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ORIGINAL ARTICLE

Persistence of Shiga toxin-producing *Escherichia coli* O26 in various manure-amended soil types

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Abstract

Aims: To evaluate the behaviour of Shiga toxin-producing *Escherichia coli* (STEC) O26 strains inoculated in manure-amended soils under *in vitro* conditions.

Methods and Results: Four green fluorescent protein (GFP)-labelled STEC O26 strains were inoculated in duplicate (at 10^6 CFU g^{-1}) in three different manure-amended soil types, including two loam soils (A and B) and one clay loam soil (C), and two incubation temperatures (4 and 20°C) were tested. STEC counts and soil physical parameters were periodically monitored. STEC O26 cells were able to persist during extended periods in soil even in the presence of low moisture levels, i.e. less than 0·08 g H_2O g^{-1} dry soil. At 4 and 20°C, STEC could be detected in soil A for 288 and 196 days, respectively, and in soils B and C for at least 365 days postinoculation at both temperatures. The ambient temperature (i.e. 20°C) was significantly associated with the highest STEC count decline in all soils tested.

Conclusions: The temperature and soil properties appear to be contributory factors affecting the long-term survival of STEC O26 in manure-amended soils.

Significance and Impact of the Study: This study provides useful information regarding the ecology of STEC O26 in manure-amended soils and may have implications for land and waste management.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are one of the most important emerged groups of food-borne pathogens. STEC strains can cause a broad spectrum of human illness ranging from uncomplicated diarrhoea to haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS) (Riley *et al.* 1983; O'Brien and Kaper 1998). Although *E. coli* O157:H7 is currently the most frequently isolated serotype from human patients, other STEC-serogroups were often isolated in Europe (Caprioli *et al.* 1997) and North America (Brooks *et al.* 2005). Among them, serogroup O26 is regarded as an important cause of STEC-associated diseases (Tarr and Neill 1996). In Germany, a cluster of HUS cases caused by STEC O26:H11 was detected, but the source of the STEC infection was unknown (Misselwitz *et al.* 2003). Furthermore, an outbreak linked to the consumption of Camembert-type cheeses contaminated by O26 EHEC was recently reported in France. Six children of less than 2 years old developed an HUS (Espie *et al.* 2006).

STEC strains with O26-serogroup were frequently found in cattle with prevalence rates of 5·1% in Japan (Fukushima and Seki 2004) or 1·7% in Australia (Cobbold and Desmarchelier 2000). In France, a recent study showed that 15% (2/13) of the dairy farms tested were positive for STEC O26 (Fremaux *et al.* 2006). The long-term survival of STEC O26 in cow faeces (up to 18 weeks at 15°C) has important health implications as they might be spread to crops during direct application of farm effluents to land (Fukushima *et al.* 1999). Nowadays, land application is becoming a routine procedure for the

disposal of animal wastes of faecal origin. The potential transfer of zoonotic agents to food through the application of animal excreta as soil fertilizers in agriculture has been extensively reviewed (Cieslak *et al.* 1993; Pell 1997).

A recent study showed that *E. coli* O157:H7 is able to persist for extended time in soil treated with contaminated manure (more than 7 months) (Islam *et al.* 2004). Both biotic and abiotic parameters could affect the persistence of faecal bacteria including *E. coli* in soil (Chandler and Craven 1980; Recorbet *et al.* 1992; Kerry 2000). Many studies described the impact of temperatures, physico-chemical (pH, moisture and organic matter content) and texture soil properties (clays content) on the survival of *E. coli* O157:H7 in soil. Jiang *et al.* (2002) and Mukherjee *et al.* (2006) showed that the *E. coli* O157:H7 decline in manure-amended soil stored at 4°C was faster than that of soil stored at ambient temperature. Moreover, Fenlon *et al.* (2000) showed that *E. coli* O157:H7 viable cells fell from approx. 10^5 CFU ml^{-1} to undetectable levels within 8 weeks in a sandy soil compared with 25 weeks in a clay soil. However, these studies were limited to the O157:H7 serotype and little information is available regarding the fate of STEC O26 in manure-amended soils.

