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Evaluation of Bacteriophage Application to Cattle in Lairage at Beef Processing Plants to Reduce *Escherichia coli* O157:H7 Prevalence on Hides and Carcasses

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Abstract

Escherichia coli O157:H7 is a major food safety concern for the beef industry. Several studies have provided evidence that cattle hides are the main source of beef carcass contamination during processing and that reductions in the *E. coli* O157:H7 load on the hides of cattle entering processing facilities will lead to reductions in carcass contamination. Bacteriophages have been proposed as a novel preharvest antimicrobial intervention to reduce the levels of *E. coli* O157:H7 on cattle hides. The objective of this study was to evaluate a commercialized phage application administered in the lairage area of commercial beef processing plants for the ability to reduce *E. coli* O157:H7 contamination of cattle hides and carcasses. Cattle lots either received phage spray treatment ($n = 289$) or did not ($n = 301$), as they entered the lairage environments in two separate experiments at two different commercial beef processing plants. Hide and carcass samples were collected and analyzed for *E. coli* O157:H7 prevalence and concentration. Cattle hides receiving phage treatment had an *E. coli* O157:H7 prevalence of 51.8%, whereas untreated hides had a prevalence of 57.6%. For carcass samples, the *E. coli* O157 prevalence in treated and untreated samples was 17.1% and 17.6%, respectively. The results obtained from these experiments demonstrated that the treatment of cattle hides with bacteriophages before processing did not produce a significant reduction of *E. coli* O157:H7 on cattle hides or beef carcasses during processing.

Keywords: *E. coli* O157:H7, bacteriophage, cattle, carcass, beef

Introduction

Escherichia coli O157:H7 is a major food safety concern for the beef industry. Several studies (Barkocy-Gallagher *et al.*, 2001; Nou *et al.*, 2003; Bosilevac *et al.*, 2005) have provided convincing evidence that cattle hides are the main source of beef carcass contamination during processing and that reductions in the *E. coli* O157:H7 load on the hides of cattle entering processing facilities will lead to reductions in carcass contamination. The prevalence of *E. coli* O157:H7 on cattle hides during the summer months in feedlot production settings frequently approaches 100% (Arthur *et al.*, 2009; Kalchayanand *et al.*, 2009). In addition, transportation from the feedlot and temporary holding at the processing plant have been shown to contribute to *E. coli* O157:H7 hide contamination (Arthur *et al.*, 2007, 2008; Dewell *et al.*, 2008), indicating that interventions applied to the live animal at the processing plant would be valuable additions to the multi-hurdle antimicrobial intervention schemes employed by commercial beef processing plants.

An alternative to pre-harvest interventions is the application of interventions at the pre-harvest/post-harvest boundary, which is the lairage environment at beef processing plants. Such an application would have the benefit that all animals would be treated and treated in a consistent manner, removing the variation in treatment caused by differing management practices among producers. Although multiple chemical processes are approved for use on cattle hides

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post-harvest, these chemicals cannot be used on live animals due to animal welfare concerns. The application of bacteriophages (phages) to cattle hides does not cause deleterious animal health issues and is approved for use before slaughter, but a determination of efficacy is lacking.

Bacteriophages (phages) are viruses that infect and potentially kill bacteria. Lytic phages have long been considered ideal biocontrol agents for bacterial pathogens due to their role as naturally occurring bacterial predators, specificity for a particular host, and the fact that they are functionally inert when interacting with eukaryotic cells (Abedon, 2009). Hence, phage application has been developed as a novel preharvest antimicrobial intervention to reduce the levels of *E. coli* O157:H7 on cattle hides before processing to reduce that pathogen load transferred to the carcass and to increase the food safety of the finished product. Coffey *et al.* (2011) reported a 1.5 log colony-forming unit (CFU)/cm^2 reduction in *E. coli* O157:H7 inoculated onto pieces of cattle hide when treated with bacteriophages and given a 1-h dwell timing. Phages are FDA approved for use in or on live cattle for control of *E. coli* O157:H7 associated with cattle. However, there are little to no definitive data demonstrating the efficacy of phage treatments in reducing the concentration or prevalence of pathogenic bacteria colonizing a live animal. The objective of this study was to evaluate a commercialized phage application administered in the lairage area of beef processing plants for the ability to reduce *E. coli* O157:H7 contamination of cattle hides and carcasses.

