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Full Length Research Paper

An evaluation of the use of probiotics and manure composting as strategies to reduce levels of Shiga toxin-producing *Escherichia coli* in sheep

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Healthy ruminants appear to be the main reservoir of Shiga toxin-producing *Escherichia coli* (STEC). Importantly, this pathogen is shed in faeces of sheep and can cause outbreaks of human illness ranging from diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) caused by Shiga toxin-producing *E. coli* (STEC) have been reported worldwide. The manure of ruminants when used as agricultural fertilizer can serve as a vehicle for STEC contamination of fruits, vegetables, water and soil. The aim of the present study was to evaluate whether the use of probiotic strains of *Ruminobacter amylophilus, Ruminobacter succinogenes, Succinovibrio dextrinosolvens, Bacillus cereus sub toyoi, Lactobacillus acidophilus* and *Enterococcus faecium,* supplemented to the daily oral food ration provided to sheep, together with composting of their feces, may be used as a strategy to reduce STEC levels on a farm. The first stage of the present study was performed during a six-week period with a total of 160 sheep distributed among four groups comprised of 40 sheep each. Group A did not receive either STEC or probiotic, Group B received probiotic alone, Group C received STEC plus probiotic and Group D received STEC alone. After the sheep were inoculated, samples of their feces were collected and the number of STEC and *E. coli* were counted. In the second stage of the study, after the six-week period, all fecal material was composted into four separate heaps. A possible protective effect of the probiotic strains against colonization by STEC was observed. It was also observed that composting was very efficient at eliminating or decreasing the STEC population. Although the number of STEC isolates was effectively decreased in all compost heaps, the Group C derived compost heap was found to have a lower amount of STEC than the Group D derived compost heap. These findings suggest that the use of probiotics, such as lactic bacteria, together with composting manure may be an efficient strategy to decrease the STEC population on a farm.

Key words: Composting, *Escherichia coli*, probiotic, Shiga-like-toxin

INTRODUCTION

Healthy ruminants appear to be the main reservoir of Shiga toxin-producing *Escherichia coli* (STEC) (Sanderson et al., 1999). Importantly, STECs are zoonotic pathogens that can cause food-borne diseases in

humans, ranging from diarrhea to hemorrhagic colitis (HC) and severe cases such as hemolytic uremic syndrome (HUS) (World Health Organization, 1998). Worldwide, sheep have been shown to be a major reservoir for STEC, including countries such as Norway (Urdahl et al., 2001), Russia (Kudva et al., 1998), Germany (Beutin et al., 2004), Spain (Rey et al., 2003) and Brazil (Vettorato et al., 2003)

Probiotics may be used as an alternative for decreasing the number of pathogenic bacteria, thereby decreasing the spread of these strains on a farm (Lema et al., 2001; Chaucheyras-Durand et al., 2010). The mechanisms by which probiotics cause microbial interference in the gut include nutrient competition, generation of an unfavorable environment and competition for attachment or adhesion sites resulting in reduced colonization by pathogenic bacteria (Caplice and Fitzgerald, 1999).

Manures composted for agricultural use have been shown to possess reduced amounts of zoonotic pathogens (Erickson et al., 2009). However, when this process is inefficiently applied or managed, fecal contamination of agricultural soil presents a potential risk of infection to humans and animals (Wu et al., 2009). Composting has been defined as intense microbial activity leading to decomposition of most biodegradable materials (Adani et al., 1997). Composting also allows the complete or partial degradation of key chemical compounds (Whitney and Lynch, 1996).

The use of probiotic strains to supplement the ration of livestock (Lema et al., 2001; Chaucheyras-Durand et al., 2010) and the use of composting to decrease the population of pathogenic microbes (Pourcher et al., 2005; Murkherjee et al., 2006; Gonçalves and Marin, 2007) have both been reported as efficient alternatives to decrease the spread of pathogenic strains on a farm. However, these studies were done separately, and there is little information about the combined use of the two practices to decrease the STEC population present on a sheep farm. Therefore, the aim of the present study was to evaluate whether the use of probiotic strains supplemented to the daily oral ration provided to the sheep, together with the composting of their feces, may be used as a strategy to reduce STEC levels on a farm.

