Chapter 10

Animal Manures: Recycling and Management Technologies

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http://dx.doi.org/10.5772/53454

1. Introduction

Many environmental problems of current concern are due to the high production and local accumulations of organic wastes that are too great for the basic degradation processes inherent in nature. With adequate application rates, animal manure constitutes a valuable resource as a soil fertilizer, as it provides a high content of macro- and micronutrients for crop growth and represents a low-cost, environmentally- friendly alternative to mineral fertilizers [1]. However, the intensification of animal husbandry has resulted in an increase in the production of manure - over 1500 million tonnes are produced yearly in the EU-27 [2] as reported by Holm-Nielsen et al. [3]- that need to be efficiently recycled due to the environmental problems associated with their indiscriminate and untimely application to agricultural fields. The potentially adverse effects of such indiscriminate applications include an excessive input of harmful trace metals, inorganic salts and pathogens; increased nutrient loss, mainly nitrogen and phosphorus, from soils through leaching, erosion and runoff-caused by a lack of consideration of the nutrient requirements of crops; and the gaseous emissions of odours, hydrogen sulphide, ammonia and other toxic gases [4]. In fact, the agricultural contribution to total greenhouse gas emissions is around 10%, with livestock playing a key role through methane emission from enteric fermentation and through manure production. More specifically, around 65% of anthropogenic N_2O and 64% of anthropogenic NH_3 emissions come from the worldwide animal production sector [5].

The introduction of appropriate management technologies could thus mitigate the health and environmental risks associated with the overproduction of organic wastes derived from the livestock industry by stabilizing them before their use or disposal. Stabilisation involves the decomposition of an organic material to the extent of eliminating the hazards and is normally reflected by decreases in microbial biomass and its activity and in concentrations

of labile compounds [6]. Composting and vermicomposting have become two of the best-known environmentally appropriate technologies for the recycling of manures under aerobic conditions [6-7], by transforming them into safer and more stabilised products (compost and vermicompost) with benefits for both agriculture and the environment. Unlike composting, vermicomposting depends on the joint action between earthworms and microorganisms and does not involve a thermophilic phase [8]. However, more than a century had to pass before vermicomposting was truly considered a field of scientific knowledge or even a real technology, despite Darwin [9] having already highlighted the important role of earthworms in the decomposition of dead plants and the release of nutrients from them.

Although microbial degradation under oxygen is usually faster and, as such aerobic processes are thermodynamically more favorable than anaerobic processes, in recent years, anaerobic digestion (AD) has become an upcoming technology for the treatment of animal manures [3, 10-13]. On the one hand, pretreatment of manure by anaerobic digestion can involve some advantages including malodor reduction, decreased biochemical oxygen demand, pathogen control, along with a reduction in the net global warming potential of the manure [4,14]. AD reduces the risk of water pollution associated with animal manure slurries (i.e., eutrophication) by removing 0.80–0.90 of soluble chemical oxygen demand and it improves human/farm cohabitation in rural regions by reducing odor emissions by 70–95% [4]. This process has other direct advantages beyond these, which are related to biogas production for renewable energy and the enrichment of mineral fractions of N and P during digestion [4,10], resulting in a more balanced nutrient mix and increased nutrient bioavalability for plants compared with undigested manure [15].

Therefore, the purpose of this chapter is to give an overview of the three major management technologies of manure recycling, including the aerobic processes of composting and vermicomposting and the anaerobic digestion for biogas production. The main changes that occur in the substrate from a chemical and microbial viewpoint during the specific phases of each degradation process are addressed, as such changes determine the degree of stability of the end product and in turn its safe use as an organic amendment. Different methods that have been proposed to evaluate compost stability are summarised. Also, the influence of the end products derived from each process on the soil microbiota and disease suppressiveness are discussed.

2. Aerobic degradation: Composting and vermicomposting processes

Under aerobic conditions, the degradation of organic matter is an exothermic process during which oxygen acts as a terminal electron acceptor and the organic materials are transformed into more stable products, carbon dioxide and water are released, and heat is evolved. Under field conditions, aerobic degradation takes place slowly at the soil surface, without reaching high temperatures; but this natural breakdown process can be accelerated by heaping the material into windrows to avoid heat losses and thus allowing for temperature increases (composting) or by using specific species of earthworms as agents for turning,

fragmentation, and aeration (vermicomposting). Although both aerobic processes, composting and vermicomposting, have been widely used for processing different types of animal manure either separately or in combination with each other (see Table 1), most of the studies are not comparable mainly due to differences in the applied experimental designs, parent material, earthworm species, as well as the length of the experiments and the parameters used for analysis, among others. Despite these limitations, all these findings have largely contributed to better understand the changes that the material undergoes during these biological stabilisation processes, which is of great importance for their optimisation, and ultimately to obtain a high quality final product. In line with this, certain chemical characteristics of the animal manures can limit the efficiency of these processes, such as an excess of moisture, low porosity, a high N concentration in relation to the organic C content or high pH values [6]. Therefore, different aeration strategies, substrate conditioning-feedstock formulation, bulking agents and process control options have been considered in manure composting and vermicomposting so as to reduce the time and costs of both processes and enhance the quality of the end-products [6-7].

2.1. The composting process

Composting is defined as a bio-oxidative process involving the mineralization and partial humification of the organic matter, leading to a stabilised final product, free of phytotoxicity and pathogens and with certain humic properties, which can be used to improve and maintain soil quality and fertility [25]. Composting of animal manures has been traditionally carried out by the farmers after manure collection for better handling, transport and management [6]. Frequently, the wastes were heaped up and very little attention was paid to the process conditions (aeration, temperature, ammonia loss, etc.) and using rudimentary methodology.

From a microbial viewpoint continuous composting processes may be described as a sequence of continuous cultures, each of them with their own physical (temperature), chemical (the available substrate), and biological (i.e., the microbial community composition) properties and feedback effects. These changes make it difficult to study the process, which is virtually impossible to simulate in the laboratory since temperature, moisture, aeration, etc., are directly related to the surface/volume ratio. However, in general, composting may be described as a four-phase process in which the energy-rich, abundant and easily degradable compounds like sugars and proteins are degraded by fungi and bacteria (referred to as primary decomposers) during an initial phase called the *mesophilic phase* (25-40 °C). Although there exists a competition between both microbial groups regarding the easily available substrates, fungi are very soon outcompeted because the maximum of specific growth rates of bacteria exceed those of fungi by one order of magnitude [26]. The importance of bacteria (with the exception of Actinobacteria) during the composting process has long been neglected, probably because of the better visibility of mycelial organisms. A review on the microbial groups involved in the first mesophilic phase is given by [27]. Provided that mechanical influences (like turning) are small, compost fauna including earthworms, mites and millipedes may also act as catalysts, thereby contributing

to the mechanical breakdown and offering an intestinal habitat for specialized microorganisms. The contribution of these animals may be negligible or, as in the special case of vermicomposting, considerable (see section 2.2). The number of mesophilic organisms in the original substrate is three orders of magnitude higher than the number of thermophilic organisms; however, the activity of primary decomposers induces a temperature rise and in turn, mesophilic microbiota is, along with the remaining easily degradable compounds, degraded by the succeeding thermophiles. The temperature rise continues to be fast and accelerates up to a temperature of about 62 °C during this second phase of composting, known as the *thermophilic phase*.

When a temperature exceeding 55 °C is reached in a compost pile, fungal growth is usually inhibited and the thermophilic bacteria and Actinobacteria are the main degraders during this peak-heating phase. Moreover, oxygen supply affects fungi to a greater extent than bacteria, and even in force-aerated systems, temporary anoxic conditions may occur. Hence, fungi play a negligible role during this phase, except for the composting of lignocellulosic residues. Bacteria of the genus *Bacillus* are often dominant when the temperature ranges from 50 to 65 °C. Moreover, members of the *Thermus*/*Deinococcu*s group have been found in biowaste composts [28] with an optimum growth between 65 and 75 °C. A number of autotrophic bacteria that obtain their energy by the oxidation of sulfur or hydrogen have been isolated from composts [28]. Their temperature optimum is at 70-75 °C and they closely resemble *Hydrogenobacter* strains, which were previously found in geothermal sites. Furthermore, obligate anaerobic bacteria are also common in composts, but up to now, there is still a gap of knowledge concerning this microbial group. It is believed that the longer generation times of archaea, in comparison with bacteria, made the archaea unsuitable for the rapidly changing conditions in the composting process. Nevertheless, in recent works, and using the right tools, a considerable number of cultivable (*Methanosarcina termophila*, *Methanothermobacter* sp., *Methanobacterium formicicum*, among others) and yet uncultivated archaea have been detected in composting processes [29-30].

