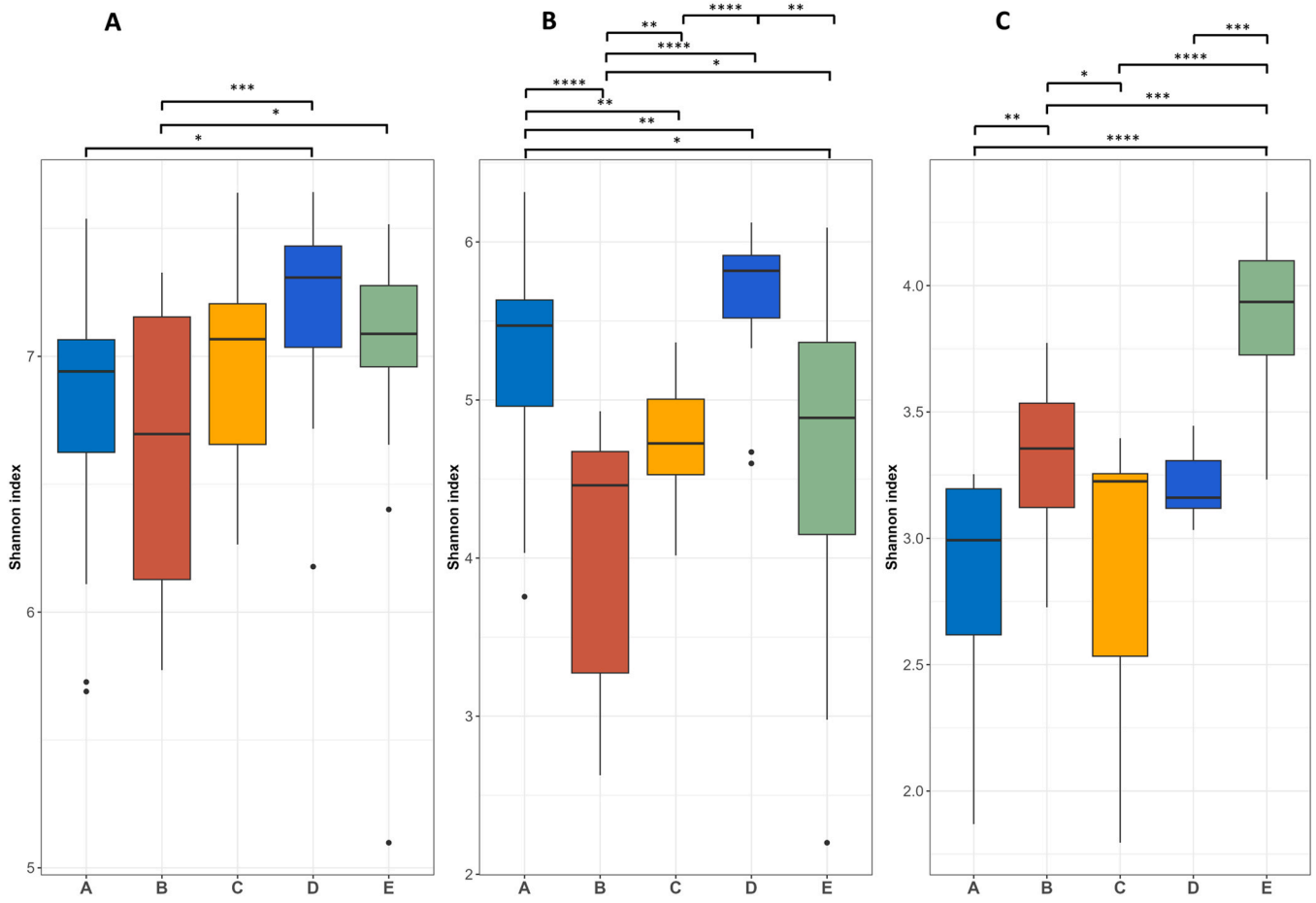


Fig. 1. Boxplots of the Shannon index in five sample matrices. **A.** Shannon index calculated from the entire dataset of the bacterial community. This panel represents the diversity of all bacteria present across the five matrices. **B.** Shannon index calculated from the pathobiome dataset. This panel shows the diversity specifically within the subset of bacteria identified as potentially pathogens. **C.** Shannon index calculated from the antimicrobial resistome dataset. This panel depicts the diversity within the subset of antimicrobial resistance genes. Outliers are indicated by dots in each boxplot. The significance of differences between the matrices is indicated by asterisks: * $p <$ **0,05**; * * $p <$ **0,01**; * ** $p <$ **0001**; * ** * $p <$ **0,0001**.

difference in terms of richness was observed in the comparison between A and B, B and D, and C and D ($p < 0.0001$), with D exhibiting highest richness and B showing the lowest richness (Fig. 1B). Similarly to what was observed in the case of the whole bacterial community, the pathobiota also exhibited a community-level structure that reflected the type of matrix analyzed (Fig. 2B).

The Venn diagram shown in Supplementary Fig. S1B revealed a clear differentiation in the composition of the resident pathobiota across the analyzed sludges. Particularly noteworthy is the presence of unique genera in B, including bacteria belonging to the genera *Campylobacter* and *Clostridium*, and the presence of *Serratia* in D, *Rickettsia* in C, and *Yersinia* in E.

