applied sciences

MDPI

Article

Vermicomposting Enhances Microbial Detoxification of Sewage Sludge, Enabling Potential Application of the Treated Product in Agroecosystems

Ana Gómez-Roel *, Manuel Aira and Jorge Domínguez

Grupo de Ecoloxía Animal (GEA), Universidade de Vigo, E-36310 Vigo, Spain; aira@uvigo.gal (M.A.); jdguez@uvigo.gal (J.D.)
* Correspondence: ana.gomez.roel@uvigo.gal

Abstract: Vermicomposting offers an eco-friendly solution to managing the sewage sludge generated in wastewater treatment plants. The objective of this study was to investigate the microbial community composition, structure and functionality during the vermicomposting of sewage sludge. We analyzed samples of sewage sludge, earthworm casts and vermicompost by applying high-throughput sequencing 16S and ITS rRNA. Most of the bacterial (95%) and fungal taxa (99%) were eliminated and subsequently replaced by other microbial taxa originating from earthworms. Further changes resulted in a vermicompost with a more diverse bacterial (but not fungal) community. In addition, the earthworm activity led to an increase in bacterial and a decrease in fungal alpha diversity, resulting in greater differences in beta diversity between sewage sludge, casts and vermicompost. We also found that bacterial pathways associated with amino acid and plant hormone biosynthesis and antibiotic synthesis were enriched. Vermicomposting successfully eliminated most of the 10 human bacterial pathogens found in the sewage sludge. Simultaneously, parasitic and pathogenic fungal taxa were removed. Overall, vermicompost derived from sewage sludge is safer for disposal on land than raw sludge, particularly regarding their respective microbial compositions. This indicates that it could potentially be used as a soil organic amendment and fertilizer.

Keywords: earthworms; sewage sludge; microbial communities; 16S RNA; ITS; PICRUSt2; vermicompost

Citation: Gómez-Roel, A.; Aira, M.; Domínguez, J. Vermicomposting Enhances Microbial Detoxification of Sewage Sludge, Enabling Potential Application of the Treated Product in Agroecosystems. *Appl. Sci.* **2024**, *14*, 7894. https://doi.org/10.3390/app14177894

Academic Editors: Antonio Calisi, Francesco Dondero and Teodoro Semeraro

Received: 5 August 2024
Revised: 30 August 2024
Accepted: 2 September 2024
Published: 5 September 2024

1. Introduction

Population growth and increased domestic material consumption have led to a global rise in waste production. As a result, waste management has gained social and political importance, and policies aimed at promoting recycling processes and discouraging direct landfill disposal have been implemented in recent years [1,2]. Large amounts of sewage sludge are generated in wastewater treatment plants (WWTPs), and the amounts produced are expected to increase even further in the near future [1]. Sewage sludge is a challenging type of waste to treat/manage because it contains several types of pollutants and also pathogenic microorganisms. Unfortunately, less than 25% of the material is effectively composted or recycled, while 50% of sewage sludge is simply dumped on agricultural or forest soils (http://ec.europa.eu/eurostat, accessed on 18 July 2024) [1,2]. An option to circumvent the high load of contaminants found in sewage sludge from WWTPs located in big cities and industrial areas is to process sewage sludge from small WWTPs belonging to medium–small cities that usually have significantly lower levels of heavy metals and other contaminants [3,4].

Vermicomposting offers an eco-friendly solution for managing sewage sludge. This process involves the combined action of earthworms and microorganisms to modify and expedite the decomposition process. It consists of an active phase and a maturation phase. The active phase consists of the transit of the substrate through the earthworm gut (referred

to as gut-associated processes or GAPs), during which microbial communities are greatly modified and excreted as earthworm casts [2,5]. The maturation phase consists of the ageing of casts (cast-associated processes, CAPs), during which microbial succession is the main process involved [2,5]. Some research has shown that vermicomposting can effectively reduce the presence of human bacterial pathogens (HBPs) [6–9], environmental pollutants, such as antibiotic resistance genes, pharmaceuticals and personal care products, and also the bioavailability of heavy metals of sewage sludge [10–13]. Furthermore, several studies suggest that at appropriate doses, vermicompost can be used as a soil conditioner and fertilizer due to its physical, chemical and biological characteristics [14–17].

One major gap in knowledge about vermicomposting is understanding the composition of microbial communities and their changes throughout the process [2,10,18]. As mentioned previously, this change in composition happens in two phases: first, earthworm gut digestion, followed by the maturation of earthworm cast (GAPs and CAPs). Most studies focusing on changes in bacterial communities during vermicomposting have shown that the bacterial communities of vermicompost differ significantly at the phylum level from those of sewage sludge [2,5,14–17]. Thus, bacterial communities of sewage sludge are commonly dominated by Campylobacterota and Proteobacteria [2] or Actinobacteriota and Bacteroidota [19]. Once earthworms have digested the sludge (i.e., after GAPs), the main bacterial phyla change to Bacteroidota and Verrucomicrobiota [2]. After CAPs, the main bacterial phyla of vermicompost are Actinobacteriota, Proteobacteria, Bacteroidota, Chloroflexi, Firmicutes and Verrucomicrobiota [2,10,19–22]. Fungal communities have been far less studied in comparison to bacteria during vermicomposting [2,19,23,24]. Vermicomposting significantly altered the fungal communities of sewage sludge, typically dominated by the phyla Basidiomycota [2], Ascomycota [19] or a combination of both [23]. GAPs promoted the rise of Ascomycota, Basidiomycota and Mortierellomycota [2], while CAPs enhanced Mortierellomycota and Basidiomycota [2], Basidiomycota and Zygomycota [19], Rozellomycota and Ascomycota [23] or Mucoromycota, Ascomycota, and Cryptomycota [24]. These divergences in the composition of bacterial and fungal communities of sewage sludge and vermicompost across studies at the phylum level were even higher at a more detailed taxonomic level, such as the genus [2,10,19–22].

Therefore, it is essential to identify the microbial species that are involved in vermicomposting and to understand how they change throughout the process, especially in fungal communities. It is also essential to determine the functional potential of the microbes involved, as this can provide insight into their activities and help explain the changes observed and some of the final properties of the vermicompost. Moreover, it is relevant to determine the impact of vermicomposting on human bacterial pathogens due to environmental health concerns when the sewage sludge or vermicompost is land disposed or used as organic amendments.

Our aim was to determine the effect of earthworms on the bacterial and fungal communities during the different stages of vermicomposting of sewage sludge. To this end, we analyzed samples of sewage sludge, earthworm casts and vermicompost by applying advanced 16S and ITS rRNA high-throughput sequencing, together with sophisticated metataxonomic analysis.

2. Materials and Methods

2.1. Sewage Sludge, Vermicomposting Set-Up and Earthworm Cast Sampling

The sewage sludge used as vermicomposting feedstock was collected from a WWTP located in Vilagarcía de Arousa (37,689 inhabitants, northwestern Spain). Vermicomposting of sewage sludge was carried out in medium-scale vermireactors as previously described [2]. Thus, we added a layer of fresh sewage sludge (120 kg fresh weight) and then collected 5 samples that were kept at −80 °C. The earthworm density (*Eisenia andrei*) exceeded 12,000 individuals m^{-2} at the start of vermicomposting. To collect fresh cast samples, we followed a previously described protocol [2]. Thus, we picked adult earthworms from the vermireactor, washed them and left in sterile Petri dishes (5 dishes with 20 individuals per

dish) in an incubator chamber for 24 h [2]. We then sampled the casts in sterile conditions. Collected casts were stored in sterile Eppendorf tubes at −80 °C. Vermicompost samples (n = 5) were collected from the vermireactor after 3 months.

2.2. Amplification, Sequencing and Analysis of 16S and ITS rRNA

We extracted DNA of samples using the MO-BIO PowerSoil® kit following the manufacturer's protocols under sterile conditions in a laminar flow hood. Sequencing was conducted at the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory (Lemont, IL, USA) following the protocol previously described in 2 × 150 and 2 × 250 Miseqs runs (16S and ITS, respectively) [2].

We used DADA2 (version 1.30) to infer the amplicon sequence variants (ASVs) present in each sample [25]. ASVs are more accurate and reproducible than OTUs defined at a constant level (97% or other) of sequence similarity [26]. DADA2 pipeline was run following the procedure described previously [2], with minor modifications. Thus, 16S forward and reverse reads were truncated at 140 nt, whereas ITS forward and reverse reads were not truncated. Bacterial ASVs were inferred from forward and reverse reads, whereas fungal ASVs were inferred from forward reads only because more ASVs were assigned than when using forward and reverse reads (502 and 363 ASVs in forward and merged forward and reverse reads, respectively). Taxonomic assignment was conducted using Silva (version 138) and UNITE (version 9.0) databases for 16S and ITS genes, with the RDP naive Bayesian classifier [27,28]. We removed ASVs unclassified at phylum level (0.85 and 15% of sequences for 16S and ITS, respectively). At the end of the DADA2 pipeline, we retained 708 bacterial ASVs that comprised 103,220 sequences (mean: 6881, SD: 1307) and 376 fungal ASVs that comprised 308,497 sequences (mean: 20,532, SD: 9778). Rarefaction curves showed that sampling depth was fine (Supplementary Figure S1). To assess the impact of earthworms on human bacterial pathogens (HBPs), we used the assignSpecies command from DADA2 to obtain the taxonomy at the species level. We set the allowMultiple option to FALSE to ensure unambiguous identifications, which is appropriate for 16S amplicon data [29]. After obtaining the taxonomic classification at the species level, we compared it with the comprehensive list of HBPs from Bartlett et al. [30] to identify which species were present in the sewage sludge and to evaluate the effect of earthworms on them. Sequence data were uploaded to the GenBank SRA database under accession numbers PRJNA1090289 (16S) and PRJNA1090297 (ITS).

