BACTERIOPHAGE MIXTURE INACTIVATION KINETICS AGAINST *ESCHERICHIA COLI* O157:H7 ON HARD SURFACES

STELIOS VIAZIS, THEODORE P. LABUZA and FRANCISCO DIEZ-GONZALEZ[1]

Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108

[1]Corresponding author.
TEL: 612-624-9756;
FAX: 612-625-5272;
EMAIL: fdiez@umn.edu

ABSTRACT

This study determined the effect of previously described phage cocktail (BEC8) on the inactivation kinetics of a mixture of *Escherichia coli* (EHEC) O157:H7 strains on food processing surface materials: stainless steel chip (SSC), ceramic tile chip (CTC) and high-density polyethylene chip (HDPEC). EHEC strains were combined and spot-inoculated on surfaces in dried or liquid form and BEC8 was applied and incubated at 12, 23, 30 and 37C. EHEC survival was determined on tryptic soy agar. D values ranged from 3.9 min at 37C to 46.7 min at 12C. Z values calculated for SSC, CTC and HDEPC resulted in 26.1, 23.7 and 26.7C for dry cells, respectively, and 23.2, 23.7 and 24.5C for liquid cells, respectively. There was a significant difference in the rate of bacterial inactivation between lower (12 and 23C) and higher temperatures (30 and 37C). These results indicated that BEC8 killed EHEC on hard surfaces rapidly following first-order kinetics.

PRACTICAL APPLICATIONS

The effect of a previously described phage cocktail (BEC8) on the inactivation kinetics of a mixture of *Escherichia coli* (EHEC) O157:H7 strains applied on hard food processing surface materials was studied. The results indicated that phages killed EHEC on hard surfaces relatively rapidly following a predictable rate. However, one must consider the long times to achieve a 5-log reduction when initial cell counts are high. It would take approximately 2–4 h for a 5-log cfu reduction of liquid cells at 23C. Both the time length and the temperature of application must fit within the industry time scales.

INTRODUCTION

Food processing equipment, surfaces and facilities can be contaminated by pathogenic bacteria, causing serious problems (Abuladze *et al.* 2008). Bacteria are able to attach to surfaces to survive in nature (Lindsay and von Holy 1999) but can also survive on utensils, days after initial contact (Scott and Bloomfield 2008). Food particles can be cleaned from surfaces when good hygienic practices are followed, but attached bacteria may escape this process and not be removed (Kusumaningrum *et al.* 2003). Decontamination presents a serious challenge due to the increased resistance of many potentially pathogenic bacteria to traditional sanitizers, such as hypochlorous acid and benzalkonium chloride (Davidson and Harrison 2002). A number of chemical sanitizers can also be corrosive and toxic, which is undesirable for a food processing environment. Because of these limitations of existing chemical sanitizers, new alternatives are needed to assist in decontaminating food processing surfaces.

Phages are obligate parasites of bacteria capable of killing specific species and offer a natural method to control contamination of foods (Callaway *et al.* 2008). Bacteria may develop resistance to phages and this consideration should be considered in phage applications. The use of cocktails of several phages can avoid resistance development by offering sufficient breadth and complementarity of targets (Brussow 2005). Phages use different protein receptors at the bacterial membrane, so the simultaneous use of several phage strains at once reduces the probability of phage resistance. Bacterial resistance mechanisms against phages and antibiotics differ, and as a result, the possible emergence of resistance against

phages will not affect the susceptibility of bacteria to antibiotics used for humans. Phage preparations can be readily modified in response to changes in bacterial pathogen populations or susceptibility, whereas antibiotics have a long and expensive development cycle.

The mixture of bacteriophages BEC8 was developed using previously described bacteriophages in combination with additional newly isolated *Escherichia coli* (EHEC) O157:H7-specific phages (Viazis *et al.* 2011a). The efficacy, specificity and partial characterization of this group of phages were first determined in pure culture against a large group of *E. coli* O157:H7 strains. The potential for application of BEC8 was evaluated in spinach and lettuce leaves, as the cocktail was capable of reducing the bacterial count more than 3 $\log_{10}$ cfu and this effect was enhanced in combination with *trans*-cinnamaldehyde (Viazis *et al.* 2011b). The lytic ability of BEC8 when applied to *E. coli* cells dried on inert surfaces was also previously investigated with almost 4 $\log_{10}$ cfu/chip reductions in viable cell counts (Viazis *et al.* 2011c).