The final temperature increase may exceed 80 °C and it is mainly due to the effect of abiotic exothermic reactions in which temperature-stable enzymes of Actinobacteria might be involved. Such high temperatures are crucial for compost hygienisation in order to destroy human and plant pathogens, and kill weed seeds and insect larvae [31]. The disadvantage of temperatures exceeding 70 °C is that most mesophiles are killed, and therefore the recovery of the decomposer community is retarded after the temperature peak. The inoculation with matter from the first mesophilic stage might, however, solve this problem.

When the activity of thermophilic organisms ceases due to the exhaustion of substrates, the temperature starts to decrease. This constitutes the beginning of the third stage of composting, called the *cooling phase* or *second mesophilic phase*. It is characterised by the recolonisation of the substrate with mesophilic organisms, either originating from surviving spores, through the spread from protected microniches, or from external inoculation. During this phase there is an increased number of organisms with the ability to degrade cellulose or starch, such as the bacteria *Cellulomonas*, *Clostridium* and *Nocardia*, and fungi of the genera *Aspergillus*, *Fusarium* and *Paecilomyces* [27]. Finally, during the *maturation phase*, the ratio of

fungi to bacteria increases due to the competitive advantage of fungi under conditions of decreasing water potential and poorer substrate availability. Compounds that are not further degradable, such as lignin-humus complexes, are formed and become predominant. Some authors have proposed a fifth composting phase, known as the *curing phase* (or *storage phase*), during which the physico-chemical parameters do not change, but changes in microbial communities still occur [32]. Therefore, the chemical and microbial changes that the substrate undergoes during the different phases of the composting process will largely determine the stability and degree of maturity of the end product and in turn, its safe use as an organic amendment. There exists a wide range of parameters that have been proposed to evaluate compost stability/maturity, as shown in the next section.

2.1.1. Evaluation of compost stability and maturity

The stability and maturity of compost is essential for its successful application, particularly for composts used in high value horticultural crops [33]. Both terms are usually used interchangeably to describe the degree of decomposition and transformation of the organic matter in compost [34], despite the fact that they describe different properties of the composting substrate. Stability is strongly related to the degree to which composts have been decomposed to more stable organic materials [35]. Unstable compost, in contrast, contains a high proportion of biodegradable matter that may sustain a high microbial activity [36]. Typically, compost stability is evaluated by different respirometric measurements and/or by studying the transformations in the chemical characteristics of compost organic matter [6]. On the other hand, compost maturity generally refers to the degree of decomposition of phytotoxic organic substances produced during the active composting stage and to the absence of pathogens and viable weed seeds [37]. This property is often characterised by germination indexes [38] and/or by nitrification [6] and has been related to the degree of compost humification. Wu et al. [37] reported that the low CO_2 evolution is not always an indicator of a non-phytotoxic compost, which suggests that a stable compost may not always be at a level of maturity suitable for its use as a growing medium for certain species of plant.

Several authors highlight that there is no one single method that can be applied successfully for determining compost stability mainly due to the wide range of raw materials used to produce compost, as reviewed by [6]. Therefore, the integration of different parameters seems to be the most reliable option for evaluating the stability/maturity stage of composted materials. For instance, physical parameters including temperature, odor and color constitute a very simple and rapid method for stability evaluation, giving a general idea of the decomposition stage reached; however, little information is achieved as regards to the degree of maturation. In addition, chemical parameters including pH, electrical conductivity, cation exchange capacity (CEC), the ratios of C to N and NH_4^+ to NO_3^- and humification parameters have also been widely used as indicators of stability [39]. Nevertheless, several drawbacks have been found regarding these parameters, thereby preventing their use as accurate indicators. According to Wu et al. [37], pH and electrical conductivity may be used to monitor compost stabilisation, as long as the source waste

composition is relatively consistent and other stability tests are conducted. Moreover, Namkoong et al. [40] established that the C to N ratio could not be considered as a reliable index of compost stability, as it changed irregularly with time. In fact, when wastes rich in nitrogen are used as the source material for composting, like sewage sludges or manures, the C to N ratio can be within the values of a stable compost even though it may still be unstable. Zmora-Nahum et al. [34] reported a C to N ratio lower than the cutoff value of 15 very early during the composting of cattle manure, while important stabilisation processes were still taking place. The increase in CEC with composting time is related to the formation of carboxyl and phenolic functional groups during the humification processes; however, the wide variation in CEC values among the initial substrates prevent to establish a threshold level and to use it as a stability indicator [41].

Biological parameters such as respiration rates (CO_2 evolution rate and/or O_2 uptake rate) and enzyme activities have been proposed to measure compost stability [6-39]. The principle of the respirometric tests is that unstable compost has a strong demand for O_2 and high CO_2 production rates as a consequence of the intensive microbial development due to the presence of easily biodegradable compounds in the raw material. Then, as composting proceeds, the decrease in the amount of degradable organic matter is accompanied by a decline in both O_2 and CO_2 respirometry. The Solvita test, which measures CO_2 evolution and ammonia emissions simultaneously have been found to be a simple and easily used procedure for quantifying soil microbial activity in comparison with both titration and infrared gas analysis [42]; this test has also been used for determining the stability degree in diverse composts [43]. Enzymatic activities have also been found suitable as indicators of the state and evolution of the organic matter during composting, as they are implicated in the biological and biochemical processes through which the initial organic substrates are transformed (Tiquia, 2005). Important enzymes during composting are related to the C-cycle (cellulases, β-glucosidase, β-galactosidase), the N-cycle (protease, urease, amidase) and/or the P-cycle (phosphatase) [44]. These latter authors established that the formation of a stable enzymatic complex, either in moist or air-dried compost samples, could represent a useful index of stabilisation. Additionally, enzymatic activities, especially dehydrogenases, are considered easy, quick and cheap stability measurements [36]; however, the wide range of organic substrates involved in the composting process makes it difficult to establish general threshold values for these parameters. The hydrolysis of fluorescein diacetate (FDA), which is a colourless fluorescein conjugated that is hydrolyzed by both free (exoenzymes) and membrane bound enzymes [45], has been suggested as a valid parameter for measuring the degree of biological stability of the composting material, as it showed a good correlation with other important stability indexes [46]. The analysis of phospholipid fatty acid (PLFA) composition has also been proposed for determining compost stability [47-48]. These authors found a positive correlation between the proportion of PLFA biomarkers for Gram-positive bacteria and the germination index during the maturation of composting of poultry manure and cattle manure, respectively. The strength of this lipid-based approach, as compared to other microbial community assays, is that PLFAs are rapidly synthesized during microbial growth and quickly degraded upon microbial death and they are not found in storage molecules, thereby providing an accurate 'fingerprint' of the current living

community [49]. The potential ability of the microbial community to utilise select carbon sources by determining the community-level physiological profiles (CLPPs) with the Biolog® Ecoplate has also been considered for compost stability testing [50]. The principle is that compost extracts are inoculated onto microtiter plates that contain 31 different C substrates [51-52]. Ultimately, molecular techniques are becoming increasingly useful in composting research. For example, as in reference [32] the authors used three different cultivation-independent techniques based on 16S rRNA gene sequences, i.e. PCR-denaturing gradient gel electrophoresis (DGGE), clone libraries, and an oligonucleotide microarray (COMPOCHIP), in order to evaluate the dynamics of microbial communities during the compost-curing phase. Specific compost-targeted microarrays are suitable to investigate bacterial [53-54] and fungal community patterns [55], including plant growth promoting organisms and plant and human pathogens.

2.1.2. Influence of compost amendments on the soil microbiota

Composted materials have gained a wide acceptance as organic amendments in sustainable agriculture, as they have been shown to provide numerous benefits whereby they increase soil organic matter levels, improve soil physical properties (increased porosity and aggregate stability and reduced bulk density) and modify soil microbial communities [56]. Substantial evidence indicates that the use of compost amendments typically promotes an increase in soil microbial biomass and activity, as reviewed in [56-57]. This enhancing effect may be attributed to the input of microbial biomass as part of the amendments [58]; however, the quantity of organic matter applied with the compost is very small in comparison with the total organic matter present in the soil and, in turn it is believed that the major cause is the activation of the indigenous soil microbiota by the supply of C-rich organic compounds contained in the composting materials [58]. Such effects on microbial communities were reported to be dependent on the feedstocks used in the process [59]. However, other authors did not find significant differences between soil plots that had been amended with four different compost types (green manure compost, organic waste compost, manure compost and sewage sludge compost) over 15 years [60]. Similarly, Ros et al. [61] observed that different types of composts had a similar effect on the fungal community and microbial biomass in soil in a long-term field experiment. This fact suggests that the soil itself influences the community diversity more strongly than the compost treatments. Such discrepancies between the previous findings may be due to differences in soil properties, land-use and compost type (i.e., different starting material and process parameters), frequency and dose of application, length of the experiment and parameters chosen for analysis, among others.