Therefore, the objective of the present study was to investigate the fate of four STEC O26 strains in manure-amended soils, and to evaluate whether the type of soils (that differed according to their pH, texture or organic matter content) and the incubation temperature (4 and 20°C) are contributory factors affecting the behaviour of STEC O26 in soils.

Material and methods

Characterization of the GFP-labelled STEC O26 strains

Four STEC strains belonging to serotype O26:H11 (S1, S2, S3 and S4) were isolated from faeces of different French dairy farms between January 2003 and August 2004 during the course of a previous study (Fremaux *et al.* 2006). The four strains were electroporated in the presence of a plasmid vector pGFPuv (Ozyme, Montigny-Le-Bretonneux, France), using a Gene Pulser II apparatus (Bio-Rad, Marnes-la-Coquette, France) at 2·5 kV, 25 μF and 200 Ω, and with an electrical pulse of 4·7 ms (Fremaux *et al.* 2007b). Virulence profiles of the four strains were also characterized by PCR (Fremaux *et al.* 2007b). All the four STEC O26 strains harboured the intimin gene *eae*, the enterohaemolysin gene *ehx* and the Shiga toxin gene *stx1*. Furthermore, genome fingerprints of each strain were characterized at the beginning and the end of the study by pulsed-field gel electrophoresis (PFGE) as previously described by Fremaux *et al.* (2006).

Physical and chemical characterizations of soil and organic waste samples

Soil samples (A, B and C) were collected, in early spring 2006, from the upper layer (0–20 cm) of three sites under permanent pasture and located in the Rhônes-Alpes region, France. Soils A and C were brunisols sampled near Thonon-les-Bains and soil B, a luvisol sampled at La Côte St André (LCSAp) (Ranjard *et al.* 1997). Soil A and B correspond to loam soils and contain, respectively, 45 and 38·2% sand, 39 and 45·1% silt, and 15·9 and 16·6% clay. Soil C corresponds to clay loam soil and contains 21·7% sand, 38·9% silt and 39·3% clay. Fresh cow manure was collected in a farm near Lyon in March 2006. Analyses of the physical and chemical characteristics of soils and fresh cow manure were all performed by CESAR (Centre Scientifique Agricole Regional, Ceyzeriat, France) using standard methods, and data were presented in Table 1. PCR on *stx* genes were performed in order to check whether soils and fresh cow manure were STEC-free. The enrichment step and the DNA preparation were performed as described previously (Fremaux *et al.* 2006). PCR-*stx* was performed on 100-fold diluted DNA extracts using degenerate primers ES149 and ES151 as described by Read *et al.* (1992).

Preparation, inoculation and sampling of manure-amended soils

Preparation of bacterial inocula

Each green fluorescent protein (GFP)-labelled STEC O26 strain was grown overnight at 37°C on Luria Bertani

Table 1 Chemical characteristics of the diverse soils and manure waste used in the present study

Material	pH	Total limestone (g kg^{-1})	Total N (g kg^{-1})	Total C (g kg^{-1})	C/N	Organic matter (g kg^{-1})	CEC	Total P (g kg^{-1})	NH_4 (g kg^{-1})
Loam soil (A)	8·19	173	1	8·8	9	15·1	69	0·07	–
Loam soil (B)	5·87	0	2·9	28·7	9	49·4	100	0·64	–
Clay-loam soil (C)	7·58	37	5·7	47·3	8	81·3	228	0·05	–
Manure waste	9·05	–	3·19	51·13	16·04	–	–	0·96	0·34

CEC, cationic exchange capacity.

plates (Sambrook *et al.* 1989) containing ampicillin (200 μg ml^{-1}) (LB-A). Then, each strain was cultivated in 1 l of LB broth supplemented with ampicillin (200 μg ml^{-1}) at 37°C for 24 h. 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 pellets were resuspended in 200 ml of phosphate-buffered saline (PBS) and centrifuged. This operation was repeated and afterwards the cell pellets were resuspended in 180 ml of sterile water supplemented with 20 ml of PBS.

