Microbiological quality assessment of a compost produced from animal waste and vegetables

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Abstract

Daily composting has been identified as the best method for final disposal of carcasses, but the potential risk of pathogen transmission seriously limits its use. In this study we assessed the microbiological quality and biosafety of a compost produced in an experimental unit of daily mortality composting at Universidade Estadual Paulista (UNESP), Brazil. We know that mature compost presents a good composition of the most important agricultural chemical components and also the aqueous solutions do not inhibit the *Solanum lycopersicum* (tomato) and *Lactuca sativa* (lettuce) germination as well its development. To study the microbiological safety, samples were evaluated to determine the presence of coliforms, *Salmonella* sp, and several soil-borne phytopathogenic fungi (*Rhizoctonia spp*, *Fusarium spp*, *Pythium spp*, *Phytophthora spp*). These evaluations were carried out using selective and differential microbiological culture media. The composition of bacterial population in mature compost was also determined by the 16SrRNA gene sequencing in the Illumina System. The presence of STEC, EHEC, and EPEC pathogenic bacteria *E. coli* genes of virulence was not verified by molecular techniques. *Salmonella* and phytopathogenic fungi presence were negative. Coliform levels were 1160 UFC/kg, and the most common bacteria observed by 16S rRNA gene were from Firmicutes and Proteobacteria phylum. The results show that a daily mortality composting method is effective to reduce pathogenic microorganisms but it does not terminate all of them. Thus it can be used as fertilizer, except to crops intended for human or animal direct consumption. Additional tests must be performed to assure the absence of some pathogens such as viruses.

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1 Introduction

The consumption and biodegradable waste discarded increasing nowadays entails a serious problem for the environment conservation and preservation due to pollution and sanitation problems caused by the residues' final disposal. Among the different methods of management of waste for agricultural purposes, the residues conversion into compost is emphasized. Composting is a natural microbiological decomposition process that has been used to recycle organic matter. This process has as a result valuable soil amendment compost. Composting organic waste is an oxidative process that occurs under controlled conditions and it promotes the mineralization and partial unification of the organic matter. The final product is free of phytotoxicity and pathogens, together to certain humic properties [1]. Furthermore, the not-mineralized carbon from the compost can be retained in the soil for a long period and it provides several important elements for fertility, as nitrogen, phosphorus and some micronutrients. This way we can say that the compost contributes indirectly to the emission of greenhouse gases reduction [2].

Composts can be made with several organic compounds, whether vegetable or animal waste. The animals' carcasses were first used in composting at the end of 80s, when chickens were totally degraded in 30 days [3]. This process was used by the poultry industry as a convenient method for disposing of animal carcasses on other occasions [4]. Composting as a methodology to disposal animal shows biosecurity by reducing pathogens. The transformed organic waste allows the destruction of pathogenic organism, virus, weed seeds and nematodes [5, 6]. However, the composting process must be well controlled, because some studies show that the levels of microbial inactivation by the temperature are limited and controversial [7]. A small percentage of the microorganism from the environment can be growth through the traditional microbiological technique, and then the use of molecular tools to study the microbial population from some samples, like composting, is important [8].

Daily composting has been identified as the best method for final disposal of carcasses, but the potential risk of pathogens transmission seriously limits its use. The Faculdade de Ciências Agrárias e Veterinárias (UNESP, Brazil) produces around 180 tons of animal waste per year, most of it is carcass. This waste is the result of teaching and researching of the veterinary hospital and other departments within the University activities [9]. Considering the need for proper disposal of this waste, the University built a pilot composting plant using such animal and vegetable waste. The mature compost obtained was used as a starting material in this study.

In this study the biosafety and the bacterial population of mature compost produced with daily mortality carcasses was assessed, using some traditional and molecular techniques.

2 Methodology

The study was carried out with samples of the experimental composting at the Universidade Estadual Paulista in Jaboticabal (SP, Brazil). The compost was made from pruning trees and peanut shells (as bulking agents) and animal carcasses (excluding those who died from infectious diseases). This animal waste was produced during teaching and researching activities of the Veterinary Hospital and other university departments. The process took over 150 days of retention and reached temperatures higher than 55°C [9].

Compost samples were randomly collected from a compost pile of 400 L, in different depths (20cm, 50cm and 100cm), in duplicate. These samples were mixed to generate a composite sample.

The chemical elements analyzed were those contemplated in the Brazilian Regulations by National Council of the Environment [10] for organic fertilizer or soil conditioner and sewage sludge. The analyses were made by both spectroscopy and colorimetric methods [11] in the Soil Chemistry Laboratory at the FCAV/UNESP - Jaboticabal.

