#757

Office of Food Additive Safety (HFS-200)
Attn: Dr. Paulette Gaynor, DBGNR (HFS-255)
Center for Food Safety and Applied Nutrition
US Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

USA

January 15th, 2018

Dear Dr. Gaynor,

For your convenience, find enclosed a hard copy and digital version of GRAS notification of the bacteriophage cocktail PhageGuard E™ for bio-control of *E. coli* O157 on beef.

Sincerely,

Dr. Steven Hagens
Chief Scientific Officer
Micreos B.V.

Office of Food Additive Safety (HFS-200)
Attn: Dr. Paulette Gaynor, DBGNR (HFS-255)
Center for Food Safety and Applied Nutrition
US Food and Drug Administration
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College Park, MD 20740

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Dear Dr. Gaynor,

In accordance with 21 CFR part 170, Subpart E (GRAS notice for human use) published in the Federal Register. I am submitting, a GRAS notification of the bacteriophage cocktail PhageGuard E™ for bio-control of *E. coli* O157 on beef.
We would also like to request the agency to grant USDA/FSIS access to the data in order to review the intended use of PhageGuard E™ as a suitable processing aid in beef products. We would request USDA to include PhageGuard E™ in FSIS directive 7120.1.

Please let me know if you have any questions,

Sincerely,

Dr. Steven Hagens
Chief Scientific Officer
Micreos B.V.

Table of contents

I. GRAS Exemption Claim

Part 1: Signed statements and certification

A. Claim of Exemption from The Requirement for Premarket Approval Requirements Pursuant to 21 CFR part 170, Subpart E.

PhageGuard E™ was determined by Micreos B.V. to be generally recognized as safe through scientific procedures, and therefore exempt from the requirement of premarket approval, under the conditions of intended use as described below. The basis for this finding is described in the following sections

Signed

(b) (6)

01/15/2018

Dr. Steven Hagens
Chief Scientific Officer
Micreos B.V.

Date

B. Name and address of Notifier

Micreos B.V.
Nieuwe kanaal 7P
6709 PA Wageningen
The Netherlands

C. Common or Usual Name of the Notified Substance

PhageGuard E™

D. Conditions of Use

The intended use of PhageGuard E™ is as an antimicrobial on foodstuffs (notably beef carcasses, subprimals, beef cuts and trimmings intended for ground beef) to control *E. coli* O157 at an application rate of up to $1x10^9$ pfu (plaque forming units) per gram of food.

E. Basis for the GRAS Determination

Pursuant to 21 CFR part 170, Subpart E, Micreos has determined that PhageGuard E™ is GRAS through scientific procedures.

F. Availability of Information

All data and information that serve as basis for this GRAS determination are maintained at the Offices of Micreos B.V. at Nieuwe kanaal 7P, and is available for the agency's review at customary business hours. Any information will be made available for the Food and Drug Administration and will be sent to the agency upon request to:

Steven Hagens s.hagens@micreos.com
Nieuwe Kanaal 7P Tel: + 31 317 421414
6709 PA Wageningen
The Netherlands

Micreos B.V. certifies that no data or information contained herein are exempt from disclosure under the Freedom of Information Act (FOIA). As such we request all information contained herein is shared with USDA/FSIS.

The undersigned, Steven Hagens – chief scientific officer (CSO) of Micreos Food Safety B.V. declares that to his knowledge all information concerning the safety evaluation of the proposed product, including favorable and unfavorable data is contained in the notification, resulting in a complete, balanced, and representative description of facts for the agency to review.

II. Part 2: Detailed information About the Identity and specifications of the Substance

A. Identity

PhageGuard E™ consists of a watery solution containing two *E. coli* O157-specific bacteriophages, EP and EP75, which are produced and purified separately and mixed in equal concentrations. The commercial product has a minimal titer of $2x10^{11}$ pfu/mL.

This solution is concentrated and will be diluted with water at application sites by a factor 10-100 to ensure application rates at a maximum of 1 $x10^{9}$ pfu/gram of treated food.

B. Method of Manufacture

The bacterial host is incubated in a bioreactor, using heat sterilized medium at temperatures that support growth. The medium consists of water (97.5%), Yeast Extract (0.5%), Soy Peptone (1%) and Sodium Chloride (1%). Either bacteriophage EP335 or EP75 solution is added and the bacteriophages multiply during incubation. After completion of the incubation process, the bacteriophages are separated from bacteria and bacterial debris by filtration. The bacteriophage solution is subsequently concentrated and purified by ultrafiltration. During this cleaning process, a substantial amount of medium components and host proteins are removed and replaced by cleaning solution. The cleaning solution consists of water (95.5%), soy peptone (0.1%) and sodium chloride (4.4 %). It is estimated that >95% of cell debris, host derived proteins as well as yeast and peptone remnants from the original medium are removed in these steps. After this step, the solution is sterile filtered.

The final solution will be prepared (sterile EP335 bacteriophage solution (~19%) and EP75 bacteriophage solution (~19%) as active ingredients and filter-sterilized tap water (~62%) as solvent), resulting in a solution containing 2 $x10^{11}$ PFU/mL. The final solution will be mixed and filled out in sterile bottles. The filled bottles will be labelled and put under quarantine. After QC testing the product will be released for the market by QA. The process is schematically presented in figure 1.