Preparation and inoculation of the manure-amended soils
Soil moistures were adjusted to two-thirds of their water-holding capacity at the beginning of the study (i.e. 81·1% for the soil A, 72·7% for the soil B and 72·8% for the soil C). Each soil was inoculated in duplicate by mixing manure with each STEC O26 strain (S1, S2, S3 and S4). Amended soils were incubated at two different temperatures, i.e. 4 and 20°C. Therefore, 48 buckets of inoculated manure-amended soils were prepared. For that purpose, 4 kg of each soil were introduced into each of 16 large sterile buckets and inoculated with 200 g of manure containing 10^8 CFU g^{-1} of each GFP-labelled STEC O26 strain. Then, the inoculated manure was mixed manually for 5 min with soil in order to disperse the inoculum associated with manure particles through the soil. The final concentration of STEC O26 was around 10^6 CFU g^{-1} of manure-amended soil at the beginning of the study. The manure-amended soils were kept at 4 and 20°C into level 2 safety rooms.

Sampling of the manure-amended soils
Five soil samples were collected from each soil bucket using a 50 ml sterile Falcon tube, and mixed in a sterile BagFilter plastic bag (Interscience, Saint Nom La Breteche, France). Twenty-five grams was then used for enumeration of STEC O26. Manure-amended soils were taken at regular time intervals, transported to the laboratory and, within the same day, assayed to determine their microbiological and physicochemical status.

Analysis

Enumeration of STEC O26 cells by direct plating assay
Each manure-amended soil sample (25 g) was added to 225 ml of buffered peptone water (bioMérieux, Marcy l'Etoile, France) supplemented with ampicillin (150 μg ml^{-1}) in a sterile BagFilter and stomached for 30 s at medium speed. Aliquots from appropriate serial dilution were spread onto selective LB-A plates, which were incubated at 37°C for 24 h. The GFP-labelled STEC O26 colonies were counted under the UV light. An anti-O26 agglutinating serum (Bio-Rad) was randomly used to confirm that fluorescent colonies screened on agar plates under UV light belonged to O26-serogroup.

Enrichment of the samples was undertaken when the first decimally diluted suspensions (BagFilter) did not allow obtaining STEC colonies on LB-A plates (<10 STEC ml^{-1}). To enrich these weakly contaminated samples, the BagFilter was incubated at 37°C for 24 h. One millilitre of the enriched suspension was plated on LB-A. The plates were incubated 24 h at 37°C and placed under UV light to confirm the presence or absence of STEC O26 strains in the sample tested.

Physicochemical assays
Physicochemical parameters were measured for soils from each bucket, at regular time intervals. Moisture content of manure-amended soil was determined by drying 5 g of soil at 105°C for 24 h and weighing the residual. The pH of manure-amended soil sample was measured after adding 5 g of soil to 200 ml of distilled water. The suspension was then stirred for 5 min and was allowed to settle for 5 min. The pH of the manure-amended soil was measured with a pH meter 330 (WTW, Champagne au Mont d'Or, France).

Statistical analysis
For each condition, STEC counts obtained, respectively, from the two manure-amended soil buckets inoculated with the same strain were compiled and the log-linear model with tailing (Geeraerd *et al.* 2000) was used to fit them:

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

where N represents the STEC density (CFU g^{-1}) observed at time t (in days), N_0 is the initial STEC density (in CFU g^{-1}), N_{res} is the residual STEC density (in CFU g^{-1}), and k_{max} is the specific inactivation rate (in day^{-1}) equal to ln(10)/D-value. Model was fitted using, respectively, the nls function of the R software version 2.0.1 (Ihaka and Gentleman 1996).