MATERIALS AND METHODS

Probiotics and STEC strain used

The probiotic bacteria used in this study were *Ruminobacter amylophilus, Ruminobacter succinogenes, Succinovibrio dextrinosolvens, Bacillus cereus* sp *toyoi, Lactobacillus acidophilus* and *Enterococcus faecium* isolated from bovine rumina and intestinal tracts following the recommendations of Hungate (1975). These bacteria have the following features: they are nonpathogenic, enzyme-producing and resistant to lactic acid and low pH (Rigobelo and Ávila, 2012). It was performed the inoculum count resulting in 3×10^8 colony forming units (CFU) per gram, and then, each strain was lyophilized and mixed all together. Each ration was supplemented with 0.2% probiotic inoculums, and the treated animals received 200 g of rations per animal per day. Water was supplied freely.

It was used a STEC strain previously isolated from the feces of healthy sheep, and the presence of virulence genes was detected by a multiplex PCR (Vidal et al., 2005) in the Laboratory of Bacteriology and Veterinary Pathology. This strain carries *stx1, stx2* and *eae* genes and belonging to O101 sorogroup.

Trial design

A total of 160 sheep were sourced from five rural properties of Dracena region (Sao Paulo, Brazil) and housed at the research facilities of Sao Paulo State University (UNESP) located in Dracena city. The sheep Santa Ines breed at the fattening stage. The animals were selected based on equivalence of body weight (41±2 kg) and age (9-12 months). All groups were fed a commercial diet (Rações Pioneira, Ribeirão do Pinhal, Brazil) of identical composition. The animals were separated into four different groups (A, B, C and D) with 40 sheep in each group and kept separated for the six weeks of the experimental work. The animals belonging to Group A, the control group, did not receive either STEC or probiotics. Group B received probiotics in the feed for six weeks. Group C received a single oral dose of an STEC strain with the same probiotics given to Group B (probiotic given in the feed for six weeks). Group D received a single oral dose of the same STEC as given to Group C. This study was conducted in accordance with the ethical guidelines for investigations involving laboratory animals and was approved by the Ethics in Animal Research Committee (EARC), protocol number is 21/2010 of UNESP- Univi Estadual Paulista, Sao Paulo, Brazil.

Inoculation of sheep with STEC

All sheep of Groups C and D received orally a 40 ml of solution containing 2×10^9 viable STEC carrying the *stx1*, *stx2* and *eae* genes in 0.9% saline solution, as previously described (Ávila et al., 1986). After inoculation, the animals were monitored daily for changes in rectal temperature and the development of diarrhea.

Identification of STEC from feces

During the six-week period that the animals were kept separate, feces were collected directly with a rectal swab from each animal from each group weekly. The feces were then transported to the laboratory aseptically in a bottle. In the laboratory, the feces were weighed, and 1 g was inoculated into a bottle containing 10 ml Brilliant Green Bile broth, and the bottles were incubated at 37ºC, for 12 h. Next, 0.1 ml of broth was collected and cultured on MacConkey agar at 37ºC for 12 h according to Wollum, (1982). Colonies suspect of *E. coli* were identified based on the colony characteristics, Gram staining and biochemical profile (Koneman et al., 1997). It was performed the DNA extraction of the samples and then analyzed by PCR for the presence of *stx1*, *stx2* and *eae* genes

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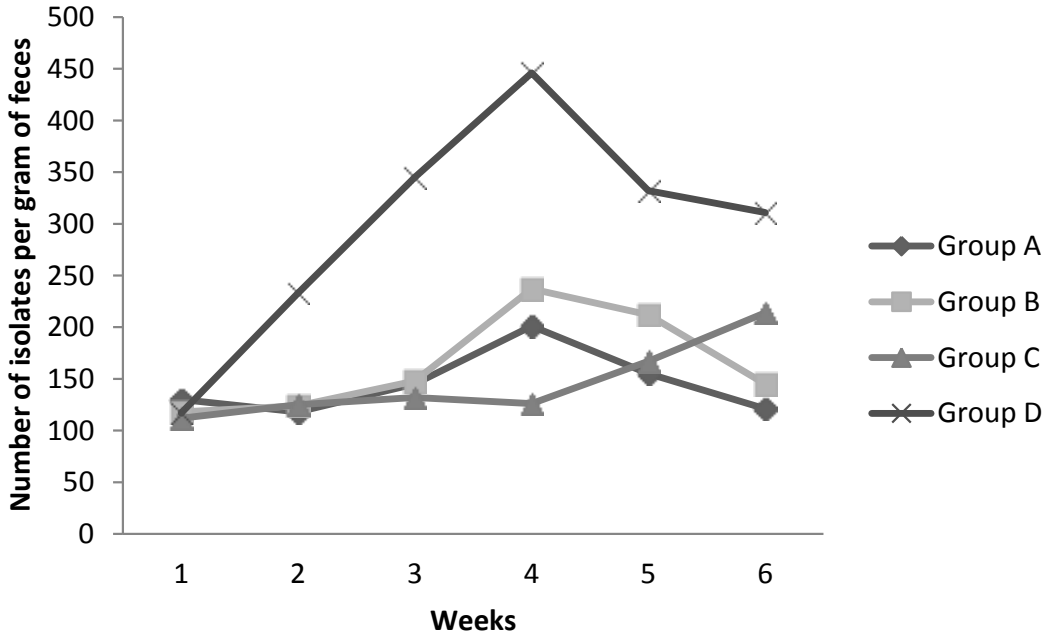

