International Journal of Food Microbiology 145 (2011) 301–310

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Transfer and internalisation of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cabbage cultivated on contaminated manure-amended soil under tropical field conditions in Sub-Saharan Africa

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ARTICLE INFO

Article history:
Received 11 November 2010
Received in revised form 5 January 2011
Accepted 8 January 2011

Keywords:
Internalisation
E. coli O157:H7
S. Typhimurium
Cabbage
Manure-amended soils
Tropics

ABSTRACT

Surface contamination and internalisation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in cabbage leaf tissues at harvest (120 days post-transplantation) following amendment of contaminated bovine manure to soil at different times during crop cultivation were investigated under tropical field conditions in the Central Agro-Ecological Zone of Uganda. Fresh bovine manure inoculated with rifampicin-resistant derivatives of non-virulent strains of *E. coli* O157:H7 and *S.* Typhimurium was incorporated into the soil to achieve inoculum concentrations of 4 and 7 log CFU/g at the point of transplantation, 56 or 105 days post-transplantation of cabbage seedlings. Frequent sampling of the soil enabled the accurate identification of the survival kinetics in soil, which could be described by the Double Weibull model in all but one of the cases. The persistence of 4 log CFU/g *E. coli* O157:H7 and *S.* Typhimurium in the soil was limited, i.e. only inocula applied 105 days post-transplantation were still present at harvest. Moreover, no internalisation in cabbage leaf tissues was observed. In contrast, at the 7 log CFU/g inoculum level, *E. coli* O157:H7 and *S.* Typhimurium survived in the soil throughout the cultivation period. All plants (18/18) examined for leaf contamination were positive for *E. coli* O157:H7 at harvest irrespective of the time of manure application. A similar incidence of leaf contamination was found for *S.* Typhimurium. On the other hand, only plants (18/18) cultivated on soil amended with contaminated manure at the point of transplantation showed internalised *E. coli* O157:H7 and *S.* Typhimurium at harvest. These results demonstrate that under tropical field conditions, the risk of surface contamination and internalisation of *E. coli* O157:H7 and *S.* Typhimurium in cabbage leaf tissues at harvest depend on the inoculum concentration and the time of manure application. Moreover, the internalisation of *E. coli* O157:H7 and *S.* Typhimurium in cabbage leaf tissues at harvest seems to be limited to the worst case situation, i.e., when highly contaminated manure is introduced into the soil at the time of transplantation of cabbage seedlings.

1. Introduction

Escherichia coli O157:H7 and *Salmonella* spp. have been implicated with increasing frequency in food-borne disease outbreaks associated with minimally processed fresh vegetables (Beuchat, 1996; Delaquis et al., 2007; Michino et al., 1999; Nguyen-the and Carlin, 2000; Sivapalasingam et al., 2004; Söderstrom et al., 2008). The increasing association of raw vegetables with outbreaks of *E. coli* O157:H7 and *Salmonella* spp. infection has been attributed in part to the widespread use of unsafe agricultural practices such as irrigation of crops with polluted water and amendment of soil with improperly treated livestock manure or sewage/sludge at/during the primary production stage (Beuchat, 2002; Wood et al., 2010). The decline in soil fertility and the exorbitant costs of mineral fertilisers in Sub-Saharan Africa have stimulated the utilisation of cattle manure in soil fertility management by small-holder farmers despite the fact that cattle are potential reservoirs of enteric pathogenic bacteria in the environment. Good Agricultural Practice (GAP) is one of the strategies that have been recommended for the management of microbiological safety of fresh produce in the field (De Rover, 1998). An important aspect of GAP is the composting of animal manure before incorporation into the soil (FDA, 1998). However, composting is a time consuming and expensive process that is not practiced by small-holder farmers in Sub-Saharan Africa due to intensive use of land. Instead, farmers

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doi:10.1016/j.ijfoodmicro.2011.01.018

prefer to dispose raw manure directly into agricultural fields to save time and off-set the costs associated with composting.

Fresh vegetables can be contaminated with *E. coli* O157:H7 and/or *Salmonella* spp. at any point along the farm-to-table production chain (Abadias et al., 2008; Garcia-Villanova et al., 1987; Johnston et al., 2006). However, pre-harvest contamination in the field is of great concern since pathogens may internalise in plant tissues and thus become protected against sanitizers used at the post-harvest stage (Cooley et al., 2003; Dong et al., 2003; Gil et al., 2009; Guo et al., 2002; Itoh et al., 1998; Seo and Frank, 1999; Solomon et al., 2002; Warriner et al., 2003a, 2003b; Watchel et al., 2002; Xicohtencatl-Cortes et al., 2009). Internalisation of human enteric food-borne pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella* spp. in plant tissues has been demonstrated with seedlings of temperate vegetable crops (lettuce, tomatoes, cress, radish, alfalfa, and mug bean) in controlled environmental conditions mimicking the temperate climate (Dong et al., 2003; Franz et al., 2007; Guo et al., 2002; Itoh et al., 1998; Jablasone et al., 2005; Johannessen et al., 2005; Solomon et al., 2002; Warriner et al., 2003a). However, no studies have yet been performed to investigate internalisation of human enteric pathogenic bacteria in vegetable crops under tropical conditions. Moreover, a major criticism of the studies that have demonstrated internalisation of *E. coli* O157:H7 and/or *S.* Typhimurium in leafy vegetable tissues is that the use of seedlings is not representative of the mature plants, and therefore such information might not be relevant for the safety of vegetable crops such as cabbage which is consumed at a mature stage. Furthermore, experiments performed under controlled environmental conditions in the laboratory may not reflect actual situations likely to occur in the field. For instance, Dreux et al. (2007) compared the population dynamics of *Listeria innocua* on the aerial surface of parsley between a controlled laboratory set-up and a field set-up and observed that the CFU number of the organism decreased more rapidly under field conditions than under controlled conditions in the laboratory. Therefore, the potential for the internalisation of *E. coli* O157:H7 and *Salmonella* in tissues of edible plant parts needs to be evaluated under realistic conditions in the field. This is particularly important for developing countries such as those in Sub-Sahara Africa where vegetable production takes place entirely in the field.

This study aims at examining the risk of surface contamination and internalisation of *E. coli* O157:H7 and *Salmonella* spp. in cabbage leaves at harvest following cultivation on soil amended with contaminated bovine manure under tropical field conditions in Sub-Saharan Africa. There are two practical events inherent in the smallholder agricultural system in Sub-Saharan Africa through which animal manure can be a conduit for contamination of vegetables with pathogenic microorganisms in the field, i.e., (i) direct incorporation of non-composted manure into the soil by farmers and (ii) deposition of faecal matter in the agricultural environment by roaming animals that graze randomly and sometimes enter into crop fields. Hence, in contrast with other studies which focus on manure application at the time of seedling transplantation, in this study, the effect of the time at which contaminated bovine manure was introduced into the soil during plant cultivation on surface contamination and internalisation of pathogen was examined.