An analysis of variance (ANOVA) was carried out in order to analyse the impact of the temperature (4 and 20°C) and the soil type (A, B and C) on the specific inactivation rate (k_{max}) of each STEC O26 strain. A mixed ANOVA model was considered, in which the temperature and the soil type were considered as fixed factors, and the strain was considered as a random factor.

Results

Genetic stability of the GFP-labelled STEC O26 strains

A first assay was performed to confirm the stability of the plasmid vector pGFPuv in nonselective conditions. The GFP-labelled STEC strains were inoculated into manure-

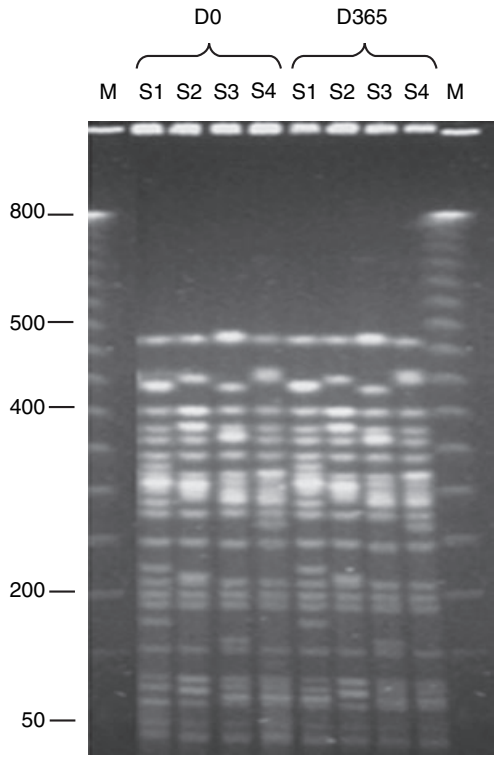

Figure 1 Pulsed-field gel electrophoresis of *Xba*I-digested genomic DNA obtained for each STEC O26 strain S1, S2, S3 and S4 recovered from the soil B held at 4°C, at the day 0 and at the end of the study (day 365). M are molecular weight markers (numbers on the left are the number of kilobase pairs).

amended soil microcosms (50 g) and enumerated up to the 16th day. At two time points (after 7 and 16 days), STEC counts obtained by direct plating assay were consistent with those obtained using a MPN-PCR *stx* method (described by Fremaux *et al.* 2007a).

Moreover, PFGE analyses performed at the beginning and the end of the study showed that no genetic change was observed for any of the STEC O26 strains tested for the duration of the study (see Fig. 1 for soil B held at 4°C as an example). PFGE of *Xba*I-digested genomic DNA gave 20–25 fragments (ranging from <50 to 500 kb) for each of the four GFP-labelled STEC O26 strains and all PFGE profiles showed at least three band differences.

Evolution of STEC O26 counts in manure-amended soils

The STEC inoculum levels ranged from 5·87 to 6·35 $\log_{10}$ CFU g^{-1}. STEC counts in each manure-amended soil decreased over time. Observed kinetics of STEC counts were correctly fitted by the log-linear model with tailing (see Fig. 2 for S1 as an example), which permits an estimation of the specific inactivation rate (k_{max}) and of the *D*-value ($\ln(10)/k_{max}$).

The ANOVA did not show any significant effect of the strain on k_{max}. The four STEC O26 strains were able to survive during a long period of time in the diverse soils tested (Table 2). At 4°C, they were recovered for at least 288 days in the soil A and more than 365 days postinoculation for the soils B and C. At 20°C, the same survival data were obtained, except for the soil A in which the pathogen was detected up to 196 days. However, the analysis of variance showed a significant effect of the temperature on k_{max} ($P < 0{\cdot}0001$). The STEC counts decrease was higher in the manure-amended soils held at 20°C than that obtained at 4°C (Figs 2 and 3). For example in soil B, the mean estimated *D*-values calculated for the four STEC strains were 18·5 days at 20°C *vs* 59·6 days at 4°C (Table 2). Furthermore, there was a significant effect of the soil type ($P < 0{\cdot}01$) and a significant interaction between temperature and soil type ($P < 0{\cdot}05$). The soil A was associated with a higher decline of STEC cells than the soils B and C at the two incubation temperatures (Fig. 3). At 20°C, STEC O26 counts decreased faster in soil C than in soil B, but an opposite finding was obtained at 4°C (Fig. 3).