To determine and count fecal coliforms the membrane filter technique was employed, using the kit for heterotrophic microorganisms (Alfakit, Florianópolis, Brazil) according to the manufacturer's specifications. Compost suspensions were prepared in sterile water (1g in 10ml, w:v) and serial dilutions prepared from $1:10^3$, $1:10^5$ and $1:10^7$, The suspensions were passed through a membrane filter with pore size of 0.45 microns, the membrane was incubated in a selective and differential medium at 37°C and 41°C from 24 to 48 hours. The CFU count was performed by the characteristic topology of bacterial colonies developed on the membrane. To determine the presence of *Salmonella sp* in the sample, 200µL of dilutions previously used for coliforms counting were grown on agar MacConkey, each one of the colonies grown with *Salmonella* topology were subjected to a biochemical identification using the kit API® 20E™(BioMerieux, France) according to the manufacture's specification.

The suspensions of compost used to determine fecal coliforms were also inoculated in a highly selective and differential microbiological media for analysis of the main soilborne phythopathogenic fungi: *Rhizoctonia spp* [12], *Fusarium spp* [13, 14], *Pythium spp*, *Phytophthora spp* [15] and *Sclerotinia spp* [15].

Compost maturity assessment was determined by variations in germinated and elongation of rootlets seeds of *Solanum lycopersicum* (tomato) and *Lactuca sativa* (lettuce) in plates with suspensions in several proportions of compost in sterile water [17]. The samples were compared to the control that contained only distilled water. All dishes remained in a germination chamber under controlled conditions of temperature (22°C) and in the absence of light for 5 days. The percentage of relative seed germination (RSG), the relative root growth (RRG) and germination index (GI) were determined [18].

RSG = (Seeds germinated in compost / Seeds germinated in control) x 100
RRG = (Mean root length in compost / Mean root length in control) x 100
GI = (RSG).(RRG)/100

To molecular studies the metagenomic DNA was extracted using the kit NucleoSpin® Soil (Macherey Nagel, Germany) fulfilling manufacturer's specifications.

The determination of pathotypes of *E. coli* EPEC, EHEC and STEC was assessed by PCR, using primers targeting the genes *stx*$_1$, *stx*$_2$ and *eae* [19, 20, 21], described in the Table 1. The amplicons obtained were observed after agarose electrophoreses done with TBE buffer (89 mM Tris; 89 mM Boric acid and 2.5 mM EDTA, pH 8.3) contained 0.5 μg mL^{-1} Etidium Bromide the gel was visualized under UV light in Gel Doc 1000 (Bio Rad, USA). In these reactions were used as a positive control DNA obtained from the isolate Ecl 6611 that has these genes [19].

Table 1: Primers used for determination of pathogenic *E. coli* (STEC, EHEC, and EPEC) and size of amplicons.

Target gene [reference]	Primers	Amplicon size
stx$_1$ [20]	B54, AGAGCGATGTTACGGTTTG B55, TTGCCCCCAGAGTGGATG	388bp
stx$_2$ [21]	B56, TGGGTTTTTCTTCGGTATC B57,GACATTCTGGTTGACTCTCTT	807bp
eae [21]	B52, AGGCTTCGTCACAGTTG B53, CCATCGTCACCAGAGGA	570bp

The assessment of bacterial diversity was carried out by sequencing the 16S rRNA gene analysis. The metagenomic DNA (0.615 ng) was used in a PCR reaction with the primers: F pA (5'-AGAG TTTGATCCTGGCTCAG-3'; *Escherichia coli* bases 8 to 27) and R PC5B (5'-TACCTTGTTACGACTT-3'; *E. coli* bases 1507 to 1492) [22], under the following parameters: 0.4 μL dNTPs [10 mM], 2 μL of 10X buffer [100 mM Tris-HCl, pH 8.8 at 25°C, 500 mM KCl , 0.8 % (v/v) of Nonidet P40], 0.6 μL of $MgCl_2$ [25 mM], 0.2 μL [10 μM] of each primer, 1 unit of Taq DNA polymerase (Fermentas, Europe), and H_2O mili-Q (Millipore) was added for a total volume of 20 μL. Optimal PCR conditions were as follows: 1 cycle of 95°C, 2 minutes, annealing at 54°C, 30 sec, and extension at 72°C, 1 minute, followed by 25 cycles of 94°C, 30 sec, 54°C, 30 sec, and 72°C, 5 minutes. The amplicon of 1486 pb was visualized on agarose gel 1.3 % by staining with ethidium bromide, and it was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, USA), according to manufacturer's instructions.

