

# Bacteriophage Isolated from Feedlot Cattle Can Reduce *Escherichia coli* O157:H7 Populations in Ruminant Gastrointestinal Tracts

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## Abstract

*Escherichia coli* O157:H7 can live undetected in the gut of food animals and be spread to humans directly and indirectly. Bacteriophages are viruses that prey on bacteria, offering a natural, nonantibiotic method to reduce pathogens from the food supply. Here we show that a cocktail of phages isolated from commercial cattle feces reduced *E. coli* O157:H7 populations in the gut of experimentally inoculated sheep. A cocktail of phages was used in order to prevent the development of resistance to the phages. In our first *in vivo* study we found that our cocktail of phages reduced *E. coli* O157:H7 populations in the feces of sheep ( $p < 0.05$ ) by 24 hours after phage treatment. Upon necropsy, populations of inoculated *E. coli* O157:H7 were reduced by phage treatment in both the cecum ( $p < 0.05$ ) and rectum ( $p < 0.1$ ). In our second *in vivo* study, several ratios of phage plaque-forming units (PFU) to *E. coli* O157:H7 colony-forming units (CFU) were used (0:1, 1:1, 10:1, and 100:1 PFU/CFU) to determine the most efficacious phage dose. A 1:1 ratio of phage to bacteria was found to be more effective ( $p < 0.05$ ) than either of the higher ratios used (10:1 or 100:1). Ruminal levels of *E. coli* O157:H7 were not significantly reduced ( $p > 0.10$ ) in any of the studies due to relatively low inoculated *E. coli* O157:H7 ruminal populations. Our results demonstrate that phage can be used as a preharvest intervention as part of an integrated pathogen reduction scheme.

## Introduction

**A**LTHOUGH THE FOOD SUPPLY in the United States is very safe and grows safer yearly, more than 76 million Americans are made ill each year with foodborne pathogenic bacteria (Mead *et al.*, 1999). One of the most significant pathogens is enterohemorrhagic *Escherichia coli* (EHEC; such as *E. coli* O157:H7), which causes severe enterohemorrhagic enteritis, renal da-

mage and failure, and death, especially among children and the elderly (Rangel *et al.*, 2005; Gyles, 2007). Enterohemorrhagic *E. coli* cause approximately 75 deaths per year in the United States and create a direct economic impact estimated at over US\$1 billion per year (ERS/USDA, 2001; Rangel *et al.*, 2005).

This critical pathogen, cattle, and ground beef are all linked, resulting in the unfortunate popular designation of human *E. coli* O157:H7 cases

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as “hamburger disease” or “barbecue syndrome” (Cassin *et al.*, 1998), and EHEC strains have cost the beef industry more than US\$2.7 billion over the past 10 years (Kay, 2003). New procedures instituted in processing plants have reduced the prevalence of *E. coli* O157:H7 on carcasses and in meat, resulting in a reduction in human illnesses caused by beef (Arthur *et al.*, 2002; Koochmaraie *et al.*, 2005); however, recent outbreaks have been linked to leafy green vegetables, such as spinach and lettuce, and crops fertilized with ruminant manure (Cody *et al.*, 1999; Manshadi *et al.*, 2001; Vugia *et al.*, 2007).

If the incidence of *E. coli* O157:H7 in live cattle can be reduced, human health and safety will be improved because there will be reduced transmission into the human food chain and therefore fewer human exposures (Hynes and Wachsmuth, 2000). Therefore, the development and implementation of novel preharvest interventions to reduce *E. coli* O157:H7 populations in cattle can improve human health and food safety via several mechanisms (Callaway *et al.*, 2004; Sargeant *et al.*, 2007). Due to increasing concerns about antibiotic resistance dissemination and animal agriculture, proposed pathogen reduction strategies should not utilize traditional antibiotics (Summers, 2001).

