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*Research Article*

# Influence of Tillage and Daily Manure Application on the Survival of Bacterial Pathogens Indicators in Soil and on Radish

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We measured *Escherichia coli*, and *Enterococcus* sp. numbers in soil and on fresh radish (*Raphanus sativus* L.) at 1, 7, 14, 28, 54, and 84 days after the addition of high and low amounts of solid dairy manure in combination with chisel tillage to a 20 cm depth (deep) or roller tillage to a 10 cm depth (shallow). When the high or low amount of solid dairy manure was added to the soil, *E. coli* populations in soil were higher in the 54 days following manure addition compared to the control treatment. Dairy manure addition increased *Enterococcus* sp. in soils compared to the control treatment for the entire 84 days sampling period. At harvest, which was 84 days after application, we did not detect *E. coli* in radish in rhizosphere soil or on radish roots. Addition of solid dairy manure increased *Enterococcus* sp. numbers in radish rhizosphere soil and on radish roots. We suggest that fresh animal manure be applied to soil at least 120 days prior to planting to allow die-off of human pathogenic bacteria and reduce the incidence of bacterial adhesion on or bacterial colonization of ready to eat vegetables.

## 1. Introduction

In the last decade, there has been a major shift in animal rearing toward large-scale confined animal feeding operations (CAFOs). CAFOs are a source of agricultural pollution and pose risks to water quality and public health due to the large amount of manure generated [1]. The US Environmental Protection Agency estimated that animal waste production in 1992 was 13 times greater on a dry weight basis than human production. Sources of water pollution from CAFOs include direct discharges, open feedlots, treatment and storage lagoons, manure stockpiles, and land application of manure. Pollution of surface flow and ground water from animal waste applied to soils has been documented [2–4]. Liquid-waste discharge onto soil initiates solute and microbe movement into the soil following ground water drainage patterns and can potentially contaminate adjoining surface water. These same bodies of water are often sources of drinking water or are used for recreational activities. Human contact with recreational waters containing intestinal pathogens is an effective method of disease transmission. Thus, employing appropriate treatment strategies to maintain the quality of lakes and streams and keep them free of pathogens is important.

Runoff and ground water from waste-treated agricultural land shows that enteric bacteria increase in spring flows and decrease in the dry period, increase in water after irrigation or after manure is applied, and rapidly decrease once manure application is halted [5–8]. Several investigators found that enteric bacteria declined rapidly when transported through dispersed soils, indicating that bacterial pollution occurs by transport via water through soil macropores [4, 9, 10]. In studies where animal waste had been continually applied for several years, enteric bacteria were found in soils and ground water [11–14]. Patogen survival time in the soil varied from 4 to >250 days [10, 15, 16] and strongly reflects the organism's ability to respond to adverse environmental conditions. Obligate parasites usually only live a few minutes outside the host, but many enteric pathogens can live in ground water and soil for months [4, 7, 8, 16]. Several factors influence the survival of pathogens in soil after waste materials are applied. Soil water content and temperature are the most

important factors [10, 14, 15, 17]. Survival of bacterial pathogens in soil increases in moist warm soil [11, 12]. Although further research is necessary, waste is continually applied to soil at least 120 days prior to crop planting to allow enteric bacteria die [1]. After land application of animal waste, the opportunity for transfer of these organisms from soils to surface and ground water, and ultimately to humans, depends in part on their ability to survive in the soil environment [11, 12].

Enteric bacteria can transfer from manure-amended soil to leaf and root vegetables [18–20]. Several studies have found enteric bacteria on or inside a wide variety of vegetables [16, 18, 21, 22] grown in soils amended with animal waste or irrigated with waste water. Standards permit no more than one fecal coliform or *E. coli* in a 100 mg sample for food to be deemed safe for human consumption [23]. *Salmonella* sp., *Listera* sp., and *E. coli* O157:H7 on ready-to-eat salad vegetables and fruits have caused several disease outbreaks [24–27].

*Escherichia coli* and *Enterococcus* sp. are sensitive and commonly used indicators of bacterial contamination. Their presence implies the potential presence of microbes pathogenic to humans. The concentrations of *Escherichia coli* and *Enterococcus* sp. in drinking and bathing water have been correlated with disease incidence [23]. Our first objective was to determine the influence of high and low amounts of solid dairy manure applied to soil in combination with disk or deep tillage on the survival of *Escherichia coli* and *Enterococcus* sp. in soil. The second objective was to determine the survival of *E. coli* and *Enterococcus* sp. on radish peel and in rhizosphere soil in relation to survival of these pathogens in soil.

