

# Evaluation of Bacterial Diversity in a Swine Manure Composting System Contaminated With Veterinary Antibiotics (VAs)

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## Research Article

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# Abstract

The objective of this work was identify microorganisms present in swine effluent composting system, under the contamination by most use veterinary drugs in Brazil. The composting took place for 150 days, where was addition of 200 liters of manure (these 25 liters initially contaminated with 17 antibiotics) in 25 kg of eucalyptus wood shavings. Microorganisms were measured at times (0 until 150 days), and were identified in the V3-V4 regions of the 16S RNAr for Bacteria, by means of next-generation sequencing (NSG). The results showing 7 different Bacteria Phyla and, 70 Bacteria Genus identified (more than 1% significance), in total there were more than 26 phyla and 585 genera of bacteria. The genus *Brucella* was found during mesophilic and thermophilic phases, this genus, not yet been reported in article involving composting process. These results suggested the potential of adaptation of the bacterial community with antibiotics denoted through the antibiotics.

## Highlights

- More than 726.00 Phylum and Bacterial genus-level sequences have been identified.
- Phylum Proteobacterias showed greater abundance during the composting process.
- Proof of adaptability microorganisms (*Clostridium* bacteria) in aerobic process.
- First identification of *Brucella* genus of bacteria in swine composting process.

## 1. Introduction

Brazil is the fourth largest pork producer in the world, behind China, the United States, and the European Union. Pig breeding in Brazil has potential to generate more than 300 million liters of manure per day, which in many cases are used as organic fertilizers, that is applied directly or indirectly to the soil (Li et al., 2015), causing several environmental impacts. The most common damages are contamination of the soil and the water resources by the residues of veterinary medicines and / or heavy metals, which can cause changes in the microbial community of the soil beyond the enhancing of microorganisms resistance to medicines for veterinary use, with risks to human health (Sui et al., 2019; Hu et al., 2018; Wang et al., 2016; Segura et al., 2015).

Composting has become one of the best alternatives for the treatment of agricultural waste, especially in the treatment of swine manure (Zhang et al., 2014). Due the high levels of efficiency in the biotransformation of this waste (Wei et al., 2018), with low operating cost and hygiene capacity, in addition to transforming a waste with a potential polluter into a value-added product (Meng et al., 2018; Wang et al., 2018; Onwosi et al., 2017).

Because it is considered an aerobic (Zhao et al., 2017; Ma et al., 2018), biochemical and heterogeneous process, composting involves the transformation of organic matter through mineralization into CO<sub>2</sub>, NH<sub>3</sub>

and water, producing a stabilized final product (Steel et al., 2018; Wei et al., 2018; Liu et al., 2017), which is intrinsically dependent on the action of microorganisms (Awasthi et al., 2017). In this process, different types of microorganisms can be found (Onwosi et al., 2017), with the predominance of bacteria and fungi, which can be divided into aerobic, thermotolerant and mesophilic (Liu et al., 2015), in addition to of anaerobic microorganisms such as, for example, bacteria of the genus *Clostridium* (Franke-Whittle et al., 2014). In a study carried out by Ma et al. (2018), in the analysis of the composting process of swine manure with wheat straw residues, these researchers identified the predominance of the following bacterial phyla in their study: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes* and *Gemmatimonadetes*, representing more than 99% of the total sequences sampled. Zhong et al. (2018) observed the predominance of six phyla: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Chloroflexi* and *Planctomycetes* in a research carried out with the composting of bovine manure.

Corato et al. (2018) in a work with the identification of microorganisms in composting municipal solid waste, observed five predominant phyla among the bacteria, these being *Firmicutes* (36.32%), *Gamma-Proteobacteria* (23.53%), *Actinobacteria* (11.83%), *Alpha-Proteobacteria* (9.21%) and *Sphingobacteria* (5.71%), and two phylum of fungi, *Ascomycota* and *Basidiomycota* (80.57% and 19.43%). Meng et al. (2018) observed the influence of swine manure on microbial diversity in composting with different substrates, and they found the presence of five fungi phyla, *Ascomycota* (predominant), *Ciliophora*, *Basidiomycota*, *Schizoplasmodiida* and *Centrohelida*.

In addition to the potential contamination of environmental matrices with residues of Veterinary Antibiotics (VAs) used in animal production, which remain in swine manure, when they are used as organic fertilizer in the soil (Mojica and Aga, 2011), many microorganisms can show resistance to VAs. Qian et al. (2016), evaluating the resistance of microorganism genes to the antibiotic oxytetracycline, observed the predominance of bacteria distributed in 5 phyla, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Proteobacteria* in the process of aerobic composting of bovine manure. Chlortetracycline, in turn, can inhibit the growth of bacteria in the soil at different concentrations (Bao et al., 2009). Likewise, genes resistant to the class of antibiotics of the Sulfonamides group were observed by Ben et al. (2017).

In this sense, the identification of microorganisms that act during the treatment of swine manure contaminated with VAs through composting is of fundamental importance with a view to knowing its dynamics and improving the composting process (Franke-Whittle et al., 2014), as well as the ability of this process to degrade the VAs (Nguyen et al., 2017). Thus, the main goal of this work was to identify the microbiological diversity of bacteria at the phylum and gender level in a composting system of swine manure contaminated with VAs.

## 2. Material And Methods

### 2.1 Description of the composting system and process

The composting system was assembled on a pilot scale, using a polyethylene box, 0.53 m high, 0.74 m in diameter (0.25 m<sup>3</sup>), where wood shavings + pig waste were added for the start up of the composting process for pig manure. 25 kg of eucalyptus wood shavings were used, with a volume of 0.20 m<sup>3</sup>. The pig slurry added to the process came from a pig-producing farm located in the municipality of Três Passos - RS in Southern Brazil (53°56 'W and 27°30' S). During the entire period of the composting process, 200 L of pig slurry were injected and distributed once a week in the system over 12 weeks. Table 01 shows the amounts of swine manure applied in the composting system, whose initial dosage of injected swine manure was 25 L / week.

