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Potential Uptake of *Escherichia coli* O157:H7 from Organic Manure into Crisphead Lettuce

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To investigate the potential transfer of *Escherichia coli* O157:H7 from contaminated manure to fresh produce, lettuce seedlings were transplanted into soil fertilized with bovine manure which had been inoculated with approximately 10^4 CFU g^{-1} *E. coli* O157:H7. The lettuce was grown for approximately 50 days in beds in climate-controlled rooms in a greenhouse. As the bacterium was not detected in the edible parts of the lettuce, the outer leaves of the lettuce, or the lettuce roots at harvest it was concluded that transmission of *E. coli* O157:H7 from contaminated soil to lettuce did not occur. The pathogen persisted in the soil for at least 8 weeks after fertilizing but was not detected after 12 weeks. Indigenous *E. coli* was detected only sporadically on the lettuce at harvest, and enterococci were not detected at all. The numbers of enterococci declined more rapidly than those of *E. coli* in the soil. *Pseudomonas fluorescens*, which inhibited growth of *E. coli* O157:H7 in vitro, was isolated from the rhizosphere.

In recent years there has been an increased awareness of fruits and vegetables as potential vehicles in the transmission of human infections (30). Several outbreaks of *Escherichia coli* O157:H7 infection (1, 3–5, 10) have raised concerns about the origins of the contamination. One hypothesis is that the use of untreated or fresh manure as fertilizer may lead to contamination of crops, as animal manure may harbor pathogens such as *E. coli* O157:H7, *Salmonella* spp., and others. In organic farming manure is frequently used as fertilizer in comparison with its use in conventional agriculture. *E. coli* O157:H7 shed from healthy cattle can survive for extended periods of time in the environment; survival times measured in days, weeks, and months have been recorded for manure, soil, water, and vegetables such as lettuce (2, 8, 15, 17, 26, 31, 32).

In a field study described by Johannessen et al. (16), where the influence of manure on the hygienic quality of lettuce was studied, *E. coli* O157:H7 was unexpectedly isolated from naturally contaminated firm manure and slurry used for fertilizing and also from soil 1 week after fertilizing. However, *E. coli* O157:H7 was not detected in lettuce grown in this soil. Nevertheless, results from other studies have shown that pathogens may be transferred from manure to the surface of vegetables via contaminated soil (11–14, 23). Additionally, Solomon et al. (29) demonstrated experimentally that *E. coli* O157:H7 could become internalized in lettuce tissue when lettuce seeds were sown in manure-amended soil.

The potential for transmission of pathogens may have serious implications for the use of fresh or untreated manure in organic or conventional production of vegetables. The few investigations on the bacteriological quality of organic fruit and vegetables that have been published have found no evidence that these products are of poorer hygienic quality, or have higher numbers of pathogenic bacteria present, than conventionally produced ones (16, 20–22, 27).

Various studies have been conducted to investigate the antagonistic effect of native soil microflora on human pathogens (19, 28). Schuenzel and Harrison (28) showed that several of the culturable microorganisms native in soil have antagonistic effects on human pathogenic bacteria, including *E. coli* O157:H7. The presence of antagonists in soil may contribute to a reduction in numbers of human pathogens. However, as the infectious dose for *E. coli* O157:H7 is low (7), survival of even a few of these bacteria may pose a risk to consumers either from ingestion of raw contaminated vegetables or from cross-contamination to other products.

Due to climatic conditions, the outdoor growing season in Norway is short compared to many other countries. Therefore, the usual procedure for commercial lettuce production is cultivation of seedlings indoors followed by transplantation into the field when the weather conditions permit.

The aim of the present study was to investigate the potential for *E. coli* O157:H7 to be transferred from soil fertilized with contaminated manure to the edible parts of organic crisphead lettuce when seedlings were transplanted into manure-amended soil. The presence of fecal indicator bacteria in lettuce was investigated. Survival of *E. coli* O157:H7 in the soil was studied, and the inhibitory effect of selected bacteria from the rhizosphere against *E. coli* O157:H7 in vitro was also demonstrated.

