Arq. Bras Med. Vet. Zootec., **v.59, n.4, p.825-831, 2007**

Fate of non O157 Shiga toxigenic *Escherichia coli* in composted cattle manure

[*Eliminação de Escherichia coli Shigatoxigênica não-O157 em compostagem de esterco bovino*]

V.P. *Gonçalves*[1], J.M. *Marin*[2*]

[1]Aluna de pós-graduação - FCAV-UNESP – Jaboticabal, SP
[2]Faculdade de Odontologia de Ribeirão Preto - USP
Av. Café, s/n
14040-904 – Ribeirão Preto, SP

ABSTRACT

To determine the fate of Shiga toxigenic *Escherichia coli* (STEC) non-O 157 in composted manure from naturally colonized cattle, fresh manure was obtained from three cows carrying non-O157 STEC strains possessing the *stx*2 gene. Two composting systems were used: a 0.6m deep cave opened in the soil and an one meter high solid manure heap in a pyramidal architecture. Every day, for the 10 first days, and every five days for a month, one manure sample from three different points in both systems was collected and cultured to determine the presence of *E. coli* and the presence of the *stx* 2 gene in the cells. The temperature was verified at each sampling. STEC non-O157 *E. coli* cells survived for 8, 25 and 30 days at 42, 40 and 38°C, respectively, in the deep cave and 4, 4 and 7 days at 65, 58 and 52°C, respectively, in the heap, during the composting manure. Temperature and indigenous microorganisms appear to contribute to pathogen disappearance in the composting system. It is concluded that both composting systems were efficient to eliminate STEC cells. Land application of composted manure should minimize environmental risk associated with the dissemination of the pathogen.

Keywords: bacterial reduction, manure, STEC, environment, composting system

RESUMO

Determinou-se o tempo necessário para a eliminação de Escherichia coli *Shigatoxigênica (STEC) não-O157 em esterco bovino composto, obtido de fezes frescas de três vacas portadoras de cepas STEC não-O157 que apresentavam o gene stx 2. Foram utilizados dois sistemas de compostagem, o primeiro foi um buraco de 0,6m escavado no solo e o segundo um monte apresentando uma arquitetura piramidal com um metro de altura. Todos os dias, durante os primeiros 10 dias e a cada cinco dias durante um mês, uma amostra de três pontos diferentes dos dois sistemas de compostagem foram coletadas e semeadas para determinar a presença de* E. coli *e a presença do gene* stx *2 nas células, sendo que em cada coleta a temperatura do sistema de compostagem foi determinada. Células de STEC não-O157 sobreviveram por 8, 25 e 30 dias nas temperaturas de 42, 40 e 38°C, respectivamente, no sistema enterrado no solo, enquanto que no sistema de monte as células foram detectadas por 4, 4 e 7 dias em temperaturas de 65, 58 e 52°C, respectivamente. A temperatura e os microrganismos presentes na microbiota do sistema de compostagem parecem ser os responsáveis pela eliminação do patógeno. Pode-se concluir que os dois sistemas de compostagem utilizados mostraram-se eficientes na eliminação de células de STEC. A aplicação de esterco após compostagem deve diminuir o risco de contaminação ambiental e a disseminação do patógeno.*

Palavras-chave: redução bacteriana, esterco, STEC, ambiente, sistema de compostagem

Recebido em 5 de outubro de 2006
Aceito em 25 de junho de 2007
*Corresponding author (*autor para correspondência*)
Email: jmmarin@forp.usp.br

INTRODUCTION

Spreading of bovine manure is a common soil fertilization procedure, often used in vegetable production in Brazil (Pereira Neto, 1996). A potential risk arising from the disposal of animal waste of fecal origin is the spreading of enteric pathogens (Peel, 1997).

Animals from which food is derived are recognized as reservoirs of many significant food-borne pathogens, including Shiga-toxigenic *Escherichia coli* O157: H7 and other non-O157 strains (Kudva et al., 1998; Elder et al., 2000). Shiga toxin-producing *E. coli* (STEC) organisms of different serotypes have been increasingly isolated from diseased humans and from domestic animals (Karmali, 1989; Beutin et al., 1993). Many of these isolates were typical STEC, belonging to serotypes O26, O111 and O157, but most belonged to serotype O157:H7, which can cause severe diseases including hemorrhagic colitis and hemolityc-uremic syndrome in humans (Paton and Paton, 1998). The Shiga toxin exists in two antigenically different forms: stx1 and stx2. The genes encoding *stx* are located in lysogenic bacteriophages. Unless appropriately processed, manure is a potential biohazard capable of transmitting infective agents to both humans and animals (Jones, 1980; Tauxe, 1997).