Table 01

Volume (L) of swine manure and the duration (weeks) of injection into the composting system

Weeks	1 - 2	3 - 4	5 - 6	7 - 8	9 - 10	11-12	13-16
Injection rate (L / kg shavings)	1.00	0.75	0.65	0.50	0.40	0.30	0.20

The pig slurry used in the first week (25 L), were contaminated with the 17 main veterinary antibiotics used in pig-producing farms, which are divided into 6 groups: Sulfamethoxazole, Sulfamethazine, Trimethoprim, Sulfadiazine, Sulfadoxine, Sulfadimethoxine and Sulfathiazole (Sulfonamides); Chlortetracycline, Doxycycline, Tetracycline and Oxytetracycline (Tetracyclines); Enrofloxacin and Marbofloxacin (Fluroquimolonas); Florfenicol (Amphenicis); Tilmicosin and Tylosin (Macrolides) and Norfloxacin (Chylonomas). The VAs were homogenized in the swine manure and after being injected into the composting system where they were mixed with wood shavings (Bohrer et al., 2020), which was manually revolved every three days in order to oxygenate the composted waste (wood shavings + swine manure + VAs), until the end of the process.

The temperature and humidity of the composting waste (wood shavings + swine manure + VAs) were monitored daily, with readings taken with a probe (Digital Thermo-hygrometer, model AK - 28, Asko brand, with temperature ranging from -50 ° C to 70 ° C, and 20 - 99% RH), placed in the layer of 20 cm from the bottom. The hydrogen potential (pH) of the composting system was measure daily using a pH meter (model PH-300, brand Instruthehrm).

The initial characterization of pig manure and the characterization of the compost (wood shavings + swine manure + VAs) were carried out at times 0, 30, 60, 90 and 150 days after the start of the composting process. Physico-chemical analyzes were performed according to the Standard Methods for the Examination of Water and Wastewater (Apha, 2012).

## 2.2 Bacterial community assessments

### 2.3 Sample collection and preparation

To assess the microbial diversity of bacteria present in the compost, samples of the raw pig manure and samples of the compost were collected during composting. The raw manure was sampled prior

contamination with the drugs (time zero). The compounds were analyzed at the following times: 0, 15, 30, 45, 60, 75, 90, 120 and 150 days after the start of the composting process.

The sampling separation of the compost for the analysis of microbial diversity was carried out in a composite manner, where several sub-samples of the compost were removed in different areas and layers of the composting system, with subsequent homogenization, which resulted in a composite sample of 200-300g. For greater representativeness, the preparation of the samples was carried out according to Fig. 1, where 60 g of the compound (in triplicates) were placed in beakers, with 200 mL of deionized water. The resulting material was stirred at 300 rpm for 3 hours on a shaking table. Subsequently, the material was sieved to remove the coarse material, and inserted in microtubes for centrifugation at 12,000 rpm for 10 minutes.

### 2.3.1 Next Generation Sequencing

For the identification of the diversity of bacteria present in the compost during the different stages of the composting process, the next-generation sequencing was carried out in the samples (NGS, Next Generation Sequencing) with coverage of 50 thousand reads. The V3-V4 regions of the 16S rRNA for bacteria. The primers used to identify bacteria were U341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). After the results were sequenced using the Illumina MiSeq platform (Illumina Inc., USA), using the 300-cycle, single-end V2 kit, following the instructions of the manufacturer (Christoff et al., 2017). The DNA sequences of the bacteria were analyzed using a proprietary pipeline (Neoprosecta Microbiome Technologies, Brazil), considering at most 1% of accumulated error in the sequencing. Subsequently, for taxonomic classification, the results were compared with the data from the KAPA Library kit of Quantification for Illumina platforms (KAPA Biosystems, Woburn, MA) (Christoff et al., 2017), and finally, the bioinformatics analyzes were loaded into the Neobiome platform for viewing.

### 2.4 Statistical analysis

For descriptive statistics were used to characterize the physical-chemical parameters evaluated during composting, with the aid of the Origin Pro 2018 software. The analysis of bacteria diversity at the level of phylum and gender was carried out according to the methodology described by Zhang et al. (2018). The bacterial diversity indices, in addition to the analysis of redundancy and correlation between environmental and antibiotic factors with the bacterial community, were calculated in the program R, version 3.5.

## 3. Results And Discussion

### 3.1 Physico-chemical properties during composting

The data regarding the physical-chemical parameters (pH, composting temperature, total nitrogen (NT), total phosphorus (FT) and carbon / nitrogen ratio (C / N), obtained during composting shown in Fig. 2.

Temperature is considered one of the crucial factors for composting, as it is linked to microbial activity in the system, and consequently in the stabilization of the final compost (Gusain et al., 2018). During composting, the initial temperature was 25°C, reaching a peak of 46.2°C on the 30th day. This increase probably occurred due to the initial increase in microbial activity (Onwosi et al., 2017), which through its enzymatic activity (Sánchez et al., 2017), produce exothermic reactions, which cause an increase in temperature with degradation and breakdown, through hydrolysis, cellulose, lignin, hemicellulose, and proteins. During the composting, the mesophilic, thermophilic and maturation phases were observed, however, there was no maintenance of the temperature in the thermophilic phase for more than 5 consecutive days.

Initially the pH declined, reaching 5.6 on the 30th day. This behavior can be justified by the volatilization of ammoniacal nitrogen, through the biological nitrification process caused by nitrifying bacteria, which release hydroxyls in the medium (Wang et al., 2016). At the end of composting (120-150 days) the pH values ranged from 6 to 6.8, staying in an optimal pH range (5.5 - 8), as observed by Chen et al. (2015) in the process of composting swine manure.