Evolution of pH and dry matter

The pH and dry matter data are expressed as mean values obtained from all manure-amended soil buckets for each soil type and each incubation temperature. As shown in Fig. 4a, the dry matter percentage of all manure-amended soils gradually increased during the course of the present study. Soil drying was more pronounced at 20°C than at 4°C. On average, the dry matter increased over time from 81·1% (±0·35), 72·7% (±0·42) and 72·8% (±0·26) at the beginning of the study to values ≥92% at its end in soils A, B and C, respectively. This is true except for the soil A incubated at 4°C, which reached a dry matter of 84% (±1·24), 365 days postinoculation.

No difference was observed between pH values reported for each soil type, according to the incubation temperature (Fig. 4b). Just after addition of manure, pH of all soils increased by approx. 0·7 pH units [i.e. 8·9 (±0·02), 6·5 (±0·02) and 8 (±0·07) for the soils A, B and C, respectively]. Thereafter, it slightly increased during the 50 first days and markedly decreased to finally reach, at the 127th day, a mean value of 7·75 (±0·03), 5·27 (±0·05) and 6·44 (±0·01) for the soils A, B and C stored at 20°C, respectively. Similar results were observed for the soils held at 4°C. Afterwards, the pH of each soil increased gradually and tended to reach, at the end of the study, a pH value close to the initial ones, because of soil buffering capacity.

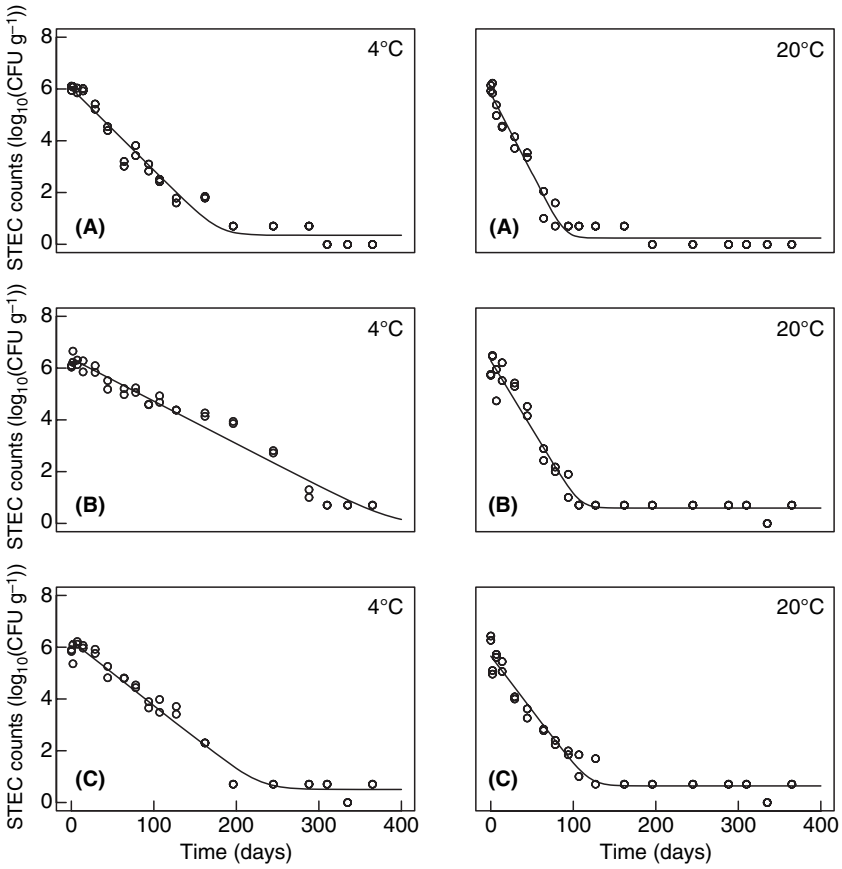

Figure 2 Survival of one STEC O26 strain (S1) in the diverse manure-amended soils (A, B and C) held at 4 and 20°C.

