RESEARCH LETTER

Influence of indigenous eukaryotic microbial communities on the reduction of *Escherichia coli* O157:H7 in compost slurry

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Abstract

Compost made from livestock manure is commonly used as a crop fertilizer and serves as a possible vehicle for the transmission of *Escherichia coli* O157:H7 to fresh produce. In this study, we hypothesized that the indigenous microbial communities present in composts adversely affects the survival of *E. coli* O157:H7. *Escherichia coli* O157:H7 was spiked into compost slurry and incubated at 25 °C. *Escherichia coli* O157:H7 exhibited a *c.* 4 $\log_{10}$ reduction over 16 days. When compost was supplemented with the eukaryotic inhibitor cycloheximide, there was a minimal decrease in *E. coli* O157:H7 counts over the same time period. Analysis of microbial communities present in the compost with denaturing gradient gel electrophoresis (DGGE) suggested minor differences in the fungal communities present in cycloheximide-treated compost, compared with untreated compost over a period of 12 days at 25 °C. However, the DGGE profiles of protists showed drastic differences in community complexity. Clone library sequence analysis of protist populations revealed significantly different species composition between treatment and control samples at different time points. This suggests that predation of *E. coli* O157:H7 by protists might be a potential mechanism for reducing *E. coli* O157:H7 in compost materials.

Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is a deadly foodborne pathogen, with fewer than 50 bacterial cells sufficient to cause diseases such as hemorrhagic colitis and hemolytic uremic syndrome (Kaper *et al.*, 2004). Every year, approximately 73 000 illnesses occur due to *E. coli* O157:H7 infections in the United States alone (Rangel *et al.*, 2005). While most cases were attributed to improperly cooked ground beef, an increasing number of cases are associated with the consumption of fresh produce (Sivapalasingam *et al.*, 2004). Cattle are known to be the primary reservoirs of *E. coli* O157:H7 (Borczyk *et al.*, 1987). These asymptomatic carriers excrete this pathogen in their feces, and thus cattle manure can serve as a vehicle for pathogen transmission to food products.

Previous research has demonstrated that *E. coli* O157:H7 survives for long periods of time in a variety of natural environments (Kudva *et al.*, 1998; Jiang *et al.*, 2002; Islam *et al.*, 2004a, b; Scott *et al.*, 2006) including cow manure. Cow manure and composted manure is commonly applied on farm lands as a fertilizer. Improper composting of farm waste can lead to the survival of pathogenic bacteria such as *E. coli* O157:H7. In order to ensure the safety of compost derived from animal manure, it is imperative to develop science-based composting procedures that minimize the survival of pathogens such as *E. coli* O157:H7.

The present study was designed to identify the class(es) of microorganisms that are antagonistic to *E. coli* O157:H7 in a cow manure compost slurry model. We determined that one or more members of the protist community negatively affected pathogen survival in our model system. Therefore, we suggest that practices that encourage protist growth and survival within the environment would reduce the transmission of pathogens such as *E. coli* O157:H7 within agricultural settings.

Materials and methods

Bacterial strain, culture conditions and preparation of inocula

An *E. coli* O157:H7 EDL933 (Perna *et al.*, 2001) derivative that is resistant to streptomycin was selected by growing the

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strain overnight at 37 °C in Luria–Bertani (LB) broth (Difco Laboratories, Detroit, MI), followed by plating approximately 10^9 CFU onto LB plates supplemented to 100 μg mL^{-1} streptomycin. The inoculum for survival studies was prepared by growing cells from a single colony on Sorbitol MacConkey agar (SMAC) plates (Becton, Dickinson and Company, Sparks, MD) in 10 mL of LB broth containing 100 μg mL^{-1} streptomycin overnight at 37 °C with agitation (300 r.p.m.). A 1-mL culture was then centrifuged (16 000 ***g***, 5 min), washed twice in phosphate-buffered saline (PBS), pH 7.4, and resuspended in PBS. Cells were adjusted with PBS to an $OD_{600\,nm}$ of 0.5 (*c.* 10^9 CFU mL^{-1}).

