Impact of Manure Fertilization on the Abundance of Antibiotic-Resistant Bacteria and Frequency of Detection of Antibiotic Resistance Genes in Soil and on Vegetables at Harvest

Romain Marti,^a Andrew Scott,^a Yuan-Ching Tien,^a Roger Murray,^a Lyne Sabourin,^a Yun Zhang,^a Edward Topp^{a,b}

Agriculture and Agri-Food Canada, London, Ontario, Canada^a; Department of Biology, University of Western Ontario, London, Ontario, Canada^b

Consumption of vegetables represents a route of direct human exposure to bacteria found in soil. The present study evaluated the complement of bacteria resistant to various antibiotics on vegetables often eaten raw (tomato, cucumber, pepper, carrot, radish, lettuce) and how this might vary with growth in soil fertilized inorganically or with dairy or swine manure. Vegetables were sown into field plots immediately following fertilization and harvested when of marketable quality. Vegetable and soil samples were evaluated for viable antibiotic-resistant bacteria by plate count on Chromocult medium supplemented with antibiotics at clinical breakpoint concentrations. DNA was extracted from soil and vegetables and evaluated by PCR for the presence of 46 gene targets associated with plasmid incompatibility groups, integrons, or antibiotic resistance genes. Soil receiving manure was enriched in antibiotic-resistant bacteria and various antibiotic resistance determinants. There was no coherent corresponding increase in the abundance of antibiotic-resistant bacteria enumerated from any vegetable grown in manure-fertilized soil. Numerous antibiotic resistance determinants were detected in DNA extracted from vegetables grown in unmanured soil. A smaller number of determinants were additionally detected on vegetables grown only in manured and not in unmanured soil. Overall, consumption of raw vegetables represents a route of human exposure to antibiotic-resistant bacteria and resistance determinants naturally present in soil. However, the detection of some determinants on vegetables grown only in freshly manured soil reinforces the advisability of pretreating manure through composting or other stabilization processes or mandating offset times between manuring and harvesting vegetables for human consumption.

Nutritionists currently endorse what mothers have forever told their children, that vegetables are an indispensable component of a healthy diet (1). Unfortunately, the frequency of food-borne outbreaks due to contaminated fresh produce is increasing, with an estimated 131 produce-related outbreaks in the United States between 1996 and 2010, for example (2–4). The Codex Alimentarius “Code of Hygienic Practice for Fresh Fruits and Vegetables,” the Canadian Food Inspection Agency “Code of Practice for Minimally Processed Ready-to-Eat Vegetables,” and new produce safety standards proposed under the Food Safety Modernization Act in the United States are examples of recommended or mandated management practices that seek to reduce microbial contamination of produce during production, processing, and distribution (5–7). A key component of good practice is to maintain adequate environmental hygiene standards, notably with respect to ensuring that produce does not come into contact with pathogens carried in raw manure that is used as a fertilizer. This can be achieved by disinfecting the manure through composting or another appropriate treatment prior to application to land or ensuring a suitable offset time between manure application and harvest of fresh produce. These practices will reduce the risk of produce contamination by manure-borne pathogenic microorganisms.

Fecal material from domestic animals, humans, and wildlife will contain bacteria that are resistant to some antibiotics (8). The abundance, types of resistance, and distribution of resistance in populations of commensal and pathogenic bacteria vary enormously, no doubt due to varied factors, in particular exposure of the enteric flora to antibiotics in human medicine or as commonly employed in commercial animal production (9, 10). Emissions of antibiotic residues and resistant bacteria from various human ac-

tivities, including animal production, fish production, wastewater treatment, and antibiotic manufacturing, will increase the burden of antibiotic resistance in exposed environmental matrices (11–16). The abundance and the mobility of antibiotic resistance genes in agricultural soils may be enhanced by various management practices, for example, the application of animal manures, wastewater, or waste treatment residues that contain antibiotic resistance genes on mobile elements and antibiotic residues (17–20). The use of antibiotics in animal production and the abundance of antibiotic-resistant bacteria and plasmids carrying antibiotic resistance determinants in manure have engendered concern that recycling of manure onto agricultural land used for crop production can potentiate and disseminate resistance to crops destined for animal or human consumption (21).

Consumption of fresh produce, particularly eating raw vegetables, represents a route of direct human exposure to soil microorganisms. In the present study, we investigated the antibiotic resistance characteristics of bacteria on tomatoes, cucumbers, peppers, carrots, radishes, and lettuce at harvest. These vegetables represent a range of roots, fruits, and leafy vegetables that vary in their soil contact and surface presentation to key environmental factors:

Received 28 May 2013 Accepted 8 July 2013

Published ahead of print 12 July 2013

Address correspondence to Edward Topp, ed.topp@agr.gc.ca.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.01682-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.01682-13

sun, rain, and wind. In order to evaluate the potential impact of fecal material used as fertilizer, vegetables were grown in soil fertilized inorganically or freshly fertilized with dairy or swine manure at agronomic rates of application. Our hypothesis was that antibiotic-resistant bacteria would be more abundant on vegetables grown in manured soil than in unmanured soil. Sowing plants or seeds directly into freshly manured soil is contrary to mandated or recommended practices but does represent a worst-case scenario for potential transfer of enteric microorganisms to fresh produce. Furthermore, soil fertilized with raw manure provides a useful contrast with respect to the abundance of antibiotic-resistant bacteria naturally found in soil that may find their way onto harvested vegetables. Antibiotic-resistant populations were evaluated by plate count, and the distributions of gene targets associated with resistance to specific antibiotics, plasmid incompatibility groups, and integrons were evaluated by PCR analysis of DNA extracted from soil and harvested vegetables.

MATERIALS AND METHODS

Field operations. Experiments were undertaken during the 2011 and 2012 growing seasons on the Agriculture and Agri-Food Canada research farm in London, Ontario, Canada (42.984°N, 81.248°W). The soil is a hodge-podge resulting from glaciolacustrine deposits and has the following properties: pH of 7.2 to 7.4, cation exchange capacity of 13.2, sand-silt-clay percentages of 57/23/19, and organic matter content ranging from 3.4 to 3.9%. Climate conditions (temperature and precipitation) during the experimental period are available in Fig. S1 in the supplemental material. Prior to the present study, the field plot area was cropped to rye in 2008 and to soybeans in 2009 and 2010 and had not been irrigated or received any manure amendments. In the present study, crops were periodically irrigated with well water obtained at the research farm. Periodic sanitary surveys (using media and methods described below) indicated that all levels of indicator and pathogenic bacteria were below the detection limit. There are no livestock farms within a radius of about 2 km from the research farm.

Dairy and swine manure was obtained from two local commercial farms, and key properties of the manures are summarized in Table S1 in the supplemental material. The dairy herd consisted of 180 Holstein cows. Dairy manure was stored in an open pit. Penicillin and Liqueamycin (injectable oxytetracycline) were used in the dairy operation in both years. The swine herd consisted of 300 farrow-to-finish pigs. Swine manure for the present study was sampled from the manure-holding pit under the barn. In both years, the swine operation used medicated feed containing Aureo S-P 250 (220 mg chlortetracycline/kg, 220 mg sulfamethazine/kg, and 110 mg penicillin/kg of feed).

