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Effects of Cattle Feeding Regimen and Soil Management Type on the Fate of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Typhimurium in Manure, Manure-Amended Soil, and Lettuce

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Survival of the green fluorescent protein-transformed human pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium was studied in a laboratory-simulated lettuce production chain. Dairy cows were fed three different roughage types: high-digestible grass silage plus maize silage (6:4), low-digestible grass silage, and straw. Each was adjusted with supplemental concentrates to high and low crude protein levels. The pathogens were added to manure, which was subsequently mixed (after 56 and 28 days for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, respectively) with two pairs of organically and conventionally managed loamy and sandy soil. After another 14 days, iceberg lettuce seedlings were planted and then checked for pathogens after 21 days of growth. Survival data were fitted to a logistic decline function (exponential for *E. coli* O157:H7 in soil). Roughage type significantly influenced the rate of decline of *E. coli* O157:H7 in manure, with the fastest decline in manure from the pure straw diet and the slowest in manure from the diet of grass silage plus maize silage. Roughage type showed no effect on the rate of decline of *Salmonella* serovar Typhimurium, although decline was significantly faster in the manure derived from straw than in the manure from the diet of grass silage plus maize silage. The pH and fiber content of the manure were significant explanatory factors and were positively correlated with the rate of decline. With *E. coli* O157:H7 there was a trend of faster decline in organic than in conventional soils. No pathogens were detected in the edible lettuce parts. The results indicate that cattle diet and soil management are important factors with respect to the survival of human pathogens in the environment.

Agricultural animals are widely recognized as reservoirs of human enteric pathogens (31, 44). These pathogens are shed in their feces, which in turn could serve as the primary source for contamination of various food products. Most cases of human infection by these pathogens have been linked primarily to the consumption of animal food products. However, various pathogens have been recovered from vegetables (3), and the number of documented disease cases associated with the consumption of raw vegetables has increased in recent years (40, 44). Outbreaks of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium have been associated with the consumption of lettuce (17, 19). Both human enteric pathogens have a principal reservoir in cattle (9, 53).

One possible mechanism of vegetable contamination with these pathogens is the land application of manure as fertilizer (33). The conditions for survival of enteric human pathogens are generally considered to be unfavorable once they are excreted from the animal (46). Possible contamination of vegetables grown in soil enriched with manure will largely depend on the survival capabilities of the pathogen in manure, in soil, and in or on plants. Differences in animal feeding regimens and the absence of synthetic fertilizers, pesticides, and routine use of antibiotics may lead to differences in pathogen prevalence and survival between organic and conventional farming systems. Because animal manure is the major source of fertilization in organic crop production, microbial safety is at the center of attention for organic vegetable production (1). However, it has not been demonstrated that the risk of contamination of fresh vegetables is higher with organic than with conventional production (29).

Diet composition, abrupt changes in diet, or fasting may influence the shedding of *E. coli* O157:H7 (43). There has been considerable debate concerning the effect of hay feeding versus grain feeding on the shedding and acid resistance of *E. coli* O157:H7. Grain feeding can create a more acidic environment in the guts of cattle, which leads to the selection for acid-resistant generic *E. coli*, which may include the considerably acid-resistant *E. coli* O157:H7 (11, 38). This dietary effect on shedding of *E. coli* O157:H7 is supported by some epidemiological data (16, 37), but other results point in another direction (39). The hypothesis has also been supported (45) or challenged (15, 20, 25, 48) by experiments conducted with ruminants inoculated with *E. coli* O157:H7. Besides affecting the shedding of pathogens, the cattle feeding regimen can be expected to affect manure composition and might thereby also affect pathogen survival capabilities in manure.

In bovine manure, *E. coli* O157:H7 is documented to survive for extended periods of time (5, 26, 28, 55). *Salmonella* serovar Typhimurium also is capable of survival for considerable periods of time in manure (18) and slurries (18, 24). Survival of excreted pathogens in freshly produced manure will be affected by the manure management system used on the farm: manure

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TABLE 1. Description of the six types of diet fed to dairy cows in an experimental, controlled setup (J. W. Reijs, personal communication)

Manure type	Roughage	Concentrate(s)	CP[a]	VEM[b]	NDF[c]	ADF[d]
GMH	60% high-digestible grass silage plus 40% maize silage	40% soy plus 60% maize	180 (H)	971	370	232
GOH	100% low-digestible grass silage	100% soy	176 (H)	799	461	320
SH	100% straw	75% soy plus 25% maize	185 (H)	772	504	334
GML	60% high-digestible grass silage plus 40% maize silage	55% maize plus 45% beet pulp	116 (L)	970	392	246
GOL	100% low-digestible grass silage	19% soya plus 81% beet pulp	104 (L)	772	524	349
SL	100% straw	21% maize plus 58% beet pulp	105 (L)	761	565	357

[a] Crude protein level of the total diet (grams kilogram [dry weight]$^{-1}$); CP = % N × 6.25. H, high; L, low.
[b] VEM, energy level of total diet (kilogram [dry weight]$^{-1}$); 1 VEM = 6.904 kJ net energy.
[c] NDF of total diet: cellulose, hemicellulose, and lignin (grams kilogram [dry weight]$^{-1}$).
[d] ADF of total diet: cellulose plus lignin (grams kilogram [dry weight]$^{-1}$).

is handled as a slurry or as solid manure, applied to fields after a range of storage times, and applied by surface spreading or injection into the soil (31). So far, the potential influence of cattle diet on pathogen survival in manure has not been the subject of research. In manure-amended soil, reported survival times of *E. coli* O157:H7 vary considerably, from several weeks (32) to several months (5, 21, 23, 28). Long-term survival has also been demonstrated for *Salmonella* serovar Typhimurium (22, 30).

E. coli O157:H7 and *Salmonella* may be transferred from manure-amended soil or manure compost-amended soil to leaf and root vegetables and can persist for long periods of time on these vegetables (21, 22, 30). Recently it has been shown that *E. coli* O157:H7 can become internalized in lettuce by entering the plant through the root system from a planting mixture of manure and soil and can migrate throughout the edible part of the plant (41). Because of the lack of chemical treatments for controlling pathogen invasion in lettuce production, suppression of pathogens must rely solely on the antagonistic capacity of the resident microflora in the different ecological niches. Functional and taxonomic diversity and biomass of soil microbial and faunal communities are frequently higher in organic than in conventional fields and have been correlated with a higher suppression of soilborne plant pathogens (50, 51).