Lytic bacteriophages are viruses that bind to specific bacterial cell surface receptors, inject their DNA, and take over the biosynthetic machinery of the bacterium to produce daughter phages, which are released via host lysis to repeat the process in other target bacteria (Guttman *et al.*, 2004; Kutter and Sulakvelidze, 2005). Phages have been isolated from many environments, including the gastrointestinal tracts of food animals, where they are natural members of the microbial ecosystem (Orpin and Munn, 1973; Klieve and Bauchop, 1988). Because phages exhibit a high degree of specificity for their host it has been suggested that they could be used as a “designer antimicrobial” to eliminate specific pathogens from the gastrointestinal microbial population, including *E. coli* O157:H7 (Greer, 2005). The present study was designed to determine if *E. coli* O157:H7-killing phages previously isolated from the feces of commercial feedlot cattle (Callaway *et al.*, 2006) could reduce gastrointestinal populations of *E. coli* O157:H7 in an artificially inoculated sheep model.

## Materials and Methods

### Bacterial cultures

*Escherichia coli* O157:H7 strain 933 (ATCC 43895) was repeatedly grown by 10% (v/v) transfer in anoxic (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub> atmosphere) tryptic soy broth (TSB) medium at 37°C. This strain was made resistant to novobiocin and nalidixic acid (20 and 25 µg/mL, respectively) by repeated transfer and selection in the presence of sublethal concentrations of each antibiotic. This resistant phenotype was stable through multiple unselected transfers in batch culture and through repeated culture vessel turnovers in continuous culture (data not shown). Overnight cultures (1 L) were harvested by centrifugation (7500 g, 10 minutes) and cell pellets were resuspended in TSB medium (150 mL total volume). Populations of *E. coli* O157:H7 in these cell suspensions were determined to be approximately 1×10<sup>9</sup> colony-forming units (CFU)/mL and 2×10<sup>9</sup> CFU/mL for studies 1 and 2, respectively, by serial dilution and plating as described below.

### Bacteriophage

Bacteriophage that lysed *E. coli* O157:H7 strain EDL 933 were previously collected and isolated from feedlot cattle feces (Callaway *et al.*, 2006). The phages (eight isolates) that produced the largest plaques on *E. coli* O157:H7 strain EDL 933 lawns at a constant plaque-forming units (PFU) concentration were selected and grown using a standard liquid amplification protocol. Individual phage isolates were grown overnight in 1-L cultures of *E. coli* O157:H7 strain 933 as described above.

Phage stocks were prepared by adding plaques (multiplicity of infection = 0.1–0.001) to an *E. coli* O157:H7 strain 933 (ATCC 43895) culture that was in early exponential growth phase (OD<sub>600</sub> < 0.3). After growth was completed (overnight) chloroform was added to cultures followed by vigorous shaking to lyse bacterial cells releasing progeny phage, and cultures were centrifuged at low speed (5000 g, 10 minutes) to remove cellular debris. Phage supernatants were serially diluted and spot tested against lawns of *E. coli* O157:H7 strain 933 to estimate phage populations. Phages (*n* = 8 isolates

at individual concentrations of approximately  $10^8$  PFU/mL) were grown individually and pooled for use as a cocktail (total phage cocktail titer of approximately  $10^9$  PFU/mL).

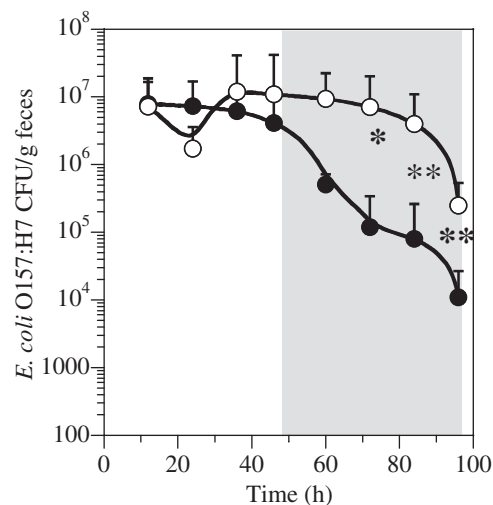
#### Sheep, rations and experimental design

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC protocol 05-001). Ramboulet/Suffolk sheep (average 60 kg body weight) were purchased from a commercial feedlot and were transported to the Food and Feed Safety Research Unit laboratory. Sheep were fed a commercial high grain ration composed of (dry matter basis): cracked corn, 74.4%; soybean meal, 9.2%; urea, 0.7%; trace mineral salts, 0.4%; and coastal bermudagrass hay, 15.3%. The diet was formulated according to National Research Council (NRC) recommendations and sheep were allowed *ad libitum* access to water.

