#724

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August 8, 2017

Paulette Gaynor, Ph.D
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: GRAS Notice-Exemption Claim for Escherichia coli-specific phage preparation

Dear Dr. Gaynor:

On behalf of my client FINK TEC GmbH, and in accordance with FDA's final rule of August 17, 2016 (81 FR 54960) and 21 CFR §170.225(c)(1), please accept this submission of a new notice of a GRAS exemption claim for the above referenced substance, *Escherichia coli*-specific phage preparation.

FINK TEC GmbH certifies that to the best of FINK TEC GmbH's knowledge this GRAS notice is a complete, representative, and balanced submission, which contains all information known to the company that is pertinent to the evaluation of the safety and GRAS status of the substance.

This GRAS notice is submitted on CD-ROM (enclosed; checked and virus free) containing: a GRAS notice exemption claim; detailed information on the notified substance; and attachments containing further referenced and substantiating information on the substance.

Further, as the use of the substance, i.e., as an anti-microbial on the surface of beef carcasses, is an USDA regulated use and, whereas USDA/FSIS and FDA have outlined the role of each Agency in the evaluation of food ingredients in the production of meat, poultry or eggs in a Memorandum of Understanding (MOU) between The United States Department of Agriculture Food Safety and Inspection Service and The United States Department of Health and Human Services, Food and drug Administration, we respectfully ask that you provide the appropriate liaison in USDA/FSIS with a copy of this GRAS notice

Paulette Gaynor, Ph.D
OFAS/GNP
August 8, 2017

for their use in making a suitability determination for the described use as provided for under Appendix A, Section C of the MOU.

Please promptly contact me should you have any questions regarding the submitted notice. I look forward to receiving acknowledgment of receipt of this notice and to a timely response regarding the noticed substance. Thank you.

Sincerely,

(b) (6)

Kevin O. Gillies

Enc.

Cc: Michael Fink, Ph.D, FINK TEC GmbH

KOG/Kg

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

Generally Recognized as Safe Notice

Escherichia coli-specific phage preparation

August 2017

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

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FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

PART 1

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Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
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5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: GRAS Notice-Exemption Claim for *Escherichia coli*-specific phage preparation

Dear Dr. Gaynor:

On behalf of my client FINK TEC GmbH, and in accordance with FDA's final rule of August 17, 2016 (81 FR 54960) and 21 CFR §170.225(c)(1) relating to the filing of generally recognized as safe (GRAS) notices, please accept this claim and the attached information, submitted on CD-ROM (enclosed; checked and virus free), for that purpose as it relates to the use of *Escherichia coli*-specific phage preparation as a processing aid anti-microbial on beef carcasses.

Specifically, FINK TEC GmbH has determined that the use of *Escherichia coli*-specific phage preparation as a processing aid anti-microbial on the surface of beef carcasses is Generally Recognized as Safe by scientific procedures and is, thereby, exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act and certifies that to the best of FINK TEC GmbH's knowledge this GRAS notice is a complete, representative, and balanced submission, which contains all information known to the company that is pertinent to the evaluation of the safety and GRAS status of the substance.

In conformity with the requirements outlined in the rule, the following information is included with this exemption claim:

§ 170.225(c)(2) - Name and Address of Notifier:

FINK TEC GmbH
Oberster Kamp 23
Hamm D-59069
Germany

§170.225(c) (3) – Appropriately descriptive term: *Escherichia coli*-specific phage preparation

§170.225(c) (4) Intended Conditions of Use: The phage preparation is intended for use as processing aid antimicrobial agent to control shiga-toxin producing *E. coli* O157:H7 as well as non-O157:H7 shiga-toxin producing *E. coli* that may be present on beef carcasses in commercial slaughter operations. The preparation is applied at processing plant ambient temperature as a spray using existing approved spray systems in meat processing facilities. The phage preparation is used as an ingredient at levels not to exceed current good manufacturing practice in accordance with 21 CFR 184.1(b). The targeted application rate will typically be approximately 1.5 X 10E11 phage particles per carcass.

§170.225(c) (5) - Statutory basis for GRAS conclusion: Scientific procedures

§170.225(c) (6) – Premarket approval: The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

§170.225(c) (7) Availability of Data and Information that are the Basis of Determination: The data and information forming the basis for FINK TEC's GRAS determination and the exemption claim asserted herein are available for FDA review and copying during reasonable business hours at the following address, or will be sent to FDA upon request:

Kevin O. Gillies
Kevin O. Gillies Consulting Services, LLC
1759 Grape Street
Denver, Colorado 80202
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§170.225(c)(8) - FOIA (Freedom of Information Act): Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification: To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to FINK TEC GmbH and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

On the basis of the information and the additional requested information as specified in the final GRAS rule as attached hereto and submitted with this letter, please accept this as FINK TEC GmbH's GRAS notification and claim of exemption from the statutory premarket approval requirements for the use of *Escherichia coli*-specific phage preparation on beef carcasses.

Should you have any questions regarding the submission of this notice, please contact me at the above number.

Thank you for your prompt consideration of, and response to, this notice.

Sincerely

(b) (6)

Kevin O. Gillies

KOG/Kg

Attachments

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

PART 2

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

1. Identity of Substance

Common and Usual Name of the Food Grade Substance: *Escherichia coli*-specific phage preparation

Chemical Name: None

Chemical Abstract Service (CAS) Registry Number: None

Empirical Formula: None

Structural Formula: None

Quantitative Composition: *Escherichia coli*-specific phage preparation is comprised of twelve (12) bacteriophages (phages) to be used six (6) at a time in the final commercial product "Secure Shield E1". All phages used in the phage preparation are strictly lytic as determined by an analysis of phage genomes.

An application of the food ingredient will use a mixture of equal proportions of six (6) phages selected from the designated twelve (12) phage preparation components, with specificity against the target bacterium *E. coli*, specifically shiga toxin-producing *E. coli* O157:H7 as well as non-O157:H7 shiga toxin-producing *E. coli*. The applied preparation will contain a total phage concentration ranging between 5×10^9 and 1×10^{10} active Plaque Forming Units per milliliter of solution (PFU/mL).

The ability to utilize a blend of selected phages in a particular processing plant is necessary to guarantee the broad scope of lytic activity of the blend against the target *E. coli* strains. In addition, a rotation in the composition of the cocktail, using a subset of the twelve (12) phages that are the subject of this notice, reduces the risk that the targeted bacterial pathogen might develop resistance against the applied preparation product. The possibility to create diversity within the preparation composition will also provide the meat producer with the means to rapidly react to outbreaks of novel shiga toxin-producing *E. coli* strains.

The final phage preparation is a colorless and odorless liquid suspension of phages that is produced as a concentrate to be diluted with water at the site of application to generate a working solution ranging in concentration from 1 x 10^5 to 1 x 10^7 PFU/mL, depending on the actual application.

The phages are specific for a wide range of *E. coli* isolates, including shiga-toxin producing *E. coli* O157:H7 as well as non-O157 shiga-toxin producing strains (Attachment 1). Eleven of the preparation phages belong to the family of Myoviridae, one bacteriophage to the family of Podoviridae. All bacteriophages are strictly lytic as determined by the analysis of their genomes.

Phages that comprise the preparation are deposited with the Leibniz Institute DSMZ–German Collection of Microorganisms and Cultures (https://www.dsmz.de/home.html) as designated below:

Order:	Caudovirales
Family:	Myoviridae
Species:	DSM 103290 (AB27)
	DSM 104013 (TB49)
	DSM 104014 (TB120)
	DSM 104015 (KRA2)
	DSM 104016 (TB69)
	DSM 104018 (BO1)
	DSM 104019 (EW2)
	DSM 104020 (TB6A)
	DSM 104021 (GWF)
	DSM 104022 (HAM53)
	DSM 104023 (MP75)

Order:	Caudovirales
Family:	Podoviridae
Species:	DSM 104017 (TB11)

Phage properties:

Host range: FINK TEC GmbH conducted host range studies on phage preparation component phages. The results of these studies demonstrated phage preparation lytic activity against 17 out of 18 tested *E. coli* strains (94.44 %). Only the *E. coli* strain O113:H4 (stx2d+) showed no phage interaction in this analysis. All of the phages were able to infect at least three *E. coli* strains.

Since the individual host ranges varied and none of the phages were lytic against all *E. coli* strains tested, the different phages complement each other,

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increasing the total coverage, which is important for the composition of an efficient targeted anti-microbial phage product. DSM 104019 (EW2), DSM 104020 (TB6A), DSM 103290 (AB27) and DSM 104021(GWF) considerably increased the coverage of the *E. coli* strains O-:H19 (stx1c+, stx2d+), O-:H25(stx2d+) and O121:H19 (stx2a+), which were not efficiently covered by the broad-spectrum phages DSM 104013 (TB49), DSM 104016 six phages out of twelve in order to produce a product with an optimal range of activity (Attachment 1).

Phage Type: All phage preparation phage components are exclusively lytic (Part 6 below). The biology of phages has been exhaustively studied in the 100 years since their discovery. Two major phage classes have been described, lytic and temperate. Temperate phages do not necessarily kill their host bacterium. They have the ability to passively invade a host and thereby are transferring their own genes from one host bacterium to the next, in a process called lysogenic conversion (Brüssow et al. 2004)(Fortier and Sekulovic 2013). As some temperate phages may carry toxin genes (Davis et al. 2000; Smith et al. 2012), they are not suited to be components of a phage cocktail produced and applied on an industrial scale.

Lytic phages, on the other hand, lack the genes responsible for lysogenic conversion and an infection by a lytic phage always leads to the death of the bacterial host. Thus, lytic phages are safe for use in food, as they do not disseminate toxin or other genes that pose risks for humans.

2. Manufacturing

Description of the manufacturing process: Manufacturing of the phage preparation involves, for each individual phage component, a two-step process (1) a well-controlled fermentation process, employing only GRAS raw materials and safe and suitable phages and host strains, and (2) filtration processes using GRAS buffer ingredients and safe filtration food contact materials. Packaging materials are approved for food use.

All processes are carried out in accordance with FINK TEC GmbH Standard Operating Procedures (Attachment 2; SOP table of contents; full text available to FDA upon request).

The individual phages are produced, as described in Fig.1, in an aerobic fermentation process in Springer®0251 medium (20g/L of water). For each phage production lot, the non-pathogenic host strain is grown at 37^{o}C to a target optical density of 0.3 at 600 nanometers (OD_{600}) in a 100L batch fermenter. Once the target OD_{600} is reached the fermentation broth is infected with the phage inoculum of 1L of phage preparation of 1 x 10^{9} Plaque Forming Units (PFU) / mL) and the combination is incubated with aeration and mixing at 37^{o}C. The OD_{600} of the

fermentation broth continues to increase to an OD_{600} of approximately 0.6 before plateauing and subsequently decreasing, indicating phage lysis of the host. After the fermentation and lysis processes are complete (OD_{600} ~ 0.1), the phage lysates are assayed in a semi-soft agar plate assay to determine the concentration of the progeny phage (Clokie & Kropinski, 2009).

Description of the phage purification and sterilization process

Purification of each phage component consists of a 3-step physical separation process: (1) continuous centrifugation, (2) tangential flow filtration, and (3) filter sterilization.

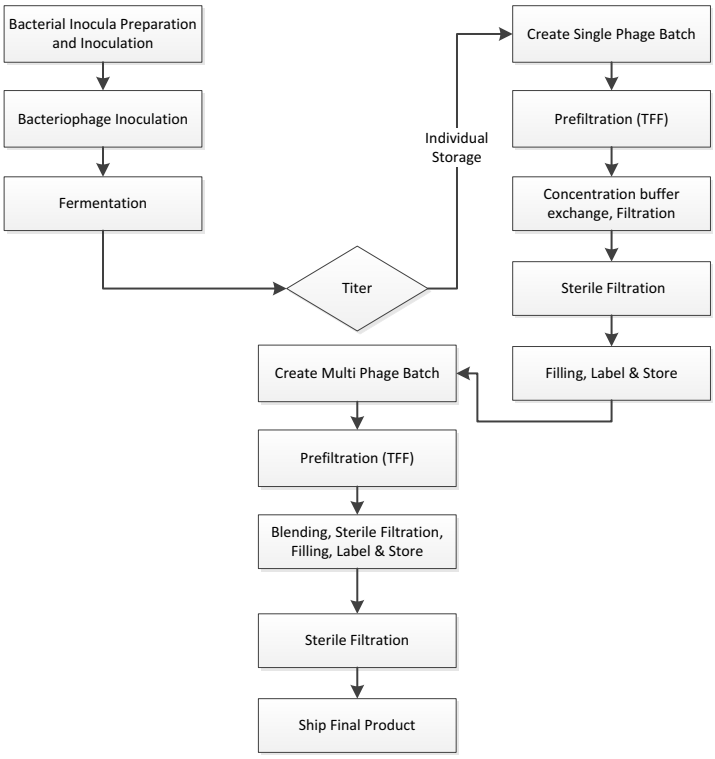
Once the fermentation broth reaches OD_{600} of approximately 0.1 indicating completion of phage lysis of the host cells, phage lysates are clarified through an initial continuous centrifugation process to remove unbroken host cells and host cell debris.

In a second Tangential Flow Filtration (TFF) step, the bacterial growth medium and other small molecule components are exchanged to phosphate-buffered saline (PBS, 100 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of di-sodium hydrogen phosphate, 0.24 g of potassium di-hydrogen phosphate di-hydrate, 898.12 g of H_20) thus insuring that there is no carryover of fermentation ingredients or host cellular materials, thereby eliminating the risk of carryover of unknown toxicants.

In the third filtration step the individual phage solutions are sterilized through a 0.22 µm filter in PBS (Lehnherr and Bartsch 2012). All purification steps are done at 22^{o}C. Filter sterilized phage production lots and final Phage preparation preparations are stored at 4^{o}C.

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

Fig. 1. Manufacturing Process flow diagram



Each individual batch of phage stock must meet the specified release parameters (Table 1, below) before it can be used as one of six components of the final phage preparation.

Table 1. Product specification for each batch of an individual phage.

Parameter	Specification
Phage titer[1]	>5 x 10^9 PFU/mL
Microbial sterility[2]	No growth
PCR identity[3]	Identical to PCR reference profile

[1] Clokie and Kropinski 2009
[2] ISO 48833
[3] Mullis et al. 1986; Methods and test validation (Attachment 3)

Maintenance of host and phage stocks to insure purity: Phage host bacteria and phage stocks are maintained to insure safety and integrity of

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the materials for use in manufacturing Phage preparation. Frozen aliquots of the non-pathogenic host strains, *E. coli* CCUG 29188 (ATCC® 43888) (Beutin et al. 2004) or *E. coli* MG1655 (ATCC® 47076) (Guyer et al. 1981), a derivative of *E. coli* K12, are stored at -80°C as glycerol stocks. For each production cycle the respective host strain is streaked out on an agar plate and a single colony is used to start a pre-culture to be used in the large-scale fermentation process.

Stocks of phage lysates are sterilized by 0.22 µm filtration and stored at 4°C. Immediately before use in the fermentation process, the phage stocks are sterilized anew by 0.22 µm filtration to minimize the risk of contamination.

Host organisms for the production of phages are safe and suitable microorganisms: Phages with exclusive specificity for the O157:H7 antigen are grown on the non-pathogenic *E. coli* host CCUG 29188 (ATCC® 43888, BioSafety Level 1), described by Castellani and Chalmers (Beutin et al. 2004). This *E. coli* strain does not produce shiga-like toxins and verotoxins, does not possess the genes for these toxins and is also negative for haemolysin.

Phages that do not show exclusive specificity for the O157:H7 antigen were grown on the *E. coli* K12 derivative MG1655 (ATCC® 47076, BioSafety Level 1). *E. coli* K12 is not considered a human, animal or plant pathogen, nor is it toxicogenic (Environmental Protection Agency (1997a). *E. coli K12* has a history of safe use in the production of specialty chemicals and human drugs and was exempted from EPA review under TSCA § 725.420 (Toxic Substance Control Act) (Environmental Protection Agency (1997b). In addition, *E. coli* K12 derivatives have been used repeatedly in the production of GRAS notified food ingredients e.g. α-cyclodextrin (GRN 000155), L-leucin (GRN 000308) and Lycopene (GRN 000299).

While, the host *E. coli* strains are safe and suitable production organisms, as noted above, viable cells, cell debris and other cellular materials are removed by centrifugation and successive filtration steps in the phage purification process, thereby eliminating any risk from unknown factors.