The objective of this study was to determine the inactivation kinetics of a mixture of growing vegetative EHEC O157:H7 strains when treated with a bacteriophage cocktail (BEC8) and applied on surfaces of materials commonly found in food processing plants.

MATERIALS AND METHODS

Phage Preparation

The bacteriophage mixture designated as BEC8 was first described by our group (Viazis *et al.* 2011a) and included eight lytic, *E. coli* O157:H7-specific phage strains: 38, 39, 41, CEV2, AR1, 42, ECA1 and ECB7, which are members of the family Caudovirales, and is highly effective against strains of *E. coli* O157:H7 as shown previously through efficiency of plating tests, spot testing and activity against high bacterial titers. The phage cocktail was suspended in a solution of tryptic soy broth (TSB; Neogen, Inc., Lansing, MI) and kept at 4C.

Bacterial Strains

The bacterial strains used to inoculate hard surfaces were *E. coli* O157:H7 ATCC 43895, an isolate from a 1982 hamburger outbreak; EK27 TWO8635, a clade 8 isolate received from Dr. Thomas Whittam (STEC Center, Michigan State University); and I-2005003658-472, a 2005 spinach outbreak isolate (Minnesota Department of Health, St. Paul, MN). Strains were stored in vials containing TSB and 10% glycerol (Sigma Chemical Co., St. Louis, MO) at −55C. For inoculum preparation, frozen suspensions were streaked on

tryptic soy agar (TSA; Neogen, Inc.) and the TSA plates were incubated at 37C for 24 h.

Hard Surfaces

Stainless steel chips (SSCs) (1 cm diameter), ceramic tile chips (CTCs) ($2.0 \pm 0.2 \times 2.3 \pm 0.4$ cm) and high-density polyethylene chips (HDPECs) ($1.5 \pm 0.4 \times 1.8 \pm 0.5$ cm) were used to represent three different food processing materials. Matrices were cleaned with 70% ethanol, autoclaved and placed in Petri plates.

Inoculation

The EHEC strains were streaked on TSA, incubated overnight at 37C, individual colonies were transferred to tubes with 9 mL of TSB and incubated overnight at 37C. Aliquots of 100 μL were transferred to fresh tubes with 9 mL of TSB and incubated at 37C for 3 h, resulting in mid-exponential phase cultures. Aliquots of 0.5 mL of each strain were combined in 1-mL microcentrifuge tubes, vortexed and centrifuged at $14,500 \times g$ for 5 min. Supernatants were decanted and fresh 1.5 mL of TSB was added and mixed. Three different levels of bacteria were added to each hard surface. Mixtures of the three EHEC strains were diluted in TSB to provide approximately 4–5 log cfu/chip of inoculum. The mixture was spot-inoculated (20 μL) onto the hard surfaces and allowed to dry for 1 h in a biosafety cabinet.

Phage Application

Aliquots (100 μL) of BEC8 (10^6 pfu/mL) or TSB (treatments and negative control, respectively) were placed with pipettes on top of areas of the hard surfaces inoculated with mixtures of the three *E. coli* O157:H7 strains that were dried, calculated to obtain a multiplicity of infection (MOI; phage/cell ratio) of 100. Liquid cells involved 20 μL of bacterial mixtures spotted on the hard surfaces and mixed with 100 μL of BEC8 without drying. Chips were then incubated inside of covered Petri plates at 12 and 23C for up to 60 min and at 30 and 37C for up to 10 min.

Processing

Samples were processed after 0, 5, 10, 20, 40 and 60 min when incubated at 12 and 23C, and after 0, 2, 4, 8 and 10 min when incubated at 30 and 37C. Treated chips were placed in 50-mL centrifuge tubes that contained 1 mL of phosphate buffer saline (PBS) (pH 7.2) and 10–15 glass beads (3 mm) using sterile forceps. The tubes were then vortexed for approximately 1 min, followed by serial dilution of PBS and plating on TSA. The TSA plates were then incubated at 37C overnight and enumerated. The bacterial

counts as cfu were calculated based on the number of colonies and dilutions plated on a per chip basis and the cfu values were transformed to logarithm base 10.

