Escuela Agricola Panamericana, Zamorano

Food Science and Technology Department

Food Science and Technology Major

Graduation Research Project

The effect of PhageGuard intervention on *Escherichia coli* O157:H7 applied to beef hides.

Student

Andres Isai Martinez Gutierrez

Advisors

Markus Miller, Ph.D.

Mindy Brashears, Ph.D.

Ligia Luna, M.Sc.

Honduras, november 2023

Authorities

SERGIO ANDRÉS RODRIGUEZ ROYO

President

ANA M. MAIER ACOSTA

Vice President and Academic Dean

ADELA M. ACOSTA MARCHETTI

Head of Food Science and Technology Department

HUGO ZAVALA MEMBREÑO

General Secretary

Acknowledgments

I thank Mindy M. Brashears, PhD and Markus F. Miller, PhD for the trust, opportunity and unconditional support they placed in me by giving me the opportunity to complete my project at Texas Tech University. I would also like to thank Ligia Luna, MSc. for her patience, attention, support and her dedication in helping me to carry out this project in the best way possible, and Karla M. Rodriguez MSc. and Rigo Soler Diaz MSc. for their attention and support along the way to carry out the project.

Content

List of Tables

List of Figures

List of Appendices

Abstract

An in-plant study was conducted to assess the antimicrobial effectiveness of the Phage Guard intervention against E. coli O157:H7 in cattle stalls before slaughter. The reduction in microbial surface area was evaluated by collecting 500-600 cm^2 hide samples from the cleanest area of the cattle during slaughter at the Gordon W. Davis Meat Laboratory. Three replicates, each comprising nine beef hide samples, were obtained. These samples were inoculated with an E. coli O157:H7 cocktail via spray application. Throughout the study, periodic collections of inoculated hide samples were performed, and bacterial loads were quantified using the Drop Dilution method and TEMPO System. The TEMPO System proved to be the most suitable and accurate method for quantifying E. coli O157:H7 on beef cattle hides. The study's findings revealed no significant differences between treatments. Potential factors contributing to this outcome included the chosen temperature and exposure time for the bacteriophage's optimal hatching, growth, multiplication, and lysis, typically 37 °C and 30 minutes. However, under the specified conditions, a substantial reduction in E. coli O157:H7 with the PhageGuard E product was not observed. In conclusion, the environmental temperature and exposure time (15 minutes), considering the minimum wait time for beef cattle before slaughter, were insufficient to produce significant reductions in bacteria.

Keywords: phage cocktail, drop dilution, phage hatching, lysis phase

Resumen

Se llevó a cabo un estudio en planta para evaluar la efectividad antimicrobiana de la intervención Phage Guard contra *E. coli* O157:H7 en los corrales de ganado antes del sacrificio. La reducción en el área de superficie microbiana se evaluó mediante la recolección de muestras de piel de 500-600 cm2 de la zona más limpia del ganado durante el sacrificio en el Laboratorio de Carne Gordon W. Davis. Se obtuvieron tres réplicas, cada una compuesta por nueve muestras de piel de res. Estas muestras fueron inoculadas con una mezcla de *E. coli* O157:H7 mediante aplicación de pulverización. A lo largo del estudio, se realizaron colecciones periódicas de muestras de piel inoculadas y se cuantificaron las cargas bacterianas mediante el método de dilución por gotas y el Sistema TEMPO. El Sistema TEMPO demostró ser el método más adecuado y preciso para cuantificar *E. coli* O157:H7 en la piel del ganado bovino. Los hallazgos del estudio no revelaron diferencias significativas entre los tratamientos. Los factores potenciales que contribuyeron a este resultado incluyeron la temperatura y el tiempo de exposición elegidos para la eclosión, crecimiento, multiplicación y lisis óptimos del bacteriófago, típicamente 37 °C y 30 minutos. Sin embargo, bajo las condiciones especificadas, no se observó una reducción sustancial de *E. coli* O157:H7 con el producto PhageGuard E. En conclusión, la temperatura ambiental y el tiempo de exposición (15 minutos), considerando el tiempo de espera mínimo para el ganado bovino antes del sacrificio, resultaron insuficientes para producir reducciones significativas en las bacterias.

Palabras claves: cóctel de fagos, dilución de gotas, eclosión de fagos, fase de lisis.

Introduction

The contamination of food by microorganisms has caused a serious problem in public health and a high economic loss worldwide. Food contamination by pathogenic microorganisms has emerged as a significant public health concern, leading to substantial economic losses on a global scale (Bintsis, 2017). One prevalent example of such contamination is the presence of foodborne pathogenic *Escherichia coli* (*E. coli*), including strains such as *E. coli* O157 and O104, which are prevalent even in developed nations (Yang et al., 2017). The occurrence of these pathogens in food products poses a serious risk to consumer health and necessitates rigorous preventive measures and interventions throughout the food production and supply chain.

Entero-hemorrhagic *E. coli (EHEC)* which produces Shiga-like toxin or also known as verotoxin, is responsible for inducing hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in the human population. These outbreaks are often associated with the consumption of bovine-derived food products. *E. coli* O157:H7 is the main serotype of EHEC and has been responsible for the most severe and widespread outbreaks across the globe (Etcheverría y Padola, 2013).