In 2011, dairy manure was applied at 6,900 gal/acre (ac) and swine manure at 3,600 gal/ac to supply 150 to 170 lb of nitrogen, the amount calibrated according to residual soil nitrogen. Dairy manure was applied the same day it was obtained, but the swine manure was stored for 22 days in the field prior to application. Based on soil tests, inorganic fertilizer was also applied to the dairy plot: 25-0-30 (N-P-K) at 380 lb/ac. For swine and control plots, 46-0-0 was applied at 359 lb/ac and 16-16-16 at 281 lb/ac. In 2012, both dairy and swine manure was applied at 8,500 gal/ac on the same day they were obtained. Based on soil tests, inorganic fertilizer was also applied to the dairy plot: 46-0-0 at 219 lb/ac and 16-16-16 at 200 lb/ac. The swine plot was supplemented with urea at 141 lb/ac and 16-16-16 at 200 lb/ac. The control plot received urea at 325 lb/ac and 16-16-16 at 200 lb/ac. Immediately following application, manures were soil incorporated to a depth of 15 cm using a disk and an S-tine cultivator. In the 2011 season, both control and treated blocks were subdivided into 20 4-m by 6-m plots. Four replicates of tomatoes, radishes, carrots, cucumbers, and peppers were planted within the plots. Individually planted blocks were separated by borders consisting of 2 m of unplanted ground. Radishes were planted late to avoid bolting because of a wetter-than-normal spring

followed by a hot summer. Plots were monitored for weeds and pest pressures on a weekly basis. Cucumbers were sprayed with Bravo (chlorothalonil; Syngenta Crop Protection Canada) to control mildew. Because of the mildew, the cucumbers were harvested earlier than the target harvest date. Based on experience in the 2011 season, peppers and cucumbers were not included, and lettuce was added in the 2012 season. Sixteen 4- by 6-m plots were delineated and planted randomly with four replicates of tomatoes, radishes, carrots, and lettuce. Because of the reduced variety of vegetables, individually planted blocks were separated by borders consisting of 10 m of unplanted ground for ease of mechanical weed control based on weed pressure identified in 2011. Plots were monitored for weeds and pest pressures on a weekly basis. Sticky traps were laid out for flea beetles in the radish plots. Each spring, all plots received a preplant application of glyphosate (Roundup) at a commercial rate of 2.0 liters/ha. Otherwise, pest management, weed control, and irrigation were done manually throughout the experiments. Vegetable varieties planted were tomatoes (*Solanum lycopersicum* var. Bellstar; 60-cm spacing), radishes (*Raphanus sativus* var. Sora; 600 seeds per row, spaced at 75 cm), carrots (*Daucus carota* var. Ibiza hybrid; 30-cm rows thinned at emergence), cucumbers (*Cucumis sativus* var. Straight Eight; 100 seeds per row, spaced at 75 cm), peppers (*Capsicum annuum* var. Early Calwonder; 60-cm spacing), and lettuce (*Lactuca sativa* var. Summertime; 100 seeds per row, spaced at 75 cm). The dates that manure application, planting, and harvest were undertaken are indicated in Table S2 in the supplemental material.

Soil and vegetable sampling. Soil cores were taken haphazardly on days 0, 7, and 30 and at harvest, with day 0 being the day of manure application. Six 2-cm-wide cores were sampled from each vegetable plot to a depth of 15 cm using a T-sampler sterilized with 70% ethanol between sampling. Cores were bulked into a labeled Ziploc bag, mixed by hand until homogenous, and transported to the laboratory in a cooler with cool packs. Samples of the edible portions of each crop were harvested when the crop was visibly ripe and ready for consumption. Four kilograms of root (carrots and radishes) and 10 individual samples of above-ground (lettuce, cucumber, tomato, and pepper) vegetables were harvested, individually bagged, and kept in a cooler on icepacks for transport to the laboratory. All implements were sterilized with 70% ethanol between plots.

Vegetable processing. Prior to the manipulations described below, excess soil was removed from all vegetables with a clean cloth and distilled water to achieve the visual cleanliness that a typical North American consumer would expect in normal food preparation. There was an additional step taken in the preparation of tomatoes, carrots, and radishes in the 2012 sampling year. Bacteria on the surface of the vegetables were washed off and recovered before the surface peel was obtained and macerated, as was done in 2011.

In the 2011 sampling season, 10 to 12 tomatoes from each block were aseptically peeled using an ethanol (70%, vol/vol)-sterilized vegetable peeler. Peels were placed aseptically into a sterile Filtra-Bag stomacher bag (Fisher Scientific, Mississauga, ON, Canada), until 50 g of peel had been collected. Eighty milliliters of sterile sodium metaphosphate buffer (2 g/liter; Fisher Scientific, Mississauga, ON, Canada) was added to the Filtra-Bag. The Filtra-Bag was then macerated for 30 s in a stomacher blender (Smasher; AES Laboratories, Kerr Lann, France). Bags were placed on ice until ready for further processing. In the 2012 sampling season, 20 tomatoes were placed into a clean Ziploc bag to which was added 200 ml of sterile Milli-Q water. The Ziploc bag was then shaken by hand for 1 min. The water was aseptically poured into sterile 50-ml conical plastic tubes (Fisher Scientific) and designated "Wash" samples. Wash fluid samples were immediately placed on ice until ready for analysis. The Ziploc was then opened, and tomatoes were aseptically peeled using an ethanol (70%, vol/vol)-sterilized vegetable peeler and processed as in the 2011 season.

Peppers and cucumbers (2011 season only; $n = 6$ to 8 peppers and cucumbers from each block) were peeled, and 50 g of peel was macerated as described for the tomatoes.

In the 2011 season, procedures for radish (20 to 25 from each block) sample collection were as described for peppers and cucumbers. In the 2012 season, about 725 g of radishes was placed into a clean Ziploc bag to which was added 300 ml of sterile Milli-Q water. The Ziploc bag was then shaken by hand for 1 min. The radishes were removed and placed into a second Ziploc bag. The first wash water was frozen for further analysis. A total of 200 ml of sterile Milli-Q water was added to the prewashed radishes in the Ziploc bag. The Ziploc bag was then shaken by hand for 1 min. The water was aseptically poured into sterile 50-ml conical tubes (Fisher Scientific, Mississauga, ON, Canada) and designated "Wash" samples. Tubes were immediately placed on ice until ready for analysis. The Ziploc bag was then opened, and radish peel was obtained and processed as described for the other vegetables.

In the 2011 season, procedures for carrot (10 to 12 per block) sample preparation were as described for the other vegetables. In the 2012 season, 20 carrots were placed into a clean Ziploc bag to which was added 300 ml of sterile Milli-Q water. The Ziploc bag was then shaken by hand for 1 min. The carrots were removed and placed into a second Ziploc bag. The first wash water was frozen for further analysis. A total of 200 ml of sterile Milli-Q water was added to the prewashed carrots in the Ziploc bag. The Ziploc bag was then shaken by hand for 1 min. The water was aseptically poured into sterile 50-ml conical tubes (Fisher Scientific) and designated "Wash" samples. Tubes were immediately placed on ice until ready for analysis. The Ziploc bag was then opened, and carrot peel was obtained and macerated as described for the other vegetables.

Lettuce heads were obtained in the 2012 season. The outer leaves of each head of lettuce were removed, and the head was cut in half using an ethanol-sterilized knife. Five grams of tissue from the center of 10 heads of lettuce was removed and placed into a sterile Filtra-Bag stomacher bag until duplicates of a composite totaling 50 g had been achieved. Each Filtra-Bag then had 100 ml of sterile Milli-Q water added to it and was then manipulated by hand for 1 min. The water was aseptically poured into sterile 50-ml conical tubes (Fisher Scientific, Mississauga, ON, Canada), avoiding transfer of tissue material to the tubes. Tubes were immediately placed on ice until ready for analysis.

Soil processing. Six cores were sampled from each vegetable plot to a depth of 15 cm by using a T-sampler sterilized with 70% ethanol between samplings. Cores were bulked into a labeled Ziploc bag and mixed by hand until homogenous. Fifty grams of soil was measured and placed aseptically into a sterile Filtra-Bag stomacher bag (Fisher Scientific, Mississauga, ON, Canada). One hundred milliliters of sterile sodium metaphosphate buffer (2 g liter⁻¹; Fisher Scientific, Mississauga, ON, Canada) was added to the Filtra-Bag. The Filtra-Bag was then shaken by hand for 1 min. Bags were then placed on ice until ready for further processing. Fifty grams of soil was also frozen at -20°C for future use.