In summary, the manufacturing process for individual phage componets and the composite phage preparation is safe and suitable for the production of phages and host strains are safe for human consumption: the fermentation medium and buffer ingredients are food grade approved food additives or GRAS substances; the filtration buffer components are approved food additives or GRAS substances; and the final purification and sterilization steps are designed to remove all fermentation materials except for the desired phages.

3. Quality Control Testing and Release

Phage preparation preparations meet or exceed established quality control specifications for food ingredients (Table 2 below).

Table 2. Final Product Specifications

Description	**Standardized bacteriophage cocktail based on naturally occurring bacteriophages, stabilized**	
Concentration	Approx. 1 x 10^9 bacteriophages/ml[1]	
Packaging	Stainless steel KEG barrels, flat fitting	
Storage	Cool and dry (recommended 4-8°C), do not store in direct sun light	
Shelf life	3 month, process immediately after opening	
Appearance	Colorless to light yellowish liquid	
Texture	Liquid	
pH	7.0-7.4	
odor / taste	Characteristic	
Microbiological Parameter		
Total plate count	ISO 48833[2]	< 50 CFU/g
Yeast and Mould	NMKL 98[3]	< 100 CFU/g
Staphylococcus	ISO 6888[2]	< 10 CFU/g
Salmonella	NMKL 71[3]	not detectable in 25 g
Enterobacteriacea	ISO 21528[2]	< 100 CFU/g
Sulfite-reducing Clostridia	ISO 15213[2]	< 1000 CFU/g
PCR Verification[4]	Complies	Yes/no (for single phage solutions)

[1] Clokie and Kropinski 2009
[2] http://www.iso.org/iso/catalogue_detail.htm?csnumber=23036
[3] http://www.nmkl.org/index.php/en/
[4] Mullis et al. 1986; Methods and test validation (Attachment 3)

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Batch records are kept for each fermentation lot as specified above and in FinkTec Standard Operating Procedure (available on request). Batch records include in-process controls, phage identity confirmation, and final Quality Control release results. Three (3) representative batch records are attached (Attachment 4, 5, 6).

4. Intended Use

The proposed phage preparation is an antimicrobial agent intended for use as a processing aid to control shiga-toxin producing *E. coli* O157:H7 as well as non-O157:H7 shiga-toxin producing *E. coli* that may be present on beef carcasses in commercial slaughter operations.

The phage preparation is applied at processing plant ambient temperature as a spray using existing approved spray systems in meat processing facilities. The envisioned use is consistent with Good Manufacturing Practices and the expected efficacious dose is approximately 1.5×10^{11} phage particles per carcass. The preparation has been shown to be effective in reducing the number of *E. coli* 0157:H7 test strain in liquid, semi-solid agar and meat model assays.

5. Processing Aid Use

The technical effect on the food of the preparation is measured by a reduction in target *E. coli* on the food and not by a measure of the absence or inactivation of phage particles on the food. It is not intended for the phage preparation to have an ongoing effect on the treated meat and technical studies using the meat model system were undertaken to demonstrate that no ongoing effect occurs under conditions of the tests.

Test data demonstrate a significant reduction in test *E. coli* populations within four (4) hours of the phage treatment. Analysis at four (4) days and six (6) days following phage treatment (samples held at $3^{o}C$ to simulate plant conditions in cold boxes) found no further reduction in *E. coli* counts thus demonstrating that there is no ongoing technical effect on the treated beef (Attachment 7, 8).

These results are consistent with the known properties of the phages and their hosts, as well as processing parameters in the meat processing plant.

Phage/host infection requires a physical contact between the phage and its host. Phages must either come into contact with the host as a direct result of the spray application or move to the host by passive diffusion in a liquid environment. Because phages and *E. coli* are not mobile on dry surfaces such

as fully chilled beef carcasses, the phage/host interaction will occur only during the time that the carcass is wet.

Typically, during processing, including treatment with anti-microbial sprays such as the phage preparation, the carcasses undergo rapid cooling by virtue of the spray chilling process and are then hung in a cold, dry environment. The spray chilling portion of the process occurs over approximately 14 hours of the initial chilling followed by approximately 10 hours in a dry chiller to finish the cooling where the carcasses dry (Savell, J. W., Beef Carcass Chilling: Current Understanding, Future Challenges, www.beefresearch.org/CMDocs/BeefResearch/BeefCarcassChilling%20White%20Paper_final.pdf accessed July 25, 2017).

Once the carcass has dried, the phages and *E. coli* hosts are essentially immobilized. Only phage/host interaction initiated while the carcass surface is wet result in host kill, thereby limiting the technical effect to the early phases of the meat production process. The common practice of spray chilling of carcasses will also reduce phage numbers on the surface of carcasses.

In addition, phage infection and killing of the host depends on host metabolic functions. As meat carcasses are chilled to 4°C and all following steps of the process occur at refrigeration temperatures, new phage infections of the host are effectively eliminated. Cold temperature coupled with a removal of approximately 80% of the treated surface during post-chill de-ribbing, trimming and cutting of carcasses to the primal and sub-primal cut stage in meat processing (beef processor communication) results in a significant reduction in residual phage numbers and, thereby, opportunity for further infection events.

USDA has determined and codified in FSIS Directive 7120.1 numerous similar applications of phages to meat products, including poultry meat immediately prior to consumer packaging (https://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 28, 2017), as processing aids requiring no labelling on the commercial meat product.

In summary, the phage preparation will not have an ongoing technical effect of beef carcasses in the envisioned application for following reasons:

- Phage infection of the *E. coli* host is limited to the early phases of the meat processing process

- Phages are removed from the beef carcass during processing at various stages depending on plant protocols

- Phage / host interaction is inhibited at refrigeration temperatures in the post-chill supply chain

- USDA has determined that similar phage technology do not have an ongoing effect and are processing aids on meat and poultry products.

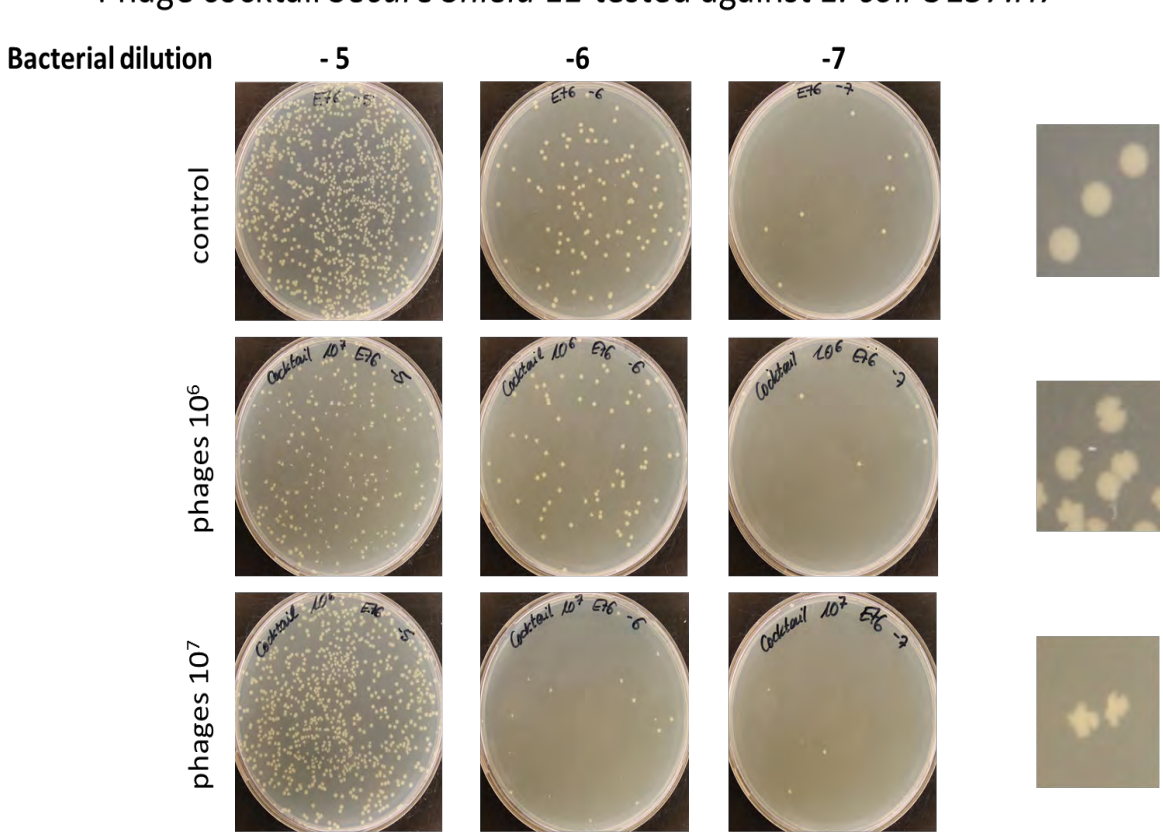
6. Efficacy of Use

Liquid Assay: Tests of the effectiveness of the phage preparation against a strain of O157:H7 *E. coli* (DSM 19206 (Leibniz Institute DSMZ) demonstrate that the phages reduce the number of the test *E. coli* in a liquid assay. Cultures with bacterial counts less than or equal to 1x106 CFU (Colony Forming Units)/mL were completely eradicated by $1x10^6$ PFU (Plaque Forming Units) /mL of phage preparation over 16 h at 37°C. The growth of cultures with higher bacterial starting counts was still significantly inhibited by 95.71 % (significant; $0.05 < p < 0.01$) and 99.06 % (highly significant; $0.01 < p < 0.001$), respectively (Attachment 9).

Semi-solid media assay: An overnight culture of an *E. coli* O157:H7 strain was serially diluted 10-fold in liquid growth medium. The dilutions were then plated onto agar plates to create three sets of plates that contain approximately 10, 100 and 1000 bacterial cells, respectively. The first set of plates served as a control for the growth of the *E. coli* strain, no bacteriophages were added.

Each plate of the second set was sprayed with 300 µl of the test phage preparation solution containing 10^6 phages per milliliter, while each plate of the third set was sprayed with a Phage preparation solution containing 10^7 phages per milliliter. All plates were then incubated for 16 hours at 37 °C. The results are shown in Figure 2 below.

Figure 2: Growth inhibition assay in semi-solid medium.



In the control experiment, all bacterial cells spread on the plates could grow into colonies, which, when viewed under the microscope appear perfectly round. When a bacteriophage cocktail with a concentration of 10^6 phages per milliliter was sprayed onto the surface of the plates, a reduction of the colony count of approximately 1 logarithmic scale could be observed. Under the microscope, the colonies appear sectorized, indicating that during colony growth they came into contact with bacteriophages present on the plate. When a concentration of 10^7 phages per milliliter was applied, a reduction of the colony count of approximately 2 logarithmic scales could be observed and the surviving colonies appear increasingly fuzzy. It can be concluded that the bacteriophages work less effectively on semi-solid medium than in liquid, most likely due to the reduction in the rate of diffusion.

Beef model system assay: A beef model system was used to evaluate the efficacy of the Phage preparation treatment on a meat substrate. Briefly, beef cubes of consistent dimensions were first inoculated with 10^3 cells of the *E. coli* test strain. Following a brief period to allow for adherence of the test

bacteria to the substrate, the treated beef cubes were treated with 1mL of a 10^6 PFU/mL suspension of the test phage preparation. After incubation, the surface bacteria were counted. The results of the study indicate that Phage preparation can reduce the population of an *E. coli* 0157:H7 test strain by approximately 79% under the test conditions (Attachment 9).

PART 3

7. Dietary Exposure

The expected dietary exposure to *E. coli*-specific phages as a result of application of the phage preparation is insignificant and below the level of toxicological concern. Calculation of incremental exposure to the commercial preparation or incremental Estimated Dietary Intake (EDI) was done according to the following:

Assuming the following:

- CEDI and EDI are assumed to be equal in this case as phage preparation described herein is a new product and has no other approved applications.
- All beef consumed in the US is treated with Phage preparation (highly conservative; see below)
- Phage particle wt. = 2 x 10^{-16} grams (Taylor, Epstein, and Lauffer 1955)(Giddings, Yang, and Myers 1977)(Mazzone, Engler, and Bahr 1980).
- Concentration phage particles per treatment = 1.5 X 10E11 phage particles per carcass (10E10 particles / liter applied)
- Typical beef carcass wt. = 350 kg
- Avg. Annual Beef Consumption/person/year in US = 35 kg (based on per capita carcass weight disappearance; (http://ers.usda.gov/datafiles/Livestock_Meat_Domestic_Data/Quarterly_red_meat_poultry_and_egg_supply_and_disappearance_and_per_capita_disappearance/Beef/WASDE_Beef.pdf; accessed July 23, 2016)

NB: Calculations are extremely conservative for the following reasons.
1. EDI assumes that all phages applied adhere to beef carcasses but the application is by spray on hanging carcasses and runoff of the phage preparation is estimated to exceed 90%.
2. EDI assumes that all consumed beef is treated with the preparation, but only the surface of beef carcasses is treated and the majority of the treated beef is removed from the carcass as trim (fascia and fatback) at the processing plant and is not sold to consumers (beef producer communication).

EDI Calculation

1. (Wt. of phage in gram X Conc. Phage particles/liter of applied phage suspension = gram phage per treated beef carcass.

2. Grams Phage per treated beef carcass / Wt. of dressed beef carcass(g) = Wt. phage/g of beef treated.

3. Wt. of phage/gram of beef treated X (Avg. beef consumption in US (g)/person/year / 365 days/year) = Incremental EDI as grams of phage consumed/person/day.

Following the above formula, we obtain:

1. **Gram phage/treated carcass** = 2x10E-16g X 1.5x10E11 phage/treated carcass = 30 **µg**
2. **Wt. phage/g of beef treated** = 30 µg phage / 3.5x10E5 = 8.6**x10E-11 g/g of beef treated**
3. **Incremental EDI** = (8.6x10E-11 g/g of beef treated X 3.5x10E4) / 365 days/year =

0.0082 µg/person/day

Because the highly conservative, calculated incremental exposure to phages in consumed beef treated with phage preparation is less than 0.5 ppb in the total diet, no toxicological safety studies were done in accordance with FDA guidance in "Guidance for Industry: Summary Table of Recommended Toxicological Testing for Additives Used in Food" June 2006 (https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/default.htm; accessed August 2, 2017) and "Guidance for Industry: Preparation of Food Contact Notifications for Food Contact Substances: Toxicology Recommendations": (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm081825.htm#iva; accessed June 9, 2016).

We further note that the incremental EDI above also implicitly assumes that every gram of beef consumed is treated, which is not the case. Specifically, the use as a spray treatment on beef carcasses means that only the surface of the beef carcass is treated. None of the interior muscle meat is intended to come in contact with the phage preparation and it is reasonable to expect that most of the treated surface, i.e. approximately 80% of the fascia and fatback will be removed from the carcass as trim prior to breaking down the carcass to consumer cuts (beef producer comment). Further, as the spray application occurs while the carcass is hung (pre-chill) in the processing plant, runoff of the application liquid could be as high as 90%. Subsequent processing of the carcasses to primal and sub-primal cuts would further reduce phage numbers.

Thus, it is quite clear that the presence of the phage preparation is at "de minimus" levels on beef that would be consumed; and it is more likely that

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consumers will be exposed to phage from the environment rather than as a result of the Phage preparation use.

Further, all other materials present in the phage preparation are either GRAS ingredients or approved food additives and thus present no risk to consumers of treated beef.

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PART 4

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8. Self-Limiting Levels of Use

The use of the phage preparation and subsequent human exposure is limited by:

a. Cost of the product to achieve the desired reduction of *E. coli* on beef carcasses is high and will limit the use of the ingredient to the efficacious dose.

b. Once the host *E. coli* in the proximity of the phages is diminished the phages will stop replicating and the phage numbers will diminish with time.

c. Phages are susceptible to a number of environmental factors, which act to decrease the number of active phage with time.

Part 5

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9. Common Use in Food Before 1958

None

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Part 6

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10. Basis of Determination of GRAS by Scientific Procedures

General Safety of phages as Antimicrobial agents for use in human food: Phages are bacterial viruses that exclusively infect bacteria with high specificity, i.e. whose host range consists exclusively of bacteria and, therefore, pose no risk of infections to humans. Phages have been characterized dating back to the early 1900s. To date there is no evidence that phages exhibit harmful effects on humans or animals (O'Mahony et al. 2011; Enderson, 2014).