People of any age can become infected with *E. coli* O157:H7 , but the groups most at risk for severe disease are children under 5 years old, adults over 65 years old, and people with weakened immune systems, such as people with HIV, diabetes, or undergoing cancer treatment (Joseph et al., 2020).

An estimated 73,480 cases of illnesses resulting from *E. coli* O157 infection are reported annually in the United States. These cases lead to approximately 2,168 hospitalizations and 61 deaths each year (Rangel et al., 2005). Furthermore, it is worth noting that this infection constitutes a significant factor contributing to acute renal failure in pediatric patients (Siegler et al., 1994).

Escherichia coli O157:H7 is present in the gastrointestinal tracts of animals, and due to fecal or food animal processing-related contamination, it is frequently detected in soil, water, and various food products. The emergence of *Enterohemorrhagic E. Coli (EHEC)* O157:H7 as a public health concern traces back to its initial recognition as a pathogen during a 1982 outbreak of illness linked to

the consumption of inadequately cooked ground *beef* (Schroeder et al., 2002). According to United States Department of Agriculture USDA, (2014) this fecal excretion in cattle is a hazard that occurs during pre-harvest and can continue in the feedlot pens. This fecal excretion can consequently contaminate the hides and the contamination can later be transferred to the carcass during cutting, making the main source of contamination of beef carcasses *Escherichia coli* O157:H7 and *Salmonella*, which are found in the hides of slaughtered animals and are the main source of contamination of beef carcasses (Arthur et al., 2007).

Applying preharvest interventions is important in the food industry as they can be applied in different control measures to avoid pathogen contamination, which can be classified into physical, chemical, and biological interventions. The biological intervention of bacteriophage (phages) in which are viral entities with the capacity to infect and potentially terminate bacterial organisms. Lytic phages, renowned for their attributes as natural bacterial predators, offer substantial promise as biocontrol agents against bacterial pathogens. This is attributed to their host-specificity, inherent capacity to target particular bacterial strains, and their non-interfering behavior in interactions with eukaryotic cells (Abedon, 2009). The application of bacteriophages (phages) has been innovatively devised as a preharvest antimicrobial strategy aimed at diminishing the prevalence of *E. coli* O157:H7 on cattle hides prior to processing. This approach seeks to curtail the transmission of this pathogenic microorganism to the carcass during processing, ultimately bolstering the safety of the end-product intended for consumption (Arthur et al., 2017).

Bacteriophages are quantifiable elements found in the natural microflora throughout the entire food production process, starting from the farm and extending to the retail outlet. These phages exhibit remarkable stability within these environments and can be easily detected and isolated from various sources such as soil, sewage, water, farm and processing plant wastewater, feces, and retail food products (Połaska y Sokołowska, 2019). During the preharvest stages of food production for both plant and animal-derived products, strategies utilizing phages as a form of biocontrol have been

primarily focused on combating plant and animal pathogens that may pose a risk to human health. These phage-based approaches aim to enhance the yield and quality of food products during these early stages of the production process. Additionally, in the context of animal-derived foods, phages can be utilized to mitigate the spread of human pathogens within the environment (Greer, 2005).

Phages life cycle generally consists of two main phases in Gram-negative bacteria (Kutter y Sulakvelidze, 2005)): the adsorption, and injection phase and hatching phase.

Adsorption: Is the process in which phage tail fibers bind to primary receptor molecules to trigger rearrangements in basal plate components, subsequently another tail protein irreversibly binds to a secondary receptor. Different phages bind to different receptors, usually an outer membrane protein as the primary receptor followed by a heptose residue from the inner core of the lipopolysaccharide (Mayer, 2008). After irreversible binding of the phage to the target cell, the phage DNA is injected into the target cell phage DNA is injected through the tail into the host cell. Once inside the cell, many phage DNAs are circularized, to avoid being substrate for exonucleases, by means of cohesive ends or have their linear ends protected (Kameyama et al., 2001). Replication is the process where new phage DNA is synthesized. For this, the synthesis of nucleic acids and proteins of the host is stopped, and its genome begins to degrade. By the mentioned events, the phage takes control of the metabolic machinery of the host, synthesizing its nucleic acids and proteins.

Assembly of the virions: Once all the building blocks that make up the phage, namely DNA, head (icosahedral) and tail proteins as well as fibers and the proteins that make up the basal plate have been synthesized, the structural proteins are assembled. The structural proteins are assembled, and the de novo synthesized DNA is packaged in condensed form into the heads by a process that exhibits both cis- and trans-regulation. Finally, the tail is attached to the head to give rise to the complete phage virion (Tomat, 2013).

Hatching Phase: The hatching phase is the final stage of the phage life cycle, during which the new viral progeny particles must exit the bacterial cell to infect new bacteria. To accomplish this,

phages utilize enzymes and proteins that degrade and weaken the cell wall of the host bacterium. Once the cell wall is weakened sufficiently, the bacterial cell ruptures or lyses, releasing the new viral progeny particles into the surrounding environment, where they can seek out and infect other bacteria (Openstax, 2005).

Intervention studies are an effective way that can be applied in a processing plant to evaluate the "before" and "after" treatments to analyze and assess with certainty how effective the microbial load reduction of the product is to be used. These interventions should be applied at the beginning of the process in the stabling zone up to the final part of the process (Zdolec et al., 2022).