2. Materials and methods

2.1. Bacterial strains, culture media and inoculum preparation

Rifampicin resistant derivatives of non-virulent *E. coli* O157:H7 ATCC 43888 (*E. coli* O157:H7-Rifr) and *Salmonella enterica* serovar Typhimurium LT2 (*S.* Typhimurium-Rifr) constructed in a previous study (Ongeng et al., accepted for publication) were used. *E. coli* O157:H7 ATCC 43888 lacks the *sxt*1 and *stx*2 genes (Kudva et al., 1998) while *S.* Typhimurium LT2 carries an attenuating allele of the *rpoS* gene (Wilmes-Riesenberg et al., 1997). The organisms were kept at −80 °C in 15% glycerol. When required, stock culture of each organism was streaked on the Luria-Bertani agar medium (LB: 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl and 14 g/L bacteriological agar; all purchased from Merck, Darmstadt, Germany) containing 100 μg/mL rifampicin and incubated aerobically for 24 h at 37 °C. A single colony of *E. coli* O157:H7-Rifr or *S.* Typhimurium-Rifr from the LB plate was inoculated in 10 mL of LB broth (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl; all from Merck) containing 100 μg/mL rifampicin and incubated for 18 h at 37 °C with agitation. The cultures were pelleted by centrifugation (4000×*g* for 10 min). The pellets were washed three times, re-suspended and diluted in 0.9% NaCl to an OD_{650} of 0.7, which corresponded with an inoculum density of approximately 9 log CFU/mL.

2.2. Experimental setup

Fresh bovine manure was obtained from the Animal Production Unit while soil was obtained from an experimental field, at the National Crops Resources Research Institute (NaCCRI) in Uganda. Both manure and soil did not contain background contaminants that could interfere with selective detection and enumeration of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, as shown by spread plating 100 μL of a 10^{-1} dilution of manure or soil on CT–SMAC–Rif100–Cy50–Ny50 (Cefixime Tellurite Sorbitol MacConkey agar containing 100 μg/mL rifampicin, 50 μg/mL cycloheximide and 50 μg/mL nystatin) and XLT4–Rif100–Cy50–Ny50 (Xylose Lysine Tergitol 4 agar containing 100 μg/mL rifampicin, 50 μg/mL cycloheximide and 50 μg/mL nystatin) followed by 24 h of incubation at 37 °C. CT-SMAC and XLT4 were obtained from Merck (Darmstadt, Germany) while rifampicin, cycloheximide and nystatin were purchased from Fluka Biochemika (Milan, Italy). CT–SMAC–Rif100–Cy50–Ny50 and XLT4–Rif100–Cy50–Ny50 were previously selected and validated for selective detection and enumeration of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, respectively in a non-sterile manure-amended soil matrix (Ongeng et al., accepted for publication). Contaminated manure was prepared by inoculating separately, *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr at a rate of 5 or 8 log CFU/g. This was achieved by adding 100 mL of 6 or 9 log CFU/mL inoculum to 900 g of manure followed by kneading the manure in a plastic bag in order to distribute the inoculum. Contaminated manure was then added to soils (1 part manure to 9 part soil) on which cabbages (*Brassica oleracea* var. *capitata* cv. Gloria) were cultivated. Three schedules of manure application were implemented, i.e., (i) at the time of transplantation of seedlings; (ii) 56 days post-transplantation and (iii) 105 days post-transplantation. At each time of manure application, the soil was loosened using a stainless steel gardening fork followed by mixing the contaminated manure with the soil using a stainless steel garden cultivator to achieve an inoculum density of 4 or 7 log CFU/g in manure-amended soil. Three weeks old cabbage seedlings were used and the experiments were performed in 6 L plastic pots. The pots were randomly placed as sets of 6 pots per replicate for each treatment in an open space in the field at NaCCRI. Each treatment was replicated 3 times (n = 18) and the pots were spaced 60 cm apart within a set and 2 m apart between sets. Cabbage cultivation lasted for 120 days and no pesticides were used. Daily precipitation and temperature minima and maxima during the experimental period were obtained from a nearby meteorological station and are shown in Fig. 1.

2.3. Microbiological analysis of soil

Microbiological analysis of soil in the vicinity of the plant roots was performed weekly and after every three days in the case of 7 and 4 log CFU/g inoculum, respectively. At each sampling moment, 5 g of soil was carefully removed from two randomly chosen pots per replicate (six in total) and suspended in sterile 50 mL Falcon tubes containing 45 mL of sterile 0.9% saline. The samples were vortexed 4 times for

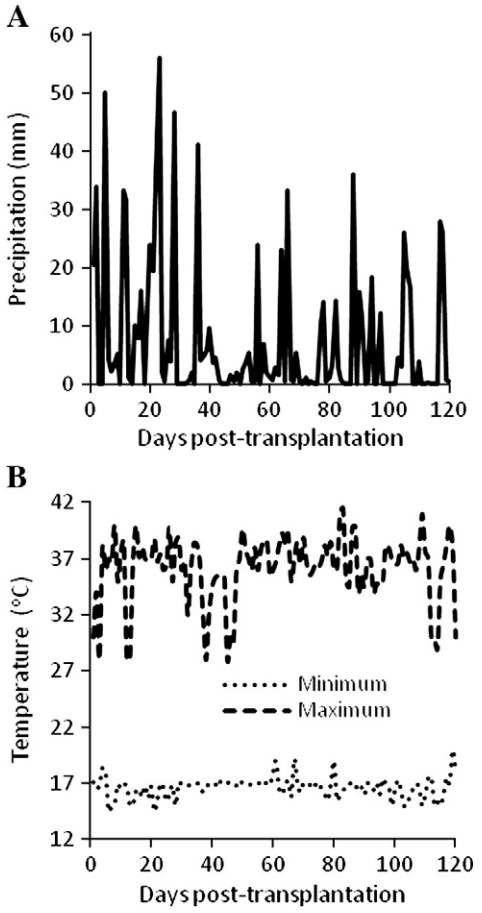

Fig. 1. Daily precipitation (A) and temperature minima and maxima (B) during the experimental period.