Sheep were housed in environmentally controlled facilities and feces from each sheep was sampled on arrival and each subsequent day ( $n = 7$  days) during the dietary/facility adaptation period to verify that no organisms capable of growth on MacConkey's agar plates supplemented with novobiocin ( $20 \mu\text{g}/\text{mL}$ ) and nalidixic acid ( $25 \mu\text{g}/\text{mL}$ ) were present in the sheep. During this period no colonies grew on any of the MacConkey's novobiocin-nalidixic acid plates. During this period, fecal samples were analyzed for bacteriophage that could lyse *E. coli* O157:H7 933 (Callaway *et al.*, 2006), the strain used in the present inoculation studies.

#### Study 1: Effect of bacteriophage on intestinal populations of *E. coli* O157:H7

Twenty sheep were randomly assigned to one of two treatment groups ( $n = 10$  control and  $n = 10$  phage-treated). Each sheep was inoculated with *E. coli* O157:H7 933 ( $1 \times 10^{10}$  CFU/sheep) via oral gavage (10 mL total volume per sheep) at 0 hours (Fig. 1). Excreted fecal samples were collected via rectal grab at 12-hour intervals after inoculation and populations of inoculated *E. coli* O157:H7 strain 933 were enumerated via serial dilution and plating. Sheep were dosed with the phage cocktail via oral gavage at 48 and 72 hours to obtain a phage dosage of approximately  $10^7$  PFU/mL intestinal contents



**FIG. 1.** Fecal populations of *E. coli* O157:H7 strain EDL 933 (colony-forming units [CFU]/g feces;  $n = 10$  sheep/treatment). Shaded area represents period of phage treatment. Open circles depict populations in untreated control sheep, filled circles indicate sheep treated with phage at 48 and 72 hours. Values that differ from their respective control by  $p = 0.047$  are indicated by (\*), those differing by  $p < 0.097$  are indicated by (\*\*). Error bars represent standard deviations.

based on the determined intestinal volume of approximately 33 L from two randomly sampled sheep prior to study initiation. This level of phage was utilized to obtain a ratio of phage to inoculated *E. coli* O157:H7 populations of approximately 1 PFU/1 CFU.

#### Study 2: Effect of concentrations of bacteriophage on intestinal populations of *E. coli* O157:H7

In the second experiment, 24 sheep that contained neither phage nor antibiotic-resistant *E. coli* were randomly assigned to one of four treatment groups of various ratios of bacteriophage to inoculated *E. coli* O157:H7 (0:1, 1:1, 10:1, 100:1). Each sheep was inoculated with *E. coli* O157:H7 strain 933 ( $2 \times 10^{10}$  CFU/sheep) via oral gavage (10 mL total volume per sheep) at 0 hours. Fecal samples were collected after inoculation and populations of inoculated *E. coli* O157:H7 strain 933 were enumerated via serial dilution and plating. Sheep were dosed with the phage cocktail containing approximately  $10^8$  PFU/mL via oral gavage at 48 and 72 hours. Phage dosage was calculated to achieve ratios of phage cocktail to targeted bacteria of 0:1, 1:1,

10:1, and 100:1 PFU/CFU of *E. coli* O157:H7 in the feces.

#### Gastrointestinal sample collection

Sheep in both studies were humanely euthanized and exsanguinated at 96 hours (Fig. 1). Feces and intestinal contents from the rumen, distal end of the cecum, and the terminal rectum prior to the anal sphincter were aseptically collected upon necropsy. Samples were diluted as described below for quantitative enumeration of intestinal *E. coli* O157:H7 strain 933. Sample aliquots and epithelial tissues were added to TSB for overnight qualitative enrichment for inoculated *E. coli* O157:H7 strain 933. Overnight enrichments were plated as described below. Gastrointestinal content pHs were determined immediately upon return to the laboratory using a Corning 430 pH meter equipped with a calomel pH meter (Acton, MA). Intestinal contents were analyzed for volatile fatty acid (VFA) concentrations (Corrier *et al.*, 1990).