Furthermore, C addition to soil seems to select for specific microbial groups that feed primarily on organic compounds. Therefore, it can be expected that the addition of organic amendments not only increases the size of the microbial community but also changes its composition, as has already been observed in previous experiments [61-63]. As shown by [62] higher amounts of composts resulted in a more pronounced and faster effect in the structure of microbial communities, as revealed by PLFA analysis, indicating that the compost application rate is a major factor regarding the impact of compost amendments on

soil microbiota. Carrera et al. [63] found that soil PLFA profiles were influenced by both the treatment with poultry manure compost and the sampling date. Ros et al. [61] observed that the date of sampling contributed more to modifications in fungal community structure, assessed by PCR-DGGE analysis, than treatment effects. However, in contrast to fungi, the bacterial community structure, both on the universal and the Streptomycetes group-specific level, were influenced by compost amendments, especially the combined compost and mineral fertilisers treatments. This seems reasonable, as bacteria have a much shorter turnover time than fungi and can react faster to the environmental changes in soil. Bacterial growth is often limited by the lack of readily available C substrates, even in soils with a high C/N ratio, and are the first group of microorganisms to assimilate most of the readily available organic substrates after they are added to the soil [64]. CLPP profiles have also been used to evaluate the impact of compost amendments on the potential functional diversity of soil microbiota, as they are considered suitable indicators for detecting soil management changes [65]. As shown by [66] different types of compost (household solid waste compost and manure compost) affected differently the substrate utilization patterns of the soil microbial community relative to unamended control soils. Contrarily, no significant changes in CLPP profiles were found by [59]. Other authors also reported that the sampling date had more weight on CLPP results than compost treatments [63,67]. All these studies together highlight the importance of a multi-parameter approach for determining the influence of compost amendments on the soil microbiota, which is of utmost importance to understand the disease suppressive activity of compost and the mechanisms involved in such suppression [68].

Since the 1980s a large number of experiments have been addressed describing a wide array of pathosystems and composts from a broad variety of raw materials. Interestingly, Noble and Coventry [69] evaluated the suppression of soilborne plant pathogens by compost in both laboratory and field scale experiments. In general, they found that the effects in the field were smaller and more variable than those observed at lab-scale. Termorshuizen et al. [70] compared the effectiveness of 18 different composts on seven pathosystems and interestingly they found significant disease suppression in 54% of the cases, whereas only 3% of the cases showed significant disease enhancement. They highlighted that the different composts did not affect the pathogens in the same way and that no single compost was found to be effective against all the pathogens. Furthermore, in a study carried out with 100 composts produced from various substrates under various process conditions, it was found that those composts that had undergone some anaerobic phase showed the best results in terms of suppressing plant disease [71].

However, up to now, there is still a general lack of understanding concerning the suppressivity of compost [68], as it depends on a complex range of abiotic and biotic factors. Such factors are reviewed by [72]. Briefly, the main mechanisms by which compost amendments exert their suppressivity effect against soil-borne plant pathogens include hyperparasitism; antibiosis; competition for nutrients (carbon and/or iron); and induced systemic resistance in the host plant [73]. The first three affect the pathogen directly and reduce its survival, whilst the latter one acts indirectly via the plant and affect the disease cycle.

Manure type	Bulking agent	Composting process	Vermicomposting process	Duration of experiment	Investigated parameters	Remarks	References
Pig manure	-	-	Vertical continuous-feeding system (*E. fetida*)	36 weeks	Microbial biomass C, basal respiration, metabolic quotient (qCO_2), CLPPs, enzymatic activities	Increase in functional microbial diversity with earthworm presence associated with a decrease in qCO_2, indicative of high metabolic efficiency Increase in cellulase activity with earthworm presence accompanied by greater C losses throughout the process	[16-17]
Pig manure	-	-	Vertical continuous-feeding system (*E. fetida*)	36 weeks	PLFAs, basal respiration	Decrease in bacterial and fungal biomass, assessed by PLFA biomarkers, associated with an increase in microbial activity in the presence of earthworms	[18]
Sheep manure	Olive waste	Turned windrow	Vermicomposting bed (*E. fetida*)	36 weeks for both composting and vermicomposting	Enzymatic activities, PCR-DGGE, real time-PCR	Higher bacterial abundance and microbial diversity was found in vermicompost relative to the initial substrate than in compost	[19]

Manure type	Bulking agent	Composting process	Vermicomposting process	Duration of experiment	Investigated parameters	Remarks	References
Cow manure	Straw	Forced-ventilation	Vermicomposting bed (*E. andrei*)	Composting: 15 d (thermophilic phase) Vermicomposting: 40 d (active phase)	Microbial biomass C, basal respiration, enzymatic activities, ergosterol	Lower levels of microbial biomass and dehydrogenase activity indicative of a higher degree of stabilisation were found in the combined treatment (composting + vermicomposting)	[20]
Cow manure	Agricultural plant waste	In-vessel system	Semicontinuously vermicomposting system (*E. fetida*)	Composting: 3-4 weeks (thermophilic phase) Vermicomposting: not specified	Substrate-induced respiration, PCR-DGGE, CompoChip	Vermicompost tea composition was influenced by production and storage conditions Carbon substrate addition during the tea production process was identified to be of major importance for obtaining a vermicompost tea with a rich and diverse microbial community	[21]
Cow manure+poultry manure	Biochar	Turned windrow	-	12 weeks	PLFAs	Changes in microbial community composition depended on the origin of manure composted with biochar	[22]
Cow manure	Sawdust	Turned windrow	-	30 d	PCR-DGGE, clone libraries	Ammonia oxidizing archaea were found to be dominant during the composting process probably because they could adapt to increasing temperature and/or nutrient loss	[23]
Cow manure+Horse manure+Pig Manure	-	-	In-container system (*E. andrei*, *E. fetida* and *P. excavatus*)	30 d (active phase)	Basal respiration, microbial growth rates, PLFAs	Species-specific effects of earthworms on microbial community structure and bacterial growth rate	[24]

Table 1. Research studies focused on the stabilization of animal manures through the aerobic processes of composting and vermicomposting.

Two mechanisms of biological control, based on antibiosis, hyperparasitism, competition and induced protection, have been reported for compost amendments. On the one hand, diseases caused by plant pathogens such as *Phytophtora* spp. and *Phytium* spp. have been eradicated through a mechanism known as "general suppression", in which the suppressive activity is attributed to a diverse microbial community in the compost rather than to a population of a single defined species augmented to infested soil. Whilst, for *Rhizoctonia solani*, few microorganisms present in compost are able to eradicate this pathogen and, in turn this type of suppression is referred as "specific suppression". Overall, all of the above reinforces that the activity of microbial communities in composts is a major factor affecting the suppression of soilborne plant pathogens. Indeed, the disease suppressive effect is usually lost following compost sterilization or pasteurization [68]. Better understanding of the microbial behaviour and structure of the antagonistic populations in the compost will provide tools to reduce its variability. In line with this, Danon et al. [32] detected, using PCR-based molecular methods, distinctive community shifts at different stages of prolonged compost curing being Proteobacteria the most abundant phylum in all the stages, whereas Bacteroidetes and Gammproteobacteria were ubiquitous. Actinobacteria were dominant during the mid-curing stage, and no bacterial pathogens were detected even after a year of curing.

The addition of antagonistic microorganisms to compost is also a promising technique to improve its suppressivity. Already in 1983, Nelson et al. [74] increased the suppressive potential of compost by adding selected *Trichoderma* strains. They found that not only the addition of the antagonist is important, but also the strategy of inoculation of the antagonist in order to efficiently colonize the substrate, as the autochthonous microbial community can inhibit it. Ultimately, predicting disease suppression on the basis of pure compost is expected to be highly advantageous for compost producers. This would enable them to optimise the composting process based on the specific disease jeopardising the target crop. For this purpose, a further step could be the development of quality control criteria based mainly on bioassays designed for a specific pathogen or disease.

2.2. The vermicomposting process

Vermicomposting is defined as a bio-oxidative process in which detritivore earthworms interact intensively with microorganisms and other fauna within the decomposer community, accelerating the stabilization of organic matter and greatly modifying its physical and biochemical properties [75]. Epigeic earthworms with their natural ability to colonize organic wastes; high rates of consumption, digestion and assimilation of organic matter; tolerance to a wide range of environmental factors; short life cycles, high reproductive rates, and endurance and resistance to handling show good potential for vermicomposting [76]. The earthworm species *Eisenia andrei*, *Eisenia fetida*, *Perionyx excavatus* and *Eudrilus eugeniae* display all these characteristics and they have been extensively used in vermicomposting facilities.