Data Analysis

Decimal reduction time (*D*) values (time to inactivate 90% of the population) and their respective 95% confidence level (CL) were determined from the linear portion of the survival curves by plotting the survival counts against incubation times at each temperature using a semi-log plot. Calculations were made using the procedures developed by Labuza and Kamman (1983) using the spreadsheet available at: http://www.ardilla.umn.edu/00fscn8334-1f/FScN8334 _Graphs%20and%20Spreadsheets.html.

Inactivation trials were performed at least twice and using duplicate samples at each time interval. Note that the Excel Trendline function allows for the calculation of slope of a linear function but does not have a built-in 95% CL function for the line slope *k*. Based on the exponential regression equation for survival plots (i.e., $N = No^{-kt}$, where *No* is the initial inoculum, *N* is the cfu count at time and *k* is the slope of the line or the death rate constant with units of reciprocal time). The *D* values were calculated from $D = 2.303/k$ and correspond to the time for 1-log reduction. The Excel spreadsheet allows for creating a plot with both the upper and the lower 95% CL as well as for projection of the future value corresponding to the limit of detection. The increase of temperature (C), which would cause a 10-fold reduction of the cell count, was determined from the slopes of the linear regression of the decimal reduction time curves (log *D* values versus temperature), where $Z = -\text{slope}^{-1}$.

RESULTS AND DISCUSSION

Kinetic studies that are focused on bacteriophage adsorption, development, lysis and propagation usually employ a one-step growth experiment. In a typical one-step growth experiment, a culture of cells is combined with a phage suspension at a low MOI to ensure one-to-one infection. Samples are then withdrawn at certain intervals, serially diluted and plated on lawns of susceptible bacteria, whereas the number of phages is calculated from the number of plaques formed following overnight incubation. This procedure is commonly used to describe the development of the T4 bacteriophage within *E. coli* under various physiological states of the host (Rabinovitch *et al.* 1999). The purpose of this study was to determine the inactivation kinetics of the bacteriophage cocktail BEC8 against a mixture of *E. coli* O157:H7 strains on hard surfaces commonly used in the food industry. To our knowledge, there has been no similar

approach in determining the inactivation kinetics of bacterial pathogens from hard surfaces using bacteriophage cocktails.

The bacteriophage cocktail used in this study was previously characterized and included the phages 38, 39, 41, AR1, 42, CEV2, ECB7 and ECA1 (Viazis *et al.* 2011a). Extensive spot testing against EHEC O157:H7 showed that the BEC8 phages were capable of causing lysis of 94–98% of the strains (*n* = 123) screened. When BEC8 phages were subjected to polymerase chain reaction for EHEC virulence factors *stx1*, *stx2*, *eaeA* and *hlyA*, none of the virulence factors were detected.

The detection limit for the recovery of cells of *E. coli* O157:H7 from the hard surfaces was 1 log cfu/chip. All of the results are presented under the assumption that the survival of bacterial mixture at levels below the detection limit cannot be quantified. Therefore, when no surviving cells were found, they were assumed to be at a 1 log cfu/chip level. Figure 1 shows an example semi-log plot of viable count as a function of time, which includes the projections to the limit of detection. Table 1 summarizes the *D* value results for all conditions tested giving the upper and lower CL as well as the R^2 for each plot. Table 2 contains the results for the time to the limit of detection (5-log$_{10}$ inactivation) and 95% CL.

When the kinetics of inactivation with BEC8 were calculated, *D* values were consistently lower for liquid cells than for dry cells, but these differences were not significant, except for the case of SSC at 30°C. *D* values of inactivation ranged from approximately 3.9 min at 37C to 46.7 min at 12C, and they were significantly different when comparing the different incubation temperatures used (Table 1). Specifically, the *D* values for the two lower temperatures (12 and 23C) were significantly and consistently longer from the two higher temperatures (30 and 37C). However, *D* values were not statistically different among the different hard surfaces. When the limits of detection were calculated, the results followed a similar pattern as the *D* values in terms of significant differences. The values were consistently shorter for liquid than for dry cells, but these differences were not significant, except for the cases of SSC and CTC at 30C. The values ranged from 15 min at 37C to 205 min at 12C. Again, the *D* values for the two lower temperatures (12 and 23C) were significantly and consistently higher than the values for the two higher temperatures (30 and 37C).

