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ORIGINAL ARTICLE

Growth and survival of non-O157:H7 Shiga-toxin-producing *Escherichia coli* in cow manure

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cow, *Escherichia coli*, manure, Shiga toxin, survival.

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Abstract

Aims: The main objective of this study was to evaluate the behaviour of non-O157:H7 Shiga-toxin-producing *Escherichia coli* (STEC) strains in cow manure.
Methods and Results: A mixture of eight green-fluorescent-protein-labelled STEC strains was inoculated around 10^6–10^7 CFU g^{-1} into four manure heaps. Two heaps were regularly turned and the two others remained unturned. STEC counts and physical parameters (temperature, pH, moisture content and oxido-reduction potential) were monitored for 1000 manure samples. The highest mean pH values were obtained near the surface at the base of all manure heaps. At the surface, the moisture content decreased from 76·5% to 42% in turned heaps. Temperatures reached 65°C near the main body of all manure heaps, and only 35°C near the superficial parts located at the base of them. These two sites (the centre and the base) were associated with *D* values for the STEC counts of 0·48 and 2·39 days, respectively. We were able to detect STEC strains during 42 days in turned manure heaps and during at least 90 days in unturned ones.
Conclusions: These results emphasize the long-term survival of non-O157:H7 STEC in cow manure.
Significance and Impact of the Study: Good management practices (e.g. turning) should be respected in order to minimize the risk of environmental contamination by STEC.

Introduction

Shiga-toxin-producing *Escherichia coli* (STEC) are emerging foodborne pathogens. STEC strains were associated with both sporadic cases and outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome in patients, especially among children and elderly persons. Although *E. coli* O157:H7 is currently the most common serotype isolated from patients and the most reported in literature, other serogroups including O26, O103 or O111 are being more and more frequently recovered from infected patients.

Ruminants are recognized as the principal reservoir of STEC, and so undercooked beef, dairy product or vegetables contaminated by the faeces of infected cattle are frequent sources of human STEC infection. 10–25% of cattle are healthy carriers of STEC but highest levels have been reported for cattle from Brazil (Moreira *et al.* 2003) or France (Pradel *et al.* 2000).

A previous study highlighted that 11% of manure samples from French dairy cattle were contaminated by STEC (Vernozy-Rozand *et al.* 2002). A composting process of animal excrements is frequently included in farming practices to convert agricultural effluents into organic fertilizers before their spreading on culture crops. The transfer of pathogens to food through the application of animal manures to agricultural land is well described (Cieslak *et al.* 1993; Pell 1997). Manure that consists of animal excrements (faeces and urine) mixed with bedding is a complex biological process with thermal variations mainly because of fermentation by indigenous bacteria. A recent study demonstrated a

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4 $\log_{10}$ CFU g^{-1} reduction of *E. coli* O157:H7 in cow manure compost after 15 min at 60°C (Jiang *et al.* 2003b). Another study revealed that *E. coli* O157:H7 strains could survive 47 days (Kudva *et al.* 1998) in cow manure heaps (volume = 100 cm^3) exposed to climatic changes. These studies were limited to the O157:H7 serotype or were performed on very small manure heaps. Therefore, little is known about the growth and survival of STEC strains in dairy cow manure prepared and treated in compliance with commonly established dairy farms practices.

The main objective of this work was to study the survival and growth of STEC strains isolated from dairy cattle in cow manure. STEC strains were transformed with the green fluorescent protein plasmid vector (pGFPuv) and inoculated into different 2 m^3 heaps of fresh cow manure. Samples were taken at regular time intervals and in different parts of the heaps for determination of pH, moisture content, oxido-reduction potential (ORP) and numeration of transformed STEC bacteria by direct plating.

Materials and methods

Transformation of STEC strains

STEC strains

Eight STEC strains, all from faeces of one dairy cattle, were isolated between January 2003 and August 2004 during the course of a previous study (Fremaux *et al.* 2006). These *E. coli* strains were characterized for the presence of genes coding virulence factors: *stx1* and *stx2*, respectively, coding the Shiga toxins 1 and 2, *eae* coding the intimin and *ehx* coding the enterohaemolysin. Five STEC strains were *stx1* (+), 6 *stx2* (+), 4 *eae* (+) and 7 *ehx* (+). Two carried the four genes: *stx1, stx2, eae* and *ehx*. None of them belonged to the O157:H7 serotype.

Preparation of the transformant STEC cells

The eight STEC strains were electroporated in the presence of a plasmid vector pGFPuv (Ozyme, Montigny-Le-Bretonneux, France) carrying ampicillin resistance gene and the green fluorescent protein (GFP) open reading frame, according to the protocol described by Delazari *et al.* (1998) with minor changes. The competent bacterial cells were electroporated in a Gene Pulser II (Bio-Rad, Marnes-la-Coquette, France) with the plasmid vector pGFPuv and an electrical pulse of 4·7 m s was applied at 2·5 kV and 25 μF with the pulse controller adjusted to 200 ω. The resulting ampicillin-resistant-transformed cells showed bright green fluorescent colour when viewed under UV light at 365 nm (Herolab, Wiesloch, Germany).

Control of stability of the plasmid vector pGFPuv in STEC cells

Each GFP-labelled STEC strain was initially inoculated at a level of 10^6 CFU g^{-1} in small manure piles of 100 g. Each pile was sampled every week for 1 month. Each GFP-labelled STEC strain was enumerated by direct plating procedure and by the most probable number (MPN)-PCR *stx* method, as described in the following section.