Vermicomposting systems sustain a complex food web that results in the recycling of organic matter and release of nutrients [77]. Biotic interactions between decomposers (i.e., bacteria and fungi) and earthworms include competition, mutualism, predation and

facilitation, and the rapid changes that occur in both functional diversity and in substrate quality are the main properties of these systems [77]. The biochemical decomposition of organic matter is primarily accomplished by the microbes, but earthworms are crucial drivers of the process as they may affect microbial decomposer activity by grazing directly on microorganisms [78-79], and by increasing the surface area available for microbial attack after the comminution of organic matter [8]. Furthermore, earthworms are known to excrete large amounts of casts, which are difficult to separate from the ingested substrate [8]. The contact between worm-worked and unworked material may thus affect the decomposition rates [80], due to the presence of microbial communities in earthworm casts different from those contained in the material prior to ingestion [81]. In addition, the nutrient content of the egested materials differ from that in the ingested material [82], which may enable better exploitation of resources, because of the presence of a pool of readily assimilable compounds in the earthworm casts. Therefore, the decaying organic matter in vermicomposting systems is a spatially and temporally heterogeneous matrix of organic resources with contrasting qualities that result from the different rates of degradation that occur during decomposition [83].

The impact of earthworms on the decomposition of organic waste during the vermicomposting process is initially due to *gut- associated processes* (GAPs), i.e., via the effects of ingestion, digestion and assimilation of the organic matter and microorganisms in the gut, and then casting [79] (Figure 1). Specific microbial groups respond differently to the gut environment [84] and selective effects on the presence and abundance of microorganisms during the passage of organic material through the gut of these earthworm species have been observed. For instance, some bacteria are activated during the passage through the gut, whereas others remain unaffected and others are digested in the intestinal tract and thus decrease in number [78]. These findings are in accordance with a recent work that provides strong evidence for a bottleneck effect caused by worm digestion (*E. andrei*) on microbial populations of the original material consumed [79]. This points to the earthworm gut as a major shaper of microbial communities, acting as a selective filter for microorganisms contained in the substrate, thereby favouring the existence of a microbial community specialised in metabolising compounds produced or released by the earthworms, in the egested materials. Such selective effects on microbial communities as a result of gut transit may alter the decomposition pathways during vermicomposting, probably by modifying the composition of the microbial communities involved in decomposition, as microbes from the gut are then released in faecal material where they continue to decompose egested organic matter. Indeed, as mentioned before, earthworm casts contain different microbial populations to those in the parent material, and as such it is expected that the inoculum of those communities in fresh organic matter promotes modifications similar to those found when earthworms are present, altering microbial community levels of activity and modifying the functional diversity of microbial populations in vermicomposting systems [80]. Previous studies have already shown that a higher microbial diversity exists in vermicompost relative to the initial substrate [19,85]. Upon completion of GAPs, the resultant earthworm casts undergo *cast- associated processes* (CAPs), which are more closely related to ageing processes, the presence of unworked

material and to physical modification of the egested material (weeks to months; Figure 1). During these processes the effects of earthworms are mainly indirect and derived from the GAPs [17].

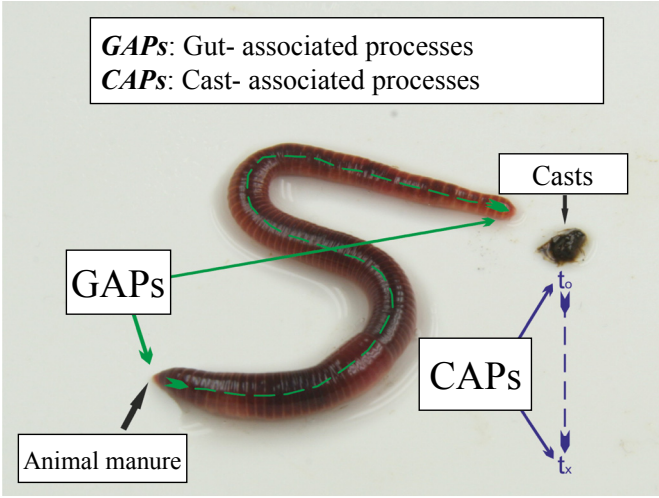

Figure 1. Earthworms affect the decomposition of the animal manure during vermicomposting through ingestion, digestion and assimilation in the gut and then casting (*gut- associated processes*); and *cast-associated processes,* which are more closely related with ageing processes.

Overall, vermicomposting includes two different phases regarding earthworm activity: (i) an *active phase* during which earthworms process the organic substrate, thereby modifying its physical state and microbial composition [86], and (ii) a *maturation phase* marked by the displacement of the earthworms towards fresher layers of undigested substrate, during which the microorganisms take over the decomposition of the earthworm-processed substrate [17-18]. The length of the maturation phase is not fixed, and depends on the efficiency with which the active phase of the process takes place, which in turn is determined by the species and density of earthworms, and the rate at which the residue is applied [8]. During this aging, vermicompost is expected to reach an optimum in terms of its nutrient content and pathogenic load, thereby promoting plant growth and suppressing plant diseases [8]. However, unlike composting, vermicomposting is a mesophilic process (<35 °C), and as such substrates do not undergo thermal stabilisation that eliminates pathogens. Nevertheless, it has been shown that vermicomposting may reduce the levels of different pathogens such as *Escherichia coli*, *Salmonella enteritidis*, total and faecal coliforms, helminth ova and human viruses in different types of waste [75]. In a recent work [78], a reduction by 98% in the number of faecal coliforms of pig slurry was detected after two weeks of processing in the presence of *E. fetida*, which indicates that the own earthworm digestive abilities play a key role in the reduction of the pathogenic load of the parent material. In a previous study, these authors found that the decrease in pathogenic bacteria (i.e. total coliforms) as a result of gut transit differed among four vermicomposting earthworm species (*Eisenia fetida*, *Eisenia andrei*, *Lumbricus rubellus* and *Eudrilus eugeniae*)

[87]. This was consistent with the fact that specific microbial groups respond differently to the gut environment, depending on the earthworm species. The pathogen considered is another important factor controlling the reduction in the pathogenic load during the process. Parthasarathi et al. [88] observed that earthworms did not reduce the numbers of *Klebsiella pneumoniae* and *Morganella morganii*, whereas other pathogens such as *Enterobacter aerogenes* and *Enterobacter cloacae* were completely eliminated. In a recent study [89] a decrease in the abundance of faecal enterococci, faecal coliforms and *Escherichia coli* was recorded across the layers of an industrial-scale vermireactor fed with cow manure; whereas no changes were reported for total coliforms, *Enterobacteria* or *Clostridium*. These findings are of great importance for the optimisation of the vermicomposting process because despite the pioneering studies of Riggle [90] and Eastman et al. [91], little is known about this process in industrial-scale systems, that is, vermicomposting systems designed to deal with large amounts of wastes. This selective effect on pathogens indicates that earthworms not only modify the abundance of such pathogenic bacteria but also alter their specific composition. According to [89], the unaffected pathogens could benefit as a result of the overall decrease in bacterial and fungal biomass across the layers of the reactor, thereby diminishing possible competition for resources.

Collectively, the aforementioned studies highlight the importance of monitoring the changes in microbial communities during vermicomposting, because if the earthworms were to stimulate or depress microbiota or modify the structure and activity of microbial communities, they would have different effects on the decomposition rate of organic matter, thereby influencing the vermicompost properties, which is critical to guarantee a safe use of this end-product as an organic amendment and thus benefit both agriculture and the environment.

2.2.1. Effects of earthworms on microbial communities during vermicomposting: a case study.

Animal manures are microbe-rich environments in which bacteria constitute the largest fraction (around 70% of the total microbial biomass as assessed by PLFA analysis), with fungi mainly present as spores [24]. Thus, earthworm activity is expected to have a greater effect on bacteria than on fungi in these organic substrates in the short-term [79]. In line with this, a significant increase in the fungal biomass of pig manure, measured as ergosterol content, was detected in a short-term experiment (72 h) with the earthworm species *E. fetida*, and the effect depended on the density of earthworms [82]. A higher fungal biomass was found at intermediate and high densities of earthworms (50 and 100 earthworms per mesocosm, respectively), which suggests that there may be a threshold density of earthworms at which fungal growth is triggered. This priming effect on fungal populations was also observed in previous short-term experiments in the presence of the epigeic earthworms *Eudrilus eugeniae* and *Lumbricus rubellus* fed with pig and horse manure, respectively [16,86]. These contrasting short-term effects on bacterial and fungal populations are thus expected to have important implications on decomposition pathways during vermicomposting because important differences exist between both microbial decomposers

related to resources requirements and exploitation [92]. This is based on the fact that fungi can immobilise great quantities of nutrients in their hyphal networks, whereas bacteria are more competitive in the use of readily decomposable compounds and have a more exploitative nutrient use strategy by rapidly using newly produced labile substrates [92].