Compost model

Commercially available completed compost (GardenPlus Compost, Archbold, OH) was used as a compost model throughout the study. The package indicated that the amount of available nitrogen, phosphate and potash in this product was 0.5%, 0.5% and 0.5%, respectively, similar to compost used in other studies (Islam *et al.*, 2004a, b). Completed commercial compost was used to reduce lot-to-lot variation, and all experiments were performed using compost from a single bag. Equal amounts of compost and autoclaved water (w/v) were combined and centrifuged at 50 ***g*** for 40 s. This resulted in a thick supernatant of compost slurry that could be transferred easily to a tube using a pipette. This preparation method also increased the repeatability of bacteria quantification by plate counts. Before inoculation, compost samples were tested for the presence of *E. coli* O157:H7 by plating 100 μL of a sample onto SMAC supplemented with streptomycin. *Escherichia coli* O157:H7 was then inoculated into a 10-mL compost slurry sample to a final cell density of *c.* 10^7 CFU mL^{-1}.

Survival studies

To test the effect of autoclaving on the reduction of *E. coli* O157:H7 in the model compost, compost slurry samples were autoclaved for 20 min, allowed to cool and then inoculated with *E. coli* O157:H7. An unautoclaved compost sample was also inoculated with *E. coli* O157:H7 and used as a control. Serial dilutions of samples were plated onto SMAC plates supplemented with streptomycin and incubated overnight at 37 °C. All survival studies were performed at least twice. Statistical analysis was performed using MINITAB (release 15.00, Minitab Inc., State College, PA). Linear regression was performed on natural log transformations of the number of CFU vs. time. ANOVA was used to compare the slopes of the regression lines generated from the survival of the pathogen. A P value of 0.05 or less was considered to be significantly different.

To determine the effect of various microbial inhibitors on the reduction of *E. coli* O157:H7 survival in compost, samples were supplemented with either crystal violet (a gram-positive bacterial inhibitor) at 100 μg mL^{-1}, streptomycin (a gram-negative bacterial inhibitor) at 100 μg mL^{-1}, amphotericin B (fungal inhibitor) at 20 μg mL^{-1} or cycloheximide (eukaryotic inhibitor) at 100 μg mL^{-1} before inoculation. The optimal concentration for the inhibitors was determined earlier by performing experiments involving a range of concentrations (data not shown).

DNA extraction, PCR amplification and denaturing gradient gel electrophoresis (DGGE) analysis

DNA was extracted from compost using the PowerSoil DNA kit (Mo Bio Laboratories Inc.). Fungal 16S–23S rRNA intergenic spacer (ITS) regions were amplified using the primer set ITS1-F (Gardes & Bruns, 1993) and ITS2 (White *et al.*, 1990). A GC-clamp (Muyzer *et al.*, 1993) was added to the 5′ end of ITS1-F to improve the melting behavior of the PCR fragments. The PCR protocols for both the fungal and the protist PCR reactions were adapted from Von Sigler's online research protocol (http://www.eeescience.utoledo.edu/Faculty/Sigler/RESEARCH/Protocols/PCR/PCR.pdf), and each 25 μL PCR reaction consisted of 1 × *Taq* polymerase buffer, 3 mM magnesium chloride, 2 mg mL^{-1} bovine serum albumin, 0.2 mM each dNTP, 0.5 μM each primer, 0.04 U μL^{-1} of *Taq* Polymerase (New England Biolabs, Ipswich, MA) and 1 μL of extracted compost DNA. PCR cycling parameters were 94 °C for 6 min, followed by 35–40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 7 min. All amplification products were electrophoresed in 1.25% w/v agarose gels stained with ethidium bromide and visualized under UV light.

DGGE was performed using the DCode™ Universal Mutation Detection system (16 cm system; Bio-Rad Laboratories, Hercules, CA). DGGE parallel gradients ranged from 20% to 70% (8% acrylamide) and were run at 100 V for 16 h at 60 °C. DNA bands were stained with ethidium bromide and visualized under UV light. The protist-specific amplicons from cycloheximide-treated samples resulted in the formation of nonspecific products; however, no effort was made to extract the *c.* 300-bp product before DGGE analysis. We chose not to do this because in these cases the specific band was not present at a high enough quantity to be recovered and observed by DGGE.

Cloning and sequence analyses

DNA was extracted from compost samples and PCR was used to amplify protist-specific DNA from four samples (Days 0, 6, 8 and 12) using the same methods mentioned above. Whenever multiple bands were observed, the expected sized band was extracted, gel purified and used for

clone library construction. Two libraries were independently created from DNA extracted at each of the four time points using the TOPO TA cloning kit in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmids containing inserts were sent for sequencing to the Nucleic Acid Facility at the Pennsylvania State University (University Park) on an ABI Hitachi 3730XL DNA Analyzer.

