Microb Ecol (2009) 58:498–508
DOI 10.1007/s00248-009-9524-x

ENVIRONMENTAL MICROBIOLOGY

Evaluating the Effect of Environmental Factors on Pathogen Regrowth in Compost Extract

Jinkyung Kim · Marion W. Shepherd Jr. · Xiuping Jiang

Received: 16 December 2008 / Accepted: 24 April 2009 / Published online: 12 May 2009

Abstract Pathogenic microorganisms may survive the composting process in low numbers and subsequently regrow to high levels under favorable conditions. The objective of this study was to investigate the regrowth potential of *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* in dairy-based composts under different environmental conditions. Water extract of commercially available dairy compost was used as a model system. Cocktails of five rifampin-resistant strains of each pathogen previously grown in reduced nutrient media (1/2 or 1/10 strength of tryptic soy broth, TSB) were inoculated into water extract of compost of different ratios (1:2,1:5, and 1:10, *w*/*v*), and then stored at 35°C or 22°C for 7 days. The strains exhibiting greatest survival or regrowth were identified by pulsed-field gel electrophoresis (PFGE). At 22°C, both *E. coli* O157:H7 and *L. monocytogenes* multiplied in all compost extracts, whereas *Salmonella* spp. regrew in both 1:2 and 1:5 compost extracts but not in 1:10. For all three pathogens, incubation at 22°C provides better conditions for regrowth than at 35°C. Both *Salmonella* and *E. coli* O157:H7 previously adapted to nutrient-limited broth (1/10 strength of TSB) regrew in compost extracts to higher populations than the control cultures grown previously in full strength of TSB. In the absence of indigenous microorganisms, all three pathogens regrew even in the most diluted sterile compost extract (1:10) with growth potentials ranging from 2.30 to 3.59 log CFU/ml. In nonsterile compost extract with ca. 5 log CFU/ml of background microorganisms, all three pathogens regrew only in the most concentrated compost extract (1:2) with much less population increases ranging from 0.70 to 1.43 log CFU/ml. Compost extract samples of all ages supported the regrowth of both *Salmonella* and *E. coli* O157:H7 with population increases ranging from 0.95 to 2.32 log CFU/ml. The PFGE patterns for *E. coli* O157:H7 isolates from sterile compost extracts matched with either the spinach outbreak strain or an avirulent B6914 strain. These results demonstrated that compost extract of dairy-based compost contained sufficient nutrients for pathogen regrowth. Cultures previously adapted to low nutrient media regrew to higher populations than control cultures; however, indigenous microflora suppressed the pathogen regrowth in compost extract, especially at 35°C.

J. Kim · M. W. Shepherd Jr. · X. Jiang (✉)
Department of Food Science & Human Nutrition,
Clemson University,
Clemson, SC 29634, USA
e-mail: xiuping@clemson.edu

Introduction

Composting is a biological decomposition process as the result of microbial activities. Composting has been used as a practical and effective way for reducing human pathogen populations in various types of animal wastes. The finished compost can then be applied to agricultural land, home gardens, ornamental nurseries, and greenhouses as the value-added fertilizer and soil amendment [23, 24]. A potential risk of pathogenic bacteria including *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* in farm environment is generally low [27, 29, 36]. However, as shown in recent fresh produce-related outbreaks due to *E. coli* O157:H7 and *Salmonella* spp. contaminations, concerns about contamination of vegetables with fecal bacteria in the agricultural environment have increased [6, 10, 34]. Raw or inadequately composted manure has been identified as a potential preharvest source of contamination [5].

Bacterial regrowth in compost indicates that either a few survived cells during composting have multiplied under favorable growth conditions or that recontamination has occurred after active composting. The majority of research on pathogen regrowth was conducted in biosolids. These studies investigated the effect of different environmental factors such as moisture content [4, 12, 40], temperature [15], available nutrients [15, 35], and the population and diversity of indigenous microorganisms [33, 35] on *Salmonella* regrowth in sterile and/or nonsterile composted biosolids. However, inoculum levels were relatively high (ca. 10^3–10^5 CFU/g) in these studies which may not be encountered in composted materials. Additionally, there was the lack of information on the regrowth of *E. coli* O157:H7 or *L. monocytogenes* in compost.

Previous studies of manure or manure-based composts were focused on the survival of pathogens such as *Salmonella* and *E. coli* O157:H7 in stored composts and/or compost-amended soils. In these studies, high populations of seeded pathogens were monitored under laboratory and field settings. A few studies reported the regrowth of pathogens during composting of animal waste under field condition [8, 32]. For example, Cekmecelioglu et al. [8] enumerated *Salmonella* populations during windrow composting of food waste and cow manure in both summer and winter. Under winter conditions, inconsistent inactivation of pathogens and regrowth to high levels on several sampling days was observed. It was not clear whether the regrowth was related to rainfall as this information was not reported. Also, there was no data indicating the possibility of fecal contamination by wild animals. Recently, Shepherd et al. [32] reported the increase of avirulent *E. coli* O157:H7 counts in surface samples during on-farm dairy manure composting for a short period of time immediately following rainfall. The increase of *E. coli* O157:H7 counts in surface samples of compost could result from the bacterial proliferation when the pathogen encountered appropriate moisture levels in compost after rainfall. Although these studies suggest the possibility of pathogen regrowth in compost, there is a lack of information on the effect of environmental factors on regrowth of pathogen from a few cells in manure-based composts.

Physiological stage of pathogenic microorganisms is an important factor affecting the fate of pathogens in compost. Most composting studies investigating the fate of pathogens were conducted with nonstressed cultures [19, 20, 24], a condition not representative of the microorganisms in complex composting systems. Benito et al. [3] evaluated the response of natural isolates of *E. coli* O157:H7 to various stresses and concluded that it is important to include stress-resistant strains of target pathogens for preservation treatment. Growth conditions for pathogen typically encountered during the composting such as limited nutrients should be addressed.

The objective of this study was to investigate the regrowth potential of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* in compost under the selected environmental conditions, using water extract of dairy compost as a model system.