From the breakdown of organic compounds by microorganisms to obtain energy (nutrients), many macronutrients, such as nitrogen, phosphorus and carbon are used to maintain the microorganisms involved in composting. The macronutrients Total Nitrogen (TN) and Total Phosphorus (FT) showed initial concentrations of 744 and 180 mg / kg and final concentrations of 12,695 mg / kg, and 99.2 mg / kg, respectively. The increase in TN values may be related to bacteria that have the ability to fix nitrogen in the compound, through the transformation of ammonia into nitrite and nitrate, other aspects such as temperature and pH may also have influenced this process, as the temperature did not reach ranges above 55°C, and the pH was in more alkaline ranges, contributing to less volatility of ammonia (Ryckeboer et al., 2003; Sánchez et al., 2017).

The C/N ratio of the compound started at 0.82, going to 58.3 (30th day), 82.8 (90th day) and at the end of the process it presented a value of 22.5. The reduction in this parameter was also observed by López-González et al. (2015) in the process of composting lignocellulosic waste, due to the use of carbon by microorganisms, as well as by Yang et al., (2015), who related the decrease in the C / N ratio with the lowest rate of nitrogen mineralization organic than the organic carbon rate. Another aspect that can also be observed in the lower values of this relationship is that the stabilization process may possibly be occurring, as according to Ezzariai et al. (2017), the C/N ratio values of the compound reduce at the end of the process and tend to stabilize.

## 3.2 Bacterial diversity in the composting process

The total number of sequences observed was 762,516, with 387,235 at the phylum level and 375,281 at the genus level, allowing the identification of the 16S rRNA. The sequences ranged from 22,014 to 67,685 sequences in the different samples. 26 phyla were observed (TS 01), however the 7 (seven) main phyla (Fig. 3) with 99% of the readings, were: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Spirochaetes*, *Tenericutes* and *Bacteria unclassified phylum*.

These data are partially compatible with Wang et al., (2018) who observed the predominance of three (*Proteobacteria*, *Bacteroidetes* and *Firmicutes*) of the seven phyla found here, in a study of the composting of swine manure. For Ma et al. (2018) in composting wheat residues with swine manure the predominance was of the following phyla: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes* and *Gemmatimonadetes*. Tian et al. (2013) observed the predominance of *Proteobacterias*, *Firmicutes*, *Chloroflexi*, *Actinobacteria* and *Bacteroidetes* in composting cattle manure. In swine manure composting, with different concentrations of copper, Yin et al. (2018) observed the dominance of the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Euryarchaeota*.

The predominant phylum throughout the composting process was one of *Proteobacteria*, with presence greater than 69% during the times (0 to 150 days). Other phyla that were also observed throughout the composting process were *Bacteroidetes* (16%), *Firmicutes* (8%) *Actinobacteria* (5%) and *Acidobacteria* (1%). The bacteria of the *Proteobacterias* and *Bacteroidetes* phyla may be linked to the degradation of organic matter, as well as in the cycling of nitrogen and organic carbon (Awasthi et al., 2017), mainly due to the phylum *Proteobacterias*, which has a great diversity of species, acting on carbon, sulfur, and nitrogen cycles (Zhong et al., 2018). According to Wang et al. (2016) the presence of the phylum *Actinobacteria* in the composting process is due the activity of these microorganisms that is linked to the maturity of the compound, which was proven in the performance of this work through the presence of the genus *Nakamurela*, *Lysimimonas* and *Leisifsonia* in samples collected 150 days after the composting process.

During the composting process, more than 585 different genera of bacteria were observed (TS 02). However, the most representative genera were grouped, with an abundance greater than 1% at certain times. The 70 genera of bacteria with relative abundance greater than 1% at certain times can be seen in Fig. 4 and (TS 03). In a study carried out with compost of swine manure, Mao et al. (2018) observed the predominance of 12 genera of bacteria and among them, *Clostridium*, *Pseudomonas* and *Sphingobacterium*.

The genders with the greatest representation over time (T) were: T zero – *Pseudomonas* (37%), T 15 - *Sphingobacterium* (24.3%), T 30 - *Devosia* (15.5%), T 45 - *Brucella* (12.2%), T 60 - *Brucela* (20%), T 75 - *Devosia* (7.5%), T 90 - *Flavisolibacter* (25.1%), T 120 - *Sphingomonas* (12.2%) and T 150 *Sphingomonas* (13.2%) and *Nitratireductor* (13%).

The presence of the genus *Pseudomonas*, where some species of nitrogen-fixing bacteria are found, whose process is carried out using the nitrogenase enzyme complex (Sánchez et al., 2017; Awasthi et al., 2017), can explain the increase in TN during the composting process (up to 60 days).

Two examples of genera, which are considered pathogens, *Escherichia* and *Pseudomonas*, were only observed at the times (0 to 60 days) and (0 to 15 days) respectively, their absence in later stages of composting, may be linked to the increase in temperature in the which would have the potential for inverting these microorganisms (Ma et al., 2018). Even with the maintenance of lower temperatures and with maintenance time with temperatures lower than those indicated for ideal composting systems, there

was inactivation of these genera in the process, since the temperatures of the compost recorded during the experiment were lower than in others works (Gou et al., 2018; Onwosi et al., 2017; Sánchez et al., 2017). Tian et al. (2013) observed that the genera *Devosia*, *Hyphomicrobium* and *Sphingopyxis*, were present during the final stages of composting, as was also observed in this work.

Another genus that draws attention is *Clostridium*, which is anaerobic and was observed in the initial time (zero) and with abundance in the order of 1% in time 30, this may be associated with the initial partial anaerobic conditions of the process, as well as with a poor oxygenation in the system (Tian et al., 2013).