Chemicals and media. The following media were used: Chromocult agar (EMD Chemicals, Cedarlane Laboratories, Burlington, ON, Canada), mEnterococcus agar (Difco, Fisher Scientific, Ottawa, ON, Canada), mEndo-LES agar (Difco), mFC agar (Difco), mFC-BCIG agar consisting of mFC basal medium (Difco) supplemented with 100 mg/liter 3-bromo-4-chloro-5-indolyl-β-D-glucuronide (cyclohexyl ammonium salt; Alere Canada, Ottawa, ON, Canada), mCP agar (Accumedia; Alere Canada), ampicillin-dextrin agar (mADA-V; Hardy Diagnostics, Alere Canada), Campy-Line agar (22, 23), *Salmonella* chromogenic agar (Oxoid, Ottawa, ON, Canada), XLD agar (Difco), azide dextrose broth (Difco), MUG-EC broth (Sigma Chemical Co., Toronto, ON, Canada), UVM *Listeria* broth (Difco), *Listeria* enrichment broth (Difco), *Listeria* chromogenic agar (Oxoid), and PALCAM agar (Difco). Antibiotics were purchased from Sigma-Aldrich (Toronto, ON, Canada) and AK Scientific (Union City, CA).

Enumeration and primary isolation of bacteria. Vegetable filtrate and soil suspensions were enumerated for the following bacteria: total coliforms, fecal coliforms, *Escherichia coli*, *Enterococcus* species, *Clostridium perfringens*, *Aeromonas* species, *Yersinia* species, *Campylobacter* species, *Salmonella* species, and *Listeria* species. The panel of media used and incubation conditions are presented in Table 1. Briefly, 100 µl of the

TABLE 1 Media and incubation conditions used for the enumeration, enrichment, and primary isolation of the indicated bacteria from vegetable, soil, and manure samples

Medium	Incubation conditions	Bacteria
Chromocult agar	37°C; 24 h	Coliforms and <i>E. coli</i>
mEnterococcus agar	37°C; 48 h	<i>Enterococcus</i> spp.
mEndo-LES agar	37°C; 24 h	Total coliforms
mFC agar	44.5°C; 24 h	Fecal coliforms
mFC-BCIG agar	44.5°C; 24 h	<i>E. coli</i>
mCP agar	44.5°C; 24 h; anaerobic	<i>C. perfringens</i>
mADA-V agar	37°C; 24 h	<i>Aeromonas</i> spp.
Campy-Line agar	42°C; 48 h; microaerophilic	<i>Campylobacter</i> spp.
XLD agar	42°C; 48 h	<i>Salmonella</i>
<i>Salmonella</i> chromogenic agar	42°C; 24–48 h	<i>Salmonella</i>
<i>Listeria</i> chromogenic agar	37°C; 24 h	<i>Listeria</i> spp.
PALCAM agar	37°C; 24 h	<i>Listeria</i> spp.

suspension was spread plated onto agar plates containing semiselective differential media and incubated at the appropriate temperatures. Bacteria were enumerated based on the colonial morphology indicated for the particular selective medium used. Only plates with 20 to 200 colonies were used for enumeration. Additionally, 2-ml aliquots of each sample were inoculated into azide dextrose broth (for detection of enterococci), EC-MUG broth (for detection of *E. coli*; primary enrichment of *Salmonella*), and *Listeria* enrichment broth. Following an initial incubation of 48 h at 37°C, *Listeria* enrichment broth cultures were transferred into UVM *Listeria* broth and further incubated for 48 h at 37°C prior to inoculation onto chromogenic *Listeria* agar (Oxoid, Ottawa, ON, Canada) and PALCAM agar (Difco, Fisher Scientific, Ottawa, ON, Canada). Following an initial incubation of 24 h at 37°C, EC-MUG broth cultures were inoculated onto mFC-BCIG agar and incubated at 44.5°C and simultaneously inoculated onto XLD agar and *Salmonella* chromogenic agar (Oxoid), which was then incubated at 42°C for 24 h. Presumptive positive *Salmonella* colonies were further tested by an oxidase test and a *Salmonella* rapid latex spot test (Oxoid, Ottawa, ON, Canada). Presumptive positive *Listeria* isolates were further identified using the API-*Listeria* identification system (bioMérieux, Montreal, QC, Canada). Where required, anaerobic or microaerophilic atmospheric conditions were established using the AnaeroPack container system (Mitsubishi Gas Chemical Co., Thermo-Fisher Scientific, Ottawa, ON, Canada) in tandem with either Pack-Anaero or Pack-MicroAero gas-generating packets, respectively. Based on the sample size processed, the detection limits for the various methods were as follows: plate counts, 400 CFU/g for manure, 160 CFU/g for soil, 160 CFU/g for vegetables; enrichments, 4 cells/g for EC-MUG manure and 2 cells/g for soil or vegetable, 4 cells/g for azide dextrose manure and 2 cells/g for soil or vegetable, 2 cells/g for *Listeria*, all matrices.

Enumeration of antibiotic-resistant coliform bacteria. Manure, soil, and vegetable macerates were spread plated onto Chromocult agar containing the following individual antibiotics at breakpoint concentrations (24) in mg/liter and incubated overnight at 37°C: amikacin (AMK), 64; amoxicillin-clavulanic acid (Augmentin), 32:16; ampicillin (AMP), 32; cefoxitin (FOX), 32; ceftiofur (TIO), 8; chloramphenicol (CHL), 32; ciprofloxacin (CIP), 4; gentamicin (GEN), 16; nitrofurantoin (NFR), 128; norfloxacin (NOR), 16; sulfamethoxazole (SMX), 512; co-trimoxazole, trimethoprim-sulfamethoxazole (SXT), 4:76; tetracycline (TET), 16; trimethoprim (TMP), 16; chlortetracycline (CTC), 64; and meropenem (MEM), 4. Ten-fold dilution series of the manures and soil samples were prepared in sterile sodium metaphosphate buffer (2 g/liter; Thermo-Fisher Scientific, Ottawa, ON, Canada) prior to plating 100 µl of each dilution. Plates were enumerated for coliforms and *E. coli* based on the manufacturer's recommendations for target colony morphology and color. For statistical validity, only plates with 20 to 200 colonies were used for enumeration.

TABLE 2 Viable populations of bacteria in manures^a

Bacterium	Log ₁₀ CFU/g wet wt (mean $\pm$ SD; <i>n</i> = 2 samples)				
	2011			2012	
	Swine manure at sampling	Swine manure	Dairy manure	Swine manure	Dairy manure
Total coliforms	4.31 $\pm$ 3.55	2.70 $\pm$ 0.00	5.24 $\pm$ 3.85	5.53 $\pm$ 4.30	5.43 $\pm$ 4.81
Fecal coliforms	3.98 $\pm$ 2.75	BDL	4.30 $\pm$ 2.85	5.27 $\pm$ 4.71	5.39 $\pm$ 5.07
<i>E. coli</i>	3.96 $\pm$ 3.45	BDL	4.63 $\pm$ 3.63	3.76 $\pm$ 3.26	5.06 $\pm$ 4.57
<i>Enterococcus</i> spp.	5.04 $\pm$ 3.45	3.40 $\pm$ 2.75	5.17 $\pm$ 4.57	5.70 $\pm$ 4.87	5.79 $\pm$ 5.15
<i>C. perfringens</i>	3.59 $\pm$ 2.89	3.63 $\pm$ 3.05	2.88 $\pm$ 1.85	6.45 $\pm$ 4.76	3.24 $\pm$ 2.71
<i>Yersinia</i> spp.	2.54 $\pm$ 2.55	BDL	5.24 $\pm$ 4.41	4.40 $\pm$ 3.67	4.26 $\pm$ 3.64
<i>S. enterica</i>	2.30 $\pm$ 2.45	BDL	4.72 $\pm$ 4.08	BDL	BDL
<i>Campylobacter</i> spp.	BDL	BDL	BDL	2.66 $\pm$ 2.17	3.36 $\pm$ 2.72
<i>Aeromonas</i> spp.	2.00 $\pm$ 2.15	BDL	3.61 $\pm$ 1.85	3.37 $\pm$ 3.32	4.19 $\pm$ 3.06
<i>L. monocytogenes</i>	BDL	BDL	BDL	BDL	BDL

^a All data were obtained on the day of manure application, with additional swine manure at the time of sampling in 2011. BDL indicates that counts were below the detection limit.

