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Metagenomic Analysis of Bacterial Community Structure and Dynamics of a Digestate and a More Stabilized Digestate-Derived Compost from Agricultural Waste

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Abstract: Recycling of different products and waste materials plays a crucial role in circular economy, where the anaerobic digestion (AD) constitutes an important pillar since it reuses nutrients in the form of organic fertilizers. Knowledge about the digestate and compost microbial community structure and its variations over time is important. The aim of the current study was to investigate the microbiome of a slurry cow digestate produced on a farm (ADG) and of a more stabilized digestate-derived compost (DdC) in order to ascertain their potential uses as organic amendments in agriculture. The results from this study, based on a partial fragment of 16S bacterial rRNA NGS sequencing, showed that there is a greater microbial diversity in the DdC originated from agricultural waste compared to the ADG. Overall, the existence of a higher microbial diversity in the DdC was confirmed by an elevated number (1115) of OTUs identified, compared with the ADG (494 OTUs identified). In the DdC, 74 bacterial orders and 125 families were identified, whereas 27 bacterial orders and 54 families were identified in the ADG. Shannon diversity and Chao1 richness indexes were higher in DdC samples compared to ADG ones (Shannon: 3.014 and 1.573, Chao1: 68 and 24.75; $p < 0.001$ in both cases). A possible association between the microbiome composition at different stages of composting process and the role that these microorganisms may have on the quality of the compost-like substrate and its future uses is also discussed.

Keywords: agro-waste; compost; microbiome; molecular characterization; taxonomy

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1. Introduction

Anaerobic digestion constitutes an adaptable technology able to process urban, industrial, and agricultural residues. Throughout this microbiological process, a complex community of microorganisms acts to decompose organic matter (OM), transforming it into two main final products: digestate and biogas [1–3]. The key reactions during the decomposition of OM have been extensively studied [4–9]. Briefly, it has been demonstrated that the degradable OM would be utilized as the energy source and few compounds such as CO_2, NH_3, and moisture were released along with large quantities of heat [4–9]. After the anaerobic digestion (AD) of a biodegradable feedstock, the residual material is known as digestate (ADG). The digestate is a secondary waste that has unpleasant features such as being viscous, containing high volatile fatty acids (VFA), and possessing an elevated level of humidity [10]. Due to its richness in nutrients and nitrogen, the ADG can be further processed and used as a fertilizer, while the biogas represents an important source of re-usable energy. The bioprocess of anaerobic digestion of biodegradable waste has widely been recognized by the EU as a recycling method by explicitly including it in Annex II of the Waste Framework Directive under point 'R3', in which a list of recovery operations was established [11–13]. Many studies have shown that the AD process is affected by several factors and is very sensitive to environmental changes [14–16]. In addition, maintaining

the stability of the microbial community in AD is of crucial importance as its equilibrium can be easily affected by any variation of the operative parameters.

Agricultural waste recycling is an important key in the circular economy concept worldwide [17–19]. In Italy, the regulations on agricultural waste recycling always follow the EU laws and recommendations on this matter. Their implementations are coordinated by the Italian Ministry of the Environment fitting within the European Circular Economy Action Plan [13,20].

In Europe, several countries successfully implemented the agricultural waste recycling processes starting from various substrates like: biogenic waste in Austria [21]; organic waste, by-products, and energy crops in Germany [22,23]; chicken manure, animal slurry, food waste, or other organic materials in the UK [24]. In Italy, the agricultural waste recycling processes start from food waste, organic municipal, wastewater, and livestock waste [19,25] and produce energy or biofertilizers/soil amendments.

Organic material can easily be decomposed by the natural process of composting and then re-used as it is rich in humus soil amendment [26–28]. Several methods are employed for performing the composting procedures. For this reason, different types of composting systems exist, such as the conventional windrow, aerated static pile, in-vessel, and the newly developed two-stage composting system [6,29]. It is important to stress that many parameters such as temperature, pH, oxygen, porosity, moisture content, etc. need to be verified during the whole process and constantly optimized for a good composting result [30–32]. Due to their ability to improve health, physical and chemical properties of soil, plant growth, and also to suppress pathogens and plant diseases, compost materials have been greatly recommended and widely used in agriculture [19,33–35].

Above all, the quality of the fertilizer obtained after the composting greatly depends on the microbial structure of the digestate or compost [36–38]. It is known that composting is a very fragile process. The results of this process could be predicted only if any modification that may arise in the microbial communities is taken into account. In addition, knowledge about the microbial community composition during the whole process of composting allows us to make the best choices for compost stabilizers and microbial agents. This also establishes the basis for future screenings of microorganisms with some important and special roles [37–39].

Despite all previous efforts made to reveal the compost structure, a full and precise description of the microbial communities present in it is still incomplete. This is mainly due to the technical limitations of the traditional microorganism culture methods, which are not suitable to isolate and characterize such complex microbial groups. As previously stated by Hultman [40], classic methods generally provide a reduction of dozens (at maximum hundreds) of Operational Taxonomic Units (OTUs). When modern techniques were applied, the presence of thousands of species in digestate and compost was revealed [41–43]. The very high levels of microbial diversity discovered indicated that the traditional methods, which are dependent by the in vitro culture of the investigated species, did not show sufficient resolution to fully understand the microbial communities from digestate, compost, or soil. This is due to the fact that the majority of the bacterial species found in these matrices are not cultivable in vitro. A useful technique has been provided by modern technology such as the new sequencing tools (e.g., pyrosequencing, Roche, Illumina). These technologies are able to provide, in a very short time frame, a huge amount of information that allows us to better understand and describe the complexity of the microbial communities from various substrates [41,44–48].

Molecular investigations on both digestate and compost microbiomes are generally rather difficult because of the particular nature of these matrices, which contain great quantities of OM (especially humic acids). Those substances are produced by bacteria, fungi, and protozoa in soil or water sediments during the degradation of plants or of other organic substances. Due to their very high molecular weight and their mainly poly-anionic nature, the humic substances easily interfere with the nucleic acid extraction methods. They also act as potent PCR inhibitors and their presence, even in very small

quantities, is able to inhibit the DNA polymerase and shift the PCR or sequencing assay results towards a complete lack of success. To overcome all these issues, specific kits and adequate molecular protocols have been developed and successfully employed in many studies [46–50]. They facilitate total DNA extraction from OM rich complex matrices such as digestate and compost.