The MPN-PCR stx protocol

The MPN procedure was performed using 96-well μl plates (Haines *et al.* 1996; Wrenn and Venosa 1996). Six-fold dilution series were prepared from all the manure samples. Fifty microlitres of each dilution were placed in 96-well μl plates, with five replicates per dilution. Then, 150 μl of enrichment broth, buffered peptone water (BPW; bioMèrieux, Marcy l'Etoile, France), was added to each well containing dilution. The plates were incubated for 24 h at 37°C. Thus, 1 μl of each well suspension was added to the PCR mixture containing bovine serum albumin (20 mg ml^{-1}) (Sigma-Aldrich, Steinheim, Germany) and dimethyl sulfoxide (Sigma-Aldrich), and subjected to the PCR *stx*. For each dilution, the *stx*-positive wells were enumerated and the MPN was estimated using standard Mac Grady tables (Hugues and Plantat 1983).

PCR detection of stx genes

The PCR was performed with degenerate primers ES149 and ES151, as described by Read *et al.* (1992). The amplification reactions were processed through 40 cycles in the PCR T3 Thermocycler (Biometra, Göttingen, Germany). After an initial denaturation step of 5 min at 94°C, the cycle program consisted of denaturation at 94°C for 30 s, annealing at 49°C for 30 s and extension by DNA polymerase at 72°C for 30 s. To ensure complete strand extension, the reaction mixture was incubated for 7 min at 72°C after the last cycle. The amplified product was visualized by ethidium bromide staining after gel electrophoresis of 10 μl of the final reaction mixture on 2·5% agarose.

Preparation and inoculation of the manure heaps

Preparation of the manure heaps

Approximately 10 m^3 of fresh cow manure was collected from one dairy farm in Rhône-Alpes region, in early spring 2005. To prove that manure was STEC free, several manure samples were collected from different parts of the initial manure heap. The enrichment of these and the DNA preparation were performed as described previously (Fremaux *et al.* 2006). A 1000-fold dilution of the DNA extract was subjected to the PCR *stx*. Then, the manure

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was divided into five heaps, each of which had a volume of 2 m^3 with a rectangular base of approx. 2 m^2 and a height of 1 m. The manure heaps were kept indoors in five separate rooms (P2 safety) for 3 months. The room temperature remained around 15°C during the course of the present study. Manure liquids were collected and evacuated by a specific trap. Three of the heaps were unturned and the two others were turned by mixing them three times during the study at interval of 7 days between each turning. Each heap was turned with a pitchfork.

STEC suspension and inoculation of the manure heaps

Each GFP-labelled STEC strain was grown overnight at 37°C on BHI plates (bioMérieux) containing ampicillin (150 μg ml^{-1}) (BHI-A). One colony of each GFP-labelled STEC strain was cultivated in 1 l of BHI broth plus ampicillin (150 μg ml^{-1}) at 37°C for 24 h (total bacterial culture, 8 l). All the cultures reached a final concentration of approx. 10^9 CFU ml^{-1}. The bacteria were sedimented by centrifugation at 5000 ***g*** for 20 min, and cell pellet was resuspended in 400 ml of phosphate-buffered saline and centrifuged. This procedure was repeated once and finally the cell pellets corresponding to the eight STEC strains were resuspended together in 800 ml of Tryptone medium (bioMérieux). The mixed suspension (800 ml) was used to inoculate four manure heaps to yield a final concentration ranging from 10^6 to 10^7 CFU g^{-1} at the start of the study. The last heap was used as a control negative heap. Each cubic metre was inoculated by pulverization with 100 ml of bacterial suspension and carefully turned and mixed to allow the uniformity of the inoculation. The latter was checked by enumerating STEC cells. Then samples of each manure heap were collected and analysed in the laboratory.

Sampling of solid and liquid manure

Different parts in each manure heap were sampled according to Fig. 1. For the unturned manure heaps, samples were collected from surface (sites A and F), the inner layers (sites B and D) and the main body (sites E and C). The corresponding sites for turned manure heaps were noted Aa to Fa. As the manure heaps could settle as a result of heat and moisture loss over a short time, the different sampling sites were relocated to appropriate relative positions. Furthermore, after each sampling occasion, each site was refilled with manure from the same location. Manure samples were taken for microbiological analysis at regular time intervals (using sterile gloves, changed between samplings). The liquids collected in the trap and on the floor near the heap were also sampled every week. Temperature results are obtained from the average of different points within each location. Inside

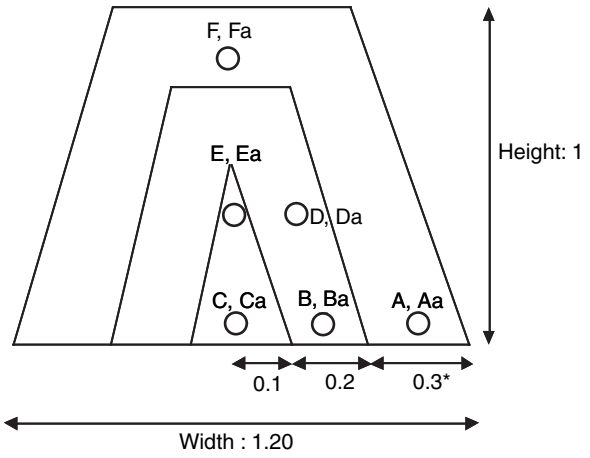

Figure 1 Cross section of a manure heap and location of the different sampling sites. The sites were noted A to F and Aa to Fa for unturned and turned manure heaps, respectively. The sites A, Aa and F, Fa were at the surface of the heaps (on the first 30 cm), the sites B, Ba and D, Da in the inner layers (30–50 cm) and the sites C, Ca and E, Ea in the main body (50–60 cm) of the heaps. *Measurements are expressed in metres.

samples from manure heaps were taken with hands protected with long sterile gloves. When the hand of the operator reached the appropriate location, a handful of manure was collected and carefully withdrawn from the heap to obviate any cross-contamination.