The sequencing of bacterial diversity from 16S rRNA gene was carried out using the Illumina MiSeq. The library was constructed using Nextera DNA Sample Preparation kit (Illumina®). Libraries were mixed with Illumina-generated PhiX (v3) control libraries (expected 20%) and our own genomic

libraries and denatured using fresh NaOH solution. The library was clustered to a density of approximately 570 K/mm^2 and was sequenced using paired end 250 base pair (2x250bp). Sequencing by synthesis was performed with MiSeq Reagent kit v2 (Illumina®) on an Illumina MiSeq using protocols defined by the manufacturer. Sequencing data was available within approximately 48 hours. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument.

The generated sequences were processed on CLC Genomics Workbench 7.0.3 (Arhaus, Denmark) for trimming of ambiguous bases and low quality regions. The high-quality reads were analyzed with EMIRGE software [23, 24] to reconstruct full-length *16S rRNA gene* sequence. EMIRGE was run for 40 iterations with default parameters (join_threshold = 0.97) designed to merge reconstructed 16S rRNA genes if candidate consensus sequences share more than 97% sequence identity in any given iteration. The Fasta sequences obtained were submitted to comparative analysis in NCBI and RDP data bank to bacterial identification.

3 Results and discussion

All chemical elements analyzed have values in average 100 times below to the maximum level of contaminants allowed in organic fertilizers in Brazilian regulation (Table 2). Furthermore, the compost has a high concentration of Calcium, Phosphorus, Boron, Nitrogen and other important elements in agriculture. These results allowed suggest that, from the chemical point of view, the compost is a biosafety product; it can be used as a soil conditioner or fertilizer, mainly in acid soils. Thus, the risk of soil pollution is almost inexistent when this organic compost is used.

The tests for germination and root growth with tomato and lettuce were used to assess the maturity of the compost. The values obtained in our study (Table 3) shows that maturity and stability were reached in the mortality compost, the germination percentages were close to 100% at all concentrations tested. The elongation of the root was as higher as the control. We could see that at the concentration [1:10] the lettuce root reached a higher elongation, as the maximum relative germination percentage. The tomato seeds root elongation was inversely proportional to the concentrations tested but always higher than the elongation of root in the control treatments. These results have indicated that the compost does not show any toxicity to the seeds germination as well the plant development.

In order to establish the presence of the most important soilborne phytopathogenic fungi in the assessed samples, several selective and different media culture were employed. None of fungi evaluated were detected or isolated (Table 4). The presence of *Salmonella* genus bacteria was not observed either. On the other hand the number of fecal coliform (220ufc/g) found was almost 5 times below than the maximum value allowable, when compared to the Brazilian regulation.

Table 2: Chemical elements of agricultural interest assessed in a sample, determined by the organic fertilizer or soil conditioner and sewage sludge normative in Brazil (CONAMA-MAPA-SDA IN 27/2006).

Element	Sample of compost ($mg.kg^{-1}$)	Maximum allowed of each element ($mg.kg^{-1}$)
Arsenic	0.009	20/40
Barium	15.84	1,300
Cadmium	0.361	3/39
Lead	3.342	150/300
Cupper	10.48	1,500
Chrome	1.484	200
Mercury	0.004	1/17
Nickel	2.22	70/420
Selenium	0.045	80/100
Zinc	16.7	2,800
Calcium	4,062,500	nd*
Phosphorus	20,699.30	nd
Boron	4.027	nd
Nitrogen	2,381.7	nd
Iron	1,851.8	nd
Sulfur	1,746.34	nd
Cobalt	11.92	nd
Manganese	68.1	nd

*nd = not defined by normative.

The PCR amplification performed to targeting *stx1* and *stx2* genes and *eae* gene were negative in the compost samples DNA. These results suggested that the fecal coliforms or E coli present in the compost don't have genes that encode for shiga toxin 1 and 2, the major virulence determinants in STEC and EHEC or for intimin, the protein responsible for the attachment of EPEC to enterocytes.

Table 3: Compost maturity tests using lettuce and tomato seeds. The germination and root elongation rate were observed to be used to estimate the Germination Index.

Lettuce	Relative germination (%)	Relative root growth (%)	Germination Index
[1:5]	83.33	185	154.2
[1: 10]	116.6	302.47	302.5
[1: 15]	100	130	130
Tomato	**Relative germination (%)**	**Relative root growth (%)**	**Germination Index**
[1:5]	100	107	107
[1: 10]	100	149.7	149.7
[1: 15]	100	156.6	156.6

Table 4: Number of total and fecal coliforms, *Salmonella* spp and phytopathogenic fungi compared with their maximum values determined by the Brazilian normative (CONAMA-MAPA-SDA IN 27/2006).