The aim of this study was to: (i) investigate the microbiome composition of a slurry cow digestate (ADG) produced on farm and taxonomically classify its microorganisms; (ii) examine the microbiome of a more stabilized digestate-derived compost (DdC), obtained after 50 days of a composting process, and determine its taxonomic entities. The role that these microorganisms may have on the quality of the DdC and its use as a final product, more suitable for agricultural purposes, is also discussed.

2. Materials and Methods

2.1. Digestate and Stabilized Digestate-Derived Compost Sources

A zoo-technical farm located in Atella (Basilicata Region, Italy) (40°51′ N; 15°38′ E) that produces biogas derived from an anaerobic digestion of livestock effluent and agricultural waste provided the anaerobic digestate (ADG) utilized in this study. All ADG samples were collected in sterile containers, transported in ice to the laboratories at the University of Basilicata, and maintained in a refrigerator at 4 °C until further use.

In order to obtain the stabilized digestate-derived compost (DdC), wheat straw was also used, in the digesters, in this experiment. Details about its origin, full description, and usage were previously reported by Vitti and co-authors [51]. In this study, the authors investigated the chemical properties and the biologic activity of an ADG and DdC from agro-waste in Southern Italy.

The digestate-derived compost (DdC) was obtained in hand-made thermally isolated semi-batch reactors of 50 L, following an extraction method that had been entirely described by Vitti et al. [51]. Briefly, each reactor had a cylindrical shape, with a 0.05 m^3 volume. It was made of plastic material and the control systems for both qualitative and quantitative parameters were present. The experiment had semi-batch sets of 12 continually stirred digesters, which were filled with 90% of ADG and 10% wheat straw. The reactions were allowed to develop for 50 days and at the end of this period the DdC samples were collected. In order to improve homogeneity, aeration, and temperature control, agitating of the composting substrates for 5 min every 3 h was applied. Each reactor was filled up to 35% of the total volume and kept constant throughout the experiment. Finally, the DdC was separated and air-dried for 4 days [51].

2.2. Molecular Analysis of Microbiota from Digestate and Stabilized Digestate-Derived Compost

2.2.1. DNA Extraction

Bacterial community composition (BCC), diversity, and taxonomy of the ADG and DdC samples were investigated using Illumina MiSeq platform and NGS-sequencing technology followed by bioinformatics analyses. From ADG and DdC samples (3 samples each of 0.25 g), the genomic DNA (gDNA) were extracted with three different methods: Qiagen-DNeasy Plant kit Mini, Omega Bio-Tek- E.Z.N.A.® Soil DNA kit and PowerSoil® kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). For our samples, the most efficient method was the PowerSoil® kit, which was used following manufacturer's instructions. The quantity and quality of the gDNA were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA). The extracted gDNA was stored at −20 °C for further analyses.

2.2.2. PCR Amplification, Sequencing, and Bioinformatics Analyses

For metagenomics analysis, the extracted gDNA was amplified and sequenced by the BMR Genomics Company (Padua, Italy) to obtain the DNA sequences of the 16S rRNA bacterial gene. A double step PCR was performed, using primers with tails for the 16S gene region [52] Pro341F:5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNB

CASCAG-3′ and Pro 805R:3′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACNVGGGTATCTAATCC-5′ amplifying a partial 16S fragment (428 bp in size). The amplification was performed using a PCR mix containing: Taq Platinum HiFi enzyme 0.2 µL (Thermofisher Scientific Inc., Waltham, MA, USA), $MgSO_4$ (50 mM) 1 µL, Forward and Reverse primers (10 µM) 1 µL each, dNTPs mix (10 mM) 0.5 1 µL, buffer 2.5 µL, genomic DNA (3–10 ng/µL) 5 µL, and double distilled water till 25 µL. PCR cycling program was the following: 94 °C for 1 min (1 cycle), followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 45 s, and a final extension at 68 °C for 7 min. Samples were performed in triplicate and a positive control of a previously tested DNA and a negative control with distilled water were always included. The PCR outcomes were verified running 5 µL of the PCR product on agarose gel electrophoresis at 1.5% stained with SYBR™ Safe DNA Gel Stain (Invitrogen, Thermo Scientific Inc., Wilmington, NC, USA) and visualized by an UV transilluminator model Euro Clone Combi Light (Euro Clone S.p.A., Milan, Italy). PCR products were sequenced using the MiSeq technology (Illumina). For that purpose, they were further purified with magnetic beads and the Index Illumina Nextera XT were linked to the universal tails in a second PCR reaction. Subsequently, the samples were normalized and multiplied until 188. The library created was loaded to MiSeq with the 300PE strategy, the quality of the run was controlled and the reads revealed in fastq format. Regarding the bioinformatics analysis, processing the raw reads started with quality check and filtering of low-quality ones ($Q < 25$). The forward and reverse sequences (R1 and R2 reads) were united with the software FLASHv1.2.11 and filtered for their quality ($Q > 30$). All reads containing ambiguous bases were removed. The clustering of the Operational Taxonomic Units (OTUs) [cluster of similar (97% identity) sequences, which represent a "bacterial species"] was based on the method pick_closed_reference_otus.py of Qiime 1.9.1 and on the database Greengenes v.13–8. The Quantitative Insights into Microbial Ecology (QIIME) pipeline was applied in order to control the data quality, including length-based filtering and read-quality filtering. The OTUs were filtered at 0.005% of abundance to eliminate the OTUs with less frequency [53,54]. Finally, the relative abundance (RA %) of each sample type (ADG and DdC), at different taxonomic levels, was calculated as the average of the three independent samples. All the outcomes of sequencing and bioinformatics analyses for both substrates were identically processed at the Laboratory of Plant Pathology of the University of Basilicata, School of Agriculture, Forestry and Environmental Sciences (SAFE) using Microsoft Excel Office 2007 advanced functions. The results were further used for the statistical analyses.