Hundred samples were collected per week (approx. 1000 samples were analysed during the course of the present study), transported to the laboratory and analysed at the same day.

Analysis

Enumeration of STEC cells by direct plating assay

Each manure sample (25 g) was added to 225 ml of BPW (bioMérieux) complemented with ampicillin (150 μg ml^{-1}) in a sterile Bagfilter (Interscience, Saint Nom La Breteche, France) and stomached for 30 s at medium speed. Aliquots from appropriate dilution series were spread onto selective BHI-A plates, which were incubated at 37°C for 24 h. The GFP-labelled STEC colonies were counted under UV light.

Concerning the liquid manure samples, 1 ml of each of them was taken and added to 9 ml of BHI broth. Aliquot of 1 ml was spread onto BHI-A plates that were incubated at 37°C for 24 h.

The enrichment of the sample was required when no colony of STEC was detected on the plate spread with the first decimal diluted suspension which means less than 10 STEC ml^{-1}. To enrich these poor STEC-contaminated samples, we incubated the first decimal diluted suspen-

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sion (Bagfilter) at 37°C for 24 h. After the enrichment step, 1 ml of the enriched suspension was plated onto BHI-A. The plates were incubated for 24 h at 37°C and placed under UV light to confirm the presence or absence of STEC strains in the sample.

Physical and chemical assays

The physical and chemical parameters were measured for each site in all manure heaps at regular time intervals. Total moisture content was determined by drying 5 g of manure at 105°C for 24 h and weighing the residual. The pH of manure sample was measured after adding 5 g of manure to 250 ml of distilled water. The suspension was then stirred for 5 min and was allowed to settle for 5 min. The pH of the liquid was measured with a pH meter 330 (WTW, Champagne au Mont d'Or, France). The ORP was measured by an ORP electrode sentix 81 (WTW). The temperature was measured with a thermic probe (WTW).

Statistical analysis

At each site and only for the unturned heaps, the log-linear model with tailing (Geeraerd *et al.* 2000) was used to fit the STEC counts observed at the beginning of the kinetics, i.e. during the first 30 days

$$N = (N_0 - N_{res})\exp(-k_{max}t) + N_{res}$$

where N represents the STEC density (CFU g^{-1}) observed at time t (days), N_0 is the STEC density (CFU g^{-1}) of the inoculum, N_{res} is the residual STEC density (CFU g^{-1}), and k_{max} is the specific inactivation rate (day^{-1}) equal to $\ln(10)/D$ value. Estimated values of k_{max} for each site were graphically reported with their respective 95% confidence intervals. This model was fitted on the decimal logarithm of the STEC counts using the Ginafit tool (Geeraerd *et al.* 2005).

Statistical comparisons of pH and redox mean values between sites were performed using a one-way ANOVA. Multiple comparisons were performed using Bonferroni's correction for P values. These statistical calculations were performed using the R software version 1.9.0 (Ihaka and Gentleman 1996).

Results

Stability of the plasmid vector pGFPuv in STEC cells

A first assay was performed to confirm the stability of the plasmid vector pGFPuv in nonselective conditions. The GFP-labelled STEC strains could be enumerated for 14 days. At the 7th and the 14th day, the level of all GFP-labelled STEC strains was around 10^4 and 10^2 CFU g^{-1}, respectively. These results were consistent with those noted using the MPN-PCR method. After 21 days, no *gfp*$^+$ STEC strains could be detected, even after an enrichment step.

STEC counts and temperature profiles in unturned cow manure heaps

As shown in Table 1, the STEC inoculum levels ranged from 5·45 to 6·81 $\log_{10}$ CFU g^{-1}. The numerations of STEC and the readings of temperatures were performed for each of the six sites of the heaps and during the whole sampling period. The results obtained for the sites A, E and F during the first month of the present study are displayed in Fig. 2. The log-linear model with tailing was fitted to STEC counts data (Fig. 2), which permits an estimation of the specific inactivation rate (k_{max}) and of the D value ($\ln(10)/k_{max}$). The specific inactivation rates with asymptotic 95% confidence intervals for each site of unturned manure heaps are shown in Fig. 3.

The specific inactivation rate of STEC progressively increases as it approaches the heart of the manure piles (Fig. 3). The estimated D values of the sites A (near the surface at the base of the heap), F (on the surface at the top of the heap), B (inner layers at the base of the heap), C (in main body at the base of the heap) and D (inner layers in the middle of the heap) were 2·39, 1·57, 1·54, 1·15 and 0·78 days, respectively. Compared with the results obtained with these precited sites, site E (main body in the middle of the heap) was associated with the

Table 1 Time of survival of GFP-labelled STEC strains in the different sites of all manure piles and maximum temperature recovered in each of them