Large numbers of phages have been found in virtually every aquatic or terrestrial habitat where bacteria exist (Gómez and Buckling 2011)(Marston and Sallee 2003)(Clokie et al. 2011). The gut of mammals and humans is an especially rich source of phages (Dalmasso, Hill, and Ross 2014), many of which have been consumed on a daily basis via various foods (Kennedy, Oblinger, and Bitton 1984)(Atterbury et al. 2003)(Hsu, Shieh, and Sobsey 2002)(Suárez and Reinheimer 2002)(Kiliç et al. 1996). This abundance of phages in the environment and the continuous exposure of humans to them could explain the absence of any adverse effects in various safety studies in humans and animals (Carlton et al. 2005)(Chibani-Chennoufi et al. 2004)(Bruttin and Brüssow 2005) as well as in long term applications in human medicine (Weber-Dabrowska, Mulczyk, and Górski 2003)(Górski et al. 2009)(Kutter et al. 2010)(Kutateladze 2015).

The idea of using these bacterial viruses as anti-bacterial agents followed and was employed extensively up to WWII and the discovery of antibiotics. Recently, because of the understanding that antibiotics are becoming less effective as their use has escalated to include non-therapeutic uses such as animal feed, there has been renewed interest in using phages as antibiotic agents in numerous applications, including direct human food use to control both pathogens and spoilage organisms and for use in animal feed as replacements for antibiotics (Endersen, 2014).

Phase I Human Clinical Trials and other Animal Safety Studies: Human and mammalian safety testing of phage exposure via the oral route has recently been reviewed by Sarker and Brussow (2016) (Summary of studies below). These tests were undertaken by the Nestle Research Center, Lausanne, Switzerland and by the International Center for Diarrhoeal Diseses Reseavh, Bangladesh. Specifically, phage safety studies using *E. coli*-specific phages have been performed on healthy and ill volunteers and no adverse effects were observed on the health of the test populations nor on the composition of subject's gut microflora (Sarker et al. 2016, Sarker and Brussow, 2016, Sarker et al. 2017, Bruttin and Brussow 2005).

E. coli phage interaction with a mammalian system has been studied in a mouse model. Phage/animal interactions were measured in a panel of tests using T4-like

phages. These studies are relevant to the safety determination of phage preparation component phages as T4-like phages are in the group Myoviridae; and 11 of 12 Phage preparation phages are in this group, showing high sequence homology to the T4 phages. One phage DSM104017(TB11) in the Phage preparation component phages is in the family Podoviridae and is related to the T7-like phages also used in the studies described below.

Dosage ranges in the mouse model tests were from 10^3 to 10^6 PFU (Plaque Forming Units)/ml (treatment levels similar to exposure estimates for the phage preparation. Passive transit of phages was observed as recovery of test phages was quantitative in a dose dependent way. Low levels of phage amplification were observed in initial phase of feeding but no amplification was observed after three (3) days.

Ampicillin resistance-marked T4 phages remained exclusively in the mouse gut lumen and histological analysis indicated no impact on gut mucosa. While phages were detected in the small intestine, cecum and colon, no phages were detected in the blood of test animals and no serum antibodies to the test phages were detected. In general, no adverse effects were observed in in treated mice vs. controls.

Human Phase I clinical trials have also demonstrated the safety of exposure to T4 (Myoviridae) and T7 (Podoviridae) *E. coli*-specific phage administered via the oral route. Healthy adult volunteers were fed 10^5-10^7 PFU and test phages in the study. Phages were detected in feces within 2 days demonstrating bioavailability and activity of the treatment. No adverse effects were linked with oral phage exposure in the trial and no antibodies to the test phages were detected in the blood. No test-related changes in liver damage markers were detected.

Significantly, the ingestion of test phages did not decrease the commensal *E. coli* fecal counts in test subjects indicating that non-target gut microflora including non-target *E. coli* are not affected by the treatment. Higher dose phase I trials in healthy adults using the same phage (10^7-10^9 PFU) confirmed the earlier findings. A series of clinical, clinical chemistry and hematology tests assessing liver and kidney function did not reveal a difference between test and placebo treatments. Again, as in the lower dose studies, 16S rRNA gene sequencing of stool samples detected no significant difference in gut microflora between treatment and placebo subject.

Phage-based pharmaceuticals are commonly used in Russia and a sample of these products was subjected to phase I trials in healthy adults. The commercial product contained both T4 and T7 phages and no adverse events were observed after application of 10^9 phages to adult volunteers as determined by a panel of clinical chemistry tests. Again, no impact on the gut microflora of tests subjects was detected using 16S rRNA sequencing of stool samples.

Follow up studies with the commercial product and T4/T7 phage cocktails demonstrated no adverse effects in children of 1-5 years and 5-9 years of age in clinical safety studies. These results led an institutional review board to approve randomized clinical trials with phages in hospitalized children.

Testing of *E. coli*-specific phages (highly related to the phage preparation phages) in a mouse model and human phase I safety trials confirm that ingestion of bacteriophages in oral dosage ranges in excess of those anticipated with the application of phage preparation are safe for human consumption as the trials have shown the phages to have no adverse effect on trial subjects (both children and adults).

In summary, there is no publically available scientific evidence indicating a health risk from consumption of lytic bacteriophages in adults or children. In fact, as noted above, humans consume vast numbers of phages daily without adverse effect. And, finally, there is no evidence of adverse effect of ingestion of bacteriophages on human gut microflora in these controlled studies. The scientific information available on the consumption of phages has also been reviewed and determined to be safe by regulatory agencies around the world, including approval for use in the US food market.

Additional safety requirements for individual phages that are to be used as antibacterial agents in food: While there is no evidence for adverse effects on humans or other animals, in general, the safety of individual phages to be used as antibacterial agents in food can be further assessed via analysis of phage genomes to insure that (1) the phage genome is free of genes encoding bacterial virulence factors such as toxins, (2) the phage is lytic, and (3) the phage does not code for known human allergens (including the so-called "big eight" or other factors known to be hazardous to humans (Endersen et al. 2014).

The genomes of the phages utilized in the phage preparation have been assessed by current state of the art bioinformatic methodologies and were found to conform to the safety standards listed.

Phage preparation phages are exclusively lytic: The biology of phages has been exhaustively studied in the 100 years since their discovery. Two major phage classes have been described, lytic and temperate. Temperate phages do not necessarily kill their host bacterium. They have the ability to passively invade a host and thereby are transferring their own genes from one host bacterium to the next, in a process called lysogenic conversion (Brüssow et al. 2004)(Fortier and Sekulovic 2013). As some temperate phages may carry toxin genes (Davis et al. 2000)(Smith et al. 2012), they are not suited to be components of a phage cocktail produced and applied on an

industrial scale. Lytic phages, on the other hand, lack the genes responsible for lysogenic conversion and an infection by a lytic phage always leads to the death of the bacterial host. Thus, lytic phages are safe for practical applications, as they do not disseminate toxin or other genes that pose risks for humans.

With the aid of comparative genomics, it is possible to clearly distinguish lytic from temperate phages and thus select only the former for a cocktail like Phage preparation. All phages in the Phage preparation preparation have been determined to be lytic phages by genomic analysis (Data available upon request).

Phage preparation phage genomes are free of genes encoding *E. coli* virulence factors: The complete phage genome sequence of each of the phage preparation phage components was analyzed using the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services) database and VirulenceFinder-1.5 Server test system (Joensen et al. 2014).

A curated database of *E. coli* toxins and virulence factors were employed for the analysis of the twelve (12) phage genomes for presence of known *E. coli* toxins and virulence factors. Initially, the database contained sequence variants for 76 genes (Joensen et al 2014); at the moment of the search it had 956 sequence variants of 103 *E. coli* genes associated with virulence (gene names are listed in the attached table). The search was performed using the lowest possible stringency provided by the search engine (85 % identity and 40% minimum length of the match) and resulted in no hits, demonstrating absence of known *E. coli* virulence genes in the genomes of the twelve phages studied (search output for every investigated genome is included as an attachment). No known *E. coli* virulence factors or stx-Holotoxin factors were found (Data available upon request).

Phage preparation phage genomes are free of genes encoding known toxins of concern as listed in 40CFR725.421(d): In the course of the bioinformatic characterization of the genomes of the phage preparation phage components, the presence of known toxin genes was analyzed by comparison of all phage genomic sequences against all proteins encoded by genomes of the species mentioned in 40CFR725.421(d), namely *Abrus precatorius*, *Adenia digitata*, *Aeromonas hydrophila*, *Androctnus australis*, *Bacillus alve*, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus laterosporus*, *Bacillus thuringiensis*, *Bordetella pertussis*, *Bungarus caeruleus*, *Bungarus multicinctus*, *Centruroides sculpturales*, *Chironex fleckeri*, *Clostridium bifermentans*, *Clostridium spp*, *Clostridium botulinum*, *Clostridium caproicum*, *Clostridium chauvoei*, *Clostridium difficile*, *Clostridium histolyticum*, *Clostridium novyi*, *Clostridium oedematiens*, *Clostridium perfringens*, *Clostridium septicum*, *Clostridium sordellii*, *Clostridium tetani*, *Corynebacterium diphtheria*,

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Corynebacterium ulcerans, *Crotalus spp*, *Dendroaspis viridis*, *Escherichia coli* and other *Enterobacteriaceae*, *Legionella pneumophila*, *Listeria monocytogenes*, *Naja naja varieties*, *Notechis scutatus*, *Oxyuranus scutellatus*, *Pseudomonas aeruginosa*, *Ricinus communis*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Vibrio cholera*, *Vibrio mimicus*, *Yersinia enterocolitica*, *Yersinia pestis* and *Proteus mirabilis*.

The comparison was done using the BLASTX program available at the website of the National Center for Biotechnology Information (NCBI) https://blast.ncbi.nlm.nih.gov/Blast.cgi and two protein sequence databases, Non-redundant protein sequences and UniProtKB, also provided by NCBI. The similarity between the compared sequences was considered significant, when the E-value of the match was 1 x 10^4, or lower, which was 10 times more stringent than the cut-off E-value parameter ≤ 10^5 used in practice (http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=528 (accessed February 11, 2017); Miller et al 2003).

No toxin-encoding sequences of concern were found in the genomes of the 12 component phages of the phage preparation.

Clinically significant antibiotic resistance genes are absent from phage preparation phage genomes: The complete phage genome sequence of each of the phage preparation phage components was analyzed using the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ResFinder/) database and ResFinder 2.1 test system (Zankari et al. 2012). Antibiotic resistance genes were not found to be encoded in phage preparation phage genomes.

Genes encoding for allergenic proteins of concern are not found in phage preparation phages: An allergen encoding gene sequence search for each phage genome was carried out against the database AllergenOnline of the University of Nebraska (version 16 released on January 27, 2016; http://www.allergenonline.org) using the recommended algorithm according to Codex Alimentarius (Codex Alimentarius Commission, 2003) and the AllergenOnline website (http://www.allergenonline.org).

According to Codex Alimentarius, IgE cross-reactivity between phage proteins and known allergens was considered a possibility when more than 35% identity in a segment of 80 amino acids (80mer) is identified.

Table 1 (below) summarizes the 80mer similarity results showing that none of the phages in Phage preparation have 80mer Open Reading Frames with homology to know allergen sequences of 35% or higher. Therefore no IgE

cross-reactivity with phage proteins is to be expected according to the standards of the Codex Alimentarius and the AllergenOnline website.

Table 1. Highest similarity scores between phage protein 80mers and the AllergenOnline database. No similarity equal or higher than 35% has been found.

Phage Name	ORFs with highest Similarity in 80mer	Highest Similarity in 80mer
DSM103290(AB27)	124	28%
DSM104018(BO1)	61	27%
DSM104019(EW2)	210	26%
DSM104021(GWF)	163	29%
DSM104022(HAM53)	146	28%
DSM104015(KRA2)	42	30%
DSM104023(MP75)	35 and 103	25%
DSM104020(TB6A)	177 and 180	24%
DSM104017(TB11)	63	29%
DSM104013(TB49)	258	29%
DSM104016(TB69)	63	27%
DSM104014(TB120)	192 and 272	26%

A sliding window of amino acid sequence 80mers of each phage protein was compared to the allergen database by local alignment search using FASTA (Version 36.3.8c Dec 2015, BL50 Blossum scoring matrix ktup:2 and gap open/ext: 20/10). All tested 80mers of phage proteins had identity to known allergens below 35%.

Therefore, the genomes of the phage preparation phages do not encode any known allergen sequences.

No Other Safety Concerns

There is no scientific evidence that Phage preparation component phages and other preparation components, i.e. buffer components, pose a risk as carcinogens in humans or other animals, and there is no reason, based on the composition and structure of the phages to suspect that the phages are carcinogenic.

In addition, incremental exposure for consumers of beef treated with the phage preparation is below 0.5 ppb. The Estimated Daily Intake of Phage preparation is 0.0082 µg/person/day or 0.003 ppb based upon a diet of 3000

g/person/day. Because the highly conservative, calculated incremental exposure to phages in consumed beef treated with Phage preparation is less than 0.5 ppb in the total diet, no toxicological safety studies were done as recommended by FDA in "Guidance for Industry: Preparation of Food Contact Notifications for Food Contact Substances: Toxicology Recommendations" (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm081825.htm#iva; accessed June 9, 2016).

Phage preparation is substantially equivalent to FDA-approved phage products: Numerous phage-based ingredients used as anti-microbial substances to control pathogens on foods have been reviewed and approved by regulatory agencies in the US and other countries.

Since the FDA in 1996 approved a *Listeria*-specific phage preparation as a food additive, several other products based on lytic phages, targeting various bacterial pathogens, have been designated GRAS in the US and/or have been cleared for food safety usage by a number of regulatory agencies.

The phage preparation is substantially equivalent to the approved, safe and suitable phage preparations listed below:

Listex™ is a phage preparation containing a single *Listeria monocytogenes* lytic phage, P100, used for bio-control of *Listeria* in susceptible foodstuffs, that is GRAS (GRAS Notice No. 000218; http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=218&sort=GRN_No&order=DESC&startrow=1&type=basic&search=218; accessed July 23, 2016)

Listex™ is also listed by the USDA FSIS for use as processing aid for use on ready-to-eat meat products (FSIS Directive 7120.1; http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 23, 2016)

Listex™ is also approved as a processing aid for susceptible foodstuffs in many countries, including Canada, by Health Canada and FSANZ, Australia and New Zealand. The Dutch Ministry of Health has issued a formal statement, confirming that Listex™ can be used as a processing aid. Additionally, Listex™ has been approved for use in Switzerland in cheese making and also as processing aid in keeping with European legislation on food safety (http://www.listex.eu/product/; accessed July 23, 2016).

Listex™ is listed by the Organic Materials Review Institute (OMRI). Listex™ may be used in the certified organic production of food, food processing and

handling according to the USDA National Organic Program Rule (http://www.listex.eu/product/; accessed July 23, 2016).

ListShield™ is a phage preparation containing six lytic *Listeria monocytogenes*-specific phages, that is a FDA-approved food additive (21 CFRF§172.785)

ListShield™ is listed by the USDA FSIS for use as processing aid with no labeling requirements when applied to various ready-to-eat meats and poultry products (FSIS Directive 7120.1; http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 23, 2016).

ListShield™ is GRAS for direct application to fish and shellfish (including smoked varieties; e.g. smoked salmon), fresh and processed fruits, fresh and processed vegetables, and dairy products (including cheese) (GRAS notice No. 000528; http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=528&sort=GRN_No&order=DESC&startrow=1&type=basic&search=528; accessed July 23, 2016)

ListShield™ is also EPA-registered for use on non-food surfaces in food processing plants to prevent or significantly reduce contamination of *Listeria monocytogenes* (EPA registration #74234-1; https://www3.epa.gov/pesticides/chem_search/ppls/074234-00001-20080618.pdf; accessed July 23, 2016).

ListShield™ is Health Canada approved for use on ready-to-eat meat and poultry, smoked salmon, fresh cut apples and long leaf lettuce (interim Letter of No Objection).

ListShield™ is National Food Service of Israel approved as a food processing aid for the treatment of ready-to-eat meat and poultry products (Ref: 70275202).

EcoShield™ is a phage preparation containing three lytic phages specific against *E. coli* O157:H7, that is FDA approved through a "Food Contact Notification" for use on red meat parts and trim, intended to be ground (FCN no. 1018; http://www.accessdata.fda.gov/scripts/fdcc/?set=FCN&id=1018&sort=FCN_No&order=DESC&startrow=1&type=advanced&search=¤¤phage%20¤; accessed July 23, 2016).