That's why the bacteriophage for *E. Coli* O157:H7 will be used in this project, in how plays a crucial role in regulating host bacterial populations, effectively maintaining control over their density. Through their ability to specifically target and destroy bacteria, lytic phages employ an important strategy for treating infectious diseases and managing detrimental pathogens within diverse environmental ecosystems (Parmar et al., 2017). Accordingly, the following objectives have been set:

To determine the antimicrobial efficacy of the PhageGuard E on *E. Coli* O157:H7 at different exposure times applied onto beef cattle hide.

To determine the log reduction of *E. Coli* O157:H7 using two different concentration levels of the PhageGuard E intervention.

To compare the results obtained with the TEMPO method versus the droplet dilution method in evaluating the results similarity of *E. Coli* O157:H7 in beef hides.

Materials and Methods

Study Site

Samples were collected from cattle processed at the Gordon W. Davis meat lab located in the Animal and Food Science Department. In addition, microbiological analysis was performed at the Food Microbiology laboratory located at the Experimental Science Building at Texas Tech University.

Phase I. Sampling at the Abattoir and Technical Specification (Beef Hides Collection)

Approximately 9 pieces were taken of hide samples from the cleanest part of the animal (Back). Samples were taken throughout the slaughter day at the Gordon W. Davis meat laboratory. A 500-600 cm^2 of hide was cut (Vargas et al., 2022), and in which it was inoculated and treated for further analysis. The cuts of beef hides were immediately chilled and placed in a Ziploc bag to be transported in coolers to the Experimental Science Building for further microbiological analysis.

Phase II. Preparation of the *E. coli* O157:H7 Cocktail

A frozen stock culture of different strains of *E. coli* O157:H7 (Table 1) were removed from the -27 °C (-80 °F) freezer, spread onto plates on TSA agar, and incubated for 18-24 h at 37 °C. A single colony of each strain (Table 1) was individually transferred to 9 mL BHI tubes and incubated at 37 °C for 18-24 h. After that, a volume of 1mL of the BHI tubes was transferred to different 9.0 mL Buffer Peptone Water (BPW) tubes in triplicate for each strain and incubated at 37 °C for 18-24h. To prepare the cocktail, each strain in BPW tubes (Table 1) was combined and the final volume will be adjusted to 70 mL BPW with an inoculum concentration of 9.5 log CFU/mL in which was verified using the nephelometer (Porto-Fett et al., 2008).

Table 1

Escherichia Coli O157:H7 Strains Description.

Organism	Serotype	ATCC	Isolation
E. Coli	O157:H7	43889	Clinical-human patient with hemolytic uremic syndrome
E. Coli	O157:H7	43895	Raw hamburger meat implicated in hemorrhagic colitis outbreak

Organism	Serotype	ATCC	Isolation
E. Coli	O157:H7	43894	Human feces from outbreak of hemorrhagic colitis, Michigan
E. Coli	O157:H7	51658	Clinical isolate, King Co., WA
E. Coli	O157:H7	43888	Human feces
E. Coli	O157:H7	51657	Clinical isolate, Mason Co., WA
E. Coli	O157:H7	35150	Feces, human

Treatments

In this study three treatments were performed (Table 2), Treatment 1 represents the water, Treatment 2 the PhageGuard E product with a concentration of 1x10^7 PFU/cm2 (low dose) and Treatment 3 the PhageGuard E product always with a concentration of 1x10^7 PFU/cm2 (double dose) and three repetitions of each treatment at different time points (before inoculation, after inoculation, 0 min, 5 min, 10 min, and 15 min) where a total of 54 experimental units were obtained.

Table 2

Samples ID taken from Beef Hides of the Treatments at Different Timepoints.

Repetitions	Trial Treatment	Pathogen	Before Inoculation sample ID	After Inoculation sample ID	0 min sample ID	5 min sample ID	10 sample ID	15 min sample ID
1	Water	O157:H7	1	2	3	4	5	6
	PG1X	O157:H7	7	8	9	10	11	12
	PG2X	O157:H7	13	14	15	16	17	18
2	Water	O157:H7	19	20	21	22	23	24
	PG1X	O157:H7	25	26	27	28	29	30
	PG2X	O157:H7	31	32	33	34	35	36
3	Water	O157:H7	37	38	39	40	41	42
	PG1X	O157:H7	43	44	45	46	47	48
	PG2X	O157:H7	49	50	51	52	53	54

Note. Treatment 1 Water, Treatment 2 Phageguard 1 dosis application (PG1X), Treatment 3 Phageguard double dosis application (PG2X). Number of samples: Fifty-four samples were taken, where in the table samples 1-18 represent replicate #1, samples 19-36 represent replicate #2, and samples 37-54 represent replicate #3. The *E. coli* O157:H7 cocktail was used and then the stipulated treatments were applied.

Phase III. Hides Cleaning, Sample Preparation, Cocktail Application, and Sample Taking

Hides Cleaning

The combination of water and dishwashing liquid to clean the beef hides gave a pH: 7.99 in which it was verified using the Ph meter and Ph: 7.5-8 with the McolorpHast.