Among the foodborne potentially pathogenic bacteria identified in the analyzed matrices, it is noteworthy that the differential abundance of *Bacillus*, *Staphylococcus*, *Escherichia*, and *Brucella* is statistically different across the various sludges (Table 2A). Particularly, although detected in every matrix, the genus *Bacillus* was more prevalent in samples derived from D. In addition, samples belonging to A displayed a higher load of *Bacillus* than B, C, and E ($p < 0.001$) and B than in E ($p < 0.05$). The genus *Staphylococcus* was significantly higher in A if

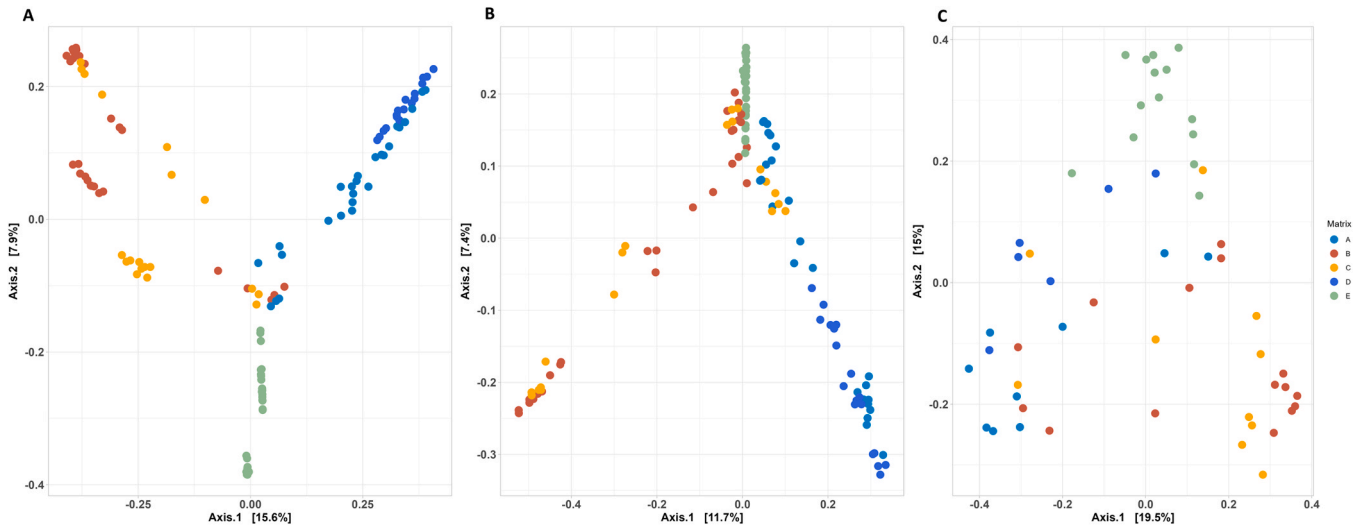


Fig. 2. PCoA plot of beta diversity. Each point represented the structure of microbial community of samples. Samples with similar composition tended to be in the same area of the plot, while points far apart from each other represent samples with dissimilar composition. Kinds of samples' matrix are identified by different colours (A: light-blue; B: red; C: yellow; D: blue; E: green). **A.** Beta-diversity calculate for whole bacterial community **B.** Beta-diversity calculate for pathobiome **C.** Beta-diversity calculate for antimicrobial resistome.