EcoShield™ is also listed by the USDA FSIS as safe and suitable for use in the production of red meat parts and trim prior to grinding as processing aid

with no labeling requirements (FSIS Directive 7120.1; http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 23, 2016).

EcoShield™ is Health Canada approved for use on red meat parts and trim prior to grinding (Health Canada interim Letter of No Objection)

EcoShield™ is National Food Service of Israel approved as food processing aid for the treatment of meat immediately before grinding (Ref: 70275202).

AgriPhage™ is a phage preparation targeting *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* that is EPA-registered for use on tomatoes and peppers. AgriPhage™ can be applied directly as foliar spray and can be used as a curative on symptomatic plants or preventively prior to visual signs of damage (EPA Reg. No. 67986-1; https://www3.epa.gov/pesticides/biopesticides/product_lists/new_ai_2006.htm; accessed July 23, 2016).

AgriPhage™ has been amended to now include organic usage on tomato and pepper plants as governed by the USDA National Organic Program (NOP).

AgriPhage-CMM™ is a phage preparation targeting *Clavibacter michiganensis* pv. *michiganensis* that is EPA-registered for use on tomatoes. AgriPhage-CMM™ can be applied directly as a foliar spray and can be used as a curative on symptomatic plants or preventively prior to visual signs of damage (EPA Reg. No. 67986-6; https://www3.epa.gov/pesticides/chem_search/ppls/067986-00006-20110930.pdf; accessed July 23, 2016).

The Canadian Pest Management Regulatory Agency (PMRA) has approved bio-pesticide AgriPhage-CMM™ for bacterial stem canker in tomato caused by *Clavibacter michiganensis* pv. *michiganensis* (30301)

Finalyse™ is a phage preparation targeting *E. coli* O157:H7, that received USDA Food Safety and Inspection Services approval for commercialization and application as a spray mist or wash on live animals prior to slaughter to decrease pathogen transfer to meat (FSIS Directive 7120.1; http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 23, 2016).

Armament™ is a phage preparation targeting *Salmonella*, that received USDA Food Safety and Inspection Services approval for the commercialization and application as a spray mist or wash on the feathers of live poultry prior to slaughter to decrease pathogen transfer to meat(FSIS Directive 7120.1;

http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 23, 2016)

Salmonelex™ is a phage preparation containing two specific phages, S16 and FO1a, for use as antimicrobial to control *Salmonella* serovars in certain pork and poultry products at levels up to 10^8 PFU/g of food, that was designated as GRAS (GRAS Notice No. 000468; http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=468&sort=GRN_No&order=DESC&startrow=1&type=basic&search=468 ; accessed July 23, 2016).
SalmoFresh™ is a phage preparation for controlling the foodborne bacterial pathogen *Salmonella enterica*, that is GRAS for direct application onto poultry, fish and shellfish and fresh and processed fruits and vegetables (GRAS Notice No. 000435; http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=435&sort=GRN_No&order=DESC&startrow=1&type=basic&search=435 ; accessed July 23, 2016).

SalmoFresh™ is also FSIS-listed as safe and suitable as antimicrobial for use in the production of poultry products as a processing aid with no labeling requirements (FSIS Directive 7120.1; http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES ; accessed July 23, 2016).

SalmoFresh™ is Health Canada approved as a processing aid for use on fish, shellfish and fresh and processed fruits and vegetables or on ready-to-eat poultry products prior to slicing and on raw poultry prior to grinding or after grinding (Health Canada interim Letter of No Objection).

SalmoFresh™ is National Food Service of Israel approved as a food processing aid for the treatment of fish, shellfish, fresh and processed fruits and vegetables and poultry immediately before or after grinding and on ready-to-eat products before slicing (Ref: 70275202).

Specifically, Phage preparation is substantially equivalent to EcoShield™, which is a FDA-approved food contact substance (FCN 1018); (http://www.accessdata.fda.gov/scripts/fdcc/?set=FCN&id=1018&sort=FCN_No&order=DESC&startrow=1&type=advanced&search=¤¤phage%20; accessed July 23, 2016). Both substances are *E. coli*-specific, lytic-type phage antimicrobial preparations designed for use on meat for human consumption.

In addition to FDA determining that the substance is safe and suitable for the described use as a food contact substance, the use of EcoShield™ was determined to have no significant effect on the quality of the human

environment and therefore exempt from preparation of an environmental impact statement (http://www.fda.gov/Food/IngredientsPackagingLabeling/EnvironmentalDecisions/ucm243606.htm; accessed July 23, 2016). Finally, the substance is listed for use as a safe and suitable antimicrobial for use on meat by the USDA (http://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES; accessed July 223, 2016).

SalmoPro® is a preparation of Salmonella-specific phages that are GRAS (GRN no. 603) for general use on food. Submitters claimed explicitly that all lytic phages were GRAS and FDA responded with a "no objection" finding.

Absence of other toxicological or carcinogenicity concerns: There are no known toxicological or carcinogenicity concerns arising from the use of phages as antimicrobial agents in or on food, generally, and none specific to the *E. coli*-specific phage preparation phages in the scientific literature.

Summary of the Basis of for determination of GRAS: The phage preparation component phages as described in detail herein were isolated from the natural environment and are part of the vast number of phages that are ubiquitous in the earth's air and water and that are consumed inadvertently daily by man. The ingestion of lytic-type *E. coli*-specific phages is considered safe in general and has been shown in scientific studies to be safe in animal studies and human clinical trials.

The phages in the *E. coli*-specific phage preparation have also undergone additional safety determinations specific to individual phage isolates. The phages are well-characterized and meet criteria posed in the scientific literature for the safe use of phage technology in food, i.e. (1) the phages are lytic-type; (2) genomic analysis indicates that the phages do not encode allergenic proteins; (3) their respective genomes do not encode bacterial or other toxins of concern that could be transferred via phage infection of target *E. coli* strains; and (4) the genomes do not encode for clinically significant antimicrobial resistance factors.

Individual phage components are manufactured in a well-controlled, food-grade fermentation process that employs GRAS food ingredients as raw materials, eliminating risk from carryover of the raw materials in the final food ingredient. Purification and sterilization of the individual phage production lots employ GRAS or approved food additive buffers which insures that there is no carry-over of host cell materials or unknown hazardous materials. Further, the host bacteria used in the manufacturing process are non-pathogenic, non-toxigenic microorganisms with a safe history of use in GRAS substance manufacturing that are safe and suitable for use in the manufacture of the component phages. Quality Control release

specifications, which include PCR identity test for each lot are stringent and appropriate for food ingredients.

Further, human exposure to phages in the preparation used as intended will be insignificant and below the level of toxicological concern. Incremental EDIs of the phage preparation components are estimated to be exceedingly low (8.2 nanogram/person/day range or approximately 0.003ppb of consumed food) at orders of magnitude below FDAs guidance thresholds for safety testing. It is also highly unlikely that these phages would survive post-treatment processing in the meatpacking environment and would almost certainly be inactivated by normal cooking temperatures employed by consumers. In fact, the final cooking step would reduce the phages to components such as proteins and nucleic acids, which are all consumed daily in a virtually all foods.

There is no evidence in the scientific literature to suggest that the use of phages in the preparation or other lytic-type phage, as described herein, poses any toxicological or carcinogenicity risk to consumers. And, the use of the phages as envisioned poses no significant risk to the environment because they are natural components of the environmental phage population and the additional phages released into the environment would be insignificant relative to the natural phage populations.

Finally, the phage preparation is substantially equivalent to other phage technologies that have been the subjects of extensive regulatory safety review and approval by regulatory agencies in the US and abroad, including FDA and USDA.

In summary, FINK TEC GmbH has utilized data available in this GRAS notice, including estimated dietary exposure under the conditions of use, the publically available information in the scientific literature which is available to experts in the field, and approvals of substantially equivalent phage technologies by competent authorities in the US and other jurisdictions and has determined that *Escherichia coli-specific phage preparation* is GRAS when used as an anti-microbial agent on beef carcasses.

FINK TEC GmbH is not aware of any data that are, or may appear to be, inconsistent with the conclusion that *Escherichia coli-specific* phage preparation described herein is GRAS for the intended use.

PART 7

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

11. Publically available data

All of the information that FINK TEC GmbH discusses in Part 6 is publically available including analytical methods as listed in the Reference section below or in the text of Part 6, except FINK TEC GmbH laboratory data, e.g. efficacy of use data, included in the Attachment section of this notice.

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ATTACHMENTS

ATTACHMENT 1

Host spectrum analysis of the Phage preparation phages based on spot test assays with non-O157:H7 and O157:H7 stx-encoding *Escherichia coli* strains.

R018-SAHL

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

PTC Phage Technology Center GmbH

Host spectrum analysis of the *Secure Shield E1* phages based on spot test assays with non-O157:H7 and O157:H7 *stx*-encoding *Escherichia coli* strains.

Study Number # R018-SAHL

PTC Phage Technology Center GmbH

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Dr. Michael Fink

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1. STUDY TITLE

Host spectrum analysis of the *Secure Shield E1* phages based on spot test assays with non-O157:H7 and O157:H7 *stx*-encoding *Escherichia coli* strains.

2. STUDY DIRECTOR

Hansjörg Lehnherr, Ph.D.

3. STUDY PERSONNEL

The following personnel contributed to the conduct and reporting of the studies reported herein:

Name:	Title:	Role:
Hansjörg Lehnherr, Ph.D.	Chief scientist	Study director
Anna Bierbrodt, M.Sc.	Research scientist	Hands-on-research

4. PERFORMING LABORATORY

PTC Phage Technology Center GmbH
Siemensstraße 42
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Tel.: 49 (0) 2383 919 174
Fax: 49 (0) 2383 919 179

5. STUDY OBJECTIVE

Determine the coverage of a collection of non-O157:H7 and O157:H7 *stx*-encoding EHEC strains by the *Secure Shield E1* phages. The host range analysis was based on spot test assays with the overlay agar method.

6. PHAGES

The phage cultures used for the host spectrum analysis are listed below:

***Table 1**: Phage data*

Phage		Date/# Ch.-B.	Titer [PFU/mL]
DSM #	Internal #		
DSM 104013	TB49	09.09.2016	$6x10^{10}$
DSM 104014	TB120	# 170820161	$3x10^{9}$
DSM 104015	KRA2	16.09.2016	$5.8x10^{10}$
DSM 104016	TB69	# 21020151/1	$6x10^{9}$
DSM 104017	TB11	21.07.2014	$2x10^{10}$
DSM 104018	BO1	26.02.2016	$2.5x10^{10}$
DSM 104019	EW2	20.07.2016	$3.6x10^{10}$
DSM 104020	TB6A	# 160920151/1	$1x10^{9}$
DSM 104021	GWF	17.02.2016	$1x10^{9}$
DSM 104022	HAM53	17.08.2016	$2x10^{7}$
DSM 104023	MP75	10.09.2014	$1.7x10^{10}$
DSM 103290	AB27	# 160920162	$3x10^{10}$

7. BACTERIAL STRAINS USED FOR HOST RANGE ANALYSIS

The following bacterial strains were used to determine the host ranges of the *Secure Shield E1* phages. Except for DSM-19206 (E76) and CCUG-29188 (E202), which served as controls, all of the strains are designated as EHEC and encoded at least one type of *stx* gene.

***Table 2**: Non-O157:H7 and O157:H7 stx-encoding E. coli strains*

Strain	Serotype	Stx gene	O157:H7	Shiga toxin
E. coli	O⁻:H48	stx2b	no	yes
E. coli	O⁻:H19	stx1c; stx2d	no	yes
E. coli	O⁻:H25	stx2d	no	yes
E. coli	O103:H25	stx2a	no	yes
E. coli	O111:H8	stx1a	no	yes
E. coli	O113:H4	stx2d	no	yes
E. coli	O121:H19	stx2a	no	yes
E. coli	O145:H25	stx2a	no	yes
E. coli O157:H7 (1)	O157:H7	stx2a; stx2c	yes	yes
E. coli O157:H7 (2)	O157:H7	stx2a	yes	yes
E. coli ISI-2107	O157:H7	not specified	yes	yes

Strain	Serotype	Stx gene	O157:H7	Shiga toxin
E. coli ISI-2391	O157	not specified	yes	yes
E. coli ISI-2411	O157	not specified	yes	yes
E. coli ISI-2435	O157	not specified	yes	yes
E. coli ISI-2444	O157	not specified	yes	yes
E. coli ISI-244 7	O157	not specified	yes	yes
DSM-19206 (E76)	O157:H7	none	yes	none
CCUG-29188 (E202)	O157	none	yes	none

8. MEDIA AND REAGENTS

All media and reagents were sterilized before usage.

- LB broth Lennox (Roth, Karlsruhe, Germany; catalog # X964.4)
- LB agar Lennox (Roth, Karlsruhe, Germany; catalog # X965.2)
- Agar-Agar, Kobe I (Roth, Karlsruhe, Germany; catalog # 5210.2)
- LB top agar (LB broth with 0.6 % Agar-Agar)

9. GENERAL OUTLINE OF STUDY

- Overnight cultures of the bacterial test strains were grown in LB medium at 37°C. The LB top agar was melted and kept in a water bath at 55°C.
- 100 µL of the overnight cultures were mixed with 4 mL melted, warm top agar. The mixture was vortexed and distributed on LB agar plates.
- After solidification 2 µL of each phage culture were spotted on the top agar overlays.
- The LB agar plates were incubated at 37°C for 16h. Clear spots in the bacterial lawn were classified as positive phage-host interactions.

10. RESULTS

1. Raw Data

***Table 3**: Coverage of a collection of non-O157:H7 and O157:H7 stx-encoding E. coli strains by the Secure Shield E1 phages.*

***E. coli* strains**		**Phages**											
		TB49	TB120	BO1	EW2	TB69	KRA2	GWF	HAM53	AB27	TB6A	TB11	MP75
Non-O157 *stx*-encoding *E. coli* strains	O⁻:H48	+	-	-	+	+	+	+	-	+	+	+	+
	O⁻:H19	-	-	-	+	-	+	+	-	+	+	+	-
	O⁻: H25	+	-	-	+	-	+	+	-	+	+	-	-
	O103:H25	+	-	-	+	+	+	+	-	+	+	+	-
	O111:H8	+	+	-	+	+	+	+	-	+	+	+	+
	O113:H4	-	-	-	-	-	-	-	-	-	-	-	-
	O121:H19	-	-	-	+	-	+	+	-	+	+	+	-
	O145:H25	+	-	-	+	+	+	-	-	+	+	+	+
O157 *stx*-encoding *E. coli* strains	O157:H7(1)	+	-	+	+	+	-	-	+	+	-	-	+
	O157:H7(2)	+	-	+	+	+	-	-	+	+	-	+	-
O157 stx-encoding *E. coli* wild isolates	ISI-2107	+	-	-	-	+	-	-	-	-	-	+	+
	ISI-2391	+	+	-	-	+	-	-	-	-	+	-	-
	ISI-2411	+	+	-	-	+	+	-	+	-	+	+	+
	ISI-2435	+	-	-	-	+	-	-	-	-	-	+	+
	ISI-2444	+	-	-	-	+	+	-	-	-	-	+	+
	ISI-2447	+	-	-	-	+	+	-	-	-	-	+	+
O157 stx-free *E. coli* strains	E76	+	-	+	+	+	+	+	+	-	+	+	+
	E202	+	-	-	-	-	-	-	+	-	-	-	+

2. Tabular presentation of results

***Table 4**: Ranked coverage*

E. coli strains	Phages												Pos. int./strain
	TB49	TB69	TB11	KRA2	MP75	EW2	TB6A	AB27	GWF	HAM53	TB120	BO1	
O111:H8	+	+	+	+	+	+	+	+	+	-	+	-	10
E76	+	+	+	+	+	+	+	-	+	+	-	+	10
O⁻:H48	+	+	+	+	+	+	+	+	+	-	-	-	9
O103:H25	+	+	+	+	-	+	+	+	+	-	-	-	8
O145:H25	+	+	+	+	+	+	+	+	-	-	-	-	8
ISI-2411	+	+	+	+	+	-	+	-	-	+	+	-	8
O157:H7(1)	+	+	-	-	+	+	-	+	-	+	-	+	7
O157:H7(2)	+	+	+	-	-	+	-	+	-	+	-	+	7
O⁻:H19	-	-	+	+	-	+	+	+	+	-	-	-	6
O⁻: H25	+	-	-	+	-	+	+	+	+	-	-	-	6
O121:H19	-	-	+	+	-	+	+	+	+	-	-	-	6
ISI-2444	+	+	+	+	+	-	-	-	-	-	-	-	5
ISI-2447	+	+	+	+	+	-	-	-	-	-	-	-	5
ISI-2107	+	+	+	-	+	-	-	-	-	-	-	-	4
ISI-2391	+	+	-	-	-	-	+	-	-	-	+	-	4
ISI-2435	+	+	+	-	+	-	-	-	-	-	-	-	4
E202	+	-	-	-	+	-	-	-	-	+	-	-	3
O113:H4	-	-	-	-	-	-	-	-	-	-	-	-	0
Pos. int./phage	15	13	13	11	11	10	10	9	7	5	3	3	

Abbreviations: Pos. int./phage Positive interactions per phage
Pos. int./strain Positive interactions per strain

3. Interpretation of results

The twelve *Secure Shield E1* phages showed positive interactions with 17 out of 18 tested *E. coli* strains (94.44 %). Only the strain O113:H4 (*stx2d*⁺) showed no phage interaction in this analysis. All of the phages were able to infect at least three *E. coli* strains. Since the individual host ranges varied, the different phages complement each other, increasing the total coverage, which is important for the composition of an efficient phage product like *Secure Shield E1*. DSM 104019 (EW2), DSM 104020 (TB6A), DSM 103290 (AB27) and 'DSM 104021 (GWF) considerably increased the coverage of the strains O⁻:H19 (*stx1c*⁺, *stx2d*⁺), O⁻:H25 (*stx2d*⁺) and O121:H19 (*stx2a*⁺), which were not efficiently covered by the broad spectrum phages DSM 104013 (TB49), DSM 104016 (TB69) and DSM 104017 (TB11). Information like

this will be used to choose six phages out of twelve in order to produce a Secure Shield E1 product with an optimal range of activity.