Sites	Maximum temperature (°C)	STEC count ($\log_{10}$ CFU ml^{-1}) at day 0	Time of survival of STEC (days)
Unturned piles			
A	35	6·17	90
B	46·5	6·67	42
C	59	5·45	23
D	56	5·57	16
E	65	6·45	9
F	58	6·03	90
Turned piles			
Aa	35	6·7	42
Ba	46·5	6·81	42
Ca	65	6·17	20
Da	58	5·81	35
Ea	68	6·07	23
Fa	59·5	5·97	23

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weakest *D* value of 0·48 days. For all the sites, the STEC cells markedly decreased during the first 10 days but residual STEC populations could be detected for at least 90 days in sites A and F, and only 9 days in site E (Fig. 2; Table 1). The initial temperature was around 20°C in all sites from the manure heaps. Thereafter, the temperature reached maximum values of 46·5°C, 59°C, 56°C and 58°C for the sites B, C, D and F, respectively, but only 35°C in site A (Table 1). Inside site E, the temperature was higher than those noted in other sites, with a maximum temperature being recorded at 65°C. Average temperatures of 37°C in site B, 51°C in sites C, D and F and 61·5°C in site E were noted between the second and the sixth days. Then the temperature declined gradually to reach its initial level (20°C) for all the sites.

STEC counts and temperature profiles in turned cow manure heaps

The turning of the heaps resulted in a noticeable fluctuation of both STEC counts and temperature in each sampled site (Fig. 2). As a consequence, no model could be adequately fitted to the data obtained. STEC strains could not be detected after 42 days from manure. As shown in the Table 1, a residual STEC population was recovered from manure heaps up to 42 days in sites Aa (a: corresponding to turned manure heaps) and Ba, 35 days in Da, 20 days in site Ca and 23 days in sites Ea and Fa.

STEC counts decreased by approx. 4 $\log_{10}$ CFU g^{-1} during the first 10 days of our study in sites Aa and Ba, but only for the first 8 days in sites Da and Fa and 5 days

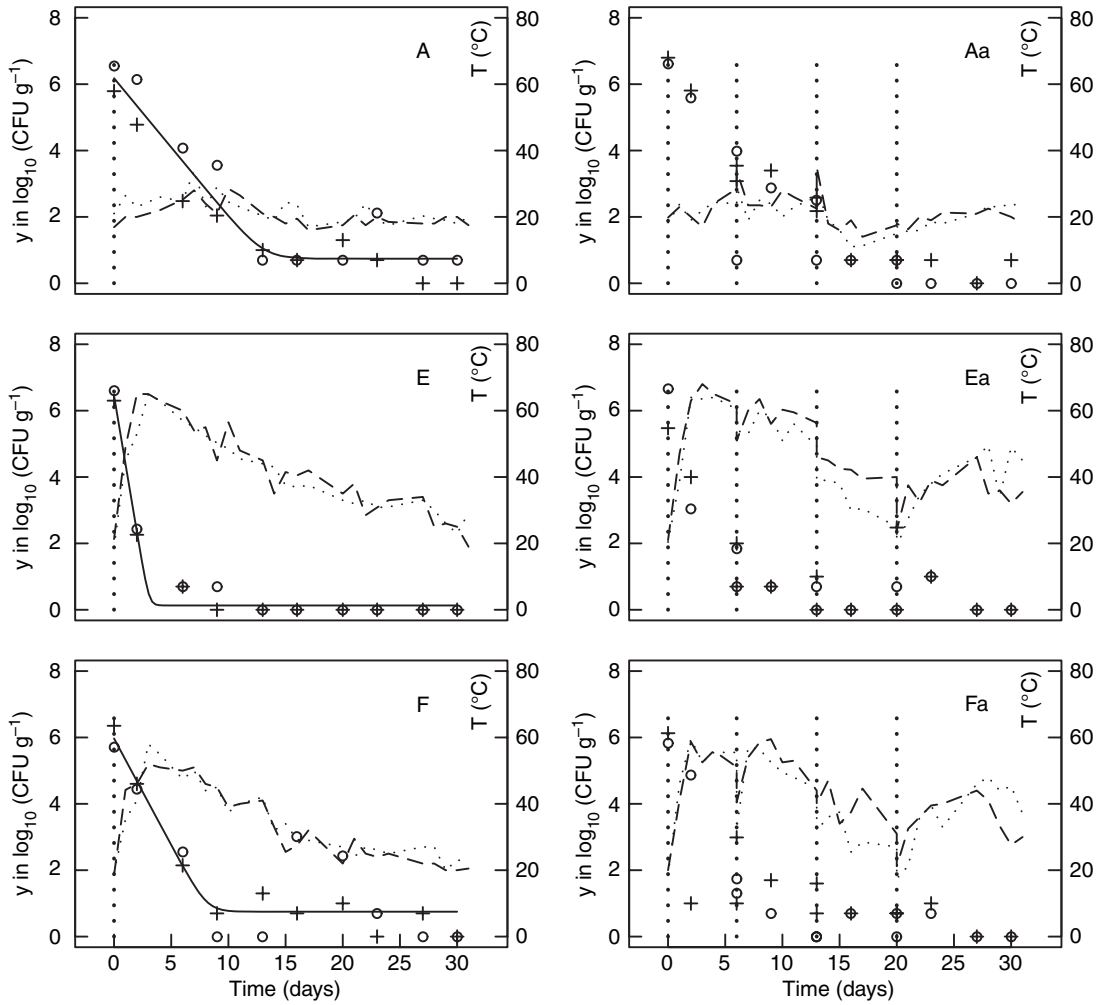

Figure 2 STEC counts obtained in the two unturned manure heaps [heap 1 (○), heap 2 (+)] for each site A, E and F and the two turned manure heaps [heap 1 (○), heap 2 (+)] for each site Aa, Ea and Fa. The dotted lines indicate each temperature profile obtained in the site of the two manure piles. The exponential decay lines represent the log-linear model with tailing only fitted to the survival data of the different sites in unturned manure heaps. The aeration times are displayed with vertical dotted lines for the turned manure heaps.