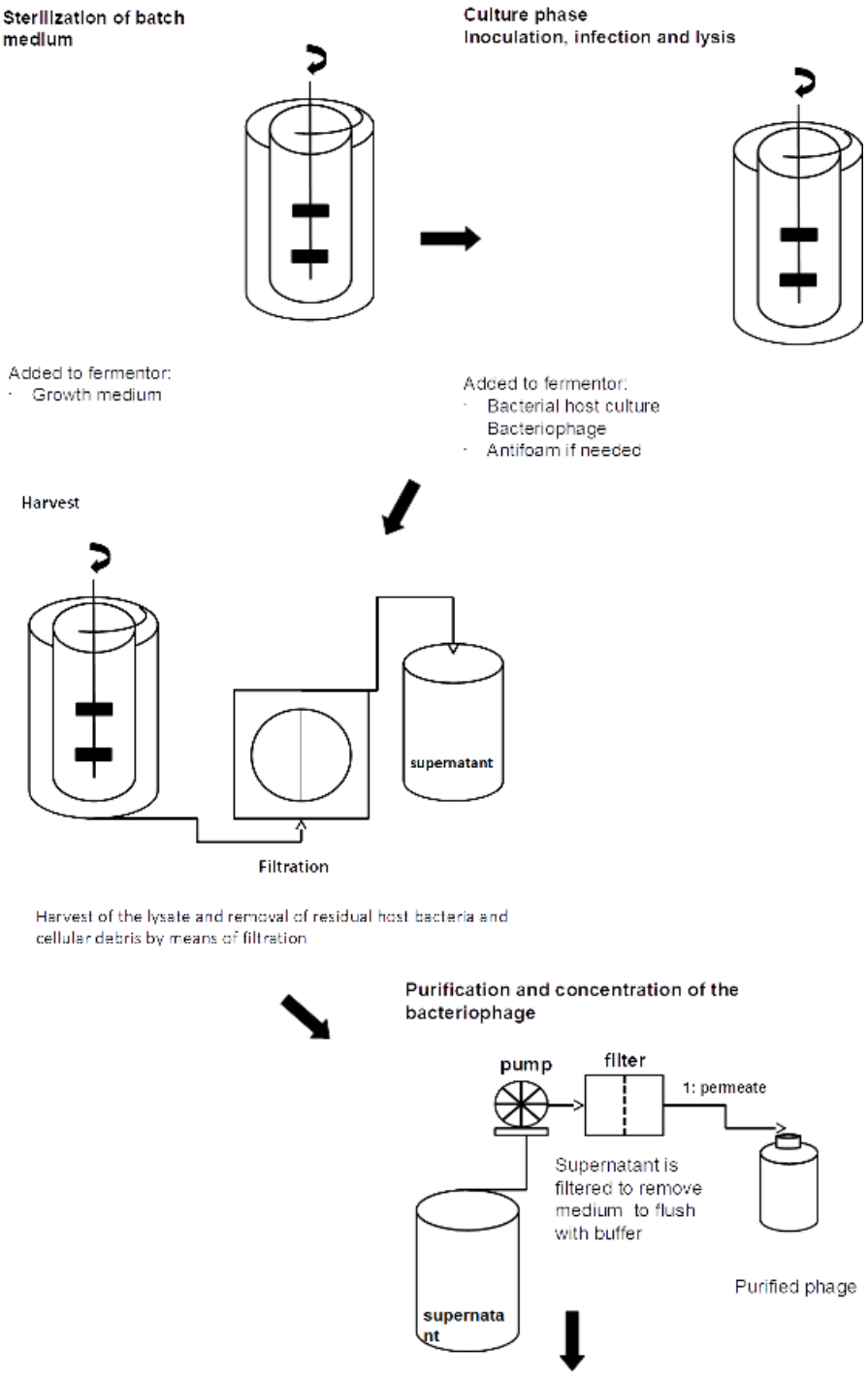

Figure 1 Schematic representation of the production process of PhageGuard E™

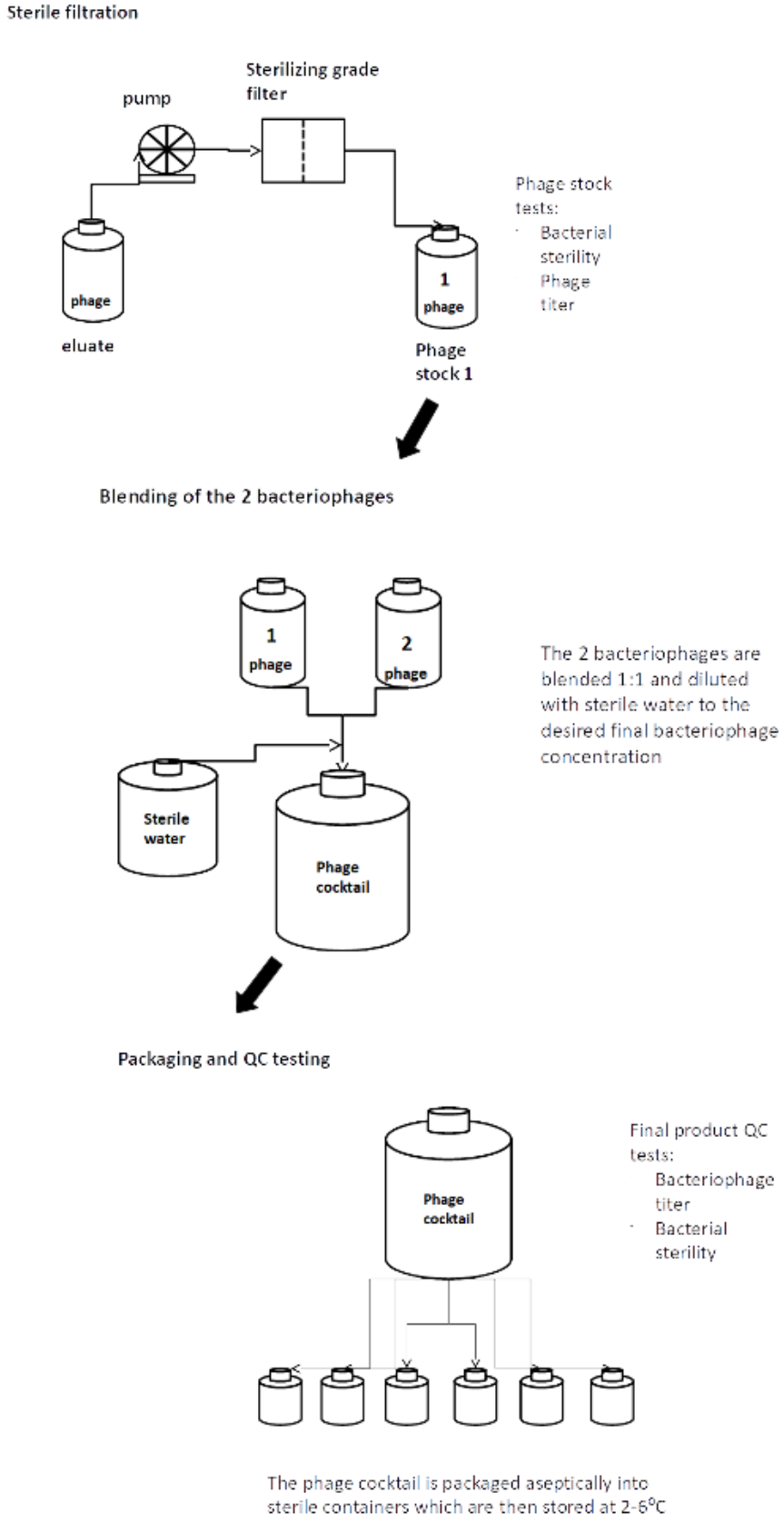

Figure 1: Continued

C) Specifications

1) Batches undergo testing to ensure they meet specifications. Standard phage titration protocols are used to ensure potency (2 $x10^{11}$ pfu/mL +/- 10%).

2) The product is tested for sterility by a 5-day enrichment of 1% of each batch in elective bacterial medium, followed and confirmed by plating of the enrichment on elective agar plates (Total plate count medium).

D) Chemical analysis

PhageGuard E™ is a clear, odorless liquid. With an average weight of the phages of ~ $1x10^{8}$ Dalton, the phage components make up 33.2 ppm of the total weight of the concentrated liquid. The production process will result in soy peptone and sodium chloride remaining in the final product at concentrations of 0.1 and 4.4% respectively.