The abundance of some genera of bacteria found during the composting process, such as *Clostridium*, *Achromobacte* and *Stenotrophomonas*, can be explained by the capacity of these genres to produce the enzyme carboxymethylcellulose. This enzyme is responsible for the degradation of polysaccharide and lignocellulosic compounds, and to withstand high doses of heavy metals (Awasthi et al., 2017), thus justifying its abundance in the composting process, as well as being a reason to explain the low influence of VAs in the abundance of some genera.

The genera found in each stage of composting is show in Fig. 5. The different stages of composting were distributed as follows: mesophilic stage at times 0, 15, 60, 75 and 90 days; the thermophilic phase at 30 and 45 days, and the maturation phase at 120 and 150 days.

During the mesophilic phase, the bacteria responsible for breaking down organic matter act in temperature conditions that vary in the range of 15 - 35 ° C. Among them, *Pseudomonas* can be mentioned, which consume soluble compounds, such as sugars, amino acids, and lipids, after their breakdown (hydrolysis process) (Awasthi et al., 2017). At this stage, the most abundant genera were *Pseudomonas* (12%), *Sphingobacterium* (8%), *Brucella* (7%), *Devosia* (6%) and *Flavisolibacter* (5%).

In the thermophilic phase, the most common genera were *Devosia* (12%), *Brucella* (8%), *Sphingomonas* and *Escherichia* (4%). For Sánchez et al. (2017), this stage had the predominance of the phylum of *Actinobacterias*, diverging from the data observed in this work, where the predominant phylum was that of *Proteobacterias*.

At the end of composting, in the maturation phase (120 - 150 days), the predominant genera found were: *Sphingomonas* (16%), *Nitratoreductor* (15%), *Devosia* (8%), *Rhizomicrobium* (5%) and *Sphingopyxis* (4%). In this stage, the remaining microorganisms break down the residues of sugars, cellulose, and hemicellulose, transforming them into humic substances (Sánchez et al., 2017).

The genus *Brucella*, one of the most prevalent during the mesophilic and thermophilic phases, had not yet been reported in articles involving the composting process. When seeking more information about the genus, and relating it to its phylum (*Proteobacterias*), it is attributed to characteristics of mesophilic and thermotolerant bacteria, due to the adaptability and great diversity in gender level of this phylum (Zhong et al., 2018). However, this genus also includes the species *Brucella abortus*, observed in this work at the species level, with 6.8% of the total sequences obtained. This genus can affect animals and humans

through brucellosis, manifesting with symptoms of osteo complications, articular, neurological, gastrointestinal in addition to potentiating abortions (Lalsiamthara and Lee, 2018), but that at the end of composting, in the maturation phase, its presence was not detected.

Another predominant genus, *Devosia* (Fig. 5) can be related to both the thermophilic and mesophilic phases of the composting process (Awasthi et al., 2017), showing adaptation characteristics during composting.

### **3.4 Relations of the bacterial community on environmental parameters and veterinary antibiotics.**

Redundancy analysis was applied to observe the physicochemical variations and influence of antibiotics on the main bacteria genera during composting. The relationships between environmental factors (pH, windrow temperature and humidity), and the antibiotics influence with the bacterial community at the genus level can be seen in Figure 6.

For the bacterial community (Fig. 6a), the two axes explain 51.7%, with 35.57% and 16.20%, respectively. The genus *Hyphomicrobium* was related to the highest measured values of humidity and temperature. On the other hand, *Pseudomonas* and *Sphingobacterium* showed the opposite pattern. This behavior is related to the ecological succession of the genera (Wang et al., 2021), since they only appear in the initial stages of composting, *Pseudomonas* (15 d) and *Sphingobacterium* (up to 60 d). This study also observed that abiotic factors (pH and Humidity) during composting did not play a fundamental role in influencing bacterial communities, with only temperature having a significant influence on most bacterial genera. For (Wang et al. (2021); Chi et al. (2020)) abiotic factors, such as pH, humidity, but mainly temperature, play an important role in the succession of bacterial genera in the composting processes. For Wang et al. (2018), a more prominent effect of moisture on the bacterial community was observed, but in compost with cattle manure.

The effects of veterinary antibiotics, as measured by the RDA, on major bacterial genera are showing in Figure 6b. To measure this relationship, only drugs (Trimethoprim, Ivermectin, Tilmicosin, Oxitetracyclin, Florfenicol and Ciprofloxacin) that was related to the others were considered, to avoid redundancy of results. The first two axes of the RDA explain 40.40% and 28.04%, totaling 68.44% of the explanations for the relationship between antibiotics and the bacterial community. As can be seen in most of the bacterial genera, they have almost neutral relationships, but they are negatively influenced by veterinary antibiotics (Oxit, Tilmic, Flor, Cipro). The antibiotic trimethoprim showed a positive relationship with some genera, especially with (*Sphingobacterium* and *Pseudomonas*). One of the facts that may be happening is, as we observed in (TS 04), the concentrations of antibiotics persist during the process, and with that, what may be happening is that the bacterial community is adapting to the antibiotics, becoming resistant (Zhang et al., 2014).

## **4. Conclusions**

The identification of bacteria by the new generation sequencing process (SNG) proved to be efficient in measuring microbial diversity in the process of composting swine manure.

The analysis of the microorganisms showed 7 (seven) different Phyla of Bacteria, with predominance of *Proteobacteria*, 70 genera with relative abundance greater than 1% at different times (0, 15, 30, 45, 60, 75, 90, 120 and 150 days) in a swine manure composting system contaminated with Veterinary Antibiotics (VAs).

Another important aspect pointed out by the work was the ability of microorganisms to adapt to the contaminated medium with high doses of VAs with changes in the bacterial communities present in the compost analyzed during the composting process (0, 15, 30, 45, 60, 75, 90, 120 and 150 days), the results of which contribute to a better understanding of the bacterial biological dynamics in swine manure composting systems.

Finally, it highlights the importance and the need for greater efforts in the search for understanding the role of different groups of bacteria in the processes and mechanisms linked to the composting process.