Materials and Methods

Preparation of Compost Extracts Commercial Black Kow dairy compost (Black Gold Compost Company, Oxford, FL, USA) was purchased at a local store. As listed on its label, the compost contains 0.5% total nitrogen, 0.5% available phosphate (P_2O_2), 0.5% soluble potash (K_2O), and no more than 1% chlorine. Compost was extracted with sterile tap water at different ratios (1:2, 1:5, and 1:10, *w*/*v*) in a shaking incubator (100 rpm, 22°C) for 24 h and centrifuged at 5,000×*g* for 20 min. The supernatant was transferred to a sterile 50 ml centrifuge tube and stored at freezer (−18°C) until used. For sterile water extract preparation, compost extracts of both 1:10 and 1:2 ratios were filtered through 0.2 μm syringe filter (Corning, NY, USA) and stored in freezer until used. Tryptic soy agar (TSA; Becton Dickinson, Sparks, MD, USA) was used to enumerate microorganisms in compost extract.

Dairy compost at different ages (days 0, 7, 14, 30, and 60 after the onset of composting) was taken from the compost heaps of a concurrent field study performed by our lab. The compost mixture consisted of a cow manure/sawdust–calf feces mixture, wasted feed, old hay, and vegetable wastes (squash and plant residues) at a ratio of 10:2:2:2, respectively. All materials used were obtained from a single dairy farm, and none of the collected was subjected to any treatment before composting. The materials were thoroughly mixed with the aid of a front-end loader, and heaps were conical shape of ca. 1.2×2 m. During composting, the heaps were on the concrete slab (25×16 m) surrounded by a gated fence and were not covered or protected from environmental conditions. The heaps were turned on days 3, 7, 14, 21, and 30 of composting with the use of a front-end loader. Compost samples from days 7, 14, and 30 were taken after the compost heaps were turned, whereas composts from days 0 and 60 were taken from the surface. Water extracts of the compost were prepared as described above.

Bacterial Cultures A five-strain mixture of each *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* was used as the inoculum. *Salmonella enteritidis* H2292, *Salmonella* Newport H9113, *Salmonella* Poona H9301 (kindly provided by Dr. Doyle, University of Georgia), *Salmonella*

Typhimurium DT 104 ATCC 700408 ISSA with green fluorescent protein (GFP; kindly provided by Dr. Sofos, Colorado State University), and avirulent *Salmonella* Typhimurium 8243 (kindly provided by Dr. Curtis, Washington University) for *Salmonella* spp., strains F06M-0923-21 (spinach outbreak strain from California Dept. of Health), F07M-020-1 (Taco John's outbreak strain from California Dept. of Health), H1730 (human isolates associated with lettuce consumption, kindly provided by Dr. Harrison, University of Georgia), ATCC 43895 ISEH GFP (kindly provided by Dr. Sofos, Colorado State University), and avirulent B6914 (kindly provided by Dr. Fratamico, Eastern Regional Research Center, USDA-ARS) for *E. coli* O157:H7, strains 201, LCDC, Scott A, 101M, and 109 from our stock cultures for *L. monocytogenes* were used. These strains were induced to be resistant to 100 μg/ml of rifampin (Fisher Scientific, Fair Lawn, NJ, USA) through gradient plate method [28] and stored at −80°C in tryptic soy broth (TSB; Becton Dickinson) with 20% glycerol. For each species, the lag phase and growth rate of rifampin resistant strains were compared to the wild-type strain. The stability of the rifampin-resistant strains was tested up to 21 generations.

Inoculum Preparation and Inoculation Procedure Each strain was streaked onto TSA supplemented with 100 μg/ml of rifampin and grown at 37°C for 24 h. Single colonies of each strain were transferred into TSB, 1/2 strength of TSB, or 1/10 strength of TSB supplemented with rifampin and grown in a shaking incubator (100 rpm, 37°C) for 24 h. Each culture was centrifuged at 5,000×*g* for 20 min, washed twice, and resuspended in 0.85% saline. After being adjusted to OD_{600} as 0.5 (ca. 10^8 CFU/ml), five equal volumes of rifampin-resistant strains were combined and serially diluted to the desired concentration (ca. 10^3 CFU/ml). The five-strain mixture of each pathogen was inoculated into compost extract with 1:100 ratio (*v*/*v*) to achieve a final concentration of ca. 10 CFU/ml of compost extract and then stored at 35°C or 22°C for 7 days. For nonsterile compost extract, samples were taken and analyzed at days 1, 3, 5, and 7 during storage, whereas 4-, 8-, 16-, and 36-h sampling intervals were added in addition to the same sampling intervals for sterile compost extract.

Microbiological Analysis The population of each pathogen in compost extract was enumerated by plating tenfold serial dilutions, in duplicate, onto Xylose lysine tergitol 4 (XLT-4, Becton Dickinson), TSA, and Oxford medium (Becton Dickinson) supplemented with rifampin (100 μg/ml) for *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, respectively. The plates were incubated at 37°C for 24~48 h for the enumeration of each pathogen. When the direct plating method failed to detect any pathogens (detection limit, 10 CFU/ml), selective enrichment method was used. One milliliter of compost extract was transferred into 9 ml of universal preenrichment broth (UPB, Acumedia Manufacturers, Lansing, MI, USA) and incubated at 37°C for 24 h, then transferred into Rappaport–Vassiliadis (RV) broth, TSB, and Fraser broth (Becton Dickinson) supplemented with rifampin for detection of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, respectively. After incubation at 37°C for 24 h, a loopful of each secondary enrichment culture was streaked onto XLT-4, sorbitol MacConkey agar (Becton Dickinson), and Oxford medium supplemented with rifampin for *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, respectively. Randomly selected colonies grown on each selective media were confirmed by a latex agglutination test (Oxoid, Basingstoke, Hampshire, UK) for *E. coli* O157:H7 and real-time polymerase chain reaction assay for *Salmonella* spp. and *L. monocytogenes* [37].

Pulsed-Field Gel Electrophoresis A five-strain mixture of each pathogen was used to address strain variation in terms of growth and survival in the compost ecosystem. The strains exhibiting extended survival or growth in compost extracts were identified by pulsed-field gel electrophoresis (PFGE). The restriction enzyme *Xba*I for *Salmonella* and *E. coli* O157:H7 and *Asc*I for *L. monocytogenes* were used to digest the DNAs extracted from the randomly selected isolates. PFGE for *Salmonella* and *E. coli* O157:H7 was performed using the protocol described by CDC/PulseNet [7], whereas *L. monocytogenes* strains were analyzed using a protocol by Graves and Swaminathan [13]. Following restrictive enzyme digestion, PFGE for *E. coli* O157:H7 and *Salmonella* was performed with the CHEF MAPPER system (Bio-Rad Laboratories, Hercules, CA, USA) in a 1% agarose gel and 0.5× Tris–borate–EDTA buffer at 6 V/cm for 18 h at 14°C, with initial and final switch times of 2.2 and 54.2 s, respectively. For *L. monocytogenes*, running time was 22 h with initial and final switch times of 4.0 and 40.0 s, respectively. Lambda DNA ladder (Bio-Rad) was used as molecular size standard. The gel was stained with ethidium bromide, the bands were visualized with UV transillumination, and the gel image was captured with the GelDoc 1000 system (Bio-Rad).