At present there is insufficient information about the influence of cattle diet and manure characteristics on the survival of human pathogens in manure. It is also not known whether organically and conventionally managed soils differ in the capability to suppress human pathogens. Moreover, the possible internalization of human pathogens in the edible parts of leafy vegetables grown in manure-amended soil is scarcely documented. Previous studies on pathogen survival in the agricultural environment focused primarily on single parts of the lettuce production chain, such as manure, soil, or manure-amended soil with crops. In the present study, we simulated the lettuce production chain in the laboratory and monitored the fate of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in three subsequent niches: manure, manure-amended soil, and plant. The objectives of the present study were to determine pathogen survival as a function of cattle diet, soil type, and soil management (organic or conventional). Furthermore, the possibility of (internal) contamination of lettuce after a period of pathogen survival in manure and manure-amended soil was investigated.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* O157:H7 strain B6-914 gfp-91 was kindly provided by Pina Fratamico (13). This strain does not produce Shiga-like toxins I and II (Stx1$^-$ Stx2$^-$) but contains the pGFP cDNA vector (Clontech Laboratories, Inc., Palo Alto, CA) expressing green fluorescent protein (GFP) and ampicillin resistance. The survival characteristics of the GFP-labeled strain were indistinguishable from those of the wild-type strain (13). In addition, Kudva et al. (26) reported no differences in survival in bovine manure and manure slurry between toxin-positive (Stx1$^+$ Stx2$^+$) and toxin-negative (Stx1$^-$ Stx2$^-$) *E. coli* O157:H7. Two phenotypes of *Salmonella enterica* serovar Typhimurium, MAE 110 (P*agfD1 rdar*; aggregate phenotype) and MAE 119 (Δ*agfD101 saw*; wild-type morphology), were kindly provided by Ute Römling (35, 36). These strains were derived from strains MAE 51 and MAE 52, respectively, and both carry kanamycin and gentamicin resistance and the GFP gene on the chromosome after transformation with the PAG408 minitransposon (42). The two strains can be distinguished by their appearance under UV light. The colony appearance of MAE 110 is larger, flatter, more ragged, and less bright than that of MAE 119. Bacteria were stored at −80°C and were checked for viability prior to use.

Cattle feeding and manure collection. Manure was obtained from an ongoing experiment on the effect of diet on manure quality by the Department of Animal Science of the Wageningen University and Research Center, The Netherlands (J. W. Reijs, personal communication). Dairy cows (Holstein Frisian, 3 to 7 years of age) were housed in one stable under identical conditions. Six pairs ($n = 2$) of animals were fed six different diets for nearly 9 weeks (from 20 January 2003 until 21 March 2003): high-digestible grass silage (60%) plus maize silage (40%) (GM), low-digestible grass silage (GO), and straw (S), each adjusted with supplemental concentrates to high (H) and low (L) crude protein (CP) levels (Table 1). Fresh manure (without urine) was collected directly from the pairs of cows (with equal amounts of manure from each individual well mixed in a bucket) at the end of the feeding trial (after 9 weeks) and stored at 5°C in 20-liter containers.

Soils. An organically managed sandy soil and a conventionally managed sandy soil, cropped to potatoes, were collected from two neighboring farms in Marknesse (Flevoland, The Netherlands). Organic and conventional loamy soils, cropped to onions, were collected from two neighboring farms in Ens (Flevoland, The Netherlands). Both organic farms were accredited by Skal (the inspection body for organic production in The Netherlands) and thus refrained from the use of artificial fertilizers or pesticides. However, both the organic and conventional farmers used animal manures as fertilizer. Throughout each field, 15 soil samples (1 to 20 cm deep) were collected between the plants with an augur and mixed. Samples were transported in plastic bags to the lab, stored at 5°C, and sieved (4 mm) before use.

Inoculation of manure. A simulation of the transitions in the lettuce production chain from manure to soil and plants was done separately for *E. coli* O157:H7 and for a mixture of both *Salmonella* serovar Typhimurium phenotypes. The inoculum was prepared in Luria-Bertani broth with 50 μg/ml ampicillin for *E. coli* O157:H7 B6-914 gfp-91 and 50 μg/ml kanamycin for the *Salmonella* serovar Typhimurium phenotypes. Both phenotypes were grown separately and mixed to equal amounts before inoculation of the manure. Cells were harvested by centrifugation at 3,000 × *g* (Hermle 2384 K) and washed with and resuspended in 0.1% peptone buffer (Oxoid) to a density of 1×10^9 CFU per milliliter. This cell density was determined spectrophotometrically, taking into account that an optical density at 630 nm of 1 would equal approximately 0.7×10^9 CFU ml^{-1}. The dry weight of the manure was determined by drying over-

TABLE 2. Chemical characteristics of six types of cattle manure, collected directly from cows fed the six diets described in Table 1

Manure	pH	N-NH_4 (mg/kg)	Dry matter (g/kg)	Total N (g/kg)	Total C (g/kg)	C/N	NDF[a] (%)	ADF[b] (%)
GMH	6.1	364.65	123.32	32.76	463.17	14.14	49.41	34.16
GOH	6.9	282.42	186.09	21.33	382.75	17.94	53.28	37.07
SH	7.0	33.12	114.11	13.57	454.45	33.49	66.45	50.32
GML	6.4	255.39	137.64	30.27	467.89	15.46	46.62	34.07
GOL	7.0	122.74	153.88	19.67	409.16	20.80	55.16	39.33
SL	7.8	48.49	137.46	19.04	341.29	17.93	64.43	46.82

[a] Cellulose, hemicellulose, and lignin in organic matter.
[b] Cellulose plus lignin in organic matter.

night at 105°C. Cells were added to a final density of 1×10^7 CFU per gram manure dry weight (gdw^{-1}). For *Salmonella* serovar Typhimurium a mixture of 0.5×10^7 CFU MAE 110 gdw^{-1} and 0.5×10^7 CFU MAE 119 gdw^{-1} was added to manure. After mixing by thoroughly kneading the manure in a plastic bag from the outside by hand, 500 g of the inoculated manure was transferred to a preweighed plastic pot (1 liter), which was closed but had the ability of gas exchange. There were three replicate pots per manure type and the same number of noninoculated pots, which functioned as blanks, with 0.1% peptone buffer added instead of bacterial suspension. The pots were weighed and incubated at 10°C in darkness. At each sampling time, pots were weighed before and after sampling to check for evaporation. The moisture content remained constant (on average around 85%) during the experiment. In addition, at each sampling time, manure samples from the blanks were dried overnight at 105°C to determine their dry weight.