## Declarations

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### Declaration of Competing Interest

The authors declare that they are not aware of competing financial interests or personal relationships that may have influenced the work reported in this article.

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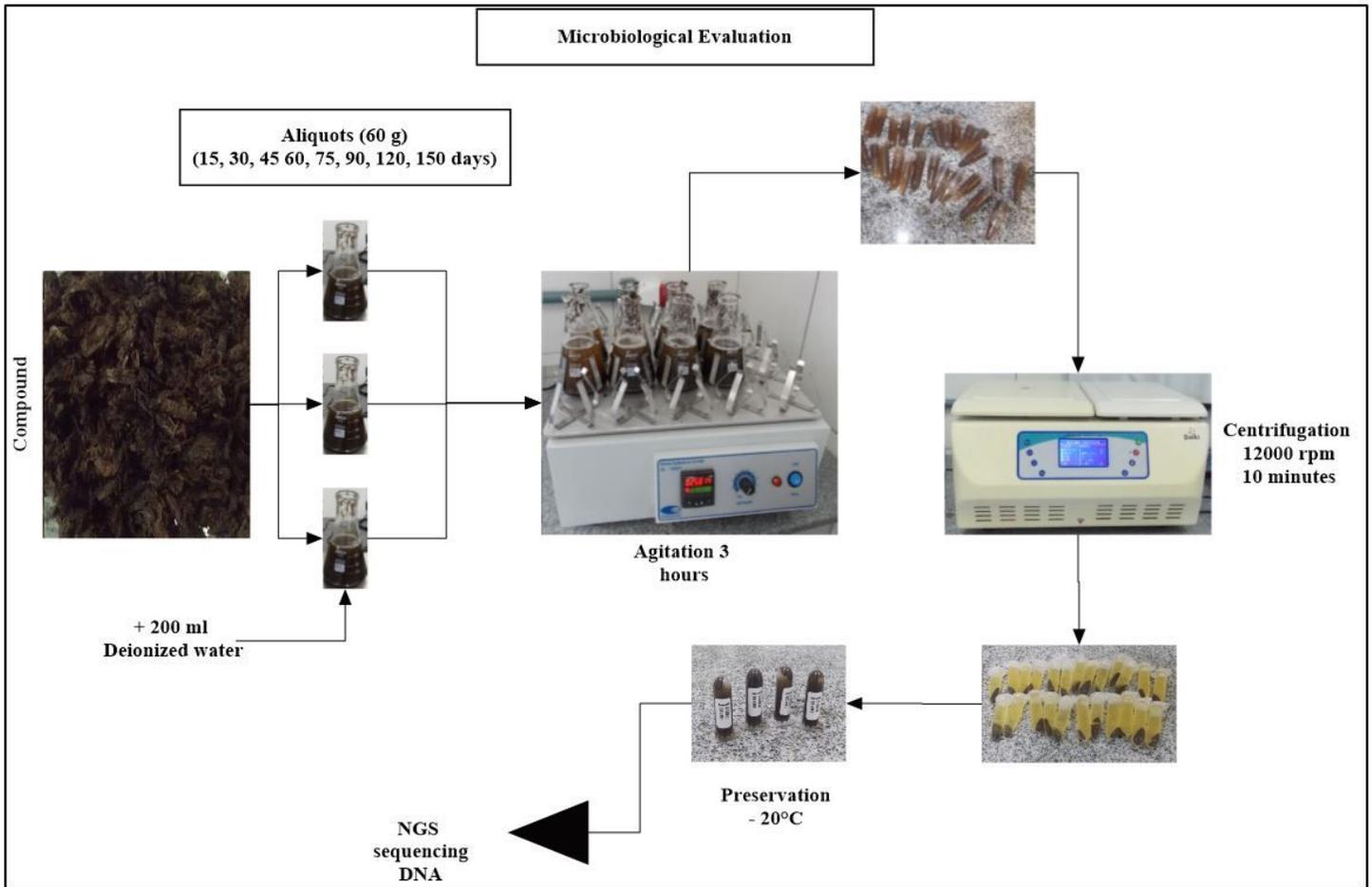
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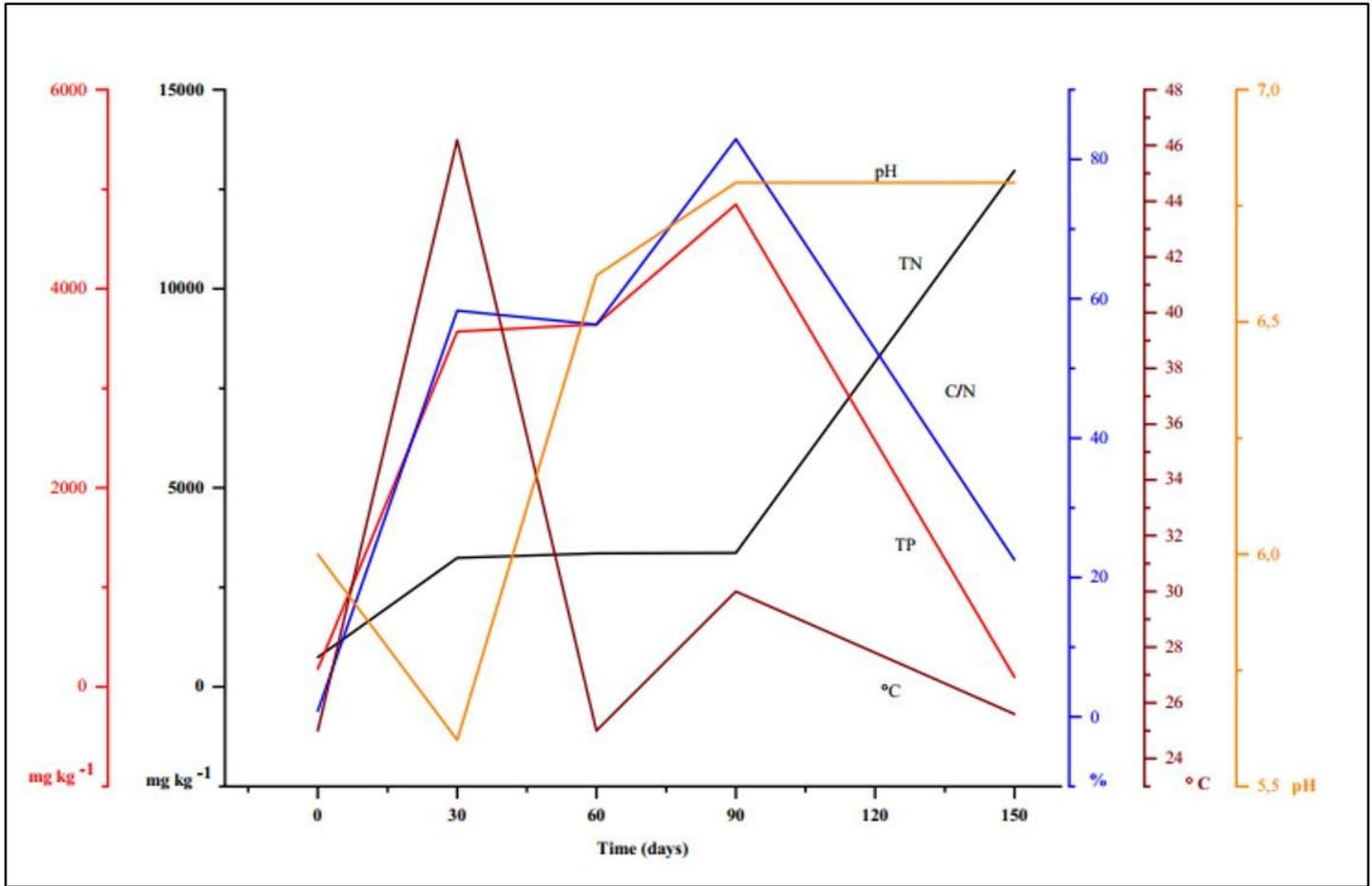
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## Figures



