Quality and microbiological safety of a daily mortality compost produced in a veterinary faculty at São Paulo state

Calidad y seguridad microbiológica de un compost de mortalidad diaria producido en una facultad veterinaria del estado de São Paulo

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ABSTRACT

Introduction: Daily and outbursts mortality composting have been identified as one of the finest methods for final disposal of animal corpses, but the probable threat of pathogens transmission truly limits its use. **Materials and Methods**: In this study we evaluated the quality and microbiological biosafety of a compost produced in daily mortality experimental unit composting at the Universidade Estadual Paulista in the state of Sao Paulo, Brazil. Settled compost sample was evaluated in order to determine the presence and counting of coliforms and Salmonella sp. and the pathotypes of *E. coli* STEC, EPEC and EHEC using culture and molecular techniques. The occurrence of frequent soil borne phytopathogenic fungi was also estimated using selective and differential microbiological culture media. **Results and Discussion**: The occurrence of pathogenic *E. coli*, *Salmonella sp* and phytopathogenic fungi were negative. Coliforms level was 3.05 $\log_{10}$/g. **Concussions**: The results showed that daily mortality composting method is effective to reduce pathogenic microorganisms, however, in order to add the product on crops or plants such as vegetables that are for direct human consumption, additional tests must be performed to assess the presence of viral pathogens and endospores forming bacteria.

RESUMEN

Introduccion: El compostaje diario ha sido identificado como el mejor método para la disposición final de las cadaveres, pero el riesgo potencial de transmisión de patógenos limita seriamente su uso. En este estudio evaluamos la calidad microbiológica y la bioseguridad de un compost producido en una unidad experimental de compostaje de mortalidad diaria en la Universidade Estadual Paulista (UNESP), Brasil. Se encontró que el compost maduro presenta una buena composición de los componentes químicos agrícolas más importantes y además las soluciones acuosas no inhiben la germinación de Solanum lycopersicum (tomate) y Lactuca sativa (lechuga) ni su desarrollo. Para estudiar la seguridad microbiológica, se evaluaron muestras para determinar la presencia de coliformes, Salmonella sp y varios hongos fitopatógenos del suelo (Rhizoctonia spp, Fusarium spp, Pythium spp, Phytophthora spp). Estas evaluaciones se realizaron utilizando medios de cultivo microbiológicos selectivos y diferenciales. La composición de la población bacteriana en el compost maduro también se determinó mediante la secuenciación del gen 16SrRNA en Illumina System. La presencia de genes de virulencia de E. coli de las bacterias patógenas STEC, EHEC y EPEC fue verificada por técnicas moleculares. Resultados La presencia de Salmonella y hongos fitopatógenos fue negativa. Los niveles de coliformes fueron 1160 UFC/kg, y las bacterias más comunes observadas por el gen 16S rRNA fueron de los filos Firmicutes y Proteobacteria. Los resultados muestran que un método de compostaje de mortalidad diaria es eficaz para reducir los microorganismos patógenos, pero no acaba con todos ellos. Por tanto, puede utilizarse como fertilizante, excepto en cultivos destinados al consumo directo humano o animal. Se deben realizar pruebas adicionales para asegurar la ausencia de algunos patógenos como virus.

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INTRODUCTION

The massive generation of organic waste from human activities, led to necessity for alternatives to reduce landfill and promote the recycling, an alternative is the composting; It's a well-established process that enables stabilization and sanitation of a large variety of organic waste. Composting occurs by accelerated microbial decomposition of organic matter under aerobic conditions, the final product the compost can be used as: soil conditioner to maintain its moisture, reduce erosion and improving runoff, moreover the compost increases the amount of organic carbon in soil by sequestering it from the atmosphere, reducing greenhouse gas emissions, additionally it's highly effective reducing pathogenic microorganisms [9].

Mortality composting began at the end of the 1980's [24], the principal objective was to prevent the spread of infectious diseases and protect the air, water and soil quality . Traditional methods of carcasses disposal included: abandonment, favoring the transmission of diseases; burning, producing uncontrolled atmospheric emissions and burial that could generate contamination of water bodies without guarantee of pathogens elimination . Mortality composting is defined as the temporary animal corpse burial in the ground surface, in a mound of material that provides supplementary carbon for allowing decomposition by thermophilic microorganisms; the heating of the pile reduces the most pathogens and digests the tissue, supplementary carbon absorbs bodily fluids and acts as biofilter to prevent the escape of odors [21].