MATERIALS AND METHODS

Experimental design. The experiment was carried out in two climate-controlled rooms in a greenhouse at The Centre for Plant Research at the Agricultural University of Norway (AUN). Seedling lettuces were transplanted into soil fertilized with slurry (11.8% dry matter) inoculated with *E. coli* O157:H7 and grown for 7 weeks. Bacteriological examinations of soil, slurry, and lettuce were carried out at the day of fertilizing (soil and slurry only), at transplanting 1 week

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after fertilizing, at 3 weeks after transplanting, at harvest 7 weeks after transplanting, and at 12 weeks after fertilizing (soil only). All the plants were sampled for bacteriological analyses.

Soil and plant beds. Surface soil, without turf, was collected from an organically grown field at the AUN, cultivated by a 6-year rotation. The soil type was between loam and silty clay loam. The soil was stored for 1 month at +4°C in wooden boxes lined with polyethylene film before the soil was placed to a depth of 17.5 cm in two large beds (80 × 180 cm) and one small bed (80 × 130 cm) in each room. The beds were made of plywood lined with 0.2-mm polyethylene sheets with drainage holes in the bottom, and each bed contained three rows of plants with a 25-cm plant-to-plant distance and a 15-cm plant-to-wall distance. The large beds had seven plants in each row, whereas the small bed had five plants in each row, giving a total of 114 plants for sampling. Clumps of soil were disintegrated, and larger stones were removed from the beds prior to manuring and transplanting.

Collection, inoculation, and incorporation of manure. Manure slurry and urine were collected directly from organic dairy cattle from one farm and stored in closed polypropylene containers at +4°C for 5 to 6 days. One week before transplantation and 1 day after filling the beds with soil, 32 kg manure and 6 liters urine were mixed in a large bucket to achieve slurry and used as fertilizer. For inoculation, a nontoxigenic strain of *E. coli* O157:H7 (NCTC 1200) that had growth rates comparable to the wild-type strains and was resistant to 100 μg ml^{-1} nalidixic acid and 1,000 μg ml^{-1} streptomycin (NAS) was used. A total of 32 ml of an overnight bacterial suspension of approximately 10^7 CFU ml^{-1} was mixed thoroughly with the fertilizer. The inoculated fertilizer was immediately spread on the soil surface (4.7 kg m^{-2}) and mixed into the upper 10 cm of soil with shovels. Three weeks after transplanting, 5 liters m^{-2} of diluted urine (1 liter urine plus 4 liters water) was spread on the soil surface as a supplementary fertilizer.

Vegetable production. Crisphead lettuce, *Lactuca sativa* L. var. *capitata* L., cultivar Coquette (organic, pelleted seeds; Rijk Zwaan, ZG De Lier, The Netherlands), was seeded into 96-well trays with synthetic growth medium approved for organic agriculture (VadheimGroplex AS, Loddefjord, Norway). Plants were raised in a climate room at 18°C with adequate amounts of water and light.

The seedlings were transplanted into the beds after 3 weeks. Care was taken to avoid contamination of the aboveground parts of the seedlings. For the rest of the growth period, the temperature was kept at 15°C during a light period of 20 h and at 12°C during a 4-h dark period (night). Light from Na and Hg lamps and sunlight gave PAR of 150 to 250 μmol m^{-2} s^{-1} in the light period. The beds were evenly watered (4 liters m^{-2}) three times per week with a watering can equipped with a sprinkler a short distance above the plants. This amount of water was sufficient for normal plant growth without leakage from the bed. Splash of soil onto the plants was carefully avoided. The water used was drinking water from the community supply that undergoes regular bacteriological testing by the municipal food control authorities. The relative air humidity was on average 65% (range, 50 to 80%). A periodically swinging fan was mounted in each room.