2.3. Statistical Analyses

For each microbial group, as the mean abundances were measured at the beginning (ADG) and at the end (DdC) of the same composting process, the differences between treatments were analyzed with a paired sample Student *t*-test using the R-3.6.2 software [55]. Species diversity (Shannon) and richness (Chao-1) indices were computed and compared using random permutation tests in PAST version 4.0 [56].

3. Results

3.1. Taxonomic Classification of Digestate and Stabilized Digestate-Derived Compost Microbiome

The physico-chemical properties of ADG and DdC have been previously reported by Vitti et al. [51]. According to the Italian Legislative Decree 75/2010 about "Reorganization and Revision of the Discipline on Fertilizers", the DdC could be considered a mixed composted amendment. Moreover, the ADG could be considered an amendment until its full maturation, such as the obtained DdC [51].

The NGS results showed 124,468 total reads and 55,691 filtered reads in the ADG, whereas, in the DdC the total reads were 51,542 and 11,301 filtered reads. A total number of 494 and 1113 OTUs have been identified in the ADG and in DdC, respectively. Both in the ADG and in the DdC, the bacterial species were the most abundant (80 and 99% of the

RA), whereas the remaining species belonged to the *Archaea* kingdom (20 and 0.01% RA) (Figure 1).

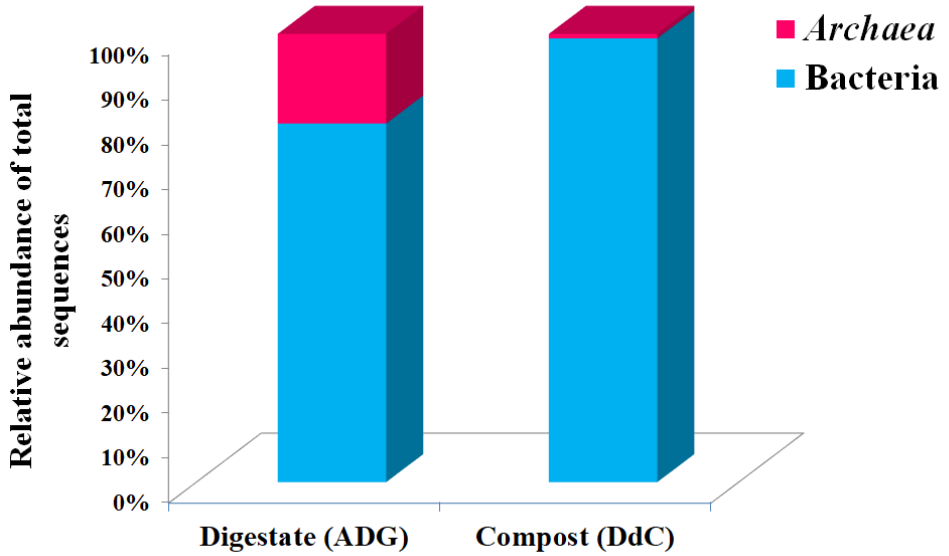

Figure 1. Relative abundance (RA%) of Bacteria and *Archaea* in digestate (ADG) and compost (DdC). The taxonomic assignments were obtained from 16S rRNA gene sequencing analysis.

The metagenomics analysis performed on the ADG and DdC samples allowed us to classify more than 90% of the high number of nucleotide sequences at various taxonomic levels. Furthermore, in our study only a lower percentage of sequences (about 1.34% in the case of ADG and around 9% in the case of DdC) remained unclassified (Table 1). In general, a great variation of the microbial community composition and diversity in the ADG and DdC matrices was observed. High-throughput 16S rRNA sequencing allowed the identification of a lower number of taxonomic orders and families inADG (27 and 54, respectively) than in DdC (74 and 125, respectively). Moreover, the ADG and DdC samples analyzed shared 23 orders and 37 families, while the remaining taxa were specific to the ADG (4 and 17) or to the DdC (51 and 88) (Table 1).

Table 1. Overview of metagenomic results based on 16S rRNA gene amplicons high-throughput sequencing of digestate and stabilized digestate-derived compost samples from agro-waste.

Total No./Taxonomic Classification	Digestate (ADG)	Compost (DdC)
Sequences obtained	55,616	11,229
Unclassified sequences (%)	1.34	9.09
Classified sequences * (%)	98.66	90.91
Orders identified	27	74
Orders included (shared by the two types of matrices and including *Archaea*)	23	23
Orders excluded	4	51
Families identified	54	125
Families included (shared by the two types of matrices)	37	37
Families excluded	17	88

* Classification was counted when it was obtained at least at the order level in allthree samples.

At order level, a high number of bacteria in the DdC compared to the ADG were identified. The taxonomic profile of the ADG microbiome identified showed 5 top orders: Bacillales (43.3% RA), Bacteriodales (22.5% RA), Methanomicrobiales (18.7% RA), Clostridiales (6% RA), and Acholeplasmatales (1.7% RA). The other orders were less abundant (<1.6% RA) (Figure 2). In the DdC microbiome, the more abundant identified orders were: Bacteriodales (14.2% RA), Alteromonadales (10.7% RA), Xanthomonadales (9.1% RA), Cytophagales (8% RA), and Rhizobiales (7% RA). The remaining orders were unclassified (Figure 2).