The *Z* values obtained on SSC, CTC and HPEC for liquid positive controls were 23.2, 23.7 and 24.5C (R^2 values >0.84), respectively, and for dry cells treated with BEC8 *Z* values were 26.1, 23.7 and 26.7C (R^2 values >0.84), respectively (Figs. 2 and 3). Thermal inactivation of bacterial pathogens, including spores, a process whereby the DNA is denatured, generally have *Z* values from about 7 to 12C (Blackburn *et al.* 1997; Montville *et al.* 2005), whereas
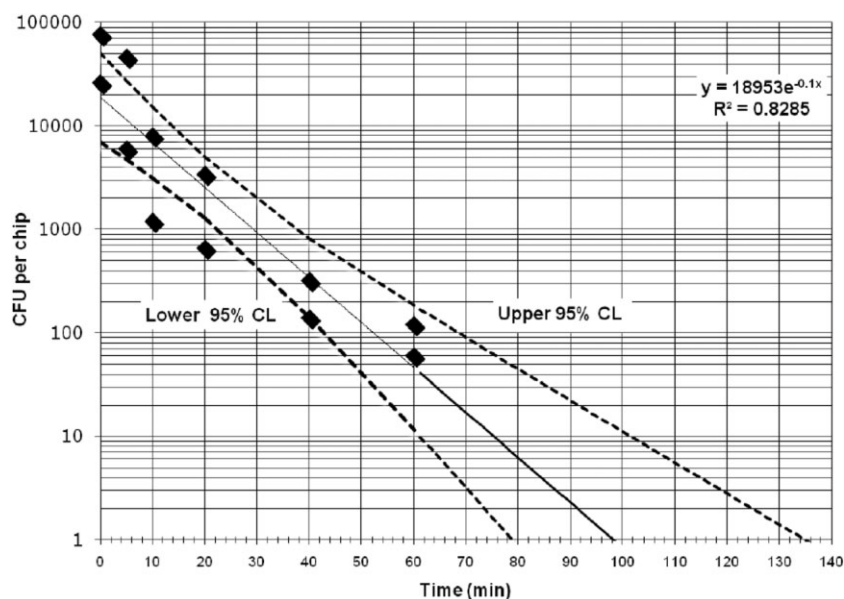
FIG. 1. SEMI-LOG PLOT OF VIABLE CELL COUNT OF THE THREE STRAINS OF *ESCHERICHIA COLI* O157:H7 MIXTURE AS A FUNCTION OF EXPOSURE TIME ON STAINLESS STEEL CHIP AT 23C WHEN INOCULATED IN LIQUID FORM WITH BACTERIOPHAGE COCKTAIL BEC8 AT A MULTIPLICITY OF INFECTION OF 100

The time at 1 cfu/chip represents the time (99 min) for a 5-log reduction, which is the limit of detection showing the 95% CL of 79–135 min.

chemical reactions such as thiamine loss or protein denaturation have Z values in the 20–30C range (Ryley and Kajda 1994; Kwok *et al.* 1998). This suggests that the mechanism of phage death kinetics is more related to the latter and is less temperature sensitive than that of heat treatment.

There have been several attempts to determine bacteriophage action on pathogenic organisms through the use of inactivation kinetics. There have been studies in the adsorption of phages on pathogens in pure culture (Yassky 1962; Moldovan *et al.* 2007), thermal destruction of phages

(Daoust *et al.* 1965), and infection and protection in infected chicks and calves (Barrow *et al.* 1998). The most recent of these studies has investigated the use of bacteriophages as self-replicating pharmaceutical agents as part of phage therapy (Payne and Jansen 2000, 2001). There have also been recent reviews that cover the advantages of using phages over traditional antimicrobial systems for foods at the preharvest (Joerger 2003) and postharvest level (Leverentz *et al.* 2001) as well as on the kinetics of phage activity as a method of biocontrol of bacteria (Abedon

TABLE 1. INACTIVATION RATE PARAMETERS (D VALUES, 95% CONFIDENCE LEVEL [CL] AND R^2) OF A MIXTURE OF THREE *ESCHERICHIA COLI* O157:H7 STRAINS TREATED WITH THE BACTERIOPHAGE COCKTAIL BEC8 ON THREE SURFACE MATERIALS (STAINLESS STEEL, CERAMIC TILE, HIGH-DENSITY POLYETHYLENE) AND TESTED AS LIQUID SUSPENSIONS (POSITIVE CONTROL) OR AFTER DRYING ON SURFACES