1 min followed by serial dilution in 0.9% saline. One hundred micro-litre aliquots of appropriate dilutions were spread plated in duplicate on CT–SMAC–Rif100–Cy50–Ny50 and XLT4–Rif100–Cy50–Ny50 to determine the CFU number of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, respectively. The CFU was counted after 24 h of incubation at 37 °C. When the detection limit of the plate count method (2 log CFU/g) was reached, enrichment was used to detect the presence of the remaining viable cells. For *E. coli* O157:H7-Rifr, 5 g of soil was aseptically added to 50 mL of modified EC broth containing novobiocin (Merck) and incubated for 24 h at 37 °C. One hundred micro-litres of the enrichment broth was surface-plated on CT–SMAC–Rif100–Cy50–Ny50 and incubated for 24 h at 37 °C. Samples were considered positive when non-sorbitol fermenting straw-coloured colonies developed on the plates. For *S.* Typhimurium-Rifr, the soil samples were enriched in selenite cystine broth (Merck) and plated on XLT4–Rif100–Cy50–Ny50. Incubation conditions were as described for *E. coli* O157:H7-Rifr. The samples were considered positive when black or black-centred colonies developed on the plates.

2.4. Microbiological analysis of cabbage leaves

Microbiological analysis of cabbage leaves was carried out at harvest (120 days post-transplantation) and all the 18 plants per treatment were used. For each plant, the leaves were separated from the stalk using a sterile scalpel and divided into two sets. One set was surface-sterilised by dipping in 1% $AgNO_3$ twice for 1 min in order to enumerate internalised cells as reported by Franz et al. (2007). The surface-sterilised leaf (SSL) samples were rinsed in sterile distilled water to remove the remaining $AgNO_3$. The effectiveness of the method for leaf-surface sterilisation was proven in a preliminary study with cabbage leaves artificially contaminated with *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr. Ten grams of SSL samples were ground in a mortar containing 90 mL of 0.9% saline followed by plating 100 μL of the plant extract on CT–SMAC–Rif100–Cy50–Ny50 and on XLT4–Rif100–Cy50–Ny50 to determine the CFU number of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, respectively. CFU were enumerated after 24 h of incubation at 37 °C. The lot of non-surface-sterilised leaf (NSSL) samples were processed and analysed for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contamination as described above for the SSL samples. When the organisms were not detected by direct plating, 100 μL of the plant extract was used for enrichment detection of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr as described above in Section 2.3.

2.5. Statistical analysis and modelling bacterial survival in soil

Survivor CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the soil were log-transformed and fitted to various model functions using the GInaFiT Excel-Add-In model fitting tool (Geeraerd et al., 2005). In principle, this is a modelling step to be performed on data obtained under static conditions. However, in this study, this procedure was used to have a quantification of survivor trends. Models were selected by making use of the goodness-of-fit and the Root Mean Sum of Squared Error (RMSE) criterion (Ratkowsky, 2003). In the case of 4 log CFU/g inocula, survival curves of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr for manure applied at the point of transplantation and 56 days post-transplantation were fitted to the Double Weibull model (Coroller et al., 2006; Eq. (1)). This was also the case for the 7 log CFU/g inocula introduced into the soil through manure amendment at the point of transplantation.

$$N(t) = \frac{N_o}{1 + 10^{\alpha}}\left[10^{-\left(\frac{t}{\delta_1}\right)^p + \alpha} + 10^{-\left(\frac{t}{\delta_2}\right)^p}\right] \quad (1)$$

By Eq. (1), the overall population of the test organism is partitioned into two sub-populations 1 and 2 based on the assumption that sub-population 1 is more sensitive to the environmental stress than sub-population 2 resulting into more rapid decay of sub-population 1. The parameters in Eq. (1) are: $N(t)$ = number of survivors (log CFU/g); N_o = initial inoculum size (log CFU/g); t = time (days); p = shape parameter (dimensionless); δ_1 = time needed for the first decimal reduction of sub-population 1 (days); δ_2 = time needed for the first decimal reduction of sub-population 2 (days); α = ratio of the fraction of sub-population 1 to the fraction of subpopulation 2 at time zero (dimensionless). Survival curves of the 7 log CFU/g inocula for manure applied 56 days post-transplantation followed the log-linear model that incorporates a shoulder parameter (log-linear-shoulder) according to Eq. (2) (Geeraerd et al., 2000):

$$N(t) = N(0).e^{-k_{max}.t}.\left[\frac{e^{k_{max}.S_l}}{1 + (e^{k_{max}.S_l}).e^{-k_{max}.t}}\right] \quad (2)$$

where $N(t)$ is the cell number (CFU/g) at any time (days), $N(O)$ is the original cell number (CFU/g), k_{max} is the first order inactivation rate constant (day^{-1}), S_l is the shoulder length (days) and e is Euler's number (the base of the natural logarithm = 2.718). For manure applied 105 days post-transplantation, survival data could not be fitted since CFU counts had not declined substantially. The *lsqnonlin* procedure of the MatLab Optimization Toolbox (The Mathworks Inc., Version 2007b; www.mathworks.com) was used to calculate the 95% confidence and prediction intervals of the data along the fitted curves. At each inoculum density and for the different manure application dates, the independent 2-sample Student *t*-test procedure of the GenStat Discovery Edition 3 (http://discovery.genstat.co.uk) was used to compare the parameter values of the fitted curves between *E. coli*

O157:H7-Rifr and *S.* Typhimurium-Rifr. The error level was fixed at 5%. The models were used to derive the time for CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in soil to reach the detection limit (*ttd*) of the plate count method in situations where such a case was observed.

3. Results

3.1. Survival of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in soil

For soils which received contaminated manure at the point of transplantation, CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr decreased with time, but the respective CFU number in the soil at harvest (120 days post-transplantation) was dependent on the inoculum density. CFU counts of the 7 log CFU/g *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr fell below the detection limit of the plate count method 77 and 84 days post-transplantation (Fig. 2A), respectively, but the organisms remained culturable by enrichment up to the time of harvest. For the case of the 4 log CFU/g inocula, CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr dropped to the detection limit of the plate count method 24 and 33 days post-transplantation (Fig. 2B), but remained culturable by enrichment only up to day 27 and 42 post-transplantation, respectively. Graphical illustrations of how the Double Weibull model fitted the survivor curves, and the 95% confidence and prediction intervals determined by the model are presented in Fig. 3. Statistical measures of the fits and parameter values of the fitted curves are shown in Table 1. In the case of the 7 log CFU/g inoculum level, the values of t_{4D}, p and α were not significantly different ($p>0.05$) while values of δ_1 and δ_2 were significantly higher for *S.* Typhimurium-Rifr than for *E. coli* O157:H7-Rifr ($p\leq 0.05$). In the case of the 4 log CFU/g inoculum density, the values of the Double Weibull parameters were not significantly different between *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr ($p>0.05$). According to the Double Weibull model, the *ttd* of *E. coli* O157:H7-Rifr in soil was significantly shorter than that of *S.* Typhimurium-Rifr irrespective of the inoculum density ($p<0.05$) (Table 2).