Growth Rate and Generation Time The growth curve of individual strain of *E. coli* O157:H7 was determined in sterile compost extract (1:2 ratio). The OD_{600} of each culture in 96-microwell plate (Corning, Corning, NY, USA) was measured at every 2 h up to 10 h and then every 4 h through 48 h from the time of culture inoculation. The viable cell counts at the selected time periods were determined using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD, USA). The growth rate and

generation time of individual strain were determined from two points in the exponential phase of the growth curves using following equations.

$$\text{Growth rate constant}(\mu) = ((\log_{10} N_t - -\log_{10} N_0) \times 2.303)/(t - t_0)$$

$$\text{Generation time}(g) = (\log_{10} N_t - -\log_{10} N_0)/0.301;$$

N_t and N_0 are the number of cells at time t and time t_0.

pH Determination The pH was determined with an Orion pH meter Model 310 (Orion Research, Boston, MA, USA).

Statistical Analysis For each trial, two compost extracts of each condition were prepared, and duplicate samples were taken from each extract at selected sampling intervals. At least two trials were conducted for each experiment. Bacterial counts were converted to log CFU/ml and subjected to analysis of variance using SAS (SAS Institute, ver. 9.1, Cary, NC, USA). *P* value below 0.05 was considered significantly different. Duncan's multiple range test was used to determine the pathogen populations differed at various nutritional concentration, growth medium, extract type (sterile or nonsterile), and compost at different ages. The same statistical tests were applied to determine if pathogen populations were significantly influenced by storage time at each condition.

Results

Determination of Pathogen Regrowth in Compost Extracts Water extract of commercially available dairy compost was used as a model system to evaluate the effect of temperature, nutrient availability, and indigenous microbial population on pathogen regrowth in compost. Rifampin resistant cultures of each species were not significantly different from the wild-type strain in duration of lag phase and growth rate. Up to 21 generations, no reversion of rifampin resistance to susceptibility was observed (data not shown).

Tables 1 and 2 presented the plate count data of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* in compost extract at 35°C and 22°C, respectively. At 35°C, both *Salmonella* spp. and *E. coli* O157:H7 regrew in compost extract with ratios of 1:2 and 1:5 but not 1:10. The population increases ranged from 0.37 to 2.07 log CFU/ml, with increased regrowth of pathogens in more concentrated compost extract (1:2; Table 1). However, no regrowth was detected for *L. monocytogenes* in all compost extracts. When tested at room temperature (22°C), all three pathogens regrew in the compost extracts with population increases ranging from 0.27 to 1.77 log CFU/ml (Table 2). Both *E. coli* O157:H7 and *L. monocytogenes* multiplied in all compost extracts at 22°C, whereas *Salmonella* spp. regrew in both 1:2 and 1:5, but not 1:10 compost extracts. The population increases were transient at 35°C compared to 22°C. For example, both *Salmonella* and *E. coli* O157:H7 at 35°C were only detectable after enrichment at 5 days,

Table 1 The potential regrowth and persistence of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* previously grown in TSB in nonsterile water extract of compost with different nutrient concentrations at 35°C

Microorganism	Ratio[a]	Mean[b]±SD (log CFU/ml) at the sampling time (day)				
		0	1	3	5	7
Salmonella spp.	1:2	BC[c]1.24±0.34 a[d]	A 2.19±0.34 a	A 1.86±0.19 a	B 1.39±0.10 a	C <1.0 a
	1:5	BC1.24±0.34 a	A 1.78±0.24 ab	A 1.76±0.20 a	AB1.54±0.20 a	C 1.15±0.17 a
	1:10	AB1.24±0.34 a	A 1.48±0.21 b	B 1.08±0.15 b	B <1.0[e] b	B <1.0 a
E. coli O157:H7	1:2	C 1.15±0.21 a	A 3.22±0.03 a	B 2.92±0.07 a	D <1.0 a	D <1.0 a
	1:5	B 1.15±0.21 a	A 1.49±0.16 b	A 1.52±0.17 b	B <1.0 a	B <1.0 a
	1:10	AB1.15±0.21 a	A 1.42±0.15 b	A 1.37±0.30 b	B <1.0 a	B <1.0 a
L. monocytogenes	1:2	A 1.15±0.21 a	A 1.08±0.15 a	A <1.0 a	B ND a	B ND a
	1:5	A 1.15±0.21 a	A 1.12±0.24 a	B ND b	B ND a	B ND a
	1:10	A 1.15±0.21 a	A 1.08±0.15 a	B ND b	B ND a	B ND a

ND not detected after enrichment

[a] Ratio of compost to water (*w*/*v*)

[b] The mean is the average of eight replicates

[c] Means with different upper case letters in a row are significantly different ($P<0.05$)

[d] For each pathogen, means with different lower case letters in a column are significantly different ($P<0.05$)

[e] <1.0 log CFU/ml, positive after enrichment

Table 2 The potential regrowth and persistence of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* previously grown in TSB in nonsterile water extract of compost with different nutrient concentrations at 22°C

Microorganism	Ratio[a]	Mean[b]±SD (log CFU/ml) at the sampling time (day)				
		0	1	3	5	7
Salmonella spp.	1:2	C[c]1.24±0.34 a[d]	A 3.01±0.43 a	B 2.17±0.17 a	B 2.11±0.11 a	B 2.10±0.05 a
	1:5	B 1.24±0.34 a	A 2.00±0.25 b	A 1.91±0.26 a	AB1.53±0.38 b	AB1.53±0.29 b
	1:10	A 1.24±0.34 a	A 1.15±0.17 c	A 1.08±0.15 b	A 1.15±0.17 b	A 1.08±0.15 c
E. coli O157:H7	1:2	D 1.15±0.21 a	A 2.61±0.04 a	A 2.56±0.06 a	B 2.30±0.19 a	C 1.99±0.10 a
	1:5	B 1.15±0.21 a	A 2.03±0.07 b	A 1.91±0.07 b	A 1.88±0.20 b	A 1.87±0.13 a
	1:10	C 1.15±0.21 a	AB1.65±0.18 c	A 1.70±0.07 c	BC1.39±0.10 c	C 1.32±0.23 b
L. monocytogenes	1:2	BC1.15±0.21 a	A 2.01±0.11 a	B 1.30±0.0 ab	B 1.27±0.20 a	C ND a
	1:5	B 1.15±0.21 a	A 1.57±0.20 b	A 1.52±0.26 a	B 1.08±0.15 ab	B ND a
	1:10	B 1.15±0.21 a	A 1.42±0.15 b	B 1.08±0.15 b	B <1.0[e] c	B ND a