Extraction of DNA from soil, vegetables, and manures. The MO-BIO PowerSoil (MO-BIO Laboratories, Mediacorp, Montreal, QC, Canada) was used to extract DNA from 250-mg portions of soil by following the manufacturer's instructions. The elution volume was 100 μ l. DNA was extracted from vegetables in the 2011 experiment as follows. Following maceration of vegetable peel samples, 50 ml of the macerate was placed in two 50-ml tubes and centrifuged at 15,000 rpm (27,000 $\times$ g) for 10 min at 4°C (2011) or 7,500 $\times$ g for 15 min at 4°C. Pellets were weighted (generally 200 to 500 mg per veggie, except tomato, for which the weight was 1 g) and stored at -20°C for DNA extraction. DNA extraction from pellets was performed using the MO-BIO PowerSoil kit by following the manufacturer's instructions. The elution volume was 100 μ l. The method for DNA extraction from vegetables in the 2012 experiment is as follows. Rinsate (95 ml) from vegetables was filtered through a 0.45- μ m-pore-size nitrocellulose membrane. Membranes were placed in 15-ml Falcon tubes containing 500 μ l of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) and stored at -80°C . DNA was extracted from each filter by using the DNeasy (Qiagen, Mississauga, ON, Canada) tissue kit by following the manufacturer's instructions, except that the proteinase K digestion step was omitted. The elution volume was 100 μ l. DNA concentrations were measured using the NanoDrop ND1000 microspectrophotometer (NanoDrop Technologies, Wilmington, DE).

PCR detection of gene targets. Primers used to detect various antibiotic resistance genes, integrase, and plasmid incompatibility groups were obtained from the literature and are detailed in Table S3a in the supplemental material. The following targets were evaluated in the present study: plasmid incompatibility groups HI1, HI2, I1, K/B, L/M, N, P, Q, T, W, X, and Y; class 1, 2, and 3 integrases; beta-lactamase class A genes *bla*_{CARB-4}, *bla*_{CTX-M}, *bla*_{KPC}, *bla*_{SHV}, *bla*_{TEM}; beta-lactamase class B (metallo- β -lactamase) genes *bla*_{GIM-1}, *bla*_{IMP}, *bla*_{IMP-1}, *bla*_{NDM-1}, *bla*_{SIM-1}, *bla*_{SPM-1}, *bla*_{VIM}, *bla*_{VIM-2}; beta-lactamase class D (oxacillinase) genes, groups 1, 2, and 3; among group 1, *bla*_{OXA-5}; among group 2, *bla*_{OXA-20}; among group 4, *bla*_{LCR-1}; not group affiliated, *bla*_{OXA-18} and *bla*_{OXA-48}; macrolide-lincosamide-streptogramin type B resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(E), *erm*(F); streptogramin type A resistance genes *vga*, *vat*, *vat*(B); streptogramin type B resistance gene *vgb*; fosfomycin resistance gene *fos*(A); fluoroquinolone resistance genes *qnr*(A), *qnr*(B), *qnr*(S); aminoglycoside resistance genes *aac*(3)IV, *aad*(A); *str*(A) and *str*(B); sulfonamide resistance genes *sul*1, *sul*2, *sul*3; tetracycline resistance genes *tet*(A), *tet*(B), *tet*(BP), *tet*(C), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), *otr*(A); vancomycin resistance genes *van*(A), *van*(B), *van*(C1), *van*(C2/C3). The reaction mixture consisted of deoxynucleoside triphosphates at 0.2 mM, primers at 250 nM each, 1 $\times$ GoTaq green master mix, 1.5 mM MgCl₂, 0.3 U GoTaq DNA polymerase (Promega, Thermo Fisher Scientific, Ottawa, ON, Canada), and 2 μ l of template DNA for a final volume of 25 μ l. Reactions were done in a C1000 thermal cycler (Bio-Rad

Laboratories, Mississauga, ON, Canada) with a program consisting of amplification for 30 cycles of 94°C for 5 min, 94°C for 30 s, the indicated annealing temperature (see Table S2 in the supplemental material) for 30 s, and 72°C for 30 s. A final extension was carried out at 72°C for 5 min. PCR products were resolved electrophoretically in 1.5% agarose gel and sized using the MassRuler DNA ladder mix, ready-to-use, 80- to 10,000-bp (Thermo Scientific) marker. The detection limit of targeted genes, determined by adding known quantities of targeted genes into a 10-fold-diluted DNA template prepared from soil or vegetables, was 1 to 10 copies/ μ l. PCR products obtained from manure DNA were sequenced in order to confirm the identity of the targeted genes on the basis of best match with sequences encoding the expected gene target. The sequences are available in Table S3b in the supplemental material. Confirmed PCR products were cloned in the pSC-A-amp/kan plasmid using the StrataClone PCR cloning kit (Agilent) and transformed into *E. coli* according to the manufacturer's instructions. Plasmids were extracted using the Qia-gen plasmid midikit (Qiagen, Mississauga, ON, Canada) and used as positive controls for PCR made on soil and vegetable DNA samples. Plasmids were also used for determination of the detection limit of the PCR method. Known quantities of cloned insert were added to DNA extracted from soil and from each vegetable type, with a final concentration ranging from 1 to 100 copies/ μ l of DNA. DNA extracts that were negative for each target were used for these experiments. Each gene target was analyzed in triplicate by PCR, and plasmid quantity giving three positive results was chosen as the detection limit. Based on these results, the detection limits for the various gene targets were in the range of 10⁴ to 10⁵ gene copies per gram of soil or per gram of vegetable.

Data analysis and statistical methods. Only viable plate counts between 20 and 200 were considered in the analysis. The frequency of resistance to antibiotics was calculated relative to viable plate counts on antibiotic-free Chromocult medium. Mean and standard deviations were imported into GraphPad Prism (version 5; GraphPad, San Diego, CA), and differences between treatments were tested by analysis of variance (ANOVA). The significance level was set at a *P* value of 0.05.

Statistically significant treatment effects were determined using Fisher's exact test. Data were treated using online tools (<http://www.vassarstats.net/tab2x2.html> [last accessed 27 May 2013]). The significance level was set at a *P* value of 0.05.

RESULTS

Microbial composition of the manure. Coliform and pathogenic bacteria were enumerated in the manure at the time of application (Table 2). Due to operational constraints and climate conditions in 2011, the swine manure used in the experiment was held in a polyethylene tank in the field for 22 days before it was applied.

TABLE 3 Frequency of resistance to the indicated antibiotics by bacteria enumerated on Chromocult agar containing the indicated antibiotics at clinical breakpoint concentrations^a

Antibiotic ^c	% resistance (mean $\pm$ SD) ^b				
	2011			2012	
	Swine manure at sampling	Swine manure	Dairy manure	Swine manure	Dairy manure
Control (log ₁₀ CFU/g wet wt)	4.34 $\pm$ 3.85	3.65 $\pm$ 2.85	5.04 $\pm$ 4.45	5.35 $\pm$ 4.28	4.64 $\pm$ 3.98
AMK	BDL	BDL	BDL	BDL	BDL
AUG	44.1 $\pm$ 10.3	4.4 $\pm$ 0.0	4.4 $\pm$ 2.2	0.7 $\pm$ 0.2	11.1 $\pm$ 0.2
AMP	34.5 $\pm$ 22.5	12.2 $\pm$ 1.6	36.7 $\pm$ 3.9	18.1 $\pm$ 7.5	34.8 $\pm$ 2.3
FOX	3.0 $\pm$ 1.0	BDL	0.3 $\pm$ 0.4	2.3 $\pm$ 0.2	13.2 $\pm$ 0.5
TIO	BDL	BDL	BDL	BDL	BDL
CHL	2.7 $\pm$ 1.3	1.1 $\pm$ 1.6	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	1.1 $\pm$ 0.0
CIP	0.5 $\pm$ 0.6	BDL	BDL	1.3 $\pm$ 0.3	BDL
GEN	BDL	BDL	BDL	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0
NFR	BDL	BDL	BDL	BDL	BDL
NOR	BDL	BDL	BDL	2.0 $\pm$ 0.2	BDL
SMX	13.9 $\pm$ 1.0	34.4 $\pm$ 17.3	0.8 $\pm$ 0.1	11.4 $\pm$ 1.6	30.8 $\pm$ 1.4
TET	58.9 $\pm$ 8.7	7.8 $\pm$ 1.6	5.2 $\pm$ 6.3	4.7 $\pm$ 0.6	32.5 $\pm$ 0.2
TMP	17.7 $\pm$ 4.5	BDL	1.6 $\pm$ 1.9	26.2 $\pm$ 3.1	16.3 $\pm$ 0.2
SXT	13.2 $\pm$ 6.4	5.6 $\pm$ 4.7	BDL	11.5 $\pm$ 0.8	19.5 $\pm$ 0.1
CTC	40.5 $\pm$ 25.1	16.7 $\pm$ 1.6	5.0 $\pm$ 0.8	44.4 $\pm$ 12.5	64.6 $\pm$ 2.1

^a For reference, the viable count on Chromocult in the absence of antibiotics is presented. All data were obtained on the day of manure application, with additional swine manure at the time of sampling in 2011. BDL indicates counts were below the detection limit.