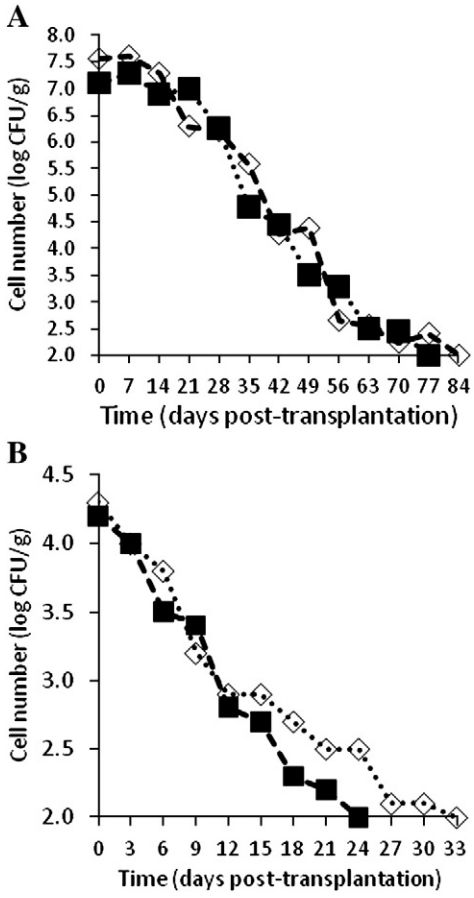

Fig. 2. Survival of *E. coli* O157:H7-Rifr (■) and *S.* Typhimurium-Rifr (◊) in soil amended with contaminated manure at the point of transplantation. (A): 7 log CFU/g; (B): 4 log CFU/g. Data points are averages of three replicates. Error bars are not shown for clarity of illustration.

Survival patterns of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in soils amended with contaminated manure 56 days post-transplantation are shown in Fig. 4. CFU numbers declined with time but the final cell concentration in the soil at harvest was dependent on the inoculum density. At 7 log CFU/g inoculum level, mean CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the soil at the time of harvest was about 3 log CFU/g (Fig. 4A). In the case of the 4 log CFU/g inoculum density, *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr CFU numbers declined to the detection limit of the plate count method 80 and 83 days post-transplantation (Fig. 4B) and could not be detected by enrichment beyond 89 and 98 days post-transplantation, respectively. Fig. 5 illustrates the model fit, and the 95% confidence and prediction intervals as determined by the Double Weibull and log-linear-shoulder model for the 4 and 7 log CFU/g inocula, respectively. Statistical measures of the fits and parameter values of the fitted curves are shown in Table 3. At 4 log CFU/g inoculum level, the values of the Double Weibull parameters were not significantly different between *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr ($p>0.05$) except, for the δ_2 parameter which was significantly higher for *S.* Typhimurium-Rifr than for *E.* coli O157:H7-Rifr by about a week ($p\leq 0.05$). In the case of the 7 log CFU/g inoculum, the values of $t_{4D,}$ k_{max} and s_l described by the log-linear–shoulder model were not significantly different between *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr ($p>0.05$). In the case of 4 log CFU/g inoculum density, the *ttd* for *E. coli* O157:H7-Rifr, according to the Double Weibull model (Table 2) was significantly shorter than that of *S.* Typhimurium-Rifr ($p\leq 0.05$). Model *ttd* was not calculated in the case of 7 log CFU/g inoculum since the CFU number was still above the detection limit of the plate count method at the time of harvest (Figs. 4A and 5A and B).

For soils that were amended with contaminated manure 105 days post-transplantation, the respective mean contamination level of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the soil at harvest for the 7 log CFU/g inoculum was not significantly different from the initial inoculum density ($p>0.05$) (Fig. 6A and B). However, for the 4 log CFU/g inoculum, CFU (mean ± SE, n = 6) of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the soil at harvest for manure applied 105 days post-transplantation was 2.94 ± 0.19 and 3.2 ± 0.08 CFU/g, respectively (Fig. 6C and D).

3.2. *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contamination of cabbage leaves at harvest

Table 4 shows the occurrence of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on/in NSSL and in SSL at harvest following incorporation of contaminated manure into the soil at different dates during crop cultivation. *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contaminations of cabbage leaves at harvest were only evident for plants cultivated on soils that received contaminated manure 105 days post-transplantation irrespective of the inoculum density, and for plants raised on soils in which 7 log CFU/g inocula were introduced through amendment of contaminated manure at the time of transplantation and 56 days post-transplantation. In the case of NSSL samples for which contamination was observed, the CFU numbers of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr were mostly at the detection limit of the plate count method except in the case of 7 log CFU/g inocula introduced into the soil 105 days post-transplantation for which mean (± SE, n = 18) CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on/in NSSL samples were 3.6 ± 0.91 and 3.40 ± 0.48 log CFU/g, respectively. However, following leaf-surface