## 2. Material and Methods

*2.1. Site Description.* The site is located at United States Department of Agriculture (USDA) Agricultural Research Service's Northwest Irrigation and Soils Research Laboratory at 42°30′00″ N, and 114°20′40″ W and is at 1300 m elevation. The climate is typified by cool, moist winters, and hot, dry summers with annual precipitation ranging from 175 to 305 mm, two-thirds of which occurs during October through March. Average annual temperature ranges from 9 to 10°C with the warmest temperatures occurring in July with an average maximum temperature of 17°C and the coolest temperatures occurring in January with an average minimum temperature of −21°C. The site was planted to dry bean (*Phaseolus vulgaris*) in 2006 after being planted to corn (*Zea mays*) the three previous years. Soil was classified as a coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcid, with 0.1–0.21 g/g clay and 0.6–0.75 g/g silt, and organic matter of approximately 13 g $kg^{-1}$. The soil has a pH of 7.6–8.0. Slope on this site is about 1.5%.

*2.2. Experimental Design.* The experimental design was a split-split plot design and was replicated four times. Main plots that consisted of shallow and deep tillage and plots were split into high solid dairy manure, low solid dairy manure, and control (dairy manure not applied) treatments. Each subplot (high solid dairy manure, low solid dairy manure) was 21 m × 50 m and waslocated across a 1.5% slope at least 50 meters apart so that there was not cross contamination from soil or irrigation water. There were a total of 24 plots which consisted of 2 tillage treatments × 3 manure treatments for 4 replications. Time was also analyzed as subplots and each sample taken was replicated three times. Dairy manure treatments were (1) high dairy manure amounts where plots received annual dairy manure applications, (2) low dairy manure amounts where plots received alternate year application, and (3) no dairy waste (control). Plots that received an annual dairy waste application received 110 Mg $ha^{-1}$ in 2004, 67 Mg $ha^{-1}$ in 2005, and 67 Mg wet weight manure $ha^{-1}$ in 2006. Plots that received dairy waste applications in alternate years received 110 Mg $ha^{-1}$ in 2004 and 67 Mg wet weight manure $ha^{-1}$ in 2006. Fresh solid dairy manure was applied to soil at a low application rate of 67 Mg wet weight manure $ha^{-1}$in May in 2005 and 2006. Fresh solid dairy manure was transported from a local dairy operation on May 20 and applied the same day. The dairy waste contained 10.1 g N $kg^{-1}$ and 2.7 g total P $kg^{-1}$ dry manure, which was equivalent to 155 kg N and 42 kg P $ha^{-1}$. The pH of the manure was 7.4 and acid detergent fiber was 304 g dry matter $kg^{-1}$. In 2004 the control plots received equivalent of 155 kg N $ha^{-1}$ as $NH_4NO_3$ and 42 kg P $ha^{-1}$ as $P_2O_5$. In 2005 and 2006 the control plots received equivalent of 95 kg N $ha^{-1}$ as $NH_4NO_3$ and 26 kg P $ha^{-1}$ as $P_2O_5$. Concentrations of *E. coli* and *Enterococcus* sp in manure were $4.0 \times 10^5$ and $2.7 \times 10^5$ organisms $g^{-1}$ soil, respectively. Immediately after manure or fertilizer application deep tillage plots were chisel plowed (20 cm depth) and shallow plots were roller harrowed (10 cm depth). Radish (*Raphanus sativus* L.) seeds were sown on May 21, 2006 in three separate 1 m long segments within each of four rows near the center of each plot. The field was sprinkler irrigated with a linear move irrigation system based on crop water use estimates for dry beans from the US Bureau of Reclamation's AgriMet model. The first irrigation was applied one day after planting. Irrigation water was obtained from the Snake River with fecal coliform concentration in this water ranging from 0 to 1812 cfu/ 100 ml water [28]. All plots were cultivated for weed control during the growing season.

*2.3. Sampling.* Three 10 cm diameter cores to a 10 cm depth were collected at random points in each plot. *Escherichia coli* and *Enterococcus* sp. populations in soil were determined in the above stated treatments at 1, 7, 28, 56, and 84 days after application of solid dairy manure and tillage treatments. We took 3 soil cores from each treatment × tillage plot on each sampling date (3 manure treatments × 2 tillage treatments × 4 replications × 3 soil samples taken in each plot). Each core was placed in sterile plastic bags, stored at 4°C in coolers, and transported to the Northwest Irrigation and Soils Research Laboratory. Samples were stored at 4°C and incubation began within 2 hours of collection. Eighty four days after planting, 24 to 30 plants were randomly harvested from each plot and analyzed for the presence of *E. coli*