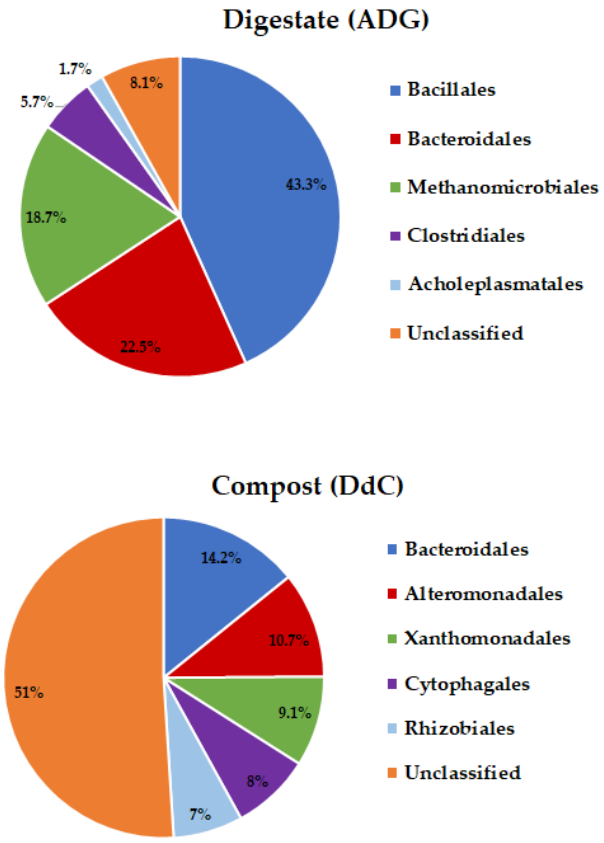

Figure 2. Top 5 bacterial orders identified in digestate (ADG) and a more stabilized digestate-derived compost (DdC) microbiome. The taxonomic profile was identified using sequencing of the amplicons of 16S rRNA gene region. Unassigned 16S sequences were excluded.

In addition, four orders were only found in the ADG, namely, Sphaerochetales (1.55% RA), Neisseriales (0.01% RA), Dehalococcoidales (0.01% RA), and Fusobacteriales (0.01% RA). However, a very complex microbiome was observed in the DdC where the abundant OTUs (≥5% RA) identified were members of Bacteroidales (14.21% RA), Rhizobiales (8.38% RA), Xanthomonadales (8.89% RA), Alteromonadales (10.38% RA), and Bacillales (5.6% RA) orders, while the other bacterial orders were less represented (0.3–4% RA). A morecomplex microbiome was then observed in the DdC than in the ADG (Figure 3). In DdC, the microbes identified were grouped in 74 taxonomical orders. In the ADG samples, the microorganisms identified were classified in 24 bacterial orders (Figure 3). The most abundant bacterial orders identified in ADG were Bacillales and Bacteroidales (22–44% RA), while in DdC the most abundant orders were Bacteroidales and Alteromonadales (10–14% RA). In the DdC samples, some orders such as Xanthomonadales (9.16% RA), Cytophagales (8.04% RA), Rhizobiales (7.05% RA), and Sphingomonadales (2.53% RA) are specific for this substrate (<10% RA) (Figure 3).

At order level, for all the microbiota found in the ADG and DdC, the mean values (±standard errors) of the relative abundance were calculated and reported in Table 2. In particular, the mean differences were statistically significant for the majority of the orders identified, except for Enterobacteriales, Methylcoccales, Aeromonadales, Fibrobacterales, Lactobacillales, Podosphaerales, and Synergistales (Table 2). Additionally, for Dehalococcoidales, Fusobacteriales, Gaiellales, Gemmatales, Legionellales, Neiseriales, Nitrospirales, and Puniceicoccales orders, it was not possible to perform the Stundent's *t*-test since their abundances were extremely low or zero (Table 2).

Table 2. Relative abundance (mean ± standard errors) of all microbiota at orders level in digestate and digestate derived-compost and *p* values of Student's *t*-test.

Order	Digestate (ADG)	Compost (DdC)	*p* (t)
Acholeplasmatales	1.7 ± 0.02	0.02 ± 0.0058	8.27×10^{-5}
Acidimicrobiales	0 ± 0	2.4 ± 0.1155	0.0023
Actinomycetales	0.08 ± 0.0058	1.25 ± 0.01	2.43×10^{-5}
Aeromonadales	0.02 ± 0.0058	0.01 ± 0	0.225
Alteromonadales	0 ± 0	10.66 ± 0.0346	1.06×10^{-5}
Anaerolineales	0 ± 0	0.03 ± 0.0058	0.035
Bacillales	43.27 ± 0.0519	5.27 ± 0.0230	5.778×10^{-5}
Bacteroidales	22.47 ± 0.4798	14.21 ± 0.060	0.004
Bedellovibrionales	0 ± 0	0.08 ± 0.0058	0.0041
Burkholderiales	1.11 ± 0.0058	0.82 ± 0.0058	0.00013
Caldilineales	0 ± 0	0.13 ± 0.0058	0.0019
Campylobacterales	0.6 ± 0.0288	0.23 ± 0.01	0.00315
Caulobacterales	0 ± 0	0.5 ± 0.0152	0.0009
Chromatiales	0 ± 0	0.32 ± 0.0058	0.00032
Chrysiogenales	0 ± 0	0.03 ± 0.0058	0.035
Chthoniobacterales	0 ± 0	0.7 ± 0.0058	0.0067
Clostridiales	5.72 ± 0.0723	2.13 ± 0.0115	0.00028
Cytophagales	0 ± 0	8.04 ± 0.0115	2.06×10^{-6}
Deferribacterales	0.01± 0	0 ± 0	0.0078
Dehalococcoidales	0.01 ± 0	0 ± 0	NA *
Desulfobacterales	0 ± 0	0.05 ± 0.0058	0.013
Desulfovibrionales	0.11 ± 0.0058	0.34 ± 0.0058	0.00063
Enterobacteriales	0 ± 0	0.03 ± 0.01	0.09
Erysipelotrichales	1.25 ± 0.0173	0.03 ± 0.0058	8.96×10^{-5}
Fibrobacterales	0.04 ± 0.0058	0.03 ± 0.01	0.22
Flavobacteriales	0.8 ± 0.0208	4.32 ± 0.01	1.08×10^{-5}
Fusobacteriales	0.01 ± 0	0 ± 0	NA *
Gaiellales	0 ± 0	0.01 ± 0	NA *
Gammatimonadales	0 ± 0	0.04 ± 0.0058	0.02
Gemmatales	0 ± 0	0.01 ± 0	NA *
Ignavibacteriales	0 ± 0	0.15 ± 0.0058	0.0015
Kiloniellales	0 ± 0	0.33 ± 0.0058	0.00148
Lactobacillales	0.08 ± 0.0058	0.07 ± 0.0058	0.225
Legionellales	0 ± 0	0.02 ± 0	NA *
Methylcoccales	0 ± 0	0.02 ± 0.0058	0.074
Methylophilales	0 ± 0	0.51 ± 0.01	0.00038
Myxococcales	0 ± 0	0.44 ± 0.02	0.0021
Natranaerobiales	0 ± 0	0.04 ± 0.0058	0.02
Neiseriales	0.01	0	NA *
Nitrosomonadales	0 ± 0	0.41 ± 0.0152	0.0014
Nitrospirales	0 ± 0	0.01 ± 0	NA *
Oceanospirillales	0 ± 0	3.64 ± 0.0230	4.03×10^{-5}
Optitutales	0 ± 0	0.24 ± 0.0058	0.00058
Phycisphaerales	0 ± 0	0.12 ± 0.0058	0.0023
Pirelullales	0 ± 0	0.37 ± 0.0058	0.00024
Planctomycetales	0 ± 0	0.08 ± 0.0058	0.0052
Podosphaerales	0.02 ± 0.01	0.02 ± 0	1
Pseudomonadales	0.5 ± 0.0058	2.13 ± 0.01	1.25×10^{-5}
Puniceicoccales	0 ± 0	0.01 ± 0	NA *
Rhizobiales	0 ± 0	7.05 ± 0.0058	6.71×10^{-7}
Rhodobacterales	0 ± 0	2.05 ± 0.01	2.38×10^{-5}
Rhodocylales	0 ± 0	0.95 ± 0.0153	0.000258
Rhodospirillales	0 ± 0	1.88 ± 0.0251	0.000179
Rhodothermales	0 ± 0	1.04 ± 0.0058	3.08×10^{-5}
Rickettsiales	0 ± 0	0.22 ± 0.0058	0.000688
Saprospirales	0 ± 0	1.64 ± 0.1001	0.0037
Solibacterales	0 ± 0	0.09 ± 0.0058	0.0041