Plate counts of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. The inoculated pots were sampled over time to determine the survival of the pathogens in manure (at time zero and after 3, 8, 16, 22, 28, 43, 56, 84, and 133 days). At each sampling time, two samples of approximately 1 g of each replica were removed from the middle of each mixture with a sterile spoon and put in separate preweighed dilution tubes with 4.5 ml of 0.1% peptone. Sampling holes were closed. Sample-containing tubes were weighed to determine the exact size of the sample. Samples were vortexed and put in a ultrasonic bath for 30 s (Branson 5200; 120-W output power, 47 kHz). The samples were vortexed again, and 10-fold serial dilutions were made. From the two highest dilutions, 50 μl was plated in duplicate on petri dishes with sorbitol-MacConkey (Oxoid) agar with ampicillin (50 μg/ml) for the enumeration of *E. coli* O157:H7 or on Luria-Bertani medium with kanamycin (50 μg/ml) for the enumeration of *Salmonella* serovar Typhimurium. The number of necessary dilutions was estimated based on preliminary counts. This resulted in two plates per dilution, four plates per sample, and thus eight plates per replica. When low cell numbers were expected, 16 or 32 plates per sample were used to increase the detection limit. Cell suspensions were spread on the surface by shaking with 2-mm sterile glass beads. The inoculated plates were sealed with Parafilm and incubated at 37°C for 24 h. Numbers of *E. coli* O157:H7 and of *Salmonella* serovar Typhimurium were determined by counting green fluorescent CFU with a dark-blue lamp (Philips PL-S 9W/08 Blacklight Blue, peak at 365-nm UV-A). Colony shape and GFP intensity enabled distinction between *Salmonella* serovar Typhimurium phenotypes 110 and 119. Colony counts were calculated as number of CFU gdw^{-1}.

Transmission to and survival in soil. To determine survival in manure-amended soil, a subset of 60 g of fresh weight (gfw) of manure was mixed with 540 gfw of each of the four soils (1:9). These mixtures were mixed thoroughly in plastic bags by hand and transferred to plastic pots (1 liter) similar to those used in the survival-in-manure part of the experiment. For *E. coli* O157:H7 this was done 56 days after inoculation with manure types GMH and GML, because the other manure types showed too low numbers of pathogens for further transition to soil at that time. With the *Salmonella* serovar Typhimurium experiment, pathogen levels allowed amending of the four soils with the two more contrasting manure types, GMH and SH, which was done after 28 days of survival in manure. The pots were incubated at 15°C in darkness. For each manure-soil combination there were three replicate pots. Soils for the noninoculated pots (blanks) were mixed in the same way as the manure blanks of the manure survival part of the experiment. Sampling of the inoculated pots to determine survival was done as described above (at time zero and after 2, 7, 13, 28, and 57 days).

Lettuce production. Two weeks after the manure was mixed with soil, aliquots of 500 gfw of mixture were transferred to plastic pots; one seedling of iceberg lettuce (*Lactuca sativa* L cv. Dublin) was planted in each pot (3 replicate pots × 8 treatments = 24 plants on inoculated soil mixtures and 24 plants on noninoculated blanks), and the pots were placed in a completely randomized manner on a greenhouse bench (15°C; relative humidity, 60%). After 3 weeks, root samples (1 to 2 gfw) and shoot samples (on average three small leaves, 1 to 2 gfw) were checked for pathogen presence. Root samples were washed in sterile water twice to remove soil particles. Both root and leaf samples were ground with a pestle and mortar in 5 ml 0.1% proteose peptone (Oxoid) and crystal sand and plated (100 μl) directly on selective media as described above. To distinguish between the epiphytic and endophytic presence of pathogens, half of the samples were surface sterilized by being dipped in 1% $AgNO_3$ for 10 s and washed two times in sterile water before grinding. Bulk soil samples were plated as described above.

Chemical measurements. Chemical characteristics were determined before starting the experiment for each manure (Table 2) and soil type (Table 3).

(i) Manure. Dried samples (40°C) were ground and analyzed for total carbon by the Dumas method followed by detection by a CHN1110 element analyzer (CE Instruments, Milan, Italy) and for fiber content (52). Total nitrogen content was determined by the Kjeldahl method (8), and ammonium content was determined in a solution of trichloroacetic acid with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY). The pH was measured in a watery suspension with an Inlab pH level 1 (WTW GmbH, Weilheim, Germany).

(ii) Soil. Total nitrogen and carbon were determined as for manure. Nitrate and ammonium contents in soil samples were determined with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY) after addition of 0.01 M $CaCl_2$ suspension. The pH of the soil samples was measured in this $CaCl_2$ suspension with an Inlab pH level 1 (WTW GmbH, Weilheim, Germany).

Statistical analysis. Microbial data (CFU counts) were log transformed, and these log numbers over time for each replica were fitted to the following logistic function by nonlinear regression (Gauss-Newton method): $CFU(t) = a + [b/1 + e(^{c - dt})]$, where $CFU(t)$ is the log number of CFU gdw^{-1} on day t, a is the lower asymptote, $(a + b)$ is the upper asymptote, d is the slope parameter (referred to as the decline rate), and c is the position parameter (referred to as the location of the inflection point). The true location of the inflection point is given by c/d, and the true maximum decline rate at the inflection point is given by $(b \times d)/2$ (SAS version 8; SAS Institute, Cary, NC). Time point zero was defined as the first sampling time, which occurred immediately after inoculation, and the upper and

TABLE 3. Physical and chemical characteristics of four soils, collected as neighboring pairs in The Netherlands, used for mixing with pathogen-inoculated cattle manure which functioned as a substrate for growth of lettuce seedlings

Location	Management	Soil type	Code	Clay (%)	Silt (%)	Sand (%)	pH	N-NO_3 (mg/kg)	N-NH_4 (mg/kg)	Total N (mg/kg)	Total C (mg/kg)	C/N
Marknesse	Organic	Sand	OS	3.2	33.3	63.5	7.1	20.92	29.69	2.28	23.78	10.43
Marknesse	Conventional	Sand	CS	3.2	32.4	64.5	7.1	5.20	30.18	1.35	14.45	10.70
Ens	Organic	Loam	OC	8.3	54.5	37.2	7.3	4.70	21.74	1.50	16.67	11.11
Ens	Conventional	Loam	CC	7.7	51.9	40.4	7.3	8.86	26.66	1.56	18.34	11.76

the lower asymptotes were kept constant at, respectively, 7 log CFU gdw^{-1} and 0. Time points which gave a CFU count of zero were included in the analysis with the value of 1 log CFU gdw^{-1}, which was the detection limit. Significance of the fit was assessed by an F test ($F = MS_{regression}/MS_{residual}$), and the goodness of fit was determined by calculating a pseudo-r^2 [$1 - (SS_{residuals}/SS_{total\ corrected})$], where MS is the mean square and SS is the sum of squares. For *E. coli* O157:H7, the number of days needed to reach the detection limit of 1 log CFU gdw^{-1} was calculated from the fitted decline function. Multivariate analysis of variance (significance level of 5%) followed by contrast analysis was conducted on the regression parameters c and d. From the second part of the multivariate analysis of variance (within-subject comparisons) the effects of roughage type and crude protein level on the decline rate (d) and the location of the inflection point (c) were assessed. Differences in decline rate between *Salmonella* serovar Typhimurium phenotypes were analyzed by two-sided t tests. Correlation tests were conducted to check for linear relationships between decline rate and chemical parameters of the manure. Stepwise multiple regressions were conducted to determine to what extent variation in chemical and biological parameters can explain variation in decline rates. Variables left in the regression model were significant at the 0.15 level, and models were restricted to a maximum of two parameters.