11. SUMMARY CONCLUSION OF THE STUDY

For the present study *E. coli* strains with high serotypical variability were chosen to create a representative collection of shiga toxin producing strains. This collection was covered to 94.44 % by the twelve component phages of *Secure Shield E1*, depending on phages with broad host ranges and complementary host preferences. Therefore *Secure Shield E1* contains a combination of phages appropriate for application against EHEC *E. coli.*

12. SIGNATURES

_____ _____

Anna Bierbrodt, M.Sc. Date
Research scientist

_____ _____

Hansjörg Lehnherr, Ph.D. Date
Study director

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11. SUMMARY CONCLUSION OF THE STUDY

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12. SIGNATURES

(b) (6)

Anna Bierbrodt, M.Sc.
Research scientist

1/12/2016
Date

(b) (6)

Hansjörg Lehnherr, Ph.D.
Study director

1/12/2016
Date

ATTACHMENT 2

Document matrix PTC GmbH

Quality System procedures

VA_EK_01 acquisition
VA_EK_02 change of supplier
VA_GF_05 training courses
VA_MS_01 document guidance
VA_MS_02 internal audits
VA_MS_03 guidance of management documents
VA_MS_04 revision and preventive measures
VA_MS_05 guidance of faulty products
VA_MS_06 identification and traceability
VA_PR_04 resource delivery for the production (chemistry)
VA_PR_07 filling biomaterials
VA_PR_08 production biomaterials
VA_PR_09 Resource delivery for the production (biomaterials)
VA_PM_01 surveillance of testing equipment
VA_QK_01 quality control biomaterials

Standard operating procedures

AA_FE_02 preparation of cryocultures
AA_FE_03 isolation of phages from environmental probes
AA_FE_04 growth of bacterial strains on agar plates
AA_FE_05 prepration of agar plates
AA_FE_06 isolation of DANN
AA_FE_07 purification of consumables
AA_FE_08 preparation of steril liquids
AA_FE_09 preparation of steril solids
AA_FE_10 disposal of S2 waste
AA_FE_11 phage isolation in the 96 well format
AA_FE_12 stability testing for bacteriophages
AA_FE_13 meat trial
AA_PR_01 reciept of goods
AA_PR_07 production of LB growth medium
AA_PR_10 handling of product packaging
AA_PR_12 production and mixing of phage cocktails
AA_PR_13 filling of phage cocktails
AA_PR_23 preparation of phage starter cultures
AA_PR_24 fermentation 100 L scale
AA_PR_25 separation
AA_PR_26 pretreatment of packaging kegs
AA_PR_27 filtration
AA_PR_28 preparation of Tris/$MgCl_2$ buffer
AA_PR_29 handling of the filter system
AA_PR_30 innoculation
AA_PR_31 sterilization and disposal of liquids
AA_PR_32 sterilization and disposal of phage cocktails
AA_PR_33 manual filling process
AA_PR_34 preparation of PBS buffer
AA_PR_35 automatic filling by keg bottling machine

ATTACHMENT 3

MS-SOP
PCR Reaction

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-Standard operating procedure

PCR reaction

FINKTEC Professional Hygiene Management

created/changed by: Lukas Lis Date: 13.07.2015	Released by: HL Date: 14.12.2015

1. Short description:

A standard procedure to detect single bacteriophages within a cocktail and to verify the identity of a single phage following production.

Specific amplification of unique DNA regions within the genome sequences of bacteriophages. (PCR according to Mullis *et al*., 1986). Detection of the generated DNA fragment on 2% agarose gels.

2. Equipment:

- Thermocycler
- Sterile 0,5 ml Eppendorf tubes
- Gel dokumentation chamber
- Elektrophoreses chamber
- Gilson PIPETMAN Classic P200 (200 µl), P20 (20 µl) und P2 (2 µl) pipettes
- Cooling block or crushed ice

3. Reagents:

- DreamTaq Green Mastermix (5 Units polymerase)
- specific primers
- Agarose 2%
- TAE-buffer
- Invitrogen DNA-ladder 100 bp
- Sterile water

4. Calibration:

See instructions by the manufacturers.

5. Procedure:

5.1 Preparation of primer stocks

Freshly synthesized primers are dissolved in an appropriate volume of TE buffer, according to the instructions of the manufacturer. This creates a primer stock with a concentration of 100 µM. This stock is then diluted to create a working stock with a concentration of 10 µM (1:10 dilution in sterile water). Both primer stocks are stored at -20 °C.

5.2 Composition of the PCR reaction mix:

During the entire mixing procedure all components are kept in a cooling block set to 0°C or on crushed ice to reduce the loss of enzyme activity and prevent the degradation of the DNA components.

5 µl of a bacteriophage lysate (ideally with a concentration of 10^9 pfu/mL) or 2 µl of a DNA template (100 ng)
0,5 µl forward primer (20 µM)
0,5 µl reverse primer (20 µM)
12,5 µl DreamTaq Green MM (5 units of polymerase)
add sterile water to an end volume of 25 µl.

For every reaction both a negative control (reaction mix without DNA template) and a positive control (100 ng of isolierte phage-DNA) is run.

1. Label the reaction tubes and pipette the various compositions together. It is recommended to start with the master mix followed by the primers and the DNA template. Mix the reactions well using the pipette.
2. Start the thermocycler and choose the option „run“.
3. Select the appropriate program for the bacteriophage tob e analyzed, load the propes into the cycler and start the amplification.
4. While the PCR runs, pour the 2% electrophoreses agarose gel and let it solidify for at least 30 minutes.
5. Prepare 250 ml TAE running buffer and completely immerse the gel with it.
6. After the thermocycler completed the PCR program, the probes are cooled to 4°C. At any point thereafter the probes can be taken from the termocycler to analyse them on the electrophoreses gel.
7. On the gel the DNA fragments are separated by applying 90 V, 400 mA für 30 minutes.
8. The gel is then analyzed in the gel documentation chamber with the help of UV light.
9. The gel is photographed and a printed copy of photo is attached to the quality control form.

ATTACHMENT 4

Batch Record

TB69

MS-sheet
Fermentation

FINKTEC Professional Hygiene Management

fermentation	production date	24.01.17			
	intermediate	TB69			
	batch	24012017 1			
	volume [ltr.]	100			
IPC1 **raw material**	LB-medium [kg]	2,0			
	pH- Wert	6,89			
		fermenter 1	*comments*	*fermenter 2*	*comments*
IPC2 **preculture bacteria**	volume [ltr.]	1,0			
IPC2a **preculture phage**	phagelysate titer [pfu/mL]	$1,8 \times 10^{10}$			
	volume phagenlysate [ltr.]	1,0			
start (time) 7 15	OD_{600} t= 0	0,031			
	OD_{600} t = 30	0,034			
	OD_{600} t = 60	0,056			
	OD_{600} t = 90	0,112			
	OD_{600} t = 120	0,170			
	OD_{600} t = 140	0,310			
	OD_{600} t = 150	0,338			
	OD_{600} t = 180	0,456			
	OD_{600} t = 210	0,521			
	OD_{600} t = 240	0,582	Ernte		
	OD_{600} t =				
	addition of phage	t= 140		t=	
fermentation-product	phage titer [pfu/ml]	$4,3 \times 10^{10}$			

executed by (date/signature): 24.01.17 (b) (6)

product release (date/signature): 24.01.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet
separation / filtration

FINKTEC Professional Hygiene Management

fermentation	produktion date	24.01.17
	intermediate	TB69
	batch	240120A1
	volume [ltr.]	100

separation		
phage titer before separation	titer [pfu/mL]	5×10^{10}
date: 25.01.17	OD_{600} before separation	0,489
start: 640	OD_{600} first separation	0,038
end: 855	OD_{600} second separation	0,018
	phage titer [pfu/ml] after separation	$4,1 \times 10^{10}$
	conductivity [mS/cm]	11,31
	volume of buffer [ltr.]	10

comments:

executed by (date / signature): 25.01.17 (b) (6)

MS-sheet

separation / filtration

FINKTEC Professional Hygiene Management

filtration						
prefilter start: end: / IPC 5	pressure [bar]	0				
	filtration time [h]	48min.				
	volume buffer [ltr.]	10				
	phage titer concentrate [pfu/ml]	$2,8 \times 10^{10}$				
endfilter module 1 Uhrzeit von: 7:10 Uhrzeit bis: 13:32 ICP 7	pressure [bar]	0,1				
	filtration time [h]	5:22				
	volume buffer [ltr.]	200	8:45	12:00		
	conductivity [mS/cm]	16,88	11,89	16,88		
	phage titer concentrate [pfu/ml]	$3,7 \times 10^{10}$				
endfilter module 2 start: 7:10 end: 13:32 ICP 7	pressure [bar]	0				
	filtration time [h]	5:22				
	volume buffer [ltr.]	200	8:45	12:00		
	conductivity [mS/cm]	16,54	11,76	16,54		
	phage titer concentrate [pfu/ml]	$4,2 \times 10^{10}$				
intermediate after sterilfiltration CCP 1	volume intermediate [ltr.]	82,2	keg 1 50	keg 2 32,2		
	pH-value	7,59				
	total germ count at 37°C [cfu/ml]	0				
	total germ count at RT [cfu/ml]	0				
	conductivity [mS/cm]	16,62				
	phage titer [pfu/ml]	$3,8 \times 10^{10}$				
	PCR analysis (√)	✓				
	Sample for endotoxin determination (√)	✓				
	consistency, smell, visual appearance (√)	o.B.				

comments:

executed by (date / signature): 25.01.17 (b) (6)

release (date / signature): 25.01.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

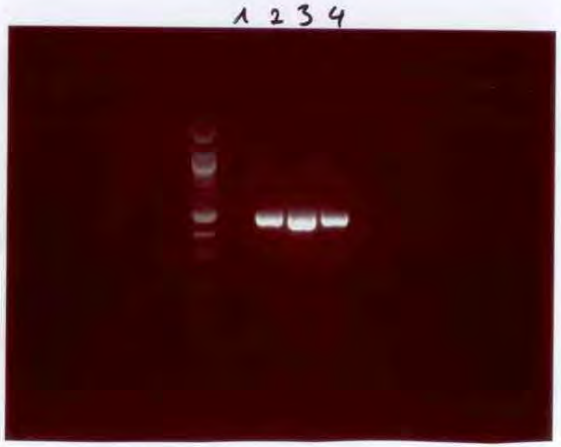
MS-sheet

PCR reaction

FINKTEC Professional Hygiene Management

PCR reaction	probe	batch	primer set	*legend*	volume [µl]
	neg. Kon.	/		*1*	25
	nach Fermentation	24012017-1	TB69-F1	*2*	25
	Produkt	24012017-1	TB69-R1	*3*	25
	pos. Kon.	07.12.16		*4*	25
				5	
				6	
				7	
				8	
				9	
				10	
				11	
				12	
				13	
				14	
				15	

Bild



execute by (date / signature): 26.01.17 (b6)

release (date / signature): 26.01.17 (b6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

ATTACHMENT 5

Batch Record
TB11

MS-sheet
Fermentation

FINKTEC Professional Hygiene Management

fermentation	production date	31.01.17			
	intermediate	TB11			
	batch	31012017 2			
	volume [ltr.]	100			
IPC1 **raw material**	LB-medium [kg]	2.0			
	pH- Wert	7,01			
		fermenter 1	*comments*	*fermenter 2*	*comments*
IPC2 **preculture bacteria**	volume [ltr.]	1,0			
IPC2a **preculture phage**	phagelysate titer [pfu/mL]	1×10^9			
	volume phagenlysate [ltr.]	1,0			
start (time) 719	OD_{600} t= 0	0,040			
	OD_{600} t = 30	0,054			
	OD_{600} t = 60	0,129			
	OD_{600} t = 90	0,287			
	OD_{600} t = 95	0,315			
	OD_{600} t = 120	0,400			
	OD_{600} t = 150	0,521			
	OD_{600} t = 180	0,571			
	OD_{600} t = 210	0,619			
	OD_{600} t = 240	0,600	Ernte		
	OD_{600} t =				
	addition of phage	t= 95		t=	
fermentation-product	phage titer [pfu/ml]	3×10^9			

executed by (date/signature): 31.01.17 (b) (6)

product release (date/signature): 01.02.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet

separation / filtration

FINKTEC Professional Hygiene Management

fermentation	produktion date	31.01.17
	intermediate	TB11
	batch	31012017
	volume [ltr.]	100

separation						
phage titer before separation	titer [pfu/mL]	4×10^9				
date: 02.02.17	OD_{600} before separation	0,455				
start: 730	OD_{600} first separation	0,030	/			
end: 930	OD_{600} second separation	0,020				
	phage titer [pfu/ml] after separation	4×10^9				
	conductivity [mS/cm]	13,07				
	volume of buffer [ltr.]	10				

comments:

executed by (date / signature): 2.2.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet
separation / filtration

FINKTEC Professional Hygiene Management

filtration							
prefilter start: end: / IPC 5	pressure [bar]	0					
	filtration time [h]	47min.					
	volume buffer [ltr.]	10					
	phage titer concentrate [pfu/ml]	4×10^9					
endfilter module 1 Uhrzeit von: 7³⁰ Uhrzeit bis: 13⁰⁰ ICP 7	pressure [bar]	0,4					
	filtration time [h]	5:10					
	volume buffer [ltr.]	200	9³⁰	11⁴⁰			
	conductivity [mS/cm]	16,63	12,73	16,63			
	phage titer concentrate [pfu/ml]	1×10^9					
endfilter module 2 start: 7³⁰ end: 13⁰⁰ ICP 7	pressure [bar]	0					
	filtration time [h]	5:10					
	volume buffer [ltr.]	200	9³⁰	11⁴⁰			
	conductivity [mS/cm]	16,66	12,40	16,66			
	phage titer concentrate [pfu/ml]	2×10^3					
intermediate after sterilfiltration CCP 1	volume intermediate [ltr.]	78,4	keg 1 50,8	keg 2 27,6			
	pH-value	7,62					
	total germ count at 37°C [cfu/ml]	0					
	total germ count at RT [cfu/ml]	0					
	conductivity [mS/cm]	16,31					
	phage titer [pfu/ml]	$9,8 \times 10^2$					
	PCR analysis (√)	✓					
	Sample for endotoxin determination (√)	✓					
	consistency, smell, visual appearance (√)	0,53					

comments:

executed by (date / signature): 02.02.17 A.F. (b) (6)

release (date / signature): 03.02.17 (b) (6)