2.3. Bioinformatic and Statistical Analysis

All of the data were analyzed and plotted using the phyloseq [31], tidyverse [32], RColorBrewer [33], patchwork [34], vegan [35], dendextend [36] and ComplexHeatmap [37] packages implemented in R version 4.4.0 [38].

We analyzed the differences in the abundances of bacterial and fungal taxa at ASV, genus and phylum levels between sewage sludge and earthworm casts and between earthworm casts and vermicompost using DESeq2 package [39], as previously described [2]. We determined taxonomic α-diversity using several indexes (observed richness and Chao1, inverse Simpson and Faith's phylogenetic diversity [40]). We determined taxonomic β-diversity using distance matrices (Bray–Curtis, Jaccard, weighted and unweighted unifrac [41]) and PCoA analysis. The effect of transit through the gut of earthworm on both α- (diversity indexes) and β-diversity (PCoA scores) was analyzed using Kruskal–Wallis tests with paired Wilcoxon test as post hoc test. *p*-values from DESEq2 and all post hoc tests were corrected for multiple comparisons (Benjamini–Hochberg FDR).

We used the previously outlined procedure [2] to assess the presence of bacterial and fungal ASVs in sewage sludge, earthworm casts and vermicompost. Briefly, we removed ASVs shared among treatments to define native ASVs of each type of sample. These are ASVs only found in sewage sludge, earthworm casts or vermicompost samples. Shared ASVs among treatments were determined by pairwise comparison of treatments.

Furthermore, for 16S sequencing data, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2 v2.5.2) [42] was used to predict functional orthologs from the KEGG database. Bacterial pathways were assumed to be complete when at least 75% of the KEGG orthologues (KOs) of each pathway were detected in the data. We selected "biosynthesis of amino acids", "furfural degradation" and "bisphenol degradation" pathways for further analysis. Additionally, we included "antibiotic synthesis", "antibiotic resistance", "plant hormone synthesis", and "nitrogen metabolism" pathways, based on KO lists from previous studies [43,44]. To standardize the data distribution, the KO abundances were z-score transformed. Contributional α and β-diversity, i.e., the taxa contributing to each KO across samples, was computed with FuncDiv [45] and KEGGREST [46] R packages. The effect of transit through the gut of earthworm on contributional α and β-diversity of bacterial communities of sewage sludge was analyzed using non-parametric Kruskal-Wallis tests. Paired Wilcoxon test was used for post hoc comparisons, with Benjamini–Hochberg FDR as a multiple test correction method.

As PICRUSt2 does not perform well with ITS sequencing data [42], we used FUNGuildR, a tool used to assign trait information based on matches with a taxonomic classification in the FUNGuild database. This database is used to compare fungal functions that can link fungal gene sequencing information with the ecological functions of fungi, as well as to identify the nutrient types used by fungi at the genus level and conduct the specific functional classifications [47]. The method categorized 81% of all taxa, and only fungal AVSs with "probable" and "highly probable" confidence rankings were chosen for further analysis. The effect of transit through the earthworm gut on the abundance of fungal guilds in the ingested sewage sludge was analyzed using non-parametric Kruskal–Wallis tests. Paired Wilcoxon test was used for post hoc comparisons, with Benjamini–Hochberg FDR as a multiple test correction method only in cases where the trophic mode was detected in more than one type of sample.

3. Results

3.1. Changes in the Composition of Bacterial and Fungal Communities

The bacterial community of the sewage sludge comprised 15 phyla, with Proteobacteria (60%), Bacteroidota (19%) and Firmicutes (13%) being the most prevalent (Table 1). The bacterial communities in fresh earthworm casts consisted of 16 bacterial phyla, with Proteobacteria (67%), Firmicutes (11%) and Bacteroidota (7%) predominating (see Table 1). A total of 21 bacterial phyla were identified in vermicompost samples, with Proteobacteria (58%), Bacteroidota (25%) and Verrucomicrobiota (4%) being the most abundant (Table 1).

Table 1. Relative abundance of the main bacterial and fungal phyla in sewage sludge, casts and vermicompost from the earthworm *Eisenia andrei*. Only bacterial phyla with an abundance >3% are displayed. Mean and SE (N = 5).

Kingdom	Phylum	Sewage Sludge	Cast	Vermicompost
Bacteria	Proteobacteria	60.13 ± 9.78	66.86 ± 1.37	57.91 ± 0.64
Bacteria	Bacteroidota	18.78 ± 4.22	7.07 ± 1.05	25.35 ± 0.64
Bacteria	Firmicutes	13.18 ± 3.19	11.66 ± 0.99	2.66 ± 0.18
Bacteria	Campylobacterota	3.96 ± 1.28	-	-
Bacteria	Verrucomicrobiota	-	5.08 ± 0.21	4.50 ± 0.17
Bacteria	Actinobacteriota	-	4.94 ± 0.7	1.91 ± 0.15
Bacteria	Planctomycetota	-	2.42 ± 0.16	3.22 ± 0.16
Fungi	Basidiomycota	87.80 ± 3.31	69.78 ± 2.44	68.53 ± 4.14
Fungi	Mortierellomycota	6.81 ± 4.21	27.68 ± 2.33	29.68 ± 4.11
Fungi	Ascomycota	5.32 ± 1.69	2.54 ± 0.28	1.80 ± 0.15
Fungi	Mucoromycota	0.06 ± 0.04	-	-

The passage of the sludge through the earthworm gut resulted in the appearance of three bacterial phyla that were not present in the sewage sludge (Verrucomicrobiota,

Actinobacteriota and Planctomycetota) and the disappearance of one bacterial phylum (Campylobacterota) (Table 1). Moreover, the gut transit significantly decreased the abundance of the bacterial phyla Fusobacteria and Bacteroidota, among others (Supplementary Table S1). Ageing of casts during vermicomposting (CAPs) decreased the abundances of the bacterial phyla Firmicutes, Chloroflexi and Verrucomicrobiota, among others, and significantly higher abundances of FCPU426, Fibrobacterota and Bacteroidota, among others (Table 1, Supplementary Table S1).

At the genus level, the passage of sludge through the earthworm gut resulted in the appearance of bacterial genera that were not present in the sludge (*Candidatus Lumbricincola*, *Rhodanobacter*, *Flavobacterium*, *Aeromonas* and *Chitinibacter*, among others) and the disappearance of bacterial genera (*Acidovorax* and *Prevotella*, among others) that were present in the sludge (Figure 1A, Supplementary Table S2). Ageing of cast during vermicomposting significantly decreased the abundances of the bacterial genera *Acinetobacter*, *Paenibacillus* and *Aeromonas*, among others, and significantly increased the abundance of *Porticoccus*, *Massilia* and *Chitinophaga*, among others (Figure 1B, Supplementary Table S2).

At the ASV level, the passage of the sludge through the earthworm gut resulted in a significant decrease in the relative abundance of 34 bacterial ASVs, mainly included in the genus *Bacteroides*, and a notable increase in the relative abundance of 90 bacterial ASVs, mainly belonging to the genus *Rhodanobacter* (Supplementary Table S3). The cast ageing processes (CAPs) significantly reduced the abundance of 12 bacterial ASVs, mainly included in the genus *Candidatus Lumbricincola*, and increased the abundance of 35 ASVs, mainly belonging to the genus *Pseudomonas* (Supplementary Table S3).

We found that there were 10 HPBs in the sewage sludge. These account for less than 5% of the total bacteria in the raw sludge and less than 0.5% of the bacteria from earthworm casts and vermicompost (Table 2). Most of the HPBs completely disappeared after transit through the earthworm gut. However, two of them appeared in vermicompost (Table 2), showing significantly reduced abundances (*Arcobacter cryaerophilus*) or not (*Collinsella aerofaciens*, Supplementary Table S3). Other three HBPs were present in earthworm cast and not in the sewage sludge, i.e., *Cellulosimicrobium cellulans*, *Turicibacter sanguinis* (ASV390) and *T. sanguinis* (ASV 130, Table 2). Vermicomposting completely removed two of them, and no significant changes were detected in the abundance of *T. sanguinis* (ASV130, Table 2).