The decline of *E. coli* O157:H7 in manure-amended soil was analyzed by fitting survival data (log CFU gdw^{-1}) of each replica to a simple exponential decline function, $CFU(t) = N_0 \times e^{st}$, where $CFU(t)$ is the log number CFU gdw^{-1} on day t, N_0 is the initial log number CFU gdw^{-1} on day 0, and s is the slope of the curve. Because all treatments showed an increase during the first 2 days (see Results), the log number CFU gdw^{-1} on day 2 was set to 100%. The subsequent log numbers CFU gdw^{-1} were relative to that on day 2. Slopes of the different treatments were compared by using two-sided t tests. When no CFU were detected, the value of the detection limit was used (0.5 log number CFU gdw^{-1}). The decline of both phenotypes of *Salmonella* serovar Typhimurium was analyzed by fitting the survival data to the same logistic function as used for the data of survival in manure because of bad fits (no convergence or low pseudo-r^2) to the exponential model.

RESULTS

Survival of *E. coli* O157:H7 in manure. In all manure types, *E. coli* O157:H7 populations dropped directly after inoculation by approximately 1.5 log CFU gdw^{-1}, followed by a period of around 16 days when it stabilized or increased by approximately 0.75 log CFU gdw^{-1} (GOL and SH) (Fig. 1). Thereafter, the pathogen declined continuously in all treatments. *E. coli* O157:H7 was not detected by plate counting after 84 days in both manures derived from a straw diet (SH and SL) and after 133 days in the other manure types.

Nonlinear logistic regression resulted in significant fits ($P < 0.001$) with high goodness-of-fit values for all six manure types (average pseudo-r^2 over three replicas: GMH, 0.92; GOH, 0.92; SH, 0.89; GML, 0.82; GOL, 0.93; and SL, 0.95). The numbers of days needed to reach the detection limit of 1 log CFU gdw^{-1} according to the logistic fits for GMH, GOH, SH, GML, GOL, and SL were, respectively, 128 ± 8, 105 ± 8, 76 ± 5, 126 ± 18, 92 ± 12, and 71 ± 8. Roughage type had a significant effect (Wilks' lambda = 0.060; $P < 0.001$) on the course of decline (effect on combined variance of both estimated parameters). Moreover, roughage type had a significant effect on the slope of decline ($P < 0.001$), and crude protein level did not. Roughage type and crude protein level showed no interaction with respect to their effect on the decline rate. The location of the inflection point was not significantly influenced by roughage type or crude protein level. Decline rates in manures based on the same roughage type, but different crude protein levels, did not differ (Fig. 2). All three roughage types differed significantly from each other with respect to the rate of decline, irrespective of crude protein level. When the manure types from the high- and low-CP groups were aggregated to roughage type, *E. coli* O157:H7 declined faster in manure derived from a diet of straw (S) compared to low-digestible grass silage (GO) ($P = 0.007$), S compared to high-digestible grass silage plus maize silage (GM) ($P < 0.001$), and GO compared to GM ($P = 0.027$).

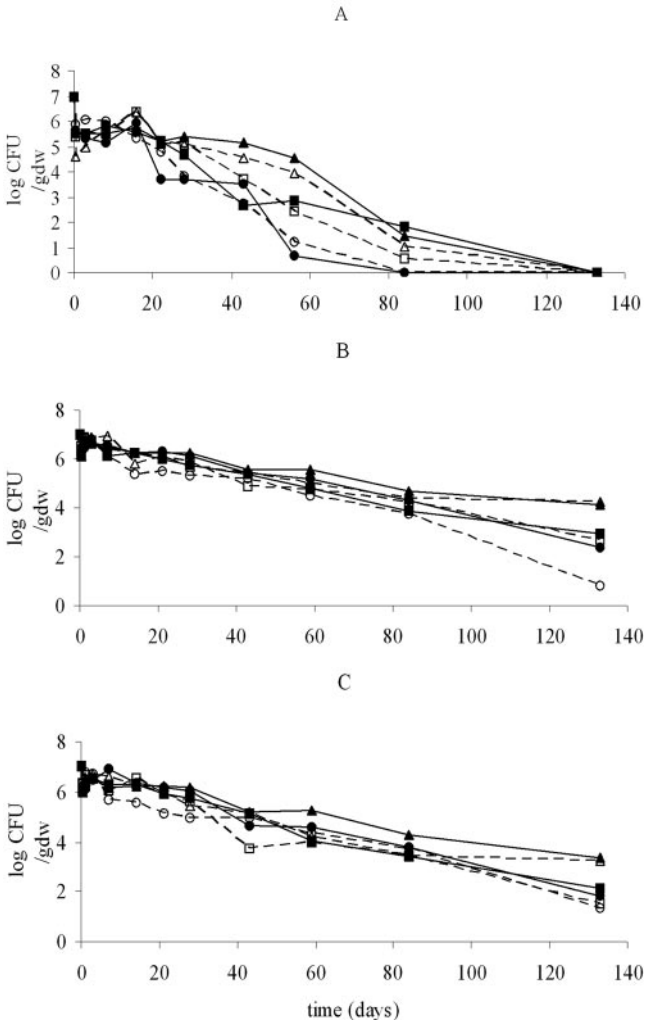

FIG. 1. Survival of *E. coli* O157:H7 (A), *Salmonella* serovar Typhimurium MAE 110 (B), and *Salmonella* serovar Typhimurium MAE 119 (C) in six different types of artificially inoculated cattle manure types resulting from three different roughage types with high (closed symbols and solid lines) and low (open symbols and dashed lines) levels of additional crude protein: high-digestible grass and maize silage (triangles), low-digestible grass silage (squares), and straw (circles).

The rate of decline (absolute value of slope) was positively correlated with pH ($P = 0.003$) and fiber content (acid detergent fiber [ADF], $P = 0.032$; neutral detergent fiber [NDF], $P = 0.017$) (Table 4). The GM manures had the lowest pHs and lowest decline rates, while the S manures had the highest pHs and the highest decline rate (Fig. 3). The GO manures had intermediate pHs and intermediate decline rates. The rate of decline showed a negative linear relationship with ammonium level ($P = 0.024$). Stepwise multiple regressions revealed that pH explained most of the variation in decline rate: slope (model $r^2 = 0.97$) = -1.80×10^{-2} (pH; partial $r^2 = 0.91$,