Sampling procedures. A total of 10 samples of soil (100 to 200 g) were collected randomly from the beds into stomacher bags at each sampling occasion (prior to fertilization and 1, 4, 8, and 12 weeks after fertilization). Slurry, before and after inoculation, was sampled directly into stomacher bags from the bucket used for mixing manure and bacterial culture (10 samples each).

Samples of two seedlings each were collected at random from the trays used for propagation (five samples for fecal indicators and a composite sample for *E. coli* O157:H7). Samples of lettuce were taken 3 and 7 weeks after transplanting. At the first sampling, 10 samples were taken, each sample comprising four lettuce plants (total of 40 plants). On the second sampling, 24 samples of two lettuce heads were collected (total of 48 plants). The lettuce was harvested by cutting the top part of the stem without including the leaves closest to the soil surface (outer leaves). The outer leaves that were left on the soil surface were also subsequently analyzed. Some of the lettuce plants were underdeveloped, and due to their small size these were analyzed as composite samples (25 plants plus 1 dead plant). All 114 plants were analyzed.

Roots from both healthy and underdeveloped lettuce were collected for analysis at both sampling occasions. The soil adhering to the roots was shaken off into stomacher bags. The roots were surface sterilized as follows: washing in sterile distilled water for 1 min followed by 1 min immersion in 70% ethanol, washing in sterile distilled water for 1 min, 1 min immersion in 20% chlorine solution, and a final washing in sterile distilled water for 1 min. The excess water was shaken off, and the roots were dried on a paper towel before they were put into stomacher bags (J. F. Hanssen, AUN, personal communication). Twenty soil samples from the roots (rhizosphere) were analyzed for the presence of fluorescent *Pseudomonas* spp. on both sampling occasions.

The samples were transported to the laboratory and processed the same day or following overnight storage at 4°C.

Bacterial analyses. Samples of soil, manure, and fertilized soil were each mixed manually before appropriate subsamples were weighed into stomacher bags for further processing. The samples from lettuce (heads and outer leaves) were cut into pieces and prepared as described by Johannessen et al. (16). For the underdeveloped lettuce and roots, all the material was cut into pieces and used for analysis. Quantitative analyses for *E. coli* and enterococci were performed using standard bacteriological methods with slight modifications (24, 25). Briefly, aliquots of a 10-fold dilution series were pour-plated in tryptic soy agar (Oxoid, Ltd., Basingstoke, Hampshire, UK) and overlaid with violet red bile agar (Difco Laboratories, Detroit, MI) or surface-plated onto Slanetz and Bartley agar (Oxoid) for the enumeration of *E. coli* bacteria and enterococci, respectively. Presumptive *E. coli* was confirmed by growth, gas formation, and fluorescence under UV light in EC broth with MUG (Oxoid). Presumptive enterococci were confirmed by negative catalase reaction and the ability to hydrolyze aeskulin on Enterococcosel agar (BBL, Becton Dickinson and Company, Sparks, MD). The detection limits were 10 and 100 CFU g^{-1} for *E. coli* and enterococci, respectively.

Detection of *E. coli* O157:H7 was performed using an automated immunomagnetic separation (AIMS) system (BeadRetriever and Dynabeads anti-*E. coli* O157; Dynal Biotech ASA, Oslo, Norway). For the samples of soil before fertilization and manure before inoculation, the samples (25 g) were each enriched in 225 ml of buffered peptone water at 41.5°C for 24 h. The rest of the samples were enriched at 37°C due to the assumption that the *E. coli* O157:H7 cells were stressed. A 1-ml volume of the enrichment broth was used for AIMS. After AIMS, 50-μl aliquots of the bead suspension were plated on agar plates. The samples from soil prior to fertilizing, the manure before inoculation, and the seedlings were plated on Chromagar O157 (CHROMagar, Paris, France) and CT-SMAC (Oxoid). The rest of the samples were plated on Chromagar O157 and blood agar (BA; Oxoid), both supplemented with 100 μg ml^{-1} nalidixic acid and 1,000 μg ml^{-1} streptomycin (NAS) and incubated at 37°C for 24 h. Presumptive colonies were cultured on BA for purity and confirmed by indole production (BBL DrySlide indole) and agglutination (DrySpot O157; Oxoid). *E. coli* O157:H7 was enumerated by adding 90 ml of peptone saline to 10 g of sample, mixed manually by shaking for 30 s, and further serially diluted in peptone saline. Aliquots of 100 μl of the appropriate dilutions were plated on Chromagar O157 and BA plates supplemented with NAS and further processed as described above.