Fungal community in the sewage sludge consisted of four phyla, dominated by Basidiomycota (87%), with minor contributions from Mortierellomycota (6%) and Ascomycota (5%). Among these fungal phyla, GAP significantly increased the abundance of Mortierellomycota (see Supplementary Table S1) and also removed phylum Mucoromycota (Table 1). The fungal community of the vermicompost comprised the same phyla as the fresh casts. In this case, there were no significant differences in the abundance of fungal phyla due to CAP (Supplementary Tables S1 and S2).

At the genus level, the passage of the sludge through the earthworm gut resulted in a significant decrease in the abundance of the fungal genera *Cutaneotrichosporon*, *Trichosporon* and *Candida* and a significant increase in the abundance of the fungal genera *Pycnopulvinus*, *Cheilymenia* and *Fusarium*. The cast ageing processes of vermicomposting did not produce significant changes in the abundance of fungal genera (Figure 1D, Supplementary Table S2).

At the ASV level, passage of the sludge through the earthworm gut significantly reduced the abundance of 57 fungal ASVs, mainly included in the fungal genera *Apiotrichum* and *Cutaneotrichosporon*, and increased the relative abundance of 49 fungal ASVs, mainly included in the fungal genera *Apiotrichum* and *Mortierella* (Supplementary Table S3). The cast ageing processes significantly reduced the abundance of 43 fungal ASVs and increased the abundance of 45 fungal ASVs, all mainly included in the fungal genera *Apiotrichum* and *Mortierella* (Supplementary Table S3).

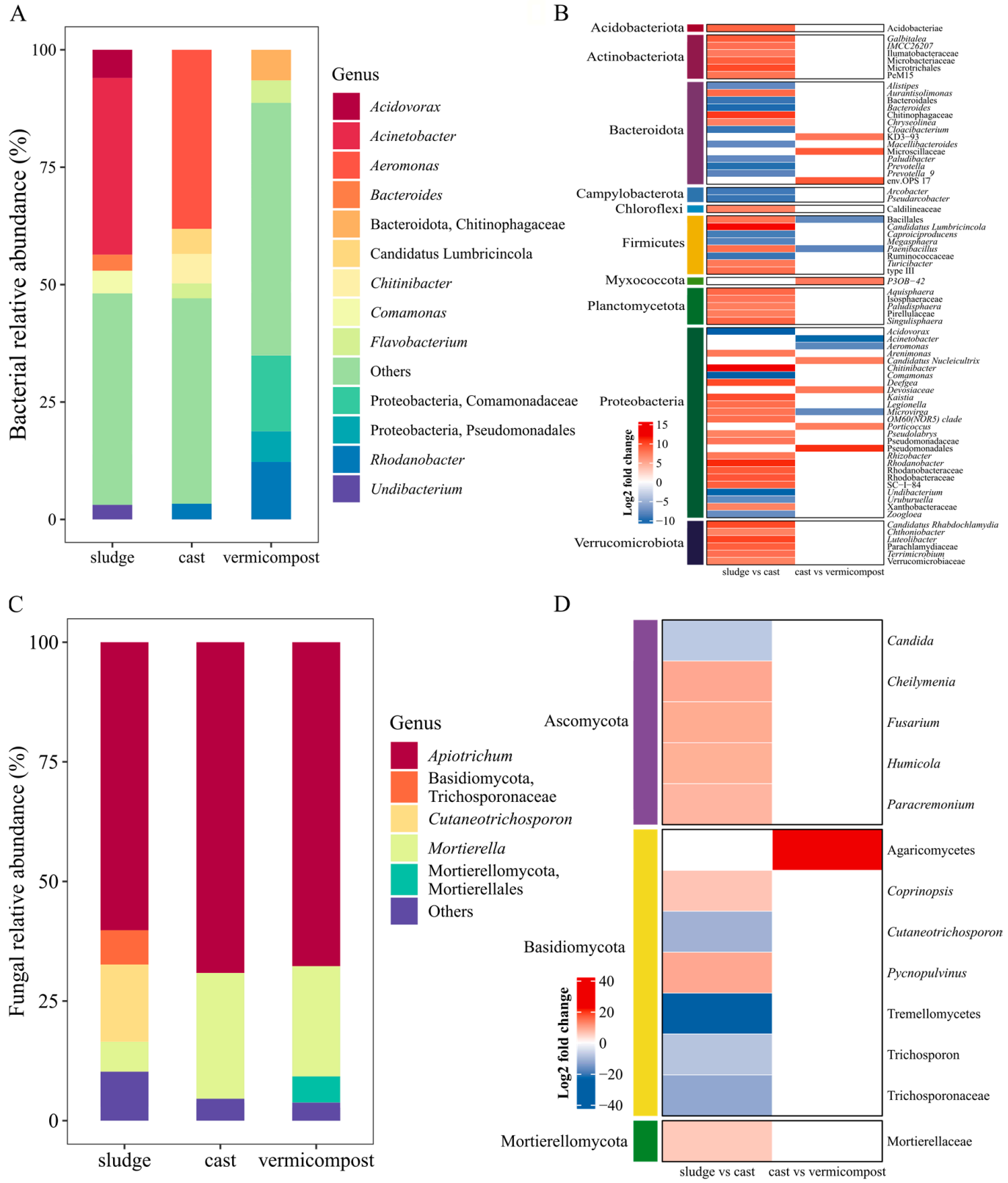

Figure 1. Relative abundance of the main bacterial (**A**) and fungal (**C**) genera found in sewage sludge, earthworm casts and vermicompost produced by the earthworm *Eisenia andrei*. Less abundant genera (less than 3% and 1% for bacterial and fungal genera, respectively) are grouped under "others". Significant differences in abundances of bacterial (**B**) and fungal (**D**) genera between sewage sludge and casts, and between casts and vermicompost (DESeq2 p-values < 0.05). Only taxa with $\log_2$ fold changes > 7 or < −7 are displayed; see Supplementary Table S2 for all genera.

Table 2. Relative abundance of the human bacterial pathogens in sewage sludge, casts and vermicompost from the earthworm *Eisenia andrei*. Mean and SE (N = 5).

ASVs	Species	Sludge	Cast	Vermicompost
ASV43	*Arcobacter cryaerophilus*	1.34 ± 0.72	-	0.01 ± 0.03
ASV57	*Bacteroides vulgatus*	0.94 ± 0.46	-	-
ASV67	*Bacteroides uniformis*	0.81 ± 0.4	-	-
ASV194	*Collinsella aerofaciens*	0.2 ± 0.19	-	0.02 ± 0.02
ASV208	*Sebaldella termitidis*	0.2 ± 0.06	-	-
ASV246	*Bacteroides massiliensis*	0.16 ± 0.12	-	-
ASV282	*Dialister invisus*	0.12 ± 0.08	-	-
ASV480	*Bacteroides caccae*	0.04 ± 0.09	-	-
ASV547	*Laribacter hongkongensis*	0.02 ± 0.06	-	-
ASV643	*Bilophila wadsworthia*	0.02 ± 0.03	-	-
ASV130	*Turicibacter sanguinis*	-	0.21 ± 0.14	0.14 ± 0.05
ASV290	*Cellulosimicrobium cellulans*	-	0.1 ± 0.13	-
ASV390	*Turicibacter sanguinis*	-	0.06 ± 0.14	-

3.2. Changes in the α- and β-Diversity of Bacterial and Fungal Communities

Passage of the sludge through the earthworm gut decreased taxonomic bacterial diversity (Inverse Simpson index, Kruskal–Wallis $p = 0.053$, Figure S2A) but did not change the phylogenetic bacterial diversity (Faith index, Supplementary Figure S2E) or bacterial richness (raw and Chao1 estimates, Supplementary Figure S2A,C). The cast ageing processes increased the taxonomic bacterial diversity (Inverse Simpson index, Kruskal–Wallis $p = 0.009$, Figure 2A), phylogenetic bacterial diversity (Faith index, Kruskal–Wallis $p < 0.05$, Supplementary Figure S2E) and bacterial richness (raw and Chao1 estimates, Kruskal–Wallis $p < 0.05$, Supplementary Figure S2A,C).

Passage of the sludge through the earthworm gut also significantly decreased fungal diversity (Inverse Simpson index, Kruskal–Wallis $p = 0.004$, Figure S2B) but did not change fungal α-diversity in terms of richness (Kruskal–Wallis $p = 0.33$, Supplementary Figure S2B) and Chao1 richness estimator (Kruskal–Wallis $p = 0.32$, Supplementary Figure S2D).

In addition to the changes in bacterial α-diversity, we observed significant changes in bacterial β-diversity during the vermicomposting process. Thus, the bacterial communities in sewage sludge, earthworm casts and vermicompost showed significant differences in PCoA 1 and PCoA 2 of Bray–Curtis (Kruskal–Wallis $p = 0.0019$ for both axes, Figure 2C), Jaccard (Kruskal–Wallis $p = 0.0019$ for both axes) and weighted and unweighted UniFrac distance matrices (Kruskal–Wallis $p < 0.01$ for both axes and distance matrices; Supplementary Figure S3A,C,D). There were also significant differences in fungal communities between sewage sludge and earthworm cast as well as between sewage sludge and vermicompost in PCoA 1. However, there were no differences in PCoA 2 for Bray–Curtis matrices (Kruskal–Wallis test, $p = 0.008$ and $p = 0.73$, respectively; see Figure 2D) or Jaccard distance (Kruskal–Wallis test, $p = 0.008$ and $p = 0.73$, respectively; see Supplementary Figure S3B).