The above-mentioned studies dealing with the effects of epigeic earthworms on microorganisms have focused on the changes before and after the active phase rather than those that occur throughout the whole vermicomposting process. Hence, in a current research study, and using a continuous-feeding vermicomposting system, we evaluated the different phases of interaction between earthworms and microorganisms and additionally, we monitored the stabilisation of the fresh manure during a period of 250 days. At the end of the experiment we obtained a profile of layers of increasing age, resembling a time profile, with a gradient of fresh-to-processed manure from the top to the bottom. This type of system allowed us to evaluate whether and when the samples reached an optimum value to be classified as vermicompost, as regards to the stabilisation of organic matter and the levels of microbial biomass and activity. Briefly, we used polyethylene reactors (n=5) with a volume of 1 m^3, which were initially comprised of a 10 cm layer of mature vermicompost (a stabilised non- toxic substrate that serves as a bed for earthworms), on which earthworms (*Eisenia fetida*) were placed and a layer containing 5 kg of fresh rabbit manure, which was placed over a plastic mesh (5 mm pore size) to avoid sampling the earthworm bedding. New layers with the same amount of fresh manure were added to the vermireactor every fifty days according to the feeding activity of the earthworm population. This procedure allowed the addition of each layer to be dated within the reactors. The reactors were divided into 4 quadrants and two samples were taken at random from each quadrant with a cylindrical corer (8 cm diameter). Each corer sample was divided into five layers of increasing age and the samples from the same layer and each reactor were gently mixed to analyse the changes in microbial communities. The structure of the microbial communities was assessed by PLFA analysis; some specific PLFAs were used as biomarkers to determine the presence and abundance of specific microbial groups [93]. The sum of PLFAs characteristic of Gram-positive (iso/anteiso branched-chain PLFAs), and Gram-negative bacteria (monounsaturated and cyclopropyl PLFAs) were chosen to represent bacterial PLFAs, and the PLFA 18:2ω6c was used as a fungal biomarker. Total microbial activity was also assessed by measuring the rate of evolution of CO_2, as modified for [17] for samples with a high organic matter content. Dissolved organic carbon was determined colorimetrically in microplates after moist digestion ($K_2Cr_2O_7$ and H_2SO_4) of aliquots of 0.5M K_2SO_4 extracts.

The earthworm species *E. fetida* had a strong effect in the decomposition of organic matter during vermicomposting, greatly modifying the structure of the microbial decomposer communities, as revealed by the phospholipid fatty acid analysis. The principal component analysis of the 27 identified PLFAs (10:0, 12:0, 13:0, 14:0, i14:0, 15:0, i15:0, a15:0, 16:0, i16:0, 17:0, a17:0, 18:0, 14:1ω5c, 15:1ω5c, 16:1ω7c, 17:1ω7c, 18:1ω7c, 18:1ω9c, 18:1ω9t, 18:2ω6c, 18:2ω6t, 18:3ω6c, 18:3ω3c, cy17:0, cy19:0, 20:0) clearly differentiated between the samples in function of the age of layers, explaining 51% of the variance in the data (Figure 2). Thus, the

upper layers (50 and 100 days old) along with the fresh manure were clearly distinguished from the intermediate (150 days old) and lower layers (200 and 250 days old) (Figure 2).

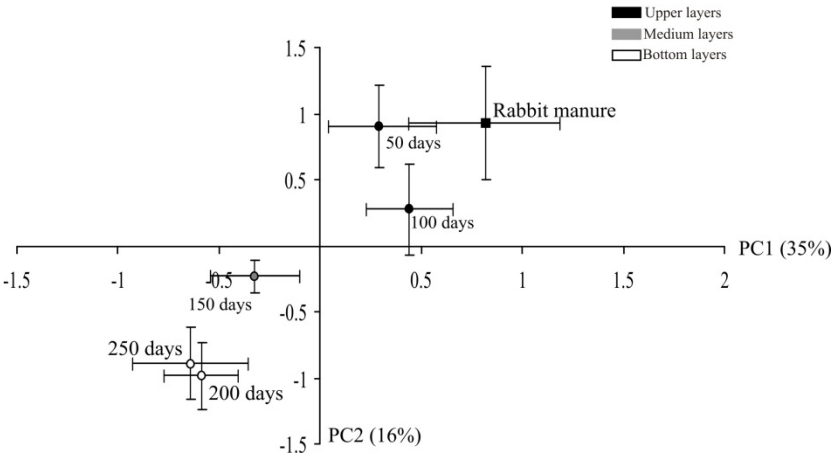

Figure 2. Changes in the microbial community structure throughout the process of vermicomposting assessed by the principal component analysis of the twenty-seven PLFAs identified in the layers of reactors fed with rabbit manure. The different layers represent different stages of the process. Values are means ± SE.

Such changes in the structure of microbial communities were accompanied by a decrease in the abundance of both Gram-positive and –negative bacterial populations with the depth of layers (Figure 3A,B), i.e. from upper to medium and lower layers; and the abundance of these groups were in the fresh rabbit manure 346 ± 49.0 and 336 ± 63 μg g^{-1} dw for Gram-positive and Gram-negative bacteria, respectively (Figure 3A,B). A similar trend was observed for fungal populations (Figure 3C), reaching an average value of 1.3 ± 0.1 at the end of the process (Figure 3C). These results are in accordance with previous studies based on PLFA profiles, with marked changes in the structure of microbial communities due to decreases in both bacterial and fungal populations throughout the process of vermicomposting [18, 89]. Recently, Fernández-Gómez et al. [94] observed that the structure of fungal communities, assessed by DGGE profiles differed at the stage of maximum earthworm biomass the most, suggesting the existence of a strong gut passage effect on the microbial communities through a continuous-feeding vermicomposting system in the presence of *E. fetida*.

Decreases in microbial activity were also detected with depth of layer (Figure 4A) and, after a maturation period for 250 d, basal respiration values dropped below 100 mg CO_2 kg^{-1} OM h^{-1} (Figure 4A), as previously shown by [18]. Accordingly, a reduction in the dissolved organic carbon content was detected from upper to lower layers (Figure 4B), reaching a value close to 7000 μg g^{-1} dw after 250 d of vermicomposting. In contrast, other authors [17] reported levels of DOC much more lower in a long-term experiment (252 days) with the epigeic earthworm *E. fetida*, with values below 1500 μg g^{-1} dw in the presence of

earthworms. Such differences could be due to the composition of the parent material (pig slurry *versus* rabbit manure) and/or to the experimental setup conditions. Unlike compost -a limit value of 4000 mg kg^{-1} is suggested for a stable compost according to [34]- there is still no threshold level of DOC for which vermicompost is to be considered stable.

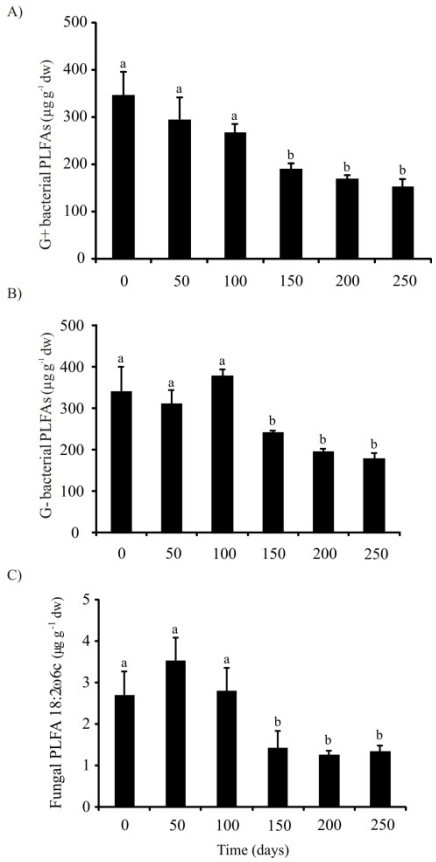

Figure 3. Changes in (a) Gram-positive bacterial, (b) Gram-negative bacterial and (c) fungal PLFAs in the layers of reactors fed with rabbit manure throughout the process of vermicomposting. The different layers represent different stages of the process. Different letters indicate significant differences between the layers based on post hoc test (Tukey HSD). Values are means ± SE.

Overall, in the present study a higher degree of stabilisation was reached in the rabbit manure after a period of between 200 and 250 days, as indicated by the lower values of microbial biomass and activity that are indicative of stabilized materials. These results underscore the potential of epigeic earthworms in the stabilisation of this type of organic substrates, which is of great importance for the application of animal manures as organic amendments into agricultural soils because, as already mentioned, it is widely recognised that the overproduction of this type of substrate has led to inappropriate disposal practices,

which may result in severe risks to the environment [6]. Furthermore, these findings constitute a powerful tool for the development of strategies leading to a more efficient process for the disposal and/or management of animal manures, thereby highlighting the continuous-feeding vermicomposting system as an environmentally sound management option for recycling such organic wastes, as previously reported by [94] for treating tomato-fruit waste from greenhouses. Ultimately, it should be borne in mind that the functioning of this type of reactors can lead to the gradual accumulation of layers and compaction of the substrate, thus minimizing earthworm- induced aeration, which can promote pathogen survival [89].