^b Values are % resistance (mean $\pm$ SD), except as otherwise noted for the control.

^c AMK, amikacin; AUG, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NFR, nitrofurantoin; NOR, norfloxacin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole; CTC, chlortetracycline.

Populations of all viable bacteria with the exception of *C. perfringens* declined significantly during this storage time, and thus viable counts for swine manure at application time were much lower in 2011 than in 2012. *Listeria monocytogenes* was not detected during the experiment, *Campylobacter* spp. was detected only in 2012, and *Salmonella enterica* was detected only in 2011.

Bacteria resistant to a panel of antibiotics were enumerated on Mueller-Hinton agar (Table 3). No resistance to AMK, TIO, or nitrofurantoin (NFR) was detected in any samples. Resistance to GEN was detected in 2012 but not 2011. Resistance to CIP was detected in swine but not dairy manure. With the exception of SMX, antibiotic-resistant bacteria were significantly less abundant in swine manure following the 2011 holding period than they were when the manure was sampled.

The presence of plasmid incompatibility groups, integrase genes, and antibiotic resistance determinants in manure was evaluated by PCR (see Table S4 in the supplemental material). Gene targets *tet*(MQST), *aad*(A), *str*(AB), *sul*1, and *erm*(ABCF) were detected in all manure samples. Several gene targets, *incW* *oriV*, *incW* *trwAB*, *tet*(ABW), *erm*(E), *bla*_{OXA1}, *bla*_{PSE}, and *bla*_{VIMgen-2} were detected in both dairy and swine manure in 2012 but not 2011. Gene targets *vat*(B) and *sul*2 were in contrast detected in 2011 and not in 2012. Overall, the distribution of many gene targets was uniform across manure type and season, whereas others varied with the year of sampling.

Pathogenic and coliform bacteria in soil and on vegetables at harvest. Viable enteric bacteria in unfertilized soils, in soils that were fertilized with swine manure, and in soils that were fertilized with dairy manure were enumerated by plate count or by enrichment culture. Data for the distribution of total coliforms, fecal coliforms, *E. coli*, *Enterococcus* spp., *C. perfringens*, *Aeromonas* spp., *S. enterica*, *Yersinia* spp., *Campylobacter* spp., and *Listeria* spp. are summarized in Table S5 in the supplemental material, and

any instances where manuring had a significant effect with respect to the abundance of viable bacteria are summarized in Table 4. *Listeria* spp. and *S. enterica* were never detected in soil. Total coliforms were frequently enriched in soils that were manured, although in two instances the viable count was lower than in unmanured soil. Populations of *Yersinia* were reduced in manured soils cropped to carrots. In the 2012 season, swine manure was associated with an increase in viable counts of *C. perfringens* in soil cropped to carrots, radishes, and lettuce.

Campylobacter spp., *Listeria* spp., and *S. enterica* were never detected on any crop at harvest regardless of fertilization method (see Table S6 in the supplemental material). There were no significant effects of fertilizer treatment on the abundance of enumerated populations, other than dairy manure increasing the number of total coliforms on peppers in 2011 (Table 4).

Abundance of antibiotic-resistant coliform bacteria in soil and on vegetables at harvest. The abundance of soil coliform bacteria resistant to a panel of antibiotics was determined by viable plate count on Chromocult containing each antibiotic at breakpoint concentrations. All of the data are presented in Table S7 in the supplemental material, and instances where manuring significantly changed the frequency of detection to specific antibiotics are summarized in Table 5. Generally, the variability in frequency of resistance to antibiotics for which resistance was detected was very large (see Table S7). Resistance to amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, chloramphenicol, and gentamicin was detected in most or all soil samples. Resistance to nitrofurantoin, norfloxacin, and co-trimoxazole was never detected, whereas resistance to ceftiofur, ciprofloxacin, sulfamethoxazole, tetracycline, and trimethoprim was detected in only one or two samples. There was no effect of manuring on the frequency of detection or resistance to the majority of antibiotics; a manuring treatment effect was detected only for amikacin, am-

TABLE 4 Instances where manuring had a significant effect on the abundance of viable populations of the indicated bacteria in soil cropped to the indicated vegetable or on the indicated vegetable at harvest^a

Bacterium	Vegetable(s)			
	2011		2012	
	Swine manure	Dairy manure	Swine manure	Dairy manure
Total coliforms	Carrot, radish, <u>tomato</u>	Carrot, radish, <u>pepper</u>, pepper	Tomato	Tomato
<i>E. coli</i>	Cucumber	Cucumber		
<i>Enterococcus</i>				
Fecal coliforms	NA	NA		
<i>C. perfringens</i>	NA	NA	Carrot, radish, lettuce	
<i>Aeromonas</i> sp.	NA	NA		
<i>Salmonella</i> sp.				
<i>Yersinia</i> sp.	NA	NA	<u>Carrot</u>	<u>Carrot</u>
<i>Campylobacter</i> sp.			Carrot	
<i>Listeria</i> sp.				

^a All data are referenced to corresponding plots fertilized inorganically. NA indicates not analyzed. Bolded script, instances where manuring increased the viable soil population enumerated; bolded and underlined script, instances where manuring decreased the detected viable soil population; plain script, instances where manuring increased the viable population enumerated from vegetables; plain underlined script, instances where manuring decreased the populations enumerated from vegetables.

picillin, cefoxitin, and gentamicin (Table 5). In 15 instances where manuring had a significant effect, 11 resulted in an increase in frequency of resistance, whereas four resulted in a decrease (Table 5). Manuring effects were evident primarily with ampicillin, cefoxitin, and gentamicin, and only one significant effect was observed with amikacin. Significant effects were observed more frequently in 2011 than in 2012 and were similar with swine and dairy manure applications.

The abundance of bacteria on vegetables that were resistant to a panel of antibiotics was determined using the same methods as those used for soil bacteria. All of the data are presented in Table S8 in the supplemental material, and instances where manuring significantly changed the frequency of detection to specific antibiotics are summarized in Table 5. Resistance to amoxicillin-clavulanic acid, ampicillin, and cefoxitin was detected in most samples. No resistance to ciprofloxacin, gentamicin, norfloxacin, sulfamethoxazole, tetracycline, or co-trimoxazole was detected. Only one or two samples revealed any resistance to ceftiofur, nitrofurantoin, or chlortetracycline (see Table S8). Manuring had a significant effect only on the abundance of amoxicillin-clavulanic acid (in only one sample), ampicillin, and cefoxitin resistance (Table 5). In 14 instances where manuring had an effect, six resulted in an increase, whereas eight resulted in a decrease in the frequency of resistance.

In every instance where manuring had a significant effect on the abundance of ampicillin- or cefoxitin-resistant bacteria recovered from carrots, it was a reduction relative to that of carrots grown in the absence of manure.

Distribution of antibiotic resistance determinants in soil and on vegetables. The distribution of 46 gene targets in manured and unmanured soils sampled at crop harvest was evaluated by PCR (see Table S4 in the supplemental material). There were eight instances where a gene target was detected significantly more frequently in manured soil than in unmanured soil ($P < 0.05$; Table 6). These gene targets were associated with the plasmid incompatibility groups Q (IncQ *repB*; IncQ *oriT*) and W (IncW; IncW *oriV*) and resistance to macrolides [*erm*(ABF)]. In only one instance [*erm*(B)] was increased frequency of detection associated with dairy manure. In contrast, swine manure increased the frequency of detection of all seven gene target, and in five of the eight instances, in the 2012 season.