Each beef hide was prewashed for 1 minute with the same amount of water and dishwashing liquid dilution and placed in a clean place for drying for 1 hour and 30 minutes in ambient laboratory temperature. Once dried, the skins were stored in Ziploc bags inside the cold room at a temperature of 4 °C.

Sample Preparation

Phageguard E Dosage.

The dilution of the product was at log 10^7 PFU/cm².

Spray bottles were prepared with the different Treatments and Cocktail (T1: Water, T2: Phageguard E 1X at 10^7 dilution, T3: Phageguard E 2X at 10^7 dilution and *E. coli* O157:H7 cocktail).

The 9 washed skins were removed from the cold room where they were marked with 6 squares of 100 cm² template per beef skin to obtain a total of 54 sampling units.

For each beef skin: 6 sampling points (before inoculation, after inoculation, 0 minutes, 5 minutes, 10 minutes, 15 minutes).

Cocktail and Treatments Application

Beef Hides were placed inside a biological safety cabinet where the cocktail and treatments were applied with the quantities show in Table 3, and the samples were taken using 25 mL EZ-Reach sponges pre-hydrated with neutralized BPW at each of the established sampling points show in Table 4.

Table 3

Spray Quantities Application on Beef Hides

Cocktail and Treatments	Spray quantities on beef hides
E. Coli O157:H7	Spray 2 times = 1mL
T1: Water	Spray 2 times = 1mL
T2: PhageGuard 1X	Spray 2 times = 1mL
T3: PhageGuard 2X	Spray 4 times = 2mL

Table 4

Application Procedure and Sample taking for each Timepoint (Squares of 100 cm^2 marked on Beef Hide)

Sampling points	Observation	Step 1	Step 2	Step 3
Before inoculation	Cleaning protocol efficiency of the beef hides	Sample taking	-	-
After Inoculation	Inoculation of *E. Coli* O157:H7 cocktail protocol efficiency of the beef hides	*E. Coli* O157:H7 cocktail application with 30 minutes of attachement	The sample was taken	-
0 minutes	Treatments efficiency in *E. Coli* O157:H7 log UFC/mL reduction on beef hides	*E. Coli* O157:H7 cocktail application with 30 minutes of attachement	Treatments application	After 0 minutes the sample was taken
5 minutes	Treatments efficiency in *E. Coli* O157:H7 log UFC/mL reduction on beef hides	*E. Coli* O157:H7 cocktail application with 30 minutes of attachement	Treatments application	After 5 minutes the sample was taken
10 minutes	Treatments efficiency in *E. Coli* O157:H7 log UFC/mL reduction on beef hides	*E. Coli* O157:H7 cocktail application with 30 minutes of attachement	Treatments application	After 10 minutes the sample was taken
15 minutes	Treatments efficiency in *E. Coli* O157:H7 log UFC/mL reduction on beef hides	*E. Coli* O157:H7 cocktail application with 30 minutes of attachement	Treatments application	After 15 minutes the sample was taken

Phase IV. Microbiological Analysis

Hide Swab Analysis for TEMPO Quantification (Enterobacteria) (Biomerieux, 2010)

Samples collected were tested by the TEMPO system to analyze total viable counts using the enterobacterial kit (TEMPO EB) for *E. coli* O157:H7. The TEMPO test consists of a vial of culture medium and a card, which are specific for each test. For the "before inoculation" samples applied 1 mL of sample to a 3 mL vial (EB) of autoclaved distilled water. For the rest of the timepoints (After inoculation, 0 minutes, 5 minutes, 10 minutes, 15 minutes) a dilution of 10^4 was performed, applying 1 mL of samples to a 9 mL tubes of Buffer Peptone Water (BPW) and in which 1 mL of the sample of the 10^4 tubes were transferred in a 3 mL vial (EB) of autoclaved distilled water. The inoculated medium in the Enterobacteria (EB) vials were mixed using the Vortex and transferred by the TEMPO Filler

instrument to the card containing 48 wells of three different volumes. The card contains 3 sets of 16 wells (small, medium and large wells) with a logarithmic difference in volume for each set of wells. The card is designed to simulate the most probable number (MPN) method (Biomerieux, 2010). The card was then hermetically sealed to avoid any risk of contamination during subsequent handling. The microorganisms present on the card reduce the substrate of the culture medium during incubation in this case at 35 °C for 22-27 hours and where they cause the appearance of a fluorescent signal, which is detected by the TEMPO Reader instrument. Depending on the number and type of positive wells, the TEMPO system calculates the number of microorganisms present in the original sample according to a calculation based on the MPN method.

Hide Swab Analysis for Plating Quantification using Drop Drop Dilution Method

Rainbow Agar O157 (Biolog, 2008) or ChromAgar (Becton, 2009) medium can be used, but in this project de ChromAgar medium with a surface area of 20 mL in Petri plates was used. When the plates were already dry, they were stored in a black bag to protect them from the light inside on the walking cooler at a temperature of 2 °C.

For this project, in order to use fewer petri plates and obtain more results, the Drop Dilution Method (Chen et al., 2003) was use in which 250 ul of each sample in the first well was placed in each row of a 96-well plate, and 10-fold serial dilutions were performed using a multichannel pipette by transferring 20 ul from the column to 180 ul of Buffer Pepton Water (BPW) medium.