Laboratory studies have shown that *E. coli* can survive for up to several months in experimentally inoculated animal feces (Kudva et al., 1998; Bolton et al., 1999; Jiang et al., 2002). The principal manner of disinfection during composting is based on time-temperature relationships that destroy pathogens, although antagonistic microorganisms may also play a role in the process (Himathongkham and Riemann, 1999; Larney et al., 2003). The temperature evolution pattern in the composting system can be divided into four different phases: mesophilic, thermophilic, cooling and maturation. After mixing of the compost material, the temperature raises rapidly reaching a maximum within nine days, after which it gradually declines to the ambient level over about 30 days (Ishii et al., 2000).

Cross-contamination of manure produce or improperly composted manure used for soil improvement may be a source of pathogen contamination during preharvesting. Competition with soil microorganisms and adverse environmental conditions can influence pathogen survival, but little information regarding the degree to which Shiga toxigenic *E. coli* non-O157 cells can survive in manure-amended soils is available. In the present study, the objective was to determine the fate of STEC non-O157 cells in cattle manure in two different composting systems.

MATERIALS AND METHODS

During June 2005, anal swabs were collected from the rectum of bovines in a herd of 50 healthy adult beef cows in the Northwestern region of São Paulo State, Brazil. The swabs were transferred to a bottle containing Stuart Transport Medium[1] and were aerobically cultured on MacConkey agar[1], as soon as possible. *E. coli* isolates were identified based on the colony characteristics, Gram staining and biochemical profile (Farmer, 1999). At least five colonies per plate were selected for further analysis. The isolates were serotyped for serotype O157 using the Latex Agglutination test kit[2]. Negative strains were considered non-O157. *E. coli* EDL 933 strain was used as a positive control for O157 serotype.

A loopfull of cells from each of the five colonies above selected was collected, mixed and overnight grown in Luria Bertani broth[3], at 37°C. Bacteria were pelleted from 1.5ml of broth, suspended in 200µl sterile distilled water and boiled for 10min. Following centrifugation of the lysate, a 150µl sample of the supernatant was stored at -20°C as a template DNA stock (Keskimaki et al., 2001). Specific primers were used to amplify sequences of *stx1* and *stx2* genes. Primer sequences, predicted sizes of the amplified products, and the specific conditions were carried out according to those described by Orden et al. (1998). Briefly, amplification of bacterial DNA was performed with 50µl volumes containing 10µl of the prepared sample supernatant; 150ng each of the oligonucleotide

[1]BBL/Becton Dickinson – Sparks, MD - USA
[2]Oxoid - Basingstoke - UK
[3]Difco – Oxford - UK

primers; 0.2mM (each) d ATP, d GTP, d CTP and d TTP; 5 µl PCR 10x reaction buffer[4] and 2.5U of Taq DNA Polymerase[4]. The PCR was performed at 94°C for 2min for one cycle, followed by 30 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1min. The amplified product was visualized by standard submarine gel electrophoresis of 10µl of the final reaction mixture on a 1.5% agarose gel. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. Molecular size markers (*Hae* III digest of X 174 DNA[4]) were included in each gel.