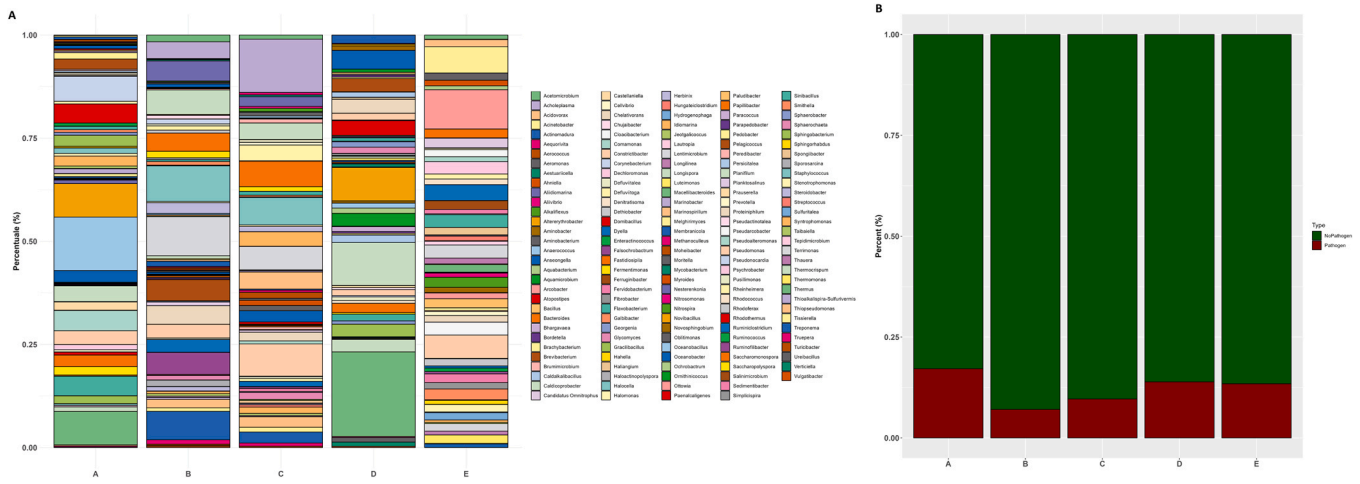


Fig. 3. A. Stacked bar charts showing relative abundance (%) of bacterial communities, annotated to the taxonomic level of genus, derived from the five different matrices in exam. Each bacterial genus is identified by a color as described in the legend. **B.** Stacked bar chart depicting % of taxa belonging to pathobiota and non-pathobiota. Red bars show the % of pathobiota, and green bar represent other taxa.

compared to B, C, and E samples ($p < 0.001$), and in D than in B ($p < 0.01$), C, and E ($p < 0001$). Additionally, *Staphylococcus* was significantly higher as well as in samples from matrix B than in C ($p < 0.05$) and E ($p < 0.01$). As regards, the genus *Escherichia* it was more prevalent in E than in the other sampled matrices ($p < 0.05$) but also in- samples belonging to B if compared to C ($p < 0.05$). The genus *Brucella* was significantly higher in D than in A and B ($p < 0.05$).

Considering the pathogenic bacterial genera, the regression model with the LASSO penalty selects 39 genera (Table S2 supplementary material). This selection was obtained considering a value of λ equal to 0.007.

The five groups of sludge were distinguishable in the RF MDS plot (Fig. 4B left panel, OOB=3 %). From this figure, B and C were more similar to each other, as for A and D, while E was different from all the others. The most relevant pathogenic bacteria genera for the sludge's classification were shown in Fig. 4B (right panel) and the first five were *Fastidiosipila*, *Brevibacterium*, *Ruminococcus*, *Corynebacterium* and *Lawsonella*..

3.4. Antimicrobial resistome composition

The Shannon index related to the resistome showed statistically significant differences based on the matrix. Specifically, the greatest differences were observed in the comparison between A and E ($p < 0.0001$), and between C and E ($p < 0.0001$) (Fig. 1C), with matrix E having, in general, the highest median Shannon index with respect with the other sludges. Looking at the results of beta-diversity analysis, the resistome structure highlighted the presence of clusters coinciding with the Compost and Digestate matrices, albeit less prominently than observed for the structure of the whole microbial community and the pathobiome (Fig. 2C).

Regarding unique and shared ARGs, the matrix showing the highest number of exclusive genes was E, where unique ARGs accounted for 59 % of genes detected in this type of sludge. The matrix with the lowest number was matrix C (11 %) (Supplementary Fig. S1C).

Among the ARG classes identified in the analyzed matrices, it is noteworthy that the differential abundance of resistance determinants to Beta-Lactams, Macrolides, Phenicols, Quinolones, and Tetracyclines is

