Acta Scientiae Veterinariae. 33(3): 283-290, 2005.

ORIGINAL ARTICLE
Pub. 637

ISSN 1678-0345 (Print)
ISSN 1679-9216 (Online)

Characterization of bacterial population during composting of municipal solid waste

Caracterização da população bacteriana em processo de compostagem de resíduos urbanos

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ABSTRACT

Composting is a natural biological process that degrades organic matter which is carried out spontaneously by aerobic microorganisms, whose metabolic action leads to the mineralization and partial humification of organic residue. The aim of this study was to investigate survival of potentially pathogenic bacterial species throughout the process and detection of enterotoxigenic *Escherichia coli* (ETEC) in the compost using PCR. The presence of heterotrophic bacteria, total total and fecal coliforms, and bacteria succession were also determined. Out of the total 222 bacterial colonies isolated, 33 different genera and 56 different species were identified, being *Bacillus, Escherichia, Enterobacter,* and *Pseudomonas*, the prevailing genera. Total coliform counts ranged between 9.0×10^4 and 3.0×10^6 cfu/g, and fecal coliform counts between 1.0×10^4 and 2.3×10^6 cfu/g. The composting process was partially efficient, since even though coliform counts were reduced, enterotoxigenic *E. coli* was detected in mature compost.

Key words: Composting, bacterial population, solid waste, enterotoxigenic *Escherichia coli* (ETEC), PCR.

RESUMO

Compostagem é um processo biológico natural de degradação da matéria orgânica, desenvolvida espontaneamente por microrganismos cuja atividade metabólica leva a mineralização e humificação parcial da matéria orgânica. O objetivo deste estudo foi investigar a sobrevivência de espécies bacterianas potencialmente patogênicas durante o processo de compostagem e a detecção de *Escherichia coli* enterotoxigênica (ETEC) do composto usando PCR. A presença de bactérias heterotróficas, coliformes totais e fecais e a sucessão da população bacteriana durante a compostagem foi determinada. Do total de 222 colônias bacterianas isoladas 33 gêneros e 56 espécies foram identificados. Os gêneros predominantes foram *Bacillus, Escherichia, Enterobacter* e *Pseudomonas*. A contagem de coliformes totais permaneceu entre $9{,}0X10^4$ e $3{,}0x10^6$ UFC/g e fecais $1{,}0x\ 10^4$ e $2{,}3x\ 10^6$ UFC/g. O processo de compostagem apresentou uma eficiência parcial, pois apesar de ter ocorrido uma redução na contagem de coliformes *E. coli* (ETEC) foi detectada no composto final.

Descritores: compostagem, população bacteriana, resíduo sólido, *E. coli* enterotoxigênica, PCR.

Received: April 2005 www.ufrgs.br/favet/revista Accepted: August 2005

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INTRODUCTION

Composting is a natural biological process of aerobic decay carried out by a wide range of microorganisms that stabilizes organic matter and generates mulch [14,29,33]. Mulch is the end product of this process and it has proved a valuable fertilizer and conditioner of depleted soils [3, 6,13,20,22]. Many factors determine the microbial community during composting, but under aerobic conditions, temperature is the major factor that defines what genera of microorganisms, will be present, species diversity, and rate of metabolic activities [15].

Microbial quality of the compost reflects the effectiveness of the composting process. Therefore, a thorough assessment of maturation is crucial because the use of an under-maturated compost may provoke serious biological damage to soils [11,17,19], along with the proliferation and spreading of potentially pathogenic microorganisms.

Enterotoxigenic *E. coli* (ETEC) strains are an important cause of diarrhea in infants and adult travellers in the human population. These strains are also responsible for diarrheal diseases of animals such as pig and cattle, and may produce types I and II heat-stable (ST) and types I and II heat-labile (LT) toxins. Most diarrhea outbreaks occurring world-wide are due to the consumption of contaminated foods and water [7].

