ORIGINAL SUBMISSION

#630

Dear Richard,

Please find enclosed an electronic submission on a CD and a signed version of page 3.

Best regards,

Steven

Dear Dr. Hagens,
Your recent submission to US FDA's GRAS Notification program for Salmonella-specific bacteriophages for use in food was received by our office January 5, 2015. I've done a pre-filing review of the submission to confirm that it is suitable for filing as a GRAS notice. I did notice that a minor administrative element is missing that will need to be provided before the submission can be filed as a GRAS notice. The submission lacks a signed and dated GRAS Exemption Claim (page 3). Also, we're not able to process electronic submissions received on USB drives at this time.

You have some options. You can either submit a new complete notice and electronic version on disc (CD or DVD ROM), or you could submit only a signed and dated page 3 (which I will place in the record as an addendum to the notice) and also, please, a copy of the complete electronic submission on a disc.

Kind regards,
Richard Bonnette

MICREOS

Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Attn. Dr. Paulette Gaynor.
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740
USA

December 11th, 2015

Dear Dr. Gaynor

In accordance with proposed 21 CFR 170 36 (Notice of a claim of for exemption based on GRAS determination) published in the Federal Register (62 FR 18938) I am submitting GRAS notification of the bacteriophage cocktail Salmonelex™ for bio-control of *Salmonella* on beef and vegetables. Both a hardcopy and an electronic version for easy distribution are provided. We have previously received a GRAS notice for this phage product for use on poultry and pork. The document remains largely unchanged as our product remains the same. Efficacy data for beef, iceberg lettuce, spinach, cucumber and zucchini are included.

We would appreciate a USDA FSIS review for the intended use of Salmonelex™ as a suitable processing aid in beef products.

Please let me know if you have any questions,

Sincerely,

(b) (6)

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

SALMONELEX™ NOTIFICATION

#630

MICREOS

GRN 000630

Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Attn. Dr. Paulette Gaynor.
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740
USA

December 11th, 2015

Dear Dr. Gaynor

In accordance with proposed 21 CFR 170 36 (Notice of a claim of for exemption based on GRAS determination) published in the Federal Register (62 FR 18938) I am submitting GRAS notification of the bacteriophage cocktail Salmonelex™ for bio-control of *Salmonella* on beef and vegetables. Both a hardcopy and an electronic version for easy distribution are provided. We have previously received a GRAS notice for this phage product for use on poultry and pork. The document remains largely unchanged as our product remains the same. Efficacy data for beef, iceberg lettuce, spinach, cucumber and zucchini are included.

We would appreciate a USDA FSIS review for the intended use of Salmonelex™ as a suitable processing aid in beef products.

Please let me know if you have any questions,

Sincerely,

(b) (6)

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

SALMONELEX™ NOTIFICATION

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Table of contents

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

I GRAS Exemption Claim

A. Claim of Exemption from The Requirement for Premarket Approval Requirements Pursuant to 21 CFR§170.36(c)(1).

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

Signed

(b) (6)

18.01.2016

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

Date

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

B. Name and address of Notifier

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

C. Common or Usual Name of the Notified Substance

Salmonelex™

D. Conditions of Use

The intended use of Salmonelex™ is as an antimicrobial on foodstuffs to control *Salmonella* at an application rate of up to $1x10^8$ pfu (plaque forming units) per gram of food.

E. Basis for the GRAS Determination

Pursuant to 21 CFR§170.30, Micreos has determined that Salmonelex™ is GRAS through scientific procedures.

F. Availability of Information

All data and information that serve as basis for this GRAS determination are available for the Food and Drug Administration review or will be sent to the agency upon request, made to:

Steven Hagens s.hagens@micreos.com
Nieuwe Kanaal 7P Tel: + 31 317 421414

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

6709 PA Wageningen

The Netherlands

II. Detailed information About the Identity and specifications of the Substance

A. Identity

Salmonelex™ consists of a watery solution containing two *Salmonella*-specific bacteriophages, FO1a and S16, which are produced and purified separately and mixed in equal concentrations. The commercial product has a minimal titer of $2x10^{11}$ pfu/mL.

This solution is concentrated and will be diluted with water at application sites by a factor 10-100 to ensure application rates at a maximum of $2 x10^{8}$ pfu/gram of treated food.

B. Method of Manufacture

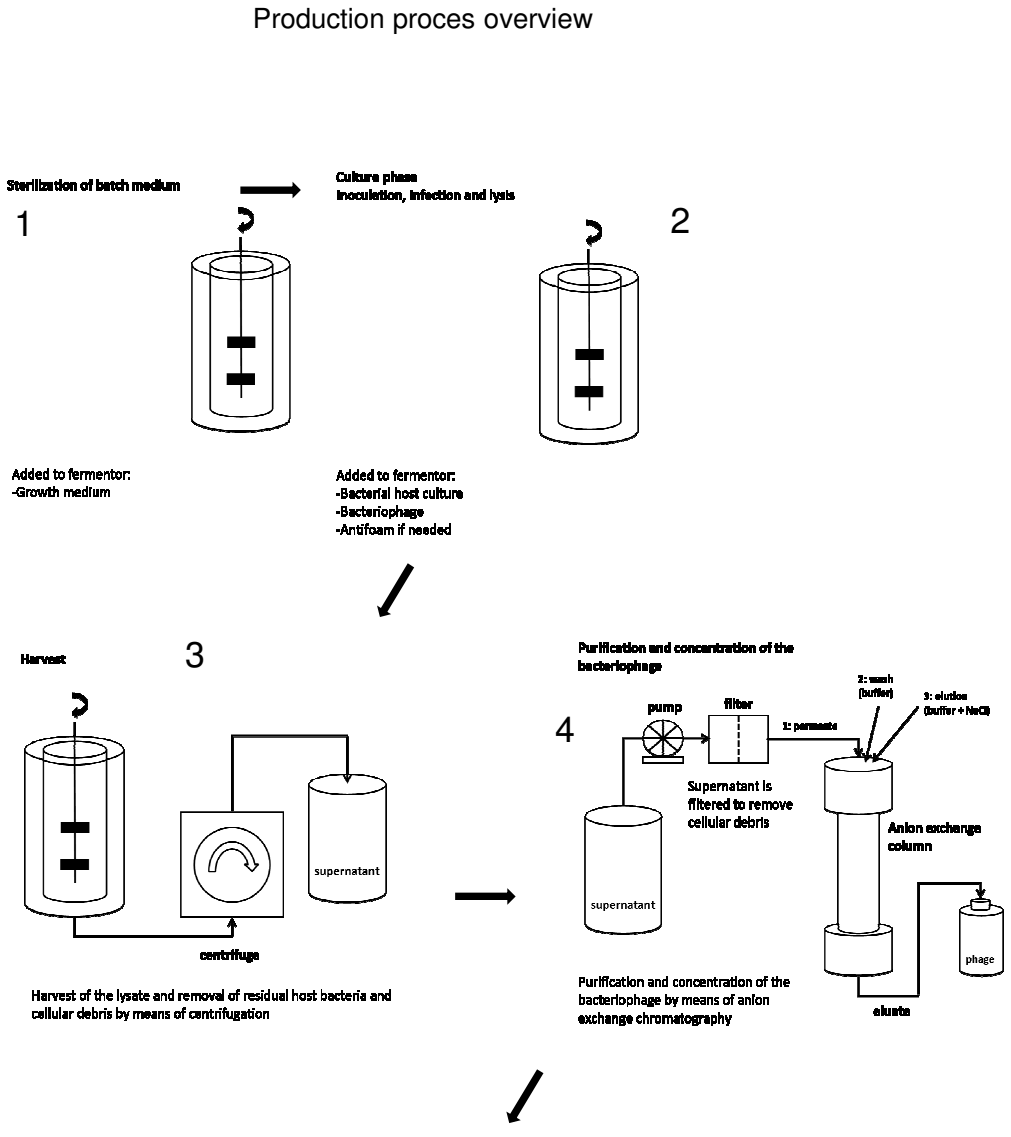
Both phages are grown separately on the same *S. bongori* production strain in a fermentor using a broth medium which is animal-product free. Phages for infecting the production strain are added at desired MOIs (multiplicity of infection) when the respective, appropriate OD_{600} values are reached. After infection the culture is further incubated under agitation and aeration conditions.