Table 2 Survival of STEC O26 in the various manure-amended soils (A, B and C) stored at 4 and 20°C

Soils	Storage temperature (°C)	Mean STEC count (log_{10} CFU ml^{-1}) at day 0*	Mean STEC *D*-values*	Time of survival of STEC (days)
Loam soil (A)	20	6·15 (±0·18)	17·1 (±1·12)	196
Loam soil (B)	20	5·87 (±0·24)	18·5 (±1·10)	>365
Clay-loam soil (C)	20	6·35 (±0·16)	25·4 (±1·66)	>365
Loam soil (A)	4	6·13 (±0·13)	26·9 (±4·1)	288
Loam soil (B)	4	6·05 (±0·16)	59·6 (±1·28)	>365
Clay-loam soil (C)	4	6·18 (±0·28)	37·8 (±3·77)	>365

*Mean values (±SD) were calculated from STEC counts obtained for the four STEC O26 strains.

Discussion

The potential presence of zoonotic agents such as STEC in soils fertilized with animal-derived organic wastes is of growing concern. Many studies described the persistence of *E. coli* O157:H7 for considerable periods in waste-amended soil, which may pose some risks of both human and animal contamination. In fact, soil and more generally the environment is one of the main pathways of STEC human infections, and a trend of environmental-outbreaks outnumbering burger-outbreaks is actually observed (Strachan *et al.* 2006). Although much information is available concerning the survival of *E. coli* O157:H7 in soil, the emergency of new STEC-serogroups implicated in human infections highlights the need for soil-survival studies performed on non-O157 STEC, and especially on STEC from the emerging disease-associated O26-serogroup.

This work was thus performed in order to evaluate the behaviour of four STEC O26 strains in manure-amended soils. In compliance with European rules set for the utilization of STEC and genetically modified organisms, the

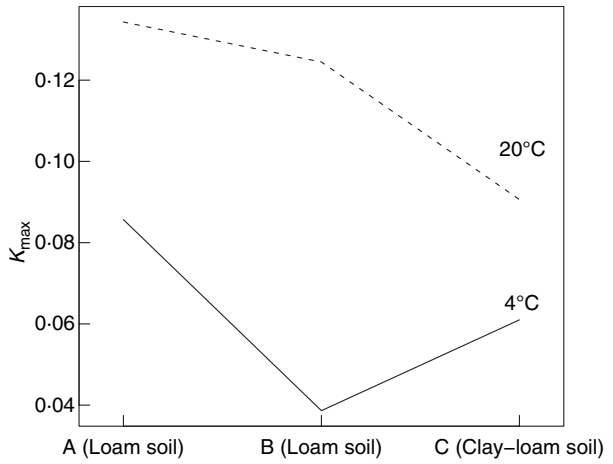

Figure 3 Interaction plot between the mean values of k_{max} obtained for the four STEC O26 strains at the two temperatures (4 and 20°C) in the three soil types (A, B and C).