The distribution of the 46 gene targets in vegetable samples at harvest was evaluated by PCR. All of the data are tabulated in Table S9 in the supplemental material, and the key findings are summarized in Table 7. Numerous gene targets were detected on vegetables grown in unmanured soil, with far more detected in the 2012 than in the 2011 season. Nine gene targets (IncP *oriV*, *sul2*,

TABLE 5 Instances where manuring had a significant effect on the abundance of antibiotic-resistant populations of viable bacteria in soil cropped to the indicated vegetable or on the vegetable at harvest^a

Antibiotic	Vegetable(s)			
	2011		2012	
	Swine manure	Dairy manure	Swine manure	Dairy manure
Amikacin		Radish		
Augmentin			Tomato	
Ampicillin	Tomato, carrot, <u>tomato</u>, pepper	Carrot, <u>tomato</u>	Carrot, <u>carrot</u>	Tomato, carrot, lettuce
Cefoxitin	Tomato, <u>carrot</u>, radish	Tomato, <u>pepper</u>, <u>carrot</u>	Tomato, <u>carrot</u> , lettuce	Lettuce, <u>carrot</u>
Gentamicin	Radish, <u>cucumber</u>	Radish, <u>tomato</u>	Radish	

^a Bacteria were enumerated on Chromocult agar containing clinical breakpoint concentrations of the indicated antibiotic and referenced to populations enumerated on antibiotic-free agar. Only those antibiotics where any treatment effect was observed are indicated. All data are available in Tables S7 and S8 in the supplemental material. Bolded script, instances where manuring increased the frequency of resistance to the indicated antibiotic in the soil population enumerated; bolded and underlined script, instances where manuring decreased the frequency of resistance to the indicated antibiotic in the soil population; plain script, instances where manuring increased the frequency of resistance in the viable population enumerated from vegetables; plain underlined script, instances where manuring decreased the frequency of the resistance population enumerated from vegetables.

TABLE 6 Gene targets that were more frequently detected in manured soil than in unmanured control soil^a

Targeted gene(s)	Presence or absence of gene				% of samples positive for the gene					
	Dairy manure		Swine manure		Dairy manure-treated soil		Swine manure-treated soil		Control soil	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
IncQ <i>repB</i>	+	+	+	+	0	0	0	81 ^b	0	0
IncQ <i>oriT</i>	+	+	+	+	0	0	0	31 ^b	0	0
<i>incW</i>	—	+	+	+	NA	62	55 ^b	100	5	56
IncW <i>oriV</i>	—	+	—	+	NA	6	NA	31 ^b	NA	0
<i>erm</i> (A)	+	+	+	+	10	0	30	81 ^b	10	0
<i>erm</i> (B)	+	+	+	+	60 ^b	75	85 ^b	87	5	75
<i>erm</i> (F)	+	+	+	+	15	12	20	62 ^b	5	6

^a Data for manures are expressed as presence (+) or absence (—) and for soils as % samples that were positive for the indicated target. In a given year, only genes that were detected in manure were analyzed in soil samples. Data for soil samples taken from all vegetable plots were pooled for the analysis; the number of soil replicates (in dairy manure-treated, swine manure-treated, or unmanured control soil) was 20 each in 2011 and was 16 each in 2012. Only those targets for which there was a significant treatment effect are presented. All of the data are available in Table S9 in the supplemental material. NA indicates not analyzed.

^b Value is significantly different from the control soil in that sampling year (Fisher's exact test, $P < 0.05$).

tet(BT), *erm*(AF), *qnr*(B), *bla*_{PSE}, and *bla*_{OXA-20}) were detected on at least one vegetable sample ($n = 4$ for each treatment in each season, except radish in 2011, where $n = 3$) but never detected in any unmanured soil sample cropped to any vegetable ($n = 20$ in 2011, $n = 16$ in 2012). Growth in dairy manure soil was associated with the detection of IncP *oriV*, *sul2*, *erm*(F), *qnr*(B), *bla*_{PSE}, and *bla*_{OXA-20}. Growth in swine manure soil was associated with the detection of *sul2*, *tet*(BT), *erm*(AF), and *bla*_{OXA-20}. In no case was a gene target detected on a vegetable in unmanured soil but not in manured soil.

DISCUSSION

In the present study, viable coliform bacteria resistant to amoxicillin-clavulanic acid, ampicillin, cefoxitin, chloramphenicol, nitrofurantoin, co-trimoxazole, and chlortetracycline were detected on vegetables grown in soil that had never been manured (see Table S8 in the supplemental material). Some of these bacteria may be intrinsically resistant to some antibiotics. However, nu-

merous genes associated with acquired resistance to aminoglycoside, tetracycline, macrolide-lincosamide-streptogramin B, sulfonamide, beta-lactam antibiotics, various plasmids, and integrons 1, 2, and 3 were detected in DNA extracted from vegetables grown in unmanured soil (Table 8). The variety of antibiotic resistance was larger, and the frequency of detection of resistant coliforms was much higher in soils at harvest than on the vegetables (see Tables S7 and S8). Presumably, the difference is accounted for by washing soil off the vegetables, as would be expected in normal culinary preparation.

These results are entirely consistent with previous studies that have detected antibiotic-resistant bacteria on vegetable products at harvest or at the retail level (25–32). Variation in laboratory methods, sampling strategy, and sample handling practices makes it difficult to compare studies, especially quantitatively. Nevertheless, viable plate counts on selective media containing antibiotics, or phenotypic screening of bacteria isolated nonselectively, consistently reveal abundant antibiotic-resistant bacteria on fresh

TABLE 7 Detection of gene targets on vegetables at harvest

Vegetable	Detected gene(s) ^a					
	No manure		Dairy manure		Swine manure	
	2011	2012	2011	2012	2011	2012
Tomato	<i>tet</i> (T), <i>str</i> (A)	IncP <i>oriT</i> , <i>incY</i> , <i>int2</i> , <i>int3</i> , <i>tet</i> (A), <i>tet</i> (S), <i>aad</i> (A), <i>str</i> (A), <i>str</i> (B), <i>erm</i> (B), <i>erm</i> (E), <i>bla</i> _{CTX-M} , ^b <i>bla</i> _{VIM} , <i>bla</i> _{TEM} ^b	IncP <i>oriV</i>	<i>tet</i> (T), <i>erm</i> (F), <i>bla</i> _{PSE} , <i>bla</i> _{OXA-20}		<i>tet</i> (T), <i>erm</i> (F)
Pepper	<i>int3</i> , <i>tet</i> (T), <i>str</i> (B), <i>sul1</i> , <i>vat</i> (B), <i>bla</i> _{OXAII}	NA				
Cucumber	IncP <i>oriT</i> , IncP <i>trfA1</i> , <i>str</i> (B), <i>sul1</i> , <i>erm</i> (B), <i>bla</i> _{OXAII}	NA	<i>sul2</i>		<i>sul2</i>	
Radish	IncP <i>oriT</i> , IncQ <i>oriV</i> , <i>int3</i> , <i>aad</i> (A), <i>str</i> (A), <i>str</i> (B), <i>sul1</i> , <i>erm</i> (B), <i>bla</i> _{OXAII}	IncP <i>oriT</i> , IncQ <i>oriV</i> , <i>int2</i> , <i>int3</i> , <i>tet</i> (A), <i>aad</i> (A), <i>str</i> (A), <i>str</i> (B), <i>sul1</i> , <i>erm</i> (B), <i>erm</i> (E), <i>bla</i> _{CTX-M} , ^b <i>bla</i> _{VIM} , <i>bla</i> _{TEM} ^b		<i>erm</i> (F)	<i>erm</i> (A)	<i>erm</i> (A), <i>bla</i> _{OXA-20}
Carrot	IncP <i>oriT</i> , IncQ <i>oriV</i> , <i>aad</i> (A), <i>str</i> (A), <i>str</i> (B), <i>sul1</i> , <i>erm</i> (C)	IncP <i>oriT</i> , IncQ <i>oriV</i> , <i>int1</i> , <i>tet</i> (A), <i>tet</i> (S), <i>sul1</i> , <i>erm</i> (B), <i>erm</i> (E), <i>bla</i> _{VIM} , <i>bla</i> _{TEM} ^b		<i>qnr</i> (B)		<i>tet</i> (B), <i>tet</i> (T), <i>bla</i> _{OXA-20}
Lettuce	NA	IncP <i>oriT</i> , IncQ <i>repB</i> , <i>incW</i> , <i>int3</i> , <i>tet</i> (A), <i>tet</i> (Q), <i>tet</i> (S), <i>aad</i> (A), <i>str</i> (A), <i>sul1</i> , <i>erm</i> (B), <i>bla</i> _{OXAII} , <i>bla</i> _{VIM} , <i>bla</i> _{TEM} ^b				

^a Shown are all gene targets that were detected on at least one vegetable sample grown in soil fertilized inorganically and those targets that were in addition detected on at least one vegetable grown with, but not without, manure. All of the data are available in Table S10 in the supplemental material. NA indicates not analyzed.