The aim of this study was to investigate the succession of bacterial population and the survival of potentially pathogenic bacteria throughout the composting process adopted in the treatment of municipal solid waste. A PCR technique was used to detect enterotoxigenic *Escherichia coli* (ETEC) in the end product.

MATERIALS AND METHODS

Sampling

Samples were collected from a municipal waste compost windrow, in a solid waste sorting and composting plant in the Greater Porto Alegre, state of Rio Grande do Sul, Brazil. The composting process consisted in forming windrows that underwent forced aeration cycles from under the bottom, for six minutes every hour. Sampling took place fortnightly for 14 weeks, and a subsequent collection happened at full maturation of the compost at the 22nd week. Samples were collected at three different locations and depths in the composting pile (lower, upper and median point) mixed, and one aliquot of 10g was used as a sample.

pH determination

The pH of each sample was determined using a suspension obtained by dispersing the compost sample in sterile distilled water 1:5 [18].

Microbiologic assays

A suspension using 10 g compost sample was prepared in 90 mL sterile distilled water. This mixture was incubated at 30^{o} C at 100 rpm in an orbital shaker for 30 min to homogenize the sample. Whereupon serial dilutions were prepared and used in microbial counts, colimetric assays and bacteria isolation for identification.

Microbial counts

The pour plate technique on plate count agar (PCA) determined the total culturable heterotrophic bacteria. Plates were incubated at 37ºC for 24-48 hours and the total number was estimated by the average number of colony forming units (CFU) per gram of compost samples. Only plates with 30 to 300 colonies were considered for the counting.

Colimetric assays

Colimetric analysis was carried out using the multiple-tube fermentation procedure as a Most Probable Number (MPN) index [2].

Bacterial isolates

Isolation of bacteria was done using seven different culture media, brain-heart infusion agar (BHI), blood base agar supplemented with ovine blood 5%, cetrimide agar, Pseudomonas agar, eosin-methylene-blue lactose saccharose agar (EMB), xylose-lysine-desoxycholate agar (XLD), xylose-lysine-tergitol 4 (XLT_4). Aliquots of 0,1mL of the last three dilutions were seeded on two plates with each of the media, incubated at 37°C for 24-48h. For the isolation of the *Salmonella* species, samples were pre-enriched in tetrathionate broth at 43°C for 16-18 h. Each dilution provided 0.1-mL aliquots that were seeded in duplicate onto plates containing XLD and XLT_4 and incubated at 37°C for 24 h. Once incubation was over, 5 squares of 1cm² were drawn over the plates and 5 colonies from each square were selected from each plate. Identification of isolates started with the Gram's method and afterwards several biochemical tests, like the oxidation/fermentation test, methyl red/Voges Proskauer assay, starch hydrolysis, citrate and indol reactivity, motility, urease reaction, arginine, lysine and ornitin decarboxylation reaction, and the reaction with diffe-

rent carbon sources were performed following the literature [16].

Contamination of the compost with enterotoxigenic *Escherichia coli* (ETEC)

Escherichia coli LT^+ ST^+ (ATCC 11105) culture was prepared in BHI broth and incubated at 37°C until it reached a 3 x 10^8 cell/mL concentration. A dilution of fully mature compost was prepared with 10 g compost dispersed in 90 mL sterile distilled water. This dilution provided 10-mL aliquots that were transferred to two 250-mL Erlenmeyer flasks containing 30 mL BHI broth. Subsequently, one of the Erlenmeyer flasks received 300 mL of the *E. coli* culture. The other flask was used as negative control, without the seedling with of the *E.coli* culture. A third flask containing 30 mL BHI broth and no compost sample received 300 mL of the *E. coli* culture and was the positive control. All flasks were then incubated at 37°C for 16-18 h. These bacterial growths provided the samples for the DNA extraction and the PCR assays.