After completion of the incubation the culture is centrifuged to remove bacterial debris. Any remaining debris is subsequently removed by filtration. The clarified phage solution is then further purified and concentrated using anion exchange chromatography which removes medium components, host proteins and a substantial amount of LPS . Bound phages are then eluted from the chromatography column using a peptone - salt buffer. The phage solution is then filter-sterilized using commercial filters. After establishing the titer of batches, phages S16 and F01a are diluted with sterile water and blended in such a manner that each phage has a final concentration of $1x10^{11}$ pfu in the commercial product. The process is presented schematically in figure 1.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Figure 1. Schematic representation of the production process of Salmonelex™

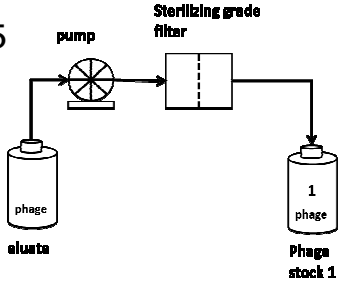


MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

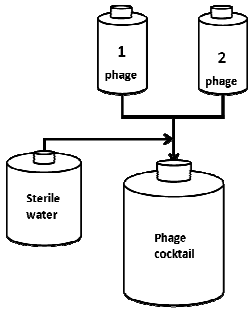
Sterile filtration

5



Phage stock tests:
-Bacterial sterility
-Phage titer

Blending of the 2 bacteriophages

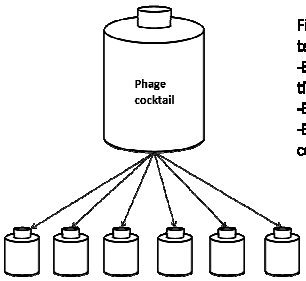


6

The 2 bacteriophages are blended 1:1 and diluted with sterile water to the desired final bacteriophage concentration

Packaging and QC testing

7



Final product QC tests:
-Bacteriophage titer
-Bacterial sterility
-Endotoxin content

The phage cocktail is packaged aseptically into sterile containers which are then stored at 2-6°C

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

C. Specifications

1) Batches undergo testing to ensure they meet specifications. Standard phage titration protocols are used to ensure potency (2 x10^{11} pfu/mL +/- 10%).
2) The product is tested for sterility by a 5-day enrichment of 1% for each batch in elective bacterial medium, followed and confirmed by plating of the enrichment on elective agar plates (Total plate count medium).

3) Each lot undergoes endotoxin testing by FDA-approved endpoint quantitative LAL assay (QCL-1000™ Endpoint Chromogenic LAL assay).
Released product specifications require endotoxin levels to be below 250,000 EU/mL for concentrated product containing 2x10^{11} pfu/mL.

D) Chemical analysis

Salmonelex™ is a clear, odorless liquid. With an average weight of the phages of ~ 1x10^{8} Dalton (what's Dalton?).
The phage components make up 33.2 ppm of the total weight of the concentrated liquid.
Three lots of Salmonelex™ have been analyzed for typical chemical composition and results of separate analysis and average values are depicted in Table 1.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Chemical Property	**Salmonelex™ batch** [redacted]	**Salmonelex™ batch** [redacted]	**Salmonelex™ batch** [redacted]	**Average values**
Total Kjeldahl Nitrogen	1,016 mg/L	1,018 mg/L	1,020 mg/L	1,018 mg/L
Total organic carbon	3,700 mg/L	3,600 mg/L	3,600 mg/L	3,633 mg/L
Arsenic	<2 µg/L	<2 µg/L	<2 µg/L	<2 µg/L
Mercury	<0.5 µg/L	<0.5 µg/L	<0.5 µg/L	<0.5 µg/L
Lead	< 8 µg/L	< 8 µg/L	< 8 µg/L	< 8 µg/L
Sulfur	81 mg/L	76 mg/L	76 mg/L	78 mg/L
Sulfate	99 mg/L	82 mg/L	89 mg/L	90 mg/L
Endotoxin level (EU)[a]	107,000 EU/mL = ~1 EU/ $2x10^6$ phages	99,000 EU/mL = ~1 EU/ $2x10^6$ phages	108,000 EU/mL = ~1 EU/ $2x10^6$ phages	105,000 EU/mL = ~1 EU/ $2x10^6$ phages

Table 1: Analysis of the chemical properties of three batches of Salmonelex™

[a]Endotoxin levels were determined by Micreos. All other analyses were performed by a certified external laboratory (Silliker Netherlands BV).