Three replicates of 10 ul of each of the three selected dilutions were then seeded onto ChromAgar medium plates, using a multichannel pipette. Plates were incubated at 37 °C for 18 hours. A Leica Darkfield Quebec colony counter was used for counting to enumerate colonies more accurately and avoid human error.

The 10 ul drops of sample were counted from 3 to 30 colonies (Herigstad et al., 2001). This criterion aligns with the SP method, where counting is performed at a sample dilution containing 30 to 300 CFUs per plate.

Experimental Design and Statistical Analysis

A Completely Randomized Design was made with repeated measures over time, with three replicates per treatment. The analysis was carried out with the Statistical Analysis System (SAS® version 9.4), making an ANOVA and Duncan mean separation. The Treatments, these being the Water application, Phageguard application with a concentration of log 10^7 PFU, and Phageguard E with a double application at a concentration of log 10^7. These three Treatments apply to four different timepoints (0 minutes, 5 minutes, 10 minutes, and 15 minutes). Finally, to compare the two methods, a Linear Regression Model was performed for each of the samples taken in the two methods with an experimental error of $P \leq 0.05$.

Results and Discussion

After applying the treatments at the sampling points “0 minutes”, “5 minutes”, “10 minutes” and “15 minutes”, the results shown Table 5 and 6, the contamination values of *Escherichia coli* O157: H7 were maintained compared with the sampling point “after inoculation” which represent the inoculated load of *E. coli* O157:H7 in where a significant increase of 5.1 log CFU/mL comparing to the sampling point “before inoculation” in which the cleaning of the hides were carried out, showing that the treatments had no significant differences at the exposure times evaluated (0 min, 5 min, 10 min, and 15 min), according with the results obtained in TEMPO methods (P-value was >0.05 with a P-value = 0.811) and Drop Dilution (P-value was >0.05 with a P-value = 0.799).

Table 5

Escherichia coli O157:H7 count by TEMPO System (log CFU/100 cm2) in beef hides samples at different timepoints

Timepoints	Water Log CFU/Ml± S.D	Phageguard 1X Log CFU/Ml± S.D	Phageguard 2X Log CFU/Ml± S.D	C.V (%)
Before inoculation	1.76±1.51^{bx}	1.78±0.45^{bx}	2.37±0.85^{bx}	45.79
After inoculation	6.88±0.69^{ax}	6.93±0.32^{ax}	6.95±0.37^{ax}	7.35
0 minutes	6.87±0.20^{ax}	7.29±0.25^{ax}	6.61±0.78^{ax}	6.44
5 minutes	7.03±0.33^{ax}	6.75±0.09^{ax}	6.83±0.19^{ax}	2.99
10 minutes	7.13±0.08^{ax}	7.23±0.16^{ax}	6.99±0.18^{ax}	1.90
15 minutes	6.98±0.43^{ax}	7.13±0.38^{ax}	6.78±0.11^{ax}	4.02

Note. S.D: Standard deviation.CV: Coefficient of variation.^{ab} Means with different lowercase letters for E. Coli log CFU/Ml indicate significant differences (P<0.05) in each column; ^{x} Means indicates no significant difference (P>0.05) in each row. Each microbiological count Log CFU/Ml of before inoculation, after inoculation, 0 min., 5 min., 10 min., 15 min. represents the mean of 3 repetitions.

Table 6

Escherichia coli O157:H7 count by Drop Dilution (log CFU/100 cm2) in beef hides samples at Different Timepoints

Timepoints	Water Log CFU/Ml± S.D	Phageguard 1X Log CFU/Ml± S.D	Phageguard 2X Log CFU/Ml± S.D	C.V (%)
Before inoculation	1.70±0.00^{bx}	2.23±0.47^{bx}	2.00±0.52^{bx}	20.50
After inoculation	5.28±3.31^{ax}	6.92±0.75^{ax}	7.01±0.40^{ax}	30.82
0 minutes	6.08±2.17^{ax}	7.25±0.31^{ax}	6.45±0.25^{ax}	19.29
5 minutes	7.05±1.17^{ax}	6.60±0.82^{ax}	7.36±0.22^{ax}	10.60

Timepoints	Water	Phageguard 1X	Phageguard 2X	C.V (%)
	Log CFU/Ml± S.D	Log CFU/Ml± S.D	Log CFU/Ml± S.D	
10 minutes	4.43±2.37^{ax}	7.09±0.34^{ax}	7.07±0.12^{ax}	22.34
15 minutes	4.83±1.16^{ax}	6.85±0.26^{ax}	7.11±0.45^{ax}	25.48

Note. S.D: Standard deviation.CV: Coefficient of variation.^{ab} Means with different lowercase letters for E. Coli log CFU/mL indicate significant differences (P<0.05) in each column; ^{x} Means indicates no significant difference (P>0.05) in each row. Each microbiological count Log CFU/mL of before inoculation, after inoculation, 0 min., 5 min., 10 min., 15 min. represents the mean of 3 repetitions.