E) Phage identity and host ranges

Name: EP75

Order: Caudovirales

Family: Myoviridae

Genus: Vi1-like viruses

EP75 was isolated by Micreos scientists in the Netherlands. Its' genome reveals a close relationship with the well-studied Vi1-like phages, especially phage PhaxI. Its 158.143 bp genome features 207 ORFs (open reading frames). None of these ORFs show homology to known virulence genes, toxins or antibiotic resistance genes, nor do they show homology to known food allergens. Host range testing again showed a very narrow host range within the species *E. coli*, infecting the majority of O157 isolates tested but no other *E. coli* strains. A summary of the host range test conducted follows:

EP75 host range:

1. O157 strains -> Plaques in high dilutions on 73/88 strains (83%)
2. O26, O45, O103, O111, O121, O145 serogroup strains -> No lysis activity on 40 strains (0%)
3. *E. coli* K-12 strains -> No lysis activity on 4 strains (0%)
4. ECOR human isolate *E. coli* -> No lysis activity on 55 strains (0%)
5. ESBL *E. coli* strains -> No lysis activity on 15 strains (0%)
6. Enterobacter -> No lysis activity on 4 strains (0%)
7. Citrobacter -> No lysis activity on 2 strains (0%)
8. Klebsiella -> No lysis activity on 17 strains (0%)
9. Morganella -> No lysis activity on 1 strain (0%)
10. Proteus -> No lysis activity on 2 strains (0%)
11. Raoultella -> No lysis activity on 1 strain (0%)

Name: EP335

Order: Caudovirales

Family: Podoviridae

Genus: PhiEco32-like viruses

Phage EP335 was isolated by Micreos scientists in the Netherlands, host-range studies were conducted both by Micreos in the Netherlands and at McGill University in Canada. Transduction experiments showing inability of the phage to transduce host DNA to other bacteria and full genome sequencing and bioinformatical analysis were performed by Micreos. EP335 is a virulent (strictly lytic) phage belonging to the PhiEco32 family of phages. Its 76.622 bp genome features 126 ORFs. None of these ORFs show homology to known virulence genes, toxins or antibiotic resistance genes, nor do they show homology to known food allergens. It exclusively infects bacteria of the species *E. coli*. The host range was found to be extremely narrow, infecting only *E. coli* O157 strains with very few exceptions.
The summary of the host range study is shown below:

EP335 host range:

1. O157 strains -> Plaques in high dilutions on 76/88 strains (86%)
2. O26, O45, O103, O111, O121, O145 serogroup strains -> Lysis activity on 3/40 strains (7.5%)
3. *E. coli* K-12 strains -> No lysis activity on 4 strains (0%)
4. ECOR human isolates of *E. coli* -> Plaques in high dilutions on 3/55 strains (5.4%)
5. ESBL *E. coli* strains -> Plaques in high dilutions on 1/15 strains (6.7%)
6. Enterobacter -> No lysis activity on 4 strains (0%)
7. Citrobacter -> No lysis activity on 2 strains (0%)
8. Klebsiella -> No lysis activity on 17 strains (0%)
9. Morganella -> No lysis activity on 1 strain (0%)
10. Proteus -> No lysis activity on 2 strains (0%)
11. Raoultella -> No lysis activity on 1 strain (0%)

The genome of EP335 revealed a close relationship to phage KBNP1711, another phiEco32-like phage exclusively infecting O157 strains. Transduction experiments passaging phages over an antibiotic resistant host and then infecting an isogenic sensitive host did not yield antibiotic resistant cells, thus strongly indicating that the phage is unable to transduce host DNA. The genomes of EP75 and EP335 accessed at GenBank under accession numbers MG748547 and MG748548 for EP75 and EP335 respectively. Fasta files can also be made available to the agency.

<u>Host range of the phage cocktail</u>

The combined host range of the two phages on 88 *E. coli* O157 strains shows that >95% of strains can be effectively killed on chilled beef, with some activity on other strains. The intended use of PhageGuard

E™ is as an additional step to increase food safety. It will be used on top of existing interventions, rather than replacing them as the specificity of this particular intervention does not reduce the risk from other (STEC) bacteria.

F) Host identity

The specificity of the phages EP75 and EP335 require the use of O157 bacteria as hosts. To reduce risks during production, the strains used for producing the bacteriophages do not contain shiga toxin genes (*stx*). The production hosts were sourced from public strain collections.

G) Undesirable Host-derived Components

The use of s*tx*-negative strains ensures that the gene cannot be induced during phage production, guaranteeing that no shiga toxin is present in the final product. The agency previously voiced no concern about residual lipopolysaccharide being present is this type of product. We do not believe any other harmful host-derived components are contained in the preparation.

III. Part 3: Dietary exposure

Estimated daily dietary intake of Phages and by-products

According to USDA information *(*www.usda.gov/factbook/chapter2.pdf*)* Americans consume approximately 195.2 lbs of meat per capita per annum. Of this, 64.4 lbs consists of beef. The consumption of beef has declined over recent decades but according to BeefUSA of the 65.8 lbs of beef consumed per capita in 2006, 27 lbs was ground beef. Using this number assuming that all cuts destined to be ground are treated with phages the following calculation can be made:

Phage intake

34 grams/beef x 1 x 10^9 pfu/g = 3.4 x 10^{10} phages/day.

Further assuming an average weight of 1 x 10^8 Da/phage the following calculation gives the total weight of phages consumed on a daily basis:

5.6 x10^{10} x 10^8 x 1.66 x 10^{-27} kg = 0.00000001328 kg/day = 6 µg/day.

Or in terms of treated product:

0.182 ppm (parts per million). This level should be considered insignificant.

Sodium

With a sodium chloride concentration of 4.4 % in the final medium a use level of 1 x 10^9 pfu/g of treated food ~8.7 µg of sodium per gram of treated ground meat would be added in an application on trimmings. Beef has a background of 720 µg of sodium per gram. We do not consider this a significant increase requiring labeling by the end user. Especially as guidelines for risk groups recommend keeping sodium intake to levels below 1500 mg/day and a 100 g portion of (unseasoned) treated product would represent 4.86% of this values compared to a 100 g portion of untreated ground beef which would represent 4.8% of that value.

These values represent levels at the maximum use level and with treatment of trimmings. Treatment of carcasses would result in significantly lower phage and sodium levels as many of the surfaces treated in that scenario would not be found in retail cuts/products. Also in reality, use levels are likely to be below the maximum use level requested.

IV. Part 4: Self-limiting Levels of Use

The proposed use of PhageGuard E™ that is the subject of this GRAS determination is as an antimicrobial processing aid for foods that are susceptible to *E. coli* O157. The purpose of PhageGuard E™ is to significantly reduce or eliminate *E. coli* O157 in the finished product.

The use of the product and potential intake would be self-limiting by two factors. First the manufacturer would use the minimum dose required to achieve the desired reduction levels for *E. coli* O157 due to the cost of PhageGuard E™. Secondly, after the host bacteria *E. coli* O157 is depleted on the food, the phage would no longer replicate and would gradually die back in viable numbers and degrade due to environmental factors such as heat and UV light.

V. Part 5: Experience based on common use in food before 1958.

While phage are common in food there was no commercial use in foods prior to 1958

The bacteriophage components of PhageGuard E™ as well as composition of the final product will be assessed in detail

VI. Part 6: Narrative

A) Background on O157 related illness

While *E. coli* O157:H7 is not the only causative agent of severe, acute hemorrhagic diarrhea and the complication of HUS (hemolytic-uremic syndrome), which can cause kidney failure or death, the percentage of patients that suffer from complications is especially high in outbreaks of *E. coli* O157:H7. This, and the existence of specific phages make it an appropriate target for an intervention.