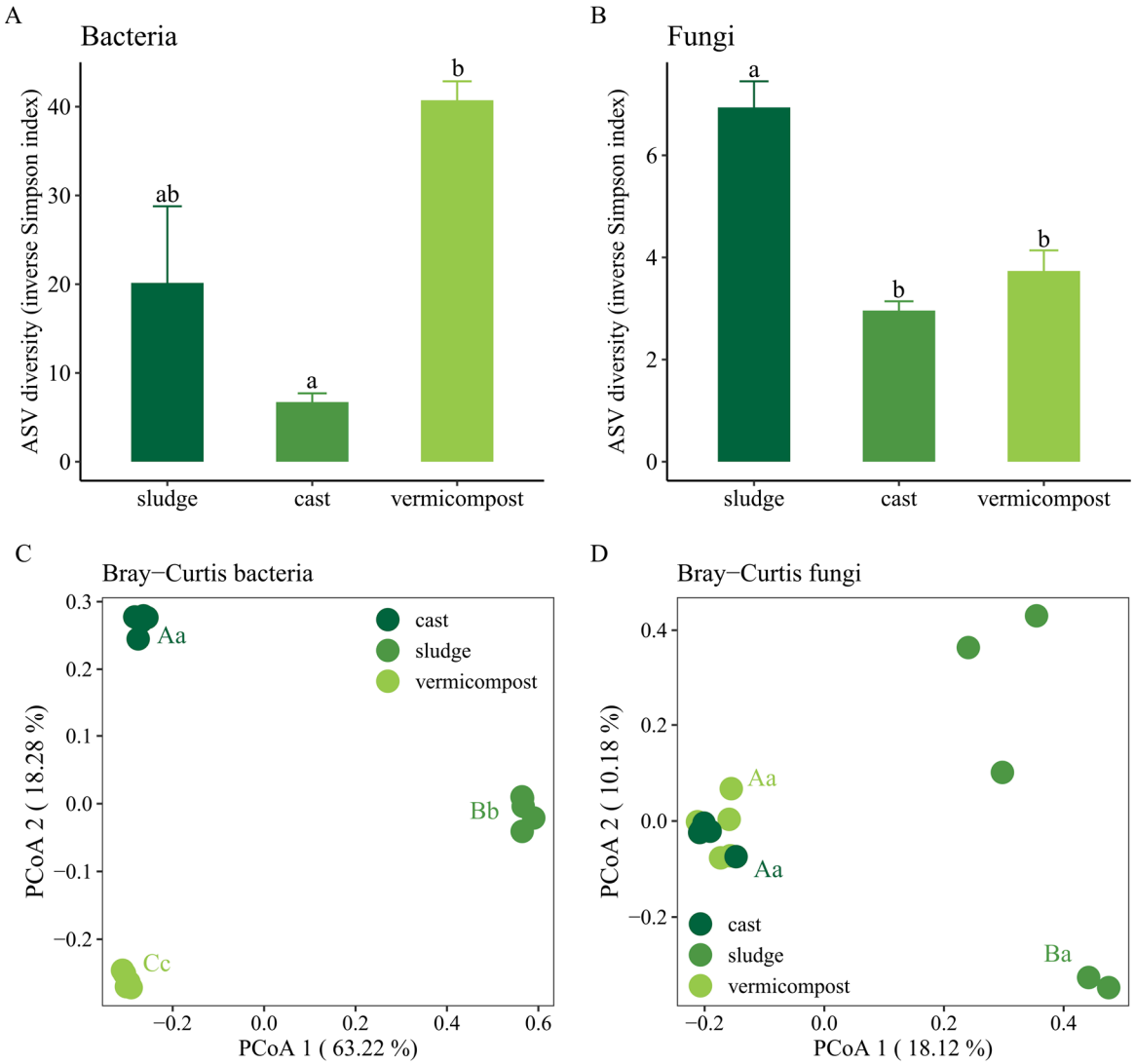

Figure 2. Changes in α-diversity (measured by the Inverse Simpson index) of bacterial (**A**) and fungal (**B**) communities and β-diversity of bacterial (**C**) and fungal (**D**) communities during vermicomposting of sewage sludge. The β-diversity was assessed using principal coordinate analysis of Bray–Curtis distances. Different letters in A and B indicate significant differences in the Inverse Simpson index between sewage sludge, fresh earthworm casts and vermicompost (paired Wilcoxon test, FDR corrected). Different capital and lowercase letters in C and D indicate significant differences between sewage sludge, casts and vermicompost in PCoA 1 and PCoA 2, respectively (paired Wilcoxon test, FDR corrected).

3.3. Shared Bacterial and Fungal ASVs among Sewage Sludge, Fresh Earthworm Casts and Vermicompost

Only 3.5% and 5% of microbial ASVs were shared among the sewage sludge, earthworm casts and vermicompost for bacterial and fungal communities, respectively (Figure 3, Supplementary Table S4). Moreover, the bacterial community of sewage sludge only shared 8% and 6% of ASVs with the earthworm cast and vermicompost communities, respectively. The fungal community of sewage sludge only shared 6% of ASVs with both the fungal communities of earthworm casts and vermicompost. On the other hand, earthworm casts and vermicompost shared more bacterial ASVs but not many fungal ASVs (Figure 3, Supplementary Table S4). Thus, cast and vermicompost shared 52% and 35% of the bacterial ASVs, which comprised 90% and 52% of their sequences but only 11% of the fungal ASVs, which comprised 2% of their sequences in both cases (Figure 3, Supplementary Table S4).

Thus, the bacterial and fungal communities of sewage sludge, cast and vermicompost samples were predominantly composed of ASVs exclusive to each type of sample (Supplementary Table S5). Overall, transit through the earthworm gut (GAP) and ageing of the earthworm casts (CAP) removed 95% of the bacterial ASVs and 99% of the fungal ASVs from the sewage sludge (Figure 3).

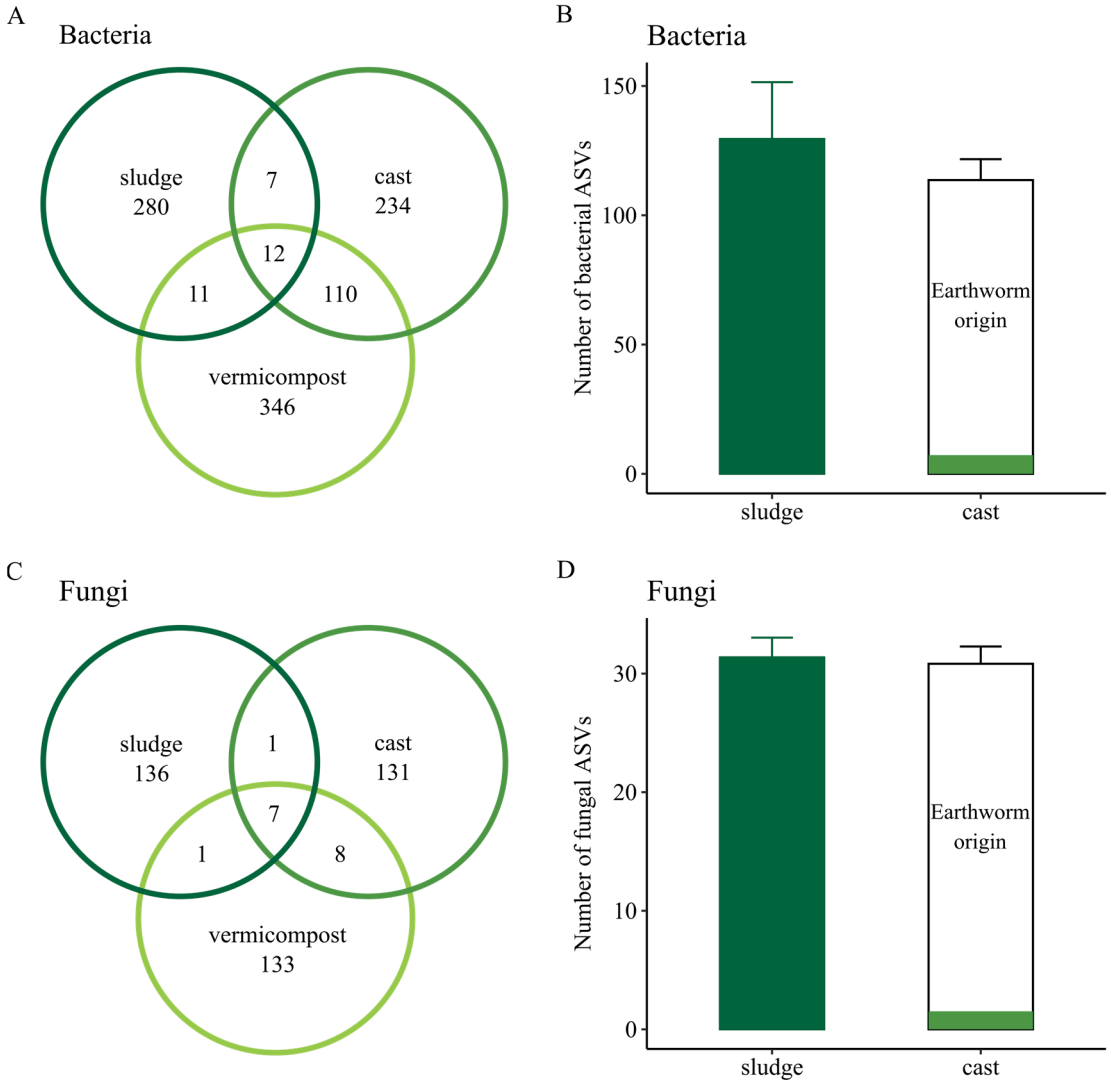

Figure 3. Changes in richness and diversity of bacteria and fungi during vermicomposting of sewage sludge. Venn diagrams showing the unique and shared bacterial (**A**) and fungal (**C**) ASVs in sewage sludge, earthworm casts and vermicompost. Effect of the transit through the earthworm gut (GAP) on the richness and diversity of (**B**) bacterial and (**D**) fungal ASVs.