Fresh manure was obtained from three cows that had been confirmed to have non-O157 STEC strains possessing the *stx2* gene. In the herd examined this was the only *stx* gene detected in the eliminated fecal bacterial cells. The collected manure was hand-mixed in a bucket, covered with a sterile plastic and added to a composting system. A 0.6m deep cave was opened in the soil and filled with three layers of grass (10cm), sawdust (1-2cm) and manure free of STEC cells (5cm) with 40% humidity respectively. This arrangement was repeated three times. On the top layer, a 10cm layer of grass was placed. To avoid cross-contamination the selected manure was introduced in three plastic tubes (PVC ¾) which were placed into the cave at 20cm (CT), 40cm (CM), 60cm (CB) in the layers described above (Fig. 1b). One side of the tube was closed by lid and the other was kept open with an adapted rubber to permit the introduction of the swabs inside the system. A thermometer was adapted at the swab to register the temperature at the collecting time. The cave was completely covered with a wide black plastic sheet. Every day, for the 10 first days, and every five days for a month, three manure samples were collected from the top, the middle and the bottom layers of the composting hole and cultured to determine the presence of *E. coli*. After the first month, samples were collected once every month for four months. The other system was a manure heap with pyramidal architecture with the same layers scheduled as in cave, but with a 20cm grass layer and a 10cm manure layer. The same schedule of sampling collecting was done using the top (HT), the middle (HM) and the bottom (HB) layers (Fig. 1a). To verify the climatic effects, a small manure pile was deposited directly on a sheet of wood over a grass pasture and left exposed to the environment. This was designated the pile, and samples were collected in the same way as those of the composting hole. Samples of 5g manure were taken to the laboratory in ice-cold nutrient broth[5]. After an overnight period of aerated incubation at 37°C, a loop of the broth was inoculated onto MacConkey agar and incubated overnight at 37°C. Ten colonies per plate were selected for *E. coli* confirmation as described above. After that, a loopfull of each colony was inoculated and grown overnight into LB broth. The template DNA stock was obtained as described above. The presence of bacteria containing the *stx* 2 gene was assayed by PCR as mentioned.

RESULTS

A survey of *E. coli* survival and the presence of *stx* 2 gene in the composting cave and composting heap systems and also in the pile is shown in Table 1. Bacteria showing the *stx* 2 gene could be detected in the top layer of the composting heap until the fourth day and in the cave until the thirstiest day; in the middle layer, until the fourth day and twenty-fifth days in heap and cave, respectively; and in the bottom layer, until the seventh and eighth days in the heap and cave, respectively. The temperature in the center of the composting hole reached 40°C, 72 hours after mixing of the manure, and reached a maximum of 45°C within 10 days; it then gradually declined reaching that of the ambient. The variation of the temperature between the top and the bottom layers was 40°C±2°C, following the same rate of decline (Fig. 2b). While the temperature in the heap top reached a maximum of 65°C between the sixth and ninth days, in the middle layer the maximum was 58°C on the ninth day and in the bottom, it was 52°C between the sixth and ninth days (Fig. 2a). However, in the pile the temperature was smaller and reached only a maximum of 33°C during the experimental period (Fig. 2c) and the bacteria carrying the *stx* 2 gene could be detected for 60 days.

[4]Amersham Biosciences - Buckinghamshire - UK

[5]Sigma Chemical Co - St Louis, Mo - USA

Table 1. Survival of *Escherichia coli* and STEC non-O157 strains showing the *stx* 2 gene in the composting systems and pile

System	Survival *E .coli*	STEC carrying *stx* 2 gene
	Duration (days)	
Composting (cave)		
Top layer	120 *	30 *
Middle layer	120	25
Bottom layer	120	8
Composting (heap)		
Top layer	120	4
Middle layer	120	4
Bottom layer	120	7
Pile	120	60

*Maximum day on which *E. coli* was detected by plating or the *stx* 2 gene was detected by PCR in DNA template.