#### Bacterial enumeration

Ruminal, cecal, and rectal contents, as well as excreted feces were serially diluted (10-fold increments) in phosphate-buffered saline (PBS; pH 6.8). Dilutions were plated on MacConkey's agar supplemented with novobiocin (20  $\mu\text{g}/\text{mL}$ ) and nalidixic acid (25  $\mu\text{g}/\text{mL}$ ) and incubated overnight at 37°C. Colonies that grew on agar plates after 24-hour incubation were directly counted (quantitative enumeration). To qualitatively confirm the presence of inoculated *E. coli* O157:H7, intestinal contents and epithelial tissue samples as well as feces were incubated overnight in TSB at 37°C and were streaked on novobiocin/nalidixic acid-supplemented MacConkey's agar plates. Plates that contained colonies after 24-hour incubation were classified as positive for experimentally introduced *E. coli* O157:H7. No colonies grew from TSB-enriched feces on the novobiocin/nalidixic acid-supplemented MacConkey's agar plates prior to the inoculation of the sheep with *E. coli* O157:H7. Random colonies were picked during the course of the study and examined via latex agglutination to verify that the colonies growing on the novobiocin/nalidixic acid-supplemented MacConkey's agar plates were indeed *E. coli* O157:H7.

#### Bacteriophage detection

Phage populations in intestinal contents of sheep were estimated by treating a 2-mL aliquot of each dilution tube (above) with chloroform to lyse bacterial cells. The layer without chloroform was spotted (5  $\mu\text{L}$ ) on a bacterial lawn of *E. coli* O157:H7 strain 933. The presence of phage in each diluted sample was determined by the presence or absence of plaques (clearing zones) in the lawn.

#### Reagents and supplies

Unless otherwise noted, all media and agar were from Difco Laboratories (Sparks, MD). Reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO).

#### Statistics

*E. coli* O157:H7 strain 933 CFU/g were  $\log_{10}$  transformed. Treatment groups were compared at each time point by the Mixed procedure of SAS (SAS Institute Inc., Cary, NC). The experimental unit was the individual sheep. Time  $\times$  treatment interactions were discounted due to the natural decay of *E. coli* O157:H7 populations in this artificially inoculated model, therefore only pointwise comparisons were performed. Significance was determined at  $p < 0.05$ .

#### Results

Sheep ( $n = 20$ ) shed  $7.5 \times 10^6 \pm 4.8 \times 10^6$  CFU *E. coli* O157:H7 strain 933/g feces at the time of bacteriophage inoculation in the first experiment (Fig. 1). Sheep were orally dosed with approximately  $10^9$  PFU of phage per sheep at 48 and 72 hours, after which populations of *E. coli* O157:H7 strain 933/g feces declined in both control and phage-treated groups as is typical in experimental inoculation studies (Fig. 1). Fecal *E. coli* O157:H7 strain 933 populations were significantly lower ( $p = 0.047$ ) in the phage-treated group compared to the controls at 72 hours (Fig. 1). At 84 and 96 hours, fecal populations of *E. coli* O157:H7 strain 933 were lower ( $p = 0.089$  and  $0.097$ , respectively) in the phage-treated group compared with the controls.