3.4. Functional Diversity of the Bacterial Communities

Passage of the sewage sludge through the earthworm gut (GAP) significantly increased the abundance of genes involved in functional pathways, such as antibiotic resistance, amino acid biosynthesis, bisphenol and furfural degradation, nitrogen metabolism and plant hormone synthesis (Figure 4, Kruskal–Wallis $p < 0.008$ for all pathways). The posterior ageing of the earthworm casts (CAPs) maintained the levels of activity of bisphenol degradation and biosynthesis of amino acids, significantly increased nitrogen metabolism and plant hormone synthesis, and significantly decreased antibiotic resistance and synthesis and furfural degradation (Figure 4A).

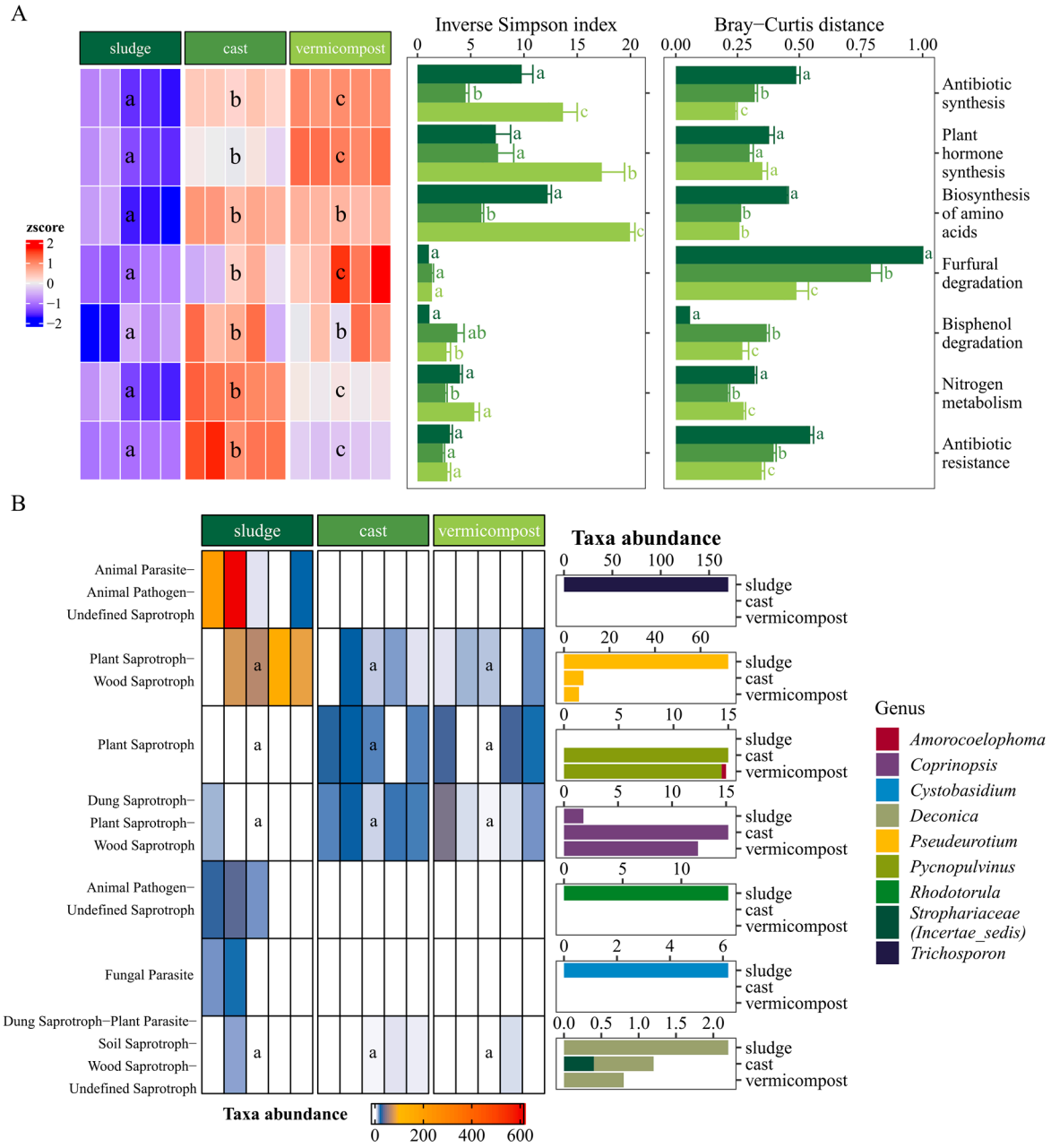

Figure 4. (**A**) Heatmap of the predicted pathways of bacterial community and corresponding changes in α and β-diversity during vermicomposting of sewage sludge. Inverse Simpson index and Bray–Curtis distance within bacterial communities of sewage sludge, earthworm casts and vermicompost associated with each pathway are shown. KEGG orthologue (KO) abundances were z-score transformed. (**B**) Heatmap of high-abundance fungal guilds present in sewage sludge, cast, and vermicompost during vermicomposting of sewage sludge, along with the mean abundance of the fungal genera they comprise. Different letters indicate significant differences between sewage sludge, fresh earthworm casts and vermicompost (paired Wilcoxon test, FDR corrected).

The contributional bacterial α-diversity, measured as the Inverse Simpson index, varied across the different pathways. Thus, it did not change in relation to furfural degradation (Kruskal–Wallis $p = 0.184$) or antibiotic resistance (Kruskal–Wallis $p = 0.767$) (Figure 4A). For bisphenol degradation, it increased during GAPs and decreased after CAPs (Kruskal–Wallis $p = 0.021$). For plant hormone synthesis, it increased after CAPs (Kruskal–Wallis $p = 0.006$). The bacterial α-diversity decreased during GAPs and increased after CAPs regarding nitrogen metabolism (Kruskal–Wallis $p < 0.001$), biosynthesis of amino acids

(Kruskal–Wallis $p < 0.001$) and antibiotic synthesis pathways (Kruskal–Wallis $p < 0.001$) (Figure 4A).

Contributional bacterial β-diversity, measured as Bray–Curtis distance within samples, was significantly different between sewage sludge and cast in all pathways studied (Kruskal–Wallis $p < 0.001$), except for plant hormone synthesis (Kruskal–Wallis $p = 0.717$). There were also significant differences between earthworm casts and vermicompost for all pathways analyzed, except plant hormone synthesis and amino acid biosynthesis (Figure 4A).

3.5. Fungal Trophic Guilds

FUNGuildR classified 336 of the 376 fungal ASVs into seven different guilds, with only 53 ASVs receiving confidence rankings of "probable" and "highly probable". The most abundant fungal guild was the "animal parasite-animal pathogen-undefined saprotroph" guild, followed by the "plant saprotroph-wood saprotroph" guild (Figure 4B). The abundance of the first fungal guild significantly decreased after the vermicomposting of sewage sludge (Kruskal–Wallis $p = 0.005$), while the others did not change significantly (Kruskal–Wallis $p = 0.569$), except the "animal pathogen-undefined saprotroph" guild (Kruskal–Wallis $p = 0.043$).

The abundance of the main fungal genus in each fungal guild decreased or increased during the vermicomposting of sewage sludge (Figure 4B, right). The "animal parasite-animal pathogen-undefined saprotroph" fungal guild mainly consisted of the fungal genus *Trichosporon*, only present in the sewage sludge. In the "plant saprotroph-wood saprotroph" fungal guild, *Pseudeurotium* was the most abundant fungal genus, but the abundance decreased after vermicomposting. The same was observed for the "animal pathogen-undefined saprotroph" fungal guild, which was mainly represented by the genus *Rhodotorula*, and for the "animal pathogen" fungal guild represented by the genus *Cystobasidium*, as both genera were present in the sewage sludge but not in the vermicompost (Figure 4B, right).