Surface materials	Temperature (C)	Liquid cells				Dry cells			
		D value (min)	Lower 95% CL	Upper 95% CL	R^2	D value (min)	Lower 95% CL	Upper 95% CL	R^2
Stainless steel	12	42.9aA	33.5	59.4	0.87	46.0aA	35.6	65.1	0.85
	23	23.0aA	17.4	33.8	0.83	30.0aA	25.3	37.1	0.93
	30	4.6bB	3.9	6.0	0.91	8.8bA	6.4	13.9	0.83
	37	4.8bA	4.7	7.8	0.94	5.9bA	4.7	7.8	0.91
Ceramic tile	12	32.6aA	23.9	51.1	0.79	46.7aA	32.1	85.5	0.71
	23	21.3aA	15.6	33.6	0.79	29.3aA	22.3	42.8	0.83
	30	3.8bA	2.7	6.1	0.82	6.6bA	4.6	12.0	0.79
	37	3.9bA	2.8	6.1	0.84	5.1bA	3.6	8.6	0.80
High-density polyethylene	12	39.1aA	26.8	72.1	0.70	46.5aA	33.5	76.1	0.77
	23	24.9aA	18.6	37.5	0.81	32.1aA	26.7	40.2	0.92
	30	5.2bA	3.7	8.5	0.81	7.0bA	4.7	14.3	0.72
	37	4.8bA	3.2	9.9	0.71	7.1bA	4.9	12.9	0.77

Note: Bacterial mixture included strains I 2005003658-472, TWO8635-EK27 and ATCC 43895.
D values with the same lowercase and uppercase letters are not significantly different within the same column and row, respectively.

TABLE 2. MEAN TIME AND 95% CONFIDENCE LEVEL (CL) FOR A 5-LOG REDUCTION (LIMIT OF DETECTION) OF DRY AND LIQUID (POSITIVE CONTROL) *ESCHERICHIA COLI* O157:H7 STRAINS INACTIVATED BY BACTERIOPHAGE COCKTAIL BEC8 ON THREE SURFACE CHIPS

Surface materials	Temperature (C)	Liquid cells			Dry cells		
		Time to 5-log reduction (min)	Lower 95% CL	Upper 95% CL	Time to 5-log reduction (min)	Lower 95% CL	Upper 95% CL
Stainless steel	12	189aA	152	254	205aA	164	281
	23	99bA	79	135	135bA	117	161
	30	20cA	16	25	37cB	28	56
	37	23cA	20	28	26cA	22	33
Ceramic tile	12	130abA	101	192	190abA	138	331
	23	85abA	67	123	110abA	89	153
	30	23cA	13	16	25cB	19	42
	37	15cA	12	22	20cA	16	31
High-density polyethylene	12	168abA	122	290	196abA	147	306
	23	101bA	80	141	129abA	110	156
	30	22cA	17	34	28cA	20	52
	37	21cA	15	37	27cA	20	45

Note: Bacterial mixture included strains I 2005003658-472, TWO8635-EK27 and ATCC 43895.
D values with the same lowercase and uppercase letters are not significantly different within the same column and row, respectively.

2009). However, there have not been studies in which the action of phages was treated as an antimicrobial, and inactivation kinetics was taken under consideration.

The most important element of inactivating bacteria by using bacteriophages is not necessarily phage replication but, instead, phage adsorption (Abedon 2009). Phages display single-hit killing kinetics, and therefore, the adsorption of a single phage into a single bacterium results in the death of both (Bull and Regoes 2006). However, more than one phage can attach to an individual bacterial cell, thus more than a one-to-one ratio of phages to bacterial cells are required for efficient inactivation of high populations of pathogenic bacteria. One could calculate the fraction of bacteria that are predicted to have not been phage adsorbed, assuming a Poisson distribution, as equal to e^{-M}, where e is the root of the natural logarithm (ln) and $M =$ MOI. If MOI = 10, then the number of bacteria left uninfected is approximately 1 in 20,000. This number is a good approximation of substantial, if not complete, bacterial killing (Abedon 2009). However, this study showed that when an even higher MOI of 100 was used, there were still bacterial survivors for both dry and liquid cells. It seems as if the surfaces provide some protective effect, as shown by the generally higher *D* and *Z* values of the dry cells as compared to the liquid cells.