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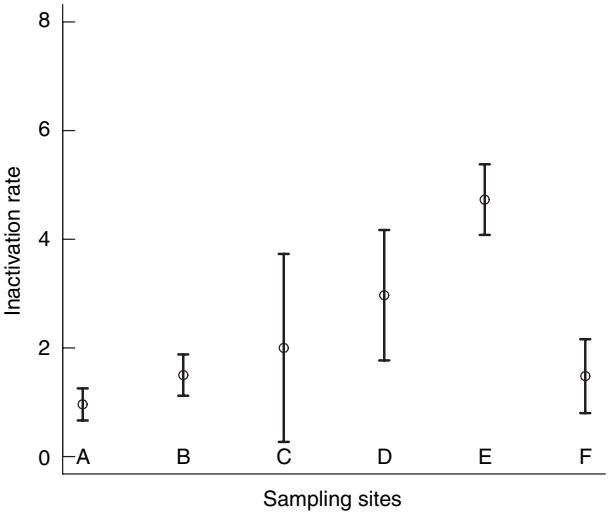

Figure 3 Specific inactivation rate in cow manure with 95% confidence interval estimated for each site in unturned manure heaps.

in sites Ca and Ea (Fig. 2). This fall of STEC concentration was concomitant to a marked increase in the temperature to maximum values of 65°C and 68°C in sites Ca and Ea with average values of 58°C and 62·2°C between the second and the eighth days, respectively. Over the same period, the sites Da and Fa had temperatures ranging from 58°C to 59·5°C with average values of 54°C and 56°C, respectively. The lowest temperatures were obtained for sites Aa with an average value of 22·5°C and Ba with 36°C. At the second turning (13th day), only residual STEC populations could be detected in manure heaps. Then the temperature decreased, ranging from 30°C to 37°C in all the sites except for the sites Aa and Ba for which the temperature reached around 20°C at the 17th day of the study (Fig. 2). The third turning led to a slight rise in the temperature that reached 40°C in both the four locations Ca, Da, Ea and Fa, and 25°C for the sites Aa and Ba, before finally decreasing to its initial level of 20°C.

STEC counts in liquid manure

The liquid manure appeared on the soil approx. 9 days after the storage of the manure heaps in different rooms. The STEC counts were superior to 2 $\log_{10}$ CFU ml^{-1} in all the sampling sites (floor near all manure heaps and recuperation traps) except for the recuperation traps close to the turned heaps in which STEC counts ranged from 1 to 2 $\log_{10}$ CFU ml^{-1}. These results were comparable to those obtained in sites A, Aa, B and Ba (approx. 2 $\log_{10}$ CFU g^{-1}). After the 35th day, a fall of STEC contamination (<0·5 $\log_{10}$ CFU ml^{-1}) was noted (a complete drying of the liquid from all manure heaps did not allow any sampling).

Determination of pH, ORP and moisture content

Results are expressed as mean values obtained on the one hand for the two unturned manure heaps and on the other for the two turned heaps. For each site, the pH and ORP parameters have only slightly fluctuated during the course of the study and were consequently expressed as mean values. The results are summarized in Fig. 4.

A significant site effect was obtained for pH values using the ANOVA test ($P < 0{\cdot}0001$). Multiple comparisons

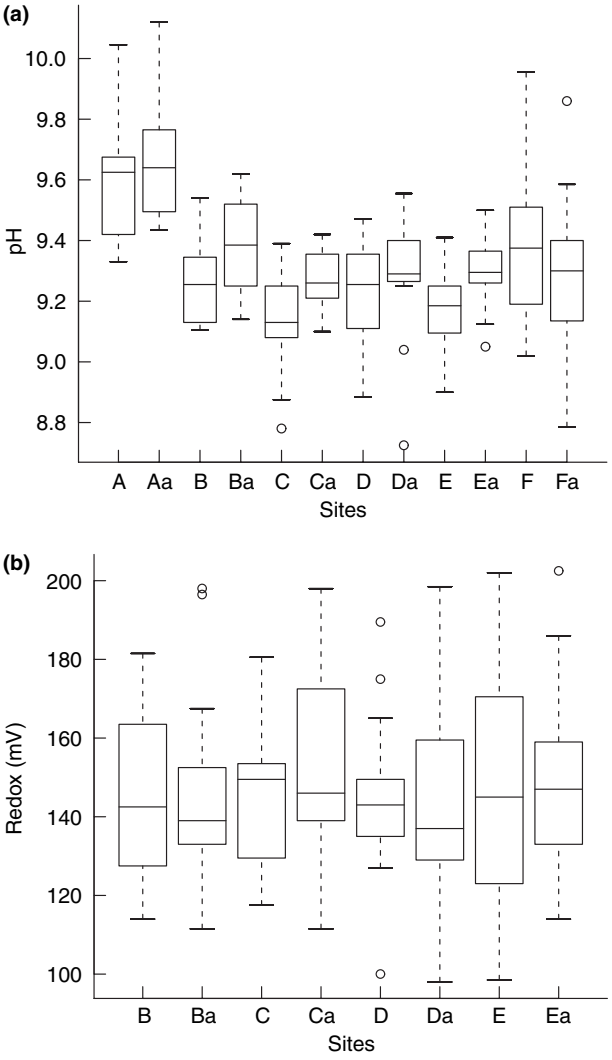

Figure 4 Box plots of pH and redox values obtained for each site in unturned and turned manure heaps. The 25th, 50th (median value) and 75th percentiles and extreme values (displayed by two tails) are shown.