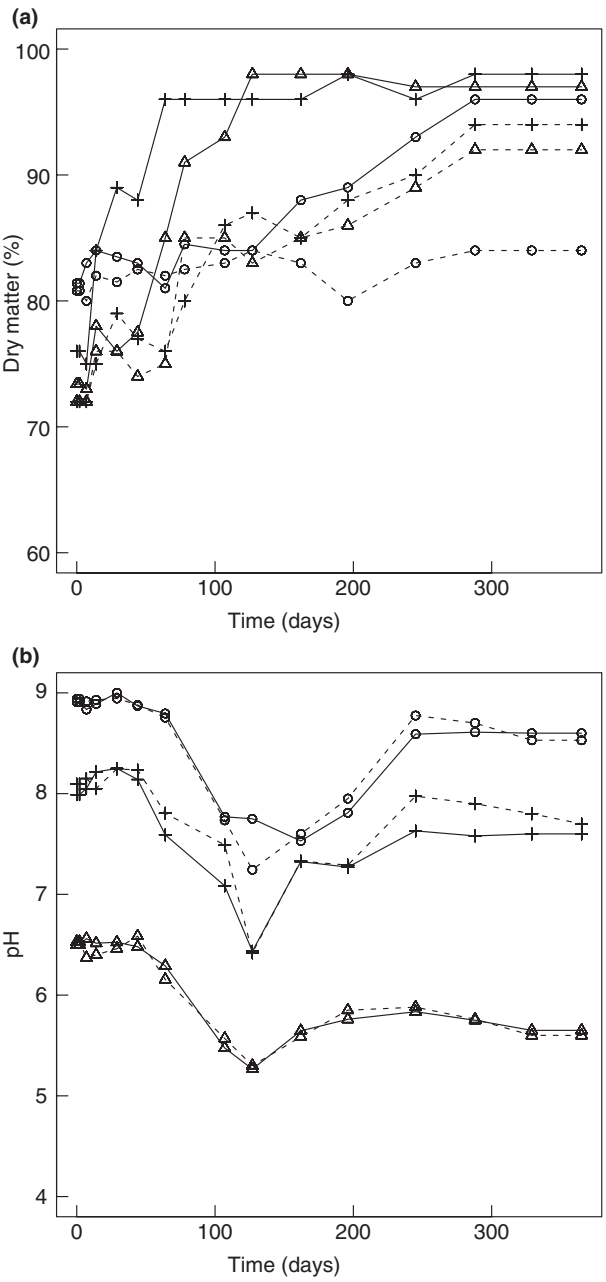

Figure 4 Dry matter content (a) and pH (b) of the various manure-amended soils (A, ○; B, △; C, +) held at the two incubation temperatures (— 20°C; - - - 4°C).

inoculated manure-amended soils were stored in a containment level 2 laboratory environment. Under these conditions, STEC O26 strains were shown to be able to survive during an extended period in all manure-amended soils. They were still detectable in manure-amended soils at least 196 days, and up to 1 year postinoculation in the majority of manure-amended soils tested whatever the soil-incubation temperature. But the manure-amended soils were not exposed to solar radiation (UV), rainfall events (responsible of bacteria leaching) and to other fluctuating environmental conditions encountered in field situation which may affect negatively STEC survival (Yaun *et al.* 2003, 2004). Moreover, in our experiment, manure was immediately mixed with soil in order to obtain a similar bacterial inoculum for all the manure-amended soil buckets at the start of the study, i.e. 10^6 CFU g^{-1} soil. In a real field situation, manure was generally left on the soil surface for typically up to 1 week before its incorporation in soil (Smith *et al.* 2001), where the bacteria can be affected by various environmental stresses. Taken in combination, the observed long-term survivals of STEC O26 strains in soils might be an overestimation of that occurring naturally in a farm environment. Many studies have reported differences in *E. coli* O157:H7 soil-survival rates according to diverse experimental conditions. Jiang *et al.* (2002) showed that *E. coli* O157:H7 cells survived for up to 231 days in manure-amended soil held under laboratory conditions at 21°C. In comparison, Mukherjee *et al.* (2006) and Ogden *et al.* (2002) reported shorter survival periods of this pathogen, which was detected for 69 days in garden plots fertilized with cattle manure and 105 days on pasture contaminated by sheep faeces, respectively. In the latter study, the *E. coli* O157:H7 soil-survival rate is likely underestimated as contaminated-sheep faeces were deposited within the fenced area 14 weeks before the sampling step began. Furthermore, Islam *et al.* (2004) showed that *E. coli* O157:H7 could survive for more than 7 months in plant-cultivated soils exposed to southern fall-winter conditions.