DNA extraction

The method employed to extract plasmid DNA was alkaline lysis described by Sambrook *et al*. [24] with modifications. From each bacterial growth previously described 6 mL were retrieved and centrifuged at 15,493g for 2 min. The pellet was washed in 1 mL 1M NaCl. After centrifugation, 200 mL of solution I was added (50mM glucose, 25mM Tris HCl , 10mM EDTA). The mixture was then homogenised and incubated in ice for 30 min. Next, 400 mL of solution II was added (1% SDS, 0,2 M NaOH), and this mixture was incubated in ice for 15 min. 300 mL of solution III (5M potassium acetate, glacial acetic acid) was added, and this final mixture was incubated in ice for 30 min. Centrifugation at 15,000 g for 15 min followed. The top aqueous phase was then transferred to a new tube and underwent purification by two consecutive extraction with equal volumes of phenol: chloroform extractions. After another centrifugation at 15,000 g for 10 min, DNA precipitation was promoted by the addition of 0.3 M sodium acetate and 2.5 v ice-cold 100% ethanol. This mixture was incubated at -20°C for 12 h, centrifuged and the pellet was washed in 70% ethanol and allowed to dry at room temperature. The DNA was resuspended in 40 mL TE (10mM Tris HCl, 1 mM EDTA).

The PCR amplification

Primers: Primers[1] used in the detection of enterotoxigenic *E. coli* were designed based on the complete sequences of the target genes of reference [12,34]. The primers LT10 and LT11 are complementary to the gene encoding the heat-labile enterotoxin and amplifies a 221-bp fragment:

LT10 (5'-CGATGGCAGGCAAAAGAGAA-3') and LT11 (5'-GTTTTCCATACTGATTGCCG-3').

Primers ST10 and ST11 are complementary to the gene encoding the heat-stable enterotoxin and amplifies a 167-bp fragment:

ST10 (5'-GTCTTTTTCACCTTTCGCTC-3') and ST11 (5' -TACAAGCAGGATTACAACAC -3').

PCR reactions took place in 2.5mL reaction buffer 1x (100 mM Tris HCl, 500mM KCl), 6.25 mL 10mM $MgCl_2$, 1 mL of a solution of the four deoxynucleotides (2.8 mM each), 300 ng bovine serum albumin (BSA), 122 ng primer ST 10, 120 ng primer ST 11, or 30 ng primers LT10 and LT 11, 1U Taq polymerase, and 30 ng template DNA, to a final volume of 25 mL. Reactions were performed in a thermal cycler[2], under the following conditions for the ST gene: 35 cycles (45 s at 95°C, 45 s at 47°C, 90 s at 72°C); and for the LT gene: 30 cycles (30 s at 95°C, 30 s at 60°C, 60 s at 72°C). The PCR product was electrophoresed in a 1,2% agarose gel containing Tris acetate EDTA (TAE) and stained with ethidium bromide, and then photographed using a Digital camera[3].

RESULTS

pH measurements

The first two samples had pH values between 5.0 and 6.0, due to the excretion of organic acids produced by microbial metabolism. Thereafter, pH values rose and remained essentially unchanged for the last three samples. The final value (8.0) was slightly alkaline (Table 1).

Number of heterotrophic bacteria

Total number of heterotrophic bacteria at the first sample collection was 5.6 x 10^6 cfu/g compost. This count dwindled during the ensuing collections, but came to rise again in the 8^{th} week of composting, reaching 8.0 x 10^7 at the end of the process (Table 2).

Coliform counts

Total coliform count fluctuated all through this experiment (Table 2), but a trend downwards was observed as compost maturated, though without an integral elimination of either total or fecal coliform. The data for fecal coliforms demonstrate a significant de-

Table 1. pH values for samples collected in municipal waste compost windrows.

Sample collection	Time of composting	pH
1	First day	5,0
2	15 days	6,0
3	30 days	7,0
4	45 days	7,0
5	60 days	8,0
6	75 days	9,0
7	90 days	8,5
8	105 days	8,0
9	154 days	8,0

Table 2. Evolution in heterotrophic microorganisms counts and of the fecal and total coliform counts per gram of solid compost, measured throughout the composting process.