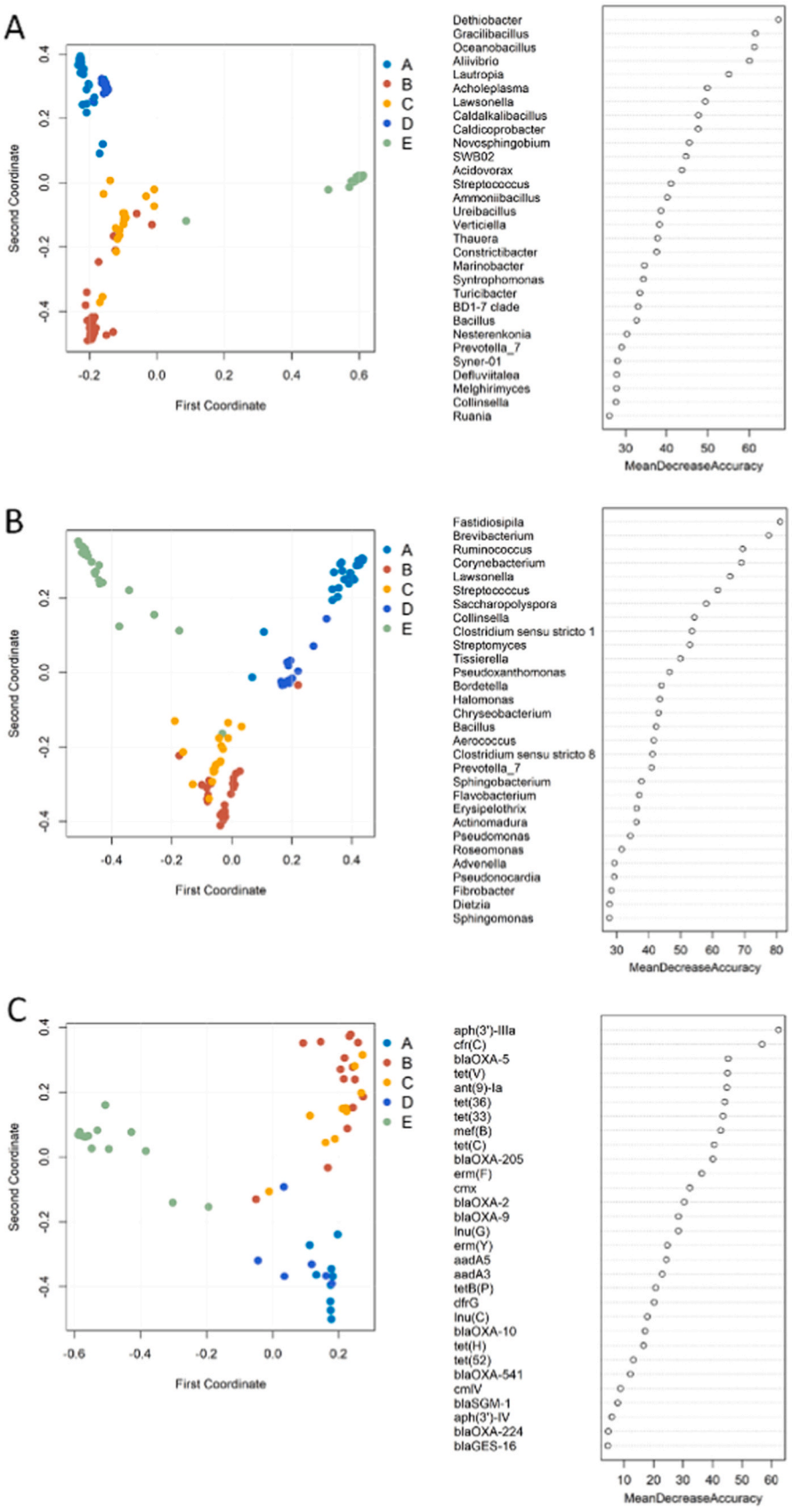


Fig. 4. Random forest output: MDS (left panel) and importance variables according to the mean decrease accuracy (right panel). A. bacterial genera characterizing the different samples; B. potentially pathogenic bacteria characterizing the different samples; C. antimicrobial resistance genes characterizing the different samples.

Table 2A
. Results of the differential abundance analysis on the pathobiome and potentially pathogenic genera with a transmission through food chain. For the single genera, only the significant ones are shown.

Differential abundance analysis					
Pathobiota					
	A	**B**	**C**	**D**	**E**
A					
B	0.577				
C	0.078	0.151			
D	0.349	0210	**0.043**		
E	4.8e−06	5,4e−05	0.184	**0.027**	
Bacillus					
	A	**B**	**C**	**D**	**E**
A					
B	7.2e−05				
C	9.8e−05	0.399			
D	**0.005**	5.6e−08	2.8e−07		
E	4.3e−07	**0.017**	0.112	4.0e−09	
Staphylococcus					
	A	**B**	**C**	**D**	**E**
A					
B	4.1e−05				
C	5.3e−06	**0.025**			
D	0.375	**0.0005**	2.8e−05		
E	3.9e−07	**0.004**	0.8218	2.0e−06	
Escherichia					
	A	**B**	**C**	**D**	**E**
A					
B	0.449				
C	0.713	**0.038**			
D	0.622	0.449	0.658		
E	**0.049**	**0.049**	**0.049**	**0.049**	
Brucella					
	A	**B**	**C**	**D**	**E**
A					
B	0.597				
C	0.639	0.877			
D	**0.048**	**0.048**	0.081		
E	0.210	0.210	0.210	0.210	

Table 2B
. Results of the differential abundance analysis on the total resitome and single antimicrobial resistance classes. For the single classes, only the significant ones are shown.

Differential abundance analysis					
Resistome					
	A	**B**	**C**	**D**	**E**
A					
B	0.087				
C	0.087	0.630			
D	0.681	0.087	0.087		
E	0.101	0.311	0.470	**0.024**	
Beta-lactamase					
	A	**B**	**C**	**D**	**E**
A					
B	0.726				
C	0.445	0.562			
D	0.062	0.110	0.230		
E	1.3e−05	5.3e−07	8.7e−06	**0.001**	
Macrolide					
	A	**B**	**C**	**D**	**E**
A					
B	0.459				
C	0.142	0.459			
D	0.459	0.142	**0.034**		
E	0.142	0.650	0.580	**0.017**	
Phenicol					
	A	**B**	**C**	**D**	**E**
A					
B	**0.001**				
C	0.541	0.100			
D	0.347	**0.001**	0.922		
E	**0.001**	0.100	0.429		
Quinolone					
	A	**B**	**C**	**D**	**E**
A					
B	**0.017**				
C	0.834	0.062			
D	0.662	**0.011**	0.662		
E	0.155	**0.005**	0.146	0.271	
Tetracycline					
	A	**B**	**C**	**D**	**E**
A					
B	**0.002**				
C	**0.009**	0.101			
D	0.069	**0.008**	**0.026**		
E	**0.009**	**0.009**	0.101	**0.024**	