ND not detected after enrichment

[a] Ratio of compost to water (*w*/*v*)

[b] The mean is the average of eight replicates

[c] Means with different upper case letters in a row are significantly different ($P<0.05$)

[d] For each pathogen, means with different lower case letters in a column are significantly different ($P<0.05$)

[e] <1.0 log CFU/ml, positive after enrichment

while at 22°C, both pathogens were still culturable after 7 days.

The population of mesophilic background microorganisms at 35°C increased rapidly by ca. 2.57 and 1.86 log CFU/ml on day 1, from 4.78 and 4.15 log CFU/ml of initial levels in 1:2 and 1:10 ratio of compost extracts, respectively, whereas at 22°C, mesophiles on day 1 regrew much slower than at 35°C with population increases of 1.86 and 1.06 log CFU/ml in 1:2 and 1:10 ratio of compost extracts, respectively. Maximum population was ca. 7.4 and 7.6 log CFU/ml on day 3 at 22°C and 35°C, respectively, regardless of compost extract ratios.

Effect of Physiological Stage of Pathogenic Inocula on the Regrowth Potential in Compost Extracts The plate count data indicated that both *Salmonella* and *E. coli* O157:H7 adapted to the reduced nutrient media regrew to higher ($P<0.05$) populations than the control cultures at 22°C (Table 3). Both *Salmonella* and *E. coli* O157:H7 previously grown in 1/10 strength of TSB increased in population by ca. 2 log CFU/ml by day 3 and remained at that level during sampling period, whereas *L. monocytogenes* grown in the same medium grew by only 0.5 log CFU/ml at day 1, and there was no significant difference in regrowth potential among previous growth media.

Effect of Indigenous Microorganisms on Pathogen Regrowth in Compost Extracts To verify the effect of competitive background microflora on pathogen regrowth in compost extract, we compared pathogen regrowth in both sterile and nonsterile compost extracts. All pathogens in sterile water extract with 1:10 ratio of compost extract started to regrow within a day at 22°C with population increases as ca. 0.99, 1.30, and 0.33 log CFU/ml for *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, respectively (Fig. 1). *E. coli* O157:H7 is the only pathogen which did not continue regrowth after 2 days of incubation. In the more concentrated compost extract (1:2), the averages of maximum population increase were 5.46, 5.79, and 4.09 CFU/ml for *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, respectively, up to 7 days after incubation. In nonsterile compost extract, both *Salmonella* spp. and *E. coli* O157:H7 regrew only in the most concentrated compost extract (1:2) with much less population increases, i.e., ca. 1.4 log CFU/ml, whereas *L. monocytogenes* cell populations increased only ca. 0.7 log CFU/ml in compost extract of 1:2 ratio.

The growth rate of each bacterium in the concentrated sterile compost extract (1:2) was approximately two times higher ($P<0.05$) than in the diluted extract (1:10) regardless of species (Table 4). Apparently, the regrowth rate for *E. coli* O157:H7 was the highest among the three species.

Strain Identification of Extended Survival in Compost Extracts The PFGE patterns of *Salmonella* for all isolates matched either serotypes Poona H9301 or Newport H9113. For *L. monocytogenes*, strains LCDC and 109 were predominant. In regard to *E. coli* O157:H7, spinach outbreak strain, FO6M-0923-21 which was originally isolated from cow feces on the implicated farm of spinach

Table 3 The potential regrowth and persistence of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* grown previously at 35°C with different growth media in nonsterile water extract of compost (1:2) at 22°C

Microorganism	Growth medium	Mean[a]±SD (log CFU/ml) at the sampling time (day)				
		0	1	3	5	7
Salmonella spp.	Full TSB	D[b] <1.0 a[c]	AB1.91±0.47 b	A 1.99±0.03 c	C 1.48±0.21 b	BC1.54±0.28 b
	1/2 TSB	C <1.0 a	A 2.16±0.22 ab	A 2.27±0.17 b	B 1.62±0.23 b	B 1.62±0.10 b
	1/10 TSB	C <1.0 a	AB2.60±0.14 a	A 2.71±0.14 a	B 2.46±0.10 a	B 2.46±0.24 a
E. coli O157:H7	Full TSB	C <1.0 a	AB2.17±0.20 c	A 2.31±0.20 b	A 2.39±0.15 b	B 1.96±0.14 c
	1/2 TSB	C <1.0 a	A 2.63±0.13 b	A 2.66±0.13 a	A 2.64±0.16 ab	B 2.37±0.12 b
	1/10 TSB	C <1.0 a	A 2.96±0.25 a	AB2.89±0.17 a	AB2.87±0.23 a	B 2.64±0.07 a
L. monocytogenes	Full TSB	B1.30±0.0 a	A1.69±0.03 a	A1.59±0.04 a	B1.26±0.13 a	B1.27±0.12 a
	1/2 TSB	C1.15±0.0 b	A1.66±0.03 a	B1.48±0.07 a	C1.17±0.13 a	D <1.0 c
	1/10 TSB	B1.15±0.0 b	A1.65±0.06 a	A1.55±0.06 a	B1.21±0.15 a	B1.10±0.05 b

[a] The mean is the average of eight replicates

[b] Means with different upper case letter in a row are significantly different ($P<0.05$)

[c] Within same sampling time, means with different lower case letter in a column are significantly different ($P<0.05$) for each pathogen

outbreak or avirulent (*stx* 1^- and *stx* 2^-) B6914 strain, was the predominant strain that regrew in the compost extract.

Strain Variation of E. coli O157:H7 Regrowth in Sterile Compost Extract The growth curve of individual strain of *E. coli* O157:H7 was determined in sterile compost extract with concentrated nutrients (1:2). Each strain had ca. 8 h of lag phase and reached maximum populations of ca. 7 log CFU/ml on day 2 (Table 5). However, there was a significant difference in growth rate and generation time among strains, showing the shortest generation time of 2.1 h for F06M-0923-21 (spinach outbreak strain; Table 5).