Table 2. *Cont.*

Order	Digestate (ADG)	Compost (DdC)	*p* (t)
Solirubrobacterales	0 ± 0	0.07 ± 0.01	0.019
Spaherochaetales	1.55 ± 0.0058	0 ± 0	1.39×10^{-5}
Sphingobacteriales	0.02 ± 0.0058	1.47 ± 0.0115	0.00011
Sphingomonadales	0 ± 0	2.53 ± 0.0404	0.00025
Spirobacillales	0 ± 0	0.04 ± 0.0058	0.02
Spirochetales	0.22 ± 0.0058	1.64 ± 0.0058	1.97×10^{-6}
Synergistales	0.09 ± 0.0058	0.09 ± 0.01	1
Syntrophobacterales	0 ± 0	0.11 ± 0.0058	0.0027
Thiotrichales	0 ± 0	0.24 ± 0.0115	0.0023
Turicibacterales	0.06 ± 0.0058	0.15 ± 0.0153	0.012
Verrucomicrobiales	0 ± 0	0.27 ± 0.01	0.00137
Xanthomonadales	0 ± 0	9.16 ± 0.0058	3.97×10^{-7}
Others *Archaea*	19 ± 0.0305	0.08 ± 0.0058	1.77×10^{-5}

* Note: NA = not applicable.

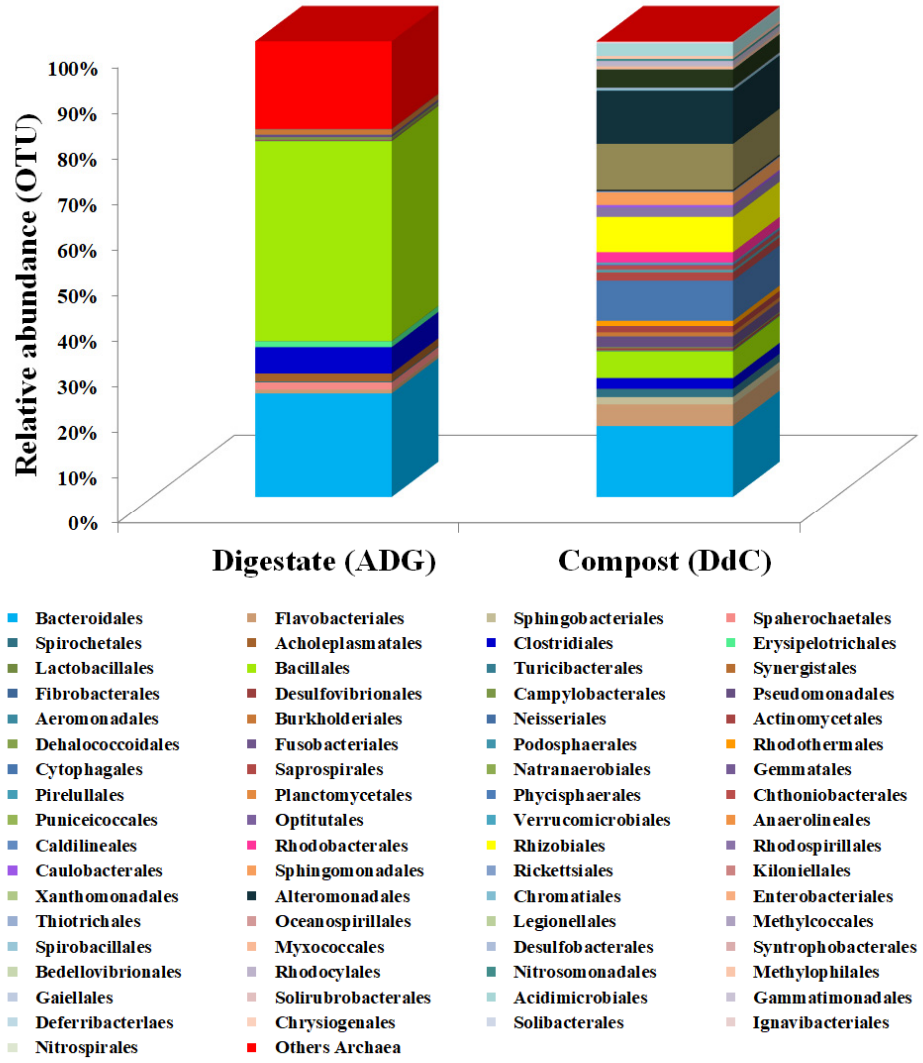

Figure 3. Microbioma composition (at order level) of the digestate (ADG) and the digestate-derived compost (DdC). The taxonomic profile is based on the sequencing of the 16S rRNA gene region. Unassigned 16S sequences were excluded.