Figure 1. Number of STEC recovered from the feces of sheep in the different groups.

as previously described (Vidal et al., 2005). It was also performed the count of all samples carrying the *stx1*, *stx2* and *eae* genes, the same pattern as present in the STEC challenge strain.

Construction of compost heaps

For six weeks, the sheep's feces accumulated in the stalls of each group of sheep. During this period, the material of each group was not mixed and they were protected against birds, because these animals can transmit STEC. At six weeks post-inoculation, the material was collected separately for construction of compost heaps. The collected fecal material was heaped into four piles of 250 kg each, and the piles were used for the construction of separate compost heaps. Each heap was formed with the following dimensions: 0.5 m high, 0.5 m wide and 1.0 m long. Three parts of dry grass to one part of sheep's feces were added to each compost heap to equilibrate the ratio of nitrogen to carbon thus facilitating the decomposition of all material. Each day, the heaps were watered up to the saturation point, which was approximately 60% wet, and all material was turned every three days. Process control was based on temperature development. The study was performed at the Campus Experimental of Dracena from January to May, 2010 when the typical ambient temperatures average was 28ºC. Under the heaps, six equidistant points were marked, from which samples were collected weekly and were transported aseptically to the laboratory for analysis. The samples were collected for seven weeks and were analyzed for temperature, total number of bacteria and whether or not the bacteria carried the *stx1*, *stx2* and *eae* genes, through the methods cited below.

Isolation and identification of *E. coli* and STEC from compost heaps

During the composting period, the feaces in stalls was removed from housing pens to avoid the re-infection by STEC strains, and 25 g of compost were collected weekly from each sampling point and transported to the laboratory. In the laboratory, the samples were inoculated into 225 ml of Brilliant Green Bile broth, mixed and incubated overnight at 37ºC for 12 h. Next, 0.1 ml was collected and spread plate on MacConkey agar. The *E. coli* isolates were identified based on colony characteristics, Gram staining and biochemical profile (Koneman, et al., 1997). A loopful of colonies in the plate was collected, mixed and grown overnight in Luria Bertani (LB) broth at 37ºC. The DNA extraction was done according to Keskimaki et al. (2001) in which bacteria were pelleted from 1.5 ml of broth, suspended in 200 µl sterile distilled water and boiled for 10 min. Following centrifugation of the lysate, a 150 µl sample of the supernatant was stored at -20ºC as a template DNA stock. A multiplex PCR to detect *stx1*, *stx2* and *eae* genes was performed as described (Vidal et al., 2005). Strains testing negative for *stx1*, *stx2* and *eae* genes were considered as *E. coli* non-STEC.

Statistical analysis

A chi-square test was performed using the SAS software (SAS Institute 2001, technical report release 8.2, Cary, NC, USA) to determine the significance of the results.

RESULTS

Throughout the six-week period, the temperature range of all animals was 38.9 to 40.0ºC. No adverse effects were observed in the animals receiving the STEC strain or probiotics during this study.

The number of STEC strains re-isolated from the feces of animals in each group (A to D) for six weeks is shown in Figure 1. The group that yielded the highest number of STEC was Group D. The number of STEC isolates from Groups A, B and C did not differ statistically. The only group that presented a significant difference was Group