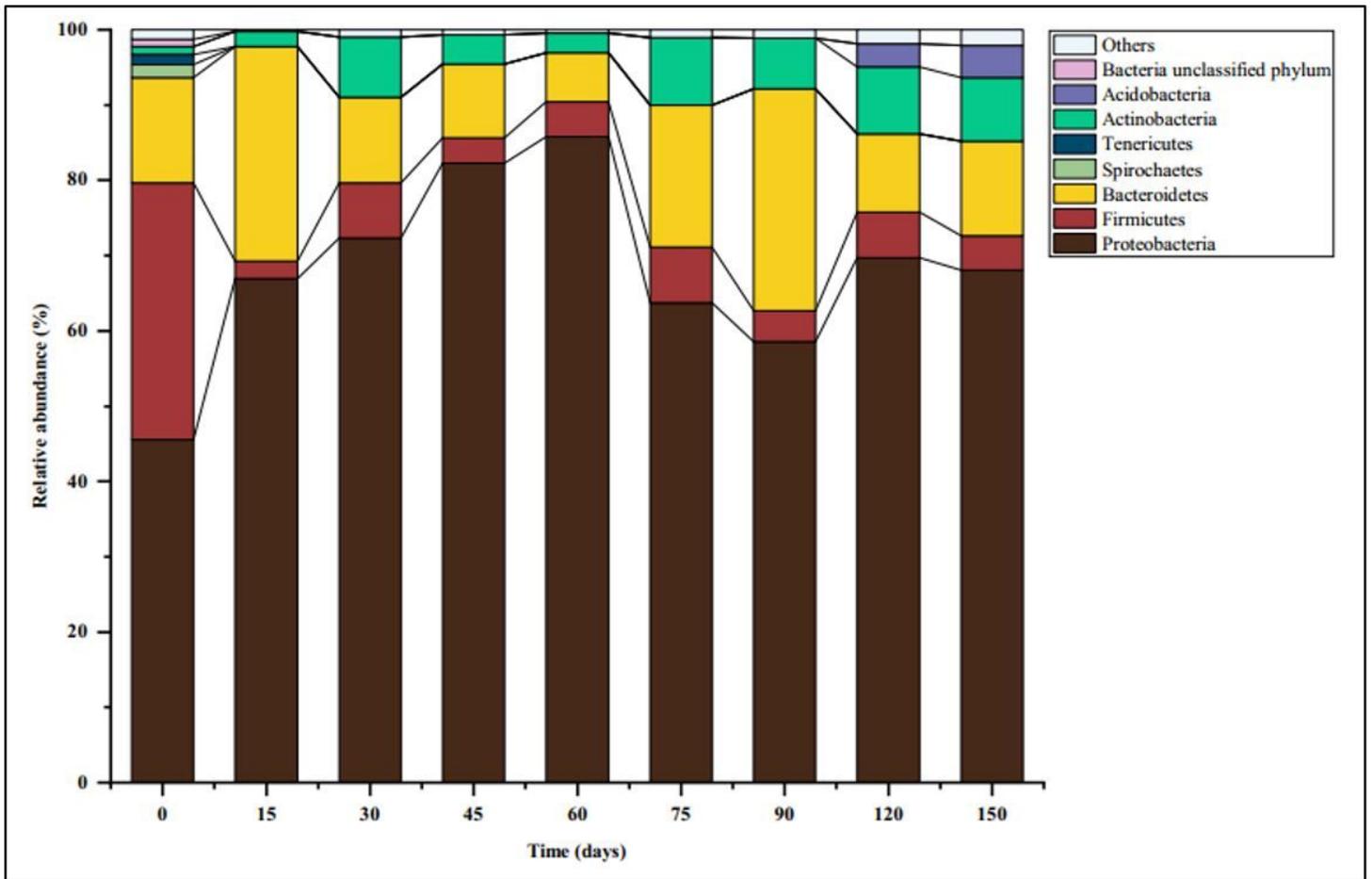
**Figure 1**

Schematic model of the main steps of preparing the samples for sending to DNA sequencing.