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

E) Phage identity and host ranges

Name: S16

Order: Caudavirales

Family: Myoviridae

Genus: T4-like viruses

Phage S16 was isolated by Micreos scientists in the Netherlands. Host-range studies were conducted both by Micreos and the Swiss Federal Institute of Technology in Zurich (ETH). Molecular analysis including identifying the receptor molecule on the *Salmonella* host, transduction experiments showing inability of the phage to transduce host DNA to other bacteria and full genome sequencing and bioinformatical analysis were performed by ETH in Zurich. S16 is a virulent (strictly lytic) phage belonging to the T4 family of phages specifically infecting strains of the genus *Salmonella*. The host range was found to be extremely broad. It infects all *Salmonella* species and subspecies but none of the 27 tested *Escherichia*, *Cronobacter*(43 strains), Enterobacter (4strains), *Citrobacter* (1strain), *Klebsiella (1 strain)*, *Vibrio* (1 strain), *Campylobacter* (1 strain) and *Pseudomonas* (3 strains) strains tested (Marti et al. 2013).

S16 specifically recognizes the *Salmonella* outer membrane protein C (ompC) which allows it to infect strains that have rough or deep rough mutations, thus not requiring intact LPS structure. S16 has a dsDNA 160 kb genome comprising 269 putative coding sequences and 3 tRNA genes. The DNA is highly modified (which allows the phage to infect strains carrying restriction modification systems, perhaps the most common and well known bacterial phage defense mechanisms (Marti et al. 2013). This recent study reports on the use of S16 as a biocontrol agent for *Salmonella* in food.

Name: FO1a

Order: Caudavirales

Family: Myoviridae

Genus: FelixO1-like phages

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

FO1a was isolated by ETH scientists in Zurich. Its' genome is almost identical (>99.99%) to the well studied original broad host-range Felix-O1 phage (Felix and Callow, 1943; Whichard et al. 2010). FelixO1 has been used in several studies to show efficacy of phage-biocontrol interventions in foods (Whichard et al. 2003; Hooton et al. 2011; Guenther et al. 2012, Marti et al. 2013). Felix-O1 like phages utilize different receptor molecules than S16, recognizing the terminal N-acetylglucosamine residue of the outer LPS core. Its genome comprises 86'155 bp and specifies 131 open reading frames and 22 t-RNAs. S16 features a complex replication mechanism and DNA packaging mode similar to the highly branched networks known from phage T4, and FO1a has fixed terminal repeats of 570 nt, ruling out the possibility for generalized transduction of host DNA.

Host range of the phage cocktail

The host range of a phage *sensu stricto* is defined as the strains any particular phage can propagate on i.e. produce progeny and thus plaques in a plaque assay. In this sense both S16 and FO1a have extremely broad host ranges being able to form plaques on the majority of strains tested. It should be considered that in terms of phage application for biocontrol death of cells after infection with phage should be considered as the host range of any particular phage instead of phage proliferation. These interventions do not rely on phage progeny for functionality but require infection and subsequent cell death of low numbers of host cells present on treated surfaces and does not rely on phage replication because any progeny phage are unlikely to be in the proximity of other targets in the intervention (Hagens and Loessner 2010). Many phage resistance mechanisms prevent phage proliferation through bacterial cell death and lack of progeny, rather than through surviving phage infection.

Testing of > 200 strains of *Salmonella enterica* did not reveal any strains that were not killed by the phage cocktail. Testing include strains of serovars *Salmonella* Infantis, Kentucky, Newport, Stanley, Hadar, Virchow, Typhimurium, Enteritidis, Agona, Anatum, Senftenberg, Montevideo, Muenster, Javiana, Heidelberg, Derby, Wien, Porci, Braederup, Panama, Newington, Livingston, Bredeney, Dublin, Cholerasuis, Give, Amherstiana, Salmone, Tennessee, Blockley, Indiana and Java and 20 non-serotyped strains. Isolates of *S. enterica* subsp. *houtenae*, *salamae*, *arizonae* and *diarizonae* were

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

analyzed and the second species in the genus, *S. bongori*, was also tested. Again no strain was able to survive infection by phages contained in the cocktail.

F) Host identity

Name of host bacteria:	*Salmonella bongori*
Authors:	Le Minor et al. 1985
Status:	New Species
Literature:	Int. J. Syst. Bacteriol. 39:371
Risk group:	2 (German classification)
Type strain and Registry numbers:	NCTC 12419, DSM 13772, ATCC 43975

Underlying the choice of using a *Salmonella bongori* strain for phage production were two lines of thought. *S. bongori* does not usually cause infection in humans. This species is associated with reptiles and amphibians rather than mammals. This lower pathogenicity significantly reduces risks for personnel in the production facility. *S. enterica* and *S. bongori* both feature similar pathogenicity island 1 (SP1), but *S. bongori* lacks pathogenicity island 2 (SP2) (Ochman and Groisman 1996). It is this pathogenicity island which produces a potentially harmful product upon ingestion, *Salmonella* enterotoxin (*stn*). While all *S. enterica* strains have been shown to possess the Stn toxin, *S. bongori* strains does not (Prager et al 1995). This rules out that Stn may be produced during phage propagation and therefore co-purify and contaminate the phage preparation.

G) Undesirable Host-derived Components

The safety of medium components, phages and ingredients added to the final product will be discussed in detail later. As discussed above *Salmonella* enterotoxin (*stn*) is not produced by *S. bongori*. While no other *Salmonella*-specific virulence factors are indicated as being harmful we consider removal of host components relevant. Ion exchange chromatography is mainly used for purifying proteins and DNA for medical purposes. Research investigating the use of phages in clinical settings has identified

the need to purify phages on large scale. Smrekar et al. (2008) suggest the use of methacrylate monolith columns for these relatively large structures. Kramberger et al. (2010) show that

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

Staphylococcus aureus bacteriophages can be effectively recovered using anion exchange chromatography in such columns resulting in reduction of host DNA by 99% and reduction of host proteins by 90%.

We have incorporated this technology in our Samonelex™ production process to remove host derived components. Lipopolysaccharide (LPS) or endotoxin is not *Salmonella*-specific but a component of the outer layer of all Gram-negative bacteria. As a consequence, endotoxin is found everywhere in the environment and consumed by humans on a daily basis. Also Gram-negative organisms releasing LPS are found in very high numbers in our intestines. In the bloodstream endotoxin can lead to toxic shock syndrome and regulations exist for medical devices that may come into direct contact with the bloodstream and medicinal preparations that are injected. No regulations exist for food. However, foodstuffs can contain high levels of endotoxins. A 1979 study by Jay et al. found endotoxin levels in ground beef in ranges of 500-75,000 EU/gram. Townsend et al. 2007 investigated the presence of endotoxin levels in infant formula and found levels ranging from 40-55,000 EU/g. A 2008 study by Gehring et al. investigated endotoxin levels in European Union milk samples. Milk from highly industrialized Nations such as Switzerland and Germany routinely contained levels ranging from 100,000 to 1,000,000 EU/mL.