statistically different across the various sludges (Table 2B). Particularly, although detected in every matrix, genes encoding for the resistance to Beta-Lactams had the highest abundance in E ($p < 0.001$) (Table 2B). Genes encoding resistance to Quinolones were significantly lesser abundant in B than in A, D, and E ($p < 0.05$); whereas, genes against Macrolides were lesser in D than in C and E ($p < 0.05$) (Table 2B). Resistance to Phenicols was significantly higher both in B, than in A and D, and in E, than in A ($p < 0.01$) (Table 2B). Resistance to Tetracyclines was lower in A and D than B, C, and E ($p < 0.05$), and in E than in B ($p < 0.01$) (Table 2B). No significant differences among matrices were found for the other resistance classes. Moreover, the total ARG abundance was higher in E than in D ($p < 0.05$) (Table 2B). Looking at the single genes, *erm*C had the highest abundance in A and *erm*F (both encoding for the resistance to Macrolides) in B and C, *aph*(6)-Id (to Aminoglycosides) in D, and *msr*E (to Macrolides) in E (Supplementary Table S7).

Considering the ARGs, the regression model with the LASSO penalty selects 57 genes (Table S7 supplementary material). This selection was obtained considering a value of λ equal to 0.025.

The five groups of sludge were distinguishable in the RF MDS plot (Fig. 4C left panel, OOB=9 %). From this figure, B and C were more similar to each other, as for A and D, while E was different from all the others. The most relevant ARGs for the sludge's classification were shown in Fig. 4C (right panel) and the first five were *aph*(3′)-IIIa, *cfr*(C), *bla*OXA-5, *tet*(V) and *ant*(9)-Ia.

3.5. Co-occurrence network

The co-occurrence network was composed of 126 nodes (52 ARGs and 74 bacterial genera) and 395 edges in A (Supplementary Figure 2**A**); 221 nodes (85 ARGs and 136 bacterial genera) and 1759 edges for B (Supplementary Figure 2B); by 209 nodes (66 ARGs and 143 bacterial genera) and 2104 edges for C (Supplementary Figure 2**C**); by 94 nodes (34 ARGs and 60 bacterial genera) and 177 edges for D (Supplementary Figure 2D); by 110 nodes (30 ARGs and 80 bacterial genera) and 150 edges for E (Supplementary Figure 2E). All the networks included several potentially pathogenic bacterial genera: *e.g.*, *Streptococcus*, *Chryseobacterium*, and *Arcobacter* in the A (Supplementary Figure 2**A**); *Treponema*, *Bacteroides*, and *Streptococcus* in B (Supplementary Figure 2B); *Mycobacterium*, *Microbacterium*, and *Bordetella* in C (Supplementary Figure 2**C**); *Aeromonas*, *Pseudomonas*, and *Brevibacterium* in the D (Supplementary Figure 2D); *Corynebacterium*, *Pseudomonas*, and *Paracoccus* in E (Supplementary Figure 2E). These genera co-occurred with ARGs. Some of the potentially pathogenic bacteria comprised genera displaying a transmission by food chain. In particular, in C, we had *Clostridium sensu stricto 1* and *Bacillus*, which co-occurred with both ARGs (*qnr*D2, *dfr*A14, and *mph*(A)) and other potentially pathogenic genera (*Microbacterium* and *Bordetella*) (Supplementary Figure 2**C**). In D, a co-occurrence of *Clostridium sensu stricto 1* with *aad*A3, *aad*A5, *aad*A8b, and *tet*(36) was observed (Supplementary Figure 2D). In E, we had *Clostridium sensu stricto 1* and *Staphylococcus*, with the former co-occurring with another potentially pathogenic genus (*Psychrobacter*) and the latter with an ARG (*cfr*) (Supplementary Figure 2E). No potentially pathogenic bacteria with food chain

transmission were found in A and B (Supplementary Figure 2**A, 2B**).

4. Discussion

Bacterial communities of the analyzed samples were differentiated based on the treatments applied (either anaerobic digestion or composting). Specifically, the bacterial community of the agro-industrial digestate resembled that obtained from the anaerobic digestion of a mixture of source-separated biowaste and green waste, in contrast to the compost samples. This result is particularly interesting, especially considering that one set of digestate samples originated from the biowaste, while a set of compost samples derived from the digestate of biowaste. This indicates that the treatment applied to the samples significantly influenced the bacterial community composition of the sludge, possibly more than the source of the sample. Indeed in both digestate samples Clostridia and Bacteroidia were among the most abundant bacteria while Bacilli and Alphaproteobacteria prevailed in compost samples, as previously observed [33,34].

The bacterial communities in sludge from WWTP samples differed from the others, with Bacteroidia and Gammaproteobacteria being prevalent. This is consistent with a previous study where these bacteria were found to be abundant within bacterial communities from inlet wastewater samples [33]. When analyzing the diversity of bacterial communities, we did not observe a specific pattern. There was no agreement between the two compost samples and the two digestate samples. Indeed, only one of the two compost samples, *i.e.*, the compost derived from the biowaste digestate, exhibited higher bacterial community diversity if compared to the other compost (and to the agro-industrial digestate). This finding is intriguing as it suggests that despite the application of two sequential treatments (anaerobic digestion and composting) to the samples derived from biowaste, alpha diversity appeared unaffected or minimally affected.