Materials and Methods

Study design

Two experiments were performed for this project. In both experiments, phages were applied to cattle hides at commercial beef processing plants through a spray application system according to the manufacturer's instructions (Finalyse®; Elanco Animal Health, Greenfield, IN). Briefly, the phage solution, containing a proprietary mixture of phages, was applied at a dose of $\sim 3 \times 10^{10}$ phage/head of cattle in one gallon of water/head with a dwell timing of at least 1 h before harvest. The spray applications were administered in the lairage environment to cattle lots shortly after cattle were unloaded from delivery trucks. Individual cattle lots either received phage application or did not; at no time were subsets of lots treated.

Experiment 1

Sample collection. Cattle hide samples were collected both before and after a phage or control application at a commercial beef processing company. Control animals ($n = 140$) received a water spray that did not contain Finalyse, whereas cattle ($n = 120$) in the treated group received a water spray containing Finalyse. To prevent carryover of phage via the application system, the system was thoroughly rinsed after application to treated animals. BEFORE samples were collected as the animals exited the transport trailer or shortly after arrival into the processing plant lairage area. AFTER samples were collected at least 1-h post phage/water application, immediately after the exsanguinated carcass was shackled and placed on the processing line. Both sample types were obtained by using a sterile sponge (Biotrace International, Inc., Bothell, WA) that was premoistened with 20 mL of buffered peptone water (Becton Dickenson, Sparks, MD), and swabbing an area of $\approx 1000\,\text{cm}^2$ on the rump. Animals were sampled over five sampling days, with 20–70 cattle being sampled per day (250 total). Animals were marked so that the BEFORE and AFTER samples were matched to an individual animal. An approximately equal number of treated and control samples were collected on each sampling day, packed on ice, and shipped to the laboratory. On arrival at the lab, the samples were analyzed for concentration and prevalence of *E. coli* O157:H7.

Experiment 2

Sample collection. A total of 340 carcasses were sampled over 3 days. Hide and pre-evisceration carcass samples were collected from 10 carcasses per lot from 34 lots. Ten lots were sampled per day on Day 1 and Day 2, and 14 lots were sampled on Day 3. Each day, half of the lots did not receive bacteriophage treatment on arrival into the lairage environment holding area and were considered CONTROL lots. The remaining lots did receive bacteriophage treatment on entry into the lairage environment and were considered TREATED lots. Bacteriophage application and dwell timing were administered by processing plant personnel according to standard operating procedures and the manufacturer's instructions. Hide sponge samples were collected after exsanguination and shackling. Hide sponge samples consisted of swabbing a 1000-cm^2 area on the front shoulder. Carcass samples were taken immediately after hide removal, but before the application of any antimicrobial interventions to the carcass surface. Carcass sponge samples were collected by swabbing a 4000-cm^2 area following the carcass midline from the navel to brisket and included the foreshank (Arthur *et al.*, 2004). No attempt was made to sample the hides and carcasses of the same animals. Samples were packed on ice and shipped to the laboratory for processing. On arrival at the lab, the samples were analyzed for concentration and prevalence of *E. coli* O157:H7.

Enumeration. *E. coli* O157:H7 was enumerated from hide samples by using a Spiral Plater (Spiral Biotech, Norwood, MA) and following previously described methods (Brichta-Harhay *et al.*, 2007). The sponge samples were homogenized by hand massage, after which 500 μL of the homogenate was transferred to a microfuge tube. After vortexing and a 3-min holding period to allow the particulates to settle, 50 μL aliquots were spiral plated on ntChromagar (Chromagar O157 [DRG International; Mountainside, NJ] supplemented with novobiocin [5 mg/L; Sigma, St. Louis, MO] and potassium tellurite [2.5 mg/L; Sigma]) plates. After incubating the plates overnight at 42°C, the plates were counted with suspect colonies confirmed by PCR. PCR was used to confirm that each *E. coli* O157:H7 isolate harbored genes for the O157 antigen, H7 flagella, γ-intimin, and at least one of the Shiga toxins (Hu *et al.*, 1999). The limit of detection in the enumeration assay was 40 CFU/100 cm^2.