Most of the raw materials to be composted contain pathogens, although composting is a well-established technology to reduce them, controlling nearly all pathogenic microorganism (viruses, bacteria, fungi, protozoa and helminths eggs) at acceptably low levels, there are important exceptions like endospore-forming bacteria and prions [21], this has limited the mortality compost use due to the risk of poten-tial contamination of agricultural products intended for human and animal consumption[35]. The bacterium E. Coli is commonly found as commensal in the lower intestinal tract of humans and animals [30], several clones of *E. coli* have acquired virulence factors, enabling adaptation to new niches and producing serious diseases as Hemolytic Uremic Syndrome being the leading cause of kidney failure in children [10], whose etiologic agent is the Shiga toxin-producing *E. coli* (STEC). Cattle are healthy carriers of STEC, that colonizes their terminal rectum [25]. Recent evidence shows that STEC and Salmonella can colonize plants as alternative hosts proliferating in plant tissues, being protected from post-harvest sanitation processes, which poses a potential health risk [6]. Enteropathogenic *E. coli* (EPEC) is an important human pathogen and a commensal of intestinal tract in cattle, was found in stool of healthy and diarrheal calves [15, 34]

In this study we evaluated the chemical quality and microbiological biosecurity of daily mortality compost produced by an experimental composting facility at the Veterinary faculty UNESP Jaboticabal, using tree pruning, peanut shells and carcasses of animal daily mortality. Was determined the number of total and fecal coliforms, the presence of STEC, EPEC, EHEC and Salmonella, the soil borne phytopathogenic fungi, the content of chemical elements of agricultural and environmental importance and finally was evaluated the compost maturity

MATERIALS AND METHOD

The study was conducted with samples of the experimental composting facility at the FCAV/UNESP, Jaboticabal, SP, Brazil, with geographic coordinates: latitude 21o14'48" S, 48o16'44" W longitude and average altitude of 557 m. The compost components were pruning trees and peanut shells (as freight forwarders)

and animal corpses (from this study, those who died from infectious diseases were rejected), these waste comes from teaching and research activities at the Veterinary Hospital and other university departments. The process was carried out for more than 150 days of retention, the highest point temperatures was above 55°C [20].

Sampling

Compost samples were taken directly from a compost pile of 400 L at dissimilar points according to the figure 1; to each selected point were taken samples in duplicate to diverse depths (20cm, 50cm and 100cm), under sterile conditions, this individual samples were subsequently joined and mixed to generate a composite sample.

Detection of enteropathogenic, enterohemorrhagic, enterotoxigenic *E. coli strains* and count of coliforms

In order to determine and determine the number of total and fecal coliforms was performed the membrane filter method by the kit for heterotrophic microorganisms (Alfakit, Florianópolis, Brazil) according to the manufacturer's specifications; briefly, several compost suspensions were prepared in sterile water, starting from a suspension (w:v) of compost 1g in 10ml of sterile water and serial dilutions prepared from $1:10^3$, $1:10^5$ and $1:10^7$. Each suspension was passed through a membrane filter with pore size of 0.45 microns, the membrane was subsequently raised in a selective and differential medium at 37 ° C and 41 ° C for 24 to 48 hours. The CFU count was conducted by representative morphology of bacterial colonies developed on the membrane.

Determination of pathotypes of *E. coli* EPEC,EHEC and STEC was evaluated by PCR, using primers directing for the genes stx_1, stx_2 and *eae* (Table 1), the metagenomic DNA was extracted using the kit NucleoSpin® Soil(Macherey Nagel, Germany) according to manufacturer's specifications. Adapted conditions used for PCR amplification (Clarissa Araújo Borges, et al 2012) were: metagenomic DNA 8.2 ng, 0.4 µl of dNTPs [10 mM], 2µL 10x buffer (100 mM Tris-HCl, pH 8.8 at 25 °C, 500 mM KCl, 0.8% [v/v] Nonidet P40), 1.6 µl $MgCl_2$ [25mM], 0.8 µl [10µM] of each primer, and 1 unit of Taq DNA polymerase (Fermentas, Europe).

Table 1. Primers used for determination of pathogenic *E. coli*

Target Gene	Primers	Amplicon size
stx1	B54, AGAGCGATGTTACGGTTTG B55, TTGCCCCCAGAGTGGATG	388bp (Beebakhee et al. 1992)
stx2	B56, TGGGTTTTTCTTCGGTATC B57,GACATTCTGGTTGACTCTCT T	807bp (Jackson et al. 1987)
eae	B52, AGGCTTCGTCACAGTTG B53, CCATCGTCACCAGAGGA	570bp (Jackson et al. 1987)

Detection of soil borne phytopathogenic fungi

Starting compost suspension (w/v), 1g in 10 mL of sterile water, sequential suspensions were prepared in ratios of 1:10, 1:100 and 1:1000, were inoculated them in extremely selective and differential media, in duplicate for examination of plant-pathogenic fungi, according to Table 2.