Analyzing the sole pathobiome, the different samples exhibited similar patterns to those observed in the whole bacterial community: digested samples clustered together, composts formed another group, while WWTP sludge samples remained distinct. Consequently, it is reasonable to reach the same conclusion as for the whole bacterial community; the treatments applied to the waste samples markedly influenced the composition of the pathobiome in different sludges. The compost from biowaste and green waste appeared to be the most human concerning sludge, exhibiting the highest abundance of the PPG and greater PPG diversity compared to all other samples, being second for pathobiome diversity only with respect to the compost derived from anaerobically digested biowaste. These findings are consistent with a previous study by Tozzoli et al., [35] which indicated that enteric viruses and pathogenic E. coli could be released into the environment through the use of sludge-derived soil improvers (TSI). This finding contrasts with the results obtained by the cultivation-dependent approaches that identified the WWTP sludges as the wastes potentially most hazardous for human health. These sludges showed a higher frequency of *Salmonella* strain isolation, if compared to the sludges available for agriculture. Currently, *Salmonella* is the only bacterial genus monitored to assess the suitability of sludge for agricultural purposes [6]. Overall, these results underscore the limitations of cultivation-dependent approaches and of the use of Salmonella as a unique indicator of soil conditioners safety, thus emphasizing the necessity of integrating both cultivation-dependent and cultivation-independent methods for comprehensive microbiological monitoring, as previously proposed [36,37]. In the pathobiome of compost derived from biowaste and green waste, *Staphylococcus*, recognized as a "significant food safety hazard" [38], was found to be significantly more abundant compared to other types of substrates. This result suggests a need for further characterization of *Staphylococcus* populations in sludges allowed for use in agriculture, to identify the virulence and the potential pathogenic behavior of these bacteria.

Contrary to the results from the pathobiome analysis, the characterization of the antimicrobial resistome identified the WWTP sludge samples as potentially more hazardous compared to the other types of sludges. This was clear by observing both a higher diversity and a greater number of exclusively detected ARGs. Notably, beta-lactamases were prevalent, including several genes, *e.g.*, $bla_{\text{GES-11}}$, $bla_{\text{OXA-10}}$, defined as high-risk ARGs for human health [39]. However, the digestate from biowaste and green waste samples shared some high-risk ARGs, *e.g.*, $bla_{\text{OXA-10}}$, with the WWTP sludge samples and their antimicrobial resistome was rich in the *erm*C gene, which has also been classified as a high-risk ARG [39]. Noteworthy, another set of samples allowed to be used in agriculture, namely agro-industrial digestate samples, showed a greater prevalence of quinolone resistance genes compared to the other samples. These ARGs are generally associated to mobile genetic elements [40] and are increasingly detected in pathogenic bacteria [41]. Furthermore, although the co-occurrence of food pathogens and ARGs was observed only in the samples categorized as not available for agriculture, potentially pathogenic bacteria and ARGs co-occurred in all the sludges, underscoring once more that also the sludges usable for agriculture might be dangerous for the transmission of potentially pathogenic and antimicrobial resistant bacteria.

Overall, our findings indicate that the current biological parameters, such as the presence of Salmonella, used to determine the suitability of soil conditioners for agricultural use are inadequate. Soil conditioners deemed compliant under these regulations may still pose greater risks to human health, especially when considering the entire pathobiome and resistome, compared to those that are prohibited for agricultural application. Furthermore, they underscore that the integration of omic approaches with classical microbiological methods can univocally define the potential risks associated with sludges for agricultural use. Nonetheless, employing metagenomic analysis for diagnostic purposes on these samples might not be straightforward. Therefore, we refined the whole bacterial community, pathobiome, and resistome to focus on a selection of a few targets that can discriminate between the different soil conditioners. These targets could be used to develop quantitative Real-time PCR or digital droplet PCR protocols for monitoring the sludge samples to be used in agriculture.

This study represents the hazard identification phase, the initial step in the risk assessment process.

The next step in this process is to evaluate the persistence of these pathogens and ARGs in soils when soil conditioners are used in real-life scenarios. This involves investigating how long these contaminants remain active and viable in the soil environment and under various agricultural conditions. Understanding the persistence of these hazards is crucial for assessing long-term risks and their potential to impact soil health and crop safety over time. If the persistence of these pathogens and resistance genes is confirmed, we will proceed to the exposure assessment phase. This phase aims to evaluate the actual rate of transfer of these biological contaminants from soil to food crops. By determining how these contaminants move through the soil and into the plants, we can more accurately gauge the potential risks to human health. This comprehensive evaluation will involve field studies and controlled experiments to monitor the uptake of pathogens and ARGs by crops under different agricultural practices.

Through this detailed risk assessment process, we aim to provide robust data to inform the development of effective regulatory measures. These measures will ensure that soil conditioners used in agriculture do not compromise food safety, thereby safeguarding public health while promoting sustainable agricultural practices.

5. Conclusion

- In the context of a circular economy, using biosolids as soil conditioners allows for the conversion of waste into valuable resources. However, as highlighted by this study, this practice necessitates a thorough risk assessment for both environmental and human health.