Additionally, Gram-negative organisms living in the oral cavity also produce endotoxin and one study shows that saliva contains 1 mg of endotoxin/mL (Leenstra et al. 1996).

The agency has previously indicated it does not consider endotoxin content critical.

H) Self-limiting Levels of Use

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

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

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

III. Basis of Determination of GRAS: by scientific procedures

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

A) Background on salmonellosis

Salmonella enterica ssp. *enterica* has been associated with a large number of food-poisoning outbreaks related to various foods. The U.S. Centers for Disease Control and Prevention (CDC) states the annual incidence of salmonellosis in the United States to be approximately 40,000 cases. However, these numbers reflect only the reported cases and CDC estimates state that actual case numbers may well be 29 timer or more higher. Salmonellosis is caused mainly by contamination of products such as soft cheeses, processed meat, poultry, and vegetables. Estimates include some 400 fatalities each year (http://www.cdc.gov/nczved/divisions/dfbmd/diseases/salmonellosis/).It is therefore desirable to introduce novel biocontrol measures to ensure food safety.

B) Phage background

The attributes of bacteriophages include the following:

- Phages kill only *live bacterial* target cells,
- Phages generally do not cross species or genus boundaries, and will therefore not affectdesired bacteria in foods (e.g., starter cultures), and commensals in the gastrointestinal tract, or accompanying bacterial flora in the environment;

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

- Phages are composed entirely of proteins and nucleic acids, so their breakdown products consist exclusively of amino acids and nucleic acids, both of which are present in abundance in food products.

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

Phages in the environment

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

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

Very few foodstuffs are completely sterile. This means that most food consumed will contain bacteria and therefore phages are likely to be present.
This holds true especially for fermented products as well as unprocessed vegetables. As an example, phages can readily be isolated from Sauerkraut (Yoon et al. 2002; Barrangou et al. 2002). In one study (Lu et al. 2003) 26 different phages were isolated from the product of 4 commercial Sauerkraut fermentation plants.
While in most commercial cheese production settings significant effort is put into ensuring that starter cultures are free from phages and to some extent resistant to phage infection, this is certainly

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

not the case for artisanal cheeses and one might even argue that as long as timing is correct, host lysis by phages and thus liberation of the proteolytic enzymes may even be desirable. Phages infecting *Propionibacterium freudenreichii* have been isolated from Swiss cheese at levels of up to 7 x 10^5 pfu/g (Gautier et al. 1995). Phages infecting thermophilic lactic acid bacteria have been isolated from Argentinian dairy plant samples at numbers of up to 10^9 pfu/ml.

More importantly, non-fermentation culture bacteriophages have also been isolated from various food sources. *E. coli* phages have been isolated from a large number of products including: fresh chicken, pork, ground beef, mushrooms, lettuce, other raw vegetables, chicken pie and delicatessen food with phage numbers as high as 10^4 per gram (Allwood et al 2004; Kennedy et al. 1986, 1987).
Also *Campylobacter* phages have been isolated at levels of 4 x 10^6 PFU from chicken (Atterbury et al. 2003) and *Brochothrix thermosphacta* phages from beef (Greer 1983).
In all these cases the researchers were looking for phages infecting one particular species, but when one considers the myriad of bacteria associated with soil and vegetables it becomes clear that in addition more phages, associated with this multitude of other species, are likely present.

Phages in biocontrol of pathogens in food.

Much research has been conducted in using phage as biocontrol agents in foodstuffs. The general mode of action, efficacy of such interventions has also been reviewed extensively in the scientific literature (Greer 2005, Hudson et al. 2005, Hagens and Loessner 2007, Goodridge 2011, Hagens and Loessner 2010).
Phages can be separated into two groups: those that can integrate into host genomes and replicate as part of the genome (temperate phages) and virulent phages (strictly lytic phages) which are not able to do this and kill their hosts after infection.
The use of temperate phages would not be effective as some hosts survive infection. While not a significant risk in everyday life some temperate phages carry undesirable genes and have been shown to transduce host genes (i.e. transfer bacterial genes from one host cell to another). No virulent phage on the other hand has to date been shown to carry undesirable genes and most virulent phages do not transduce host genes. Some virulent phages have shown ability for generalized transduction and afety

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

data should include not only genome sequence data but experimental and/or theoretical proof that the candidate phages cannot transduce.

If these properties are found in candidate phage and considering their natural presence in the environment, in and on humans such phages should be considered GRAS.

Phages contained in Salmonelex™

The identity of the two phages S16 and FO1a and their properties including host ranges are described in detail in section II.

Both phages are virulent (strictly lytic). Neither phage carries undesirable genes (toxins, virulence factors or antibiotic resistance genes). Experimental data or genome organization excludes the possibility of either phage transducing host genetic material to subsequent hosts.

The host ranges of both phages are substantial within the genus *Salmonella.* With the exception of a few atypical *E. coli* strains (in the case of FO1a) neither phage shows activity on other genera of bacteria.

The selected phages are ideal candidates for biocontrol of *Salmonella* in foodstuffs. The level of analysis in terms of behaviour and genetic properties ensures they are safe and should therefore be considered GRAS.

Substantial equivalence to other phage products

Salmonelex™ is already considered GRAS for use in poultry processing and on pork meat.

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

Other phage products have been approved in food related applications in the US as cleaning agents or for decontamination of food animals prior to slaughter or for use in agricultural settings.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

C) GRAS status of starting material

The growth medium for producing Salmonelex™ contains only GRAS ingredients/processing aids. The main components of the medium are Soy peptone, which is GRAS affirmed as well as Yeast extract (Gras affirmed) and Sodium chloride.

The antifoaming agent used is organic sunflower oil (OMRI listed) and sodium hydroxide and/or hydrochloric acid is used to adjust pH of the medium only during fermentation.

These components moreover are removed to a great extent in the anion-exchange chromatography step in down-stream-processing.

Allergenicity

I. Phage components

Bacteriophages consist of proteins and nucleic acids. The former could in theory be allergenic. In practice this is however not relevant. The most potent known food allergen is peanut protein. The threshold dose for individuals with the highest sensitivity is 100 µg (Wensing et al. 2002). Assuming the unlikely scenario that all phage proteins (capsid proteins, tail proteins, tail fibers and tail spike proteins and base plate components) of both phages would be equally allergenic as the peanut allergen, estimated daily intake (see below) indicate that approximately 18 lbs of treated food would need to be consumed in a single sitting in order to ingest 100 µg of phage proteins (approximately half the weight of a phage is made up of proteins). We therefore consider the allergenicity potential of Salmonelex™ application due to the phage components negligible.