Table 2. Selective media used for the determination of soil borne phytopathogenic fungi

Media culture	Soil borne phytopathogenic fungus	Reference
ko and Horas	*Rhizoctonia spp*	Ko and Hora (1971)
PCNB	*Fusarium spp*	Nash and Snyder (1962)
SFA	*Fusarium sp*	Tio et al. (1977)
PARP	*Pythium spp*	Mircetich and Kraft (1973)
PARP-V8	*Phytophthora spp*	Mircetich and Kraft (1973)

Compost maturity assessment

Based on the methodology of Zucconi et al (1985), was prepared suspensions in proportions 1:5 1:10 to 1:15, from an initial suspension (w/v) of 1 g of compost in 10 ml of sterile water. 15 ml of each suspension were placed in petri dishes in duplicate, 5 seeds of *Solanum lycopersicum* (tomato) and *Lactuca sativa* (lettuce) were subsequently added per petri dish, the samples were matched with the control (distilled water). All dishes were maintained in germination chamber under controlled conditions of temperature (22°C) and in the dark for 5 days. Variations in germinated seeds and elongation of rootlets was registering daily. (seeds germinated: when radicle length was 2mm or more.) The percentage of relative seed germination (RSG), the relative root growth (RRG) and germination index (GI) was determined under follow the Tiquia methods (2000):

$$RSG = \frac{\text{Number germinated seeds in compost}}{\text{Number germinated seeds in control}} \times 100$$

$$RRG = \frac{\text{Mean root length in compost}}{\text{Mean } \sqrt{\text{root length in control}}} \times 100$$

$$GI = \frac{(\%RSG).(\%RRG)}{100\%}$$

Chemical analyses of macro and micro elements

The chemical elements examined were those contemplated under the Brazilian regulations. CONAMA (Nacional council of the Environment) resolution No 375/2006 and were made in the soil chemistry laboratory at the FCAV/UNESP -Jaboticabal.

RESULTS

Detection of enteropathogenic *E.coli* STEC, EPEC and EHEC and count of coliforms

The PCR amplification implemented to aiming stx_1, stx_2 genes responsible for production of for shiga toxin 1 and 2, the major virulence determinants in STEC and EHEC and *eae* gene coding for intimin, protein responsible for the attachment of EPEC to enterocytes, showed the absence in the samples assessed of the enteropatagonic *E. coli.* (fig.1). The number of counts of total and fecal coliforms and Salmonella are show in a table 3.