DNA sequences were trimmed using MEGA 4.0 (Tamura *et al.*, 2007) to remove the vector and primer sequences. For each time point, there were a pair of libraries that consisted of the cycloheximide-untreated (Day-U; control) and the cycloheximide-treated samples (Day-T; treated). Comparative sequence analysis was conducted by BLAST (Altschul *et al.*, 1997) against the GenBank database (Benson *et al.*, 2010) to obtain the taxonomic identity of all the clones. The sequences were converted to FASTA format, imported into the software platform MOTHUR (Schloss *et al.*, 2009) and aligned against the eukaryotic SILVA database (Pruesse *et al.*, 2007). Distance matrices were generated using PHYLIP (http://evolution.genetics.washington.edu/phylip.html) and pairwise comparisons of all the sequences were carried out between the control and the treatment within each time point to establish operational taxonomic units (OTUs) for each library (OTUs established at ≥ 97% similarity) at 95% confidence using MOTHUR. The coverage of each library was calculated by dividing the number of OTUs by the nonparametric richness estimator Chao1 (Chao, 1984). LIBSHUFF (Singleton *et al.*, 2001) was used to statistically compare the two libraries (control and treatment) for each time point.

Results

Culture-dependent analysis. Reduction of *E. coli* O157:H7 in sterilized and nonsterilized compost slurry

Previous studies have demonstrated that foodborne pathogens exhibit long-term survival in compost and soil and undergo a gradual die-off (Kudva *et al.*, 1998; Jiang *et al.*, 2002; Islam *et al.*, 2004a, b). The decline in cell numbers has been attributed to temperature, moisture, pH, nutrient competition, antimicrobials as well as indigenous microbial communities, but we are unaware of any study that has correlated specific members of compost microbiota with a reduction of *E. coli* O157:H7. Our primary objective was to initiate studies that would ultimately relate pathogen survival with the composition of the compost microbial communities. In the initial experiments, the reduction of *E. coli* O157:H7 was studied in autoclaved and unautoclaved compost incubated at 25 °C. This temperature was chosen as the cycloheximide used in this study was found to be stable under these conditions, while the effectiveness of this antimicrobial decreased at higher temperatures (data not shown). The abundance of *E. coli* O157:H7 in autoclaved compost remained essentially constant throughout the test period (Fig. 1). In marked contrast, within 16 days of incubation at 25 °C in unautoclaved compost, *E. coli* O157:H7 underwent a *c.* 4 $\log_{10}$ reduction. The means of linear regression slopes between the autoclaved and the unautoclaved samples were significantly different ($P = 0.005$). This strongly suggested that background microbial communities significantly reduced *E. coli* O157:H7 in compost at 25 °C.

Identification of background microbial communities

Compost naturally contains high levels of bacteria, fungi and protists (Beffa *et al.*, 1996). The experiment comparing the survival of *E. coli* O157:H7 in sterile and nonsterile compost (Fig. 1) suggested that the autochthonous microbial communities have an antagonistic effect on *E. coli* O157:H7. In order to determine which class of microorganisms was reducing *E. coli* O157:H7, compost samples were individually treated with antimicrobials that targeted gram-positive bacteria (crystal violet), gram-negative bacteria (streptomycin) and eukaryotic species (amphotericin B and cycloheximide) (Fig. 2). The survival of *E. coli* O157:H7 improved significantly in the presence of the eukaryotic inhibitor cycloheximide and the CFU mL^{-1} remained relatively constant throughout the incubation period. No significant differences were observed in the reduction of *E. coli* O157:H7 compared with the control in the presence of crystal violet, amphotericin B or streptomycin. Statistical comparisons between the slope means between each