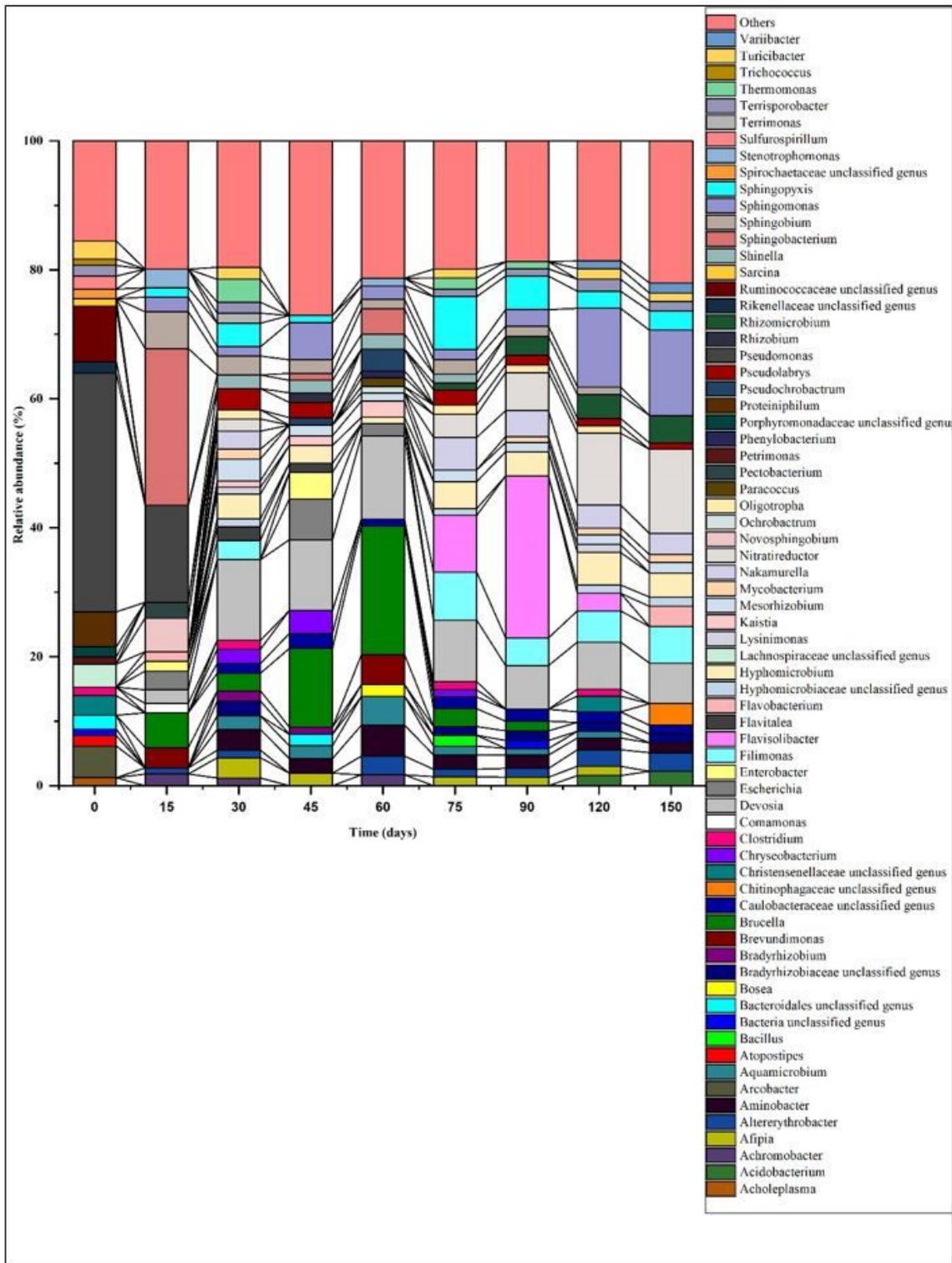
**Figure 2**

Physical-chemical parameters during composting at 0, 30, 60, 90 and 150 days.