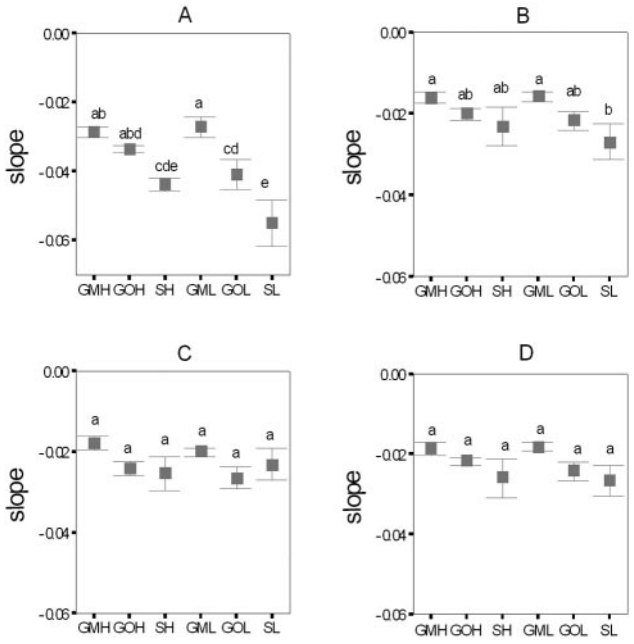

FIG. 2. Values of the estimated slope parameter for the survival of *E. coli* O157:H7 (A), *Salmonella* serovar Typhimurium phenotype MAE 110 (B), *Salmonella* serovar Typhimurium phenotype MAE 119 (C), and *Salmonella* serovar Typhimurium total counts (D) in six different types of artificially inoculated cattle manure types resulting from three different roughage types with high (H) and low (L) levels of additional crude protein: high-digestible grass and maize silage (GMH and GML), low-digestible grass silage (GOH and GOL), and straw (SH and SL). Error bars show standard errors of the means. Treatments with identical letters do not significantly differ.

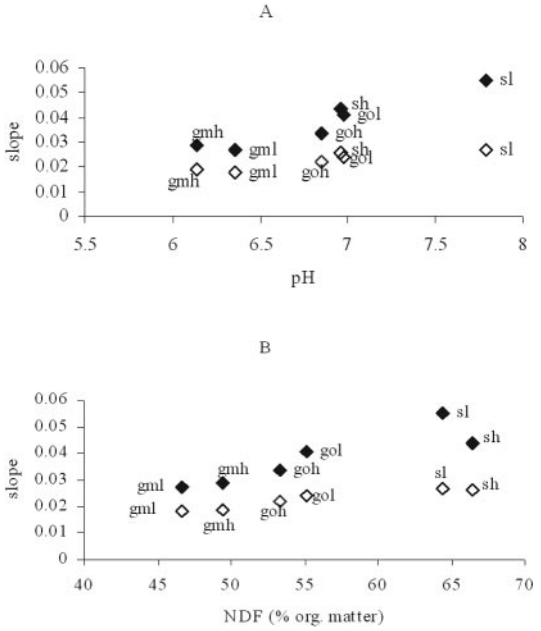

FIG. 3. Relationship between pH (A) or NDF content (B) and the rates of decline of *E. coli* O157:H7 (closed symbols) and *Salmonella* serovar Typhimurium (open symbols) in six different types of artificially inoculated cattle manure resulting from three different roughage types with high (h) and low (l) levels of additional crude protein: high-digestible grass and maize silage (gmh and gml), low-digestible grass silage (goh and gol), and straw (sh and sl).

$P = 0.003$) + 1.06×10^{-4} (dry matter content; partial $r^2 = 0.06$, $P = 0.056$) + 7.03×10^{-2} (intercept). Alternatively, when excluding pH, the neutral detergent fiber (NDF) content was best at explaining the variation in decline rate: slope (model $r^2 = 0.93$) = -2.19×10^{-3} (NDF; partial $r^2 = 0.80$, $P = 0.016$) + 7.05×10^{-4} (C/N ratio; partial $r^2 = 0.13$, $P = 0.093$) − 3.35×10^{-3} (intercept). The pH and NDF content were not significantly correlated (Table 4).

Survival of *Salmonella* serovar Typhimurium in manure. The two phenotypes MAE 110 and 119 showed rather similar survival curves, and both clearly survived longer in all manure types than *E. coli* O157:H7 (Fig. 1). CFU counts dropped directly after inoculation with 0.5 log CFU gdw^{-1}, followed by an increase of 0.5 log CFU gdw^{-1} within a few days. Thereafter, the pathogen declined continuously but at a lower rate than *E. coli* O157:H7. After 133 days, *Salmonella* serovar Typhimurium could still be detected at levels of 2 to 4 log CFU gdw^{-1} by the normal plating procedure, depending on the manure type.

TABLE 4. Pearson correlation coefficients between the absolute slope values of the fitted logistic decline curve for manure and chemical characteristics of the six types of manure ($n = 18$)

Parameter	Pearson correlation coefficient with:										
	Slope				pH	Total N	Total C	$N\text{-}NH_4$	C/N	Dry matter	NDF
	E. coli O157:H7	*Salmonella* serovar Typhimurium									
		MAE 110	MAE 119	Total counts							
pH	0.96[a]	0.97[a]	0.63	0.90[a]							
Total N	−0.13	0.00	0.33	0.16	0.13						
Total C	−0.72	−0.76	−0.45	−0.69	−0.86[a]	−0.52					
$N\text{-}NH_4$	−0.87[a]	−0.87[a]	−0.72	−0.87[a]	−0.81	0.27	0.39				
C/N	0.42	0.48	0.65	0.60	0.30	−0.36	0.13	−0.71			
Dry matter	−0.76	−0.84[a]	−0.90[a]	−0.94[a]	−0.75	−0.05	0.46	0.86[a]	−0.81[a]		
NDF[b]	0.89[a]	0.91[a]	0.61	0.85[a]	0.79	−0.29	−0.45	−0.88[a]	0.75	−0.88[a]	
ADF[c]	0.85[a]	0.86[a]	0.60	0.81[a]	0.74	−0.37	−0.35	−0.81[a]	0.81	−0.88[a]	0.99[a]

[a] Significant correlation ($P < 0.05$).
[b] Cellulose, hemicellulose, and lignin in organic matter.
[c] Cellulose plus lignin in organic matter.