B) Phage background

The attributes of bacteriophages include the following:

- they kill only *live bacterial* target cells,
- they generally do not cross species or genus boundaries, and will therefore not affect desired bacteria in foods (e.g., starter cultures), and commensals in the gastrointestinal tract, or accompanying bacterial flora in the environment; moreover, phages are composed entirely of proteins and nucleic acids, so their breakdown products consist exclusively of amino acids and nucleic acids, both of which are present in abundance in food products.

Bacteriophages thus are not xenobiotics, and, unlike antibiotics and antiseptic agents, their introduction into, and distribution within a given environment can be seen as a natural process.

<u>Phages in the environment</u>

With respect to their application for the biocontrol of undesired pathogens in foods, feeds, and related environments, it should be considered that phages are the most abundant self-replicating units in our environment, and are present in significant numbers in water and foods of various origins, in particular fermented foods (reviewed by Sulakvelidze and Barrow, 2005). On fresh and processed meat and meat products, more than 10^8 viable phages per gram are often present (Kennedy and Bitton, 1987). It is a fact that phages are routinely consumed with our food in high numbers. Moreover, phages are also normal commensals of humans and animals, and are especially abundant in the gastrointestinal tract (Furuse et al. 1983; Breitbart, 2003).

In conclusion, bacteriophages are known to be harmless for all other organisms and are very specific for a certain bacterial species, strains within this species or, more rarely, for an entire genus. Phages are also naturally present in foods.

Very few foodstuffs are completely sterile. This means that most food consumed will contain bacteria and therefore phages are likely to be present.

This holds true especially for fermented products as well as unprocessed vegetables. As an example, phages can readily be isolated from Sauerkraut (Yoon et al. 2002; Barrangou et al. 2002). In one study (Lu et al. 2003) 26 different phages were isolated from the product of 4 commercial Sauerkraut fermentation plants.

While in most commercial cheese production settings a lot of effort has been put into ensuring that starter cultures are free from phages and to some extent resistant to phage infection, this is certainly not the case for artisanal cheeses and one might even argue that as long as timing is correct, host lysis by phages and thus liberation of the proteolytic enzymes may even be desirable. Phages infecting *Propionibacterium freudenreichii* have been isolated from Swiss cheese at levels of up to 7×10^5 pfu/g (Gautier et al. 1995). Phages infecting thermophilic lactic acid bacteria have been isolated from Argentinian dairy plant samples at numbers of up to 10^9 pfu/ml.

More importantly, non-fermentation culture bacteriophages have also been isolated from various food sources. *E. coli* phages have been isolated from a large number of products including: fresh chicken, pork, ground beef, mushrooms, lettuce, other raw vegetables, chicken pie and delicatessen food with phage numbers as high as 10^4 per gram (Allwood et al 2004; Kennedy et al. 1986, 1987).

Also *Campylobacter* phages have been isolated at levels of 4×10^6 PFU from chicken (Atterbury et al. 2003) and *Brochothrix thermosphacta* phages from beef (Greer 1983).

In all these cases the researchers were looking for phages infecting one particular species and often one particular strain, but when one considers the myriad of bacteria associated with soil and vegetables it becomes clear that in addition more phages, associated with this multitude of other species, are likely present.

<u>Phages in biocontrol of pathogens in food.</u>

Much research has been conducted in using phage as biocontrol agents in foodstuffs. The general mode of action and efficacy of such interventions has also been reviewed extensively in the scientific literature (Greer 2005, Hudson et al. 2005, Hagens and Loessner 2007, Goodridge 2011, Hagens and Loessner 2010).

Phages can be separated into two groups: those that can integrate into host genomes and replicate as part of the genome (temperate phages) and virulent phages (strictly lytic phages) which are not able to do this and kill their hosts after infection.

The use of temperate phages would not be effective as some hosts will survive infection. While not a significant risk in everyday life, some temperate phages carry undesirable genes and have been shown to transduce host genes (i.e. transfer bacterial genes from one host cell to another). No virulent phage on the other hand has to date been shown to carry undesirable genes and most virulent phages do not

transduce host genes. Some virulent phages have shown ability for generalized transduction and safety data should include not only genome sequence data but experimental and/or theoretical proof that the candidate phages cannot transduce.
If the desired properties are found in candidate phage and considering their natural presence in the environment, in and on humans such phages should be considered GRAS.

Phages contained in PhageGuard E™ and safety evaluation

The identity of the two phages, EP335 and EP75, and their properties including host ranges are described in detail in section II.
Both phages are virulent (strictly lytic). Neither phage carries undesirable genes (toxins, virulence factors or antibiotic resistance genes). Experimental data excludes the possibility of either phage transducing host genetic material to subsequent hosts. As such they possess all the necessary trait of phages suitable for bio-control.

However, in the case of *E. coli* the target is potentially a commensal organism and care needs to be taken when targeting such organisms. There are a number of studies that suggest that ingestion of *E. coli* phages has no impact on intestinal *E. coli* bacteria, even if the phages consumed can infect these strains (Brutin and Brussow 2005, Sarker et al. 2012, 2016, 2017). The selected studies were mostly carried out in healthy individual or in patients suffering from *E. coli* diarrhoea. In these studies phage consumption had no negative impact on healthy individual or the patients and in the latter case also no beneficial effect. However no studies exist on the impact of phage consumptions in individuals suffering from severe intestinal dysbiosis. In contrast, we deem an O157-specific phage preparation to be absolutely safe.
The host ranges of both phages are substantial within the serovar *E. coli* O157. Both phages show no activity on other genera of bacteria, and have a very narrow host range within the genus *E. coli*, infecting O157 strains but having no serious impact on desirable *E. coli* strains.
The selected phages are ideal candidates for biocontrol of *E. coli* O157 in foodstuffs. The level of analysis in terms of behaviour and genetic properties ensures they are safe and should therefore be considered GRAS.

Substantial equivalence to other phage products

Two other Micreos phage products has already acquired GRAS status. Listex™ is a phage preparation used for biocontrol of *L. monocytogenes* in susceptible foodstuffs. It has also received status as a processing aid by USDA FSIS for use in RTE meat products. It is approved as a processing aid for susceptible foodstuffs in many countries, including approval by Health Canada and FSANZ in Australia

and New Zealand. Salmonelex™, a product to combat *Salmonella*, similarly has GRAS status and is approved for use on a large number of food products in the US and other countries.
Other phage products have been approved in food related applications in the US as cleaning agents or for decontamination of food animals prior to slaughter or for use in agricultural settings. Another O157 specific phage product has approval for use on trimmings with a FDA food contact notification (FCN 1018).

C) GRAS status of starting material

The growth medium for producing PhageGuard E™ contains only GRAS ingredients/processing aids. The main components of the medium are Soy peptone, which is GRAS affirmed as well as Yeast extract (Gras affirmed) and Sodium chloride (a compound so obviously GRAS it is not listed).

Furthermore, the antifoaming agent used is food grade, and sodium hydroxide and/or hydrochloric acid are used to adjust pH of the medium only during fermentation.

These components moreover are removed to a great extent in the purification steps in down-stream-processing.