Table 1: *Escherichia coli* and *Enterococcus* sp. mean numbers in the 0–5 cm of soil after application of inorganic fertilizer (control) and solid dairy manure and chisel or disc tillage.

| Dairy waste | Tillage | Day 1 | | Day 7 | | Day 28 | | Day 54 | | Day 84 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *E. coli* | *Enterococcus* | *E. coli* | *Enterococcus* | *E. coli* | *Enterococcus* | *E. coli* | *Enterococcus* | *E. coli* | *Enterococcus* |
| | | colony forming units/$g^{-1}$ dry weight of soil | | | | | | | | | |
| Control | Chisel | 0 d w | 73 c | 0 d w | 1 e w | 0 d w | 0 d w | 0 d w | 2 d | 0 b w | 55 d |
| Control | Roller | 0 d w | 2 d w | 1 d w | 5 e w | 0 d w | 0 d w | 0 d w | 1 d w | 0 b w | 52 d w |
| Low | Chisel | 122 b x | 82 c x | 711 a w | 550 c w | 12 b y | 78 b x | 72 b x | 217 b wx | 0 b z | 401 b w |
| Low | Roller | 2284 a w | 255 b y | 724 a x | 862 b y | 2 c z | 40 b y | 2 c z | 73 c y | 0 b z | 103 c y |
| High | Chisel | 153 b y | 320 a y | 113 b w | 4133 a x | 35 a y | 7732 a w | 4 c z | 56 c y | 0 b z | 73 d y |
| High | Roller | 2 c z | 224 b y | 32 c y | 136 d y | 8 b z | 10 c z | 223 a y | 1398 a x | 12 a z | 3573 a w |

(a) In each column, values followed by the same letter (a,b, c, d, or e) are not significantly different as determined by the least square means test ($P \leq .05$; $n = 18$). (b) In each row, within each *Escherichia coli and Enterococcus* sp. population, values followed by the same letter (w, x, y, or z) are not significantly different as determined by the least square means test. ($P \leq .05$; $n = 18$). (c) *Escherichia coli and Enterococcus* sp. populations were transformed using logarithms to achieve normal distributions; *Escherichia coli* and *Enterococcus* sp. populations are reported in untransformed numbers.

and *Enterococcus* sp. on the exterior and interior of the radish skin. The 5.0 mm of soil surrounding the radish skins (rhizosphere soil) was sampled in the manner described below.

*2.4. Escherichia coli and Enterococcus sp. Procedures.* A 10 g subsample of each soil was taken and gravimetric water content was determined. To enumerate desired soil bacterial populations, a 1 g dry weight equivalent subsample of soil was placed in 99 ml of sterile Butterfield's buffer [23] in a 160 ml sterile bottle and shaken for 20 minutes on a rocking platform shaker at 100 revolutions $min^{-1}$. The blanks were then removed and further diluted to $10^{-2}$ to $10^{-5}$ with Butterfield's buffer. *Escherichia coli* and *Enterococcus* sp. were analyzed with the membrane filter technique [23]. A 100 ml sample of the final dilution of each sample was vacuum-filtered through a sterile 0.45 $\mu$m filter and placed on mTEC medium (Fisher Scientific, Pittsburgh, PA) to enumerate *E. coli* or mEnterococcus medium (Fisher Scientific, Pittsburgh, PA) to enumerate *Enterococcus* sp. Radishes were stored at ambient temperatures and incubation began within 2 hours of collection. The samples were tested for *E. coli* and *Enterococcus* sp. on the exterior of the radish. To enumerate the *E. coli* and *Enterococcus* sp. numbers on the exterior of the radish, 3 unwashed radishes from each plot were selected, weighed, quartered, and placed in a 500 ml wide mouth sterile bottle containing Butterfield's buffer. They were shaken for 30 seconds to remove the surface soil and other debris. The solution was plated at $10^0$, $10^{-2}$, and $10^{-4}$ onto 3 M Petrifilm (St. Paul. MN), for *E. coli*/Em *Enterococcus* plates. To enumerate the population within the peel, 3 radishes were randomly selected, weighed, and washed with tap water at 25°C. The outer 4–6 mm of the radish [29] was removed by placing the radish in a 80 mm sterile Petri dish and removing the peel with a sterile knife. Fifteen grams of the peel was added to a 90 ml dilution blank of sterile Butterfield's buffer. The mixture was blended for two minutes in a sterilized blender. The blended mixture was plated at $10^0$, $10^{-2}$, and $10^{-4}$ and placed on mTEC medium (Fisher Scientific, Pittsburgh, PA) to enumerate *E. coli* or mEnterococcus medium (Fisher Scientific, Pittsburgh, PA) to enumerate. *Escherichia coli* were incubated at 44.0 ± 0.02°C for 24 hr; *Enterococcus* sp. were incubated at 45.0 ± 0.02°C for 24 hr.