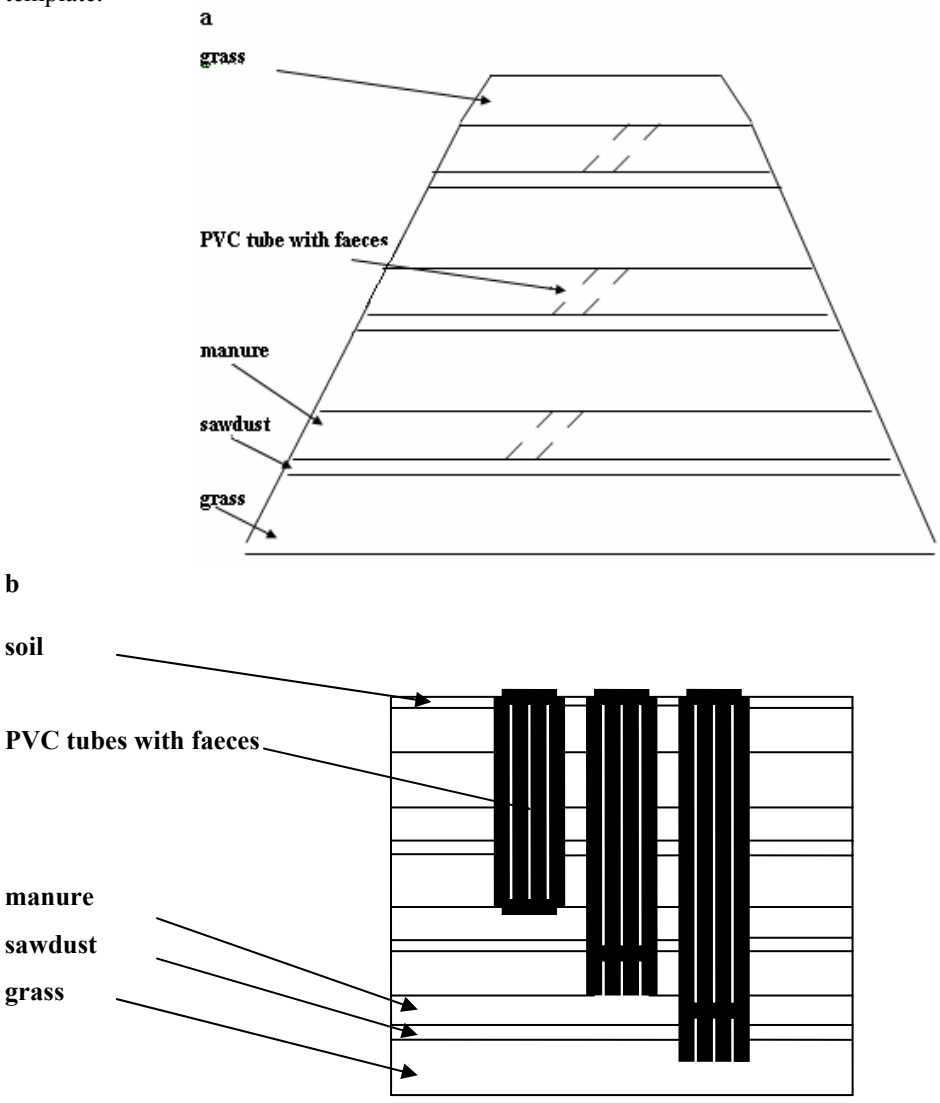

Figure 1. Cross section of the composting systems: (a) heap; (b) cave.