There are two ways by which a high enough MOI can be achieved that can successfully eliminate the target bacteria; either by adding sufficient phages or by allowing phage replication to supply adequate densities. The first option has been described as a passive treatment, whereas the second option is an active treatment (Payne and Jansen 2000). The present study used the passive treatment, which has been found to be relatively successful in other phage application

studies and provides greater guarantee that sufficient phage numbers will be provided to combat target bacteria (O'Flynn *et al.* 2004; Sharma *et al.* 2005; Abuladze *et al.* 2008; Abedon 2009). This study used the phage cocktail as a chemical antimicrobial in its approach to determining *D* and *Z* values and the survival and propagation of the phages was not taken under consideration. Furthermore, the findings presented in this set of experiments is in agreement with Rabinovitch *et al.* (2002), who reported that bacteriophage T4 development in *E. coli* and therefore *E. coli* inactivation was growth rate dependent.

The growth temperature and the physiology in general (Hadas *et al.* 1997) of the bacterial host play an important role in phage adsorption and propagation. In this study, there was no significant difference between the *D* values of the two lower temperatures of 12 and 23C. Also, there was no significant difference between the two higher temperatures of 30 and 37C. However, there was a significant difference between the *D* values at lower and higher incubation temperature ranges. In addition, the *D* values obtained at lower temperatures were significantly higher than those of the higher temperatures.

In a study by Woody and Cliver (1995), one-step replication experiments indicated that the coliphage replicative cycle is prolonged and that fewer progeny are released as the temperature decreases. Furthermore, phage replication was decreased as a consequence of fewer cells infected as the temperature was lowered or as host cells entered the stationary phase. However, the numbers of phage particles released from infected cells did not change regardless of host growth temperature. In addition, O'Flynn *et al.* (2004) also found that the bacterial viable numbers of *E. coli* O157:H7 strain P1432 during a 12C challenge test were not reduced by the
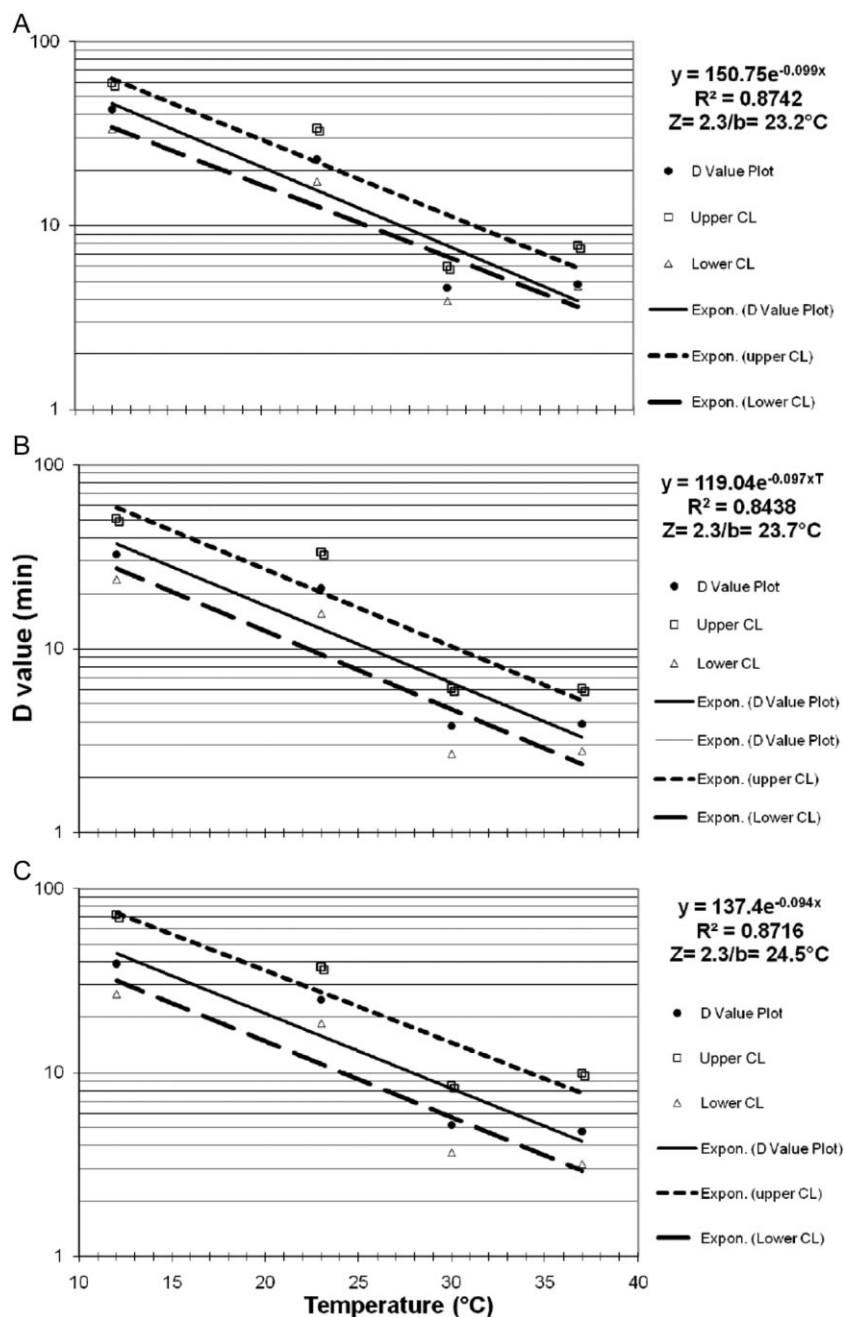
FIG. 2. D-PLOTS SHOWING THE *Z* VALUES OF THE INACTIVATION OF A STRAIN MIXTURE OF LIQUID CELLS OF *ESCHERICHIA COLI* O157:H7 BY PHAGE COCKTAIL BEC8 ON (A) STAINLESS STEEL, (B) CERAMIC TILE AND (C) HIGH-DENSITY POLYETHYLENE CHIPS