Sample	Heterotrophic count cells/g	Total coliform count cells/g	Fecal coliform count cells/g
1	$5,6 \times 10^6$	$3,0 \times 10^6$	$2,3 \times 10^6$
2	$8,0 \times 10^5$	$2,3 \times 10^5$	$2,0 \times 10^4$
3	$2,3 \times 10^6$	$5,0 \times 10^5$	$4,0 \times 10^4$
4	$1,17 \times 10^7$	$3,0 \times 10^5$	$1,3 \times 10^5$
5	$1,4 \times 10^6$	$3,0 \times 10^5$	$2,0 \times 10^4$
6	$4,80 \times 10^7$	$3,6 \times 10^5$	$4,0 \times 10^4$
7	$1,20 \times 10^7$	$2,3 \times 10^5$	$4,0 \times 10^4$
8	$5,40 \times 10^7$	$5,0 \times 10^4$	$2,6 \times 10^4$
9	$8,00 \times 10^7$	$9,0 \times 10^4$	$1,0 \times 10^4$

crease in cells/g of compost for this group of microorganisms: 2.3×10^6 to 1.1×10^4 cells/g

Biochemical identification of microorganisms

Results showed a great bacterial diversity throughout the process. Out of the 222 colonies isolated, 33 genera and 56 species were identified. The most commonly detected genera were: *Bacillus* (19.36%), *Escherichia* (14.86%), *Enterobacter* (14.41%), and *Pseudomonas* (10.36%), which altogether accounted for 59% of the total number of isolates. Prevalence of the *Bacillus* genus was seen chiefly in the first two sample collections (Figure 1). *Bacilllus pumilus* was the most common species of the genus identified in this study, comprising 41.8% of the total *Bacillus* genus identified (data not shown).

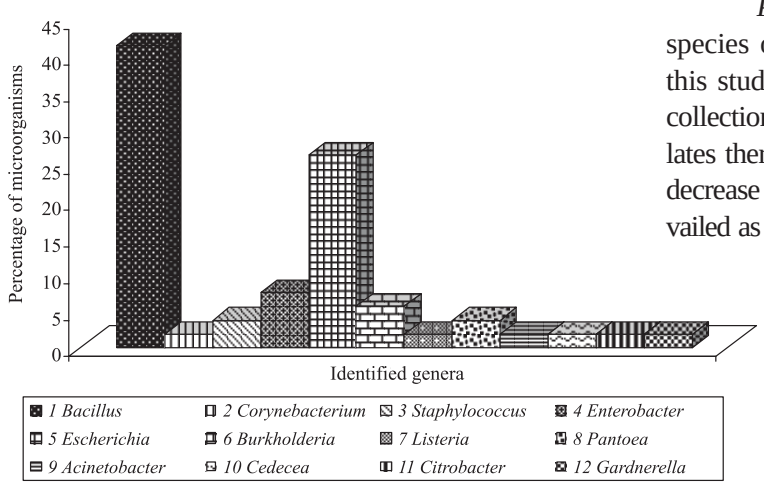

Figure 1. Total number of identified genera from sample collections 1 and 2 (15 and 30 days) respectively. N=53. The genera are read from left to right on each line.

Escherichia coli is the bioindicator to establish the presence of fecal coliforms in a given sample. In Table 2 a slight rise can be seen in the coliform count measured for the fourth sample collection as compared with the second. This rise was confirmed by the identification of a greater number of *E. coli* isolates at the fourth sample collection, which accounted for 19.35% of the total number of isolates, as opposed to all other collections (Figures 1, 2, 3 and 4).

Since *Enterobacter* is one of the genera belonging to the total coliform group, its presence also works as an indication of a likely fecal contamination. Table 2 shows a slight increase in the total coliform counts for the third and six sample collections compared to the second. Figures 1, 2, and 3 indicate a general rise in the occurrence of *Enterobacter* isolates.