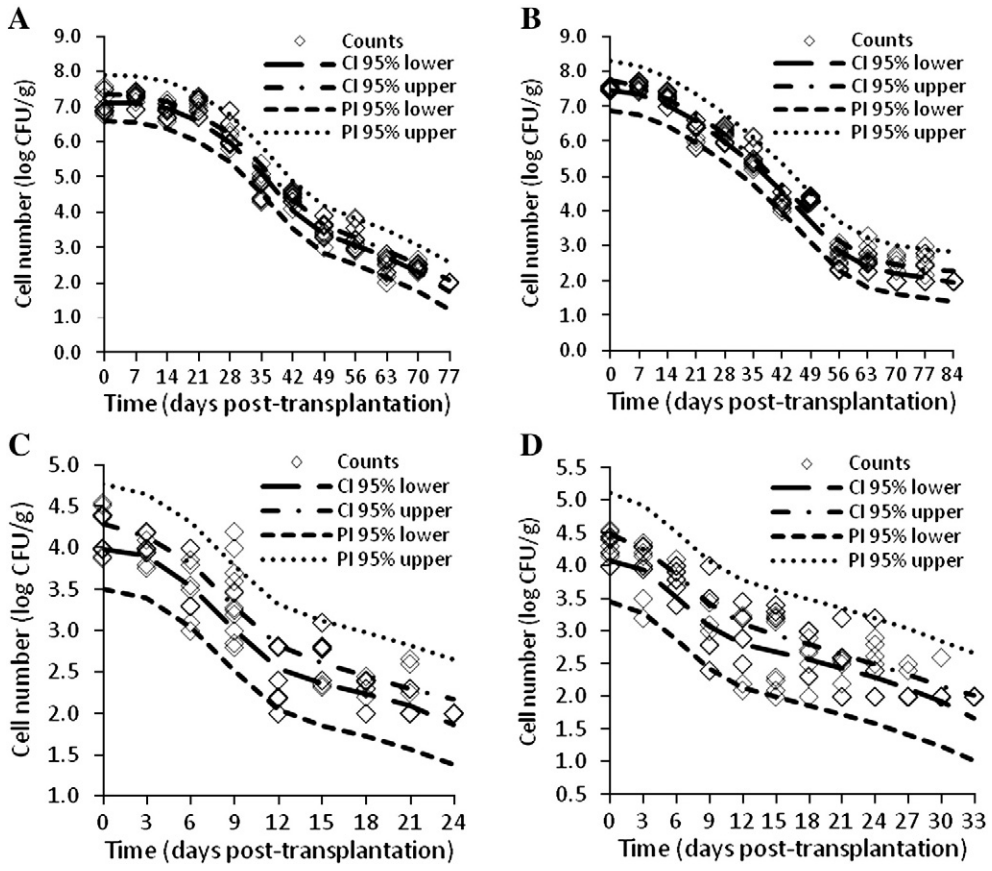

Fig. 3. Model fit, confidence interval (CI) and prediction interval (PI) of the survival curves of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in soil for manure applied at the point of transplantation according to the Double Weibull model. (A): 7 log CFU/g *E. coli* O157:H7-Rifr; (B): 7 log CFU/g *S.* Typhimurium Rifr; (C): 4 log CFU/g *E. coli* O157:H7-Rifr; (D): 4 log CFU/g *S.* Typhimurium-Rifr. All replicates are shown.

sterilisation with 1% $AgNO_3$, only plants cultivated on soil contaminated with 7 log CFU/g inocula at the point of transplantation remained positive for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr by enrichment.

4. Discussion

In this study, we investigated the risk of *E. coli* O157:H7 and *S.* Typhimurium contamination of cabbage at harvest following the application of contaminated bovine manure during cabbage cultivation under tropical field conditions. We followed the survival of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the soil in a similar way as was performed in a previous study (Ongeng et al., re-submitted after minimal revision), with one main difference, i.e., in the current study, soil samples were taken from within the vicinity of the plant roots whereas in the previous study, no plants were included in the experimental set-up and bulk soil was used. In that study, we observed that *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr survived for 21 and 35 days at 4 log CFU/g inoculum and for 84 and 98 days at 7 log CFU/g inoculum, respectively. Interestingly, the survival times of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the manure-amended soil during crop cultivation (Section 3.1) appear to be longer than their survival times in manure-amended bulk soil observed previously (Ongeng et al., re-submitted after minimal revision) thus suggesting a possible effect of the rhizosphere. Previously, studies that investigated the effect of the rhizosphere on the survival of enteric pathogenic bacteria in soil reported enhanced persistence in the rhizosphere compared to bulk soil (Gagliardi and Karns, 2002; Ibekwe et al., 2006;

Table 1
Statistical measures and parameter values of the fitted models describing the survival of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the soil following manure application at the point of transplantation according to the Double Weibull model.

Organism	RMSE	AdjR^2	t_{4D}	*No*	α	δ_1	δ_2	*p*
4 log CFU/g inoculum								
E. coli O157:H7-Rifr	0.31	0.86	NA	4.14 ± 0.1^a	1.37 ± 0.11^a	7.0 ± 0.4^a	23 ± 2.0^a	2.6 ± 0.5^a
S. Typhimurium-Rifr	0.39	0.78	NA	4.23 ± 0.1^a	1.21 ± 0.12^a	6.0 ± 0.5^a	22 ± 1.0^a	2.9 ± 0.6^a
7 log CFU/g inoculum								
E. coli O157:H7-Rifr	0.33	0.97	51 ± 3.0^a	7.3 ± 0.10^a	3.0 ± 0.22^a	22 ± 1.0^a	56 ± 3.0^a	2.5 ± 0.2^a
S. Typhimurium-Rifr	0.35	0.97	55 ± 2.5^a	7.60 ± 0.10^a	4.7 ± 0.4^a	26 ± 0.4^b	95 ± 3.3^b	1.7 ± 0.1^a

For each inoculum density, means (±SE, n = 3) reported in the same column and followed by the same superscripts are not significantly different ($p > 0.05$). RMSE: root mean sum of squared error; AdjR^2: adjusted R^2; t_{4D}: time (days) to attain 4 log reduction; *No*: initial cell count (log CFU/g); α: parameter that relates the fraction of the first sub-population to the second sub-population at time zero; δ_1: time (days) for first decimal reduction of sub-population 1; δ_2: time (days) for first decimal reduction of sub-population 2; *p*; shape parameter.

Table 2
Time to reach detection limit (*ttd*) of the plate count method (2 log CFU/g) for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr during cultivation of cabbage on contaminated manure-amended soil according to the Double Weibull model.

Manure application date (days post-transplantation)	Initial inoculum density (log CFU/g)	Predicted time to reach detection limit of the plate count method (days)	
		E. coli O157:H7-Rifr	*S.* Typhimurium-Rifr
0	4	24 ± 0.33^{a}	33 ± 0.00^{b}
0	7	75 ± 0.00^{a}	83 ± 0.58^{b}
56	4	24 ± 0.82^{a}	30 ± 0.67^{b}

For each manure application date, means (±SE, n = 3) reported in the same row and followed by the same superscripts are not significantly different ($p > 0.05$).

Semenov et al., 2009), except in one case where rhizosphere effect was not observed (Williams et al., 2007). This is a subject of future research.

We had shown in a previous study that the Double Weibull model fitted survivor curves of 7 log CFU/g inocula in manure and in manure-amended soils very well, however, survivor curves obtained with 4 log CFU/g inocula could not be fitted to any model due to the inadequate number of data points showing CFU above the detection limit of the plate count method (Ongeng et al., re-submitted after minimal revision). Therefore, in this study, we increased the sampling frequency for soils contaminated with a 4 log CFU/g inoculum and successfully fitted the survival data to the Double Weibull model. By looking at Figs. 3C and D and 5A, B, C and D, a high degree of scatter can be observed. The large variation in CFU number at each data point could be a consequence of poor distribution of inocula in the survival matrix. We do acknowledge that achieving uniform distribution of cells in the growth matrix for this kind of experiment was a challenge. Other authors have reported similar problems in other studies (Hutchison et al., 2004; You et al., 2006). It was interesting to note that survivor curves for the 7 log CFU/g inocula introduced into the soil at the point of transplantation followed the Double Weibull model despite the possible effect of the rhizosphere while for inocula introduced 56 days post-transplantation followed the log-linear–shoulder model. The difference in survival pattern between the two inoculation dates for the 7 log CFU/g inocula could be due to differences in the final cell concentration at the end of the experiment (dictated by crop harvest time). Since we did not sample beyond the time of harvest, we do not know what kind of model the survivor curves of the 7 log CFU/g inocula introduced 56 days post-transplantation would follow had sampling continued until the detection limit of the plating technique was reached. However, in treatments where CFU counts dropped to or below the detection limit of the plate count method, the Double Weibull model adequately determined the *ttd* values (Figs. 2A and B and 4B and Table 2). Model-derived *ttd* values for *S.* Typhimurium-Rifr were significantly larger than those of *E. coli* O157:H7-Rifr, and is in agreement with observed situations.