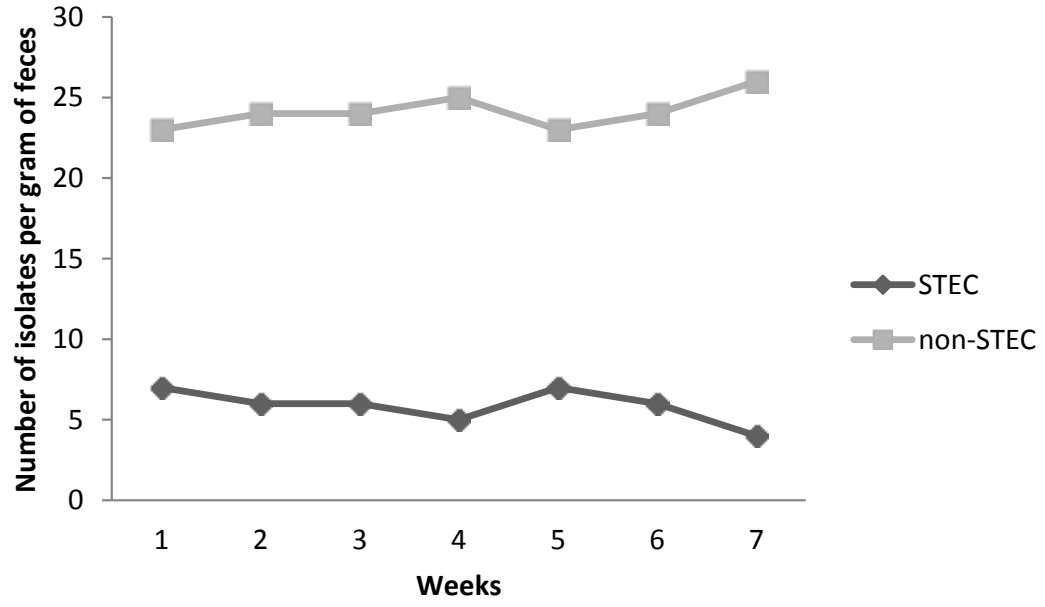

Figure 2. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group A.

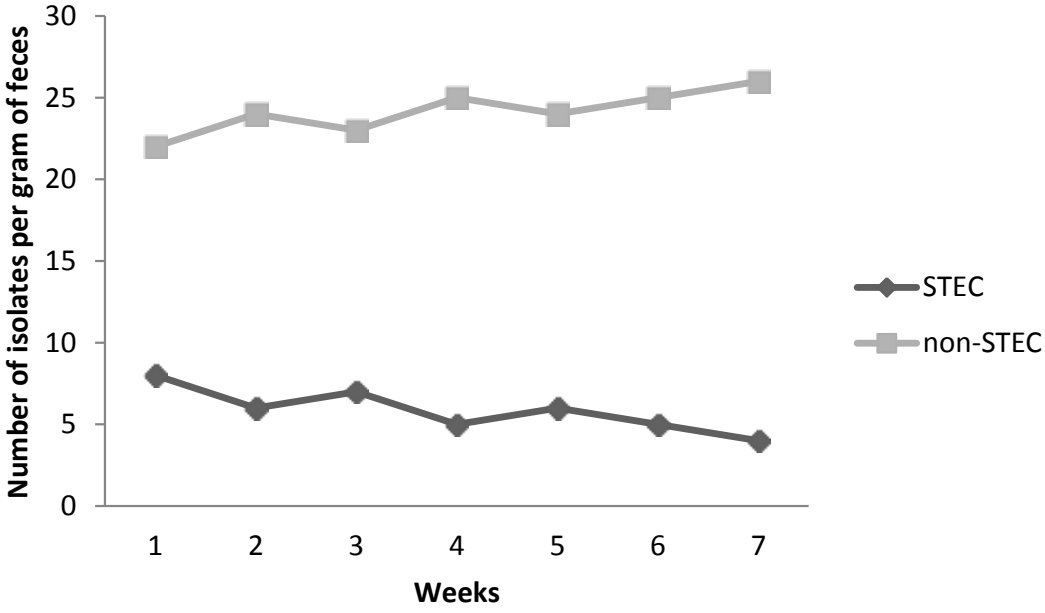

Figure 3. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group B.

D, which received one single dose of inoculum at a concentration of 2 x 10^9 cfu per ml of STEC.

During seven weeks, five *E. coli* strains were isolated from six equidistant points on the compost heaps, totaling 30 *E. coli* strains per heap per week. The number of STEC and *E. coli* non-STEC isolated weekly from all groups is shown in Figures 2 to 5. Both the control Group A and Group B were the groups from which the lowest number of STEC strains were isolated. Group C had the third lowest number of STEC strains. The group from which the largest number of STEC was isolated was Group D. However, the numbers of STEC isolates did not differ statistically between the groups. The compost heap that presented the largest initial number of *E. coli* strains was Group D. This fact was observed in spite of Group C having received the same concentration of STEC and the