FB_PR_32_separation/filtration_eng 16.10.2014 Revision 1 FB.PR.32, page 2 of 2

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet

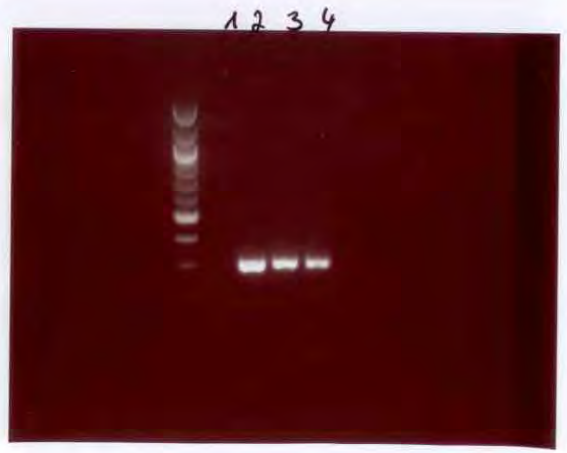
PCR reaction

FINKTEC Professional Hygiene Management

PCR reaction

probe	batch	primer set	*legend*	volume [µl]
neg. Kon.	/	TB11-FGA	*1*	25
nach Fermentation	31012017.2		*2*	25
Produkt	31012017.2	TB11-RGB	*3*	25
pos Kon.	25.11.16		*4*	25
			5	
			6	
			7	
			8	
			9	
			10	
			11	
			12	
			13	
			14	
			15	

Bild



execute by (date / signature): 03.02.17 (b) (6)

release (date / signature): 03.02.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

ATTACHMENT 6

Batch Record

MP75

MS-sheet
Fermentation

FINKTEC Professional Hygiene Management

fermentation	production date	31.01.17			
	intermediate	MP75			
	batch	31012017/1			
	volume [ltr.]	100			
IPC1 **raw material**	LB-medium [kg]	2,0			
	pH- Wert	7,01			
		fermenter 1	*comments*	*fermenter 2*	*comments*
IPC2 **preculture bacteria**	volume [ltr.]	1,0			
IPC2a **preculture phage**	phagelysate titer [pfu/mL]	6×10^9			
	volume phagenlysate [ltr.]	1,0			
start (time) 7 10	OD_{600} t= 0	0,030			
	OD_{600} t = 30	0,037			
	OD_{600} t = 60	0,062			
	OD_{600} t = 90	0,145			
	OD_{600} t = 120	0,251			
	OD_{600} t = 140	0,325			
	OD_{600} t = 150	0,336			
	OD_{600} t = 180	0,396			
	OD_{600} t = 210	0,458			
	OD_{600} t = 240	0,494	Ernte		
	OD_{600} t =				
	addition of phage	t= 140		t=	
fermentation-product	phage titer [pfu/ml]	6×10^9			

executed by (date/signature): 31.01.17 (b) (6)

product release (date/signature): 31.01.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet

separation / filtration

FINKTEC Professional Hygiene Management

fermentation	produktion date	31.01.17
	intermediate	MP75
	batch	31020171
	volume [ltr.]	100

separation		
phage titer before separation	titer [pfu/mL]	9×10^9
date: 01.02.17	OD_{600} before separation	0,593
start: 7⁰⁰	OD_{600} first separation	0,057
end: 9⁰⁰	OD_{600} second separation	0,016
	phage titer [pfu/ml] after separation	3×10^9
	conductivity [mS/cm]	11,72
	volume of buffer [ltr.]	10

comments:

executed by (date / signature): 01.02.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet
separation / filtration

FINKTEC Professional Hygiene Management

filtration						
prefilter start: end: / IPC 5	pressure [bar]	0				
	filtration time [h]	1,0				
	volume buffer [ltr.]	10				
	phage titer concentrate [pfu/ml]	8×10^9				
endfilter module 1 Uhrzeit von: 7⁴⁰ Uhrzeit bis: 12⁰⁰ ICP 7	pressure [bar]	0,4				
	filtration time [h]	04:20				
	volume buffer [ltr.]	200	8⁵⁰	10⁴⁵		
	conductivity [mS/cm]	16,87	11,74	16,87		
	phage titer concentrate [pfu/ml]	$1,6 \times 10^9$				
endfilter module 2 start: 7⁴⁰ end: 12⁰⁰ ICP 7	pressure [bar]	0				
	filtration time [h]	04:20				
	volume buffer [ltr.]	200	8⁵⁰	10⁴⁵		
	conductivity [mS/cm]	16,87	11,55	16,87		
	phage titer concentrate [pfu/ml]	$1,1 \times 10^9$				
intermediate after sterilfiltration CCP 1	volume intermediate [ltr.]	80,2	keg 1: 50	keg 2: 30,2		
	pH-value	7,61				
	total germ count at 37°C [cfu/ml]	0				
	total germ count at RT [cfu/ml]	0				
	conductivity [mS/cm]	16,59				
	phage titer [pfu/ml]	1×10^9				
	PCR analysis (√)	✓				
	Sample for endotoxin determination (√)	✓				
	consistency, smell, visual appearance (√)	o.B.				

comments:

executed by (date / signature): 01.02.17 (b) (6)

release (date / signature): 02.02.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

MS-sheet

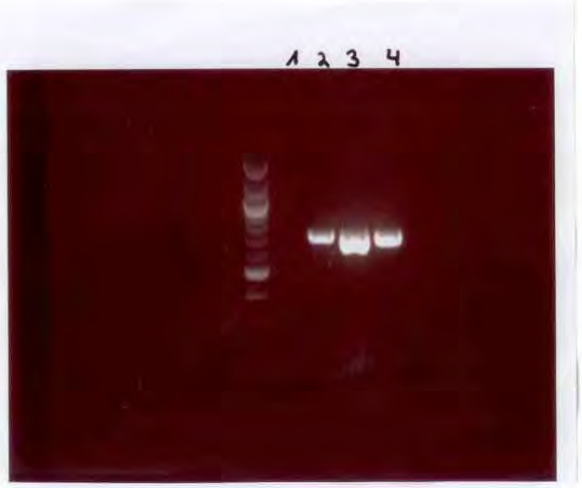
PCR reaction

FINKTEC Professional Hygiene Management

PCR reaction

probe	batch	primer set	*legend*	volume [µl]
neg. Kon.	/	HP75-F2C	*1*	25
nach Fermentation	31012017 1		*2*	25
Produkt	31012017 1	HP75-R2C	*3*	25
pos. Kon.	8122016		*4*	25
			5	
			6	
			7	
			8	
			9	
			10	
			11	
			12	
			13	
			14	
			15	

Bild



execute by (date / signature): 02.02.17 (b) (6)

release (date / signature): 02.02.17 (b) (6)

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

ATTACHMENT 7

Evaluation of the presence or absence of a continued technical effect of Phage preparation on experimentally contaminated raw beef.

RO18-TE

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

Evaluation of the the presence or absence of a continued technical effect of *Secure Shield E1* on experimentally contaminated raw beef.

Study Number # R018-TE

PTC Phage Technology Center GmbH

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Dr. Michael Fink

FINK TEC GmbH

Oberster Kamp 23

D-59069 Hamm

Tel.: 49 (0) 2385 730

1. STUDY TITLE

Evaluation of the presence or absence of a continued technical effect of *Secure Shield E1* on experimentally contaminated raw beef.

2. STUDY DIRECTOR

Hansjörg Lehnherr, Ph.D.

3. STUDY PERSONNEL

The following personnel contributed to the conduct and reporting of the studies reported herein:

Name:	Title:	Role:
Hansjörg Lehnherr, Ph.D.	Chief scientist	Study director
Anna K. Bierbrodt, M.Sc.	Research scientist	Hands-on-research

4. PERFORMING LABORATORY

PTC Phage Technology Center GmbH
Siemensstraße 42
D- 59199 Bönen
Tel.: 49 (0) 2383 919 174
Fax: 49 (0) 2383 919 179

5. STUDY OBJECTIVE

Determine the bacterial load reduction of the non-EHEC O157:H7 *E. coli* strain DSM-19206 on raw beef, resulting from the application of *Secure Shield E1* after 15 min, 1 d and 4 d, 7 d and 10 d, to evaluate the presence or absence of a continued techical effect of the bacteriophage product.

6. TEST MATRIX

The used raw beef was obtained from a butchery in Hamm-Rhynern, NRW, Germany. Samples were not washed or pre-treated prior to the studies.

7. COCKTAIL LOT AND APPLICATION

Secure Shield E1 lot # 6122016A
Secure Shield E1 titer: $1x10^8$ PFU/mL per phage component.

The application titer was $1x10^6$ PFU/mL per phage component.

8. BACTERIAL STRAINS USED TO EVALUATE COCKTAIL EFFICIENCY

Each beef sample was challenged with 10^4 CFU/cm² of the *E. coli* strain indicated below. The total reduction in *E. coli* bacterial load was evaluated.

- O157:H7 *E. coli* (*stx1*$^-$, *stx2*$^-$) DSM-19206 from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures) hereinafter referred to as E76.

9. MEDIA AND REAGENTS

- LB broth Lennox (Roth, Karlsruhe, Germany; catalog # X964.4)
  with 17 µg/mL chloramphenicol (Roth, Karlsruhe, Germany; catalog # 3886.2)
- LB agar Lennox (Roth, Karlsruhe, Germany; catalog # X965.2)
  with 17 µg/mL chloramphenicol (Roth, Karlsruhe, Germany; catalog # 3886.2)
- PBS (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4; pH 7.5)

10. GENERAL OUTLINE OF STUDY

- Original beef pieces were cut into 55 quadratic samples (4 cm²/sample) and stored at 4°C.
- *Secure Shield E1* was diluted with PBS buffer to an application titer of 10^6 PFU/mL.
- 50 samples were homogenously contaminated with 10^4 CFU/cm² of an overnight culture of the test strain E76. Five samples were not treated with bacteria and served as the uncontaminated, untreated controls.
- All samples were incubated for 10 min at room temperature.

- 25 contaminated beef samples were treated with 100 µL of *Secure Shield E1* with an application titer of 10^6 PFU/mL (100 µL PBS buffer were applied to the remaining 25 samples as the contaminated, untreated controls.
- Incubation periods were 15 min at room temperature and additional 1 d, 4 d, 7 d and 10 d at 3°C (±1°C), respectively.
- The numbers of viable *E. coli* were determined after 15 min, 1 d, 4 d, 7 d and 10 d, by adding 5 mL PBS and vortexing the samples for ten seconds.
- The suspensions were diluted 1:10 with PBS and 100 µL of each the undiluted and the corresponding diluted samples were plated on LB agar plates.
- LB agar plates were incubated at 37°C for 16 h and the numbers of viable *E. coli* were determined by counting colonies.
- The tests were performed with five replicates (n=5).

11. RESULTS

1. Raw Data

Table 1: *E. coli* counts for Study #R018-TE

RT room temperature

Time	Treatment	Surface (cm^2)	Temperature (°C)	Bacteria	Number of samples	CFU/cm^2
15 min	PBS	4	21	Yes	5	14000 16875 14625 16125 12875
	10^6 PFU/cm^2	4	21	Yes	5	5375 5875 5000 4750 4875
1 day	PBS	4	4	Yes	5	11875 16875 9750 8875 10750
	10^6 PFU/cm^2	4	4	Yes	5	9625 8250 12000 9625 9500
4 days	PBS	4	4	Yes	5	12875 12500 10875 8875 11250
	10^6 PFU/cm^2	4	4	Yes	5	9250 6500 6375 4125 9375

Table 2: *E. coli* counts for Study #R018-TE - continued

Time	Treatment	Surface (cm²)	Temperature (°C)	Bacteria	Number of samples	CFU/cm²
7 days	PBS	4	4	Yes	5	19750 10125 18500 17500 13625
	10^6 PFU/cm²	4	4	Yes	5	7125 5875 9750 6250 10125
10 days	PBS	4	4	Yes	5	11625 7750 16125 12125 20000
	10^6 PFU/cm²	4	4	Yes	5	7500 8625 9125 11750 7375

2. Tabular presentation of the results

Table 3: Reduction of *E. coli* counts on beef samples treated with *Secure Shield E1* phage solution (1x10^6 PFU/mL).

** highly significant with 0.001 < p 0.01

Time	Treatment	Replicates	Mean CFU/cm²	% Reduction	Significance
15 min	PBS	4	14900	65,27	Yes**
	10^6 PFU/cm²	4	5175		
1 day	PBS	4	11625	15,7	No
	10^6 PFU/cm²	4	9800		
4 days	PBS	4	11275	36,81	Yes**
	10^6 PFU/cm²	4	7125		
7 days	PBS	4	15900	50,79	Yes**
	10^6 PFU/cm²	4	7825		
10 days	PBS	4	13525	34,38	No
	10^6 PFU/cm²	4	8875		

3. Graphical presentation of results

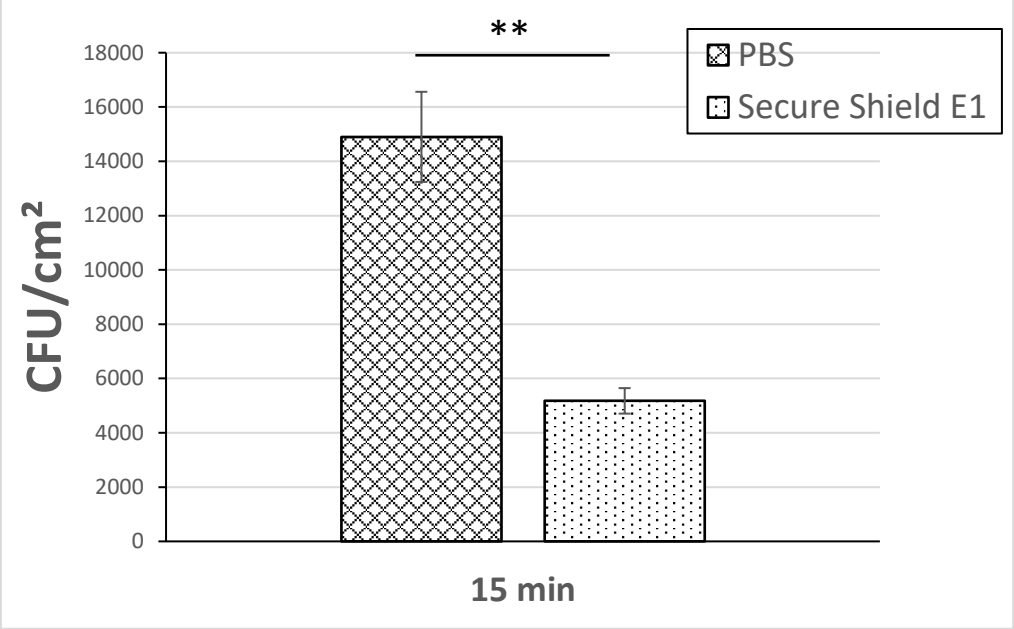

Figure 1: Initial reduction of viable *E. coli* on beef samples treated with *Secure Shield E1* phage solution (1x10^6 CFU/cm²) after 15 min at RT. Error bars indicate the confidence intervals with α = 0.01.

** highly significant with 0.001 < p < 0.01

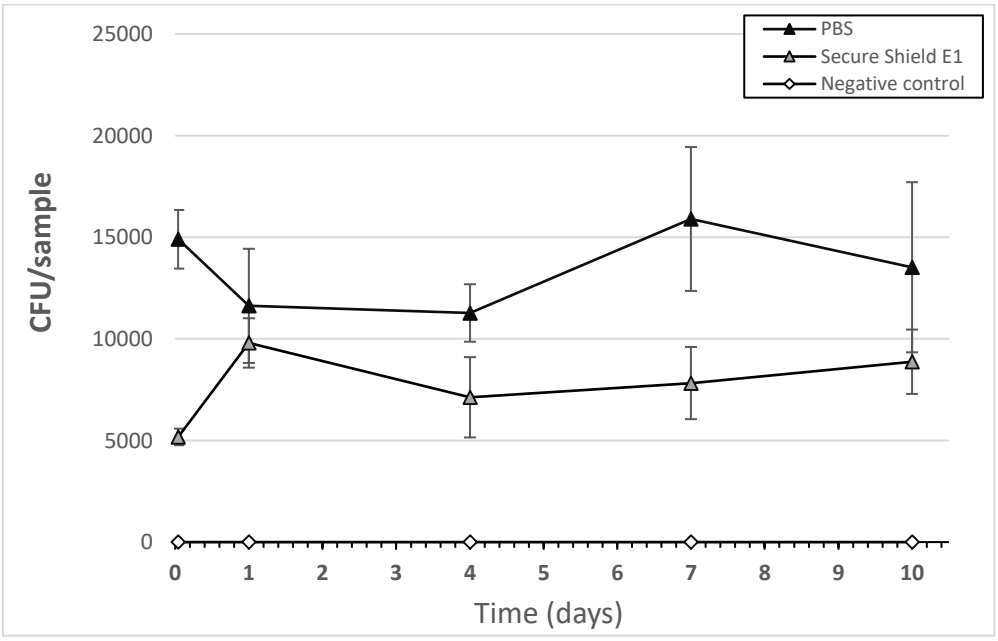

Figure 2: Reduction of *E. coli* counts on beef samples treated with Secure Shield E1 phage solution (1x10^6 CFU/cm²) over time. Error bars indicate the standard deviation.