Evaluation of Pathogen Regrowth in Compost Extract of Different Ages During composting, the levels of available nutrients, diversity, and populations of indigenous microflora in compost can vary significantly. To determine the regrowth of pathogens during composting, dairy compost samples at different ages were taken from the compost heaps of our field study. Average temperatures in the compost heaps were above 50°C for 11 days of the first 14 days of composting, with a maximal average temperature of 60.5°C on day 11 (data not shown). Compost samples (0, 7, 14, 30, and 60 days after the onset of composting) were evaluated. There was no noticeable difference in pHs and total mesophillic bacterial counts among composts of different ages (Table 6). Populations of total coliforms and *E. coli* were high initially and declined rapidly during composting. Compost extracts of all ages supported the regrowth of *Salmonella* spp. and *E. coli* O157:H7 with least increase of plate counts in 7-day-old compost extract, whereas *L. monocytogenes* grew only in the compost extract of 0 and 60 days of composting (Fig. 2). The regrowth potential of all pathogens in different ages of compost extracts ranged from 0.22 to 2.32 log CFU/ml with most population increases in initial compost extracts (Table 6). *L. monocytogenes* had the least regrowth potential among the three pathogens.

Discussion

Compost is an excellent nutrient source for agricultural crops, but it is also known as a potential source of contamination with food-borne pathogens such as *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. Generally, the risk of pathogens in compost is very low; however, a few cells which may survive during composting or be introduced to the finished compost may multiply and increase the risk of contamination of crops when compost is used as an organic fertilizer. Previous regrowth studies have been conducted in biosolids with initial inoculum more than 10^3 CFU/g, focusing on *Salmonella* regrowth. There are no studies, to date, providing any information that the pathogens can regrow from a few cells in manure-based compost. In this study, we investigated the effect of various environmental factors on pathogen regrowth in compost extracts.

Compost tea is a water extract of compost which is produced by mixing compost and water for a defined period, either actively aerating or non-aerating and with or without nutrient additives [26]. Compost tea is used as a spray for biocontrol of foliar diseases or soil drench to promote plant growth and/or control root diseases [2, 14, 39]. A few studies have examined the microbiological safety of compost tea.

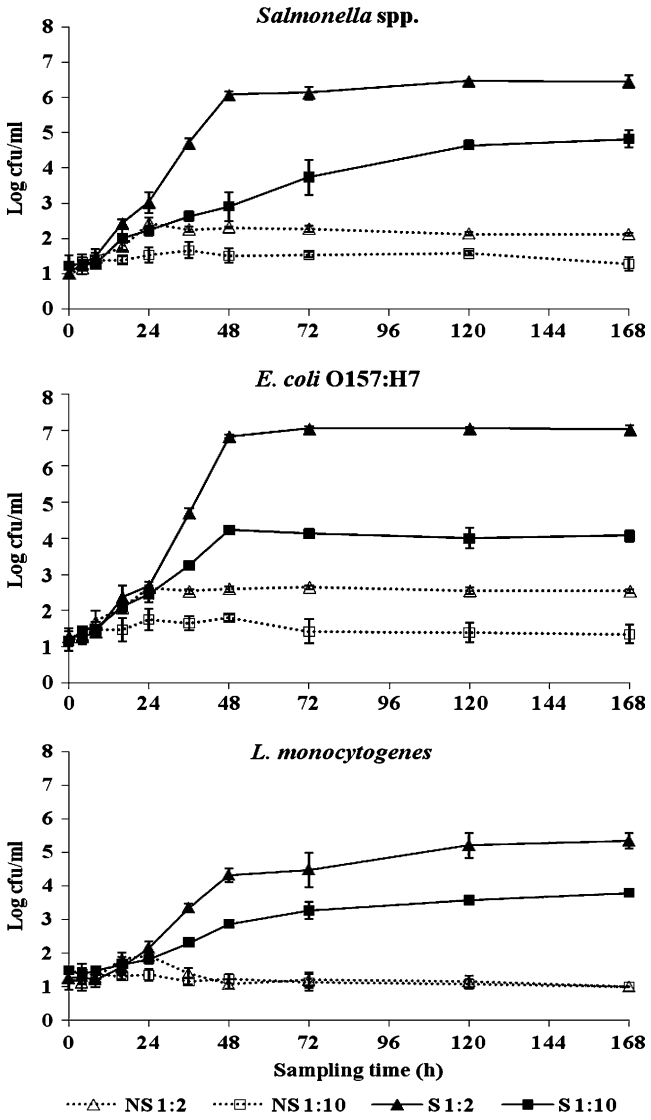

Figure 1 The potential regrowth and persistence of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* previously grown in 1/10 strength of TSB in sterile (*solid line*) and nonsterile (*dashed line*) compost extract at 22°C (n=8, error bars=SD)

Duffy et al. [11] reported that the numbers of *E. coli* O157:H7 and *Salmonella* Thompson were increased in compost tea made from various types of compost with over 0.5% molasses, showing different regrowth potentials with pathogen type and compost. More recently, Ingram and Millner [17] revealed that compost tea supplemented with commercially available nutrient solution such as blend of molasses, bat guano, sea bird guano, soluble kelp, citric acid, Epsom salts, ancient scabbed minerals, and calcium carbonate and the mixture of powdered soluble kelp, liquid humic acids, and glacial rock dust caused population increase of *E. coli* O157:H7 or *Salmonella* by 10–1,000 folds, whereas compost tea itself did not support the growth of both pathogens during aerated or non-aerated brewing cycle. These studies suggest that compost tea could serve as a potential vehicle to transmit *E. coli* O157:H7 and *Salmonella* to fresh produce. Although the compost extract was used as a model system in this study for pathogen regrowth in compost, it has some relevance to compost tea in terms of composition (compost mixed with water) and passively aerated system (sterile flask, loosely closed in a shaking incubator for 24 h) [26]. However, our study revealed various regrowth potential among bacterial species in compost extract without additional nutrients. For example, both *Salmonella* spp. and *E. coli* O157:H7 grew at both incubation temperatures in more concentrated water extracts (1:2 and 1:5 ratios), whereas *L. monocytogenes* grew only at 22°C, not 35°C (Tables 1 and 2).