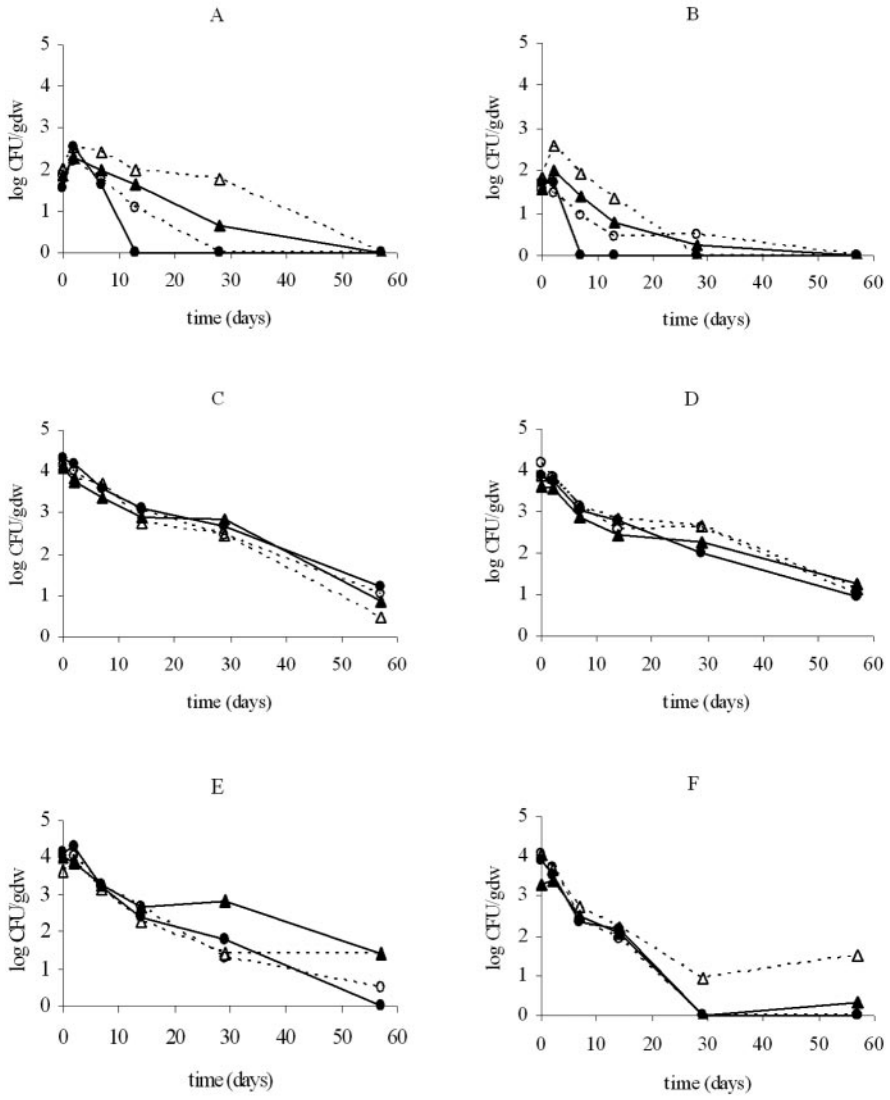

FIG. 4. Survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in four different soils: organic sand (●), conventional sand (○), organic loam (▲), and conventional loam (△). (A) Survival of *E. coli* O157:H7 in soils amended with manure GMH. (B) Survival of *E. coli* O157:H7 in soils amended with manure GML. (C) Survival of *Salmonella* serovar Typhimurium MAE 110 in soils amended with manure GMH. (D) Survival of *Salmonella* serovar Typhimurium MAE 110 in soils amended with manure SH. (E) Survival of *Salmonella* serovar Typhimurium MAE 119 in soils amended with manure GMH. (F) Survival of *Salmonella* serovar Typhimurium MAE 110 in soils amended with manure SH.

As with *E. coli* O157:H7, nonlinear logistic regression resulted in significant fits ($P < 0.001$) with high goodness-of-fit values for phenotype MAE 110 (average pseudo-r^2: GMH, 0.84; GOH, 0.84; SH, 0.89; GML, 0.71; GOL, 0.86; and SL, 0.85) and MAE 119 (average pseudo-r^2: GMH, 0.90; GOH, 0.94; SH, 0.90; GML, 0.83; GOL, 0.86; and SL, 0.87). *Salmonella* serovar Typhimurium MAE 110 and 119 showed no difference in slope over all treatments ($P = 0.223$). Since both phenotypes behaved similarly, the effects of roughage type and CP level were assessed by summing the CFU counts of phenotypes 110 and 119. There was a significant multivariate effect of roughage type (Wilks' lambda = 0.300; $P = 0.008$) and CP level (Wilks' lambda = 0.516; $P = 0.026$) on the combined variance of both regression parameters but no significant effects of roughage type and CP level separately on the decline rate or the location of the inflection point. Contrast analysis legitimated the pooling of manure types based on the same roughage type but different CP levels (Fig. 2). When grouped by roughage type, *Salmonella* serovar Typhimurium declined significantly faster in the manure resulting from the straw (S) diet compared to the high-digestible grass silage plus maize silage (GM) diet ($P = 0.020$). The rate of decline was positively correlated with pH ($P = 0.017$) and fiber content (NDF, $P = 0.005$; ADF, $P = 0.012$) (Table 4 and Fig. 3). The rate of decline showed a negative linear relationship with ammonium level ($P = 0.012$) and dry matter content ($P = 0.010$). Stepwise multiple regressions revealed that NDF content explained most of the variation in decline rate: slope (model $r^2 = 0.97$) = -2.97×10^{-4} (NDF; partial $r^2 = 0.91$, $P = 0.003$) $- 2.46 \times 10^{-3}$ (pH; partial $r^2 = 0.06$, $P = 0.114$) + 0.01081 (intercept).

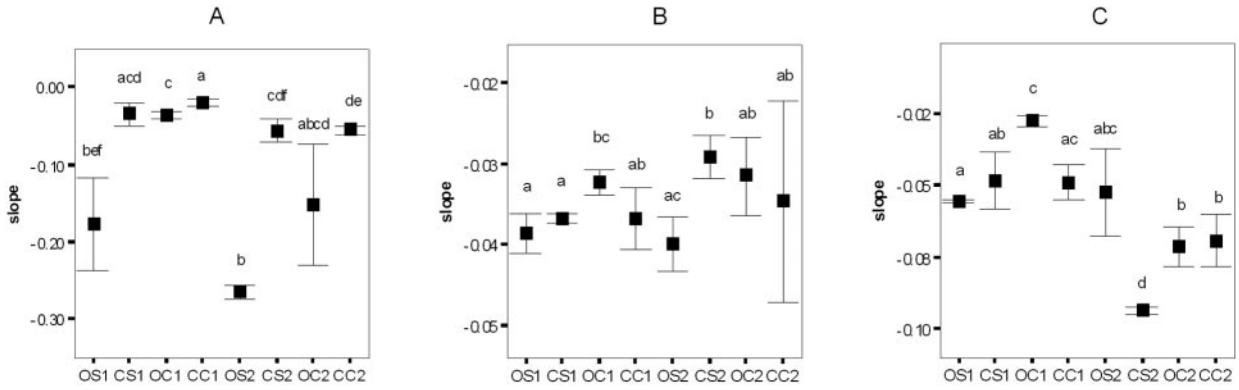

FIG. 5. Values of the estimated slope parameter for the survival of *E. coli* O157:H7 (A) and of *Salmonella* serovar Typhimurium MAE 110 (B) and MAE 119 (C) in four different soils: organic sand (OS), conventional sand (CS), organic loam (OC), and conventional loam (CC). For *E. coli* O157:H7, these four soils were amended with manure type GMH (1) and GML (2) and fitted to an exponential-decline model, while for *Salmonella* they were amended with GMH (1) or SH (2) and fitted to a logistic decline model as with the survival in manure. Error bars show standard errors of the means. Treatments with identical letters do not significantly differ.