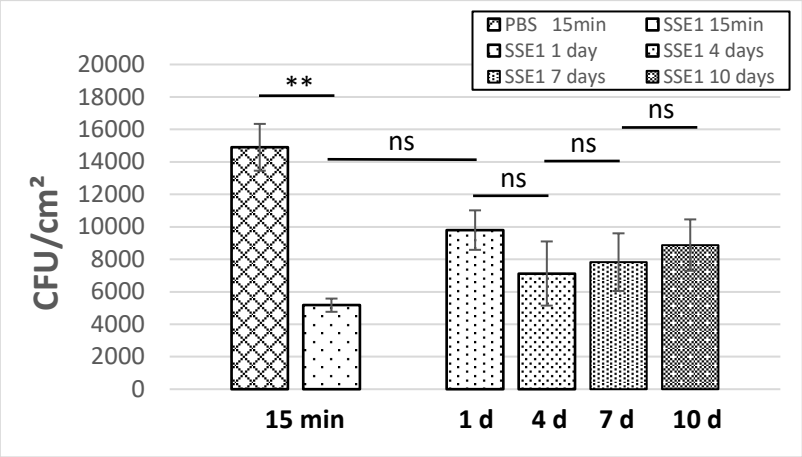

Figure 3: Reduction of *E. coli* counts on raw beef treated with *Secure Shield E1*. Significance levels were determined by ANOVA and a post-hoc Tukey HSD test. Error bars indicate the standard deviation.

** highly significant with $0.001 < p < 0.01$
ns not significant

4. Statistical analysis

Statistical analysis was performed using Office 2013 Excel for Windows (Microsoft Corporation, Redmond, WA) and the online ANOVA/Tukey HSD calculation tool of Navendu Vasavada (17.01.2017: http://astatsa.com/OneWay_Anova_with_TukeyHSD/).

The reduction of viable *E. coli* on raw beef after treatment with *Secure Shield E1* was determined by comparing the bacterial counts after 15 min, 1 d, 4 d, 7 d and 10 d, respectively. Results were analyzed by a One-way ANOVA with a post-hoc Tukey HSD test.

Table 4: Descriptive statistics of the independent treatments.

Time	Treatment	Statistical group
15 min	PBS	A
	10^6 PFU/cm²	B

1 d	PBS	C
	10^6 PFU/cm²	D
4 d	PBS	E
	10^6 PFU/cm²	F
7 d	PBS	G
	10^6 PFU/cm²	H
10 d	PBS	I
	10^6 PFU/cm²	J

Table 5: One-way ANOVA of the independent treatments.

source	sum of squares	degrees of freedom ν	mean square MS	F statistic	**p-value**
treatment	5.47E+08	9	6.08E+07	9.1391	2.39E-07
error	2.66E+08	40	6.65E+06		
total	8.13E+08	49			

The results of the One-way ANOVA suggested that one or more of the experimental treatments were significantly different (***; $p = 2.39x10^{7}$). A post-hoc Tukey HSD test was performed to identify which of the pairs of the treatment were statistically significant.

Table 6: Tukey HSD results for the reduction of viable *E. coli* on raw beef after treatment with *Secure Shield E1*.

** highly significant with 0.001 < p < 0.01

* significant with 0.01 < p < 0.05

treatment pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
A vs B	8.43	0.0010053	** p<0.01
B vs D	4.0091	0.1595329	insignificant
B vs J	3.2073	0.4338172	insignificant
D vs F	2.3188	0.7980881	insignificant
F vs H	0.6068	0.8999947	insignificant
H vs J	0.9102	0.8999947	insignificant

The number of viable *E. coli* detectable on raw beef was significantly reduced after a 15 min incubation at 21°C in comparison to the PBS treated control (A vs. B; P = 0.0010053). The number of viable *E. coli* did not increase or decrease in a significant manner after this timepoint. No statistically significant reduction of the cell count was observed from day 1 to day 10 of the treatment with *Secure Shield E1* (B vs J: insignificant; p = 0.4338172). No complete elimination of the *E. coli* cells was observed and no further technical effect of the bacteriophage product was detectable.

12. SUMMARY CONCLUSION OF THE STUDY

The study showed that *Secure Shield E1* provided a significant antibacterial effect at the moment of application to raw beef, assayed after 15 min in these experiments. However, in case there is no complete elimination, target bacteria that survived the intial treatment were still present and viable days after the treatment. This absence of any further technical effect after day 1, that would have resulted in a continuous reduction of the target bacteria, clearly argued against an ongoing effect of the bacteriophage product *Secure Shield E1*. This result, combined with the fact that *Secure Shield E1* is present in insignificant levels on the finished meat product indicated that *Secure Shield E1* is a processing aid, as defined by the FDA in section 21 CFR101.100 (a) (3).

13. SIGNATURES

(b) (6)

Anna Bierbrodt, M.Sc.
Research scientist

18.01.2017
Date

(b) (6)

Hansjörg Lehnherr, Ph.D.
Study director

18.01.2017
Date

ATTACHMENT 8

Evaluation of the presence or absence of a continued technical effect of Secure Shield E1 on experimentally contaminated raw beef.

R018-LTA

Evaluation of the presence or absence of a continued technical effect of *Secure Shield E1* on experimentally contaminated raw beef.

Study Number # R018-LTA

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Dr. Michael Fink

FINK TEC GmbH

Oberster Kamp 23

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Tel.: 49 (0) 2385 730

1. STUDY TITLE

Evaluation of the presence or absence of a continued technical effect of *Secure Shield E1* on experimentally contaminated raw beef.

2. STUDY DIRECTOR

Hansjörg Lehnherr, Ph.D.

3. STUDY PERSONNEL

The following personnel contributed to the conduct and reporting of the studies reported herein:

Name:	**Title:**	**Role:**
Hansjörg Lehnherr, Ph.D.	Chief scientist	Study director
Anna K. Bierbrodt, M.Sc.	Research scientist	Hands-on-research

4. PERFORMING LABORATORY

PTC Phage Technology Center GmbH
Siemensstraße 42
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Tel.: 49 (0) 2383 919 174
Fax: 49 (0) 2383 919 179

5. STUDY OBJECTIVE

Determine the bacterial load reduction of the non-EHEC O157:H7 *E. coli* strain DSM-19206 on raw beef, resulting from the application of *Secure Shield E1* after 4 h, 4 d and 6 d, to evaluate the presence or absence of a continued techical effect of the bacteriophage product.

6. TEST MATRIX

The used raw beef was obtained from a butchery in Hamm-Rhynern, NRW, Germany. Samples were not washed or pre-treated prior to the studies.

7. COCKTAIL LOT AND APPLICATION

Secure Shield E1 lot # 6122016A
Secure Shield E1 titer: $1x10^8$ PFU/mL per phage component.

The application titer was $1x10^6$ PFU/mL per phage component.

8. BACTERIAL STRAINS USED TO EVALUATE COCKTAIL EFFICIENCY

Each beef sample was challenged with 10^3 cells of the *E. coli* strain indicated below. The total reduction in *E. coli* bacterial load was evaluated.

- O157:H7 *E. coli* (*stx1*$^-$, *stx2*$^-$) DSM-19206 from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures) hereinafter referred to as E76.

9. MEDIA AND REAGENTS

- LB broth Lennox (Roth, Karlsruhe, Germany; catalog # X964.4)
  with 17 µg/mL Chloramphenicol (Roth, Karlsruhe, Germany; catalog # 3886.2)
- LB agar Lennox (Roth, Karlsruhe, Germany; catalog # X965.2)
  with 17 µg/mL Chloramphenicol (Roth, Karlsruhe, Germany; catalog # 3886.2)
- PBS (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4; pH 7.5)

10. GENERAL OUTLINE OF STUDY

- Original beef pieces were cut into 45 quadratic samples (42.25 cm²/sample).
- *Secure Shield E1* was diluted with PBS buffer to an application titer of 10^6 PFU/mL.
- 42 samples were homogenously contaminated with 10^3 cells of an overnight culture of the test strain E76. Three samples were not treated with bacteria and served as the uncontaminated, untreated controls.
- All samples were incubated for 10 min at room temperature.

- 21 contaminated beef samples were treated with 1 mL of *Secure Shield E1* with an application titer of 10^6 PFU/mL (1 mL of PBS buffer was applied to the remaining 21 samples as the contaminated, untreated controls.
- Incubation periods were 4 h at room temperature (21°C) and additional 4 d or 6 d at 3°C (±1°C), respectively.
- The numbers of viable *E. coli* were determined after 4 h, 4 d and 6 d by placing the beef samples upside down on LB agar plates and removing them after 10 sec (n=7; one uncontaminated, untreated control).
- LB agar plates were incubated at 37°C for 16h and the numbers of viable *E. coli* were determined by counting colonies.
- The tests were performed with seven replicates (n=7).

11. RESULTS

1. Raw Data

Table 1: *E. coli* plate counts for Study #R018-LTA

<fn> After initial 4 h at 21°C

Time	Treatment	Surface (cm^2)	Temperature (°C)	Bacteria	Number of samples	Total CFU
4 hours	$1x10^6$ PFU/mL	42.25	21	Yes	7	23 50 57 57 64 70 73
	PBS	42.25	21	Yes	7	78 95 141 145 153 174 179
4 hours + 4 days	$1x10^6$ PFU/mL	42.25	2.5<fn>	Yes	7	9 10 13 14 16 23 45
	PBS	42.25	2.5<fn>	Yes	7	113 116 116 127 132 142 147

Table 2: *E. coli* plate counts for Study #R018-LTA - continued

<fn> After initial 4 h at 21°C

Time	Treatment	Surface (cm²)	Temperature (°C)	Bacteria	Number of samples	Total CFU
4 hours + 6 days	$1x10^6$ PFU/mL	42.25	2.5<fn>	Yes	7	1 4 10 11 13 30 49
	PBS	42.25	2.5<fn>	Yes	7	106 108 111 118 135 143 147

2. Tabular presentation of results

Table 3: Reduction of *E. coli* counts on beef samples treated with *Secure Shield E1* phage solution ($1x10^6$ PFU/mL).

** highly significant with 0.001 < p 0.01

Time	Treatment	Replicates	Mean CFU/sample	% Reduction	Significance
4 hours	$1x10^6$ PFU/mL	7	56.29	59.17	Yes**
	PBS	7	137.86		
4 hours + 4 days	$1x10^6$ PFU/mL	7	18.57	85.44	Yes**
	PBS	7	127.57		
4 hours + 6 days	$1x10^6$ PFU/mL	7	16.86	86.41	Yes**
	PBS	7	124.00		

3. Graphical presentation of results

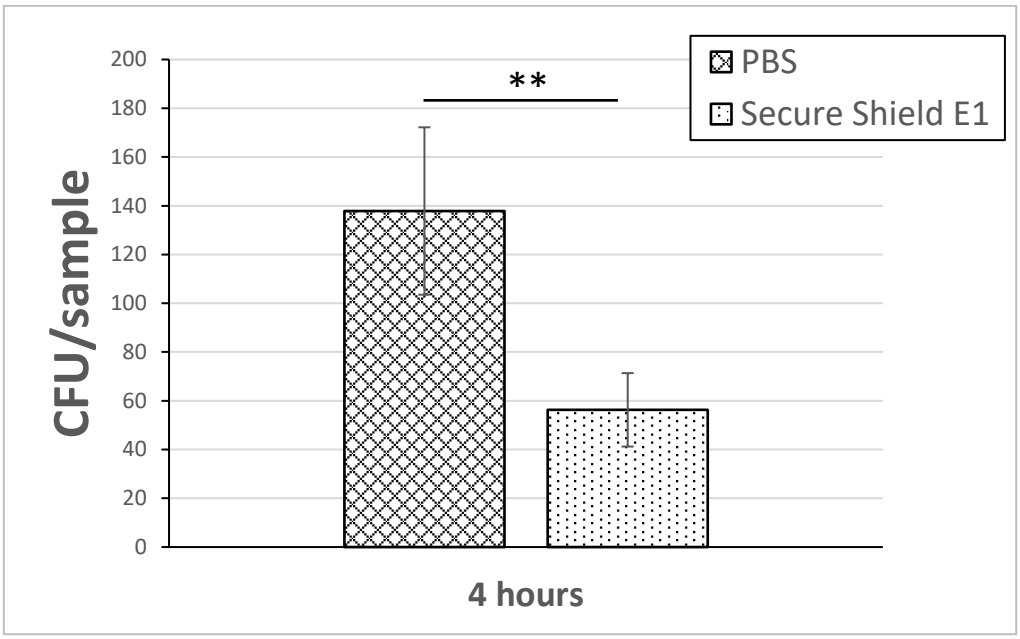

Figure 1: Initial reduction of viable *E. coli* on beef samples treated with *Secure Shield E1* phage solution (1x10^6 CFU/sample) at RT (4 h).

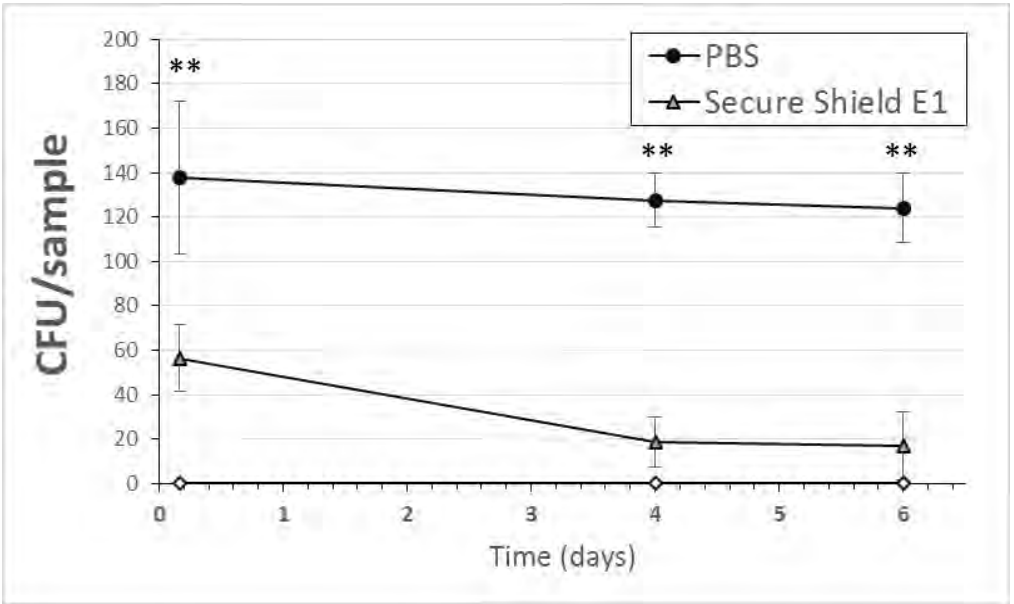

Figure 2: Reduction of *E. coli* counts on beef samples treated with *Secure Shield E1* phage solution (1x10^6 CFU/sample) over time.

Error bars indicate the confidence intervals with significance level $\alpha = 0.01$.