II. Relevant Medium Components

1. Soy Pepton

The only medium component with allergenicity potential is soy peptone. A hydrolyzed soy protein concentrate, the hydrolyzation step significantly reduces any potential allergenicity. The producer ELISA and PCR testing point out that the main allergens are absent in this soy pepton, within the

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

limits of detection . Micreos also confirms negative allergenicity on incoming product using the ELISA testing performed by an accredited laboratory. The anion exchange chromatography step used to purify the phages will furthermore remove >99% of all proteins including medium components.

D) Estimated daily dietary intake of Phages and by-products

According to USDA information (www.usda.gov/factbook/chapter2.pdf) Americans consume approximately 195.2 lbs of meat per capita per annum. Of this 64.4 lbs consists of beef. In addition Americans consume some 164 lbs of vegetables excluding potatoes per capita per annum. In the case of these products *Salmonella* contaminations are on the surface. While not all types of vegetable will be treated the calculation is based on the above consumption level.

Phage intake

80 grams/beef x 2 x 10^8 pfu/g = 1.6 x 10^{10} phages/day.

200 grams/vegetables x 2 x10^8pfu/g = 4 x 10^{10}/phages/day

Further assuming an average weight of 1 x10^8 Da/phage the following calculation gives the total weight of phages consumed on a daily basis:

5.6 x10^{10} x 10^8 x 1.66 x 10^{-27} kg = 0.000000009296 kg/day = 9.3ug/day.

Or in terms of treated product:

33 ppb or 0.033 ppm (parts per billion/parts per million). This level is insignificant.

By-products

Salt/Sodium

The phages are eluted from the anion exchange column using as solution containing 0.5 M NaCl. Subsequently this fluid is diluted in order to ensure correct potency of the phage cocktail. Recent improvements in fermentation assure that the salt concentration of final product is below 0.1M.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

At a phage level of 2x 10^8 pfu/g and treatment of 280 grams of foodstuffs and a salt concentration of 0.0059g/mL (O.1 M NaCl) of phage solution the following calculation can be made:

0.0059g/Salt x 280g/treated foodstuffs/day x 0.001 mL phage solution = 0.00165 g sodium chloride/day/serving.

The sodium content consumed (Molecular weight Chloride = 35.45 and Sodium = 22.9) would amount to 1 mg. This amount represents 0.04% of the recommended daily intake levels and thus would not change nutritional content labeling by the end user.

E) Quality Control

Phage Identity

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

Working stocks are subjected to host range testing (plaque formation behavior on several stains) and restriction fragment length polymorphism (RFLP). The results are compared to historical data and must match completely for working stocks to be approved for use in producing Salmonelex™. After production of each batch identity of the phages contained is checked by host range testing on strains exclusive for one of the two phages.

Phage numbers

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

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

Sterility

Sterility is tested by enrichment of the blended product containing the desired number of phages after packaging. 1% of final product after packaging is enriched in elective medium for 5 days prior to being plated on elective agar plates. No growth is required for product release. Batches failing this requirement will be destroyed.

Endotoxin levels

Each batch undergoes endotoxin testing by FDA-approved endpoint quantitative LAL assay (QCL-1000™ Endpoint Chromogenic LAL assay). Levels in the final concentrated product must be below 250,000 EU mL. Lots exceeding this requirement will not be released to the market.

F) Efficacy data at the intended levels of use

Data on the efficacy of FelixO1 and a combination of FelixO1 with other phages is available in the public domain.

Studies employing FelixO1 show that phage application can result in 2 log reductions on frankfurters (Whichard et al. 2003). Hooton et al. (2011) show a >99% of *Salmonella* on pig carcasses employing a phage cocktail including FelixO1. Guenther et al. (2012) provide evidence for a 3-5 log *Salmonella* reduction on turkey deli meat, chocolate milk and mixed seafood at refrigeration temperatures.

Experiments at elevated temperatures of 15°C as opposed to refrigeration temperatures show high levels of reduction but also show that *Salmonella* does re-grow after initial reduction. Re-growth rates are similar to the growth rates in un-treated controls showing that phage application results in an initial effect but has no prolonged activity beyond this.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Marti et al. (2013) repeated some of these experiments exactly and show that individually phages S16 and FelixO1 have the same effect on susceptible host strains in terms of kinetics. Addition of either phage will result in the same level of reduction if the strain is susceptible. The appendix contains data showing the effect of Salmonelex™ on relevant foodstuffs

In summary, application of phages at levels of $1x10^7$ pfu/cm^2 and $2\ x10^7$ pfu/cm^2, show the effect Salmonelex™ application on beef and vegetables contaminated with strains susceptible to both S16 and FO1a or only one of the two is demonstrated. We will show that application at this rate will result in a minimum of 1 log reductions in all cases. We expect that market demands will find this reduction level more than satisfactory but all risk analysis and daily dietary intake levels as a result of Salmonelex™ use are based on a usage levels minimal 5 times higher than the data presented in the Appendix, containing efficacy data. The higher usage level is requested in case market demands require *Salmonella* reduction levels to be far higher than 1 log in certain applications. The information in the appendix will show that Salmonelex™ application is highly effective for the relevant foodstuffs and it will show that the efficacy of the phages is very limited in time. This shows that Salmonelex™ has no function in the final product and should be considered a processing aid.

G) Summary Salmonelex™ and GRAS

Bacteriophage preparations for biocontrol of pathogens have previously been affirmed as GRAS.
The current phage preparation Salmonelex™ should also be considered GRAS. Genetic analysis and experimental evidence show that the individual phages contained in the preparation are safe in terms of being a) virulent (strictly lytic), b) not containing any undesirable genes c) being unable to transduce host DNA from one host strain to another.
Salmonelex™ is moreover highly effective in reducing *Salmonella* contaminations on beef and vegetables.
Based on these findings, Salmonelex™ should also be considered GRAS for beef and vegetable application. Or indicate that Micreos believes self affirms or believes that Sx is also GRAS for beef and veggies

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

References:

1. Allwood, P. B., Malik, Y. S., Maherchandani, S., Vought, K., Johnson, L. A., Braymen, C., Hedberg, C. W. & Goyal, S. M. (2004). Occurrence of *Escherichia coli*, noroviruses, and F-specific coliphages in fresh market-ready produce. *J Food Prot* **67**, 2387-90.

2. Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E. & Connerton, I. F. (2003). Isolation and characterization of *Campylobacter* bacteriophages from retail poultry. *Appl Environ Microbiol* **69**, 4511-8.