phage cocktail or individual phage addition because the bacterial culture did not grow during this period. This helps explain, in part, the higher *D* values of the lower incubation temperatures observed in this study.

It should be noted that in a food plant, it is likely that phages would be sprayed onto the contact surfaces as opposed to dropping the phage onto the surface. This is important because it addresses the issue of contact time between the phage and the bacterial cell, as it is probable that spraying the surfaces would lead to less contact time

between the phages and the bacteria. This is especially noted when compared to dropping a phage mixture directly onto the bacteria. Nonetheless, the experimental design in this study aimed to provide a proven method of quantifying the amount of phages that are applied onto the inoculated surfaces. Furthermore, the MOI would likely be affected when using a spray application, while dropping the phage mixture directly onto the inoculated surface kept the MOI at a constant level. It is important to mention that this study used actively growing vegetative cells. In reality, the bacteria are
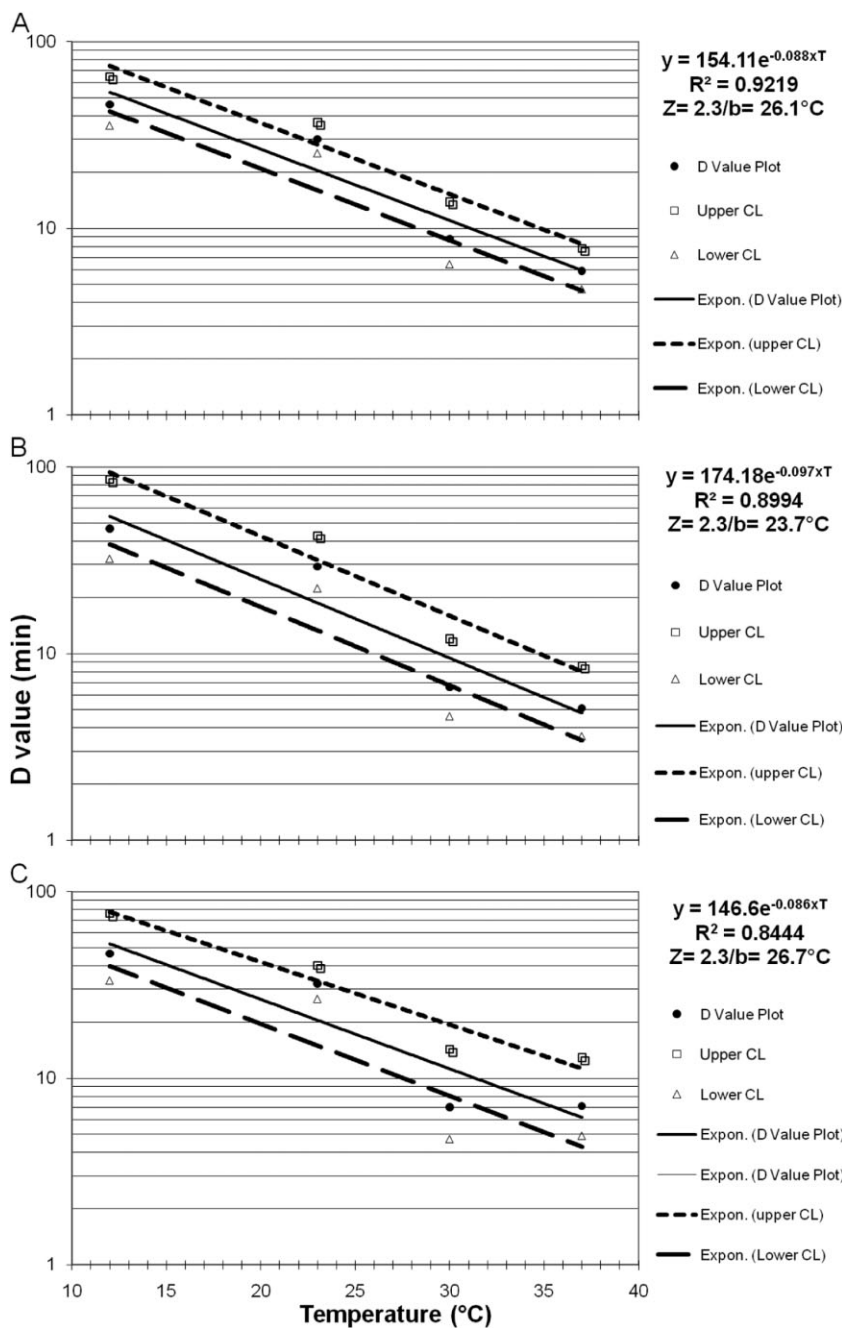
A