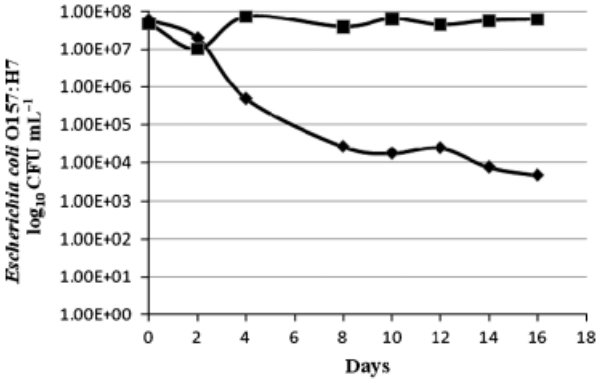

Fig. 1. Reduction of *Escherichia coli* O157:H7 in autoclaved (■) and unautoclaved (◆) compost at 25 °C. Reduction of *E. coli* O157:H7 was compared in compost that was sterilized by autoclaving with a sample that was not sterilized. Samples were incubated at 25 °C and the pathogen was enumerated by plating on SMAC supplemented with streptomycin (100 μg mL^{-1}) for 16 days. Data shown are representative of two biological replicates.

treatment and the control yielded $P = 0.578$ (crystal violet), $P = 0.258$ (streptomycin), $P = 0.993$ (amphotericin B) and $P = 0.002$ (cycloheximide). These data suggest that cycloheximide-sensitive eukaryotic species were primarily responsible for the observed decline of *E. coli* O157:H7.

Culture-independent analysis

DGGE

DGGE analysis was used to monitor the shifts in populations in compost samples (Fig. 3). At 25 °C, the banding patterns of fungal species remained similar between cycloheximide-treated and -untreated samples, except for some bands that repeatedly appeared on day 8 and day 10 of the cycloheximide-treated samples. In contrast, a dramatic shift in protist populations was observed at 25 °C between untreated and treated samples (Fig. 3). Notably, none of the prominent bands seen in control compost samples from days 4 to 12 were observed in the lanes for the cycloheximide-treated samples.

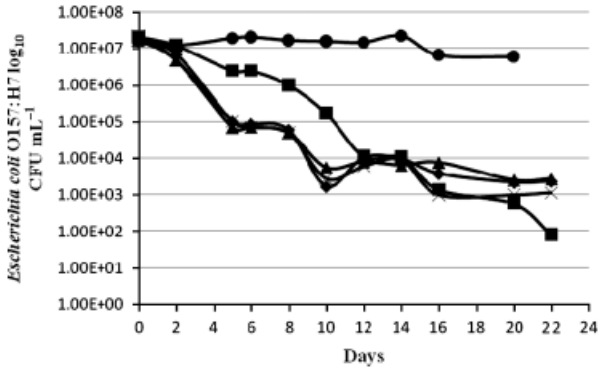

Fig. 2. Effect of inhibitors on the reduction of O157:H7. Treatments include: no inhibitor (◆), crystal violet (■), streptomycin (▲), amphotericin B (×) and cycloheximide (●). The survival of *Escherichia coli* O157:H7 in the presence of these inhibitors was monitored for 22 days. Compost samples were incubated at 25 °C and the average concentrations of *E. coli* O157:H7 were determined by culture methods using SMAC supplemented with 100 μg mL^{-1} streptomycin. Data shown are representative of two biological replicates.

Protist diversity estimation based on cloning and sequencing

Protist clone libraries were created from cycloheximide-treated and -untreated compost samples that were incubated at 25 °C. This set of samples was chosen for further analysis because DGGE results suggested that protists play a more significant role in *E. coli* O157:H7 decline in our compost model than the fungal species. The numbers of OTUs (observed richness) present in the control library at day 0 were 19 compared with 17 OTUs in the cycloheximide-treated library (Table 1). The Chao1 estimator (predicted richness) estimated that the two day 0 libraries had 28 and 35 OTUs, respectively. Therefore, we estimate that 68% and 49% of the species present in the control and the cycloheximide-treated samples at day 0 were covered by the clone libraries. LIBSHUFF was used to determine the differences between the clone libraries at each time point. Pairwise comparisons between the two day 0 libraries generated P values of 0.58 and 0.67, suggesting that the two libraries are statistically similar (Fig. 4). Similar analyses were performed on days 6, 8 and 12 samples and all control libraries were statistically different from the corresponding cycloheximide-treated sample libraries (Fig. 4). From the BLAST analysis, days 0, 8 and 12 control libraries were largely composed of the ciliophora *Arcuospathidium cultriforme* (Day 0) and *Onychodromopsis flexilis* (Days 8 and 12). At