Allergenicity

I. Phage components

Bacteriophages consist of proteins and nucleic acids. The former could in theory be allergenic. In practice this is however not relevant. The most potent known food allergen is peanut protein. The threshold dose for individuals with the highest sensitivity is 100 µg (Wensing et al. 2002). Assuming the unlikely scenario that all phage proteins (capsid proteins, tail proteins, tail fibers and tail spike proteins and base plate components) of both phages would be equally allergenic as the peanut allergen, estimated daily intake (see below) indicates that aproximately 18 lbs of treated food would need to be consumed in a single sitting in order to ingest 100 µg of phage proteins (approximately half the weight of a phage is made up of proteins). We therefore consider the allergenicity potential of PhageGuard E™ application due to the phage components negligible. Nonetheless, analysis of the individual open reading frames of both phages show no homology with known food allergens.

II. Relevant Medium Components

Soy Pepton

The only medium component with allergenicity potential is soy peptone. A hydrolyzed soy protein concentrate, the hydrolyzation step significantly reduces any potential allergenicity. According to the

supplier of the soy pepton, ELISA and PCR testing point out that the main allergens are absent in this soy pepton, within the limits of detection. Micreos also confirms negative allergenicity on incoming product using the ELISA testing performed by an accredited laboratory.

The downstream processing steps used to purify the phages will furthermore remove >95% of all proteins including medium components.

D) Quality Control

Phage Identity

Batches of the two phages are produced separately. The working stock used in fermentation of each separate phage is derived from the original master stock in a classical pyramid form. Seed stocks are produced from the original master stock. These seed stocks are used to make working stocks which are in turn used to produce individual batches.

Working stocks are subjected to host range testing (plaque formation behavior on several stains). The results are compared to historical data and must match completely for working stocks to be approved for use in producing PhageGuard E™. After production of each batch, identity of the phages contained is checked by host range testing on at least one strain exclusive for one of the two phages.

Phage numbers

After fermentation and downstream processing of the separate phages, they are tested for potency by classic phage titration. The individual phages are subsequently diluted with sterile water and blended to obtain a final phage preparation containing 1 $x10^{11}$ pfu of each phage/mL.

Sterility

Sterility is tested by enrichment of the blended product containing the desired number of phages after packaging. 1% of total final product after packaging is enriched in elective medium for 5 days prior to being plated on elective agar plates. The absence of microbial growth is required for product release. Batches failing this requirement will be destroyed.

E) Efficacy data at the intended levels of use

Data on the efficacy of the proposed cocktail on beef is provided in Appendix 1. Shortly the data conclusively shows that phage application can reduce *E. coli* O157 levels by 1-2 logs on chilled beef. Data also confirms that activity is short-lived and that activity is limited to a period of maximal 8 to 24 hours with no additional effects after this period. We believe that PhageGuard E™ should therefore be classified as a processing aid (as is the case for other phage products available).

F) Summary PhageGuard E™ and GRAS

Bacteriophage preparations for biocontrol of pathogens have previously been affirmed as GRAS. The current phage preparation PhageGuard E™ should also be considered GRAS. Genetic analysis and experimental evidence show that the individual phages contained in the preparation are safe in terms of being:

a) virulent (strictly lytic);
b) not containing any undesirable genes;
c) being unable to transduce host DNA from one host strain to another;
d) will not possibly affect the human microbiome due to the extremely narrow host range of the component phages. While this information is non-public, as host ranges were determined by Micreos B.V., we believe experts in the field given this information would agree with our safety assessment. In the research leading to this dossier, candidate phages with broader host ranges were discarded as options for a final product.

PhageGuard E™ is moreover highly effective in reducing *E. coli* O157 contaminations on beef. Based on these findings, PhageGuard E™ is considered GRAS for beef applications (notably beef carcasses, subprimals, beef cuts and trimmings intended for ground beef).

VII. Part 7: List of supporting data and information in your GRAS notice

1) Generally available: References of scientific literature

2) Not generally available: Appendix 1: Efficacy data of the proposed phage cocktail on refrigerated beef

References:

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Appendix I. Challenge Study Report: PhageGuard E™ Food Application

1. Introduction

A challenge study was performed to evaluate the effect of PhageGuard E™ phages on beef inoculated with *E. coli* O157. The challenge testing was performed at McGill University, Quebec, Canada. Beef samples (sourced from local retail) were treated with two phage concentrations $3x10^7$ pfu/cm² and $3x10^8$ (1.5×10^7 or 1.5×10^8 pfu/cm² of each phage). A contact time of 24 hours was chosen to evaluate the initial effect of the treatment, as other phage applications showed that phages have a very limited time of activity. Samples were incubated at 4°C. Duplicate samples were tested for each treatment.

In order to show that there is only an initial reduction with no further activity after 24 hours, two additional time trial experiments were performed. One experiment was performed at 4°C to show that no further reductions are observed after the initial reduction. Another experiment was performed where the beef samples were placed at 4°C during the first 24 hours post treatment, after which all the samples were transferred to 20°C for the remainder of the experiment. This last experiment was performed to observe whether growth in the treated samples is identical to growth in the control (non-treated) samples.

2. Materials and methods

2.1 Materials

Samples

Beef- Purchased at a local supermarket

Bacteria/bacteriophage

- *E. coli* O157 overnight cultures were prepared in liquid LB medium, and where indicated supplemented with 500 µg/mL streptomycin, for inoculation of samples.

Media

LB broth

LB agar plates

1 x PBS buffer (Phosphate buffered saline preparation)

1 x SM buffer

0.1% peptone water (+ 5g sodium chloride/L)

0.85% NaCl buffer

Streptomycin stock solution (500mg/mL)

2.2 Methods

2.2.1 Challenge study: PhageGuard E™ efficacy on *E. coli* O157 inoculated meat samples

Bacterial overnight cultures

One colony of the respective *E. coli* O157 strain was inoculated in 5 ml LB broth and incubated overnight at 37ºC shaking.

Preparation of samples

Beef sample pieces of 3x3(x1) cm were prepared to achieve a 5 cm² surface to be contaminated (A_{con}) and a surface of 9 cm² to be treated with phages ($A_{treated}$). Samples were placed and stored in sterile petri dishes.

Artificial contamination of beef samples

An appropriate dilution of the overnight culture is prepared in PBS buffer to allow the contamination of the samples with a final concentration of approximately 1 $x10^5$ cfu/cm² (5µL liquid/ cm²).
In the laminar flow hood 5 µl/cm² of the dilution is transferred to each sample and rubbed in evenly with the pipette tip.

Treatment with PhageGuard E™

To allow the treatment of the beef samples with a final concentration of 3 $x10^7$ or 3 $x10^8$ pfu/cm², dilutions of PhageGuard E™ were prepared in SM buffer. In the fume hood, 10 µl/cm² was transferred onto the samples. The liquid was distributed with the pipette tip. The petri dishes were closed and incubated at 4°C for 24 hours, before bacterial enumeration. Bacteria were retrieved by stomaching the beef samples with 20 ml of 0.85% NaCl buffer for 180 seconds. Dilutions as indicated in table 1 below were plated on LB agar plates. Bacteria were enumerated on two different beef samples per treatment at 0 hours and 24 hours after treatment.