4. Discussion

4.1. Composition of Bacterial and Fungal Communities during Vermicomposting of Sewage Sludge

The bacterial and fungal communities in vermicompost differed significantly from those in sewage sludge at the phylum level immediately after GAPs, and the differences in composition increased after CAPs. Bacterial communities in vermicompost were mainly composed of the phyla Proteobacteria, Bacteroidota and Verrucomicrobiota, while the fungal communities were predominated by the phyla Basidiomycota, Mortierellomycota and Ascomycota. Similar results were previously obtained for bacteria and fungi [2], and Proteobacteria and Basidiomycota were also the most abundant phyla. Other vermicomposting studies have found similar bacterial compositions at the phylum level after vermicomposting white, red and distilled grape marc [48–50], as well as Scotch broom and silver wattle [51,52].

The changes in the microbial communities during vermicomposting of sewage sludge were even more pronounced at the genus and ASV levels than at the phylum level. Thus, the composition and abundance of various bacterial and fungal genera were substantially altered. For example, earthworm casts were predominantly populated by the bacterial genera *Aeromonas*, *Chitinibacter* and *Candidatus Lumbricincola*, as well as by the fungal genera *Apiotrichum* and *Mortierella*. These findings are similar to those reported in a previously mentioned study [2] but differ regarding the predominant microbial genera found in the previously mentioned vermicomposting studies [44,51]. Other vermicomposting studies involving the closely related earthworm species *E. fetida* indicated different bacterial and fungal compositions at the phylum and genus levels following GAPs and CAPs compared to those described here [22,23,53]. For instance, the fungal communities were largely dominated by *Rozellomycota* and unidentified fungal genera, while the bacterial communities were dominated by *Chloroflexy*, *Saccharibacteria*, *Flavobacterium* and *Thermomonas* [22,23,53].

On the one hand, the previously mentioned bacterial taxa were not identified in vermicompost samples, i.e., after CAPs, while in the previous study, the abundance of predominant bacterial genera in the casts only decreased during CAPs [2]. On the other hand, as the fungal genera did not vary during CAPs, our findings are not consistent with those of the previous study, in which the abundance of predominant fungal taxa decreased [2]. Despite the fact that, at coarse taxonomic levels, earthworms increase the similarity of microbial communities of vermicompost independently of ingested substrates [54], this pattern disappeared when the taxonomic resolution was increased. This indicates that the ingested substrate plays a predominant role in the microbial composition of the resulting vermicompost.

As expected, since most of the bacterial ASVs from sewage sludge did not appear in earthworm samples (cast and vermicompost), most of the HBPs disappeared readily after gut transit. This effect was even increased during vermicomposting. This result is the expected output of vermicomposting, which significantly reduces or completely eliminates pathogens [6]. It is important to note that HBPs made up a small fraction of the bacterial communities in sewage sludge, accounting for less than 5%. None of these harmful bacteria were *E. coli* or *Salmonella* spp., which are the only harmful bacteria specified in the European regulations for waste and recycled byproducts [55,56]. It is also worth noting that all HBPs found belonged to the established category, meaning there were more than three reported cases of infecting humans [30]. The other two HBPs, *B. massiliensis* and *T. sanguinis*, were categorized as putative (i.e., with less than three reported cases of infecting humans) [30].

4.2. Changes in the α- and β-Diversity during Vermicomposting of Sewage Sludge

We found that the impact of vermicomposting on microbial α-diversity varied depending on the vermicomposting phase. Thus, in general terms, passage through the earthworm gut (GAPs) decreased the diversity of bacteria and fungi, while the ageing of earthworm casts (CAPs) increased bacterial diversity. These findings are partly consistent with those of the previously mentioned study [2], which reported a decrease in bacteria and an increase in fungi following the GAP and CAP vermicomposting phases. Other studies involving the earthworm *E. fetida* have reported different bacterial and fungal compositions at the phylum and genus levels after GAPs and CAPs, which are different from those described here [22,23,53]. In contrast to our findings, other studies have shown continuous increases in bacterial α-diversity when using different earthworm species and sewage sludge [22,23,53]. Overall, the findings indicate that the effect of vermicomposting on the diversity of microbial communities depends on the type of sewage sludge and also on the earthworm species used and how the vermicomposting is carried out. Earthworm activity led to significant changes in composition at the ASV level. Each type of sample (sewage sludge, earthworm casts and vermicompost) mainly comprised its own specific ASVs. Consequently, we observed significant differences in the β-diversity of bacterial and fungal communities, as previously observed [2]. Significant changes in microbial composition during vermicomposting were reported for sewage sludge with other earthworm species [53] and with other parent vermicomposting substrates [43,44,48–50,52]. Hence, the significant changes in microbial diversity may be an inherent property of the vermicomposting process. However, we did not observe any clear differences in fungal β-diversity between earthworm casts and vermicompost as in previous studies with sewage sludge and the same earthworm species [2], an effect that can be attributed to a lack of differences in observed α-diversity.

4.3. Bacterial Functional Diversity and Fungal Trophic Guilds

In addition to substantial changes in bacterial community composition and diversity, metagenomic predictions using PICRUSt2 and FUNGuildR revealed distinct microbiome functional profiles and a significant increase in microbial functional diversity after vermicomposting of sewage sludge. Thus, significant changes were found in important bacterial functional pathways and fungal guilds. The increase in biosynthesis pathways indicates

heightened metabolic activity in microbial communities of cast and vermicompost, with various different bacterial taxa fulfilling different roles [43,44,51]. This is consistent with the increase in the bacterial α-diversity in these pathways. Our findings on antibiotic biosynthesis could be attributed to recent findings, indicating that vermicomposting may reduce antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in sewage and dewatered sludge [57–61]. This could be due to the activity of earthworms and their endosymbiotic microbes during earthworm digestion, as pathogens could be exposed to physical disruption by antimicrobial substances, microbial antagonists or could even be enzymatically digested and assimilated directly [2,6]. Fungal guilds associated with pathogens and parasites are almost absent from earthworm casts, disappearing during GAPs. The saprotroph-related guilds did not undergo any significant changes. As far as we know, the fungal communities in vermicompost have not been studied. However, similar results were observed for compost made from sewage sludge, with a decrease in animal pathogens and the saprotroph guild predominating at the end of the process [62]. Overall, vermicomposting improves the safety of sewage sludge for application on agricultural and forest land by reducing the amounts of potentially harmful organisms the waste material contains.

5. Conclusions

This study explored the changes in microbial communities in sewage sludge during vermicomposting. The influence of earthworms during vermicomposting is clearly demonstrated, as the most significant changes in microbial diversity and composition occurred during earthworm ingestion and digestion processes (GAPs), driving posteriorly further microbial changes during cast ageing processes (CAPs). The changes in microbial composition and structure also led to alterations in functional diversity. Bacterial biosynthesis pathways were enriched, while pathogenic fungal guilds were eliminated. The resulting vermicompost was almost pathogen-free and also had properties that promote plant growth and the decomposition of organic matter. The assessment of bacterial and fungal communities during vermicomposting could help to better understand the mechanisms involved, as well as to determine which pathogens or pollutants could be targeted. Moreover, this approach could help to establish vermicomposting as an environmentally friendly way of managing sewage sludge or even transforming it into organic fertilizer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14177894/s1, Figure S1: Rarefaction curves indicating the number of bacterial and fungal amplicon sequence variants (ASVs) detected in each sample of sludge and cast and vermicompost produced by the earthworm *Eisenia andrei*, Figure S2: Changes in alpha diversity of bacterial and fungal communities of sludge, cast and vermicompost from the earthworm *Eisenia andrei*. Estimated richness (number of observed ASVs and Chao 1) and phylogenetic diversity (Faith) indexes are shown, Figure S3: Changes in beta diversity of bacterial and fungal communities of sludge, cast and vermicompost from the earthworm *Eisenia andrei*. Beta-diversity was measured as principal coordinate analysis of Jaccard, weighted and unweighted unifrac distances. Different capital and lowercase letters indicate significant differences between sludges and casts in PCoA 1 and PCoA 2 respectively (paired Wilcoxon test, FDR corrected), Table S1: Differential abundance in bacterial and fungal phyla of slugde and casts and vermicomposts of earthworm species *Eisenia andrei* analyzed using negative binomial models as implemented in the package DESeq2 [39]. Positive and negative log2FoldChange indicate significant increase and decrease in abundance of cast over sludge and cast over vermicompost, Table S2: Differential abundance in bacterial and fungal genera of slugde and casts and vermicomposts of earthworm species *Eisenia andrei* analyzed using negative binomial models as implemented in the package DESeq2 [39]. Positive and negative log2FoldChange indicate significant increase and decrease in abundance of cast over sludge and cast over vermicompost, Table S3: Differential abundance in bacterial and fungal ASVs of slugde and casts and vermicomposts of earthworm species *Eisenia andrei* analyzed using negative binomial models as implemented in the package DESeq2 [39]. Positive and negative log2FoldChange indicate significant increase and decrease in abundance of cast over sludge and cast over vermicompost, Table S4: Full taxonomy

and identity of shared bacterial and fungal ASVs present in sludge, cast and vermicompost samples, Table S5: Full taxonomy and identity of bacterial and fungal ASVs present only in sludge, cast or vermicompost samples, Table S6: Bacterial pathways predicted by PICRUSt2.