Sample processing for prevalence. Samples were processed according to methods previously described (Barkocy-Gallagher *et al.*, 2002, 2005; Nou *et al.*, 2006), with slight modifications. After removing the 500 μL aliquot for

enumeration, the sponge samples were enriched with 80 mL of Tryptic Soy Broth (Becton Dickinson Microbiology Systems, Sparks, MD) and incubated at 25°C for 2 h, 42°C for 6 h and then held at 4°C overnight. After incubation, 1 mL from each enrichment was subjected to anti-O157 immunomagnetic separation. Enrichments (1 mL) received 20 μL of anti-O157 beads (Thermo Fisher Scientific, Inc., Waltham, MA). Thereafter, the beads were extracted from enrichment samples and washed three times in phosphate-buffered saline-Tween 20 (Sigma) by using an automated magnetic particle processor (KingFisher 96; Thermo Fisher Scientific, Inc.). The final bead-bacteria complexes were spread-plated onto ntChromagar and incubated at 37°C overnight. After incubation, up to three suspect colonies were picked for confirmation. PCR was used to confirm that each *E. coli* O157:H7 isolate harbored genes for the O157 antigen, H7 flagella, γ-intimin, and at least one of the Shiga toxins (Hu *et al.*, 1999).

Statistical analysis

Statistical analysis was performed by using Stata/SE 13.1 (StataCorp, College Station, TX). The unit of statistical analysis was the individual animal. The effect of phage application on the prevalence of *E. coli* O157:H7 on the cattle hides (experiment 1), and the hides and carcasses (experiment 2) was modeled with generalized estimating equations (GEE) accounting for the clustering of observations by lots. In both experiments, sampling day was included in the GEE model as a fixed effect. In experiment 1, sampling point (before and after phage application) was included as a fixed effect to account for the repeated measurements. Model-adjusted prevalence for *E. coli* O157:H7 was obtained from the marginal predicted values. Significance was declared at a $p < 0.05$.

Results and Discussion

A considerable amount of research and development has resulted in highly effective post-harvest antimicrobial interventions, which are currently utilized in the beef processing industry. These interventions are applied in a multi-hurdle approach to sequentially reduce bacterial contamination that is transferred from cattle hides to beef carcasses during processing (Arthur *et al.*, 2004; Callaway *et al.*, 2004; Koohmaraie *et al.*, 2005; Brichta-Harhay *et al.*, 2007). However, conditions continue to arise where the incoming contamination load on cattle hides exceeds the reducing capacity of the post-harvest interventions, resulting in finished product contamination (Vosough Ahmadi *et al.*, 2007; Arthur *et al.*, 2014). Therefore, effective pre-harvest interventions are needed to aid in reducing the incoming contamination load on cattle hides.

To date, multiple pre-harvest technologies have been developed (vaccines, direct fed microbials, and ingestible phage inoculants) for use in cattle production to reduce the shedding of *E. coli* O157:H7 in the feces (Fairbrother and Nadeau, 2006; Sargeant *et al.*, 2007; Berry and Wells, 2010). By reducing the prevalence and concentrations of *E. coli* O157:H7 in the feces, it would be possible to reduce the amount of *E. coli* O157:H7 contamination on cattle hides (Cobbold and Desmarchelier, 2000; Chase-Topping *et al.*, 2008; Arthur *et al.*, 2009). However, although several studies have found these applications promising for future development, none have shown efficacy in consistently reducing *E. coli* O157 shedding in the feces of cattle (Greer, 2005; Van Donkersgoed *et al.*, 2005; Callaway *et al.*, 2008; Moxley *et al.*, 2009; Goodridge and Bisha, 2011; Wileman *et al.*, 2011; Cull *et al.*, 2012). Also, additional contamination is acquired on the hide after cattle leave production settings when they are transported to and held at beef processing plant lairage environments (Arthur *et al.*, 2007, 2008; Dewell *et al.*, 2008).

Prevalence of E. coli *O157:H7 in hide samples collected before and after phage application*

In Experiment 1, the *E. coli* O157:H7 prevalence on cattle hides as they were unloaded at the processing plant lairage area was 6.4% and 13.3% for the control and treated groups, respectively (Table 1). The prevalence of *E. coli* O157:H7 increased significantly ($p < 0.001$) for both control and treated cattle from the time they entered lairage and phage was applied to the point where the animals were stunned, exsanguinated, and shackled. The resulting *E. coli* O157:H7 prevalence on the hides was 42.0% and 38.7% for the control and treated groups, respectively. Although the treated group did not have as large of an increase in *E. coli* O157:H7 prevalence as did the control group, phage application in the lairage environment did not have a significant ($p = 0.547$)