- The parameters set by current legislation appear to be too restrictive. Soil conditioners that are deemed compliant for agricultural use may still pose potential public health risks when considering the entire resistome and pathobiome.
- The combined use of cultivation-dependent and cultivation-independent methods could significantly improve the assessment of the biological risk of biosolids used as soil conditioners.
- Integrating various omic approaches has enabled the identification of specific targets for each matrix. These targets could be used to develop real-time PCR or digital droplet PCR protocols for a rapid monitoring to discriminate the diverse types of biosolid conditioners.

Funding

This research was supported by EU funding within the NextGenerationEU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT) and by Ministero della Salute, Ricerca Corrente 2019, Grant Number RC IZSVe 02/2019.

CRediT authorship contribution statement

Sara Petrin: Writing – original draft, Investigation. **Aurora Boscolo:** Writing – original draft, Visualization, Formal analysis, Data curation. **Andrea Di Cesare:** Writing – original draft, Methodology. **Marzia Mancin:** Writing – original draft, Visualization, Methodology, Formal analysis. **Arianna Peruzzo:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Lisa Barco:** Writing – original draft, Funding acquisition, Conceptualization. **Patrizia Danesi:** Writing – original draft, Investigation. **Carmen Losasso:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Mari Cristina Lavagnolo:** Writing – original draft, Methodology, Conceptualization. **Raffaella Sabatino:** Writing – original draft, Visualization, Investigation. **Giulia Baggio:** Writing – original draft, Software, Formal analysis, Data curation. **Giovanni Beggio:** Writing – original draft, Investigation.

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.

Data Availability

Data will be made available on request.

Acknowledgments

Raffaella Sabatino was supported by the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of December 16, 2021, rectified by Decree n.3175 of December 18, 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU (code CN_00000033, Concession Decree No. 1034 of June 17, 2022 adopted by the Italian Ministry of University and Research, CUP B83C22002930006, Project title “National Biodiversity Future Center – NBFC.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135567.

References

[1] Cárdenas-Talero, J.L., Silva-Leal, J.A., Pérez-Vidal, A., Torres-Lozada, P., 2022. The influence of municipal wastewater treatment technologies on the biological stabilization of sewage sludge: A systematic review. Sustainability 14 (10), 5910.
[2] Pivato, A., Beggio, G., Bonato, T., Butti, L., Cavani, L., Ciavatta, C., et al., 2022. The role of the precautionary principle in the agricultural reuse of sewage sludge from urban wastewater treatment plants. Detritus 19.
[3] Pakdel, M., Olsen, A., Bar, E.M.S., 2023. A review of food contaminants and their pathways within food processing facilities using open food processing equipment. J Food Prot 86 (12), 100184.
[4] Papajová, I., Šmigová, J., Gregová, G., Šoltys, J., Venglovský, J., Papaj, J., et al., 2022. Effect of wastewater treatment on bacterial community, antibiotic-resistant bacteria and endoparasites. Int J Environ Res Public Health 19 (5), 2750.
[5] Communities, E., English, Edition, 1986. J Eur Communities 29.
[6] Gazzetta Ufficiale Available online: https://www.gazzettaufficiale.it/eli/id/1992/02/15/092G0139/sg (accessed on 17 April 2024).
[7] Gigliucci, F., Brambilla, G., Tozzoli, R., Michelacci, V., Morabito, S., 2017. Comparative analysis of metagenomes of Italian top soil improvers. Environ Res 155, 108–115.
[8] Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., et al., 2013. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat Commun 4 (1), 2151.
[9] Käppler, A., Fischer, D., Oberbeckmann, S., Schernewski, G., Labrenz, M., Eichhorn, K.J., et al., 2016. Analysis of environmental microplastics by vibrational microspectroscopy: FTIR, Raman or both? Anal Bioanal Chem 408, 8377–8391.
[10] Sorinolu, A.J., Tyagi, N., Kumar, A., Munir, M., 2021. Antibiotic resistance development and human health risks during wastewater reuse and biosolids application in agriculture. Chemosphere 265, 129032.
[11] Bartlett, A., Padfield, D., Lear, L., Bendall, R., Vos, M., 2022. A comprehensive list of bacterial pathogens infecting humans. Microbiology 168 (12), 001269.
[12] Hennebert, P., Beggio, G., 2021. Sampling and sub-sampling of granular waste: size of a representative sample in terms of number of particles. Detritus 17, 30–41.
[13] ISO/TS 13136:2012 - Microbiology of Food and Animal Feed — Real-Time Polymerase Chain Reaction (PCR)-Based Method for the Detection of Food-Borne Pathogens — Horizontal Method for the Detection of Shiga Toxin-Producing Escherichia coli (STEC) and the Determination of O157, O111, O26, O103 and O145 Serogroups Available online: https://www.iso.org/standard/53328.html (accessed on 17 April 2024).
[14] ISO 17604:2015 - Microbiology of the Food Chain — Carcass Sampling for Microbiological Analysis Available online: https://www.iso.org/standard/62769.html (accessed on 17 April 2024).
[15] ISO/TR 6579–3:2014 - Microbiology of the Food Chain — Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella — Part 3: Guidelines for Serotyping of Salmonella Spp. Available online: https://www.iso.org/standard/56714.html (accessed on 17 April 2024).
[16] Grimont, P.A., Weill, F.X., 2007. Antigenic formulae of the Salmonella serovars. WHO Collab Cent Ref Res Salmon 9.
[17] Issenhuth-Jeanjean, S. , Roggentin, P. , Mikoleit, M. , Guibourdenche, M. , De Pinna, E. , Nair, S. , & et al. . (2014). Supplement 2008–2010 (no. 48) to the white–Kauffmann–Le minor scheme. Research in microbiology, 165(7), 526–530.
[18] ISO 10272–1:2017/Amd 1:2023 - Microbiology of the Food Chain — Horizontal Method for Detection and Enumeration of Campylobacter Spp. — Part 1: Detection Method — Amendment 1: Inclusion of Methods for Molecular Confirmation and Identification of Thermotolerant Campylobacter Spp., the Use of Growth Supplement in Preston Broth and Changes in the Performance Testing of Culture Media Available online: https://www.iso.org/standard/77639.html (accessed on 17 April 2024).
[19] Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13 (7), 581–583.
[20] Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41 (D1).
[21] Yin, X., Chen, X., Jiang, X.T., Yang, Y., Li, B., Shum, M.H.H., et al., 2023. Toward a universal unit for quantification of antibiotic resistance genes in environmental samples. Environ Sci Technol 57 (26), 9713–9721.
[22] CRAN - Package Binom Available online: https://cran.r-project.org/web/packages/binom/index.html (accessed on 22 April 2024).
[23] CRAN - Package Rstatix Available online: https://cran.r-project.org/web/packages/rstatix/index.html (accessed on 22 April 2024).
[24] McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 8 (4), e61217.
[25] Chen, L., Reeve, J., Zhang, L., Huang, S., Wang, X., Chen, J., 2018. GMPR: a robust normalization method for zero-inflated count data with application to microbiome sequencing data. PeerJ 6, e4600.
[26] Glmnet: Lasso and Elastic-Net Regularized Generalized Linear Models Available online: https://www.researchgate.net/publication/285707231_Glmnet_Lasso_and_elastic-net_regularized_generalized_linear_models (accessed on 22 April 2024).
[27] Breiman, L., 2001. Random forests. Mach Learn 45, 5–32.
[28] R: Ggplot2: Create Elegant Data Visualisations Using the Grammar… Available online: https://search.r-project.org/CRAN/refmans/ggplot2/html/ggplot2-package.html (accessed on 22 April 2024).