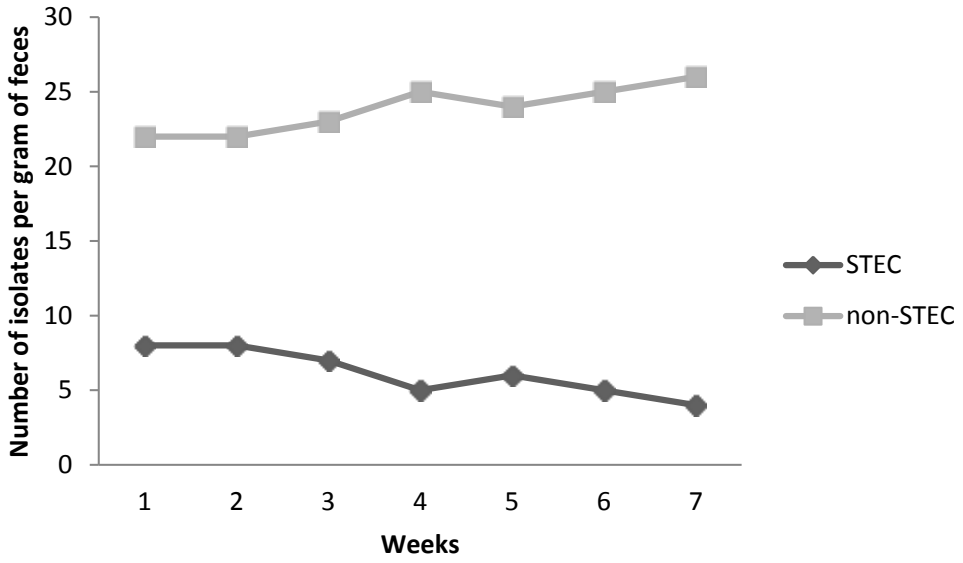

Figure 4. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group C

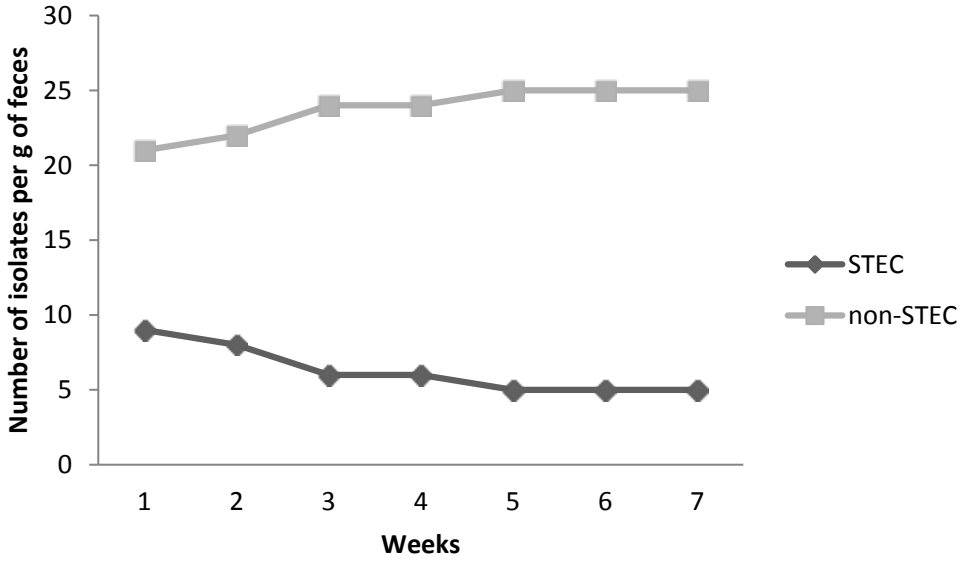

Figure 5. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group D.

probiotic strains. In this group, there was a lower number of strains spread into the feces, and these numbers were similar to those measured in Groups A and B.

There were no statistical differences between the numbers of STEC isolated from the compost heaps. All groups were efficient in the elimination of pathogenic strains. Group D had the largest initial number of STEC strains compared to the other groups. However, the composting process was efficient in the elimination high microbial concentration. The number of *E. coli* isolates began to decrease during the fourth week and had their lowest values during the sixth week. The composting process occurred normally among the compost heaps, that is, there was no difference in either temperature or the quality of materials composted. The average temperatures ranged from 12 to 65ºC for all treatments.