Pseudomonas pseudoalcaligenes was the only species of the *Pseudomonas* genus to be isolated in this study. Its presence was detected from the fourth collection on, with a gradual rise in the number of isolates thereafter (Figures 2, 3 and 4), accompanied by a decrease in the occurrence of the other genera that prevailed as of the first sample collections (Figures 1 and 4).

The present study showed a great diversity for bacterial genera characterized in compost samples. The results indicate that no considerable oscillations occurred in this diversity apart from shifts in the prevalence of some genera throughout the process.

PCR amplification

Figure 5 and 6 show the amplification products of the compost samples inocu-

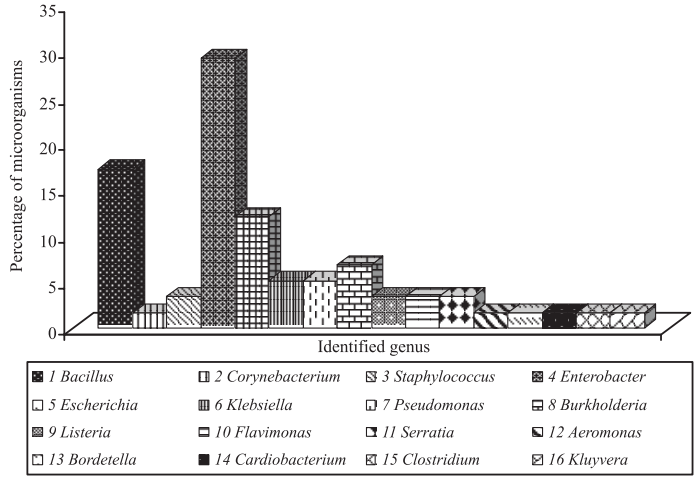

Figure 2. Total number of identified genera from sample collections 3 and 4 (45 and 60 days) respectively. N=58. The genera are read from left to right on each line.

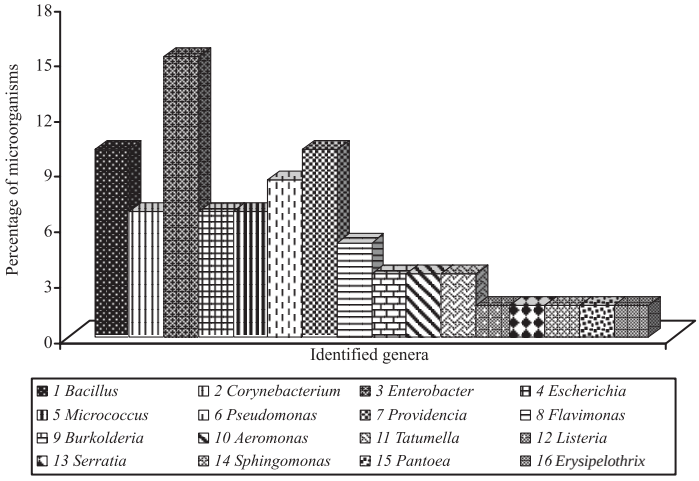

Figure 3. Total number of identified genera from sample collections 5 and 6 (60 and 75 days) respectively. N=59. The genera are read from left to right on each line.

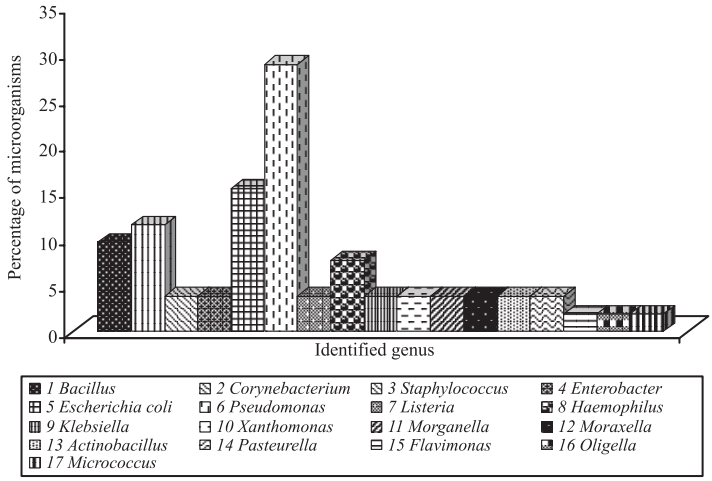

Figure 4. Total number of identified genera from sample collections 7 and 8 (90 and 154 days) respectively. N=52. The genera are read from left to right on each line.