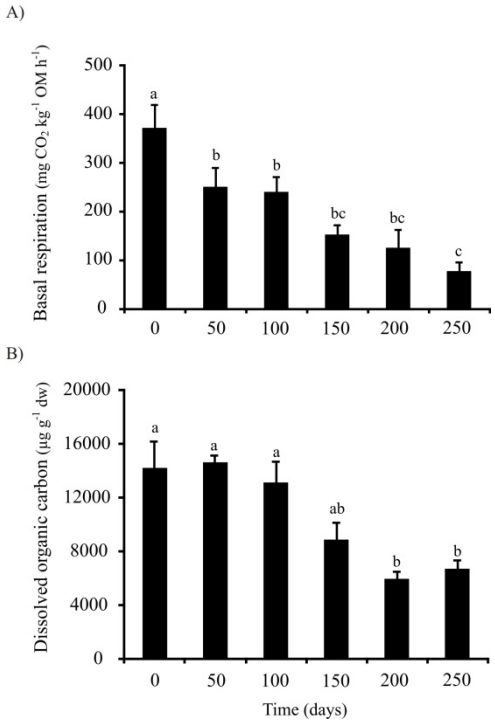

Figure 4. Changes in (a) microbial activity assessed by basal respiration, and (b) dissolved organic carbon content in the layers of reactors fed with rabbit manure throughout the process of vermicomposting. The different layers represent different stages of the process. Different letters indicate significant differences between the layers based on post hoc test (Tukey HSD). Values are means ± SE.

2.2.2. Influence of vermicompost amendments on the soil microbiota

As occurred with compost amendments, vermicompost has also been found to provide manifold benefits when used as a total or partial substitute for mineral fertiliser in peat-based artificial greenhouse potting media and as a soil amendment in field studies [95]. Among the advantages of vermicompost as a soil amendment is its potential to maintain soil

organic matter, foster nutrient availability, suppress plant diseases and increase soil microbial abundance and activity. However, although several studies have tried to disentangle the complex interactions between vermicompost application and soil microbial properties, most of them are frequently not comparable to each other due to differences in the experimental design, the land-use and vermicompost type (i.e., different starting material and earthworm species), the dose of application as well as the duration of experiments, among others. Despite these limitations, some recent findings have been made, thereby contributing to better understand whether and to what extent vermicompost amendments affect soil microbial biomass, activity and community structure. For instance, Arancon et al. [96] observed that a single application of vermicompost to a strawberry crop resulted in a significantly higher increase in soil microbial biomass than the application of an inorganic fertiliser, regardless of the dose used. Increases in the microbial activity and in the activity of the soil enzymes involved in the release of the main plant macronutrients with vermicompost amendments, have also been signalled in several studies [96-98]. Such increase could be due to the fact that soil microorganisms degrade organic matter through the production of a variety of extracellular enzymes and, in turn an input of organic matter is expected to be accompanied by a higher enzymatic activity. Moreover, the added material may contain intra- and extracellular enzymes and may also stimulate microbial activity in the soil [99]. Additionally, vermicompost has been found to promote the establishment of a specific microbial community in the rhizosphere different from that of plants supplemented with mineral fertilisers or other types of organic fertilisers such as manure [100]. Inorganic fertilisation only supplies N, P and K, whereas organic fertilisers also supply different amounts of C and macro- and micronutrients, which can select for microbial communities with different nutritional requirements [95]. Moreover, microbial communities in vermicompost are metabolically more diverse than those in manure [17], and may be incorporated, at least in the short-term, to soils [101]. Interestingly, Aira et al. [100] observed that the effect of the addition of vermicompost occurred despite the low dose used (25% of total fertilisation), and despite the short duration of the experiment (four months). Jack et al. [102] also examined how different organic transplant media amendments, including vermicompost, thermogenic compost and industry standard amendments affected the rhizosphere bacterial communities of organically produced tomato plants. They found differences in the bacterial community structure between the different amendments and these differences persisted for at least one month after seedlings were transplanted to the field. Since both compost and vermicompost were made from the same parent material, such differences could be due to the way in which the organic matter was processed prior to the amendment [102]. Previous comparisons between vermicompost and compost with respect to microbial communities [103-105] are difficult to interpret because different feedstocks were used for each process. Compost feedstocks are known to alter the material's effects on the structure of the microbial communities [86], so it is essential to use composts made from the same feedstock in order to draw valid comparisons between the two biological processes. Furthermore, it may be expected that different hybrids or plant genotypes will respond differently to vermicompost, considering that plant genotype determines important differences in nutrient uptake capacity, nutrient use efficiency and

resource allocation within the plant. Different genotypes may therefore enhance root growth or modify root exudation patterns in order to increase nutrient uptake [100], and all of these strategies will determine the establishment of different interactions with the microbial communities at the rhizosphere level. Indeed, after the application of vermicompost to sweet corn crops, these authors found important differences in the rhizosphere microbial community of two genotypes from cultivars of maize, with the sugary endosperm mutation (*su1*) and with the shrunken endosperm mutation (*sh2*), which differ in their C storage patterns.

Furthermore, recent studies have demonstrated the presence of various bacteria, which are useful for different biotechnological purposes, in diverse vermicomposts [106-107], reinforcing that the biological component (i.e., the microbial community composition) of a vermicompost largely determines its usefulness in agriculture and other applications, such as soil restoration and bioremediation. For instance, Fernández-Gómez et al. [106] detected the presence of *Sphingobacterium*, *Streptomyces*, Alpha-Proteobacteria, Delta-Proteobacteria and Firmicutes in diverse vermicomposts, irrespective of the parent material used for the process, by applying DGGE and COMPOCHIP, thereby demonstrating the usefulness of both techniques to assess the potential of vermicomposts as bioactive organic materials. Indeed, disease suppressiveness is obviously linked to the microbiota added with the vermicompost, along with the biological and physicochemical characteristics of the native soil microbial community. However, despite the large body of scientific evidence showing the positive effects of vermicompost regarding the suppression of soil-borne plant fungal diseases (reviewed in [75,108]), it is still necessary to obtain a deeper understanding of the mechanisms involved and the main factors influencing such suppressing effects. According to the mechanisms proposed for compost [68-69], disease suppression by vermicompost may be attributed to either direct effects or to the induction of systemic resistance in the plant. Direct suppression of the pathogen by the vermicompost-associated microflora and/or microfauna may be general or specific, depending on the existence of a single suppressive agent or the joint action of several agents, and the proposed mechanisms are competition, antibiosis and parasitism. Some of the indirect effects of vermicompost have been related to changes in the microbiological properties of the soil or the potting media. Processing by earthworms during vermicomposting has a strong effect on the microbial community structure and activity of the initial waste [8]. Vermicompost therefore has a rather different microbial community structure than the parent waste, with lower biomass but enhanced metabolic diversity [18]. Application of such a microbiologically active organic substrate may thus have important effects on the microbial and biochemical properties of soil or greenhouse potting media thereby influencing plant growth. Moreover, vermicompost may affect directly the plant growth via the supply of nutrients, as it constitutes a slow-release fertiliser that supplies the plant with a gradual and constant source of nutrients, and/or through the supply of plant growth regulating substances [95].

3. Anaerobic digestion

The process of anaerobic digestion (AD) has been extensively studied in natural and engineered ecosystems for more than a century. In natural habits, the anaerobic degradation

of organic matter takes place in sediments, waterlogged soils and animal intestinal tracts, in which the oxygen access is restricted; whilst in engineered environments it refers to the biotechnological process by which organic matter (i.e., organic waste, wastewater and/or a renewable resource) is degraded in the absence of oxygen for the commercial production of biogas that can be used as an eco-friendly energy source [109], thereby representing an important asset in times of decreasing fossil fuel supplies. According to [4], the anaerobic digestion from swine, bovine and poultry slurries resulted in the production of biogas at average rates of 0.30, 0.25 and 0.48 L/g volatile solids, respectively. Another valuable co-product derived from this process is the anaerobic digested slurry, which can be applied as an organic amendment into soil either in agricultural and non-agricultural lands [110](section 3.1). It is for this reason along with the production of biogas and the reduction in greenhouse gas emissions [10] that anaerobic digestion is becoming increasingly popular as a methodological alternative for manure recycling, which in turn has increased the number of farm-scale anaerobic bioreactors up to 4200 in central and northern Europe [111].