Among the three pathogens tested, *L. monocytogenes* had the least regrowth potential. It might be explained by the fact that gram-positive bacteria generally have more requirements for nutrients than gram-negative bacteria [18]. The psychrotrophic nature of *L. monocytogenes* might allow regrowth of this microorganism only at 22°C. Both *Salmonella* and *E. coli* O157:H7 at 35°C were only detectable after enrichment at 5 days, while at 22°C, both pathogens were still detectable after 7 days. Available nutrients in the concentrated compost extract (1:2) could

Table 4 Growth rate constant and generation time of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* cocktail previously grown in 1/10 strength of TSB in sterile compost extract

Ratio[a]	Microorganism	Growth rate constant (h^{-1})	Generation time (h)
1:2	*Salmonella* spp.	0.26±0.01 b[b]	2.64±0.04 b
	E. coli O157:H7	0.33±0.01 a	2.12±0.06 a
	L. monocytogenes	0.19±0.01 c	3.68±0.24 c
1:10	*Salmonella* spp.	0.12±0.02 b	6.08±0.87 b
	E. coli O157:H7	0.16±0.01 a	4.23±0.14 a
	L. monocytogenes	0.09±0.00 b	7.61±0.21 b

[a] Ratio of compost to water (*w*/*v*)

[b] Mean±SD; the mean is the average of eight replicates. Means with different lower case letter in a column are significantly different (P<0.05) for each compost extract

Table 5 Growth rate constant and generation time of individual strain of *E. coli* O157:H7 previously grown in 1/10 strength of TSB in sterile compost extract (1:2)

Strain	Lag phase (h)	Growth rate constant (h^{-1})	Generation time (h)
F07M-020-1	8	0.25±0.00 bc[a]	2.73±0.01 bc
F06M-0923-21	8	0.33±0.01 a	2.12±0.06 a
H1730	8	0.25±0.01 c	2.83±0.16 c
ATCC 43895 ISEH GFP	8	0.32±0.00 a	2.19±0.01 a
Stx 1^-, *stx* 2^- B6914	8	0.28±0.02 b	2.49±0.14 b

[a] Mean±SD; the mean is the average of eight replicates. Means with different lower case letter in a column are significantly different ($P<0.05$)

allow initial rapid regrowth of both pathogenic and indigenous microorganisms, and then pathogens may be outcompeted when nutrients became limited at 35°C. Lower incubation temperature (22°C) results in slower growth of mesophilic microorganisms and may balance the growth of pathogenic microorganisms and the background microflora.

Previous studies reported similar findings that pathogen regrowth was possible in nonsterile biosolids in the presence of indigenous microorganisms [22, 30]. Russ and Yanko [30] reported that *Salmonella* did not grow extensively but remained transient. According to Hussong et al. [15], *Salmonella* died off within 7 days in non-irradiated composted biosolids at 36°C, while in irradiated and moist compost, the pathogen regrew to 8 log CFU/g. Soares [35] revealed when sufficient moisture levels (e.g., 30%) were allowed in composted biosolids, *E. coli* and total coliforms multiplied in nonsterile compost with the addition of external carbon source. In the present study, each pathogenic bacterium grew from a few cells to maximum 2 log CFU/ml within 1 day and thereafter declined. Although direct comparison of above studies with our present study was not possible due to differences in compost materials (biosolids vs. manure-based compost) and compost types (solid vs. water extract), these results clearly indicate that pathogen regrowth is a complex response as affected by multiple environmental factors. Furthermore, as shown in present study, potential regrowth of pathogens may become higher when sterilization is used as an alternative final treatment for compost, due to the lack of indigenous microorganisms in compost.

In preharvest environments, microorganisms are usually exposed to some stresses including, but not limited to, poor nutrient availability, suboptimal temperatures, and desiccation. Sublethal exposure to various stresses may enhance the survival of bacteria under the subsequent stress conditions and result in cross-protection against other stresses [1]. For example, acid adaptation at pH 4.8 for 18 h at 37°C for *E. coli* O157:H7 enhanced subsequent acid tolerance, heat tolerance, and freeze–thaw tolerance [21, 31]. In order to simulate the nutrient conditions in the compost environment, the bacterial cultures were grown in the reduced nutrient media (1/2 and 1/10 strength of TSB) and then inoculated into water extract of compost (1:2 ratio) to observe the regrowth at 22°C. Cultures of both *Salmonella* and *E. coli* O157:H7 grown previously in reduced nutrient media (1/10 strength of TSB) regrew to higher ($P<0.05$) populations than in the control (full

Table 6 The physical and biological characteristics of compost with different ages

Compost ages (day)	pH	Log CFU/ml			Regrowth potential[a] (log CFU/ml)		
		Mesophiles	Coliform	*E. coli*	*Salmonella* spp.	*E. coli* O157	*L. monocytogenes*
0	7.93±0.09	8.02±0.43	4.71±0.03	3.20±0.05	A[b] 2.31±0.03 a[c]	A 2.32±0.04 a	B 1.12±0.14 a
7	8.37±0.04	8.26±0.21	2.56±0.23	<1.0[d]	A 1.08±0.33 c	A 0.95±0.11 d	B 0.34±0.08 b
14	8.40±0.05	8.48±0.11	<1.0	<1.0	A 2.05±0.18 a	B 1.69±0.06 b	C 0.22±0.33 b
30	8.00±0.04	8.56±0.14	<1.0	<1.0	A 1.60±0.31 b	A 1.34±0.16 c	B 0.24±0.36 b
60	8.42±0.02	8.11±0.38	<1.0	<1.0	A 1.36±0.19 bc	A 1.55±0.20 b	B 0.57±0.12 b

[a] Maximum population increase (log CFU/ml) in compost extract. Pathogenic microorganisms previously grown in 1/10 strength of TSB were inoculated into nonsterile water extract of compost (1:2, *w/v*) and incubated at 22°C for 7 days

[b] Mean±SD; the mean is the average of eight replicates. Means with different upper case letters in a row within regrowth potential are significantly different ($P<0.05$)

[c] Means with different lower case letters in a column are significantly different ($P<0.05$) in regrowth potential for each pathogen