*2.5. Statistical Analyses.* All dependent variables were tested for normal distribution. Data were then analyzed by means of analysis of variance procedures (ANOVA) for a split plot design with Statistical Analysis Systems [30]. *Escherichia coli* and *Enterococcus* sp. populations were transformed using loge to achieve normal distributions. Statistical comparisons of *Escherichia coli* and *Enterococcus* sp. populations were made for manure treatments × tillage × time since application because these interactions were significant in the GLM models [31, 32]. Residuals were equally distributed with constant variances. Differences reported throughout are significant at $P \leq .05$, as determined by the protected Least Squares Means (LSM) test [31, 32]. *Escherichia coli* and *Enterococcus* sp. populations are reported in untransformed numbers.

## 3. Results

When the high or low amount of solid dairy manure was added to the soil combined with deep or shallow tillage, *E. coli* populations in soil were higher in the 54 days following manure addition compared to the control treatment (Table 1). When the low amount of solid dairy manure was added to the soil combined with deep or shallow tillage, *Enterococcus* sp. populations in soil were higher in the 84 days following manure addition compared to the control treatment. When the high amount of solid dairy manure was added to the soil combined with deep or shallow tillage, *Enterococcus* sp. populations in soil were higher from 1–84 days after manure addition compared to the control treatment. When the low amount of dairy manure was added and then shallow tilled, *Enterococcus* sp. populations were higher in soil on days 1 and 7 but lower on days 54 and 84

Table 2: *Escherichia coli* and *Enterococcus* sp. mean numbers on radish roots (*Raphanus sativus* L.) and in rhizosphere soil after 84 days when growing in soil amended with inorganic fertilizer (control) and solid dairy manure with deep (chisel) tillage to a 20 cm depth or shallow (roller) tillage to a 10 cm depth.

| Dairy waste | Tillage | Rhizosphere Soil | | Radish Peel | |
|---|---|---|---|---|---|
| | | *E. coli* | *Enterococcus* | *E. coli* | *Enterococcus* |
| | | colony forming units/$g^{-1}$ dry weight of peel | | | |
| Control | Deep | 0 a | 418 d | 0 a | 12 c |
| Control | Shallow | 0 a | 224 d | 0 a | 8 c |
| Low | Deep | 0 a | 930 c | 0 a | 39 b |
| Low | Shallow | 1 a | 2106 b | 0 a | 74 b |
| High | Deep | 0 a | 3187 b | 0 a | 42 b |
| High | Shallow | 0 a | 11078 a | 0 a | 247 a |

(a) In each column, values followed by the same letter are not significantly different as determined by the least square means test ($P \leq .05$; $n = 18$). (b) *Escherichia coli and Enterococcus* sp. populations were transformed using logarithms to achieve normal distributions; *Escherichia coli* and *Enterococcus* sp. populations are reported in untransformed numbers.

than when the soil was deep tilled. When the high amount of dairy manure was added and then shallow tilled, *Enterococcus* sp. populations were higher in soil on days 1, 7, and 28 but lower on days 54 and 84 than when the soil was deep tilled. *Escherichia coli* were found in soil without dairy manure applied on days 7 and 54. Dairy manure addition increased *Enterococcus* sp. in soil compared to the control treatment for the entire 84 day sampling period. The numbers of *E. coli* and *Enterococcus* sp. in soil increased from 1 to 7 days after addition of dairy manure regardless of tillage but declined after 54 days. When the low amount of dairy manure was added and then shallow tilled, *Enterococcus* sp. populations were higher in rhizosphere soil than when the soil was deep tilled (Table 2). When the low amount of dairy manure was added and then shallow tilled, *Enterococcus* sp populations where higher in radish peel than when the soil was deep tilled.