[29] Barberán, A., Bates, S.T., Casamayor, E.O., Fierer, N., 2012. Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J 6 (2), 343–351.
[30] Revelle, W. (2017). Using the psych package to generate and test structural models. Recuperado de http://bioconductor. statistik. tu-dortmund. de/cran/web/packages/psych/vignettes/psych_for_sem. pdf.
[31] Bastian, M., Heymann, S., Jacomy, M., 2009, March. Gephi: an open source software for exploring and manipulating networks. Proc Int AAAI Conf web Soc Media Vol. 3 (No. 1), 361–362.
[32] Newman, M.E., 2006. Modularity and community structure in networks. Proc Natl Acad Sci 103 (23), 8577–8582.
[33] Ziganshin, A.M., Liebetrau, J., Pröter, J., Kleinsteuber, S., 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl Microbiol Biotechnol 97, 5161–5174.
[34] Liu, D., Li, M., Xi, B., Zhao, Y., Wei, Z., Song, C., et al., 2015. Metaproteomics reveals major microbial players and their biodegradation functions in a large-scale aerobic composting plant. Microb Biotechnol 8 (6), 950–960.
[35] Tozzoli, R., Di Bartolo, I., Gigliucci, F., Brambilla, G., Monini, M., Vignolo, E., et al., 2017. Pathogenic Escherichia coli and enteric viruses in biosolids and related top soil improvers in Italy. J Appl Microbiol 122 (1), 239–247.
[36] Di Cesare, A., Corno, G., Manaia, C.M., Rizzo, L., 2020. Impact of disinfection processes on bacterial community in urban wastewater: Should we rethink microbial assessment methods? J Environ Chem Eng 8 (5), 104393.
[37] Ranjard, L., Poly, F., Nazaret, S., 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Res Microbiol 151 (3), 167–177.
[38] Brauge, T., Bourdonnais, E., Trigueros, S., Cresson, P., Debuiche, S., Granier, S.A., et al., 2024. Antimicrobial resistance and geographical distribution of Staphylococcus sp. isolated from whiting (Merlangius merlangus) and seawater in the English Channel and the North sea. Environ Pollut 345, 123434.
[39] Zhang, A.N., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., et al., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. Nat Commun 12 (1), 4765.
[40] Jacoby, G.A., Strahilevitz, J., Hooper, D.C., 2015. Plasmid-mediated quinolone resistance. Plasmids: Biol Impact Biotechnol Discov 475–503.
[41] Azargun, R., Gholizadeh, P., Sadeghi, V., Hosainzadegan, H., Tarhriz, V., Memar, M.Y., et al., 2020. Molecular mechanisms associated with quinolone resistance in Enterobacteriaceae: review and update. Trans R Soc Trop Med Hyg 114 (10), 770–781.