[d] <1.0 log CFU/ml, positive after enrichment

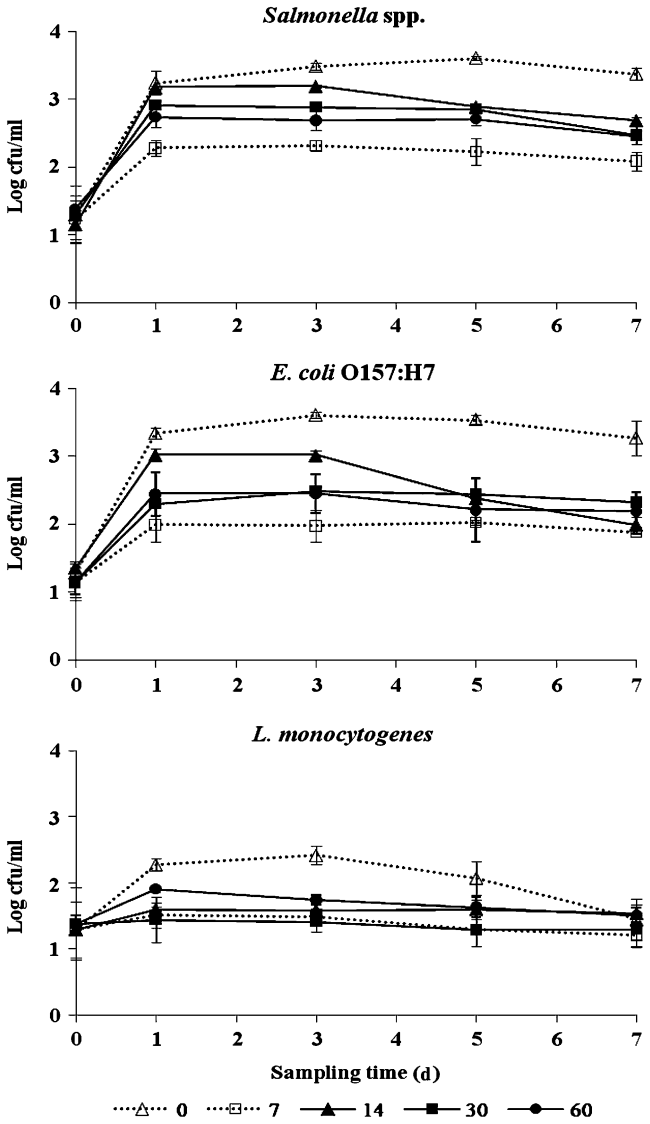

Figure 2 The potential regrowth and persistence of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* previously grown in 1/10 strength of TSB in nonsterile water extract of compost with different ages at 22°C (n=12, error bars=SD)

strength of TSB), whereas there was no difference (P> 0.05) in population of *L. monocytogenes* among growth medium. Bacterial adaptation to limited nutrients requires changes in gene expression that allows the microorganisms to utilize nutrients more efficiently and extends survival [9]. The sigma factor RpoS, which is widely present in many gram-negative bacteria including *E. coli* O157:H7 and *Salmonella*, is important for adaptation under nutrient-limited conditions [16, 38]. Previous exposure of *Salmonella* and *E. coli* O157:H7 to reduced nutrient media might result in a substantial increase in RpoS expression which then contributes to the regrowth of these pathogens in water extract with limited nutrient availability.

Available nutrients and microbial communities are expected to change with the composting process. Sidhu et al. [33] observed the decline in *Salmonella* growth in composted biosolids with maturity along with the decline in available organic carbon and nitrogen with maturity. Millner et al. [25] reported that the selected fractions of compost microorganisms had different suppression of *Salmonella* growth in compost at different temperatures. Our results demonstrate different regrowth potentials for each pathogen in composts at different ages. These observations indicate that the differences of nutrient availability and microbial communities among composts of different ages might have a combined effect on suppressing the pathogens in compost. Further studies of competitive inhibition of indigenous background microflora against pathogen regrowth in compost need to be explored.

In conclusion, dairy compost extract, even for finished ones, contains sufficient nutrients for pathogen regrowth. However, pathogen regrowth in compost was suppressed by indigenous microflora to a high extent. Lower temperature (22°C) may balance the growth of pathogenic microorganisms and the background microflora. Pathogens adapted to low nutrients can regrow well in compost extract. Regrowth potentials of all pathogens were ca. 0.7–1.4 and ca. 4–6 log CFU/ml for nonsterile and sterile compost extracts (1:2 ratio), respectively. Both *Salmonella* and *E. coli* O157:H7 regrew easier than *L. monocytogenes* under the same experimental conditions. These results demonstrate that nutrient availability, species and physiological stage of pathogens, competitive microbial flora in compost, and incubation temperature were important factors affecting the regrowth of food-borne pathogens in the compost ecosystem. Meeting the time and temperature criteria for thermophilic composting does not guarantee the complete destruction of all pathogens. Due to the limitation of sensitivity for pathogen detection, false negative results for compost analysis may occur. Our results clearly indicate the pathogen regrowth could occur from a few survived cells to hazardous levels in compost under appropriate conditions. Even with both the time–temperature exposure data and before/after composting test results, appropriate storage conditions for finished compost are necessary for compost producers to document that their process meets the standards for pathogen limits established for the use of composted fecal materials as a soil amendment. Furthermore, the use of compost tea needs to be cautious since current study shows pathogen could regrow in nonsterile compost extract without additional nutrients. Further studies on determining the combination of these environmental factors and specific competitive inhibitory microorganisms in compost that would suppress or inhibit the growth of pathogens are currently undertaken in our lab.

Acknowledgment This project was funded through a grant from the Fresh Express Produce Safety Initiative.