3.2. Species Diversity and Richness

Shannon diversity and Chao-1 richness indices for all microbiota found in digestate and compost were reported in Table 3. In particular, the Shannon diversity index was statistically higher in the DdC than in the ADG samples (3.014 and 1.573 respectively, $p < 0.001$) and Chao-1 richness index followed the same pattern, with registering higher values in DdC than in ADG (68 and 24.75, respectively, $p < 0.001$). The Chao-1 and the Shannon indexes increased with time since the compost showed higher values than the digestate, suggesting that the quantity and the composition of the microorganisms had increased following the composting process.

Table 3. Average values of the Alpha diversity indices at order level in digestate and digestate derived-compost.

Diversity Index	ADG	DdC
Shannon	1.573	3.014
Chao-1	24.75	68

3.3. Shared Microbioma Composition

Twenty-three orders (including those belonging to *Archaea*) constitute the core microbiota shared by the ADG and the DdC. The large majority of these OTUs (≥80% RA) were included in Bacteriodales (22.47% RA in the ADG and 14.21% RA in the DdC) and Bacilalles (43.27% RA in the ADG and 5.27% RA in the DdC) (Figure 4). *Archaea* were found at 20% RA in the ADG, whereas they were less abundant in the DdC (Figure 4).

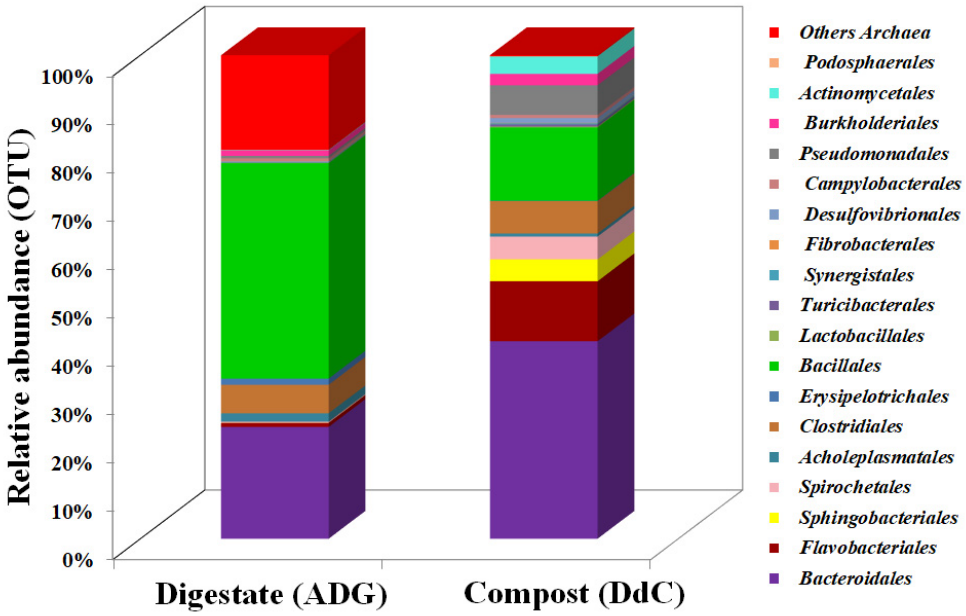

Figure 4. Shared microbiome composition (at order level) of the digestate (ADG) and the digestate-derived compost (DdC). The taxonomic profile is based on the sequencing of the 16S rRNA gene region. Unassigned 16S sequences were excluded.

The mean relative abundances of the shared orders in the ADG and in the DdC has been reported in Table 4. Specifically, the mean differences were statistically significant for the majority of the orders identified, except for four namely, Fibrobacterales, Lactobacillales, Podosphaerales, and Synergistales (Table 4).

Table 4. Relative abundance of the shared orders in digestate and digestate derived-compost and results of the statistical Student's *t*-test.

Order	Digestate (ADG)	Compost (DdC)	*p* (t)
Acholeplasmatales	1.7 ± 0.0577	0.22 ± 0.0058	0.0013
Actinomycetales	0.08 ± 0.0058	1.25 ± 0.0058	8.094×10^{-8}
Bacillales	43.27 ± 0.5832	5.27 ± 0.0115	0.00024
Bacteroidales	22.47 ± 0.4798	14.21 ± 0.0682	0.0041
Burkholderiales	1.11 ± 0.0058	0.82 ± 0.0058	0.00119
Campylobacterales	0.6 ± 0.0577	0.23 ± 0.0058	0.0214
Clostridiales	5.72 ± 0.0321	2.33 ± 0.0058	0.00012
Desulfovibrionales	0.11 ± 0.0058	0.34 ± 0.0058	0.00188
Erysipelotrichales	1.25 ± 0.0058	0.03 ± 0.0058	6.72×10^{-5}
Fibrobacterales	0.04 ± 0.0058	0.03 ± 0.0058	0.225
Flavobacteriales	0.8 ± 0.0208	4.32 ± 0.01	1.07×10^{-5}
Lactobacillales	0.08 ± 0.0058	0.07 ± 0.0058	0.422
Podosphaerales	0.02 ± 0.0058	0.02 ± 0	1
Pseudomonadales	0.5 ± 0.0577	2.13 ± 0.0058	0.00139
Sphingobacteriales	0.02 ± 0.0058	1.47 ± 0.0115	0.00011
Spirochetales	0.22 ± 0.0058	1.64 ± 0.0058	1.04×10^{-6}
Synergistales	0.09 ± 0.005	0.09 ± 0	1
Turicibacterales	0.06 ± 0.0058	0.15 ± 0.0058	0.004
Others Archaea	19 ± 0.0577	0.07 ± 0.0058	0.00092