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showed a significant difference ($0{\cdot}0001 \leq P \leq 0{\cdot}004$) between samples collected near the surface at the base of all manure heaps (sites A and Aa) that presented the highest mean pH values (9·59 and 9·67) and the other sites. Concerning these other sites, a mean value of 9·3 was noted for both turned and unturned manure heaps.

The ORP values ranged from −97·5 to −200 mV in the inner layers and in the middle of the cow manure heaps, with no significant difference between all the sites ($P > 0{\cdot}05$). Mean values of −152 mV was obtained for turned and unturned heaps, indicating anaerobic conditions in the middle and inner layers.

The moisture content was high and remained steady during the course of the study with values ranging from 72% to 83% for inner layer and middle of all manure heaps (sites B, Ba, C, Ca, D, Da and E, Ea) (Fig. 5). At the surface (sites A, Aa and F, Fa), it decreased over time from 76·5% to 42% for turned heaps and from 77·5% to 37% for unturned heaps, showing a drying at the surface of the heaps, with a fastest drying observed near the surface at the base of all manure heaps.

Furthermore, the same trends for pH, redox and moisture content profiles were observed for the negative control manure heap (data not shown).

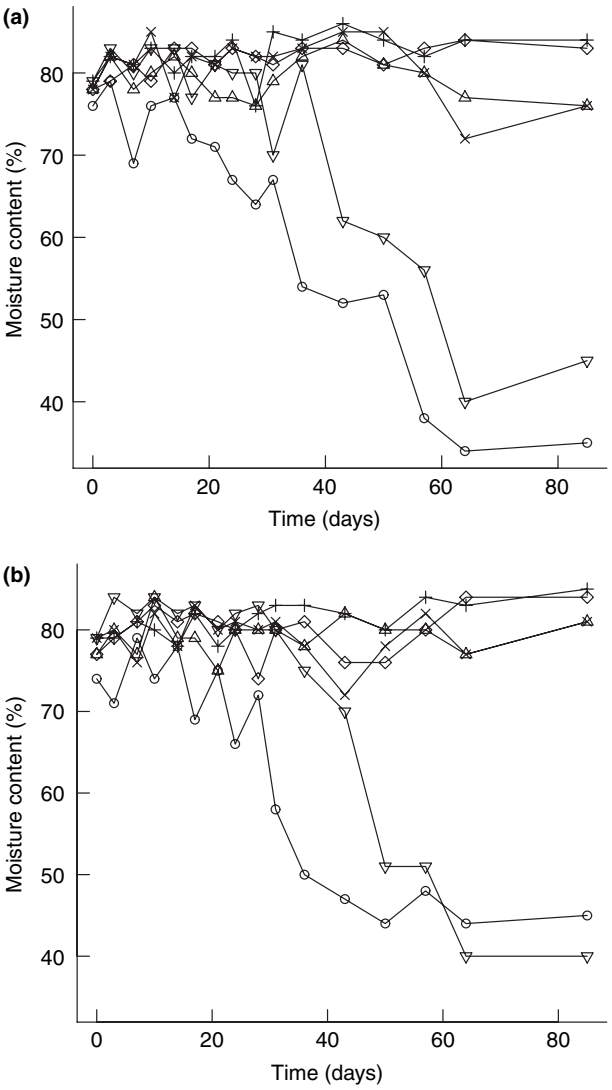

Figure 5 Mean values of moisture content profiles obtained during the course of the study for each site in unturned manure heaps (a: ○, A; △, B; +, C; ×, D; ◇, E; ▽, F) and turned manure heaps (b: ○, A; △, Ba; +, Ca; ×, Da; ◇, Ea, ▽, Fa).

Discussion

As the prevalence of cattle carrying STEC in their faeces may be high, 6% reported in a US study (Cray *et al.* 1996) to 71% in a French study (Pradel *et al.* 2000), and as STEC is known to survive for long periods in cow faeces (126 days at 15°C) (Fukushima *et al.* 1999), manure that is a mix of animal excreta (faeces plus urine) and bedding could constitute an important vehicle for environmental STEC contamination (Pell 1997).

The objective of our study was to examine the survival of eight GFP-labelled STEC strains inoculated in cow manure. We also monitored the physical and chemical parameters such as temperature, pH, moisture content and ORP. Previous works studied the survival of *E. coli* O157:H7 in cow manure, but none of them described the behaviour of non-O157:H7 strains (Kudva *et al.* 1998; Himathongkham *et al.* 1999; Jiang *et al.* 2003a; Nicholson *et al.* 2005). Furthermore, we wanted that our experiment was closed to real farming practices. Consequently, we used heaps of fresh manure with a volume of 2 m^3. This size was larger than those used by Himathongkham *et al.* (1999), Jiang *et al.* (2003a) and Kudva *et al.* (1998), with 120 g, 15 dm^3 and 100 cm^3 of manure heaps, respectively. The STEC inoculum level was fitted to 10^6–10^7 CFU g^{-1} in order to be in conditions simulating a high STEC quantity in faeces, which can reach 10^8 CFU g^{-1} (Fukushima and Seki 2004). Moreover, a large diversity of STEC strains isolated from faecal samples of a same dairy farm was previously reported (Beutin *et al.* 1997; Nielsen *et al.* 2004).Consequently and in an attempt to be closed to dairy farm conditions, a mix of eight STEC strains was used to inoculate all manure heaps. In compliance with European rules set for the utilization of STEC and genetically modified organisms, we placed the manure heaps in a lazaret of P2 safety level to ensure environmental protection.