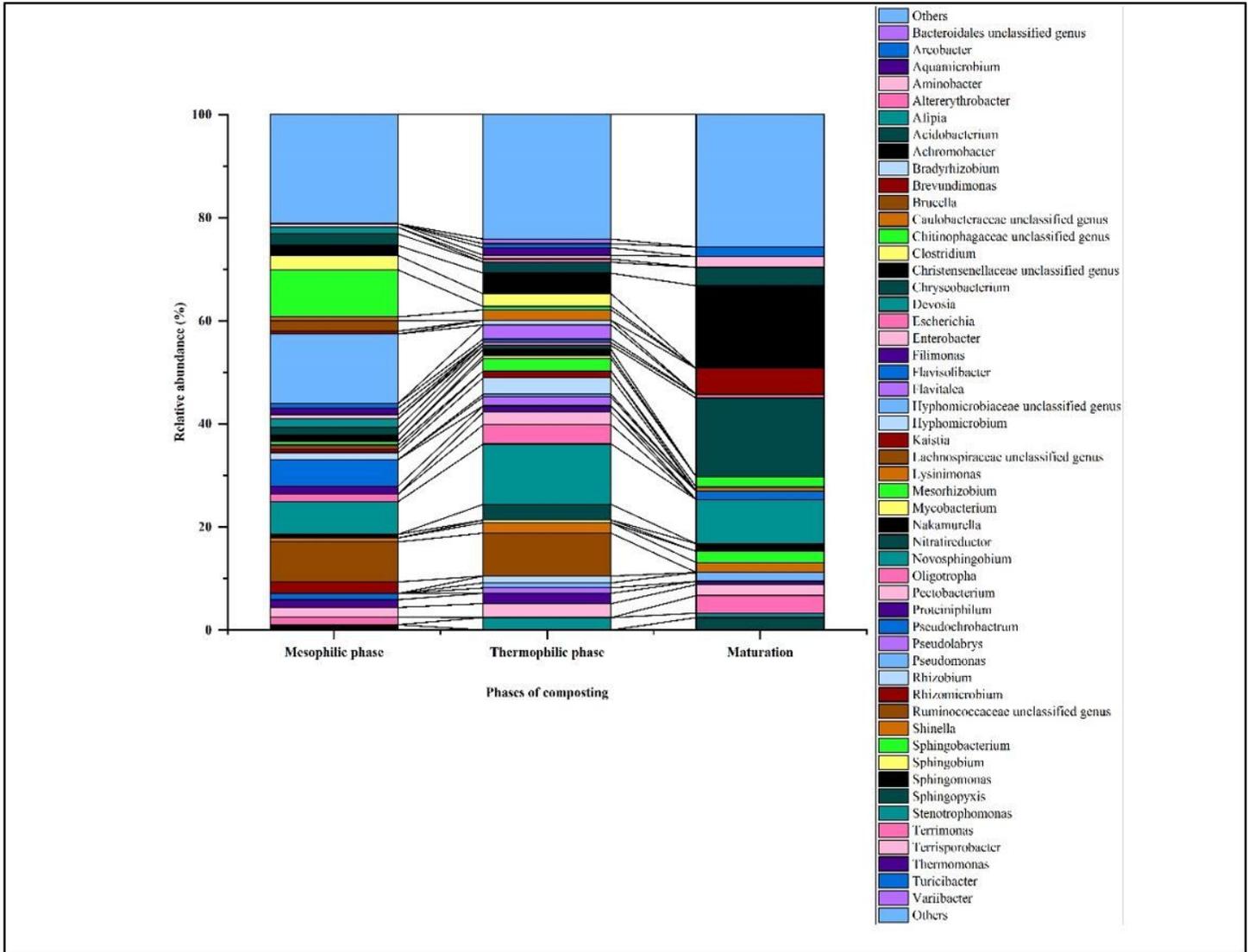
**Figure 3**

Diversity of most abundant bacteria at the phylum level during the composting of swine manure at times 0, 15, 30, 45, 60, 75, 90, 120 and 150 days.



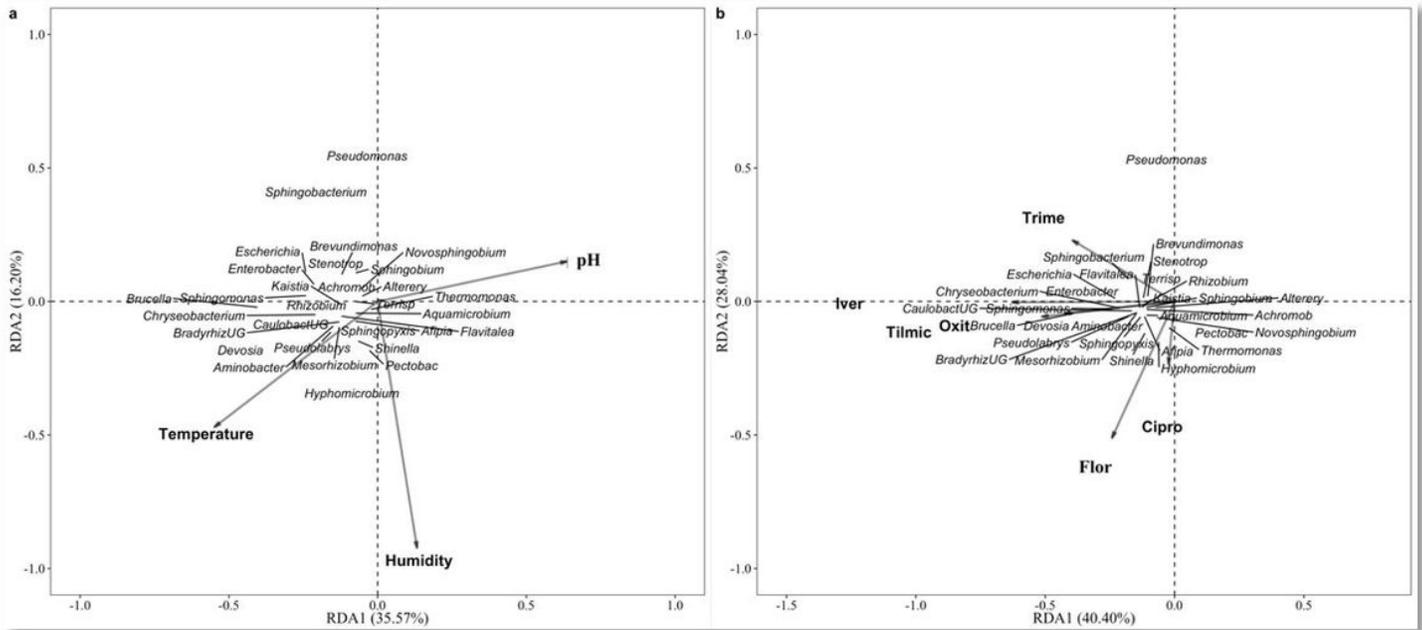
**Figure 4**

Genera of bacteria observed during the composting of swine manure contaminated with VAs at times 0, 15, 30, 45, 60, 75, 90, 120 and 150 days.



**Figure 5**

Genera of bacteria during the stages of composting with percentages of relative abundance greater than 1%.



**Figure 6**

(a). Redundancy analysis of the relationships between environmental factors and bacterial genera during co-composting. (b). Redundancy analysis of the relationships between antibiotics and bacteria genera during composting.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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