Bacteria represent over 80% of the total diversity in anaerobic digesters [112], and they are mainly composed by the phyla Firmicutes, Proteobacteria and Bacteroidetes; whereas most of the archaeal representatives belong to the phylum Euryarchaeota, which includes all the known methanogens. Anaerobic eukaryotes - particularly, fungi and protozoa- have received less attention probably because they are slower growers than bacteria and, as such, their abundance is lower in anaerobic reactors. As occurs with composting, anaerobic digestion may be described as a four-phase microbiologically driven process. Briefly, the first and rate limiting step of the anaerobic food chain is the *depolymerization* and *hydrolysis* of complex biopolymers, such as polysaccharides, lipids, proteins and nucleic acids into their corresponding structural units (sugars, fatty acids, amino acids, purines and pyrimidines) through the joint action of a complex community of fibrolytic bacteria and fungi, which produce extracellular hydrolitic enzymes (i.e., cellulases, xylanases, proteases, lipases) responsible for the disassembling of such polymers. Since polysaccharides, mainly cellulose, are the most abundant structural and storage compounds of biomass, their hydrolysis is considered as the most determinant enzymatic process regarding the efficiency of anaerobic reactors. The rate and efficiency of cellulose hydrolysis depends greatly on the particular microbial species composition involved [113], and under anaerobic conditions it proceeds slowly due to the heterogeneity of forms in which cellulose is present in nature and to the complexity of the hemicelluloses and lignin matrices in which it is embedded [113]. Similarly, protein hydrolysis to peptides and amino acids takes place slowly, whilst lipid hydrolysis into glycerol and long-chain fatty acids occurs rapidly compared to their subsequent fermentation or β-oxidation. As mentioned above, bacterial populations are more abundant and diverse and, hence, they are responsible for the majority of hydrolytic reactions, being *Clostridium*, *Acetivibrio*, *Bacteroides*, *Selenomonas* or *Ruminococcus* common examples of hydrolytic bacteria found in anaerobic reactors [112,114].

The monomeric compounds released after the hydrolysis of biopolymers can be taken up by microbial cells, in which they are either fermented or anaerobically oxidised into alcohols, short-chain fatty acids, CO_2 and molecular hydrogen (H_2). This step is known as *fermentation*

(*acidogenesis*) and usually occurs through the production of an energy-rich intermediate that is used to synthesise ATP, rendering a fermentation product that is excreted out of the cell. Since these products are typically acidic substances, the fermentative reactions are accompanied by a decrease in the extracellular pH. This fact along with the increase in short-chain fatty acids represents the most common reasons for reactor failure. Thus, for maintaining the pH balance of the system, it is of great importance to bear in mind the equilibrium between fermentative-acidogenic bacteria and acid scavenging microbes. Typically, the bacteria from the same group that hydrolyze biopolymers take up and ferment the resulting monomers. For instance, *Clostridium* sp. and enteric bacteria are common sugar fermenters in anaerobic reactors. *Streptococcus* sp. and *Lactobacillus* sp. also ferment sugars, producing lactate or lactate and ethanol, plus CO_2 and molecular hydrogen [114]. Fermentation of amino acids and purines and pyrimidines in anaerobic environments is mainly carried out by species of *Clostridium* [115].

Then, during *acetogenesis*, the fermentation products are mainly oxidised to acetate, formate, CO_2 and H_2 by acetogenic bacteria, most of them belonging to the low G+C branch of *Firmicutes* [116]. Certain acetogenic reactions are thermodynamically unfavourable under standard conditions, which make necessary a syntrophic relationship between the acetogen and a H_2-consuming methanogens in order to degrade the substrate and, in turn to obtain a net energy gain [117]. Finally, the last and most sensitive step during the anaerobic digestion of organic matter is the *methanogenesis*, i.e. the formation of methane from acetate, H_2/CO_2 and methyl compounds by the action of methanogenic organisms belonging to the phylum Euryarchaeota [118]. The orders Methanobacteriales, Methanococcales, Methanomicrobiales and Methanosarcinales include known methanogens commonly found in aerobic reactors. Members of the first three orders use CO_2 and H_2 as an electron acceptor and donor, respectively. Some species from these orders can also use formate and/or secondary alcohols (i.e., isopropanol or ethanol), but they cannot use acetate or C1 compounds such as methanol and methylamines (with the exception of the genus *Methanosphaera* from the order Methanobacteriales). However, Methanosarcinales are more diverse metabolically, and they can use acetate, hydrogen, formate, secondary alcohols and methyl compounds as energy sources. It is believed that the predominance of hydrogenotrophic or acetotrophic methanogens depends on the levels of their substrates and their tolerance to diverse inhibitors, including ammonia, hydrogen sulphide, or volatile fatty acids [119]. The aforementioned steps involved in the anaerobic digestion are explained in more detail by [109].

3.1. Influence of anaerobic digested slurry on the soil microbiota

The changes that occur in the parent waste during the process of anaerobic digestion largely depend on the dynamics of the abovementioned microbial groups, ultimately influencing the quality of the final products, i.e. biogas and anaerobic digested slurry. As mentioned before, anaerobic slurries are rich in partially stable organic carbon and can be used as organic amendments for crop production. However so far, many environmental issues relevant when these co-products are applied to agricultural land still have to be studied,

especially those related to the impact of the anaerobic digested slurry on the soil microbiota. In a recent work, Walsh et al. [120] observed that its application affected the fungal and bacterial growth in a very similar way to the application of mineral fertilizers in a 16- week greenhouse experiment. They found a pronounced shift towards a bacterial dominated microbial decomposer community, and such effects were consistent in different soils and different crop types. They conclude that mineral fertiliser could be thus exchanged for anaerobic digested slurry with limited impact on the actively growing soil microbial community, which is of great importance in the regulation of soil processes and consequently in soil fertility and crop yield. Recently, Massé et al. [4] gave an overview of the agronomic value of anaerobic digestion treated manures. In line with this, previous findings showed that the AD of animal slurries improved their fertilizer value [3], thereby leading to an increased forage yield and N uptake relative to raw liquid swine manure and mineral fertilizers [121]. Bougnom et al. [122] found a 20% increase in grass yield compared to conventional manure. Liedl et al. [123] also found that digested poultry litter resulted in similar or superior grass and vegetable yields versus N fertilizers. Therefore, all of the above provides evidence that the anaerobic digested slurry acts similarly to mineral fertiliser and should be considered as such in its application to land. Additionally, the enrichment of mineral fractions of N and P during digestion ultimately results in a higher concentration of plant-available nutrients compared with undigested manure and a subsequently elevated plant growth promotion ability, suggested to be similar to mineral fertilisers [4,123]. These latter authors also found that AD reduced swine manure total and volatile solid concentrations by up to 80% resulting in improved manure homogeneity and lowered viscosity allowing more uniform land application [4]. Nevertheless, the higher levels of mineral N found in the slurry, mainly ammonia, may also lead to an increase in the level of phytotoxicity of the slurry, thereby affecting seed germination and plant growth after land- spreading of this co-product into soils [124]. The presence of other phytotoxic substances, such as volatile fatty acids (i.e., acetic, propionic and butyric acids), as well as the high content of soluble salts may contribute to the slurry phytotoxicity [125]. Furthermore, Goberna et al. [126] found that amending soils with slurry resulted in greater nitrate losses during the first 30 d of a 100 d incubation period in 20 cm-depth lysimeters. In fact, around 23 and 45% of the total N contained in the soil (natural + added) was lost from soils amended with cattle manure and anaerobic slurry, respectively. Other authors also observed that N leaching was, along with NH_3 volatilisation, one of the most important sources of N leakage to the environment in a field-scale experiment, after having quantified the amount of mineral N at 1.7 m depth from grass cultivated plots amended with anaerobic digested slurry and mineral fertiliser [127]. Thus, the use of this co-product as an organic amendment should accurately match crop N demand because if not taken by the plants, nitrates could be drained to surface waters, leached to ground waters or denitrified into gaseous forms and emitted to the atmosphere.

The presence of pathogenic bacteria in agricultural amendments also represents a potential threat and their screening is thus of great importance mainly in those produced from animal manures, as it has been shown that such pathogenic organisms constitute a common fraction of the microbial community in manure [128]. In fact, it has been shown that some

pathogenic bacteria can survive the process of anaerobic digestion and persist in the slurry, as previously reported by [129]. In line with this, those microorganisms with a spore-forming capacity such as *Clostridium* and *Bacillus* species, which are commonly found in the intestinal flora of most warm-blooded animals and can harbor some highly pathogenic members for animals and humans [12,129], cannot be reduced during the process [130]. Accordingly, Olsen and Larsen [131] observed that the spores of *Clostridium perfringens* were not inactivated in either mesophilic or thermophilic biogas digesters. Similar results were observed by other authors [132-133] in a reactor operating under mesophilic and thermophilic conditions, respectively. It is acknowledged that bacterial spores can survive in extreme conditions and germinate after long periods, when the conditions become more favourable [131]. The non-hygienic conditions of the storage/transporting tanks can also favour pathogen regrowth [134]. The composition of the substrate fed into the reactor, as well as the reactor conditions such as pH, digestion temperature, slurry hydraulic retention time, ammonium concentration, volatile fatty acids content and nutrient supply are expected to have a significant influence on the sanitation of the end-product [130]. This indicates that there exists a potential risk of spreading potentially pathogenic microbes after the application of anaerobic slurries into soil. Indeed, Crane and Moore [135] stated that amending soils with raw and treated manures, even with a low pathogenic load, still posed a threat for the environment because a period of regrowth of some pathogens including *Escherichia coli*, enterococci, faecal streptococci and *Salmonella enterica* have been shown after manure deposition to soil [136]. Goberna et al. [126] also found that the levels of *Listeria* in soils amended with either cattle manure or anaerobic slurry were significantly higher than those in the control treatment after having been incubated for a month. They observed, however, that the cultivable forms of *Listeria* in the studied soils could correspond to *L. innocua* instead of *L. monocytogenes*, as shown by the polymerase chain reaction assays. However, as recently summarised by [137], anaerobic digestion generally reduces the pathogen risk when compared to untreated substrates.