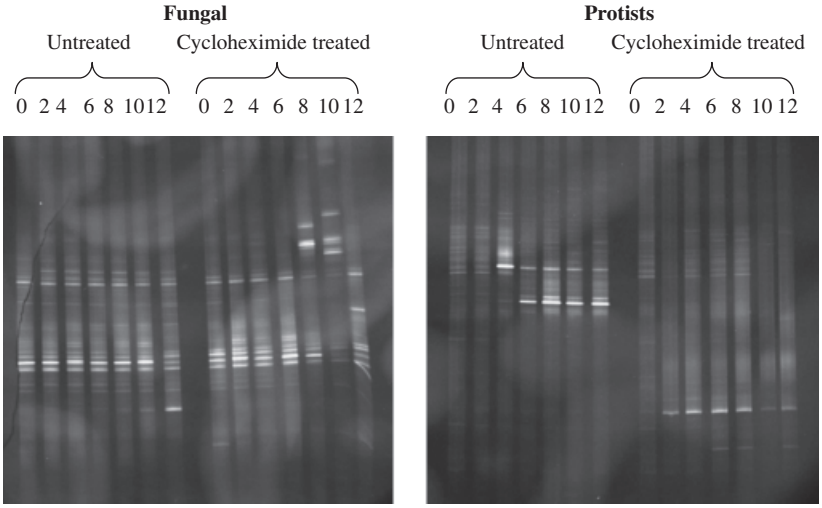

Fig. 3. DGGE patterns of fungal and protist species at 25 °C. DGGE patterns of ITS regions of fungal species and 18S rRNA of protist species in the model compost system were examined over a time course at 25 °C. DNA extracted from samples on alternate days from day 0 to day 12 was subjected to PCR with fungal or protist rDNA-specific primers. Amplicons were separated on a 20–70% denaturing gradient in a DGGE gel overnight at 100 V. Lane numbers indicate the sampling days.

Table 1. Species richness and clone library coverage

Sampling days	OTU		Chao1		% Coverage	
	Control	Treatment	Control	Treatment	Control	Treatment
Day 0	19	17	28	35	68	49
Day 6	13	15	31	30	42	50
Day 8	4	19	7	45	57	42
Day 12	7	18	7	36	100	50

The OTUs and predicted values (Chao1) were calculated using MOTHUR. Coverage of the library was determined by dividing the OTU value by Chao1 estimated value.

day 6, these libraries consisted largely of the chaonoflagellate *Volkanus costatus*. The libraries from cycloheximide-treated samples were more diverse, and consisted of a variety of species that included *A. cultriforme*, *Acanthamoeba*, *Sterkiella histriomuscorum*, *Spathidium stammeri*, *O. flexilis*, *V. costatus*, *S. stammeri* and the fungal species *Galactomyces geotrichum*.

Discussion

In our experimental model system, the reduction of *E. coli* O157:H7 in nonsterile cow manure compost was significantly faster than that observed in a sterile sample (Fig. 1), strongly suggesting that the naturally present microbial communities played a major role in the decline of *E. coli* O157:H7 cell numbers. Our most significant finding in this study was that the addition of cycloheximide, which is a protein synthesis inhibitor in eukaryotes, significantly improved the survival of *E. coli* O157:H7 in compost at 25 °C. Previous research has also observed an improvement in the survival of microorganisms such as *Xanthomonas campestris* and *E. coli* K-12 in soil amended with cycloheximide (Habte & Alexander, 1975; Johannes Sørensen *et al.*, 1999); however, we are unaware of any previous studies suggesting that cycloheximide-sensitive microorganisms were capable of inhibiting *E. coli* O157:H7 in compost.

While cycloheximide is a general inhibitor of eukaryotic populations, we feel that two pieces of data suggest that the protist populations, and not the fungal populations, have the most dramatic effect on *E. coli* O157:H7 reduction in our model system. First, the DGGE patterns do not show very remarkable differences in the complexity of the fungal populations at 25 °C (Fig. 3) between cycloheximide-treated and -untreated samples. Second, survival of *E. coli* O157:H7 improves in compost models that have a lower moisture content than the one used here (data not shown), and lower moisture is expected to promote the growth of fungal species over protists (Kouyeas, 1964; Bardgett & Griffiths, 1997). The survival in low moisture was not improved by the addition of cycloheximide, suggesting that in dry