^b Primers for *bla*_{CTX-M} and *bla*_{TEM} were used only in 2012, whereas all others were used in both years.

produce. This is concluded with varied produce from Europe, the Gulf States, North America, and Central America (25–32). Antibiotic-resistant bacteria are found in vegetables obtained at the farm, at wholesale or retail outlets, produced using conventional or organic methods, and sold fresh or bagged. Bacteria from vegetables carry resistance determinants commonly found in clinical isolates. For example, bacteria with an extended-spectrum beta-lactamase (ESBL) phenotype, notably *Rahnella* spp., were found to be widespread on various vegetables and carried *bla*_{RAHN-1} on the basis of a positive reaction with gene-specific PCR primers (28). Packaged spinach carried bacteria resistant to cefotaxime and ceftazidime, including a *Pseudomonas teessidea* strain that on the basis of sequence analysis harbored *bla*_{CTX-M-15} and *Rahnella aquaticus* that carried genes similar to *bla*_{RAHN-2} (30). *Pseudomonas* spp. from lettuce or spinach carried class 1 and 2 integrons (27). Gentamicin-resistant populations from lettuce carried plasmids from the IncP1 incompatibility group, and oxytetracycline-resistant populations carried plasmids of the IncQ group (26). Taken together, these data indicate that antibiotic-resistant bacteria are ubiquitous on vegetables, regardless of where they are produced, local climate conditions, farming practice, or vegetable type. This is consistent with studies of DNA obtained from permafrost soils or extremely deep caves that conclude that antibiotic resistance genes are ubiquitous and predate any anthropogenic effects due to antibiotic production or use or other human activities (33–36). Sequence identity between antibiotic resistance genes amenable to horizontal transfer in clinical pathogens and in environmental bacteria indicate that the former recruit these genes from the latter (20, 37, 38).

Food consumption represents only one exposure route of humans to environmental antibiotic resistance and also for the interaction of humans with the natural resistome (39, 40). The relative risks of environmental, community-acquired, and nosocomial exposure are largely unknown and ideally would be considered in a human health risk assessment framework (41). Nevertheless, some investigators have commented that antibiotic resistance transfer via vegetables represents a risk to human health that is of sufficient concern that it should be managed, by peeling and carefully washing produce, for example. We take the position that ingestion of antibiotic-resistant bacteria naturally found in soil is inevitable through consumption of raw vegetables and fruits, as recommended in a healthy diet and practiced in a normal household setting. Furthermore, the benefits of eating raw produce far outweigh any concern from exposure to natural soil bacteria that would limit the consumption of fruits or vegetables. The human microbiome is crucial for human health and wellness (42, 43). It is not unreasonable to speculate that exposure to soil microorganisms through food consumption, particularly for urban residents that have negligible contact with soil in their daily lives, places the enteric microbiome in contact with environmental bacteria. Deliberate geophagy is long practiced and thought to confer health benefits, perhaps through ingestion of beneficial microorganisms (44). What specific role the incidental consumption of small amounts of soil through food ingestion may have on health and wellness is a matter for conjecture.

There were substantial yearly differences in the detection frequency of gene targets in DNA recovered from vegetables (Table 8). This is undoubtedly due to different methods of DNA extraction that were used in the two seasons of study (Materials and Methods). In 2011, DNA was extracted from macerated vegetable

material, whereas in 2012, bacteria were removed from the vegetable matrix by being shaken in buffer and then extracted. On this basis, we recommend recovery of bacteria from vegetables prior to DNA extraction as the method of choice.

Manuring increased the abundance of viable antibiotic-resistant coliform bacteria in soil but did not do so consistently on vegetables (Table 6). Manuring also increased the frequency of detection of some gene targets in soil (Table 7), and some gene targets were detected on vegetables only when harvested from manured soil (Table 8). It has not been established in the present study that gene targets detected more frequently in manured soils were carried into the soil by the manure or were present in soil bacteria that were enriched by the addition of manure. Nevertheless, as a common-sense precaution, crop production must be undertaken such that the natural burden of antibiotic resistance on harvested crops is not increased by unsafe production practices. Notably, risk to antibiotic resistance exposure via produce consumption should best be managed by ensuring that practices designed to protect vegetables from contamination with pathogenic microorganisms are also protective with respect to exposure to antibiotic-resistant bacteria selected for in the digestive tract of animals or humans. Thus, further work is required to determine the efficacy of waste treatment practices (composting, anaerobic digestion, lime stabilization) in eliminating antibiotic resistance determinants of concern from manures or human waste (45–47). Likewise, the efficacy of offset times between the application of fecal materials in crop production and harvest need to be validated under different climate conditions to ensure that they are of sufficient length to be protective of crop quality with respect to antibiotic resistance determinants (48, 49).

ACKNOWLEDGMENTS

This research was funded by the AAFC SAGES program.

We thank T. J. Henderson, Z. Findlay, J. Anderson, and A. Belfall for valued technical assistance. We are very thankful to the AAFC farm staff and our farm cooperators, C. and M. Bontje, who provided manure.

REFERENCES

1. Health Canada. 25 April 2013, accession date. Canada's Food Guide. Health Canada, Ottawa, ON, Canada. <http://www.hc-sc.gc.ca/fn-an/food-guide-aliment/basics-base/index-eng.php>.
2. Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, Frankel G. 2010. Minireview: fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ. Microbiol.* 12:2385–2397.
3. Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, Opsteegh M, Langelaar M, Threlfall J, Scheutz F, van der Giessen J, Kruse H. 2010. Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* 139(Suppl):S3–S15.
4. Kozak GK, MacDonald D, Landry L, Farber JM. 2013. Foodborne outbreaks in Canada linked to produce: 2001 through 2009. *J. Food Prot.* 76:173–183.
5. Food and Drug Administration. 25 April 2013, accession date. Food safety standards. FDA, Washington, DC. <http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm304045.htm>.
6. Codex Alimentarius. 26 April 2013, accession date. Code of hygienic practice for fresh fruits and vegetables. World Health Organization, Geneva, Switzerland. http://www.codexalimentarius.org/download/standards/10200/CXP_053e.pdf.
7. Canadian Food Inspection Agency. 2009, posting date. Code of Practice for minimally processed ready-to-eat vegetables. Canadian Food Inspection Agency, Ottawa, ON, Canada. <http://www.inspection.gc.ca/english/fssa/frefra/safsal/minproe.shtml#s2>.
8. Yost CK, Diarra MS, Topp E. 2011. Animals and humans as sources of