STEC strains do not have common biochemical characteristic allowing the use of specific media for their isolation. Instead of using a genetical method including a *stx* PCR on enriched broths followed by a hybridization step with *stx* probe to mark the STEC colonies on the

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plate, which is a time-consuming and difficult-to-perform method, we transformed eight STEC strains with a pGFP plasmid. These STEC strains were previously isolated from faecal samples and immediately stocked at −80°C in order to prevent the loss of survival characteristics and particularly the ability to attach to surfaces. The stability of the *gfp* gene used to label a large variety of bacterial species has been checked under different high-pressure conditions in previous studies (Fratamico *et al.* 1997; Delazari *et al.* 1998; Ehrmann *et al.* 2001; Vialette *et al.* 2004). The plasmid did not affect the intrinsic characteristics of the *E. coli* O157:H7 strains under nonfavourable environmental conditions, i.e. high concentrations of NaCl or high temperatures, and no significant difference of behaviour was noted between the transformed *E. coli* O157:H7 strains and their parent strains (Ehrmann *et al.* 2001; Vialette *et al.* 2004). To make sure that the eight transformed STEC strains would not lose their plasmid during our study, we made a preliminary experiment using small manure heaps (100 g) inoculated with 10^6 CFU g^{-1} of each of our eight STEC strains. An MPN-PCR *stx* assay was performed in parallel and confirmed the counts obtained with the direct plating method, demonstrating a persistence of the plasmid inside the STEC cells during the course of this preliminary work, i.e. 21 days. An MPN-PCR *stx* assay was also used at some steps of the present study to check the possible loss of the plasmid or the none expression of the *gfp* gene. As in the preliminary work, similar results were obtained by direct plating method and MPN-PCR *stx* assay (data not shown). Finally, PCR detections of *stx* genes were randomly performed to confirm that fluorescent colonies screened on agar plates under UV light were STEC colonies.

Faeces composting, i.e. manure, is frequently included in farming practices to recycle organic materials mainly because it allows the transformation of organic compounds into biologically stable humic substances that make excellent soil amendments. This process also leads to the reduction of the contamination by numerous pathogenic micro-organisms. This clearance is readily explained by a thermal destruction. More precisely, the composting process may be divided into three distinct phases: a mesophilic phase, a thermophilic phase, during which common pathogens are killed, followed by a gradually decrease of temperature, called curing phase. The temperature increase is mainly because of the action of indigenous aerobic flora (fungi, bacteria and protozoa) and is associated with an important release of water, CO_2 and ammonia. Composting with some turning results in aerobic conditions allowing the increase of the aerobic flora activity and then a more efficient decomposition of the faecal materials (Rynk 1992).

In our experiment, composting process included a short mesophilic phase of approx. 2 days, followed by a thermophilic phase of 1 week for unturned manure heaps and 2 weeks for turned ones, with a marked increase in temperature for sites C, D, E and F of all manure heaps. However, only sites Ca, E and Ea showed temperatures superior or equal to 65°C. Sites A and B of all manure heaps showed lower temperatures with maximum values of 35°C in both the sites A and Aa, and 46·5°C in both the sites B and Ba. These results might be due at least in part to the loss of heat from the superficial parts (A and Aa) and to the uneven distribution of oxygen and nutrients that might influence the microbial activity. Furthermore, the heat release towards the top of the manure heap explained the fact that site D or F displayed a higher temperature than the one noted in site B.

The highest pH value was noted near the surface at the base of all manure heaps (9·59 and 9·67). On the contrary, Himathongkham *et al.* (1999) or Jiang *et al.* (2003a) found higher pH values (8·97 and 8·45, respectively) near the top of the heaps. We have no explanation for those discrepant results. The moisture contents were fairly steady in our study for the middle and inner layers but decreased on the surface of the manure heaps. The values ranged from 77·5% to 37% for unturned heaps and from 76·5% to 42% for turned manure heaps. Similarly, Himathongkham *et al.* (1999) and Jiang *et al.* (2003a) indicated lowest moisture content at the surface of the heap, near the top of the heap (59·3%) for the first ones and near the base of the manure heap (44·7%) for the second ones. As far as the ORP is concerned, it was negative for all manure heaps, regardless of the turning, demonstrating anaerobic conditions in main body and inner layers of the manure heap. Similar observations have already been reported, with ORP values that ranged between −70 and −230 mV (Himathongkham *et al.* 1999).