3. Barrangou, R., Yoon, S. S., Breidt Jr, F., Jr., Fleming, H. P. & Klaenhammer, T. R. (2002). Characterization of six *Leuconostoc fallax* bacteriophages isolated from an industrial sauerkraut fermentation. *Appl Environ Microbiol* **68**, 5452-8.

4. Breitbart, M., Hewson, I., Felts, B., Mahaffy, J. M., Nulton, J., Salamon, P. & Rohwer, F. (2003). Metagenomic analyses of an uncultured viral community from human feces. *J Bacteriol* **185**, 6220-3.

5. Felix, A. & Callow, B. R. (1943). Typing of Paratyphoid B Bacilli by Vi Bacteriophage. *Br Med J* **2**, 127-30.

6. Furuse, K., Osawa, S., Kawashiro, J., Tanaka, R., Ozawa, A., Sawamura, S., Yanagawa, Y., Nagao, T. & Watanabe, I. (1983). Bacteriophage distribution in human faeces: continuous survey of healthy subjects and patients with internal and leukaemic diseases. *J Gen Virol* **64 (Pt 9)**, 2039-43.

7. Gautier, M., Rouault, A., Sommer, P. & Briandet, R. (1995). Occurrence of *Propionibacterium freudenreichii* bacteriophages in swiss cheese. *Appl Environ Microbiol* **61**, 2572-6.

8. Gehring, U., Spithoven, J., Schmid, S., Bitter, S., Braun-Fahrlander, C., Dalphin, J. C., Hyvarinen, A., Pekkanen, J., Riedler, J., Weiland, S. K., Buchele, G., von Mutius, E., Vuitton, D. A. & Brunekreef, B. (2008). Endotoxin levels in cow's milk samples from farming and non-farming families - the PASTURE study. *Environ Int* **34**, 1132-6.

9. Goodridge, L. D. & Bisha, B. (2011). Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage* **1**, 130-137.

10. Greer, G. G. (1983). Psychrotrophic *Brochothrix thermosphacta* bacteriophages isolated from beef. *Appl Environ Microbiol* **46**, 245-51.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

11. Greer, G. G. (2005). Bacteriophage control of foodborne bacteria. *J Food Prot* **68**, 1102-11.

12. Guenther, S., Herzig, O., Fieseler, L., Klumpp, J. & Loessner, M. J. (2012). Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. *Int J Food Microbiol* **154**, 66-72.

13. Hagens, S. & Loessner, M. J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Appl Microbiol Biotechnol* **76**, 513-9.

14. Hagens, S. & Loessner, M. J. (2010). Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Curr Pharm Biotechnol* **11**, 58-68.

15. Hooton, S. P., Atterbury, R. J. & Connerton, I. F. (2011). Application of a bacteriophage cocktail to reduce *Salmonella* Typhimurium U288 contamination on pig skin. *Int J Food Microbiol* **151**, 157-63.

16. Hudson, J. A., Billington, C., Carey-Smith, G. & Greening, G. (2005). Bacteriophages as biocontrol agents in food. *J Food Prot* **68**, 426-37.

17. Jay, J. M., Margitic, S., Shereda, A. L. & Covington, H. V. (1979). Determining endotoxin content of ground beef by the Limulus amoebocyte lysate test as a rapid indicator of microbial quality. *Appl Environ Microbiol* **38**, 885-90.

18. Kennedy, J. E., Jr., Wei, C. I. & Oblinger, J. L. (1986). Methodology for enumeration of coliphages in foods. *Appl Environ Microbiol* **51**, 956-62.

19. Kennedy JE, Bitton G (1987). Bacteriophages in foods. In S. M. Goyal, C. P. Gerba, and G. Bitton (ed.), Phage Ecology. John Wiley & Sons, New York.

20. Kramberger, P., Honour, R. C., Herman, R. E., Smrekar, F. & Peterka, M. (2010). Purification of the *Staphylococcus aureus* bacteriophages VDX-10 on methacrylate monoliths. *J Virol Methods* **166**, 60-4.

21. Leenstra, T. S., van Saene, J. J., van Saene, H. K. & Martin, M. V. (1996). Oral endotoxin in healthy adults. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **82**, 637-43.

22. Lu, Z., Breidt, F., Plengvidhya, V. & Fleming, H. P. (2003). Bacteriophage ecology in commercial sauerkraut fermentations. *Appl Environ Microbiol* **69**, 3192-202.

23. Marti, R., Zurfluh, K., Hagens, S., Pianezzi, J., Klumpp, J. & Loessner, M. J. (2013). Long tail fibres of the novel broad-host-range T-even bacteriophage S16 specifically recognize *Salmonella* OmpC. *Mol Microbiol* **87**, 818-34.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

24. Ochman, H. & Groisman, E. A. (1996). Distribution of pathogenicity islands in Salmonella spp. *Infect Immun* **64**, 5410-2.

25. Prager, R., Fruth, A. & Tschape, H. (1995). Salmonella enterotoxin (stn) gene is prevalent among strains of *Salmonella enterica*, but not among *Salmonella bongori* and other Enterobacteriaceae. *FEMS Immunol Med Microbiol* **12**, 47-50.

26. Smrekar, F., Ciringer, M., Peterka, M., Podgornik, A. & Strancar, A. (2008). Purification and concentration of bacteriophage T4 using monolithic chromatographic supports. *J Chromatogr B Analyt Technol Biomed Life Sci* **861**, 177-80.

27. Sulakvelidze, A. (2005). Phage therapy: an attractive option for dealing with antibiotic-resistant bacterial infections. *Drug Discov Today* **10**, 807-9.

28. Townsend, S., Caubilla Barron, J., Loc-Carrillo, C. & Forsythe, S. (2007). The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat. *Food Microbiol* **24**, 67-74.

29. Wensing, M., Penninks, A.H., Hefle, S.L., Koppelman, S.J., Bruinzeel-Koomen, C.A., Knulst, A.C. (2002) The distribution of individual thershold doses eliciting allegic reactions in a population with peanut allergy. *J Allergy Clin Immunol* **110**, 915-20

30. Whichard, J. M., Sriranganathan, N. & Pierson, F. W. (2003). Suppression of *Salmonella* growth by wild-type and large-plaque variants of bacteriophage Felix O1 in liquid culture and on chicken frankfurters. *J Food Prot* **66**, 220-5.