2.2.2 Time trial studies: PhageGuard E™ efficacy at prolonged times post treatment

Bacterial overnight cultures

One colony of a streptomycin resistant *E. coli* O157 strain was inoculated in 4 mL LB broth supplemented with 500 µg/mL streptomycin, and incubated overnight at 30 °C.

Sample preparation, and artificial contamination

Sample preparation and artificial contamination were done as described above.

Treatment with PhageGuard E™

Inoculated beef samples were treated with PhageGuard E diluted in SM buffer to reach a final phage concentration of 3 $x10^8$ pfu/cm². In the fume hood, 10 µL/cm² was applied onto the beef samples, after

which the liquid was evenly distributed on the beef surface with a pipette tip. The petri dishes were closed and stored as described below for the two different time trial studies:

- *Time trial 1: Prolonged storage at refrigerating temperature.* All samples were stored at 4°C for up to 72 hours post phage treatment
- *Time trial 2: Prolonged storage at abusive temperature.* After phage treatment. all samples were first stored for 24 hours at 4°C, after which all samples were transferred to 20°C for a maximum of 54 hours post phage treatment

At the indicated time points, bacteria were enumerated on two beef samples per treatment by stomaching the beef samples with 20 mL of 0.1% peptone water for 180 seconds. Dilutions as indicated in the tables below were plated on LB agar plates supplemented with 500 µg/mL Streptomycin.

3. Results

3.1 Challenge study: PhageGuard E™ efficacy on *E. coli* O157 inoculated meat samples

The following figure 1 shows the reduction levels achieved by both phage concentrations on chilled beef samples after incubation of 24 hours at refrigeration temperature. More than a log reduction can be achieved on all mixes of *E. coli* O157 cultures depending on concentration.

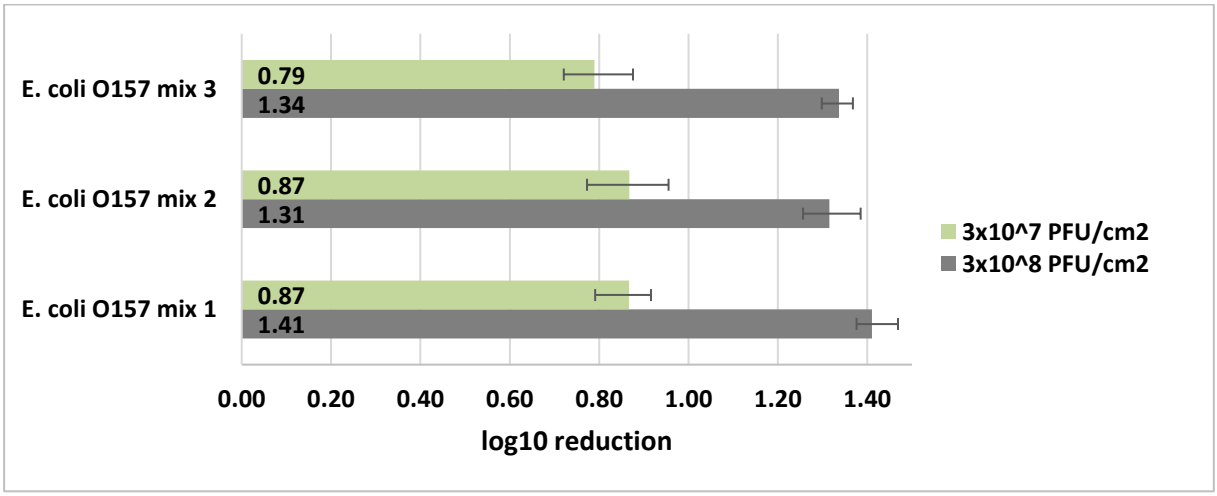

Figure 2 Efficacy of PhageGuard E on cold fresh beef after 24 h

3.2 Time trial studies: PhageGuard E™ efficacy at prolonged times post treatment

3.2.1 Time trial 1: Prolonged storage at refrigerating temperature

Figure 2 shows bacterial numbers at various timepoints for a period of 72 hours at refrigeration temperature. The figure clearly shows that maximum reduction is achieved after 24 hours and that no further activity can be observed after this timepoint.

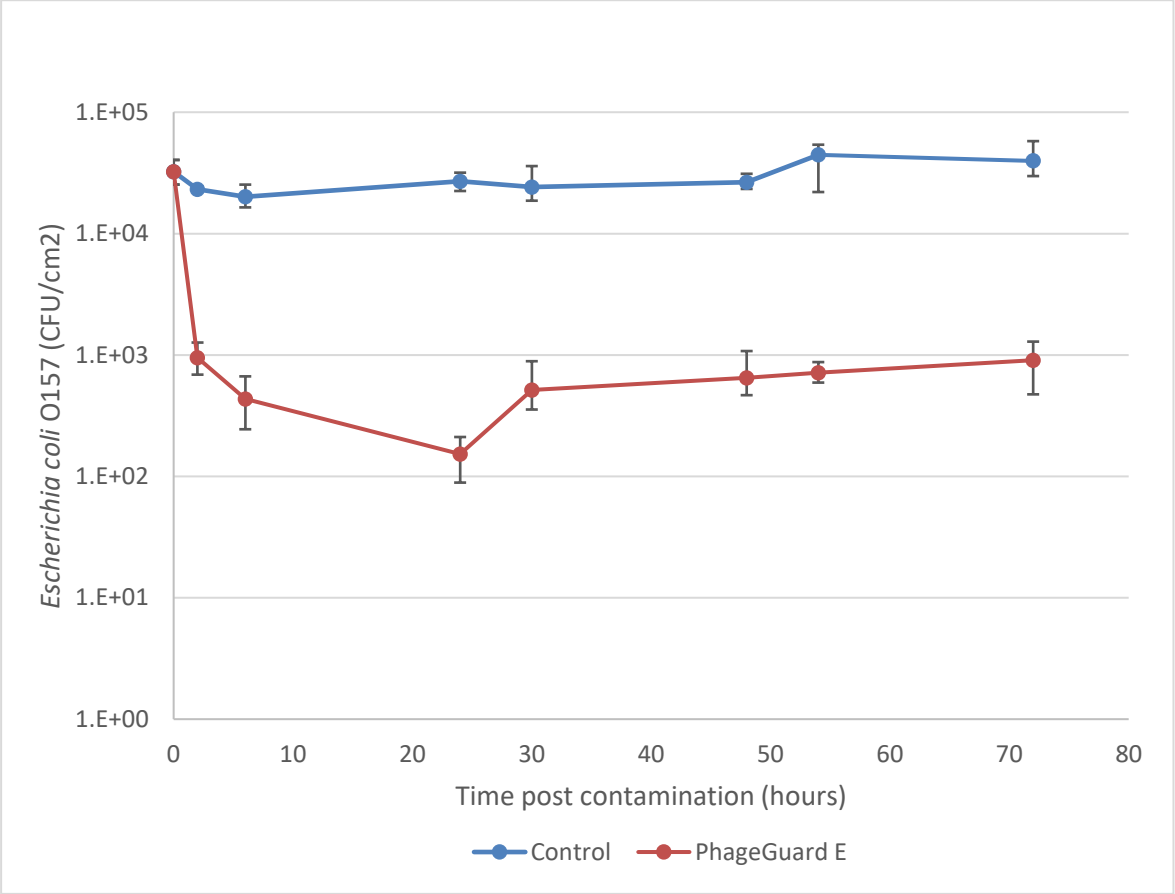

Figure 3 Bacterial load on contaminated cold fresh beef treated with PhageGuard E and stored at 4°C

3.2.2 Time trial 2: Prolonged storage at abusive temperature

Figure 3 demonstrates outgrowth of bacteria after 24 hours when placed at an abusive temperature of 20 °C up to 54 hours. Bacterial growth resumes in both control and treated beef underlining the fact that

phages are no longer active after the initial reductions have taken place. Growth rates in the phage treated product is more than equal to that in the untreated control.