The factor of time may have exerted an influential role, as indicated by the lack of statistically significant differentiation in the reduction of log CFU/mL in *Escherichia coli* O157:H7. Consequently, the bacteriophage, designated as Phageguard E, underwent an autonomous multiplication phase lasting up to 15 minutes as observed in Tables 4 and 5. Thus, there is a reasonable presumption that heightened effectiveness could have been achieved if the multiplication period was extended, given the precedent set by prior research, such as the study conducted by Viazis et al. (2011). In this study, a targeted point inoculation technique was employed, involving the application of specific bacteriophages tailored for E. coli O157:H7. This method yielded an observable augmentation in bacterial reductions of 3.36 log CFU after 24-hour timeframe, contrasting with the shorter durations of 10 minutes obtaining reduction values of 0.37 log CFU, under tested storage temperature of 37 °C.

On the other hand, the hatching phase of the bacteriophage could be another factor that affects the loads reduction of E. coli O157:H7 in all the treatments. This may be of great interest since in a study by Tomat (2013) in which the time and number of hatching in lambda (λ) phages used were determined, the hatching times of both phages were approximately 33 min at a temperature of 37 °C. Therefore, the time used in the Treatments in this study could have an impact in the activity of the phage, because was consider a maximum time of 15 min, which was not long enough for the PhageGuard E product to conclude its hatching phase. This process involves enzymes called endolysins, an enzyme capable of breaking down bacterial peptidoglycan molecules, and holins whose function is to assemble pores in the plasma membrane for the passage of endolysins. The end result of the action of these enzymes is the release of mature phage particles that have the ability to infect

new host cells to begin a new cycle of lysis. The number of phage particles released per infected bacterium constitutes the hatch number, while the time it takes for a de novo virion to emerge is the hatch time (Augusto, 2011).

In addition, temperature conditions assume significance for pivotal processes such as attachment, penetration, and replication. These factors, in turn, determine the viability and persistence of phages Pradeep et al. (2022). In this research, the temperature conditions were not examined, nor was a specific temperature of the beef hides maintained at the time of application of the bacteriophage in its different timepoints. In the case of the Phageguard E product, the optimum growth rate is 37°C as this is the optimum temperature for phages that directly attack the *Escherichia coli* O157:H7 bacterium Kameyama et al. (2001). This is also reinforced by Tomat (2013) study where the phages used in his study to attack *E. coli* were most effective at the highest temperature evaluated (37 °C), obtaining biocontrol values of up to 6.38 log CFU compare to 4 °C obtaining values of 3.79 log CFU after 24 h. The ideal temperature is 37 °C because bacteriophages can only replicate most effectively at the permissive temperature for bacterial growth (Kudva et al., 1999).

However, it is plausible to deduce that the temperature of the beef hides influenced the efficacy of the bacteriophage. The beef hides were stored in a cold room at a temperature of 4 °C and then at room temperature of 22 °C when the hide where inside the safety biological cabinet after the beef hides cleaning protocol was performed. In a previous study conducted by Pradeep et al. (2022), in which the influence of temperature on phage stability and antimicrobial activity against bacteria was analyzed, a significant decrease ($p<0.05$) in phage propagation was observed at temperatures below 26 °C and above 45 °C compared to those cultured between 26 °C and 45 °C. This is mentioned in relation to the removal of the beef hides used in the study, where they were previously kept for less than 24 hours at a temperature ranging from 4 °C in the refrigeration chamber, and it can be assumed that the beef hides where at temperature below 22-25 °C. That could explain the results in which there are no significant differences between the treatments in the results ($p>0.05$).

The dosis levels of the PhageGuard E Treatment on the other hand did not have an influence in the effectiveness of the reduction of the contamination of the *Escherichia coli* O157:H7 as it can be observe in Tables 4 and 5 in where the log UFC/mL of the samples points (0 min., 5 min., 10 min., and 15 min.) in the Treatments "PhageGuard E 1X"(recommended dosis level 1x107 PFU/cm²) and "PhageGuard E 2X"(double dosis level with the same dilution 1x107 PFU/cm² as a double coating) are similar demonstrating no significant difference between them.

The high dose level with the low dose level at the same concentration did not obtain differences because even at lower concentrations the method of action of the bacteriophage was saturated and that at lower concentrations could still be effective, according to results obtained by Worley-Morse et al. (2014) where different concentrations of phages from 105 to 108 PFU mL-1 were compared and where they produced a similar bacterial inhibition ($p > 0.05$). For some phages, higher phage concentrations may be useful up to a certain threshold, beyond which higher phage concentrations have no additive effect so it would make no difference to add more phages according to Zhang y Hu (2013).

Comparison Between the Methods TEMPO Analysis and Drop Dilution

A linear regression was performed to evaluate the relationship between two quantification methods (TEMPO Analysis and Drop Dilution) of *E. coli* O157:H7 concentration in two different Treatments (PhageGuard 1X and PhageGuard 2X). It was investigated how the values obtained by the droplet dilution method were related to the values obtained by TEMPO analysis for each of the treatments.

As a result, the data obtained from both methods are very similar in PhageGuard 1X and PhageGuard 2X, but in the Treatment of Water in case of Drop Dilution there were various plates without counting (values <1.7 log UFC/mL, which affected the results of the other Treatments (PhageGuard 1x and PhageGuard 2X) obtaining coefficient of variance more than 10% (Table 6). This leads to definition of the more precise method for counting *Escherichia coli* O157:H7 was TEMPO

System. Obtaining results in Water, PhageGuard 1X and PhageGuard2X Treatments a slope of -2.718, 0.57558, and 1.41137. This suggests that the Drop Dilution has a potential of quantifying bacteria, but TEMPO System shows more accurate results.