31. Whichard, J. M., Weigt, L. A., Borris, D. J., Li, L. L., Zhang, Q., Kapur, V., Pierson, F. W., Lingohr, E. J., She, Y. M., Kropinski, A. M. & Sriranganathan, N. (2010). Complete genomic sequence of bacteriophage Felix O1. *Viruses* **2**, 710-30.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Appendix I. Challenge Study Report: Salmonelex™ Food Application

1 Introduction

A challenge study was performed to evaluate the effect of Salmonelex™ phages on *Salmonella* serovar Enteritidis (*Se13*) inoculated beef, iceberg lettuce, cucumbers , spinach and zucchini. The challenge testing was performed at Micreos, Wageningen, The Netherlands. Beef samples were treated with two phage concentrations $1x10^7$ pfu/cm² and $2x10^7$ pfu/cm² to provide data for *Salmonella* strains sensitive for only one or both phages in the Salmonelex™ phage formulation. Vegetables were treated at a concentration of $2x10^7$ pfu/cm². Contact times of 24 hours and 48 hours were chosen to evaluate the initial effect of the treatment and 6 days as reasonable time point to resemble the shelf life for fresh meat products and vegetables. Samples were incubated at 4°C. Triplicate samples were tested for beef and duplicate samples for vegetables and the challenge studies were performed twice.

As *Salmonella* does not grow at refrigerator temperatures the challenge study as described above does show that phages only have an initial effect on *Salmonella* cells. After an initial reduction no further reduction in *Salmonella* counts was observed.

For the testing a streptomycin resistant mutant of *Salmonella* strain *Se13* (resistant to 500µg/mL) was used as available *Salmonella* selective media are rather poor in specificity. Other bacteria present in the food sample are also able to grow on this media posing a problem in the evaluation of agar plates. By using the streptomycin resistant strain and by adding streptomycin to retrieval buffer and agar this problem can be reduced significantly.

2. Materials and methods

2.1 Materials

Samples

Beef

Iceberg lettuce*

Cucumbers*

Zucchini*

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

Spinach*

* Purchased at a local supermarket

Bacteria/bacteriophage

- *Salmonella* serotype Enteritidis *Se13* Streptomycin resistant mutant (500µg/mL) (Se13 STREP3 MUTANT) → titer Overnight (ON) culture on selective agar plates + streptomycin: ~1.13x10^9 cfu/mL
- Bacteriophage formulation Salmonelex™

Media

LB broth

LB agar plates

Selective agar plates

1 x PBS buffer (Phosphate buffered saline preparation)

1 x SM buffer

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

Streptomycin stock solution (100mg/mL)

2.2 Methods

2.2.1 Challenge study: Salmonelex™ efficacy on Se13 STREP3 MUTANT inoculated food samples

Bacterial overnight cultures

One colony of *Salmonella* Se13 STREP3 MUTANT was inoculated in 4ml broth (+ 500µg streptomycin/mL) and incubated overnight at 30ºC shaking.

Preparation of samples

For Beef, iceberg lettuce, cucumbers, spinach and zucchini sample pieces of 6x3 (x1) cm were prepared to achieve a 10cm² surface to be contaminated (A_{con}) and a surface of 18cm² to be treated with phages ($A_{treated}$). Samples were placed and stored in sterile petri dishes. *Beef, vegetables*

An appropriate dilution of the overnight culture is prepared in PBS buffer to allow the contamination of the samples with a final concentration of approximately 2 x10^4 cfu/cm² (2µL liquid/ cm²).

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

To control the concentration of the dilution used to contaminate the samples, the titer is determined by plating appropriate dilutions on selective agar plates. In the laminar flow hood 2µl/cm² of the dilution is transferred to each sample and rubbed in evenly with the pipette tip.

Sprouts

An appropriate dilution of the overnight culture is prepared in PBS buffer to allow the contamination of the samples with a final concentration of approximately 2 $x10^5$ cfu/g (20µL liquid/ g).

In the laminar flow hood 20µl/g of the dilution is transferred to each sample and the sprouts are wrapped tightly in the cling foil and placed at 4 °C.

Treatment with Salmonelex™

To allow the treatment of the beef and vegetable samples with a final concentration of 1 $x10^7$ or 2x107 pfu/cm² dilutions of Salmonelex™ were prepared in sterile tap water. In the fume hood 5µl/cm² was transferred onto the samples (sample treatment schemes see Table 1). The liquid was distributed with the pipette tip. The petri dishes were closed and incubated at 4°C for 24 hours, 48 hours and 6 days.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Table 1: Sample treatment schemes
A: Incubation of samples at 4°C; **B:** Incubation of samples at room temperature (~22°C)

A

Sample	Nr.samples
t=0	
beef	2
iceberg lettuce	2
cucumbers	2
spinach	2
zucchini	2
t=24h, 48h and 6d	
beef	6*
iceberg lettuce	4*
cucumbers	4*
spinach	4*
zucchini	4*
TOTAL Nr. SAMPLES	60

* Half of the samples not phage-treated (control), the other samples treated with Salmonelex™

Retrieval of *Salmonella*

The samples were placed in separate stomacher bags with a sterile tweezers. To allow a high and homogenous retrieval rate, peptone water (+ 200µg streptomycin/mL) was added to the bags and samples were homogenized in a stomacher for 180 seconds.

20µL of the untreated controls and 200µL of the treated samples were plated in duplicate on selective agar plates (+ 200µg streptomycin/mL).

Plates were incubated for 24 to 48 hours at 37ºC.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

3 Results

3.1 Challenge study: Salmonelex™ efficacy on Se13 STREP3 MUTANT inoculated vegetable samples

Figures 1 to 9 show the percentage reduction of *Salmonella* on beef, cucumbers, iceberg lettuce, spinach and zucchini when treated with phage at an incubation temperature of 4°C.

Beef

On beef cell counts dropped by approximately 91% (~1.1log) when samples were treated with $1x10^7$ pfu/cm² Salmonelex™ and by ~95% (~1.3log) when applying $2x10^7$ pfu/cm². No significant difference was observed between the different contact times (Figures 1 and 2).

Cucumbers

With a phage concentration of $2x10^7$ pfu/cm², cell numbers could be reduced by 94% (corresponding to 1.3 log) with a contact time of 24 hours. The number of cells did not change significantly upon retrieval after 48 hours and 6 days of contact time (see Figure 3 and 4).

Iceberg lettuce

On iceberg lettuce *Salmonella* cell reduction of 94% (~1.3 log reduction) could be achieved when Salmonelex™ was applied in a concentration of $2x10^7$pfu/cm² after 24 hours. No significant difference between the different contact times was observed. (Figures 5 and 6)

Spinach

On spinach *Salmonella* cell reduction of 94% (~1.3 log reduction) could be achieved when Salmonelex™ was applied in a concentration of $2x10^7$pfu/cm² after 24 hours. No significant difference between the different contact times was observed. (Figures 7 and 8)

Zucchini

With a phage concentration of $2x10^7$ pfu/cm², cell numbers could be reduced by 96% (corresponding to 1.4 log) with a contact time of 24 hours. The number of cells did not change significantly upon retrieval after 48 hours and 6 days of contact time (see Figure 9 and 10).Cell numbers further declined over the storage period but this is occurred also in the untreated controls in all vegetable samples.