Author Contributions: Conceptualization, J.D.; formal analysis, A.G.-R. and M.A.; investigation, A.G.-R. and M.A.; writing—original draft, A.G.-R., M.A. and J.D. All authors reviewed and edited the final version. All authors have read and agreed to the published version of the manuscript.

Funding: This research received funding from the Spanish Ministerio de Ciencia e Innovación (PID2021-124265OB-100), the Xunta de Galicia (Grant number ED431C 2022/07) and the MCIN/AEI and European Union Next Generation_EU (project TED2021-129437B-100). AG acknowledges support from the European Union Next Generation_EU Programme-funded project (project TED2021-129437B-100) through the award of a predoctoral research contract.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequence data have been uploaded to the GenBank SRA database under accession numbers PRJNA1090289 and PRJNA1090297 for bacterial and fungal sequences, respectively.

Acknowledgments: The authors are grateful to Hugo Martínez, Alejandro Figueroa, Alberto Da Silva and Ainhoa Doval for assistance with the vermicomposting process, sample collection and DNA extraction.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Souza, A.C.Z.; Santos, J.E.; Marin-Morales, M.A.; Mazzeo, D.E.C. Ecotoxicological aspects and environmental implications of the use of water and sewage treatment sludges. *Int. J. Environ. Sci. Technol.* **2024**, *21*, 3527–3552. [CrossRef]
2. Domínguez, J.; Aira, M.; Crandall, K.A.; Pérez-Losada, M. Earthworms drastically change fungal and bacterial communities during vermicomposting of sewage sludge. *Sci. Rep.* **2021**, *11*, 15556. [CrossRef] [PubMed]
3. Košnář, Z.; Mercl, F.; Pierdonà, L.; Chane, A.D.; Míchal, P.; Tlustoš, P. Concentration of the main persistent organic pollutants in sewage sludge in relation to wastewater treatment plant parameters and sludge stabilisation. *Environ. Pollut.* **2023**, *333*, 122060. [CrossRef] [PubMed]
4. Wluka, A.-K.; Huang, Y.; Coenen, L.; Dsikowitzky, L.; Schwarzbauer, J. Structural Diversity of Organic Contaminants in Sewage Sludge: A Comparison of Sewage Fingerprints from Germany and China. *Discov. Water* **2021**, *1*, 4. [CrossRef]
5. Domínguez, J.; Gómez-Brandón, M. Vermicomposting: Composting with earthworms to recycle organic wastes. In *Management of Organic Waste*; Kumar, S., Ed.; InTech: London, UK, 2012; ISBN 978-953-307-925-7.
6. Swati, A.; Hait, S. A Comprehensive Review of the Fate of Pathogens during Vermicomposting of Organic Wastes. *J. Environ. Qual.* **2018**, *47*, 16–29. [CrossRef]
7. Rodríguez-Canché, L.G.; Cardoso Vigueros, L.; Maldonado-Montiel, T.; Martínez-Sanmiguel, M. Pathogen Reduction in Septic Tank Sludge through Vermicomposting Using *Eisenia Fetida*. *Bioresour. Technol.* **2010**, *101*, 3548–3553. [CrossRef] [PubMed]
8. Hénault-Ethier, L.; Martin, V.J.J.; Gélinas, Y. Persistence of *Escherichia Coli* in Batch and Continuous Vermicomposting Systems. *Waste Manag.* **2016**, *56*, 88–99. [CrossRef]
9. Lv, B.; Xing, M.; Yang, J. Exploring the effects of earthworms on bacterial profiles during vermicomposting process of sewage sludge and cattle dung with high-throughput sequencing. *Environ. Sci. Pollut. Res.* **2018**, *25*, 12528–12537. [CrossRef]
10. Chen, J.; Xia, H.; Huang, K.; Li, J.; Xie, J. Earthworms restructure the distribution of extracellular antibiotics resistance genes of sludge by modifying the structure of extracellular polymeric substances during vermicomposting. *J. Hazard. Mater.* **2023**, *452*, 131315. [CrossRef]
11. Zhu, L.; Wu, Z.; Chen, J.; Huang, K. Removal of Antibiotic Resistance Genes in Sewage Sludge Vermicomposting. In *Occurrence and Behavior of Emerging Contaminants in Organic Wastes and Their Control Strategies*; Elsevier: Amsterdam, The Netherlands, 2024; pp. 169–177, ISBN 978-0-443-13585-9.
12. El-Hassanin, A.S.; Samak, M.R.; Ahmed, S.M.; Afifi, M.M.I.; Abd El-Satar, A.M. Bioaccumulation of heavy metals during composting and vermicomposting processes of sewage sludge. *Egypt J. Chem.* **2022**, *65*, 1155–1162. [CrossRef]
13. Dume, B.; Hanč, A.; Švehla, P.; Michal, P.; Pospíšil, V.; Grasserová, A.; Cajthaml, T.; Chane, A.D.; Nigussie, A. Influence of earthworms on the behaviour of organic micropollutants in sewage sludge. *J. Clean. Prod.* **2023**, *416*, 137869. [CrossRef]
14. Lim, S.L.; Wu, T.Y.; Lim, P.N.; Shak, K.P.Y. The use of vermicompost in organic farming: Overview, effects on soil and economics. *J. Sci. Food Agric.* **2015**, *95*, 1143–1156. [CrossRef] [PubMed]
15. Kumar, A.; Prakash, C.H.B.; Brar, N.S.; Kumar, B. Potential of Vermicompost for Sustainable Crop Production and Soil Health Improvement in Different Cropping Systems. *Int. J. Curr. Microbiol. Appl. Sci.* **2018**, *7*, 1042–1055. [CrossRef]