In the ADG, 54 bacterial families of microorganisms were identified. Furthermore, the identified taxa grouped in five prevalent families, namely, Planococcaceae (42.86% RA), Porphyromonadaceae (5.65% RA), Bacteroidaceae (5.56% RA), Tissierellaceae (1.94% RA), and one *Archaea* family, Methanocorpusculaceae (18.62% RA). In the DdC samples, the five most abundant families were: Marinilabiaceae (11.49% RA), Chromatiaaceae (9.42% RA), Xanthomonadaceae (8.03% RA), Cytophagaceae (7.56% RA), and Hyphomicrobiaceae (3.56% RA), while the others were less abundant (<2.5% RA). Even at the family level, the taxonomic profiles of the ADG and the DdC samples were very different. In the DdC, a strong change in the abundance of some of the core families along with the presence of many new ones has been detected. For example, the Planococcaceae, which were abundant at the beginning of the transformation process (42.86% RA in the ADG) dropped in the final stages (2.22% RA in the DdC). The same trend was observed for the Bacteroidaceae (5.56% RA in the ADG and 0.03% RA in the DdC) and for some *Archaea* (18.62% RA in the ADG but not identified in the DdC). An opposite trend was observed for the members of the Marinilabiaceace family: they were less abundant at the first stage of the agricultural waste transformation process (0.17% RA in the ADG) compared to the final stages (11.49% RA in the DdC).

4. Discussion

4.1. Microbiota Diversity and Characterization of the Bacterial Community

An earlier study by Thummes et al. [57] on thermophilic methanogenic *Archaea* in compost material showed that cattle manure used for soil fertilization is known to contain anaerobic methanogens. The compost material acts as an effective carrier for the distribution of thermophilic methanogens by fertilization and wind [57]. Moreover, in the process of composting, *Archaea* ammonia-oxidizing (AOA) coexists with ammonia-oxidizing bacteria as shown by the Oshi et al. study. [58]. The authors demonstrated that the predominant AOA in cattle manure compost can grow and can probably oxidize ammonia under moderately thermophilic conditions [58]. In the present study, the microbiome of a slurry cow digestate produced on the farm (ADG) and of a more stabilized digestate-derived compost (DdC) were investigated. Our results on the microbial composition showed that the predominant species belonged to the bacteria Kingdom, whereas *Archaea* were less abundant, in agreement with Antunes et al. [59]. These authors, using metagenomic and

metatranscriptomic approaches, investigated the microbial community structure and dynamics in thermophilic composting and showed that the Bacteria were always dominant compared to *Archaea*, regardless of the composting age [59].

In our study, in all the ADG samples the top five Phylum identified were Firmicutes (50.4% RA), Bacteroidetes (23.3% RA), Tenericutes (2% RA), Proteobacteria (2.3% RA), and Euryarchaeota (19.8% RA), while in all the DdC samples the most abundant phyla identified were Bacteroidetes (30.74% RA), Firmicutes (5.89% RA), Tenericutes (5.89% RA), Proteobacteria (50.02% RA), and Actinobacteria (2.78% RA). It was also observed that the Euryarchaeota were very less abundant (0.09% RA) in the DdC compared to the ADG. Our results on the microbial composition observed in the ADG is in agreement with what has been previously reported by Liu et al. [41]. These authors showed that, within an anaerobic digester, Bacteroidetes and Firmicutes increased in their relative abundance. In addition, the same authors reported that Proteobacteria was the most dominant phylum in feedstock samples. This shift in the composition of the microbial community could be explained as an outcomeof their adaptation to the digested environment [41].

4.2. Environmental Factors Influencing Micobioma, the ADG, and the DdC

In our study, Firmicutes were found to be more dominant (50.4% RA) than Proteobacteria (2.3% RA). Similar results were found by Xia et al. [60] in thermophilic digesters. It is well known that community composition in digesters is mostly influenced by operational temperatures [41,61,62]. Overall, Firmicutes and Proteobacteria diversity is greatly influenced, among other factors, by temperature variation during the composting process [63].

Zhang et al. [64] pointed out that in digesters operating at lower temperatures, Proteobacteria were more dominant while in thermophilic digesters and Firmicutes were the most abundant. Regarding the roles played by these organisms, several studies showed that Proteobacteria contains members thatare able to convert glucose, butyrate, propionate, and acetate [61,65–67]. The Firmicutes, which dominated the chicken manure compost microbiota, are considered to be degraders with strong amino acid metabolism, secreting a diverse array of proteases. It has been suggested that they mainly participate in the composting process [68]. Our results seem to confirm this statement since Firmicutes were more abundant in the ADG compared to the DdC.

Overall, the results of this study are in agreement with other studies showing that the most abundant phyla and orders (those with an elevated number of reads) throughout the composting process are: Firmicutes, Proteobacteria, Bacteroidales, and Actinobacteria [59,63,68–71].

We also obtained different taxonomic profiles where some orders were dominant over the others. These differences have been influenced by the composting period (initial stage in the ADG; a more mature stage in the DdC). This finding is in agreement with what has been previously reported by Li et al. [38]. These authors stated that the abundance of some microbial species increased with the compost fermentation. At the beginning of the process, the phyla of Firmicutes and Actinomycetes were dominant while at the maturity stage, Proteobacteria, Bacteroidetes, Firmicutes, and Chloroflexi but not Actinomycetes were identified. The same authors also showed that some bacteria involved in lignocellulose degradation such as Streptomyces, Rhodococcus, and Mycobacterium were found to be more abundant in the maturity stages of composting [38]. The Firmicutes and Proteobacteria were present when the temperature was high, generally at the early stage of composting, whereas Bacteroidetes increased their abundance during the cooling period.