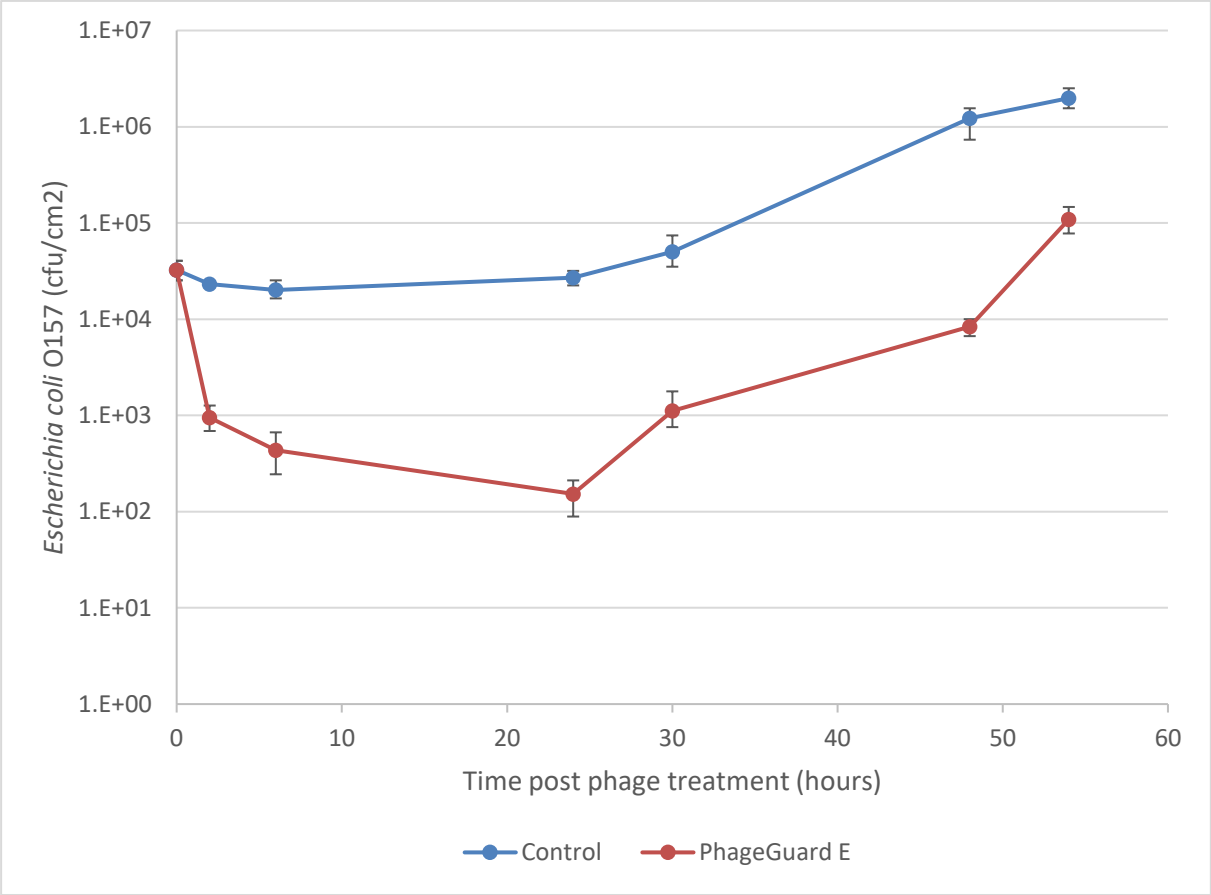

Figure 4 Bacterial load on contaminated cold fresh beef treated with PhageGuard E™ and stored for the first 24 hours at 4°C, and subsequently transferred to 20°C.

The following tables (Table 1 – 3) contain the raw data for the experiments represented in the above figures.

Table 1 Challenge study PhageGuard E™ efficacy on cold fresh beef

		Type of sample		
		Control (Not treated)	**PhageGuard E $3x10^7$ PFU/cm^2**	**PhageGuard E $3x10^8$ PFU/cm^2**
***E. coli* O157 mix 1**	**Amount of homogenate plated***	50 µL of D1 dilution	100µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	312	81	210
		320	75	256
		321	80	234
		283	100	260
	Average CFU/plate	309	84	240
	Average CFU/cm^2	2.47E+05	3.36E+04	9.60E+03
	% reduction		86.41%	96.12%
	Log reduction		0.87	1.41
***E. coli* O157 mix 2**	**Amount of homogenate plated***	50 µL of D1 dilution	100µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	329	68	335
		319	67	313
		277	91	249
		284	102	274
	Average CFU/plate	302.25	82	292.75
	Average CFU/cm^2	2.42E+05	3.28E+04	1.17E+04
	% reduction		86.44%	95.16%
	Log reduction		0.87	1.31
***E. coli* O157 mix 3**	**Amount of homogenate plated***	50 µL of D1 dilution	100µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	326	102	344
		352	91	293
		353	121	298
		336	130	325
	Average CFU/plate	341.75	111	315
	Average CFU/cm^2	2.73E+05	4.44E+04	1.26E+04
	% reduction		83.76%	95.39%
	Log reduction		0.79	1.34

Table 2:Time trial 1 enumeration of bacteria after longer incubation at refrigeration temperature

		Type of sample	
		Control (Not treated)	**PhageGuard E**
2 hours	**Amount of homogenate plated***	50 µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	51	47
		52	36
		55	57
		51	31
	Average CFU/plate	52.25	42.75
	Average CFU/cm²	2.32E+04	9.50E+02
	% reduction		97.06%
	Log reduction		1.5
6 hours	**Amount of homogenate plated***	50 µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	37	25
		46	30
		41	11
		57	12
	Average CFU/plate	45.25	19.50
	Average CFU/cm²	2.01E+04	4.33E+02
	% reduction		98.66%
	Log reduction		1.9
24 hours	**Amount of homogenate plated***	100 µL of D1 dilution	200 µL of D0 dilution
	CFU/plate	134	17
		143	19
		107	11
		101	8
	Average CFU/plate	121.25	13.75
	Average CFU/cm²	2.69E+04	1.53E+02
	% reduction		99.53%
	Log reduction		2.3
30 hours	**Amount of homogenate plated***	100 µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	103	19
		86	40
		162	18
		84	16
	Average CFU/plate	108.75	23.25
	Average CFU/cm²	2.42E+04	5.17E+02
	% reduction		98.40%
	Log reduction		1.8