## 4. Discussion

Fall application of solid dairy manure increased *E. coli* and *Enterococcus* sp. numbers compared to control soil which received only inorganic N-P fertilizer for at least 54 days. We found that *E. coli* populations in soil decreased with time when the high amount of dairy manure was applied and deep tilled and when the low amount of dairy manure was applied and shallow tilled. When the high amount of dairy manure was applied and shallow tilled, *E. coli* populations increased from day 1 to day 7, then decreased from day 7 to day 28, and then increased again on days 54 and 84. When the low amount of dairy manure was applied and deep tilled, *E. coli* and *Enterococcus* sp. populations increased from day 1 to day 7and then decreased from day 7 to day 84. Lau and Ingham [33] found that after fresh dairy manure was added to Wisconsin soils, *E. coli* and Enterococcus sp. numbers increased 10–100-fold and then decreased. Watering did not affect *E. coli* or *Enterococcus* sp. numbers in soil. Reddy et al. [34] found that enteric bacteria die-off did not follow first-order kinetics and that the two most important factors influencing survival were moisture and temperature. Soil moisture seems to be the most important of these factors [10, 12, 14, 15, 17]. Entry et al. [12] found that decreasing soil moisture with increasing soil temperature substantially decreased survival of total and fecal coliform bacteria. Detection is based on survival but the viable numbers of pathogenic bacteria in soil that can be cultured increase when the soil is moist. We applied solid dairy manure to an irrigated western soil during a dry period (surface moisture concentrations of 0.07–0.14 $g^{-1}$ water $g^{-1}$ soil) and then soil and plants received irrigation water based on plant water use. Soil temperature also exerts a major influence on the survival of coliform bacteria. Survival of pathogenic bacteria primarily reflects the organism's ability to respond to nonparasitic and adverse environmental conditions.

*Escherichia coli and Enterococcus* sp. numbers in soil after a high amount of dairy manure was applied and shallow tilled and the low amount of dairy manure was applied and deep tilled seemed to follow a cyclic decreasing and increasing pattern which could be related to increasing soil moisture from crop irrigation and/or cyclic soil temperatures. Mold board plow tillage to a depth of 30 cm may have decreased the survival of these bacteria. The influence of tillage on the survival of enteric bacteria in agricultural soils after manure addition has not been documented. Entry et al. [16] found that addition of dairy compost or solid dairy manure at rates to meet crop phosphorus uptake did not consistently increase *E. coli and Enterococcus* sp. and fecal coliform bacteria in the soil. However, they also found that fresh potato skins grown in that soil had higher *Enterococcus* sp. and fecal coliform numbers when solid dairy manure was added to soil compared to compost, inorganic N fertilizer or inorganic N-P fertilizer treatments.

*Salmonella enterica* and *Listeria* sp. were found in higher numbers in barley (*Hordeum vulgare*) rhizosphere soil, but the bacteria did not colonize root tissue [35]. *Escherichia coli* O157:H7 survived in soil for 35 days, but growth and survival were not different between bulk and rhizosphere soils [36]. The growth and survival of enteric bacteria in rhizosphere soils necessarily compete for resources including carbon sources, amino acids, nutrients, and moisture with the soil microbial community in that rhizosphere.

We found that solid dairy manure application to soil increased *Enterococcus* sp., but not numbers in radish rhizosphere soil and on radish peels. However, *E. coli* numbers in radish rhizosphere soil and on radish peels were not affected. The higher *Enterococcus* sp. and fecal coliform numbers in potato rhizosphere soil, compared to bulk soil, may have been affected by increased sugar and nutrient content commonly seen in rhizosphere soils [37–39]. Rhizosphere soils favor rapidly growing microorganisms with short generation times [40, 41], which may favor growth and survival of enteric bacteria, which can gain entry into plant tissue [42, 43]. Both high and low amounts of solid dairy manure addition increased *Enterococcus* sp. numbers in bulk soil after 84 days, in rhizosphere, and on radish peels. However, the addition of both high and low amounts of solid dairy manure did not consistently increase *E. coli* numbers in bulk soil after 84 days. We did not detect *E. coli* in radish rhizosphere soil or on radish peels after 84 days. Standards require that if as few as one fecal coliform bacteria or *E. coli* are detected in a 100 mg tissue sample, the food must be deemed unsafe for human consumption [23]. Farm managers and consumers need to be aware that bacterial pathogens have been found on and inside radish even when manure had not been applied to soils and that these pathogens can potentially cause disease.

Solid dairy manure application to soil at high rates increased *E. coli* and *Enterococcus* sp. populations in bulk soil and *Enterococcus* sp. in radish rhizosphere soil and on radish peels. However, when solid dairy manure is applied to soil at rates that met plant phosphorus uptake, *E. coli*, *Enterococcus* sp., and fecal coliform numbers in bulk soil were not affected [16].Under USDA organic standards, manure must be subjected to proper composting where manure is allowed to reach a sterilizing temperature. If raw animal manure is used, 120 days must pass before the crop is harvested if the final product comes into direct contact with the soil and products which do not come into direct contact with soil 90 days must pass prior to harvest [1]. We suggest that animal manure be applied to all soil in accordance with the USDA organic standard of 120 days prior to planting to allow die-off of human pathogenic bacteria and reduce the incidence of bacterial adhesion on or bacterial colonization of ready-to-eat vegetables.

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