For detection of *Pseudomonas* spp., 100 μl of appropriate dilutions was plated onto Kings Agar B (proteose peptone 20.0 g, Difco; Bacto agar 15.0 g, Oxoid; glycerine 10.0 g, Merck, Darmstadt, Germany; KH_2PO_4 1.5 g, Merck; $MgSO_4$-$7H_2O$ 1.5 g, Merck) supplemented with 50 μg ml^{-1} penicillin G (Panpharma, Luitré Fougéres, France), 50 μg ml^{-1} novobiocin (Sigma, St. Louis, Missouri), 75 μg ml^{-1} cycloheximide (Sigma), and 15 μg ml^{-1} chloramphenicol (Sigma) (PNCC; J. F. Hanssen, AUN, personal communication) and incubated at 20°C for 3 days. Colonies (2 to 3 from each sample) that fluoresced under UV light were picked and plated on new Kings Agar B with PNCC and incubated as described above and checked for fluorescence again. Fluorescent colonies were plated on blood agar and checked for oxidase and catalase activity and further identified using API 20NE (Biomerieux, Marcy l'Etoile, France).

Inhibition of *E. coli* O157:H7 by *Pseudomonas* spp. Inhibition testing of *Pseudomonas* sp. isolates in vitro was performed using a modified version of the method described by Schuenzel and Harrison (28). *Pseudomonas* isolates and *E. coli* O157:H7 were grown individually in TSB for 24 h at 25°C with shaking and at 37°C without shaking, respectively. A lawn of *E. coli* O157:H7 was made by placing 1 ml of a 10^6 CFU ml^{-1} TSB culture in a 9-cm petri dish onto which molten plate count agar was poured. After the agar had solidified, aliquots of 2 μl of the *Pseudomonas* cultures were spotted onto the surface of the agar plates. Each isolate was spotted three times on each of two agar plates, giving six repetitions for each isolate. The plate count agar plates were incubated at 25°C for 24 h and checked for inhibition. Inhibition was demonstrated either by a clear inhibition zone surrounding the *Pseudomonas* colonies or by absence of growth directly beneath the colony. When an isolate showed inhibiting effect at 25°C, it was also tested at 10°C, 15°C (isolates subjected to both 10 and 15°C were incubated for 1 week), 20°C (incubated for 3 days), and 30°C (incubated for 24 h).

Statistical analyses. The descriptive statistics were conducted using a Microsoft Excel 2002 spreadsheet. The 95% confidence limits for the prevalence of *E. coli* O157:H7 in the samples were estimated by assuming a Poisson distribution.

TABLE 1. Concentrations of *E. coli* and enterococci in manure, soil, seedlings, and lettuce

Material	*n*	Concn of[c]:	
		E. coli	Enterococci
Soil prior to fertilizing	10	<10	<100
Manure prior to inoculation	10	2.8×10^5 (1.9×10^5–5.2×10^5)	7.5×10^5 (5.0×10^5–2.0×10^6)
Soil 1 wk after fertilizing	10	2.5×10^4 (3.4×10^3–4.0×10^5)	1.7×10^3 (7.0×10^2–7.0×10^3)
Seedlings	5	<10	<100
Soil 4 wk after fertilizing	10	2.8×10^3 (5.9×10^2–7.7×10^3)	9.5×10^2 (5.0×10^1–5.3×10^3)
Lettuce at harvest	24	10[a]	<100
Soil 8 wk after fertilizing	10	3.4×10^2 (2.0×10^1–5.8×10^2)	1.0×10^2–1.1×10^4[b]

[a] One positive sample.
[b] Two positive samples.
[c] Results are given as the median CFU g^{-1} wet weight (min – max).