lated and not inoculated with *Escherichia coli* (ETEC) when amplified with the primers LT10, LT11 and ST10, ST11 respectively.

Figure 5 shows the result obtained for the amplification of the LT gene, with a 221 bp product. The compost samples LTA1, LTA2, LTA3 were inoculated with *E. coli* (ETEC) and amplified the expected product. Two of the four compost samples (A1 and A2) that were not inoculated also amplified the same product even though the band was less intense.

Figure 6 shows the amplification product of the ST gene with a size of 167 bp. Three of the four compost samples inoculated with the *E. coli* (ETEC), STA1, STA2 and STA3, have amplified the fragment with the molecular weight of the target gene (167 bp). Likewise, for the four non-inoculated compost samples, A1, A2, A3 and A4, amplified fragments expected.

DISCUSSION

The pH values in composting process of domestic residue, usually starts with a more acidic pH, that is the result of the initial metabolic activity of the bacteria population producing organic acids and carbon dioxide. Throughout the composting process, the elimination of carbon dioxide and the degradation of acids and proteins promotes the increase in pH values [19,25]. The pH results obtained in this study are in agreement with the results obtained by other workers [4,10].

In the initial stages of the composting process mesophilic bacteria are the main agents of biodegradation. Mesophilic bacteria are partly killed or inactivated at the outcome of the thermogenic phase, in which thermophilic and thermotolerant species such as fungi, some bacteria, and actinomycetes emerge. Average counts of heterotrophic bacteria in this work was similar to the counts found by Sidhu *et al.* [26] and Tiquia & Tam [28].

A drop in coliforms counts is expected in composting process because of the sanitization of the process. In this work there

was no fall in the number of total and fecal coliform counts, and comparable results had been found by other authors [15] with a decrease in fecal coliform from 2.0 x 10^7 to 3.1 x 10^3 cells/g. Such results point to the effectiveness of the composting process to produce a better final compost.

The drop in coliform counts for the waste under composting has been ascribed to the high temperatures in the thermophilic phase or to the loss of humidity from within the compost [15,23] respectively. These authors also state that a simple reduction or even the whole elimination of fecal coliforms are not alone useful to establish the biological quality of composts, and point to the importance of an investigation for other potentially pathogenic microorganisms that may be more resistant than the usual indicators detected in composting environments. Moreover, the full elimination of indicators from the compost is very difficult to accomplish [23]. On the other hand, some workers witnessed the complete elimination of coliforms even before the end of the first four weeks of composting [10,28].

In this study, a recontamination with coliforms was observed, possibly due to the sprinkling of the compost with runoff waste slops for the control of temperature. This is a strong evidence that the whole process needs adjusting, with certain modifications in order to increase composting efficacy.