Some core orders identified in our study were present at different times during the composting process. In particular, at the beginning of the transformation process we found that Bacteroidales, Bacillales, Clostridiales, and Acholepasmatales registered higher relative abundance values in digestate than in compost. Other orders such as Flavobacteriales, Campylobacterales, Pseudomonadales, and Actinomycetales were found to be more abundant at the end of the process, thus being more present in the DdC than in the ADG samples.

The relatively high abundance of Bacillales in the ADG and, to some extent, in the DdC is in accordance with what has been reported by Franke-Whittle et al. [72] and by Ryckeboer et al. [73]. This order was the most abundant group of bacteria in composting during the thermophilic phase and throughout the entire composting process. The Bacillales presence observed at the beginning of the transformation process, in our study, is also similar with what has been reported by Varma et al. [74]. The authors noted their abundance in the initial stages of the composting and showed that these organisms are involved in the degradation of cellulose and lignocellulose residues.

In our research, the variation in the orders of the Actinomycetales and Pseudomonadales (more abundant at the end of the transformation stages) showed that the degradation of cellulose and lignocellulose residues occurs at the end of the process. The greater abundance of Actinomycetales and Pseudomonadales and their involvement in the degradation of the material was also reported by Antunes et al. [59]. We also showed the presence of Bacteroidaceae only at the first stage of the transformation of the agricultural waste residues in compost. These results confirm that, in thermophilic composting, the microbial communities structure and dynamics change over time.

4.3. Microbial Community Changes over Time

Warma et al. [74], while examining the bacterial community structure during in-vessel composting of agricultural waste, demonstrated that a similar microbial composition was found in cow manure compost. An earlier study by Chandna et al. [75] on the biochemical analysis of the bacterial diversity during composting of agricultural by-products reported different taxonomic profiles based on the composting stage along with a higher diversity of species found in the final compost. They described the presence of microorganisms like Firmicutes and Proteobacteria at earlier stages of the composting during the mesophilic phase, while the presence of Firmicutes and Actinobacteria was reported in the thermophilic phase, thus confirming our results.

Meng et al. [76], while exploring the microbial community succession during cow manure and corn straw composting using the high throughput sequencing technology, showed that the major phyla identified were Proteobacteria, Bacteriodetes, Firmicutes, and Actinobacteria. These phyla were also found with similar higher percentages of OTUs in our study. During the composting process, their evolution over time was strictly linked to temperature variation. Initially, Bacteriodetes and Proteobacteria were the dominant phyla, followed by Firmicutes. In the latest stage, Firmicutes decreased. In addition, an earlier study of Ishii and Takki [77] on the microbial communities during four different composting processes, showed that the main factor affecting the microbial communities in the composting process is the concentration of dissolved organic materials. They detected Gram negative bacteria, Actinobacteria and various Bacillus species in four different composting processes. Similarly, Fracchia et al. [78], while looking at the bacterial diversity in finished compost and vermicompost by cultivation, described the abundant presence of Gram positive organisms belonging to Firmicutes and Actinobacteria, confirming the results of the present study. Basically, many studies reported that microorganism variation found in different composting systems depends on the initial materials composted and on the composting process used [58,75–77].

4.4. Role of the Microbial Composition in Controlling Phytopathogens

The higher abundance of microbial genera/families from the DdC samples compared to the ADG ones revealed that the present study could be used as an effective biological control strategy of soil-borne phytopathogens. This statement is supported by a recent study by De Corato [47], which reported a high diversity of bacteria in compost with positive effects on soil-borne plant pathogens.

Literature data [51,79–82] demonstrates thatDdC plays a role in the suppression of important phytopathogens such as *Rhizoctonia solani, Pythium aphanidermatum*, *Fusarium solani*, and *Verticillium dahliae*. DdC also increases the growth and development of tomato,

beans, or cabbage plants [47,51,79–82]. The maturation process of waste materials is then able to provide a compost that is useful for agricultural purposes. In small farms using self-built semi-batch reactors, this compost could be considered as an ideal alternative to chemical fertilizers and pesticides.

Environmental sustainability is a fundamental concept for circular economy and is very important in the evaluation of the reuse of wastes responsible for environmental contamination by xenobiotic compounds, microorganisms, and all the waste produced by agro-industrial processes, as well as household activities. Therefore, applying better methodologies is necessary to satisfy the energy needs of the agro-industrial productive areas through the exploitation of renewable sources available on site, and their transformation into organic matter, such as digestate and DdC, to be used in agricultural systems. Starting with cow slurry digestate (ADG) produced on-farm and/or a stabilized digestate-derived compost (DdC), with an anaerobic composting process in self-built semi-batch reactors, it is then possible to obtain an organic amendment useful for agricultural purposes. However, a short anaerobic process determines that the digestate cannot be considered as a good amendment, but it requires a further maturation process.

5. Conclusions

The NGS sequencing of 16S rRNA gene uncovered significant data about the relative abundances of different microorganisms at various taxonomic levels. Highly relevant changes were observed in the relative abundance of the microbiome components in ADG and in DdC, showing a different dynamic over time. Overall, the elevate dabundance of microbial biomass found in the DdC, demonstrates the presence of a rich and complex microbial community, which could also be a good indicator of a superior quality, a valuable feature, which can influence its further uses.

However, further investigation on all bacteria identified in this study but also on the archaeal community seem necessary to provide additional information for a better understanding and characterization of the biological processes involved in anaerobic digesters. Our results provided new data, which can be used for the benefit of the local economy, creating additional value for waste. Furthermore, the complete and updated knowledge about digestate and compost microbiome from agricultural waste is useful for its future manipulation since the application of compost and compost like-derivatives can help in case of soil-borne plant disease suppression in various cropping systems [79–86]. This knowledge is also useful to enhance soil chemical and biological quality [87,88], which can be a successful part of the circular economy.

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