RESULTS

***E. coli* O157:H7.** The manure slurry used for fertilizing contained 3.8×10^4 CFU g^{-1} of *E. coli* O157:H7 (range, 2.5×10^4 to 5.0×10^4 CFU g^{-1}). After 1 week of application of manure, the *E. coli* O157:H7 numbers in the soil were below the detection limit of the enumeration method (<100 CFU g^{-1}) and a standard enrichment procedure followed by AIMS was used to detect *E. coli* O157:H7. *E. coli* O157:H7 was then detected in 10 of 10, 3 of 10, and 2 of 10 samples of soil at 1, 4, and 8 weeks after application of manure, respectively. Samples collected 12 weeks after application of manure were negative. *E. coli* O157:H7 was not isolated from any of the lettuce, outer leaves, or root samples analyzed. The 95% confidence limit for the prevalence of *E. coli* O157:H7 was estimated to be 0 to 2.6%.

Fecal indicator bacteria. The results from the analyses of fecal indicators in soil, fertilizer, and lettuce are listed in Table 1. The results show that the level of fecal indicators present in the lettuce at harvest was low. Indigenous *E. coli* was detected in 10 of 10 samples of soil after 8 weeks at levels in excess of 100 CFU g^{-1}, whereas enterococci were only detected in 2 samples.

Inhibition of *E. coli* O157:H7. Fluorescent pseudomonads were present in numbers around 10^5 to 10^6 CFU g^{-1} of soil from the rhizosphere, and a total of 42 isolates were tested for their ability to inhibit growth of *E. coli* O157:H7 (NCTC 1200) in vitro. All isolates, except for one that was identified as *P. putida*, were identified as *P. fluorescens*. Five *P. fluorescens* isolates inhibited growth of *E. coli* O157:H7 at 25°C. For the remaining 37 isolates, no clear inhibition zone around or beneath the colonies was detected. The isolates that inhibited growth at 25°C also suppressed growth of *E. coli* O157:H7 at 10, 15, 20, and 30°C. The zones of inhibition were larger at 10 and 15°C than at the other temperatures.

DISCUSSION

In the present study, transmission of *E. coli* O157:H7 from contaminated manure slurry via soil to lettuce apparently did not occur. These results concur with those of a field study in which manure naturally contaminated with *E. coli* O157:H7 was used as a fertilizer on lettuce, and *E. coli* O157:H7 was not isolated from the lettuce (16). In contrast, other studies that have investigated the risks of using manure as fertilizer on vegetables have found that transmission may occur under experimental conditions both in greenhouses and in the field (11–14, 23, 29). However, use of a range of a different experimental parameters in these other studies means that none are directly comparable to the present study. Solomon et al. (29) sowed lettuce seeds in small soil beds in which the soil had been fertilized with inoculated manure and demonstrated that cells of *E. coli* O157:H7 could be recovered from the seedlings. It has also been shown that *Salmonella enterica* serovar Typhimurium could be isolated from different types of vegetables after sowing seeds in soil amended with artificially inoculated manure or composts (13, 23). In our study, the pathogen was introduced at a time when the plant had reached the seedling stage of growth and may have been less susceptible to uptake of human pathogenic bacteria. Results from Warriner et al. (33) indicate that the interaction of *E. coli* with spinach was dependent on the stage of introduction of the bacteria. In contrast, Islam et al. (11, 12, 14) demonstrated in a series of experiments both in a growth chamber and in the field that after seedlings were planted in soils that were amended with artificially inoculated manure-based composts, both *E. coli* O157:H7 and *S. enterica* serovar Typhimurium persisted for at least 2 months on the surface of the vegetables. These studies used a higher concentration of pathogens (10^7 CFU g^{-1} compost) in the manure than the present study (10^4 CFU g^{-1}), which may suggest that the concentration of pathogens also plays a role in the contamination of vegetables from manure. Solomon et al. (29) used three different concentrations of *E. coli* O157:H7, and the use of low concentrations of cells (10^4 CFU g^{-1}) resulted in two samples out of six being positive after 9 days of growing. The results from the present study together with the results from others indicate that both the time of introduction and the concentration of pathogen present might influence the uptake of bacteria.