16. Scaglia, B.; Nunes, R.R.; Rezende, M.O.O.; Tambone, F.; Adani, F. Investigating organic molecules responsible of auxin-like activity of humic acid fraction extracted from vermicompost. *Sci. Total Environ.* **2016**, *562*, 289–295. [CrossRef] [PubMed]
17. Nsiah-Gyambibi, R.; Essandoh, H.M.K.; Asiedu, N.Y.; Fei-Baffoe, B. Valorization of fecal sludge stabilization via vermicomposting in microcosm enriched substrates using organic soils for vermicompost production. *Heliyon* **2021**, *7*, e06422. [CrossRef]
18. Vuković, A.; Velki, M.; Ečimović, S.; Vuković, R.; Štolfa Čamagajevac, I.; Lončarić, Z. Vermicomposting—Facts, Benefits and Knowledge Gaps. *Agronomy* **2021**, *11*, 1952. [CrossRef]
19. Lei, X.; Cui, G.; Sun, H.; Hou, S.; Deng, H.; Li, B.; Yang, Z.; Xu, Q.; Huo, X.; Cai, J. How do earthworms affect the pathway of sludge bio-stabilization via vermicomposting? *Sci. Total Environ.* **2024**, *916*, 170411. [CrossRef] [PubMed]
20. Srivastava, V.; Squartini, A.; Masi, A.; Sarkar, A.; Singh, R.P. Metabarcoding Analysis of the Bacterial Succession during Vermicomposting of Municipal Solid Waste Employing the Earthworm *Eisenia fetida*. *Sci. Total Environ.* **2021**, *766*, 144389. [CrossRef]
21. Xing, M.; Zhao, R.; Yang, G.; Li, Z.; Sun, Y.; Xue, Z. Elimination of Antibiotic-Resistant Bacteria and Resistance Genes by Earthworms during Vermifiltration Treatment of Excess Sludge. *Environ. Sci. Pollut. Res.* **2024**, *31*, 7853–7871. [CrossRef]
22. Zhang, H.; Li, J.; Zhang, Y.; Huang, K. Quality of Vermicompost and Microbial Community Diversity Affected by the Contrasting Temperature during Vermicomposting of Dewatered Sludge. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1748. [CrossRef]
23. Hu, X.; Zhang, T.; Tian, G.; Zhang, L.; Bian, B. Pilot-scale vermicomposting of sewage sludge mixed with mature vermicompost using earthworm reactor of frame composite structure. *Sci. Total Environ.* **2021**, *767*, 144217. [CrossRef]
24. Zhang, Y.; Yuan, H.; Peng, S.; Wang, Z.; Cai, S.; Chen, Z.; Yang, B.; Yang, P.; Wang, D.; Guo, J.; et al. Vermicomposting Preferably Alters Fungal Communities in Wasted Activated Sludge and Promotes the Production of Plant Growth-Promoting Biostimulants in the Vermicompost. *Chem. Eng. J.* **2024**, *495*, 153232. [CrossRef]
25. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
26. Callahan, B.J.; McMurdie, P.J.; Holmes, S.P. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* **2017**, *11*, 2639–2643. [CrossRef]
27. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
28. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef] [PubMed]
29. Edgar, R.C. Updating the 97% Identity Threshold for 16S Ribosomal RNA OTUs. *Bioinformatics* **2018**, *34*, 2371–2375. [CrossRef]
30. Bartlett, A.; Padfield, D.; Lear, L.; Bendall, R.; Vos, M. A Comprehensive List of Bacterial Pathogens Infecting Humans. *Microbiology* **2022**, *168*, 001269. [CrossRef]
31. McMurdie, P.J.; Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
32. Wickham, H.; Averick, M.; Bryan, J.; Chang, W.; McGowan, L.; François, R.; Grolemund, G.; Hayes, A.; Henry, L.; Hester, J.; et al. Welcome to the Tidyverse. *J. Open Source Softw.* **2019**, *4*, 1686. [CrossRef]
33. Neuwirth, E. R Color Brewer: Color Brewer Palettes 2022. Available online: https://cran.r-project.org/web/packages/RColorBrewer/RColorBrewer.pdf (accessed on 18 March 2024).
34. Pedersen, T.L. Patchwork: The Composer of Plots 2024. Available online: https://cran.r-project.org/web/packages/patchwork/patchwork.pdf (accessed on 18 March 2024).
35. Oksanen, J.; Simpson, G.L.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'Hara, R.B.; Solymos, P.; Stevens, M.H.H.; Szoecs, E.; et al. Vegan: Community Ecology Package 2022. Available online: https://www.researchgate.net/publication/360782912_vegan_community_ecology_package_version_26-2_April_2022 (accessed on 18 March 2024).
36. Galili, T. Dendextend: An R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics* **2015**, *31*, 3718–3720. [CrossRef] [PubMed]
37. Gu, Z. Complex heatmap visualization. *iMeta* **2022**, *1*, e43. [CrossRef] [PubMed]
38. R Core Team R: A Language and Environment for Statistical Computing 2023. Available online: https://www.R-project.org/ (accessed on 18 March 2024).
39. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef] [PubMed]
40. Faith, D.P. Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* **1992**, *61*, 1–10. [CrossRef]
41. Lozupone, C.A.; Hamady, M.; Kelley, S.T.; Knight, R. Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* **2007**, *73*, 1576–1585. [CrossRef]
42. Douglas, G.M.; Maffei, V.J.; Zaneveld, J.; Yurgel, S.N.; Brown, J.R.; Taylor, C.M.; Huttenhower, C.; Langille, M.G.I. PICRUSt2: An improved and extensible approach for metagenome inference. *BioRxiv* **2019**, 672295. [CrossRef]
43. Rosado, D.; Lores, M.; Ramos-Tapia, I.; Crandall, K.A.; Pérez-Losada, M.; Domínguez, J. Integrated Fertilization with Bagasse Vermicompost Changes the Microbiome of Mencía Must and Wine. *Fermentation* **2022**, *8*, 357. [CrossRef]
44. Rosado, D.; Ramos-Tapia, I.; Crandall, K.A.; Pérez-Losada, M.; Domínguez, J. Grapevine treatment with bagasse vermicompost changes the microbiome of Albariño must and wine and improves wine quality. *OENO One* **2022**, *56*, 219–230. [CrossRef]

45. Douglas, G.M.; Kim, S.; Langille, M.G.I.; Shapiro, B.J. Efficient computation of contributional diversity metrics from microbiome data with FuncDiv. *Bioinformatics* **2023**, *39*, btac809. [CrossRef]
46. Tenenbaum, D. KEGGREST: Client-Side REST Access to the Kyoto Encyclopedia of Genes and Genomes (KEGG). R Package Version 1.44.0. Available online: https://bioconductor.org/packages/release/bioc/html/KEGGREST.html (accessed on 3 June 2024).
47. Nguyen, N.H.; Song, Z.; Bates, S.T.; Branco, S.; Tedersoo, L.; Menke, J.; Schilling, J.S.; Kennedy, P.G. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* **2016**, *20*, 241–248. [CrossRef]
48. Kolbe, A.R.; Aira, M.; Gómez-Brandón, M.; Pérez-Losada, M.; Domínguez, J. Bacterial succession and functional diversity during vermicomposting of the white grape marc Vitis vinifera v. *Albariño. Sci. Rep.* **2019**, *9*, 7472. [CrossRef] [PubMed]
49. Gómez Brandón, M.; Aira, M.; Kolbe, A.R.; de Andrade, N.; Pérez-Losada, M.; Domínguez, J. Rapid Bacterial Community Changes during Vermicomposting of Grape Marc Derived from Red Winemaking. *Microorganisms* **2019**, *7*, 473. [CrossRef] [PubMed]
50. Gómez-Brandón, M.; Aira, M.; Santana, N.; Pérez-Losada, M.; Domínguez, J. Temporal Dynamics of Bacterial Communities in a Pilot-Scale Vermireactor Fed with Distilled Grape Marc. *Microorganisms* **2020**, *8*, 642. [CrossRef] [PubMed]
51. Domínguez, J.; Aira, M.; Kolbe, A.R.; Gómez-Brandón, M.; Pérez-Losada, M. Changes in the composition and function of bacterial communities during vermicomposting may explain beneficial properties of vermicompost. *Sci. Rep.* **2019**, *9*, 9657. [CrossRef]
52. Rosado, D.; Pérez-Losada, M.; Aira, M.; Domínguez, J. Bacterial Succession during Vermicomposting of Silver Wattle (*Acacia dealbata* Link). *Microorganisms* **2021**, *10*, 65. [CrossRef]
53. Yang, J.; Huang, K.; Peng, L.; Li, J.; Liu, A. Fate of Functional Bacterial and Eukaryotic Community Regulated by Earthworms during Vermicomposting of Dewatered Sludge, Studies Based on the 16S rDNA and 18S rDNA Sequencing of Active Cells. *Int. J. Environ. Res. Public Health* **2021**, *18*, 9713. [CrossRef]
54. Lores, M.; Gómez-Brandón, M.; Pérez-Díaz, D.; Domínguez, J. Using FAME profiles for the characterization of animal wastes and vermicomposts. *Soil. Biol. Biochem.* **2006**, *38*, 2993–2996. [CrossRef]
55. Commission Decision (EU) 2022/1244 of 13 July 2022 Establishing the EU Ecolabel Criteria for Growing Media and Soil Improvers (Notified under Document C(2022) 4758) (Text with EEA Relevance). 2022; Volume 190. Available online: https://www.isprambiente.gov.it/it/attivita/certificazioni/files/ecolabel/criteri/ammendanti/eur-lex-32022d1244-en-eur-lex.pdf (accessed on 29 August 2024).
56. Directive (EU) 2015/2366 of the European Parliament and of the Council of 25 November 2015 on Payment Services in the Internal Market, Amending Directives 2002/65/EC, 2009/110/EC and 2013/36/EU and Regulation (EU) No 1093/2010, and Repealing Directive 2007/64/EC (Text with EEA Relevance). 2024. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015L2366 (accessed on 29 August 2024).
57. Cui, G.; Bhat, S.A.; Li, W.; Wei, Y.; Kui, H.; Fu, X.; Gui, H.; Wei, C.; Li, F. Gut digestion of earthworms significantly attenuates cell-free and -associated antibiotic resistance genes in excess activated sludge by affecting bacterial profiles. *Sci. Total Environ.* **2019**, *691*, 644–653. [CrossRef]
58. Huang, K.; Xia, H.; Zhang, Y.; Li, J.; Cui, G.; Li, F.; Bai, W.; Jiang, Y.; Wu, N. Elimination of antibiotic resistance genes and human pathogenic bacteria by earthworms during vermicomposting of dewatered sludge by metagenomic analysis. *Bioresour. Technol.* **2020**, *297*, 122451. [CrossRef]
59. Duan, Z.; Zhu, Y.; Xia, H.; Huang, K.; Peng, L. A Novel Strategy for Eliminating Antibiotic Resistance Genes during Fertilization of Dewatered Sludge by Earthworms: Vermicomposting Practice Using Chinese Herbal Residues Derived from Lianhua Qingwen as a Bulking Material. *J. Environ. Manag.* **2024**, *349*, 119444. [CrossRef]
60. Huang, K.; Xia, H.; Wu, Y.; Chen, J.; Cui, G.; Li, F.; Chen, Y.; Wu, N. Effects of earthworms on the fate of tetracycline and fluoroquinolone resistance genes of sewage sludge during vermicomposting. *Bioresour. Technol.* **2018**, *259*, 32–39. [CrossRef] [PubMed]
61. Xia, H.; Chen, J.; Chen, X.; Huang, K.; Wu, Y. Effects of tetracycline residuals on humification, microbial profile and antibiotic resistance genes during vermicomposting of dewatered sludge. *Environ. Pollut.* **2019**, *252*, 1068–1077. [CrossRef] [PubMed]
62. Wang, K.; Mao, H.; Li, X. Functional characteristics and influence factors of microbial community in sewage sludge composting with inorganic bulking agent. *Bioresour. Technol.* **2018**, *249*, 527–535. [CrossRef] [PubMed]