- fecal indicator bacteria, p 67–92. In Sadowsky MJ, Whitman RL (ed), The fecal bacteria. ASM Press, Washington, DC.
9. Hawkey PM, Jones AM. 2009. The changing epidemiology of resistance. *J. Antimicrob. Chemother.* 64:i3–i10.
  10. McEwen SA. 2012. Quantitative human health risk assessments of antimicrobial use in animals and selection of resistance: a review of publicly available reports. *Rev. Sci. Tech.* 31:261–276.
  11. Lanthier M, Scott A, Lapen D, Zhang Y, Topp E. 2010. Frequency of virulence genes and antibiotic resistances in *Enterococcus* spp. isolates from wastewater and feces of domesticated mammals and birds, and wildlife. *Can. J. Microbiol.* 56:715–729.
  12. Chen B, Zheng W, Yu Y, Huang W, Zheng S, Zhang Y, Guan X, Zhuang Y, Chen N, Topp E. 2011. Class 1 integrons, selected virulence genes, and antibiotic resistance in *Escherichia coli* isolates from the Minjiang River, Fujian Province, China. *Appl. Environ. Microbiol.* 77:148–155.
  13. Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegård B, Söderström H, Larsson DGJ. 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS One* 6:e17038. doi:10.1371/journal.pone.0017038.
  14. Jury KL, Khan SJ, Vancov T, Stuetz RM, Ashbolt NJ. 2011. Are sewage treatment plants promoting antibiotic resistance? *Crit. Rev. Environ. Sci. Technol.* 41:243–270.
  15. Pruden A, Arabi M, Storteboom HN. 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ. Sci. Technol.* 46:11541–11549.
  16. Suzuki S, Hoa PTP. 2012. Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in India. *Front. Microbiol.* 3:67.
  17. Jechalke S, Kopmann C, Rosendahl I, Groeneweg J, Weichert V, Kröger-recklenfort E, Brandes N, Nordwig M, Ding G-C, Siemens J, Heuer H, Smalla K. 2013. Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. *Appl. Environ. Microbiol.* 79:1704–1711.
  18. Gaze WH, Zhang L, Abdoulsalam NA, Hawkey PM, Calvo-Bado L, Royle J, Brown H, Davis S, Kay P, Boxall ABA, Wellington EMH. 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J.* 5:1253–1261.
  19. Heuer H, Schmitt H, Smalla K. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14:236–243.
  20. Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis.* 13: 155–165.
  21. Gaze WH, Krone SM, Larsson DGJ, Li X-Z, Robinson J, Simonet P, Smalla K, Timinouni M, Topp E, Wellington EM, Wright GD, Zhu Y-G. 2013. Influence of humans on evolution and mobilization of environmental antibiotic resistance. *Emerg. Infect. Dis.* doi:10.3201/eid1907.120871.
  22. Line JE. 2001. Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. *J. Food Prot.* 64:1711–1715.
  23. Kiess AS, Parker HM, McDaniel CD. 2010. Evaluation of different selective media and culturing techniques for the quantification of *Campylobacter* ssp. from broiler litter. *Poult. Sci.* 89:1755–1762.
  24. Government of Canada. 2010. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2007. Public Health Agency of Canada, Guelph, ON, Canada.
  25. Boehme S, Werner G, Klare I, Reissbrodt R, Witte W. 2004. Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs. *Mol. Nutr. Food Res.* 48:522–531.
  26. Rodríguez C, Lang L, Wang A, Altendorf K, García F, Lipski A. 2006. Lettuce for human consumption collected in Costa Rica contains complex communities of culturable oxytetracycline- and gentamicin-resistant bacteria. *Appl. Environ. Microbiol.* 72:5870–5876.
  27. Bezanson GS, MacInnis R, Potter G, Hughes T. 2008. Presence and potential for horizontal transfer of antibiotic resistance in oxidase-positive bacteria populating raw salad vegetables. *Int. J. Food Microbiol.* 127:37–42.
  28. Ruimy R, Brisabois A, Bernede C, Skurnik D, Barnat S, Arlet G, Momčilovic S, Elbaz S, Moury F, Vibet M-A, Courvalin P, Guillemot D, Andrement A. 2010. Organic and conventional fruits and vegetables contain equivalent counts of Gram-negative bacteria expressing resistance to antibacterial agents. *Environ. Microbiol.* 12:608–615.
  29. Hassan SA, Altalhi AD, Gherbawy YA, El-Deeb BA. 2011. Bacterial load of fresh vegetables and their resistance to the currently used antibiotics in Saudi Arabia. *Foodborne Pathog. Dis.* 8:1011–1018.
  30. Raphael E, Wong LK, Riley LW. 2011. Extended-spectrum beta-lactamase gene sequences in Gram-negative saprophytes on retail organic and nonorganic spinach. *Appl. Environ. Microbiol.* 77:1601–1607.
  31. Schwaiger K, Helmke K, Hölzel CS, Bauer J. 2011. Antibiotic resistance in bacteria isolated from vegetables with regards to the marketing stage (farm vs. supermarket). *Int. J. Food Microbiol.* 148:191–196.
  32. Walia S, Rana SW, Maue D, Rana J, Kumar A, Walia SK. 2013. Prevalence of multiple antibiotic-resistant Gram-negative bacteria on bagged, ready-to-eat baby spinach. *Int. J. Environ. Health Res.* 23:108–118.
  33. D'Costa VM, McGrann KM, Hughes DW, Wright GD. 2006. Sampling the antibiotic resistome. *Science* 311:374–377.
  34. Wright GD. 2010. Antibiotic resistance in the environment: a link to the clinic? *Curr. Opin. Microbiol.* 13:589–594.
  35. Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74:417–433.
  36. Bhullar K, Wagelchner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One* 7:e34953. doi:10.1371/journal.pone.0034953.
  37. D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. 2011. Antibiotic resistance is ancient. *Nature* 477:457–461.
  38. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337:1107–1111.
  39. Sannes MR, Belongia EA, Kieke B, Smith K, Kieke A, Vandermause M, Bender J, Clabots C, Winokur P, Johnson JR. 2008. Predictors of antimicrobial-resistant *Escherichia coli* in the feces of vegetarians and newly hospitalized adults in Minnesota and Wisconsin. *J. Infect. Dis.* 197: 430–434.
  40. Zhang L, Kinkelaar D, Huang Y, Li Y, Li X, Wang HH. 2011. Acquired antibiotic resistance: are we born with it? *Appl. Environ. Microbiol.* 77: 7134–7141.
  41. Ashbolt NJ, Aézquita A, Backhaus T, Borriello SP, Brandt KK, Collignon P, Coors A, Finley R, Gaze WH, Heberer T, Lawrence JR, Larsson DGJ, McEwen SA, Ryan JJ, Schönfeld J, Silley P, Snape JR, Van den Eede C, Topp E. 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ. Health Perspect.* doi:10.1289/ehp.1206316.
  42. Robinson CJ, Bohannon BJM, Young VB. 2010. From structure to function: the ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev.* 74:453–476.
  43. Human Microbiome Project. 2012. A framework for human microbiome research. *Nature* 486:215–221.
  44. Sing D, Sing CF. 2010. Impact of direct soil exposures from airborne dust and geophagy on human health. *Int. J. Environ. Res. Public Health* 7:1205–1223.
  45. Topp E, Scott A, Lapen DR, Lyautey E, Duriez P. 2009. Livestock waste treatment systems for reducing environmental exposure to hazardous enteric pathogens: some considerations. *Bioresour. Technol.* 100:5395–5398.
  46. Ma Y, Wilson CA, Novak JT, Riffat R, Aynur S, Murthy S, Pruden A. 2011. Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class I integrons. *Environ. Sci. Technol.* 45:7855–7861.
  47. Pruden A, Larsson DGJ, Amézquita A, Collignon P, Brandt KK, Graham DW, Lazorchak JR, Suzuki S, Silley P, Snape JR, Topp E, Zhang T, Zhu YG. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Health Persp.* doi:10.1289/ehp.1206446.
  48. Ingham SC, Fanslau MA, Engel RA, Breuer JR, Wright TH, Reith-Rozelle JK, Zhu J. 2005. Evaluation of fertilization-to-planting and fertilization-to-harvest intervals for safe use of noncomposted bovine manure in Wisconsin vegetable production. *J. Food Prot.* 68:1134–1142.
  49. Merchant LE, Rempel H, Forge T, Kannangara T, Bittman S, Delaquis P, Topp E, Ziebell KA, Diarra MS. 2012. Characterization of antibiotic-resistant and potentially pathogenic *Escherichia coli* from soil fertilized with litter of broiler chickens fed antimicrobial-supplemented diets. *Can. J. Microbiol.* 58:1084–1098.