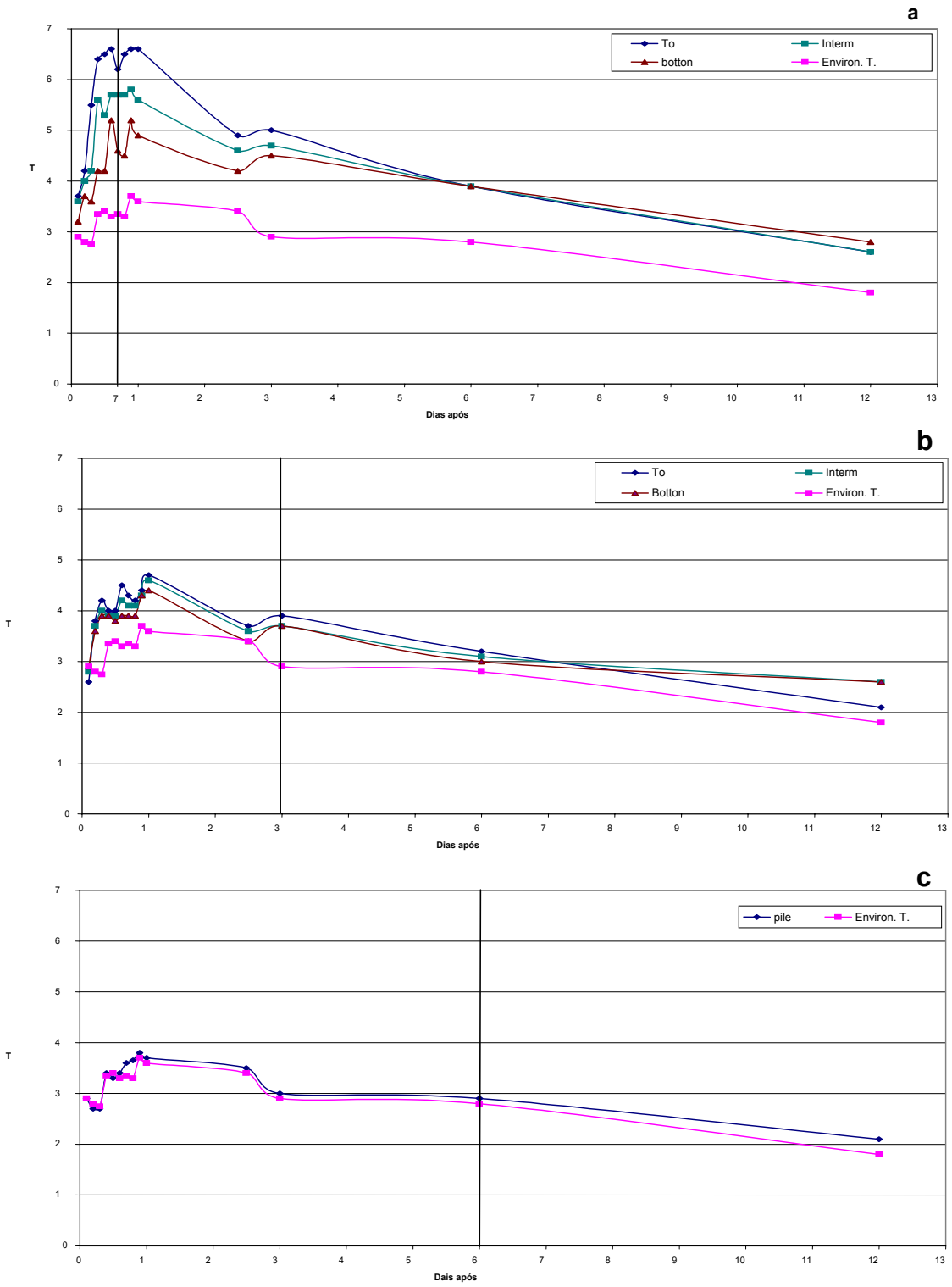

Figure 2. Temperature evaluation in the composting systems, the pile and the environment: (a) heap; (b) cave; (c) pile. The vertical line indicates the minimum time necessary to elimination of 100% of pathogenic STEC cells.

DISCUSSION

There is a growing public concern about the link between livestock production and soil contamination by pathogenic bacteria. This is especially true for the application of raw manure, which is potentially capable of spreading pathogens to a wider environment (Peel, 1997; Kudva et al., 1998). The percentage of STEC serotypes in cattle faeces ranged from 6% in a US study (Cray et al., 1996) to 71% in a French study (Pradel et al., 2000). Absence of routine cultural detection methods for non-O157:H7 means that many countries do not have data on the prevalence of non-O157:H7 and it is certain that there is considerable underreporting on these pathogens (Duffy, 2003).

E. coli O157:H7 in faeces outdoors on grass under ambient conditions in Ireland was capable of long-term survival (99 days) in faeces and in the underlying soil. The pathogen could be recovered directly from faeces on the grass for 50 days (Bolton et al., 1999). Using inoculated samples, a maximum survival time of 70 days in manure was observed by Wang et al. (1996). Mukherjee et al. (2006) reported an acquisition of *E. coli* O157:H7 by a child from garden soil recently fertilized with cattle manure and verified that the bacteria survived for more than two months in the grass. These data agree with the 60 days reported in the present work

Although the elimination of pathogens by composting has been well documented (Deportes et al., 1998; Tiquia et al., 2002; Larney et al., 2003), composting times and temperatures required to achieve elimination or reduction of the number of *E. coli* vary widely. Turner (2002) demonstrated inactivation of *E. coli* in farmyard manure, pig feces, and cereal straw already after 2h at 55^{o}C. In contrast, Lau and Ingham (2001) reported that *E. coli* could be cultured from bovine manure kept for 19 weeks at 21^{o}C. In the present study, *E. coli* could be cultured at every condition tested after up to 120 days, however the non-O157 STEC- *stx2*-gene strains were not found after 30 days, indicating that competition among the bacteria associated with the temperature seem to be very important aspects. Moreover, apparently the STEC strains are more sensitive to high temperatures than ordinary *E. coli*.