Microrganism	**Compost Sample**	**Maximum levels of contaminants allowed**
Fusarium, Phytophtora, Pythium, Rhizoctonia e Sclerotinia	0	Absence
Fecal coliforms	220ufc/g	< 1,000ufc/g
Salmonella	0	Absence in 10g

To assess the microbial diversity in compost produced with vegetable waste and animal carcasses we applied a sequence-based metagenomic approach. The total DNA extract from compost samples were used to amplify the *16SrRNA* gene, this amplicons were sequenced by Illumina MiSeq System. Total DNA sequencing has generated 183,618 sequences in pairs, with an average length of 100pb and PHRED quality > 35.

After EMIRGE analysis, it was identified only 6 OTUs. All these sequences obtained belong to an organism from the Domain Bacteria, one was classified in the phylum Firmicutes, Bacilli class and the other five belong to the phylum Proteobacteria, Gammaproteobacteria class (analysis from RDP). The sequence, when submitted to NCBI for Blastn comparison, showed similarity to the sequences described in Table 5.

Table 5: Analysis from Blastn of 16SrRNA sequences obtained from the compost.

Name	Sequence ID (length)	E value	Identity	Gaps
Rummeliibacillus pycnus str. NBRC 101231	ref|NR_041521.1 (1475)	00	1,396 (91%)	17
Escherichia fergusonii ATCC 35469	ref|NR_074902.1 (1542)	0.0	1,449 (99%)	0
	ref|NR_074902.1 (1542)	0.0	1,422 (87%)	36
Shigella flexneri 2a str. 301	ref|NR_074882.1 (1541)	0.0	777 (99%)	0
Shigella flexneri str. ATCC 29903	ref|NR_026331.1 (1488)	00	1,520 (94%)	39
Shigella sonnei Ss046	ref|NR_074894.1 (1542)	0.0	960 (99%)	1

These results show that there are few organisms identified in this material. It can be considered as a consequence of the composting process. Several studies of composters focused on the thermophilic phase as an economic way to ensure sanitation of compost from pathogens. However, the scientific community begs further investigation about the thermophilic phase [25]. The most of the studies about biosafety of compost use a product done based on vegetables waste; only few are related with animal waste composting. As reviewed by Zhao Chen and Xiuping Jiang [26] thermal processing is a good choice for inactivating pathogens in chicken litter or chicken litter-based organic fertilizers prior to land application. However, some populations may develop heat resistance through cross-protection during subsequent high temperature treatment [27, 28]. In our results we could see the presence of important, opportunistic, and pathogenic bacteria, when the *16SrRNA gene* sequences were analyzed and compared in a data bank.

Rummeliibacillus pycnus [29, 30] is an endospore-forming soil bacterium that had been described to growth *in vitro* from 5 to 45°C and described without pathogenic activity. On the other hand we can observe sequences close to *Escherichia fergusonii*, *Shigella flexneri* and *Shigella sonnei* characteristics.

Whereas the origin and distribution of *Escherichia coli* has been the subject of numerous studies, little is known about the ecology of other *Escherichia* species such as *E. fergusonii*. This species has been recognized as an important emerging opportunistic pathogen in both animals and humans. *E. fergusonii* has been isolated from pigs, sheep, cattle, goats, horses, reindeer, ostriches, turkeys, and chickens with several infectious symptoms [31]. Therefore, *E. fergusonii* has a potential to become an important animal opportunistic zoonotic pathogen what is important in food safety and public health. Also this bacterium was reported for its heavy metal resistance, production of lipopeptide biosurfactant [32] and be able to degrade crude oil on culture [33].

Shigella flexneri and *Shigella sonnei* are the most important agents of bacillary dysentery (shigellosis). Shigellosis arises mainly from food [34]. The contamination usually occurs due to poor personal hygiene by an infected food handler. These bacteria could be considered as natural organism in the human's intestinal tract but they are isolated from other mammals like poultry and waste poultry and they can survive in soil and water for a while.

The results of the studies performed in this work showed that despite daily composting being an important method for final disposal of carcasses, there is a potential risk of pathogens transmission that seriously limits its use. The chemical composition of the analyzed compound shows that it has properties to be used as a soil conditioner and/or fertilizer. The compost did not present toxicity for seed germination; it was demonstrated with the absence of pathogenic soil fungi or *Salmonella* spp. The fecal coliforms number rate was acceptable and the presence of pathogenic genes for *E. coli* was not detected. However, the *16SrRNA* genes sequencing from metagenomic DNA showed the presence of organism with great identity with pathogenic bacteria, mainly *Shigella flexneri* and *Shigella sonnei*. Then the method for composting carcasses should be improved. At the present time the use of mortality compost may be

restricted and its application may be limited to soils on crops not intended for human or animal consumption, or maybe to crops intended for human consumption that require cooking.

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