** highly significant with $0.001 < p < 0.01$

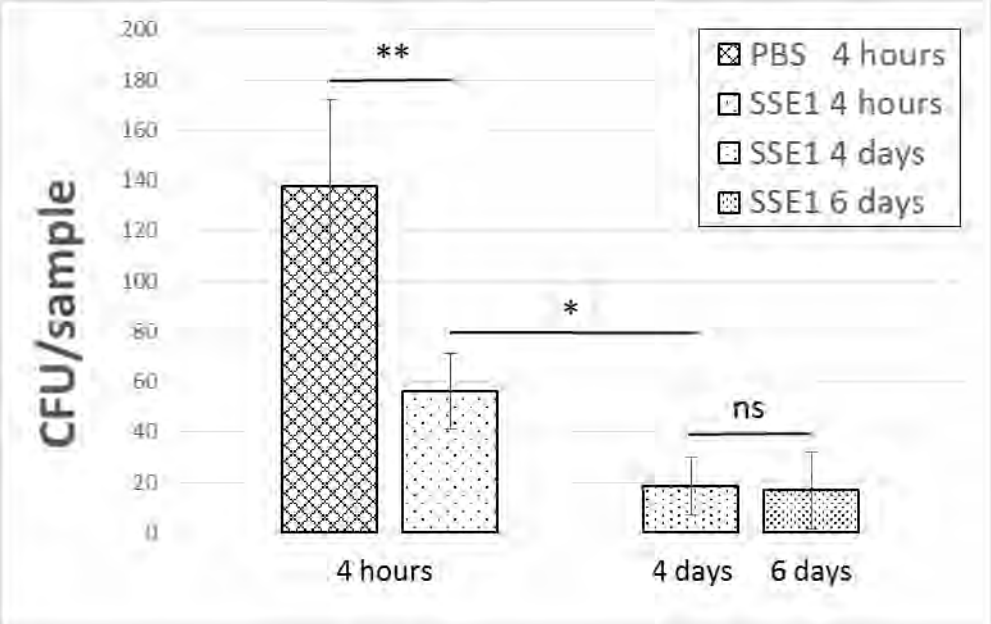

Figure 3: Reduction of *E. coli* counts on raw beef treated with *Secure Shield E1.*

Error bars indicate the confidence intervals with significance level α = 0.01.
** highly significant with 0.001 < p < 0.01
ns not significant

1. Statistical analysis

Statistical analysis was performed using Office 2013 Excel for Windows (Microsoft Corporation, Redmond, WA) and the online ANOVA/Tukey HSD calculation tool of Navendu Vasavada (21.12.2016: http://astatsa.com/OneWay_Anova_with_TukeyHSD/).

The reduction of viable *E. coli* on raw beef after treatment with *Secure Shield E1* was determined by comparing the bacterial counts after 4 h, 4 d and 6 d, respectively. Results were analyzed by a One-way ANOVA with post-hoc Tukey HSD test.

Table 4: Descriptive statistics of the independent treatments

Time	Treatment	Statistical group
4 hours	$1x10^6$ PFU/mL	A
	PBS	B
4 hours + 4 days	$1x10^6$ PFU/mL	C
	PBS	D
4 hours + 6 days	$1x10^6$ PFU/mL	E
	PBS	F

Table 5: One-way ANOVA of the independent treatments.

source	sum of squares	degrees of freedom ν	mean square MS	F statistic	**p-value**
treatment	76750.57	5.00	15350.11	34.70	8.32E-13
error	15926.57	36.00	442.40		
total	92677.14	41.00			

The results of the One-way ANOVA suggested that one or more of the experimental treatments were significantly different (***; $p = 8.32 \times 10^{-13}$). A post-hoc Tukey HSD test was performed to identify which of the pairs of treatment were statistically significant.

Table 6: Tukey HSD results for the reduction of viable *E. coli* on raw beef after treatment with *Secure Shield E1*.

** highly significant with $0.001 < p < 0.01$

* significant with $0.01 < p < 0.05$

treatment pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
A vs B*	10.2607	0.0010053	** p<0.01
B vs D	4.744	0.0214273	* p<0.05
D vs F	0.2156	0.8999947	insignificant

The number of viable *E. coli* detectable on raw beef was significantly reduced after a 4 h incubation at 21°C in comparison to the PBS treated control (A vs. B; P = 0.0010053). Until day 4 the number of bacterial cells further decreased in a significant manner (B vs. D; p = 0.0214273). No statistically significant reduction of the cell count was observed after day 4 of the treatment with *Secure Shield E1* (D vs. F: insignificant; p = 0.8999947). No complete elimination of the *E. coli* cells was observed and no further technical effect of the bacteriophage product was detectable.

12. SUMMARY CONCLUSION OF THE STUDY

The study showed that *Secure Shield E1* provided a significant antibacterial effect at the moment of application to raw beef, assayed after 4 hours in these experiments. However, in case there is no complete elimination, target bacteria that survived the intial treatment were still present and viable days after the treatment. This absence of any further technical effect after day 4, that would have resulted in a continuous reduction of the target bacteria, clearly argued against an ongoing effect of the bacteriophage product *Secure Shield E1*. This result, combined with the fact that *Secure Shield E1* is present in insignificant levels on the finished meat product indicated that *Secure Shield E1* is a processing aid, as defined by the FDA in section 21 CFR101.100 (a) (3).

13. SIGNATURES

(b) (6)

Anna Bierbrodt, M.Sc.
Research scientist

21.12.2016
Date

(b) (6)

Hansjörg Lehnherr, Ph.D.
Study director

21.12.2016
Date

ATTACHMENT 9

Evaluation of the ability of Phage preparation to inhibit the growth of an O157:H7 Escherichia coli (DSM-19206) bacterial culture in a liquid assay.

#R018-001

FINK TEC GmbH
Oberster Kamp 23, Hamm D-59069, Germany

PTC Phage Technology Center GmbH

Evaluation of the ability of *Secure Shield E1* to inhibit the growth of an O157:H7 *Escherichia coli* (DSM-19206) bacterial culture in a liquid assay.

Study Number # R018-001

PTC Phage Technology Center GmbH

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1. STUDY TITLE

Evaluation of the ability of *Secure Shield E1* to inhibit the growth of an O157:H7 *Escherichia coli* (DSM-19206) bacterial culture in a liquid assay.

2. STUDY DIRECTOR

Hansjörg Lehnherr, Ph.D.

3. STUDY PERSONNEL

The following personnel contributed to the conduct and reporting of the studies reported herein:

Name:	**Title:**	**Role:**
Hansjörg Lehnherr, Ph.D.	Chief scientist	Study director
Anna Bierbrodt, M.Sc.	Research scientist	Hands-on-research

4. PERFORMING LABORATORY

PTC Phage Technology Center GmbH
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Tel.: 49 (0) 2383 919 174
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5. STUDY OBJECTIVE

To measure the efficiency of Secure Shield E1, when applied at a concentration of 10^6 PFU/ml to a bacterial culture of the non-EHEC O157:H7 *E. coli* strain DSM-19206 in a liquid growth medium.

6. TEST MATRIX

The liquid inhibition test was performed in sterile 96-well microtiter plates.

7. COCKTAIL LOT AND APPLICATION

Secure Shield E1 lot # 12920161
Secure Shield E1 titer: $1x10^8$ PFU/ml per phage component.

The application titer was $1x10^6$ PFU/ml per phage component.

8. BACTERIAL STRAINS USED TO EVALUATE COCKTAIL EFFICIENCY

- O157:H7 *E. coli* (*stx1*$^-$, *stx2*$^-$) DSM-19206 from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures), hereinafter referred to as E76.

The total reduction in the *E. coli* bacterial load in a bacterial 1:10 dilution series by a constant amount phage was evaluated.

9. MEDIA AND REAGENTS

- LB broth Lennox (Roth, Karlsruhe, Germany; catalog # X964.4)
- LB agar Lennox (Roth, Karlsruhe, Germany; catalog # X965.2)

10. GENERAL OUTLINE OF STUDY

- The phage cocktail *Secure Shield E1* was diluted 1:100 with sterile LB medium.
- 180 µl of the cocktail dilution were applied in a vertical row of a 96-well microtiter plate. Another row was equally filled with sterile LB medium as a control.
- 20 µl of an overnight culture of the bacterial test strain were added to the first well of each row.
- 1:10 dilution series of the bacteria were prepared in the first (with phages) and the second row (LB control, without phages). Both the test series and the control series were incubated for 16 h at 37°C and shaking at 600 rpm.
- Bacterial growth was documented by measuring the optical densities at 620 nm (OD_{620}) with an appropriate 96-well-microplate reader.
- The numbers of viable *E. coli* were determined by further diluting the single dilutions of the two dilution series and plating aliquots (100 µl) on LB agar plates.
- LB agar plates were incubated at 37°C for 16h and the numbers of viable *E. coli* in the single dilutions of both dilution series were determined by enumerating colonies as follows:

$$Bacterial\ count\ [\frac{CFU}{mL}] = \frac{CFU}{plated\ volume\ [mL] * dilution}$$

- All tests were done in triplicates.

11. RESULTS

1. Raw Data

***Table 1**: E. coli counts [CFU/mL] in a 1:10 dilution series of E76 after 16 h incubation at 37°C.*

Dilution		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
CFU/mL preincubation		$1x10^{8}$	$1x10^{7}$	$1x10^{6}$	$1x10^{5}$	$1x10^{4}$	$1x10^{3}$	$1x10^{2}$	$1x10^{1}$
CFU/mL postincubation	Control	$1,1x10^{9}$	$1,4x10^{9}$	$1,4x10^{9}$	$1,2x10^{9}$	$1,2x10^{9}$	$9,2x10^{8}$	$1,1x10^{9}$	$8,5x10^{8}$
		$8,1x10^{8}$	$1,2x10^{9}$	$9,8x10^{8}$	$9,0x10^{8}$	$1,0x10^{9}$	$8,9x10^{8}$	$1,3x10^{9}$	$7,7x10^{8}$
		$1,3x10^{9}$	$1,2x10^{9}$	$1,7x10^{9}$	$1,3x10^{9}$	$1,4x10^{9}$	$1,4x10^{9}$	$1,1x10^{9}$	$8,9x10^{8}$
	Secure Shield E1	$3,0x10^{7}$	$4,0x10^{7}$	0	0	0	0	0	0
		$3,7x10^{4}$	$7,0x10^{7}$	0	0	0	0	0	0
		$6,7x10^{4}$	$5,0x10^{7}$	0	0	0	0	0	0

2. Tabular presentation of results

***Table 2**: Inhibition of E. coli growth in liquid medium by $1x10^{6}$ PFU/mL Secure Shield E1.*

CFU/mL preincubation	**Mean CFU/mL postincubation**		**Replicates**	**% Inhibition**	**Significance**
	Control	*Secure Shield E1*			
$1x10^{8}$	$1,6x10^{9}$	$1x10^{7}$	3	99.06	Yes*
$1x10^{7}$	$1,2x10^{9}$	$5,3x10^{7}$	3	95.71	Yes **
$1x10^{6}$	$1,4x10^{9}$	0	3	100	Yes *
$1x10^{5}$	$1,1x10^{9}$	0	3	100	Yes **
$1x10^{4}$	$1,2x10^{9}$	0	3	100	Yes **
$1x10^{3}$	$1,1x10^{9}$	0	3	100	Yes *
$1x10^{2}$	$1,1x10^{9}$	0	3	100	Yes **
$1x10^{1}$	$8,4x10^{8}$	0	3	100	Yes **

3. Graphical presentation of the results:

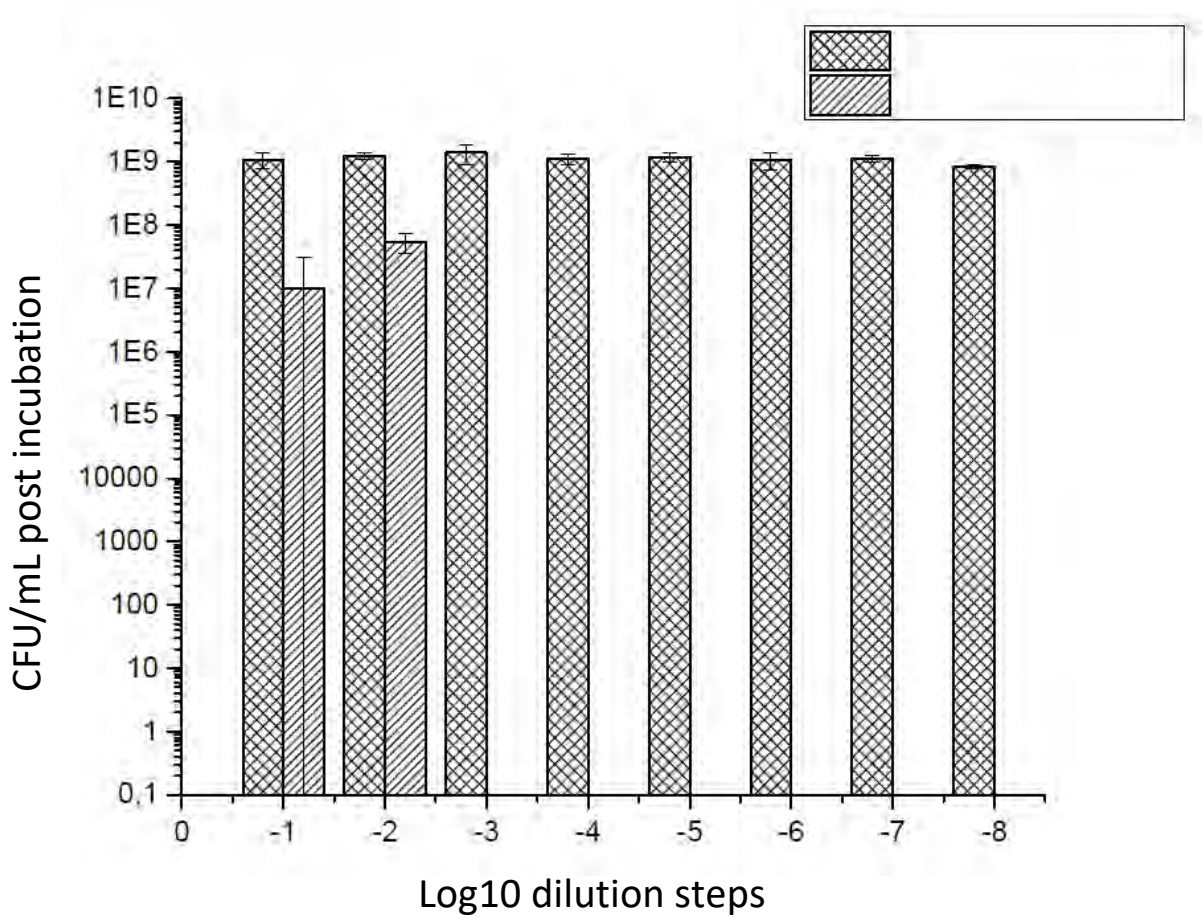

Figure 1: Inhibition of *E. coli* growth by *Secure Shield E1* (1x10^6 CFU/sample) with varying *E. coli* starting counts [CFU/mL].

Error bars indicate the confidence intervals with significance level α = 0.01.

4. Statistical analysis

Statistical analysis was performed using Office 2013 Excel for Windows (Microsoft Corporation, Redmond, WA).

The efficacy of *Secure Shield E1* in inhibiting the growth of *E. coli* in liquid culture, with varying bacterial starting counts, was evaluated by comparing the data obtained for E76 dilution series with and without $1x10^6$ PFU/mL *Secure Shield E1*.

***Table 3**: Analysis of Secure Shield E1 induced E. coli inhibition in liquid media by independent samples t-test.*

CFU/mL preincubation	Mean CFU/mL postincubation		p value t-test	Significance	Summary
	Control	*Secure Shield E1*			
$1x10^8$	$1,6x10^9$	$1x10^7$	0.0160	Yes	*
$1x10^7$	$1,2x10^9$	$5,3x10^7$	0.0029	Yes	**
$1x10^6$	$1,4x10^9$	0	0.0246	Yes	*
$1x10^5$	$1,1x10^9$	0	0.0093	Yes	**
$1x10^4$	$1,2x10^9$	0	0.0067	Yes	**
$1x10^3$	$1,1x10^9$	0	0.0215	Yes	*
$1x10^2$	$1,1x10^9$	0	0.0032	Yes	**
$1x10^1$	$8,4x10^8$	0	0.0018	Yes	**

* significant with $0.05 < p < 0.01$

** highly significant with $0.01 < p < 0.001$

Cultures with bacterial counts less than or equal to $1x10^6$ CFU/mL were completely eradicated by $1x10^6$ PFU/mL *Secure Shield E1* over 16 h at 37°C. The growth of cultures with higher bacterial starting counts was still significantly inhibited by 95.71 % (significant; $0.05 < p < 0.01$) and 99.06 % (highly significant; $0.01 < p < 0.001$), respectively.

12. SUMMARY CONCLUSION OF THE STUDY

Secure Shield E1 ($1x10^6$ PFU/mL) can significantly inhibit the growth of an O157:H7 *E. coli* strain in liquid medium right up to total eradication of all viable cells, depending on the bacterial starting count. The highest E76 count completely eradicated by *Secure Shield E1* with a given titer of $1x10^6$ PFU/mL and an incubation period of 16 h at 37°C were $1x10^6$ PFU/mL, which corresponds to a multiplicity of infection (MOI) of 1.

13. SIGNATURES

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Research scientist

29/11/2016
Date

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Hansjörg Lehnherr, Ph.D.
Study director

29/11/2016
Date