Alternatively, when excluding neutral detergent fiber content and the parameters with which it was significantly correlated (ADF and dry matter content) (Table 4), the pH was best at explaining the variation in decline rate: slope (model $r^2 = 0.95$) $= -4.98 \times 10^{-3}$ (pH; partial $r^2 = 0.81$, $P = 0.015$) $- 2.08 \times 10^{-4}$ (C/N ratio; partial $r^2 = 0.14$, $P = 0.056$) $+ 1.56 \times 10^{-2}$ (intercept). The pH and NDF content were not significantly correlated (Table 4).

Survival of *E. coli* O157:H7 in soil. Survival of *E. coli* O157:H7 in the four soils amended with both manures derived from high-digestible grass silage plus maize silage diets (GMH and GML) varied between 2 and 56 days, depending on the soil (Fig. 4). Fitting the survival data to an exponential decline function resulted in good fits (average r^2 over all treatments of 0.87 ± 0.17). The values of the estimated rate of decline are shown in Fig. 5. The kind of manure applied to the soil made no difference except for the conventionally managed loam soil, where rate of decline was higher when GMH was amended than when GML was amended ($P = 0.012$). *E. coli* O157:H7 declined significantly faster ($P < 0.05$) in all organically managed soils then in the conventionally managed neighboring soils, except for loam soil amended with GML. *E. coli* O157:H7 disappeared exceptionally rapidly in the organic sandy soil (Fig. 4 and 5).

The rate of decline in soils was positively correlated with total nitrogen content ($r = 0.86$, $P = 0.006$), nitrate content ($r = 0.81$, $P = 0.014$), and total carbon content ($r = 0.82$, $P = 0.012$). Stepwise multiple regression first resulted in a model solely including the total nitrogen content: slope (model $r^2 = 0.80$) $= -2.15 \times 10^{-1}$ (total nitrogen; partial $r^2 = 0.80$, $P = 0.105$) $+ 2.29 \times 10^{-1}$ (intercept). When the total nitrogen content and parameters correlated with it (nitrate content and total carbon content) were excluded, no variable was strong enough to enter the model, thus explaining a significant part of the variation in the decline rate of *E. coli* O157:H7 in soil.

Survival of *Salmonella* serovar Typhimurium in soil. The density of *Salmonella* serovar Typhimurium declined more steadily than that of *E. coli* O157:H7, and *Salmonella* serovar Typhimurium was in most cases still detected at 56 days after application of the manure to the soils (Fig. 4). The decline rates of *Salmonella* serovar Typhimurium could not be compared with those of *E. coli* O157:H7 directly because a different decline model was used. The two *Salmonella* serovar Typhimurium phenotypes showed quite different patterns of decline rate over the treatments: the two phenotypes differed significantly from each other in decline rate in five of the eight treatments ($P < 0.05$) (Fig. 5). With *Salmonella* serovar Typhimurium phenotype 110, none of the manure-soil treatments was exceptional with respect to the decline rate. Phenotype 119 showed an exceptionally fast decline in conventional sand amended with manure GH and a relative slow decline in organic loam with GMH, compared to the other treatments. No consistent differences were found between organic and conventional soils.

The rate of decline of phenotype 110 in soils amended with SH was positively correlated with nitrate content ($r = 0.95$, $P = 0.049$), total nitrogen content ($r = 0.95$, $P = 0.047$) and total carbon content ($r = 0.99$, $P = 0.007$). The rate of decline in soils amended with GMH did not show any correlations with soil characteristics. The rate of decline of phenotype 119 showed no correlations with any of the chemical parameters. The variation in the rate of decline of phenotype 110 over all treatments was best explained by a model solely including the nitrate content: slope (model $r^2 = 0.99$) $= -4.30 \times 10^{-4}$ (total nitrogen; partial $r^2 = 0.99$, $P = 0.071$) $- 3.03 \times 10^{-2}$ (intercept). For phenotype 119 and the total *Salmonella* counts, no parameter entered the regression model.

Presence on or in lettuce. Only one root sample of a lettuce crop grown on conventional loam amended with manure type GMH showed the presence of *E. coli* O157:H7 (1.5 log CFU gdw^{-1}). Because this sample was not surface sterilized, it is not clear whether the pathogen was present in the rhizosphere, attached on the root surface, or internalized in the root tissue. None of the samples were positive for *Salmonella* serovar Typhimurium phenotypes 110 and 119.

DISCUSSION

The potential presence of human pathogens such as *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in vegetables grown in soils enriched with manure is of growing concern. We simulated the lettuce production chain in three consecutive

steps, monitoring the fate of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure, manure-amended soil, and lettuce. In this way the pathogens experience three different niches and two niche transitions, which is a more realistic setup compared to focusing on survival in one particular niche or determining the association of pathogens with vegetables by planting them directly on inoculated manure-amended soil. We investigated the effects of different cattle diets, soil types, and soil management types on pathogen survival in manure and soil.

We showed that the roughage type, but not the dietary crude protein level, influences the survival capabilities of both *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Decline of *E. coli* O157:H7 was faster in manure derived from a pure straw diet (higher pH and higher fiber content) than in manure derived from a high-digestible grass silage plus maize silage diet (lower pH and lower fiber content). The decline found in manure derived from a low-digestible grass silage diet was intermediate. Persistence of *Salmonella* serovar Typhimurium in manure was better than that of *E. coli* O157:H7. Roughage type showed no effect on the rate of decline of *Salmonella* serovar Typhimurium, although the decline was significantly faster in the manure derived from straw than in the manure from the grass silage plus maize silage diet. The decline rates of both pathogens were mainly determined by the pH and fiber content of the manure. After the first niche transition from manure to manure-amended soil, both pathogens declined further, and again *E. coli* O157:H7 declined faster than *Salmonella* serovar Typhimurium. *E. coli* O157:H7 declined exceptionally rapidly in the organically managed sandy soil. After survival in manure and manure-amended soils, the final and most likely more realistic bacterial loads in the soils used in this experiment did not result in the presence of *E. coli* O157:H7 or *Salmonella* serovar Typhimurium in or on the edible parts of lettuce.

The survival times of *E. coli* O157:H7 reported in this study, ranging between 56 and 133 days at 10°C, resemble earlier published persistence times of *E. coli* O157:H7 in bovine manure (5, 26, 28). *Salmonella* serovar Typhimurium clearly survived longer than *E. coli* O157:H7 and was still present after 133 days. Theoretical elimination times of *Salmonella* serovar Typhimurium of 151 days at 4°C, 85 days at 20°C, and 14 days at 37°C in bovine manure could be derived from linear regression equations (18). In general it is very difficult to compare survival studies, due to the variety of experimental setups used. Moreover, as we showed with this study, survival times depend not only on temperature but also on the manure composition, which is determined by the feeding regimen.