The adjusted R-squared (adjusted R-squared) measures the proportion of the variation in "LogCFU/mL in the TEMPO assay" that can be explained by the variation in "LogCFU/mL in the droplet dilution method". Since the R-squared was not close to 1 (approximately -.223 in "Water", 0.4171 in PhageGuard 1X, and 0.1971 in "PhageGuard 2X" in the results shown in Figure 1), it indicates that the model does not fit the data well and is not able to effectively predict or explain the relationships between variables. This is because the repetitions obtained in this study were not sufficient to have expected results in the linear regression analysis which is a predictive analysis.

The p-values obtained in the study, with a p-value >0.05, suggest that there is not enough evidence to claim that there is a significant relationship between the independent variables and the dependent variable. This is because p-values of (0.6401, 0.1441 and 0.2539) were obtained for Water, PhageGuard 1X and PhageGuard 2X, respectively. Therefore, based on these results (Figure 1), there is no relationship between LogCFU/mL in the TEMPO analysis and LogCFU/mL in the Drop Dilution method across Treatments.

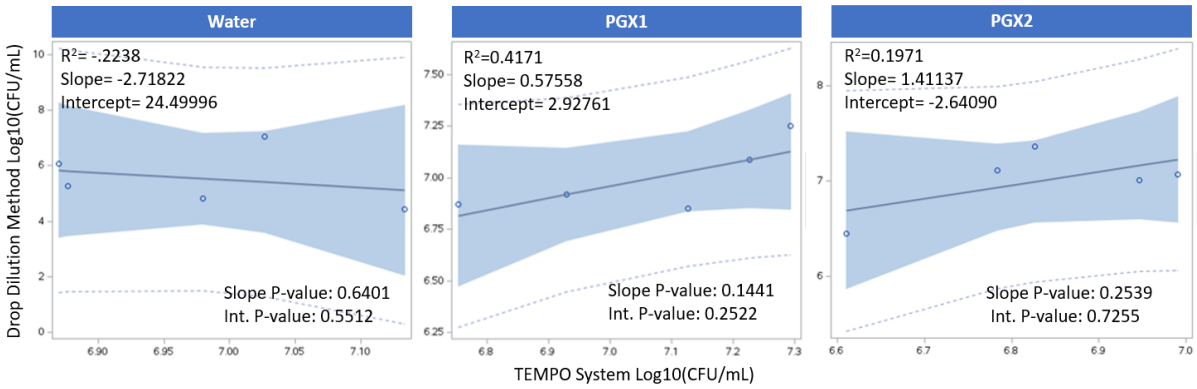
A study conducted by Cayer et al. (2020) in which a linear regression was performed between the TEMPO equipment and the plate count method Comparative linear regression analyses demonstrate good correlations between the two methods ($R2 > 0.9$). At lower levels of bacterial contamination, the TEMPO® method precision (C.V. < 8%) and accuracy (> 83%) were comparable to plate.

In another study conducted by Naghili et al. (2013) in which a linear regression was performed between the drop dilution method and spread plate method in which successive dilutions of the second subculture of *Lactobacillus casei* and *Salmonella* Typhimurium were transferred to selective agar, the Spearman's rho correlation coefficient (r) across both methods due to the data distribution

patterns for the enumeration of *Salmonella Typhimurium* and *L. casei,* respectively, was 0.62 and 0.87, which represented a moderately strong and strong relationship between the two methods, respectively. *Salmonella Typhimurium* and *L. casei* were 0.62 and 0.87, respectively, representing a moderately strong and strong relationship between the two methods, respectively. In addition, there was a strong and significant positive correlation ($p < 0.001$) between the spread-plate and drop-plate procedures.

Figure 1

Drop Dilution and TEMPO Analysis Correlation



Note. Escherichia Coli O157:H7: LogCFU/mL. R2: Measure used to describe the strength of the relationship between Drop Dilution Method (y-axis) and TEMPO system (x-axis) measurements. Slope: As the values of the first method (TEMPO system) increase, the values of the second method (Drop Dilution Method) also increase. P-value: A p-value of <0.05 suggests that the relationship between TEMPO and DROP is statistically significant. Intercept: The predicted value of the dependent variable (DROP) when the independent variable (TEMPO) is equal to zero.

Conclusions

PhageGuard E exhibited no effectiveness in reducing *E. coli* O157:H7 contamination on beef cattle hides. The study revealed no differences between PhageGuard E Treatments and control Treatment (Water) across various exposure times and different doses. Consequently, PhageGuard E did not significantly reduce bacterial levels on the hide surface.

The 15 minutes multiplication period proved insufficient for achieving significant bacterial reductions on beef hide. This limitation is likely due to the bacteriophage not having adequate time to complete its lysis phase. Furthermore, the lack of temperature control within the beef hide during the study may have hindered the phage's ability to target and reduce *E. coli* O157:H7 effectively.

The study showed a strong linear regression in the PhageGuard 1X and PhageGuard 2X Treatments, but a moderate regression in the Water Treatment. This indicates that the Drop Dilution method has potential, but TEMPO Analysis is the most suitable and accurate method to quantify *E. coli* O157:H7 on beef cattle hide.