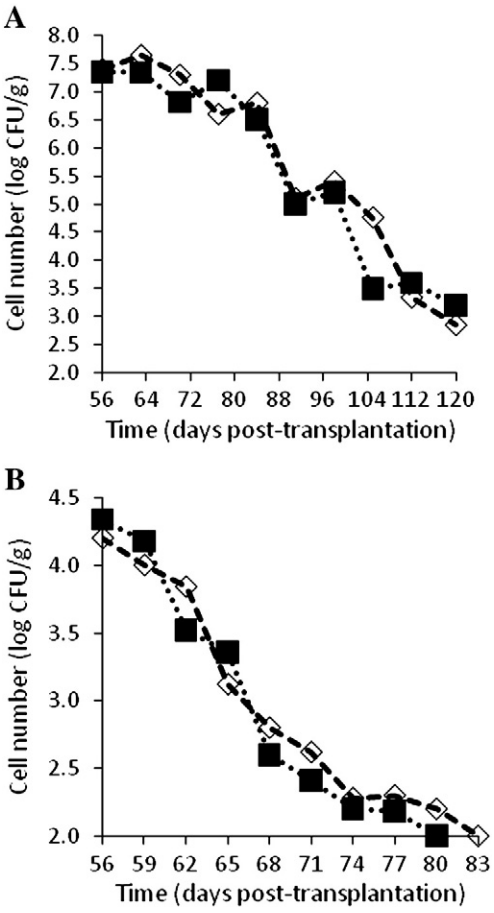

Fig. 4. Survival of *E. coli* O157:H7-Rifr (■) and *S.* Typhimurium-Rifr (◊) in soil amended with contaminated manure 56 days post-transplantation. (A): 7 log CFU/g; (B): 4 log CFU/g. Data points are averages of three replicates. Error bars are not shown for clarity of illustration.

Our data revealed that surface contamination and internalisation of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in cabbage leaf tissues at harvest depended on inoculum density and time of manure application during crop cultivation (Table 4). *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contamination of cabbage leaves at harvest correlated with the length of their survival in the soil vis-à-vis the length of cultivation. This was clearly shown by the fact that *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contamination of cabbage leaves was evident only in treatments in which the organisms survived in the soil until harvest (see Section 3.1 and Table 4). Natvig et al. (2002) planted radishes, arugula and carrots in manure-amended soils containing 4–5 log CFU/g *S.* Typhimurium, however, the organism was not detected on any of the vegetables planted 60 days post-manure application at harvest (72 days after planting), but was found on radishes and arugula planted 45 days post-manure application and harvested 42 days after planting instead which is in line with our observation on cabbage in this study. Although Natvig et al. (2002) did not show any evidence of internalisation of *S.* Typhimurium in tissues of any of the vegetables used in their study, the observed presence of the organism on some vegetables planted 45 days post-fertilisation and harvested 42 days after planting showed that the time at which the plant came into contact with the pathogens during growth determined the possibility of vegetable contamination at harvest and our results have explicitly demonstrated this aspect for cabbage under tropical field conditions.

The presence of surface-attached *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on cabbage leaves for treatments in which the organisms were present in the soil at the time of harvest could be due to splashes from the soil. However, several factors appear to be responsible for the adherence of enteric foodborne pathogenic bacteria on the foliage. Solomon and Mathews (2006) demonstrated identical adherence pattern for fluorescent microspheres, live and dead cells of *E. coli* O157:H7 on aerial parts of lettuce. This observation prompted the authors to suggest that the uptake of *E. coli* O157:H7 onto lettuce was governed by the plant and independent of any bacterial process. However, Xicohtencatl-Cortes et al. (2009) showed later that *E. coli* O157:H7 colonized the leaf surface via flagella and the type 3 secretion system independently of the production of shiga toxin, thus suggesting the role of flagella and the T3SS in colonization of leafy green produce. Results of Barak et al. (2005) revealed that *S. enterica* genes important for virulence in animal systems were also required for colonization of alfalfa sprouts. Boyer et al. (2007) reported in another study that overall hydrophobicity and cell charge