Cattle diet has been considered a potentially important factor in controlling the presence of *E. coli* O157:H7 and *Salmonella* in cattle, given that it likely affects gut microbial populations (34), but results are not unambiguous. Considerable attention has been paid to the controversial effect of cattle diet on pathogen shedding by the animal (11, 20, 25, 38, 45, 48). Roughage type may be important not only in controlling shedding but also with respect to pathogen survival in manure. We showed that the human pathogens *E. coli* O157:H7 and *Salmonella* are more persistent in manure derived from cattle fed a diet characterized by a higher energy and lower fiber content (high-digestible grass silage plus maize silage) than in manure derived from a diet characterized by a lower energy and higher fiber content (straw). Feeding hay to cattle may be a way to reduce shedding of acid-resistant *E. coli* (11). Diets high in grain are thought to create a more acidic rumen environment because the starch is incompletely digested and is fermented in the colon, which in turn should lead to the selection of more acid-tolerant *E. coli* (11, 38). It is known that both *E. coli* O157:H7 and *Salmonella* serovar Typhimurium possess several systems for surviving exposures to low pH and therefore can be considered to be quite acid resistant (6, 12). Extrapolating to pathogenic *E. coli*, the results reported by Diez-Gonzalez et al. (11) seem to be supported by some experimental studies (7, 45) and several epidemiological studies (10, 16, 37) which found a positive association between *E. coli* O157:H7 prevalence and the feeding of barley, corn silage, and grains. *Salmonella* prevalence in dairy heifers was also found to be lower when hay was fed (27). In contrast, some epidemiological studies (39, 47) and various studies using artificially inoculated animals seem to contradict the idea that more forage feeding (hay) compared to grain feeding is a mechanism to reduce selection for increased acid resistance and *E. coli* O157:H7 shedding by ruminants (15, 20, 25, 48).

Although conditions in excreted manure are likely to be different from those encountered in the rumen environment, our results seem to agree with the proposition that a high-energy diet containing grains/starch favors the proliferation and survival of *E. coli* O157:H7. We also showed the importance of a high fiber content of the diet and the resulting manure with respect to the elimination of human pathogens. This might be related to the combination of a relative slow release of readily available nutrients in manure with higher fiber content and the more copiotrophic nature of *E. coli* and *Salmonella*. In practice, feeding starch in the form of grains or maize is a common practice in dairy farming in order to fulfill the energy need of high milk production. However, there is a trend in more sustainable and organic dairy farming of feeding a diet with increased fiber content consisting of lower concentrations of cytoplasmic carbohydrates (sugars and starch) and more so-called cell wall carbohydrates (hemicellulose, cellulose, and lignin). This is often accompanied by a higher C/N ratio, consequently reducing nitrogen losses to the environment (49). According to our findings, this should result in lower survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium and consequently in a lower risk of transfer of these pathogens into the vegetable production chain.

The land application of infected manure is a major transition for human pathogens, since soil can be considered to be a hostile environment for bacteria that have the gastrointestinal tracts of mammals as their primary habitat. Although pathogen levels gradually decline with increased storage time and after land application, it is recommended that an interval of at least 120 days (2) or even 6 months (31) should be observed between manure spreading and harvest of the crop. Our results for *E. coli* O157:H7 survival between 2 and 56 days in manure-amended soil are comparable with earlier reported survival times of 34 days in sandy loam soil amended with cow manure at a similar temperature and manure-to-soil ratio (23). Others reported longer *E. coli* O157:H7 survival times of between 154 and 217 days in soils amended with inoculated compost (21) and *Salmonella* serovar Typhimurium persistence of between

203 and 231 days (22). However, those studies relied on inoculating the substrate with relatively high densities ($>10^5$ CFU gdw^{-1}). In the present study we started monitoring the fate of the pathogens in manure-amended soil after they declined to relatively low and more realistic levels in manure (approximately 10^2 CFU gdw^{-1} for *E. coli* O157:H7 and 10^4 CFU gdw^{-1} for *Salmonella* serovar Typhimurium). As with survival in manure, it must be stressed that comparison between studies is difficult, as different substrates and experimental setups are used. Persistence seems to depend on factors such as temperature (23), manure-to-soil ratio (23), and soil type (32). We showed that decline of *E. coli* O157:H7 was faster in the organically managed soil than in its conventionally managed neighbor in three out of four cases and was exceptionally fast in the organic sandy soil treatments. The latter may be more due to the relative high levels of nitrate, total nitrogen, and total carbon in this specific organic sandy soil. This might have increased the activity of the native microbial population, which decreased the competitive success of the introduced pathogen. The extremely fast decline in this particular soil was not observed for *Salmonella* serovar Typhimurium, which may have a higher competitive ability. More research with more pairs of soils is needed in order to differentiate between organic and conventional soils with respect to human pathogen suppression.

The third transition, the planting of lettuce, did not eventually result in the presence of *E. coli* O157:H7 or *Salmonella* serovar Typhimurium on or in the edible parts of iceberg lettuce. Some experimental studies demonstrated that these pathogens can become associated with vegetables (21, 22, 30, 54, 56). However, a wide variety of experimental setups were used (seedlings or seeds grown hydroponically or in soil), and most of these studies proved only surface contamination. Solomon et al. (41) showed that *E. coli* O157:H7 can enter the lettuce plant from contaminated manure through the root system and can migrate throughout the edible part of the plant. Recently, our laboratory also confirmed the possibility of internalization of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in iceberg lettuce grown hydroponically and in inoculated soil (E. Franz, A. A. Visser, A. D. van Diepeningen, M. M. Klerks, A. J. Termorshuizen, and A. H. C. van Bruggen, submitted for publication). However, the numbers of bacteria used in these studies were far greater than what may be found in an agricultural field. In the current experiment the pathogen densities in the bulk soil at the time the lettuce was planted were approximately 10 to 100 CFU gdw^{-1} for *E. coli* O157:H7 and 100 to 1,000 CFU gdw^{-1} for *Salmonella* serovar Typhimurium. These densities might be more realistic. Most likely, the population pressure was too low to allow the pathogens to enter the plants. Indeed, the results of Solomon et al. (41) showed an increased number of positive samples with increasing pathogen density of the inoculum (10^4, 10^6, and 10^8 CFU gdw^{-1}).

This study showed for the first time the fate of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium through subsequent niches: manure, manure-amended soil, and manure-amended soil with lettuce. The results indicate that the cattle feeding regimen must be recognized as an important factor determining the survival of these pathogens in manure. Since manure is the primary fertilizer in organic vegetable production and is frequently used in conventional production, these results are of importance with respect to microbial safety in vegetable production. Our results indicate that although manure is more frequently used in organic production, this does not automatically imply a higher risk of pathogen transfer to vegetable production. More work has to be done on how differences between organic and conventional farming may lead to differences in pathogen survival, not only in manure but in the whole farm ecosystem.

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