There have been few studies on the persistence of *E. coli* non-O157: H7 serotype in animal feces. However, one study which investigated the survival of *E. coli* O26, O111 and O157 in bovine feces (Fukushima et al., 1999) reported that storage at 5, 15, and 25^{o}C permitted the pathogens to survive for up to eight weeks at 25^{o}C. This agrees with the results obtained from the pile in this study. Recently, Fremaux et al. (2007) reported the elimination of non-0157 STEC strains submitted to composting in manure heaps after nine and 16 days at 65 and 56^{o}C, respectively, also similar with the results obtained in the present study.

Kuhnert et al. (2005) in Switzerland reported that 45.0 % of bovine STEC-positive faeces contained the *stx* 2 gene. This agrees with reports on Australian dairy cattle (Cobbold and Desmarchelier, 2001) and also with is reported in the present study. The *stx* 2 gene is assumed to be more important in developing disease than the *stx* 1 gene. Significantly, the *stx2* gene is also associated with the majority of STEC strains causing human disease (Boerlin et al., 1999).

It is well-known that STEC is widespread in animal feces; therefore, application of untreated manure to vegetable cultures may pose a risk for the transmission of STEC to food. Large quantities of livestock waste are spread over agricultural soil in Brazil. Thus, small reductions in the prevalence of zoonotic agents would significantly lower the risk of pathogen dissemination following the spreading of manure. To conclude, it was shown that STEC strains non-O157 carrying the *stx* 2 gene can survive in a composting system for four to 30 days, depending on the temperature reached during composting. This system should be a good alternative to minimize the risk of pathogens spreading over agricultural soil.

REFERENCES

BEUTIN, L.; GEIER, D.; STEINRUCK, H. et al. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J. Clin. Microbiol.,* v.31, p.2483-2488, 1993.

BOERLIN, P.; McEWEN, S.A.; BOERLIN-PETZOLD, F. et al. Associations between virulence factors of Shiga toxin-

producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol*., v.37, p.497-503, 1999.

BOLTON, D.J.; BYRNE, C.M.; SHERIDAN, J.J. et al. The survival characteristics of a non-toxigenic strain of *Escherichia coli* O157:H7. *J. Appl. Microbiol.,* v.86, p.407-411, 1999.

CRAY, W.C.; THOMAS, L.A.; SCHNEIDER, R.A et al. Virulence attributes of *Escherichia coli* isolated from dairy heifer feces. *Vet. Microbiol*., v.53, p.369-374, 1996.

COBBOLD, R.; DESMARCHELIER, P. Characterization and clonal relationship of Shiga-toxigenic *Escherichia coli* (STEC) isolated from Australian dairy cattle. *Vet. Microbiol.,* v.79, p.323-335, 2001.

DEPORTES, I.; BENOIT-GUYOD, J.L.; ZMIROU, D. et al. Microbial disinfection capacity of municipal solid waste (MSW) composting. *J. Appl. Microbiol*., v.85, p.238-246, 1998.

DUFFY, G. Verocytoxigenic *Escherichia coli* in animal faeces, manures and slurries. *J. Appl. Microbiol*., v.94, p.94S-103S, 2003.

ELDER, R.O.; KEEN, J.E.; SIRAGUSA, G.R. et al. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. USA*, v.97, p.2999-3003, 2000.

FARMER, J.J. Enterobacteriaceae: Introduction and identification. In: MURRAY, P.R.; E.J. BARON, M.A.; PHALER, F.C. et al. (Eds). *Manual of clinical microbiology.* 7ed. Washington: ASM, 1999.

FREMAUX, B.; DELIGNETTE-MULLER, M.L.; PRIGENT-COMBARET, C. et al. Growth and survival of non-O157: H7 Shiga-toxin-producing *Escherichia coli* in cow manure. *J. Appl. MIcrobiol*., v.102, p.89-99, 2007.

FUKUSHIMA, H.; KEN, H.; GOMYODA, M. Long-term survival of Shiga Toxin-producing *Escherichia coli* O26, O11 and O157 in Bovine feces. *Appl. Environ. Microbiol*., v.65, p.5177-5181, 1999.