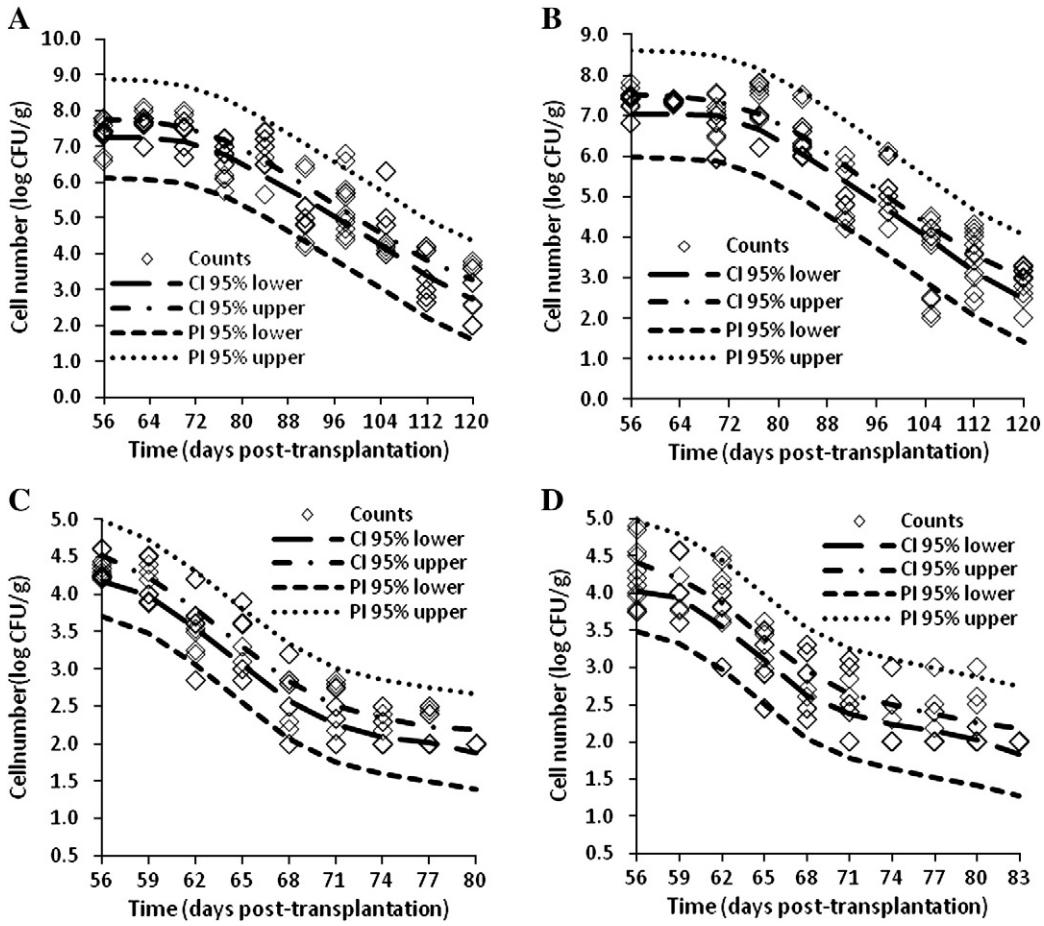

Fig. 5. Model fit, confidence interval (CI) and prediction interval (PI) of the survival curves of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in soil during cultivation of cabbage for manure applied 56 days post-transplantation. (A): 7 log CFU/g *E. coli* O157:H7-Rifr according to the log-linear–shoulder model; (B): 7 log CFU/g *S.* Typhimurium-Rifr according to the log-linear–shoulder model; (C): 4 log CFU/g *E. coli* O157:H7-Rifr according to the Double Weibull model; (D): 4 log CFU/g *S.* Typhimurium-Rifr according to the Double Weibull model. All replicates are shown.

in *E. coli* O157:H7 strains as well as the presence of curli did not influence the attachment of *E. coli* O157:H7 cells to produce items. On the other hand, Lapidot and Yaron (2009) demonstrated that transfer of *S.* Typhimurium from contaminated irrigation water to parsley was dependent on curli and cellulose; the components of the biofilm matrix. Additionally, long-term colonisation of plant surfaces has been linked to the ability of the organism to compete for limited moisture and nutrients under harsh conditions typical of the phyllosphere (Beattie and Lindow, 1999; Kinkel, 1997), a property which *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr appeared to exhibit when introduced at high inoculum levels into the soil.

It was clearly shown that potential transfer of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr from manure-amended soil to internal locations in cabbage leaf tissues at harvest was only observed when plants were challenged with high density inocula and when manure was amended at the time of transplantation. Plants derived from soils amended with contaminated manure 56 and 105 days post-transplantation exhibited evidence of surface contamination as the organisms were detected only on/in NSSL samples. The absence of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in SSL samples derived from plants cultivated on soils contaminated with 7 log CFU/g inocula 56 and 105 days post-transplantation suggests that older plants could

Table 3
Statistical measures and parameter values of the fitted models describing the survival of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in soil following manure application 56 days post-transplantation according to the Double Weibull and log-linear–shoulder model.

Organism	RMSE	AdjR^2	t_{4D}	N_o	α	δ_1	δ_2	p	k_{max}	s_l
4 log CFU/g inoculum[*]										
E. coli O157:H7-Rifr	0.29	0.89	NA	4.36 ± 0.1^a	1.90 ± 0.22^a	7.0 ± 0.4^a	26 ± 0.5^a	1.8 ± 0.3^a	NA	NA
S. Typhimurium-Rifr	0.36	0.82	NA	4.23 ± 0.10^a	1.60 ± 0.15^a	6.0 ± 0.5^b	32 ± 0.8^b	2.1 ± 0.5^a	NA	NA
7 log CFU/g inoculum[**]										
E. coli O157:H7-Rifr	0.68	0.85	57 ± 4.0^a	7.5 ± 0.13^a	NA	NA	NA	NA	0.23 ± 0.01^a	17 ± 3.0^a
S. Typhimurium-Rifr	0.65	0.86	57 ± 6.5^a	7.3 ± 0.12^a	NA	NA	NA	NA	0.24 ± 0.01^a	18 ± 2.0^a

For each inoculum density, means (±SE, n = 3) reported in the same column and followed by the same superscripts are not significantly different ($p > 0.05$). RMSE: root mean sum of squared error; AdjR^2: adjusted R^2; t_{4D}: time (days) to attain 4 log reduction; N_o: initial cell count (log CFU/g); α: parameter that relates the fraction of the first sub-population to the second sub-population at time zero; δ_1: time (days) for first decimal reduction of sub-population 1; δ_2: time (days) for first decimal reduction of sub-population 2; p; shape parameter; k_{max} : decay rate constant; s_l: shoulder length; NA: not applicable.

[*] According to the Double Weibull model.

[**] According to the log-linear-shoulder model.