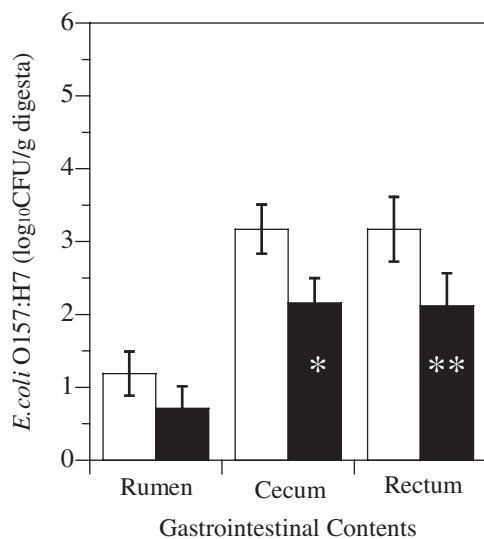
Phage treatment reduced gut populations of *E. coli* O157:H7 in the rumen, cecum, ( $p = 0.049$ ), and rectum ( $p = 0.095$ ) of sheep (Fig. 2). Phage

treatment did not alter pH, VFA concentrations, or VFA profiles of the intestinal contents (data not shown). *E. coli* O157:H7-infecting phages were recovered from all phage-treated sheep at concentrations of approximately  $10^2$  to  $10^3$  PFU/g cecal and rectal contents, but from the ruminal contents of only two phage-treated sheep.

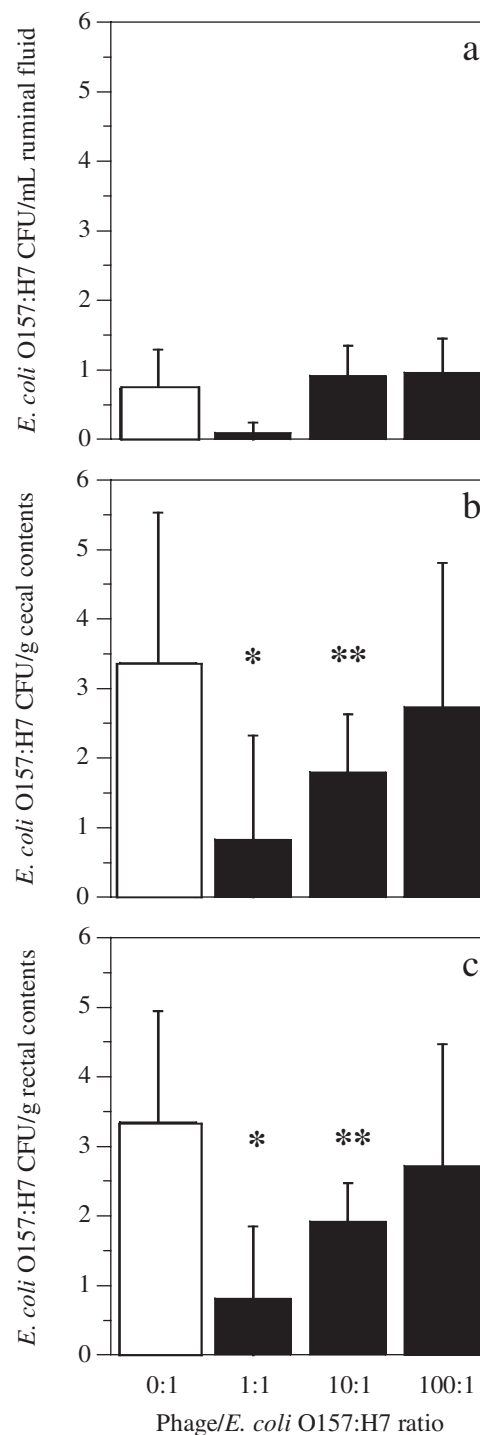
In the second study, phage treatment reduced *E. coli* O157:H7 populations in the rumen of sheep ( $n = 24$ ) compared to controls, but did not approach statistical significance ( $p < 0.09$ ; Fig. 3a). Phage-treated cecal and rectal contents contained lower counts of *E. coli* O157:H7 at ratios of 1:1 ( $p < 0.03$ ) and 10:1 PFU to CFU ( $p < 0.084$ ; Figs. 3b and 3c) than did controls. Across all three intestinal tissues, phage added at a 1:1 ratio reduced *E. coli* O157:H7 populations ( $p < 0.01$ ) and the ratio of 10:1 reduced populations ( $p = 0.051$ ; data not shown).