TABLE 1. PREVALENCE (%) OF *ESCHERICHIA COLI* O157:H7 ON THE HIDES OF BEEF CATTLE BEFORE AND AFTER PHAGE APPLICATION (EXP I)

Sampling day	*Control*					*Treated*				
	Before	n[a]	*After*	n	p-*value*[b]	*Before*	n	*After*	n	p-*value*
1	0[c] (0–30.8)	10	36.4 (10.9–69.2)	11	0.09	0 (0–30.8)	10	10 (0.3–44.5)	10	1
2	7.5 (1.6–20.4)	40	80 (61.4–92.3)	30	<0.001	30 (14.7–49.4)	30	69.0 (49.2–84.7)	29	0.004
3	0 (0–11.6)	30	46.7 (28.3–65.7)	30	<0.001	10 (1.2–31.7)	20	50 (27.2–72.8)	20	0.014
4	3.3 (0.08–17.2)	30	23.3 (9.9–42.3)	30	0.052	0 (0–11.6)	30	26.7 (12.3–45.9)	30	0.005
5	16.7 (5.6–34.7)	30	20 (7.7–38.6)	30	1	16.7 (5.6–34.7)	30	23.3 (9.9–42.3)	30	0.748
Overall	6.4 (3.0–11.9)	140	42.0 (33.4–50.9)	131	<0.001	13.3 (7.8–20.7)	120	38.7 (29.9–48.0)	119	<0.001

[a]*n* represents the number of cattle sampled.
[b]The *p*-values compare the prevalence of *Escherichia coli* O157:H7 between ''before'' and ''after'' samples within each group (control and treated).
[c]Prevalences were presented as point estimate (95% confidence interval).

Table 2. Prevalence (%) of *Escherichia coli* O157:H7 on Hides of Cattle With or Without Phage Treatment (Exp II)

	Control		*Treated*		
Sampling day	n[b]	*Prevalence*[a] *(95% CI)*	n	*Prevalence (95% CI)*	p-*value*[c]
1	50	98 (89.4–99.9)	50	86 (73.6–94.2)	0.208
2	50	62 (47.2–75.3)	50	48 (33.7–62.6)	0.430
3	70	25.7 (16.0–37.6)	70	30 (19.6–42.1)	0.753
Overall	170	57.6 (49.8–65.2)	170	51.8 (44.0–59.5)	0.644

[a]Prevalences were presented as point estimate (95% confidence interval).
[b]*n* represents the number of hides sampled.
[c]The *p*-values compare the prevalence of *E. coli* O157:H7 between the control and treated groups.

effect on the prevalence of *E. coli* O157:H7 on the hide samples (Table 1).

Previous research has described the potential for hide contamination to increase as cattle progress through processing plant lairage environments (Arthur *et al.*, 2007, 2008; Dewell *et al.*, 2008). This occurs when large numbers of cattle pass through common spaces and deposit fecal matter laden with bacterial pathogens. As subsequent cattle pass through these spaces, they acquire additional contamination on their hides through splashing or lying down on contaminated surfaces. Due to this potential to accumulate hide contamination after arrival at the processing plant, it was determined that hide sampling both before and after phage application in the lairage environment was not a useful evaluation of the phage application efficacy. It was determined that a better evaluation of the intervention efficacy would be attained by comparing the *E. coli* O157:H7 prevalence and concentrations on the hides and carcasses of treated cattle post-phage application and not collecting any BEFORE samples.

Effect of phage application on the prevalence of E. coli *O157:H7 on hide and carcass samples*

In Experiment 2, the *E. coli* O157:H7 prevalence for the first two sampling days was numerically slightly lower on treated cattle hides when compared with control cattle hides, but not on the third sampling day (Table 2). Overall, phage treatment did not have any significant ($p = 0.644$) effect on the prevalence of *E. coli* O157:H7 in hide samples. Similarly, the *E. coli* O157:H7 prevalence for the first two sampling days was numerically slightly lower on carcasses from treated cattle when compared with control cattle carcasses, but not on the third sampling day (Table 3). Again, phage treatment did not have any significant ($p = 0.928$) effect on the overall prevalence of *E. coli* O157:H7 in carcass samples.