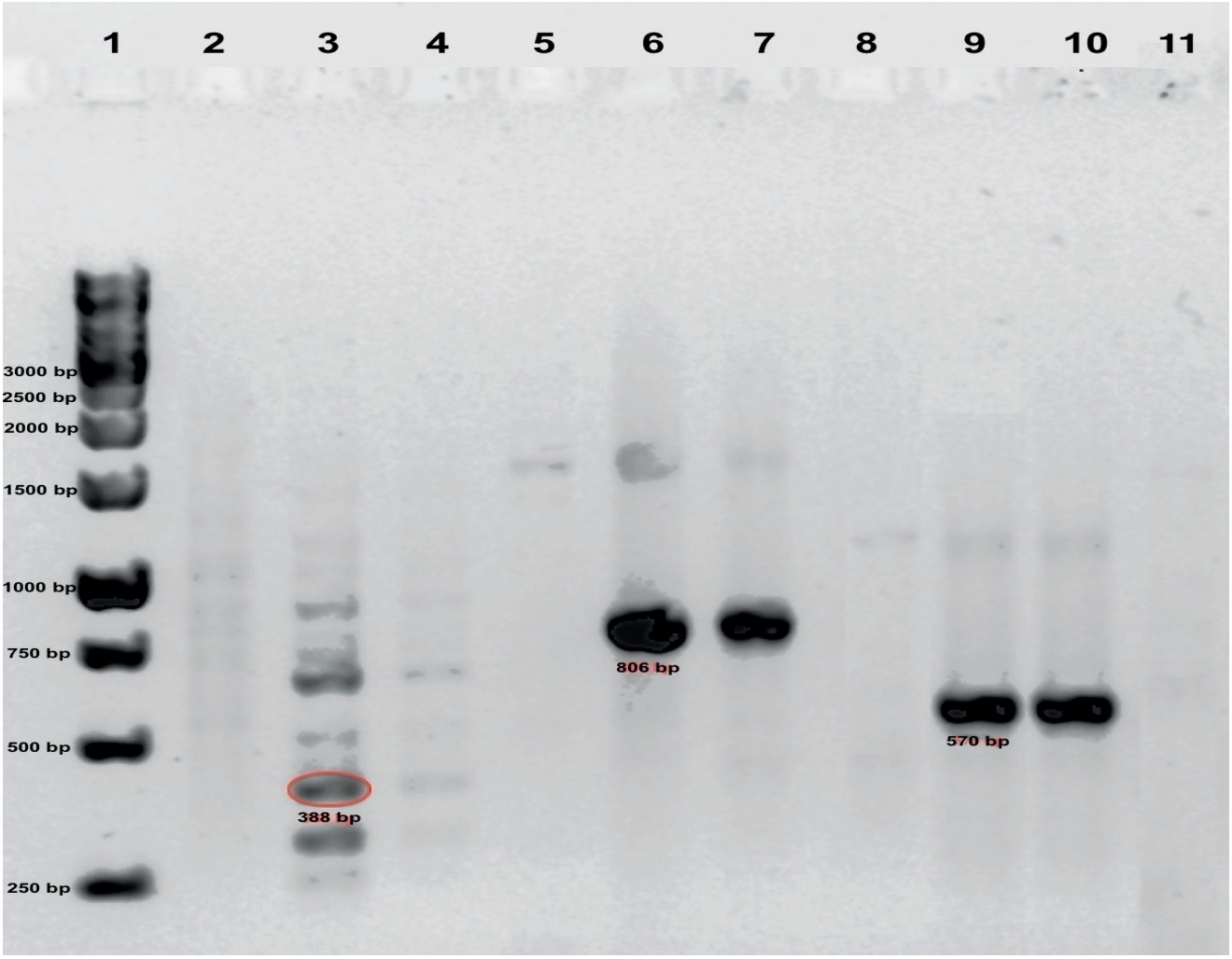

Figure 1 Agarose gel show PCR amplification gens of pathogenic *E. coli*

Agarose gel electrophoresis 1.3% with ethidium bromide lanes: 1. molecular weight marker 1kb. 2. stx1 Sample. 3. stx1 positive control. 4. Sample plus stx1 positive control. 5. *stx*2 Sample 6. *stx2* positive control 7. Sample plus stx2 positive control. 8. eae sample. 9. eae positive control. 10 eae sample plus *eae* positive control. 11 negative control.

Table 3. Count of total and fecal coliforms and Salmonella spp

Bacteria	CFU	*1 Sewage sludge or derived product	*2organic fertilizer or soil conditioner	Compost Canadian normativity
Total coliforms	1160/g	ND		
Fecal coliforms	220/g	< 1000/g	< 1000/g	< 1000/g
Salmonella	0	absence	Absence in 10g	<3CFU in 4g

ND no data, * Brazilian normativity 1Conama Res375/206 2MAPA-SDA in 27/2006.

Detection of soil-borne phytopathogenic fungi

In order to determine the presence of soil borne phytopathogenic fungi in the samples evaluated. Several selective and differential media cultures were used. none of fungi evaluated was detected or isolated.

Assessment compost maturity and Chemical elements of agricultural interest

Seed germination and root growth was tests used to assess the maturity of the compost in plant, the chosen plants to test were tomato and lettuce, the results gotten in our study demonstrated that the maturity and stability was reached in mortality compost, the germination percentages were above 100%, At wholly concentrations assayed and the grown of the root above the control was displayed. However, at the concentration [1:10] lettuce root reached greatest elongation and also the maximum relative germination percentage, despite, in tomato seeds root grown was inversely proportional to the concentrations tested but always exceeding to the controls. Tables 4a and 4b. The values of chemical elements evaluated in a sample are show in a table 5.