References

1. Abee T, Wouters JA (1999) Microbial stress response in minimal processing. Int J Food Microbiol 50:65–91
2. Akanbi WB, Adebayo TA, Togun OA, Adeyeye AS, Olaniran OA (2007) The use of compost extract as foliar spray nutrient source and botanical insecticide in *Telfairia oxxidentalis*. World J Agric Sci 3:642–652
3. Benito A, Ventoura G, Casadei M, Robinson T, Mackey B (1999) Variations in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Appl Environ Microbiol 65:1564–1569
4. Brandon JR, Burge WD, Enkiri NK (1977) Inactivation by ionizing radiation of *Salmonella enteritidis* serotype Montevideo grown in composted sewage sludge. Appl Environ Microbiol 33:1011–1012
5. Buck JW, Walcott RR, Beuchat LR (2003) Recent trends in microbiological safety of fruits and vegetables (On-line). Plant Health Progress. doi:10.1094/PHP-2003-0121-01-RV
6. Centers for Disease Control, Prevention (2006) Ongoing multi-state outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach—United States, Sep. 2006. Morb Mortal Wkly Rep 38:1045–1046
7. CDC/PulseNet One-Day (24–28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE) Available at: http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf. Accessed on January 5, 2008
8. Cekmecelioglu D, Demirci A, Graves RE, Davitt NH (2005) Optimization of windrow food waste composting to inactivate pathogenic microorganisms. Trans ASAE 48:2023–2032
9. Dong T, Joyce C, Schellhorn HE (2007) The role of RpoS in bacterial adaptation. In: El-Sharoud W (ed) Bacterial physiology —a molecular approach. Springer, Berlin, pp 313–337
10. Doyle MP, Erickson MC (2008) Summer meeting 2007—the problems with fresh produce: an overview. J Appl Microbiol 105:317–330
11. Duffy B, Sarreal C, Ravva S, Stanker L (2004) Effect of molasses on regrowth of *Escherichia coli* O157:H7 and *Salmonella* in compost teas. Compost Sci Util 12:93–96
12. Epstein E, Wilson GB (1975) Composting raw sludge. In: Municipal Sludge Management and Disposal. Information Transfer, Inc., Rockville
13. Graves LM, Swaminathan B (2001) PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int J Food Microbiol 65:55–62
14. Giskin M, Nerson H (1984) Foliar nutrition of muskmelon: I: Application to seedlings greenhouse experiments. J Plant Nutr 7:1329–1339
15. Hussong D, Burger WD, Enkiri NK (1985) Occurrence, growth, and suppression of *Salmonellae* in composted sewage sludge. Appl Environ Microbiol 50:887–893
16. Ibanez-Ruiz M, Robbe-Saule V, Hermant D, Labrude S, Norel F (2000) Identification of RpoS (σ^S)-regulated genes in *Salmonella enterica* serovar Typhimurium. J Bacteriol 182:5749–5756
17. Ingram DT, Millner PD (2007) Factors affecting compost tea as a potential source of *Escherichia coli* and Salmonella on fresh produce. J Food Prot 70:828–834
18. Jay JM (2000) Intrinsic and extrinsic parameters of foods that affect microbial growth. In: Modern food microbiology. 6th ed. Aspen Publisher, Inc., Gaithersburg, pp 35–41
19. Jiang X, Morgan J, Doyle MP (2003) Fate of *Escherichia coli* O157:H7 during composting of bovine manure in a laboratory-scale bioreactor. J Food Prot 66:25–30
20. Jiang X, Morgan J, Doyle MP (2003) Thermal inactivation of *Escherichia coli* O157:H7 in cow manure compost. J Food Prot 66:1771–1777
21. Leenanon B, Drake MA (2001) Acid stress, starvation, and cold stress affect post stress behavior of *Escherichia coli* O157:H7 and nonpathogenic *E. coli*. J Food Prot 64:970–974
22. Lemunier M, Francou C, Rousseaux S, Houot S, Dantigny P, Piveteau P, Guzzo J (2005) Long-term survival of pathogenic and sanitation indicator bacteria in experimental biowaste composts. Appl Environ Microbiol 71:5779–5786
23. Litterrick AM, Harrier L, Wallace P, Watson CA, Wood M (2004) The role of uncomposted materials, composts, manures, and compost extracts in reducing pest and disease incidence and severity in sustainable temperate agricultural and horticultural crop production—a review. Crit Rev Plant Sci 23:453–479
24. Lung AJ, Lin CM, Kim JM, Marchall MR, Nordstedt R, Thompson NP, Wei CI (2001) Destruction of *Escherichia coli* O157:H7 and *Salmonella enteritidis* in cow manure composting. J Food Prot 64:1309–1314
25. Millner PD, Powers KE, Enkiri NK, Burge WD (1987) Microbially mediated growth suppression and death of *Salmonella* in composted sewage sludge. Microb Ecol 14:255–265
26. National Organic Standards Board (2004) Compost tea task force report. Available at: http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5058470. Accessed on February 15, 2009
27. Rahn K, Renwick SA, Johnson RP, Wilson JB, Clarke RC, Alves D, Mcewen S, Lior H, Spika J (1997) Persistence of *Escherichia coli* O157:H7 in dairy cattle and the dairy farm environment. Epidemiol Infec 119:251–259
28. Rice LB, Bonomo RA (2005) Genetic and biochemical mechanisms of bacterial resistance to antimicrobial agents. In: Lorian V (ed) Antibiotics in laboratory medicine, 5th edn. Lippincott Williams & Wilkins, Baltimore, pp 441–508
29. Rodriguez A, Pangloli P, Richards HA, Mount JR, Draughon FA (2006) Prevalence of *Salmonella* in diverse environmental farm samples. J Food Prot 69:2576–2580
30. Russ CF, Yanko WA (1981) Factors affecting *Salmonellae* repopulation in composted sludges. Appl Environ Microbiol 41:597–602
31. Ryu JH, Beuchat LR (1999) Changes in heat tolerance of *Escherichia coli* O157:H7 after exposure to acidic environments. Food Microbiol 16:317–324
32. Shepherd MW, Liang P, Jiang X, Doyle MP (2007) Fate of *Escherichia coli* O157:H7 during on-farm dairy manure-based composting. J Food Prot 70:2708–2716
33. Sidhu J, Gibbs RA, Ho GE, Unkovich I (2001) The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. Water Res 35:913–920
34. Sivapalasingham S, Friedman CR, Cohen L, Tauxe RV (2004) Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J Food Prot 67:2342–2353
35. Soares HM (1996) Pathogen indicator regrowth potential as a method to evaluate compost stability. Ph. D. Dissertation, University of Massachusetts at Amherst, Department of Civil and Environmental Engineering
36. Ueno H, Yokota K, Arai T, Muramatsu Y, Taniyama H, Iida T, Morita C (1996) The prevalence of *Listeria monocytogenes* in the environmental of dairy farms. Microbiol Immunol 40:121–124

37. Wang X, Jothikumar N, Griffiths MW (2004) Enrichment and DNA extraction protocols for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR. J Food Prot 67:189–192
38. Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma factor selectivity. J Bacteriol 187:1591–1603
39. Weltzien HC (1990) The use of composted materials for leaf disease suppression in field crops. Monogr Br Crop Prot Counc 45:115–120
40. Yeager JG, Ward RL (1981) Effects of moisture content on long term survival and regrowth of bacteria in wastewater. Appl Environ Microbiol 41:1117–1122