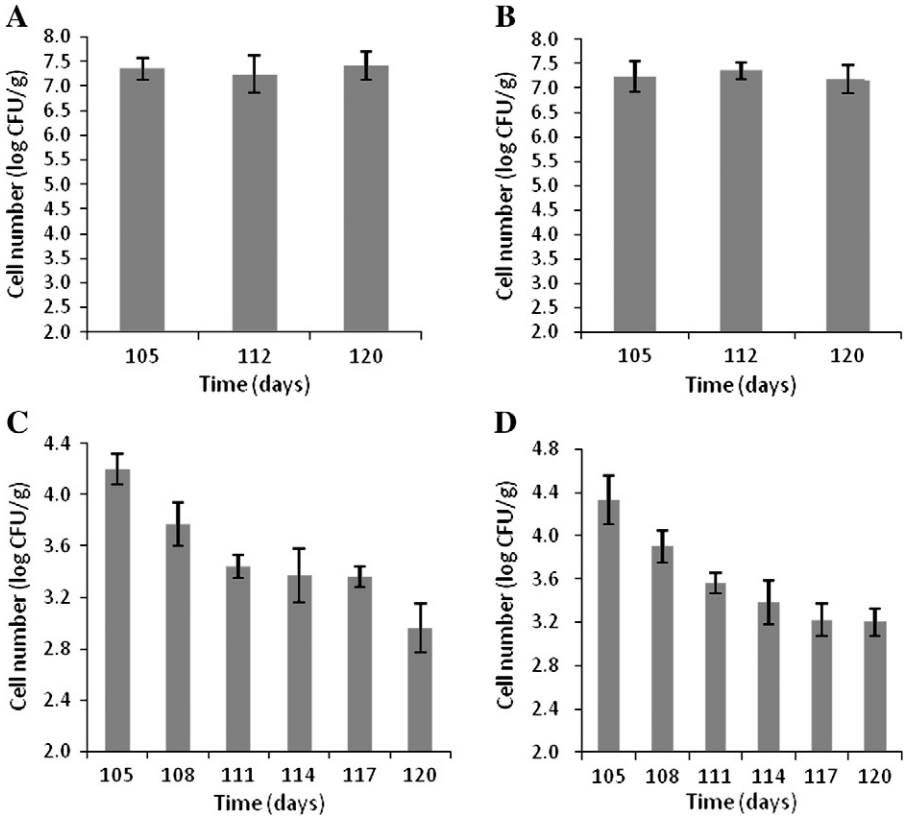

Fig. 6. Cell number (CFU/g) of *E. coli* O157:H7-Rifr and S. Typhimurium-Rifr in the soil as a function of time following incorporation of contaminated manure 105 days post-transplantation. (A): 7 log CFU/g *E. coli* O157:H7-Rifr; (B): 7 log CFU/g *S.* Typhimurium-Rifr; (C): 4 log CFU/g *E. coli* O157:H7-Rifr; (D): 4 log CFU/g *S.* Typhimurium-Rifr.

have already developed mechanisms to restrict entry and internal colonisation of the leaves (Dixon, 2001) by the two enteric bacteria. The potency of the 7 log CFU/g *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr introduced into the soil at the point of transplantation to contaminate internal locations in cabbage leaves at harvest was in contrast with the case of the 4 log CFU/g inocula for which *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr were absent in SSL samples of all the 18 plants tested. It is plausible to suggest that the 4 log CFU/g *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr introduced into the soil at the point of transplantation were out-competed by indigenous organisms present in the manure-amended-soil–plant ecosystem. Consequently the bacterial pathogens were prevented from accessing internal locations in the foliage. Cooley et al. (2003) inoculated *S. enterica* and *E. coli* O157:H7 on roots of *Arabidopsis thaliana* in the presence and absence of an *Enterobacter asburiae* strain isolated from *A. thaliana*. They observed that *S. enterica* and *E. coli* O157:H7 were capable of moving within the plant in the absence of *E. asburiae* which suppressed the growth of both organisms under gnotobiotic conditions. Johannessen et al. (2005) investigated the potential transfer of *E. coli* O157:H7 from contaminated manure to fresh produce using lettuce seedlings transplanted into soils fertilised with bovine manure containing 4 log CFU/g *E. coli* O157:H7 and did not observe any evidence of pathogen internalisation in lettuce nor contamination of outer leaves and the roots at harvest which is consistent with our findings with *E. coli* O157:H7-Rifr for cabbage cultivated on soils fertilised on day 0 and 56 post-transplantation at a similar initial inoculum level.

The occurrences of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on/in NSSL and in SSL samples were similar irrespective of the time of manure application or inoculum density (Table 4). Therefore, lack of differences between *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr with respect to contamination of cabbage leaves based on manure

Table 4
The occurrence of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on/in NSSL and in SSL samples at harvest following soil amendment with contaminated manure on various dates during crop cultivation.

MAD (DPT)	Occurrence (number of positive plants/total number tested)							
	E. coli O157:H7-Rifr				*S.* Typhimurium-Rifr			
	4 log CFU/g		7 log CFU/g		4 log CFU/g		7 log CFU/g	
	NSSL	SSL	NSSL	SSL	NSSL	SSL	NSSL	SSL
0	(0/18)	(0/18)	(18/18)[a]	(18/18)[b]	(0/18)	(0/18)	(18/18)[a]	(18/18)[b]
56	(0/18)	(0/18)	(18/18)[a]	(0/18)	(0/18)	(0/18)	(17/18)[a]	(0/18)
105	(18/18)[a]	(0/18)	(18/18)[a]	(0/18)	(18/18)[a]	(0/18)	(18/18)[a]	(0/18)

MAD: manure application date; DPT: days post-transplantation; SSL: surface-sterilised leaves; NSSL: non-surface-sterilised leaves.
[a] Observed with plating.
[b] Observed after enrichment.

amendment schedule tested in this study suggests that the likelihood of vegetable contamination with *E. coli* O157:H7 and *S.* Typhimurium would be the same if contaminated manure is applied to soil during cabbage cultivation. Survival of the 7 log CFU/g *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr over a complete production cycle of cabbage as observed in this study is not an isolated situation. Islam et al. (2004) reported recovery of *E. coli* O157:H7 from the edible portions of mature lettuce and parsley 77 and 177 days, respectively following cultivation of the crop on manure-amended soil containing approximately 6 log CFU/g inoculum. The results of this study and those of Islam et al. (2004) invalidate the minimum 120 days fertilisation-to-harvest interval recommended by the National Organic Programme of the United States (Ingham et al., 2004) thus signifying that guidelines for manure use should be designed for climate-specific environments.

Finally, it was observed that the 4 log CFU/g inocula introduced 56 days post-transplantation and the 7 log CFU/g inocula introduced at all the three manure application dates survived until harvest and resulted in plant contamination. From a practical point of view, the occurrence of such a residual population of pathogens in the soil post-harvest might be of great importance for small-holder vegetable production in Sub-Saharan Africa because of the need to re-use the field for subsequent crop under intensive land management systems. Future studies should therefore establish whether such residual population would cause contamination of the subsequent crop.

5. Conclusions

This study demonstrated that surface contamination and internalisation of *E. coli* O157:H7 and *S.* Typhimurium in cabbage leaves at harvest following cultivation on contaminated manure-amended soil depended on the inoculum density and time of manure application. The concentrations of *E. coli* O157:H7 and *S.* Typhimurium shed by cattle in faeces under tropical field conditions in Sub-Saharan Africa are not known. However, if in a worst case situation we consider that a soil contamination level of 7 log CFU/g is likely to occur, then internalisation of *E. coli* O157:H7 and *S.* Typhimurium in cabbage leaf tissues at harvest under tropical field conditions in Sub-Saharan Africa may be exhibited when manure is amended to soil at the point of transplantation. On the other hand, if an inoculum density of 4 log CFU/g represents a more realistic soil contamination level following manure amendment, then manure application may only lead to surface contamination of cabbage leaves and would only be possible when contaminated manure is introduced into the soil near harvest.

Acknowledgements

This research was sponsored by the International Foundation for Science (Ref. C/4294-1) and the Belgian Development Agency (UNI2006/01).

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