Table 4a. % the relative root growth

Lettuce	(%)Relative germination		Germination Index
[1:5]	83,33	185	154,2
[1: 10]	116,6	302,47	302,5
[1: 15]	100	130	130

Table 4b. % Relative germination and Germination index

Tomato	(%)Relative germination	(%) the 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 5. Chemical elements of agricultural interest assessed in a sample

Element	Sample (mg/kg, dry weigh)	Maximum permissible concentration in sewage sludge or derived product (mg/kg, dry weigh)	Reference values for soil quality (mg / kg dry weight)	Maximum levels of contaminants allowed in organic fertilizers (mg / kg)
Arsenic	0,009	41	3,5	20
Barium	15,84	1300	75	---
Boron	4,027	---	---	---
Cádmium	0,361	39	<0,5	3
Calcio	---	---	---	---
Lead	3,342	300	17	150
Cobalt	11,92	---	13	---
Copper	10,48	1500	35	---
Chrome	1,484	1000	40	200
Sulfur	1746,34	---	---	---
Iron	1851	---	---	---
Phosphorus	2071,79	---	---	---
Manganese	680	---	---	---
Magnésium		---	---	---
Mercury	0,004	17	0,05	1,0
Molibdenum		50	<4	---
Níckel	2,218	420	13	70
Nitrogênio		---	---	---
Potassium		---	---	---
Selenium	0,045	100	0,25	80
Zinc	16,692	2800	60	---

DISCUSSION

The group of *E. coli* STEC is diverse serologically more than 100 serotypes are linked to human infections [27], however, the serologic detection based solely on serotype O157: H7 excludes the detection of a large number of other pathogenic serotypes. The PCR method has proven to be widely used for the rapid detection of EPEC, EHEC and STEC from clinical samples, allowing detection of *stx* and *eae* genes from microbiologically complex samples [33]. Among the genes stx_1 and stx_1, stx_2 is considered the most important virulence factor associated with human disease because a shiga toxin encoded by stx_2 is approximately 400 times more toxic to mice that encode by stx_1 [16], the prevalence in São Paulo calves of stx_2 is 50% .

The inactivation of pathogenic microorganisms directly dependent on chemical and environmental factors; the results observed in this work, based on the absence of genes *stx*$_1$ *stx*$_2$ and *eae* showed that a ratio C/N around 20 [20] enable the inactivation of *E. coli* STEC, EHEC and EPEC, maintaining the thermophilic phase by more than a week at temperatures between 55 and 68 °C, in agreement with that observed in studies of survival after inoculation of the pathogen in samples of fresh compost. [28], moreover, studies have shown that *E. coli* reaches the soil via manure or runoff from a point source, the bacteria could survive, reproduce and move up to two months, threatening this environment [11]. Recent studies have shown that pathogenic bacteria may be introduced into the plant in different ways during the growth process [12, 13], this increased the concern about the potential internalization of *Salmonella spp*. and *E. coli* in various fresh vegetables, unless the manure is properly composted, the practice of everyday application of crude manure in the soil is a potential biohazard, capable of transmitting infectious agents, including pathogenic bacteria to humans and animals.

Most phytopathogenic fungi are sensitive to temperatures above 50 °C when it is maintained for more than 72h. Suppressing activity against plant soil borne diseases is generally a desirable property for corrective substrates added to soils [4,2] this feature present in compost may add value to product. The stability and maturation of the compost are integral properties indicating the degree of decomposition of organic matter and potential phytotoxicity caused by insufficient composting. Many of the substances found in the immature compost could produce reduction of germination rate of seeds. The values of maturity obtained in our study show that was reached maturity and stability of the compost, the germination index was above 100%, indicating that the compost does not show any toxicity that may inhibit germination.

Since there is no definitive version of specific regulation for assessing the microbiological biosecurity and chemical quality of mortality compost[35]; our microbiological and chemical values found in samples were compared with reference values of normativity for sewage sludge, organic fertilizers and soil quality in the Brazilian legislation. (CONAMA Res 375/2006, MAP-SDA IN.27/2009, CETESB IN 195/2005)

CONCLUSIONS

Biosecurity agencies in Australia, New Zealand, USA and Canada have recognized the potential benefits of using mortality composting like a preferred method for the disposal animal carcass, for outbreaks and daily mortality in a livestock and poultry industry. [7]. The destruction of pathogens and control of vectors that can transmit pathogens are crucial for a successful composting operation. The use of mortality compost is restricted due to fear of contamination and recontamination by pathogens, limiting their application to soils on crops not intended for human or animal consumption. Although the compost sample analyzed is free of pathogenic E. coli, Salmonella sp. and soil borne phytopathogenic fungi, to ensure safety their application in fresh vegetables cultures intended for human consumption, microbiological and molecular determinations should be made in mature compost, soil and plant to proof the absence of viral pathogens and endospores forming bacteria like *Bacillus anthracis*. Based in our chemical and microbiological results, the application of compost should be restricted of pasture crops, to crops intended for human consumption that requiring cook, and to feed new compost piles of mortality compost.

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