$$y = 154.11e^{-0.088 \times T}$$
$$R^2 = 0.9219$$
$$Z = 2.3/b = 26.1°C$$

● D Value Plot

□ Upper CL

△ Lower CL

— Expon. (D Value Plot)

- - - Expon. (upper CL)

— Expon. (Lower CL)

B

$$y = 174.18e^{-0.097 \times T}$$
$$R^2 = 0.8994$$
$$Z = 2.3/b = 23.7°C$$

● D Value Plot

□ Upper CL

△ Lower CL

— Expon. (D Value Plot)

- - - Expon. (upper CL)

— Expon. (Lower CL)

C

$$y = 146.6e^{-0.086 \times T}$$
$$R^2 = 0.8444$$
$$Z = 2.3/b = 26.7°C$$

● D Value Plot

□ Upper CL

△ Lower CL

— Expon. (D Value Plot)

— Expon. (D Value Plot)

- - - Expon. (upper CL)

— Expon. (Lower CL)

FIG. 3. D-PLOTS SHOWING THE *Z* VALUES OF THE INACTIVATION OF A STRAIN MIXTURE OF DRY CELLS OF *ESCHERICHIA COLI* O157:H7 BY PHAGE COCKTAIL BEC8 ON (A) STAINLESS STEEL, (B) CERAMIC TILE AND (C) HIGH-DENSITY POLYETHYLENE CHIPS

likely to be stressed, especially since a separate cleaning step would most likely be applied to remove organic debris before the phage mixture was applied, and this would presumably make phage infection and killing more difficult. Lastly, it should be noted that the longest drying time was 60 min, which is short for drying to occur. Longer drying times would have allowed for the question of stress, due to desiccation, to be explored and also would have more adequately mimicked what happens in industry, such as the

potentially long time between a food contact surface contamination event and cleaning and sanitation. This study aimed to determine the inactivation kinetics of a mixture of growing vegetative EHEC O157:H7 strains when treated with a bacteriophage cocktail and applied on surfaces of materials commonly found in food processing plants. Future studies could shed light on the effectiveness of the phage mixture on stressed and nonvegetative cells by taking the aforementioned parameters under consideration.

E. coli O157:H7 is a prevalent pathogen with a low infectious dose. Bacteriophages have been shown capable of reducing contamination of food processing equipment and surfaces by bacterial pathogens. The data presented in this study suggest that *E. coli* O157:H7-specific phages can be a potential intervention against foodborne pathogens. However, one must consider the long times to achieve a 5-log reduction when initial cell counts are high if one considers the 95% CL. It would take approximately 2–4 h, depending on the surface for a 5-log cfu reduction of liquid cells at 23C; thus, both the time length and the temperature of application must fit within the industry time scales.

ACKNOWLEDGMENT

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