Recommendations

Perform a comparison of the BAX quantification, Drop Dilution method and TEMPO Analysis methods for microbiological quantification.

Increase the number to 15-20 repetitions to have lower variability and higher potential in future experiments respectively.

To obtain more accurate results of the efficiency of PhageGuard E bacteriophage, it is recommended to take into consideration the environmental conditions of the beef hide as a temperature of 37 °C and the optimal pH of 7-8.

Extend the multiplication time of the PhageGuard E. bacteriophage to 30 minutes, 45 minutes and 1 hour or longer to obtain more accurate results of bacterial reduction of *Escherichia coli* O157:H7.

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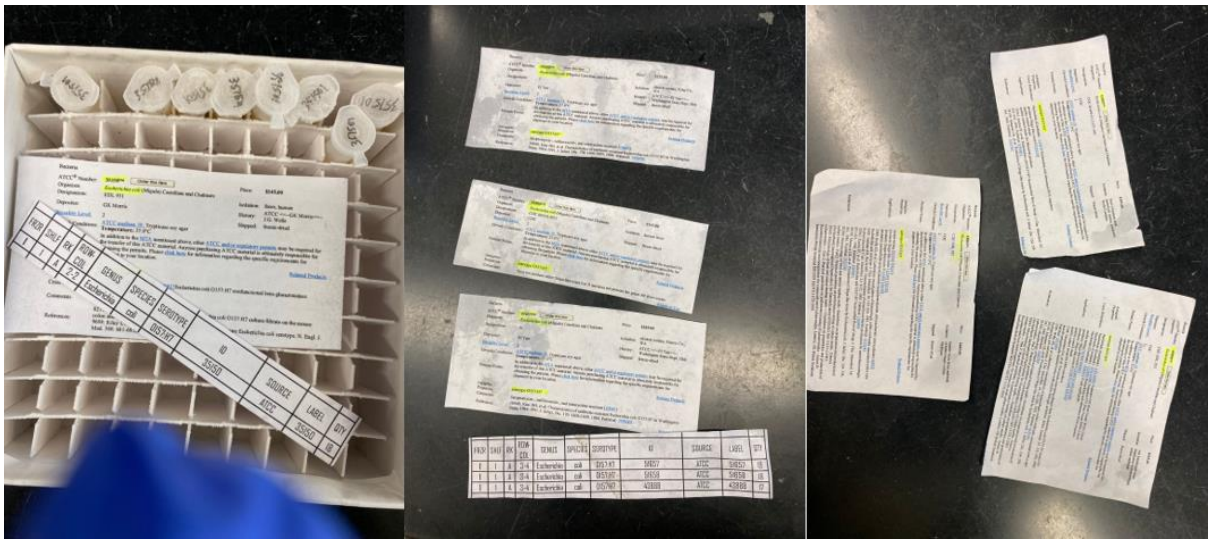
Appendices

Appendix A

Escherichia Coli O157:H7 Strain (35150, 51658, 43888, 51657, 43889,43895,43894

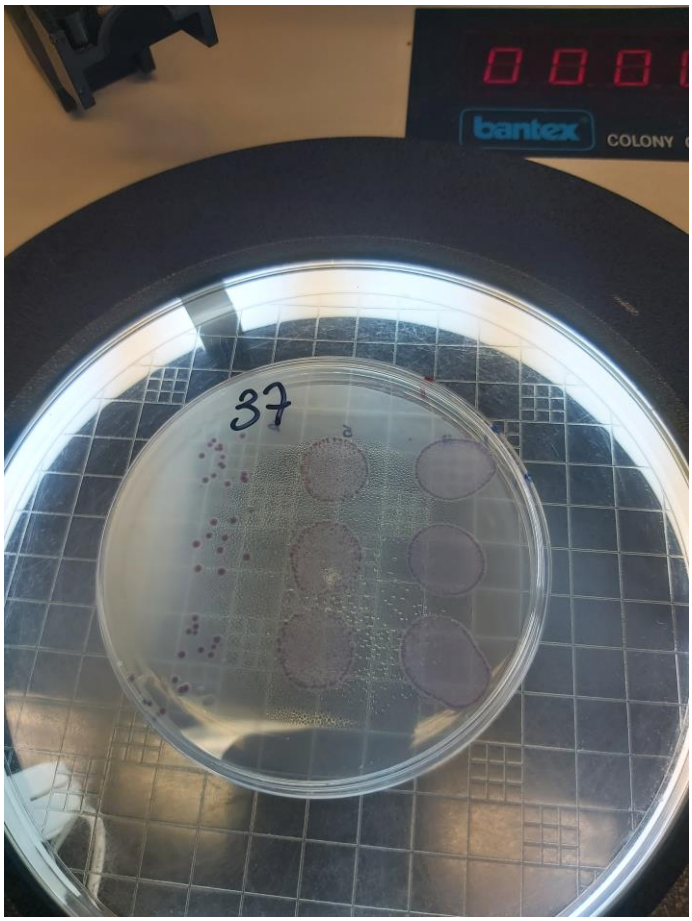


Appendix B

Beef Hides inside the Safety Cabinet with their respective timepoints

Appendix C

Drop Dilution Quantification using ChromAgar



Appendix D

Treatments (PhageGuard E and Water) and Cocktail Escherichia Coli O157:H7