The GFP-labelled STEC strains were detected during 42 days in the periphery of turned manure heaps and 90 days in unturned ones. These results confirm the turning effect and were similar to the earlier findings of Kudva *et al.* (1998), who detected *E.coli* O157:H7 in turned cattle manure heaps during 47 days. However, another study reported that *E. coli* O157:H7 could survive no more than 8 days in unturned manure heaps and 4 days in turned manure heaps (Nicholson *et al.* 2005). Likewise, Lung *et al.* (2001) showed that *E. coli* O157:H7 could survive for 4 days in 560 g of cow manure placed inside Pyrex glass chamber and incubated at 45°C. This short duration could be explained by the low inoculum size and the small manure heaps used by the authors and strongly contrasts with the composting process carried out in farming practices. All the more, as several factors could affect the

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recovery of STEC from manure heaps and thus influenced the survival data obtained in our experiment. Especially, the existence of inaccessible niches to the sampling as a result of the formation of microcolonies, by attachment to the surface of manure fibres, could influence the removal and the undistribution of the bacteria from the heap. Moreover, the possibility of the unicellular protozoa feeding on the bacteria cannot be dismissed (Kuczynska and Shelton 1999). A previous study demonstrated that *E. coli* O157:H7 may still be able to survive for an extended period after being consumed by protozoa (Barker *et al.* 1999).

During the mesophilic phase, no evidence of multiplication of the STEC strains was noted for all manure heaps, contrary to other studies that indicated a slight growth of *E. coli* O157:H7 (Delazari *et al.* 1998; Himathongkham *et al.* 1999; Jiang *et al.* 2003a). For example, Delazari *et al.* (1998) reported an increase of *E. coli* O157:H7 counts from 7·84 to 8·25 $\log_{10}$ CFU g^{-1} for the first 5 days at 37°C. During the active phase of the composting, we noted the highest decrease of STEC counts in site E for unturned manure heaps and in sites Ca and Ea for the turned heaps. The high temperature noted in these sites (≥65°C) readily explained the marked inactivation or the death of the inoculated STEC strains. In fact, Jiang *et al.* (2003b) showed a decrease of *E. coli* O157:H7 concentration by 4 $\log_{10}$ CFU g^{-1} for 3 h at 55°C, only 15 min at 60°C and 3 min at 65°C. These results highlighted that a temperature exceeding 60°C is linked to a far more rapid clearance of STEC cells. But our *D* values (≥0·48 days) greatly exceeded those noted in Jiang *et al.* (2003b) study. This could be explained by the smaller manure heap used by these authors implying a too rapid temperature increase that could prevent an adaptation of some *E. coli* O157:H7 cells to a thermal stress. As far as the sites C, D, F of the unturned manure heaps and Da, Fa of the turned manure heaps are concerned, the temperatures ranged from 55°C to 60°C and partially contributed to the inactivation of STEC in these sites. As the sites A, Aa, B and Ba where the temperatures are not high enough (<47°C) to explain the STEC inactivation, other factors such as high pH, antagonistic flora or low moisture content could lead to this reduction. Himathongkham and Riemann (1999) suggested that an increase of pH by gassing with ammonia or drying resulted in less than 2 $\log_{10}$ reduction of *E. coli* O157:H7 after 24 h. Drying followed by gassing with ammonia resulted in a 4 $\log_{10}$ CFU g^{-1} reduction after the same time. Conversely, Jiang *et al.* (2003a) reported a large difference between the reduction of *E. coli* O157:H7 populations in cow manure placed in a bioreactor at 21°C and 50°C with reduction values of 0·5 $\log_{10}$ CFU g^{-1} after 1 week *vs* 4·5 $\log_{10}$ CFU g^{-1} after 24 h, respectively. This finding is not in accordance with our results and could be explained by the small size of the manure heap used in their experiment. Moreover, the heaps were not placed in an outside situation and thus not exposed to fluctuating environmental conditions. In winter, the temperature often reaches negative values and presumable survival would be far longer (Wang *et al.* 1996; Kudva *et al.* 1998). The antimicrobial efficacy of solar radiation (UV) on STEC cells and the rainfall events could not be neglected (Yaun *et al.* 2003, 2004). Finally, in the farm situation, fresh material was added to the manure over the time, leading to possible long-term survival of STEC.

During the curing phase, STEC counts slightly decreased for all manure heaps. In fact, it has been shown that treatment of manure by thermophilic digestion caused a rapid initial decline in the numbers of viable *E. coli*, but this decrease was followed by a period during which the residual population was maintained for an extended period (Kearney *et al.* 1993). The gradual stress conditions in some zones, particularly near the surface of manure heaps, could explain the survival of this STEC population linked to the expression of heat shock genes, thereby resulting in increased resistance of pathogens during composting (Jiang *et al.* 2003a).

Finally, the liquid manure that lately appeared on the soil (ninth day) had a similar STEC concentration to that of the manure heap at the same time. As a result, it might be a good indicator to assess the STEC contamination of the manure heaps.

In conclusion, our study demonstrated a long-term survival of non-O157:H7 STEC strains in cow manure. The manure spreading on culture crops may represent a way for pathogens to travel further up the human food chain if the composting process was unadapted. European Union rules (CE no. 1774/2002) recommend minimum 1 h of composting at least to 70°C. These rules were more adapted to indoor process (bioreactor, wastes transformation factory, etc.), where the external temperature can be monitored. This temperature is not reached for all locations of the manure heaps in farm situation. Consequently, STEC strains would be able to survive during long periods.

In order to minimize the risk of environmental contamination by STEC, during the storage and following the amendment of manure to land, good management practices should be followed. According to Fenlon *et al.* (2000) results, the manure should be stored on a cement floor for a sufficient period (minimum 2 months with turning) and preferentially in a large heap form allowing a great temperature rise, and turned to sanitize the manure heap. All liquid manure should be collected in a specific trap. These farming practices minimize run-off and leaching of liquid manure into groundwater. A no-grazing

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or cutting period for grass following manure application was also recommended. Finally, farm manures should not be applied to fruit or vegetable crops that will be eaten raw.

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