Effect of phage application on concentration of E. coli *O157:H7 on cattle hides and carcasses*

For Experiment 1, there were no BEFORE hide samples harboring concentrations of *E. coli* O157:H7 greater than the lower limit of detection (40 CFU/100 cm^2) for the enumeration assay used in this study. There were seven AFTER samples (four treated and three control) harboring enumerable *E. coli* O157:H7 (data not shown). From Experiment 2, there were numerically more samples with *E. coli* O157:H7 concentrations ≥40 CFU/100 cm^2 on the control hides as opposed to the treated hides (25 vs. 16), but the difference was not significant ($p = 0.133$). For the carcass samples, there were more treated carcass samples with enumerable *E. coli* O157:H7 than control carcass samples (4 vs. 2), but this difference was not statistically significant ($p = 0.685$).

Previous applications of phage for the purposes of reducing *E. coli* O157:H7 associated with cattle have focused on killing the pathogen in the bovine gastrointestinal (GI) tract to reduce the concentrations of *E. coli* O157:H7 shed in the feces. These applications, which typically use either orally or rectally inoculated phage or a combination of both, have achieved only modest reductions in *E. coli* O157:H7 shedding. Sheng *et al.* (2006) employed a dual phage cocktail via multiple rectal applications and a continuous supply of phage to the drinking water to reduce the concentrations of *E. coli* O157:H7 being shed by inoculated calves. This treatment was not successful in completely eliminating the pathogen, as most animals continued to shed *E. coli* O157:H7 at low levels

Table 3. Prevalence (%) of *Escherichia coli* O157:H7 on Beef Carcasses With or Without Phage Treatment (Exp II)

	Control		*Treated*		
Sampling day	n[b]	*Prevalence*[a] *(95% CI)*	n	*Prevalence (95% CI)*	p-*value*[c]
1	50	16 (7.2–29.1)	50	8 (2.2–19.2)	0.272
2	50	38 (24.7–52.8)	50	34 (21.2–48.8)	0.712
3	70	4.3 (0.9–12.1)	70	11.4 (5–21.3)	0.161
Overall	170	17.6 (12.2–24.2)	170	17.1 (11.7–23.6)	0.928

[a]Prevalences were presented as point estimate (95% confidence interval).
[b]*n* represents the number of carcasses sampled.
[c]The p-values compare the prevalence of *E. coli* O157:H7 between the control and treated groups.

at the end of the study. Because *E. coli* O157:H7 have been shown to colonize the recto-anal junction (Naylor *et al.*, 2003; Low *et al.*, 2005; Cobbold *et al.*, 2007; Fox *et al.*, 2008), it has been speculated that optimal application for a phage-based intervention would bypass the bovine rumen and target the lower GI tract. Stanford *et al.* (2010) developed a polymer encapsulation technology that would protect the orally inoculated phage formulation from the microbial milieu that exists in the rumen and release the phages in a viable state as they entered the lower GI tract. This application failed to reduce *E. coli* O157:H7 shedding overall, but it did shorten the duration of shedding (Stanford *et al.*, 2010). Although several hypotheses have been given (improper phage:host ratios, inability of phage to penetrate mucous layers, etc), it is not known why susceptible host strains are not substantially reduced by phage-based interventions in the GI tract.

One potential reason for the lack of efficacy when phages are applied to cattle hides is the large amount of organic matter and pathogen contamination that cattle encounter as they move through the lairage environment. Because the phage treatment is applied before the cattle are exposed to the majority of the lairage environment contamination, it may be that the phages are simply overwhelmed by additional contamination. Further research is needed to determine whether moving the point of application closer to the exit of lairage would increase the efficacy. In addition, it may be beneficial to develop a phage treatment that is applied to carcasses immediately after dehiding to minimize the organic load and have a higher phage-to-target ratio.

To conclude, many studies have shown that during beef processing cattle hides represent the most significant source of bacterial pathogen contamination of the carcass (Barkocy-Gallagher *et al.*, 2001; Nou *et al.*, 2003; Bosilevac *et al.*, 2005). The beef industry would benefit from having additional antimicrobial interventions that can be applied before the cattle entering the processing plant facility. The application of phage to cattle hides in the lairage environment was seen as a potential intervention technology that could be used on the live animal for the improvement of food safety. However, through the experiments conducted here, it was demonstrated that the treatment of cattle hides with bacteriophages before passing through the lairage environment and before processing did not produce a significant reduction in the levels or prevalence of *E. coli* O157:H7 on hides or beef carcasses during processing; hence, it is doubtful that this application is improving the food safety of the beef supply.

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