HIMATHONGKHAM, S.; RIEMANN, H. Destruction of *Salmonella typhimurium, Escherichia coli* O157:H7 and *Listeria monocytogenes* in chicken manure by drying and/or gassing with ammonia. *FEMS Microbiol. Lett.,* v.171, p.179-182, 1999.

ISHII, K.; FUKUL. M.; TAKIL, S. Microbial succession during a composting process as evaluated by denaturing gradient gel electrophoresis analysis. *J. Appl. Microbiol*., v.89, p.768-777, 2000.

JIANG, K.; MORGAN, J.; DOYLE, M.P. Fate of *Escherichia coli* O157:H7 in manure-amended soil. *Appl. Environ. Microbiol*., v.68, p.2605-2609, 2002.

JONES, P.W. Animal health today-problems of large livestock units. Disease hazards associated with slurry disposal. *Br. Vet. J*., v.136, p.S29-S40, 1980.

KARMALI, M.A. Infection by verocytotoxin-producing *Escherichia coli. Clin. Microbiol. Rev*., v.2, p.15-38, 1989.

KESKIMAKI, M.; ATTILA, L.; PELTOLA, H. et al. EPEC, EAC and STEC in stool specimens: Prevalence and molecular epidemiology of isolates. *Diag. Microbiol. Infect. Dis.,* v.40, p.151-156, 2001.

KUDVA, I.T.; BLANCH, K.; HOYDE, C.J. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Appl. Environ. Microbiol.,* v.64, p.3166-3174, 1998.

KUHNERT P.; DUBOSSON, C.R.; ROESCH, M. et al. Prevalence and risk-factor analysis of Shiga toxigenic *Escherichia coli* in faecal samples of organically and conventionally farmed dairy cattle. *Vet. Microbiol.,* v.109, p.37-45, 2005.

LARNEY, F.J.; YANKE, L.J.; MILER, J.J. et al. Fate of coliform bacteria in composted beef cattle feedlot manure. *J. Environ. Qual*., v.32, p.1508-1515, 2003.

LAU, M.M.; INGHAM, S.C. Survival of faecal indicator bacteria in bovine manure incorporated into soil. *Lett. Appl. Microbiol*., v.33, p.131-136, 2001.

MUKHERJEE, A.; CHO, S.; SCHEFTEL, J. et al. Soil survival of *Escherichia coli* O157: H7 acquired by a child from garden soil recently fertilized with cattle manure. *J. Appl. Microbiol*., v.101, p.429-436, 2006.

ORDEN, J.A.; RUIZ-SANTA-QUITERIA, J.A.; CID, D. et al. Verotoxin-producing *Escherichia coli* (VTEC) and *eae*-positive non –VTEC in 1-30-days-old diarrhoeic dairy calves. *Vet. Microbiol.,* v.63, p.239-248, 1998.

PATON, J.; PATON, A. Pathogenesis and Diagnosis of Shiga Toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev.,* v.11, p.450-479, 1998.

PEEL, A.N. Manure and microbes: public and animal health problem. *J. Dairy Sci.,* v.80, p.2673-2681, 1997.

PEREIRA NETO, J.T. *Manual de compostagem, processo de baixo custo*. Belo Horizonte: UNICEF, 1996.

PRADEL, N.; DE CHAMPS, C.; PALCOUX, J.B. et al. Verotoxin-producing *Escherichia coli* infections: study of its prevalence in children in the Auvergne region. *Arch. Pediatr*., v.3, suppl., p.544S-550S, 2000.

TAUXE, R.V. Emerging foodborne diseases: an evolving public health challenge. *Emerg. Infect. Dis*., v.3, p.425-434, 1997.

TIQUIA, S.M.; WAN, J.H.C.; TAM, N.F.Y. Microbial population dynamics and enzyme activities during composting. *Compost Sci. Land Util*., v.10, p.150-161, 2002.

TURNER, C. The thermal inactivation of *Escherichia coli* in straw and pig manure. *Bioresour. Technol*., v.84, p.57-61, 2002.

WANG, G.; ZHAO, T.; DOYLE, M.P. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Appl. Environ. Microbiol*., v.62, p.2567-2570, 1996.