In order to determine the impact of temperatures on O26 soil-survival, the manure-amended soils were sepa-

rately held at constant temperatures of 4 and 20°C. These temperatures are comparable to temperatures recovered in spring, a main season for applying manure to fields in France. Although this model system bears little resemblance to a real world situation (where the temperature of soil cycles diurnally as well as seasonally), it allows us to show that STEC O26 cells were able to survive longer in all soils held at 4°C than those held at 20°C. It can be explained by a slowdown of competitive and antagonistic activities of indigenous bacteria. Some researchers had already described the positive effect of low temperatures on *E. coli* O157:H7 survival (Gagliardi and Karns 2002; Islam *et al.* 2004). However, these results differ with many recent studies. More precisely, Jiang *et al.* (2002) reported that *E. coli* O157:H7 strains inoculated in manure-amended soil were inactivated more rapidly at 5°C (i.e. 42 days) than at 21°C (193 days). Similarly, Mukherjee *et al.* (2006) showed that the *E. coli* O157:H7 counts decline was faster at 4°C than in ambient conditions (92 *vs* 10 days).

Physicochemical properties of soil are also important factors that could influence the STEC O26 soil-survival. It is clearly admitted that soil moisture favours the survival of bacteria. Berry and Miller (2005) showed that *E. coli* O157:H7 numbers either stay unchanged or increase at all but the lowest moisture levels examined, i.e. 0·11 g H_2O g^{-1} dry feedlot surface material. In our study, no further water was introduced to the buckets of soil during the incubation in order to evaluate the persistence of STEC O26 at critical moisture content values (<10%). Results revealed that STEC O26 can survive for extended periods of time in manure-amended soil even under very dry conditions, i.e. more than 92% dry matter at the end of the study. This result was consistent with that obtained by Jiang *et al.* (2002). Moreover the high STEC decline noted in the soil A held at 4°C, which was associated with the lower dry matter, suggested that moisture content was not a main factor influencing STEC O26 survival in soil.

All manure-amended soils used in this study contained high carbon content that might sustain bacterial growth (Table 1). It has been suggested that a higher level of organic matter could lead to a better adherence of pathogens to soil micro-aggregates, and therefore, increase their survival (Kearney *et al.* 1993). In soil A, where the lowest organic matter content was observed (Table 1), a markedly STEC O26 inactivation rate was obtained. However, the high pH values (around 9 units) reported for this soil during the course of the present study might also explain this result. Nauta and Dufrenne (1998) have estimated that the maximal pH value enabling the growth of 75 *E. coli* O157:H7 strains was around 9·4. On the contrary, Jiang *et al.* (2002) showed that basic soil pH values (i.e. pH 8) favoured the survival of *E. coli* O157:H7. Finally, soil texture can in part influence the behaviour of STEC O26 in soil. The soil C showed the highest clays content compared with soils A and B, and this might contribute to the greatest survival of STEC O26, at 20°C, in this soil. Clays favour the adsorption of bacteria to soil particles and create a barrier against microbial predators or parasites (Roper and Marshall 1978; Santamaria and Toranzos 2003).

In conclusion, a better understanding of the behaviour of STEC O26 in soil was obtained, and important factors that may contribute to the persistence of STEC O26 in soils were identified. Thus, low temperature, presence of clays and probably high organic matter content may favour the survival of STEC O26 in soils, whereas relative high pH seems to speed up the pathogen decline. Under laboratory conditions, STEC O26 survived during extended period of time in manure-amended soils at 4 and 20°C. Despite the probable overestimation of the survival times of STEC O26 strains in manure-amended soils compared with field conditions, this zoonotic agent may nevertheless be able to persist durably in this type of ecosystem. Considering this persistence, appropriate management of farm waste is indeed required to limit the spread of this pathogen to vegetable and crops.

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