Table 2 continued

48 hours	**Amount of homogenate plated***	100 µL of D1 dilution	200 µL of D0 dilution
	CFU/plate	105	42
		123	97
		140	45
		110	50
	Average CFU/plate	119.5	58.50
	Average CFU/cm²	2.66E+04	6.50E+02
		% reduction	97.99%
		Log reduction	1.7
54 hours	**Amount of homogenate plated***	100 µL of D1 dilution	150 µL of D0 dilution
	CFU/plate	235	40
		225	47
		243	59
		99	47
	Average CFU/plate	200.50	48.25
	Average CFU/cm²	4.46E+04	7.15E+02
		% reduction	97.79%
		Log reduction	1.7
72 hours	**Amount of homogenate plated***	50 µL of D1 dilution	150 µL of D0 dilution
	CFU/plate	67	47
		68	32
		130	78
		92	87
	Average CFU/plate	89.25	61.00
	Average CFU/cm²	3.97E+04	9.04E+02
		% reduction	97.21%
		Log reduction	1.6

Table 3: Time trial 2 outgrowth of bacteria in control and phage-treated beef at abusive temperature

		Type of sample	
		Control (Not treated)	**PhageGuard E**
2 hours	**Amount of homogenate plated***	50 µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	51	47
		52	36
		55	57
		51	31
	Average CFU/plate	52.25	42.75
	Average CFU/cm²	2.32E+04	9.50E+02
6 hours	**Amount of homogenate plated***	50 µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	37	25
		46	30
		41	11
		57	12
	Average CFU/plate	45.25	19.50
	Average CFU/cm²	2.01E+04	4.33E+02
24 hours	**Amount of homogenate plated***	100 µL of D1 dilution	200 µL of D0 dilution
	CFU/plate	134	17
		143	19
		107	11
		101	8
	Average CFU/plate	121.25	13.75
	Average CFU/cm²	2.69E+04	1.53E+02
30 hours	**Amount of homogenate plated***	50 µL of D1 dilution	100 µL of D0 dilution
	CFU/plate	167	37
		93	34
		112	50
		79	80
	Average CFU/plate	112.75	50.25
	Average CFU/cm²	5.01E+04	1.12E+03

Table 3 continued

48 hours	**Amount of homogenate plated***	100 µL of D3 dilution	100 µL of D1 dilution
	CFU/plate	33	39
		53	45
		70	30
		64	36
	Average CFU/plate	55.00	37.50
	Average CFU/cm²	1.22E+06	8.33E+03
54 hours	**Amount of homogenate plated***	100 µL of D3 dilution	100 µL of D2 dilution
	CFU/plate	74	40
		70	35
		100	66
		113	55
	Average CFU/plate	89.25	49.00
	Average CFU/cm²	1.98E+06	1.09E+05

Wageningen 14th of May 2018

1) Please provide more information on the host strains used in the phage production, specifically: Genus, species, and strain, their culture repository ID, whether they express any virulence factors, antibody resistance, etc.

Ad 1)

The host strain used belongs to the genus Escherichia, specifically it concerns the species *E. coli* O157. It was sourced from a culture collection (NCTC) with designation NCTC 13125 where it was determined that the strain lacks verotoxins. In addition we verified this by performing a PCR that is designed to detect both shiga toxins (Stx1 and Stx2). A third PCR showing that the strain carries the O157 antigen was also performed. Positive controls using DNA of O157 strains carrying Stx1 and Stx2 were also performed. A detailed report of the experiments carried out is attached as Appendix 1.

2) On Page 10, you state: "We do not believe any other harmful host-derived components are contained in the preparation." Please explain how or why you come to this conclusion and make this statement definitive.

Ad 2)

To our knowledge none of the metabolites, proteins (including virulence factors and toxins) produced by E. coli are harmful upon ingestion. Toxin delivery to the human host during infection requires close association of the bacterium with epithelial cells of the gut. That explains why strains carrying shiga toxins do not cause disease if they lack the ability for close association with the epithelial cells. Moreover, the production process includes a continuous diafiltration step. The lysate is concentrated and then the volume is increased to the original amount. This step is performed 7 times. The molecular cutoff of the diafiltration filter is 500kD. All known toxins are smaller that and small molecules are removed in this process. Attached is Appendix 2 which explains the process in general and allows calculating the amount of small molecules that are removed when applying multiple rounds of diafiltration. The product will not contain harmful host-derived components.

3) You state that soy peptone was used in the growth medium and cleaning solution during the production of the preparation. Consequently, please state if allergens remain in the final product or if there are no allergens in the final product, please describe how this was determined.

Ad 3)

Every batch of soy peptone that is received by Micreos is tested for the presence of allergens using an ELISA test: ES soy protein residue kits (BioMerieux, Durham, NC, USA) with an LOQ of 2.5 ppm soyprotein. This test is used extensively to detect the presence of soy proteins in food products. The concentration tested is identical to the amount of soy used in the production medium of PhageGuard Listex (which is the medium richest in soy peptone). To date all incoming product has tested negative using this assay, indicating that the antigens recognized by the antibodies of the test are not present.

We have also performed a risk analysis on the basis of PhageGuard Listex using the worst case scenario which assumes that peptic digestion of soy does not reduce allergenicity and that the USP and DSP processes do not result in a decrease in peptone. The latter two assumptions are certainly not true. Bacteria consume part of the peptone in the process and diafiltration of the lysate does remove the majority of medium components. However, even in a worst case scenario we can show that use of PhageGuard products does not hold the risk of allergic reactions in consumers. The risk analysis is attached as Appendix 3.

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Lastly it should be noted that we inform our customers of the use of Soy peptone in our process.

4) The intended use describes PhageGuardE™ as an antimicrobial on foodstuffs to control E. coli at an application rate up to 1x109 PFU/g of food. However, there was no description of the application method or any associated details regarding application.

a. Please describe the application method, e.g., to be applied as a spray, mist, rinse, wash or a mix of all the application methods.

b. Please provide the parameters of application, e.g., optimum pressure range for spray application, dwell or contact times, etc.

c. Please make it clear that PhageGuardE™ is used as an additional step and must be used in addition to existing interventions.

Ad 4)

a) PhageGuardE™ can be applied as a spray, mist, wash or by dipping. The method of choice depends on the production process and where the intervention takes place. Whole carcasses are not amenable to dipping whereas trim can be treated in a variety of ways.
b) The most important parameter is an even distribution of the phages on the surface. Conventional spaying systems, ultrasonic spraying systems, and electrostatic spraying system require very different settings to achieve a good distribution of the liquid. Micreos application specialist will always work together with industry to realize customized solution fitting the production process in question. Phages have a limited period of activity after application with the majority of activity observed shortly application. The optimal dwell time depends on the desired level of reduction and can be shortened by applying more phages. The design of the intervention will be customized in all cases.
c) Since PhageGuardE™ only removes *E. coli* 0157 it can only be used as an additional step on top of current interventions used by industry.

(b) (6)

Steven Hagens, PhD

Chief Scientific Officer

Micreos Food Safety

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