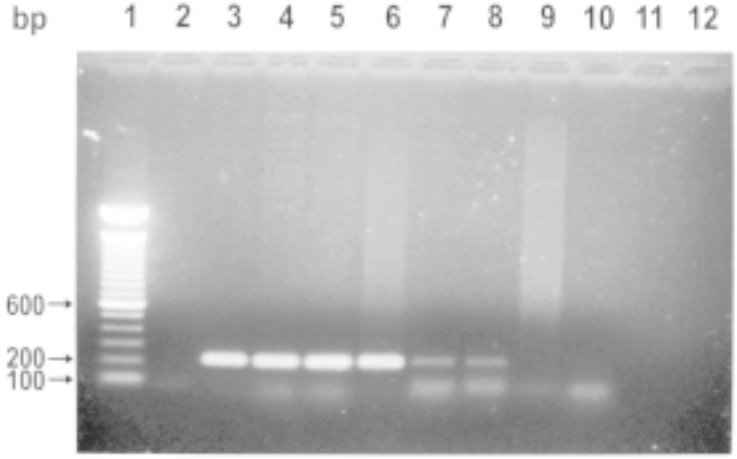

Figure 5. Agarose gel with the amplification products using LT10 and LT11 primers. Line (1): 100pb ladder; line (3): positive control - *E. coli* LT^+(LT1); lines (4-6): compost inoculated with *E. coli* LT^+ (LTA1, LTA2, LTA3); lines (7-10): compost without inoculation (A1, A2, A3, A4); line (11): negative control of the reaction.

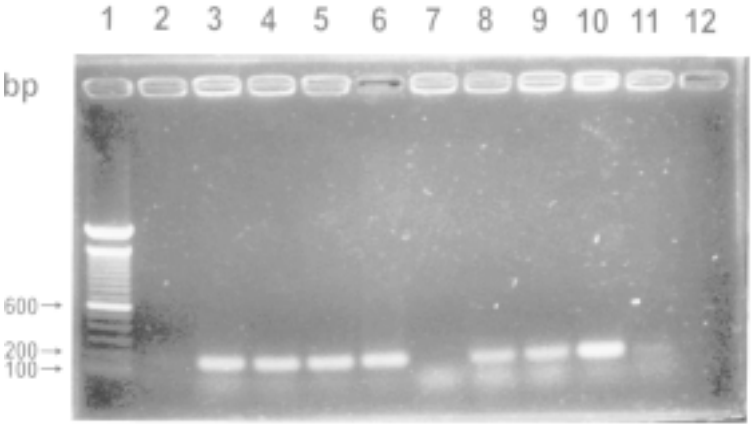

Figure 6. Agarose gel with the amplification products using ST10 and ST11 primers. Line (1): 100pb ladder; line (3): positive control - *E. coli* ST^+ (ST1); lines (4-6): compost inoculated with *E. coli* ST^+ (STA1, STA2, STA3); lines (7-11): compost without inoculation (A1, A2, A3, A4); line (12): negative control of the reaction.

The classic biochemical identification techniques rendered possible the establishment of a profile for the bacterial community present in the windrow of municipal waste under composting.

Several of the genera identified in this study had likewise been frequently found in municipal wastes [10].

The *Bacillus* genus was the most common genus identified in this work. This had also been observed by Blanc *et al*. [5], and Dees & Ghiorse [9] who stated that this prevalence happens due to the ability of the genus *Bacillus* to produce endospores which are extremely resistant to the high temperatures developed throughout the composting process.

The high presence of the *Pseudomonas* genus from the middle to the end of the process may be ascribed to a paucity of nutrients at the final phases of composting. Since this genus is metabolically very resourceful and has a high capacity to degrade renitent substances its presence in the final phases is expected [8,30].

Results showed that the compost samples A 3 and A 4 did not amplify for the LT gene and that sample STA4 followed suit for the ST gene. These results may be ascribed to the presence of a substance acting as inhibitor for the reaction, which commonly happens in DNA investigations of natural environment samples [1,21,27,31,32,35]. Since the compost analyzed was the same, only the DNA extraction happened in different experimental systems.

Results obtained for enterotoxigenic *Escherichia coli* in the samples of mature compost indicate that the PCR protocol is an important tool complementary to the classic biochemical assays that do not set apart species encoding or not virulence factors.

Acknowledgements. We thank the Brazilian government agency CAPES/PROF for the student's MS grant and the financial support to this study.

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[2]Minicycler™ MJ Research. Watertown, Massachusetts, USA.
[3]Kodak Digital Science camera. Progama Kodak versão 35.2.

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