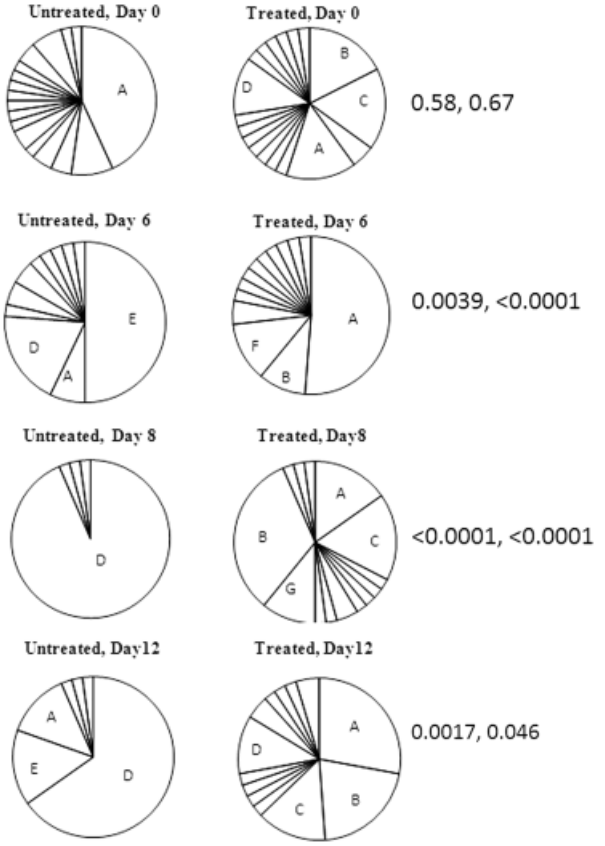

Fig. 4. Protist community diversity. Clone library generated sequences for the cycloheximide-treated and control samples for each time point (Days 0, 6, 8, 12; top to bottom) were classified using BLAST to pick the most similar species that has been cultured. The slices in the pie charts represent the relative abundance of different species. The most dominant species in each library is labeled as A, *Arcuospathidium cultriforme*; B, *Acanthamoeba*; C, *Sterkiella histriomuscorum*; D, *Onychodromopsis flexilis*; E, *Volkanus costatus*; F, *Spathidium stammeri*; G, *Galactomyces geotrichum*. To the right of each pair of pie charts are the LIBSHUFF-generated *P* values indicating significance between the two libraries comparing the cycloheximide-treated samples with untreated samples at day 0, and the converse comparison, respectively.

environments, the protists play a less significant role in pathogen reduction (data not shown). As our system likely has a much higher moisture content than that routinely present during commercial or on-farm composting, future work is needed to identify what moisture levels promote the protist-mediated decline of *E. coli* O157:H7 counts.

Clone library sequence analysis revealed significant diversity within the cycloheximide-treated samples that initially seemed to contradict DGGE data (Fig. 3). We speculate that in the absence of cycloheximide, a limited number of *E. coli* O157:H7 antagonistic protist species dominate and that this correlates with the lower diversity observed. Cycloheximide treatment may have eliminated the dominant inhibitory species, but not the low-abundance species that cannot be

visualized by DGGE. The coverage values (Table 1) suggest that all the species were not identified by this method and, therefore, other species inhibitory to *E. coli* O157:H7 might have been missed. Increasing the size of the clone libraries would help provide more conclusive data on the identity of the protist species involved. The clone library analysis showed that the Day 0 cycloheximide-treated and -untreated libraries were statistically similar as expected, validating our approach. At other time points, the treatment and control libraries were statistically different, indicating that cycloheximide did affect the protist ecology, which correlated with the improvement in the survival of *E. coli* O157:H7.

In conclusion, our data point toward the role played by the protists in the reduction of *E. coli* O157:H7. We identified a number of protists that were present in our model compost, and it remains to be determined whether any of these species were responsible for the decline in observed *E. coli* O157:H7 counts. The isolation and identification of the protist(s) that mediate this effect was beyond the scope of this study; however, this is an active area of investigation in our laboratory. Whether similar protist species are present in other composts, such as cow, pig, and horse manure, or in raw manure, is poorly understood and will be investigated in the future as well. Further work is also needed to determine how different temperatures and moisture levels would affect protist-mediated killing of *E. coli* O157:H7. Composting conditions designed to support the proliferation of protists, as well as bacteria and fungi, that are antagonistic to *E. coli* O157:H7 may provide improved methods for bioremediation.

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