## Discussion

In recent years, there has been an increasing focus on preharvest intervention strategies to reduce pathogenic bacteria in food animals prior to slaughter (Sargeant *et al.*, 2007). While



**FIG. 2.** Ruminal, cecal, and rectal populations of *E. coli* O157:H7 strain EDL 933 in sheep. Open bars depict populations in untreated control sheep ( $n = 10$  sheep per treatment), filled bars indicate sheep treated with phage at 48 and 72 hours ( $n = 10$  sheep). Columns that differ from their respective control by  $p = 0.049$  are indicated by (\*), those differing by  $p = 0.095$  are indicated by (\*\*). Error bars represent standard deviations.



**FIG. 3.** Ruminal, cecal, and rectal populations of *E. coli* O157:H7 strain EDL 933 in sheep treated with four ratios of phage. Sheep ( $n = 24$  total) were treated with phage to obtain a ratio of phage to *E. coli* O157:H7 of 0:1 (control), 1:1, 10:1, and 100:1 PFU/CFU. Open bars represent untreated control sheep; filled bars indicate sheep treated with phage at various levels 24 and 48 hours prior to slaughter. Bars marked with (\*) differ from controls within the same intestinal tissue by  $p = 0.03$ , and bars marked with (\*\*) differ by  $p < 0.084$ ; error bars represent standard deviations.

the introduction of new strategies to reduce pathogens in processing plants have been largely successful (Koochmaraie *et al.*, 2005), too many foodborne illnesses still occur. Reducing the pathogen burden entering the processing plant could enhance the effectiveness of current and future in-plant intervention strategies (Hynes and Wachsmuth, 2000). Recently, processing plant directors stated that if the *E. coli* O157:H7 burden entering the plant could be reduced to  $10^3$  cells, then the plants can “take care of the rest” through existing in-plant interventions (personal communication).

Indirect routes of transmission contribute to human illnesses and have increasingly become of concern in recent years. Water runoff from cattle facilities can contain coliform bacteria, including *E. coli* O157:H7 (Sargeant *et al.*, 2003; Gyles, 2007), which can contaminate water used for crop irrigation (Manshadi *et al.*, 2001; Thran *et al.*, 2001) and/or drinking (Anonymous, 2000; LeJeune *et al.*, 2001). Petting zoos and open farms also are routes by which individuals, specifically children (one of the most susceptible groups), have been infected by *E. coli* O157:H7 (Keen *et al.*, 2007). These various routes emphasize that there are more critical points where intervention strategies can be implemented to reduce human exposure to pathogens than just within the processing plant.

Prior to the antibiotic revolution, phages were widely researched as a cure for human illnesses; however much of this research was of poor quality and lacked a clear understanding of interplay between phage and host. Phages have been and still are widely used in Eastern Europe in place of antibiotics and have been described as an “infectious cure for infectious disease” (Barrow, 2001). Several *E. coli* O157:H7-infecting phages have been isolated from a variety of sources (Morita *et al.*, 2002; Callaway *et al.*, 2006), but in some cases these phages were only active under highly aerated conditions, such as would be useful during processing of foods (e.g., on leafy green vegetables or sprouts) rather than in the anaerobic gastrointestinal tract of food animals (Kudva *et al.*, 1999). In other studies, phages reduced *E. coli* O157:H7 populations *in vitro*, but were less effective when used in experimentally infected animal systems (Bach

*et al.*, 2002; Tanji *et al.*, 2005; Sheng *et al.*, 2006). Other researchers found that oral phage dosing caused no reduction of intestinal *E. coli* O157:H7 populations in sheep, but did reduce *E. coli* O157:H7 populations in mice (Sheng *et al.*, 2006). The primary site of *E. coli* O157:H7 colonization in cattle is the recto-anal junction (Naylor *et al.*, 2003); when a phage was added directly to the recto-anal junction and also supplied in the drinking water, *E. coli* O157:H7 populations were reduced significantly but not eliminated in the experimental cattle (Sheng *et al.*, 2006). In ruminants, a bacteriophage isolated from rangeland sheep feces significantly reduced *E. coli* O157:H7 populations in the rectum and cecum of sheep experimentally infected with *E. coli* O157:H7 (Raya *et al.*, 2006).