In a current study, we evaluated, at a microcosm level, the short and long-term effects of the anaerobic digested slurry on soil chemical and microbiological properties compared to its ingestate (i.e., raw manure) and the two widely-recognized products, compost and vermicompost. All of the organic substrates were mixed with soil by turning at a rate of 40 mg N kg^{-1} soil (dry weight). A control treatment that consisted of soil without the addition of any organic amendment was also included. A total of 45 experimental units (5 amendment levels x 3 incubation times x 3 replicates) were set-up in the present study. After an equilibration period of 4 days at 4 °C, 15 columns were dismantled and the sample was collected to analyze (incubation time 0 days). The remaining thirty columns were then maintained in a room at 22 °C, which is the average temperature of the hottest and wettest month in this area and the most suitable for the survival of pathogens. These columns were destructively sampled after 15 and 60 d incubation corresponding to short and medium-term effects. The survival of selected pathogens was then determined according to standard protocols [138-140] (ISO 16649-2, 2001 for *Escherichia coli*; ISO 4832, 1991 for faecal coliforms; and ISO 7937, 2004 for *Clostridium perfringens*) in all the organic materials and amended soils.

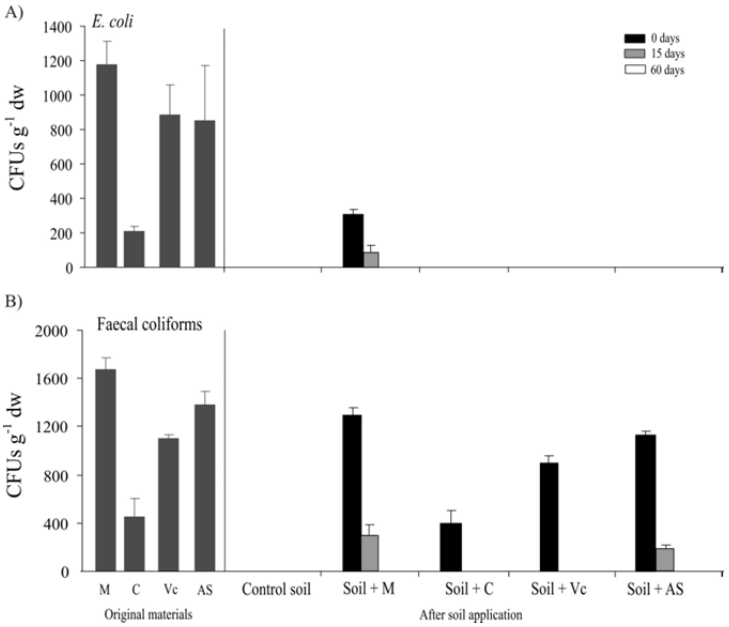

Figure 5. Abundance of *Escherichia coli* and faecal coliforms in the original materials (manure (M), compost (C), vermicompost (Vc) and anaerobic digested slurry (AS)) and in the unamended and amended soils at the three incubation times (0, 15 and 60 days). Values are means ± SE.

Briefly, culturable forms of both faecal coliforms and *E. coli* were isolated from all the initial materials, although their levels were greatly lower in compost relative to the other substrates (Figure 5). This is not surprising taking into account that the composting process, unlike vermicomposting, involved a four-day thermophilic phase, during which the process reached a temperature of 70 °C. Those pathogens were also detected in the anaerobic digested slurry after 40 days of anaerobic digestion (Figure 5). This fact suggests that feeding the reactor with four to five m^3 cattle manure d^{-1} could have provided enough nutrients to maintain a large population of the studied pathogens. Indeed, nutrient availability is one of the major factors influencing pathogen survival in biogas digesters, as previously reported by [130]. Once applied to soils, *E. coli* CFUs were detected in manure-amended soils at the start of the experiment and after incubation for 15 d (Figure 5A); whilst faecal coliforms CFUs were recorded in both manure and slurry-amended soils in the short-term, even though at lower values in comparison with the start of incubation (Figure 5B).

However, the spore-forming *C. perfringens* persisted in all the amended soils (Figure 6), which supports the fact that this bacterium has more resistance to environmental stresses and the capacity to outcompete the native soil microbiota. After 60 d CFU of *C. perfringens* were much closer to those in the control in the slurry-amended soils (Figure 6), which suggests that this time period could be considered as a safe delay between land-spreading the product into soil and crop harvesting with respect to its pathogenic load.

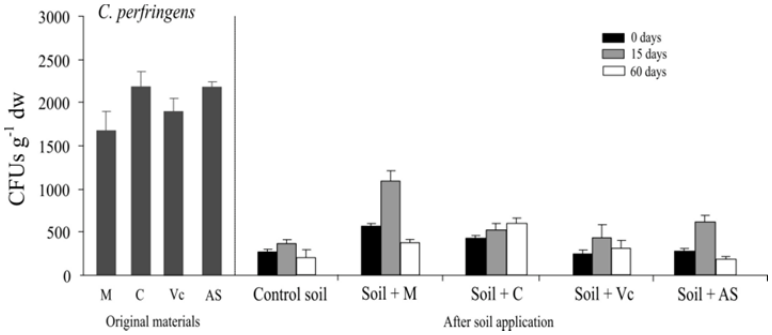

Figure 6. Abundance of *Clostridium perfringens* in the original materials (manure (M), compost (C), vermicompost (Vc) and anaerobic digested slurry (AS)) and in the unamended and amended soils at the three incubation times (0, 15 and 60 days). Values are means ± SE.

4. Conclusions

The intensity and concentrated activity of the livestock industry generate huge amounts of biodegradable wastes, which must be managed with appropriate disposal practices to avoid a negative impact on the environment. Composting is one of the best-known processes for the biological stabilization of solid organic wastes under aerobic conditions. Vermicomposting, i.e. the processing of organic wastes by earthworms under aerobic and mesophilic conditions, has also proven to be a low-cost and rapid technique. Although aerobic processes are thermodynamically more favorable, the manure treatment by anaerobic digestion has become increasingly important due to its energetic potential. A stabilised end product that can be used as an organic amendment is obtained under both aerobic and anaerobic conditions. A multi-parameter approach applying diverse methods constitutes the best option for evaluating the stability/maturity degree of the organic matter, which is of utmost importance for its safe use for the agriculture and the environment. During biodegradation all organic matter goes through the microbial decomposer pool and thus, further knowledge about the changes occurring during the process from a microbial viewpoint will contribute to further develop efficient strategies for the management of animal manures.

5. Outlook

Waste management continues to be a topic of increasing importance. Deeper knowledge of the different biological processes involved in the recycling and recovery of waste components is thus of utmost importance in order to contribute towards more sustainable production and consumption systems. For example, biowaste may be used as a resource to produce high quality lactic acid and protein, as well as biogas in a cascade procedure. Briefly, biowaste is separated into two phases, i.e. a solid phase that is used to feed *Hermetia illucens* larvae that may be harvested as an excellent source of protein for feeding chicken or fish, and the liquid phase that is microbially fermented to the platform chemical lactic acid.

The remaining residuals may eventually be used for biogas production, a cascade process that utilizes the organic waste at its highest level. Furthermore, although an interest in vermicompost research and technology has been increasing over recent years, and the body of knowledge available is quite large, there are still some important topics to be investigated. During vermicomposting, earthworm activity helps microbial communities to use the available energy more efficiently and plays a key role in shaping the structure of the microbial communities during the process. Hence, it is of future interest to evaluate whether the changes in the composition of microbiota in response to earthworm presence are accompanied by a change in the microbial community diversity and/or function. Ultimately, this knowledge will help us to understand the functional importance of earthworms on the stabilization of organic matter from a microbial viewpoint, thereby contributing to minimize the potential risks related to the use of animal manures an organic amendments.

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Acknowledgement

Marina Fernández- Delgado Juárez is in receipt of a PhD fellowship from Doktoratsstipendium aus der Nachwuchsförderung der Universität Innsbruck. María Gómez Brandón is financially supported by a postdoctoral research grant from *Fundación Alfonso Martín Escudero*. The authors acknowledge Paul Fraiz for his highly valuable help in language editing.

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