DISCUSSION

A range of enteric zoonotic pathogens, such as STEC, are present in animal manure when it is applied to agricultural land as a fertilizer, and these pathogens can be transmitted to both humans and animals (Borczyk et al., 1987; Pell, 1997). These fertilizers are provided by the livestock industry at large volumes annually (Haug,

1993; Pell, 1997; Sasaki et al., 2006; Mukherjee et al., 2006). STECs are zoonotic pathogens that can cause food-borne diseases in humans, ranging from diarrhea to HC and severe cases such as HUS (World Health Organization, 1998). Moreover, there are several studies showing the prevalence of STEC in sheep in Brazil, and because of it, the control of STEC has a great importance in public health (Martins et al., 2015; Vettorato et al., 2003).

The reasons leading to the colonization of the ruminant gut by STEC are unknown. Elucidating the relationships between ruminants and STEC may allow the development of interventions to prevent colonization by STEC, thereby eliminating STEC from the feces (Magnuson et al., 2000; Grauke et al., 2002). Moreover, the frequency, magnitude, duration and transmissibility of STEC strains in sheep are different in relation to other pathotypes, suggesting that the STEC strains are better adapted to persist in the alimentary tract of sheep (Cornick et al., 2000).

Rambaud et al. (1993) suggested that the protective effects caused by probiotics such as lactobacilli occur because the lactobacilli act as an adherence barrier to the surface of the gut. Probiotics may also produce their effects with viable as well as nonviable bacteria, suggesting that metabolic or secreted factors or structural cellular components may mediate their immune modulatory activities (Borches et al., 2009). The exact mechanism of balancing and interference of probiotic strains with the intestinal microbiota in sheep is unknown. More studies are necessary to investigate these relations. Lactic bacteria supplemented in the ration has been used as a strategy for decreasing the spread of *E. coli* in sheep (Lema et al., 2001; Guarner and Malagelada, 2003; Chaucheyras-Durant et al., 2010). In the present study, the group that received the probiotic had reduced colonization by STEC, resulting in a lower number of STEC being recovered from the feces of these animals, probably because of the lactic bacteria present at probiotic. This suggests that there was a protective effect against colonization by STEC. The protective effect of lactic bacteria occurs through a mechanism of competitive exclusion, including competition for nutrients and adhesion sites in the gut (Guarner and Malagelada, 2003; Millette et al., 2007). Several studies show that STEC was found in healthy ruminants, like sheep, cows and goats (Djordjevic et al., 2001; Zschöck et al., 2000), maybe because of it, the STEC inoculum used in the current study did not cause diarrhea. More studies are needed to explain whether this same effect would occur with a higher dose inoculum.

Together with the use of lactic bacteria to decrease the spread of pathogenic strains, it is possible to use of compost heaps as another means of decreasing the microbial population of the sheep manure. The composting process helps to ensure the hygiene of the final compost product (Pourcher et al., 2005; Murkherjee et al., 2006; Gonçalves and Marin, 2007). Previous studies have shown a long-term survival of more than 21 months for *E. coli* O157:H7 in manure held under a variety of environmental conditions (Kudva et al., 1998). In the current study, the survival of STEC strains was not greater than non-Shiga toxin producing *E. coli* as determined by comparing the number of isolates for each during the seven weeks. Gonçalves and Marin (2007) observed that the STEC strains seemed to be more sensitive to the action of temperature than the ordinary strains. All treatments that cause a decrease of the pathogen population of manure will certainly contribute to reduced dissemination of these pathogens on a farm as well as a reduced occurrence of outbreaks caused by these pathogens. Many studies have reported the efficiency of composting in decreasing the microbial population (Islam et al., 2004; Klein et al., 2010; Alexander et al., 2011). However, there is little information regarding the degree to which non-O157 STEC cells can survive in manure. Fukushima et al. (1999) reported the survival of STEC for up to 12 weeks when the temperature remained at 25ºC. Recently, Fremaux et al. (2007) reported the elimination of non-O157 STEC strains when submitted to composting in manure heaps after nine and 16 days at 35 and 56ºC, respectively. These results were similar to the present study in which the maximum temperature of the compost heaps was 65°C.

Although the number of STEC isolates was effectively reduced among all the compost heaps, the initial contamination of Group C by STEC during the first week that received probiotics together was lower than the Group D that received STEC alone. This high initial contamination by STEC in the feces might present a potential risk of contamination and spread of STEC on a farm, and the probiotics may contribute to decreasing this concern. Our findings suggest that the use of probiotics such as lactic bacteria, together with composting manure, may be an efficient strategy to decreasing the population of STEC on a farm.

Conflict of Interests

The authors have not declared any conflict of interest.

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