In our study *E. coli* O157:H7 persisted in the soil for 8 weeks after application of manure but was not detected 12 weeks after fertilizing. Bearing in mind that the soil type was between loam and silty clay loam, the survival time was relatively short compared to the findings of Fenlon et al. (8) in a study in which *E. coli* O157 was detected in artificially inoculated loam and clay soils for over 20 weeks. Results from Islam et al. (12) indicate that the type of vegetable being grown plays a role with regard to the persistence of *E. coli* O157:H7 in soil. Gagliardi and Karns (9) also showed that *E. coli* O157:H7 persisted longer with some cover crops and also with the presence of clay.

The use of fresh manure as fertilizer was not reflected in

high counts of fecal indicators on the lettuce in our study. This concurs with results from a similar study in which *E. coli* was isolated only sporadically from organically grown lettuce (16). Additionally, Loncarevic et al. (20) detected low concentrations of thermotolerant coliform bacteria in organic lettuce produced in Norway. Mukherjee et al. (22) found a significantly higher prevalence of *E. coli* in organic (both certified and noncertified) than in conventional produce. They also found that the use of cattle manure and the use of manure that had been stored for less than a year resulted in a higher prevalence of *E. coli* than that seen with the farms that used older manure and other types. Whether the manure was applied in the autumn or the spring did not play a role for the occurrence of *E. coli* in the product.

In our study the fecal indicators persisted in the soil for at least 8 weeks. The more rapid decline in the numbers of enterococci compared to *E. coli* is similar to the results of Lau and Ingham (18) in a study in which *E. coli* survived for longer in the soil than enterococci. This may suggest that *E. coli* is a better indicator when investigating the hygienic quality of produce whose edible parts may have been in contact with soil. Warriner et al. (33) showed that the numbers of *E. coli* increased progressively in the soil during the growth period of spinach when seedlings were transplanted into contaminated soil.

The results from the present study showed that *Pseudomonas* spp. that inhibit growth of *E. coli* O157:H7 in vitro were present in the soil shaken off the lettuce roots. A study by Cooley et al. (6) showed the importance of the soil microflora on the colonization of *Arabidopsis thaliana* with *S. enterica* serovar Newport and *E. coli* O157:H7. The survival of the pathogens was reduced when the plant was grown in nonautoclaved soil or amended soils. In the soil, the pseudomonads are in abundance and should compete out the added *E. coli* O157:H7, when the bacterium is present in low levels, particularly as the pseudomonads are adapted to the soil environment.

As *E. coli* O157:H7 was isolated neither from the roots nor the lettuce, it seems reasonable to assume that the pseudomonads present may have had an antagonistic effect on this pathogen. However, since persistence of the pathogen in the soil over time was observed, the antagonistic effect of the pseudomonad present must not be overestimated. Cultivation of lettuce in other soil types and under other environmental conditions may lead to other results. Interestingly, the larger inhibition zones observed in vitro were at temperatures similar to those used during cultivation (10 and 15°C).

In conclusion, transmission of *E. coli* O157:H7 from manure to lettuce was not observed when seedlings were transplanted into soil fertilized with manure inoculated with low concentrations of the pathogen. The results also indicated that some of the organisms native in the soil microflora have antagonistic effects against pathogenic bacteria introduced into soil.

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