If phages are to be a preharvest intervention strategy to kill *E. coli* O157:H7 within the gut of cattle, then phages that can survive and infect *E. coli* O157:H7 in the gut must be selected. The phages most fit for use in the gut of cattle should, therefore, originate from the gut of cattle, because the fitness of most organisms is typically greatest in their natural environment. In the present study, the individual phages in our cocktail were isolated from ruminant (cattle) feces and they effectively reduced *E. coli* O157:H7 throughout the intestinal tract of experimental sheep (a smaller ruminant which models the bovine intestinal tract) with the maximum efficacy in the cecum, although populations in the rectum were also reduced. The number of sheep that were positive for *E. coli* O157:H7 was reduced by phage treatment, but this pathogen was not eliminated from all phage-treated sheep.

Our phage cocktail was comprised of eight phage isolates that were selected *in vitro* for their activity against the specific strain of *E. coli* O157:H7 that was used in these animal studies. Because bacteriophages recognize specific receptors on bacteria, the bacteria can mutate, giving rise to phage-resistant mutants; therefore in our studies we used a cocktail of phages to prevent the emergence of phage-resistant inoculated *E. coli* O157:H7. In our studies, no phage-resistant *E. coli* O157:H7 were detected. It should also be noted that any real-world phage

cocktail should also contain phages that are active against a variety of other EHEC strains, not only *E. coli* O157:H7.

Contrary to the commonsense belief "if a little bit is good, then a lot must be better" phages have a critical threshold ratio relative to the host (targeted pathogen) populations. The fact that the initial ratio of 1:1 PFU/CFU was the most effective in our study was somewhat surprising, given that some phages were undoubtedly killed by passage through the acidic abomasum of the sheep. The reduced efficacy at higher host/phage ratios may be due to competitive interference between phages, also known as "lysis from without" (Delbrück, 1940; Kutter and Sulakvelidze, 2005). When a bacterium is infected by multiple phages nearly simultaneously, repeated initiation of replication can interfere with the ongoing replication process, causing the bacterium to lyse via cell wall degradation. Although the infected bacterium dies daughter phages are not produced, thus the "infectious cure" is unable to maintain its necessary self-sustaining chain reaction.

It is important to note that the present studies utilized an artificially inoculated model to demonstrate the efficacy of a short-term phage cocktail treatment. Following artificial inoculation of ruminants with *E. coli* O157:H7 intestinal populations decline in a manner that is not identical to the colonization of the intestinal tract near the recto-anal junction (Naylor *et al.*, 2003). Furthermore, this study examined *E. coli* O157:H7 populations in the gut after only 48 hours of phage treatment. The use of phages is currently envisioned as a short-term intervention strategy for use immediately pretransport and slaughter, thus the model used in the present studies addresses that usage, but not questions about longer usage in persistently *E. coli* O157:H7-colonized ruminants.

Interestingly, fecal counts of *E. coli* O157:H7 taken immediately premortem were approximately 2 log<sub>10</sub> CFU/g higher than in samples collected from the rectum postmortem in both control and phage-treated groups (Figs. 1 and 2). This was surprising given the temporal closeness of sample collection. However, given the fact that *E. coli* O157:H7 colonizes the lymphoid tissue in the terminal rectum (Naylor *et al.*, 2003;

Low *et al.*, 2005), it is possible that the rectal contents collected postmortem did not pass through the colonized tissue and receive an outer "coating" of *E. coli* O157:H7 as the excreted feces did.

Based upon our data, we conclude that phages are a viable strategy to reduce *E. coli* O157:H7 in ruminant animals before harvest. If we are to implement this technology in the human food chain a great deal of further research needs to be performed to determine the most efficacious dosing strategies and the most effective combinations of phages targeting the diverse EHEC population, not just *E. coli* O157:H7. Phage treatment is not a panacea to control all foodborne illness, however phage can be utilized in an integrated, multi-hurdle system aimed at reducing the passage of EHEC from farm to fork.

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