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

For all vegetable samples cell numbers declined over time but this happened in untreated control samples at similar rates.For detailed results see Appendix Tables 2 to 6.

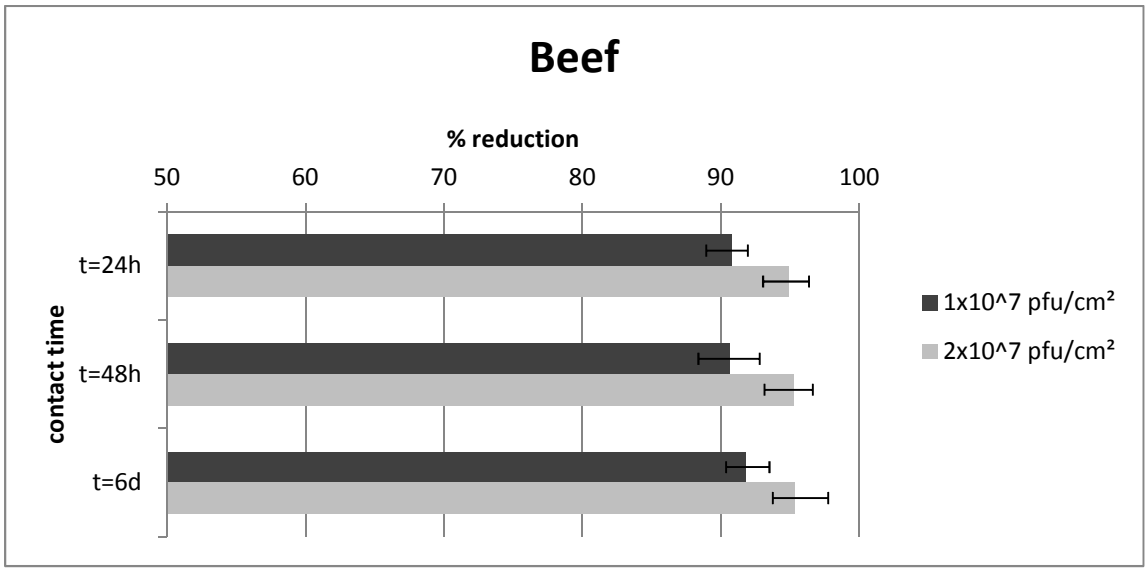


Figure 1: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex treated BEEF samples. Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

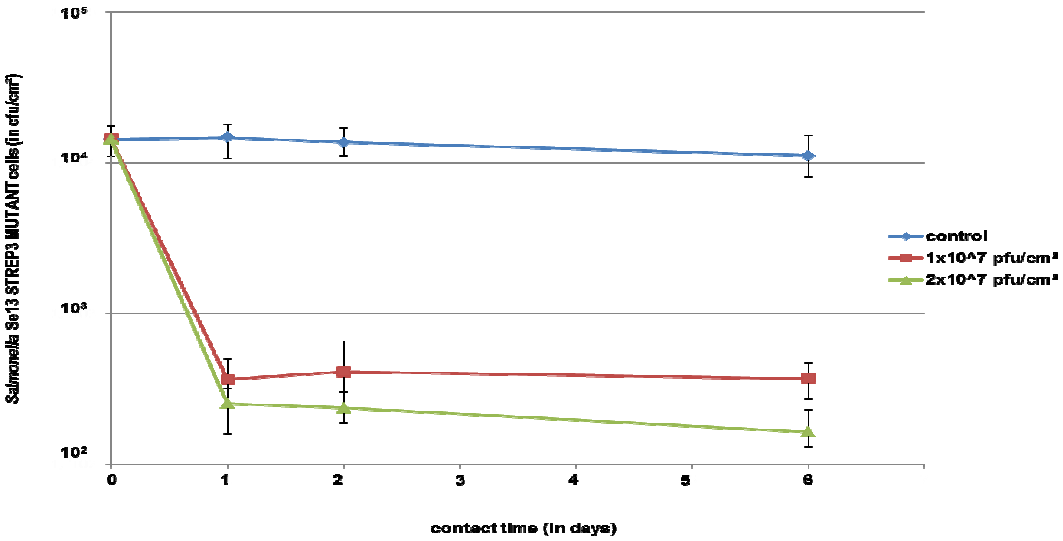


Figure 2: Effect of Salmonelex on growth of *Salmonella* strain Se13 STREP3 MUTANT on BEEF at 4°C over 6 days Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

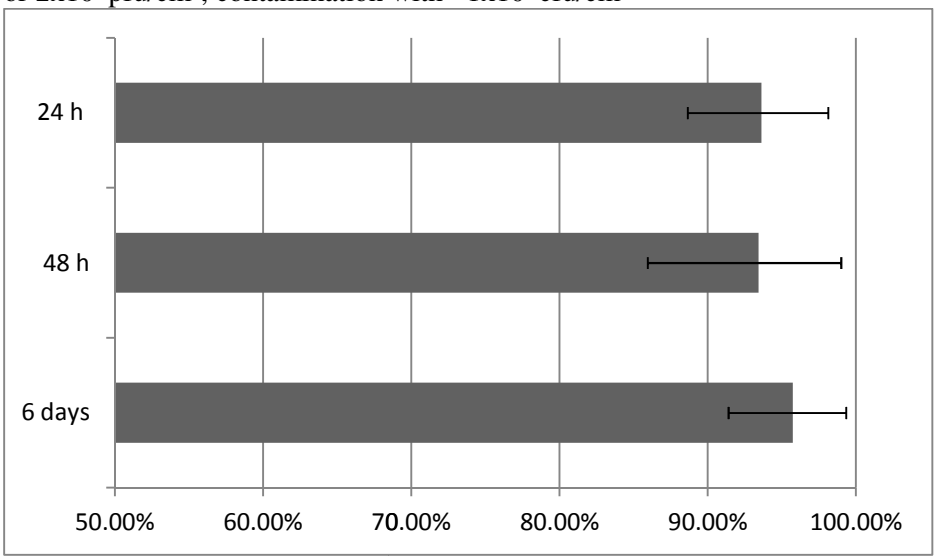


Figure 3: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated CUCUMBER samples
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

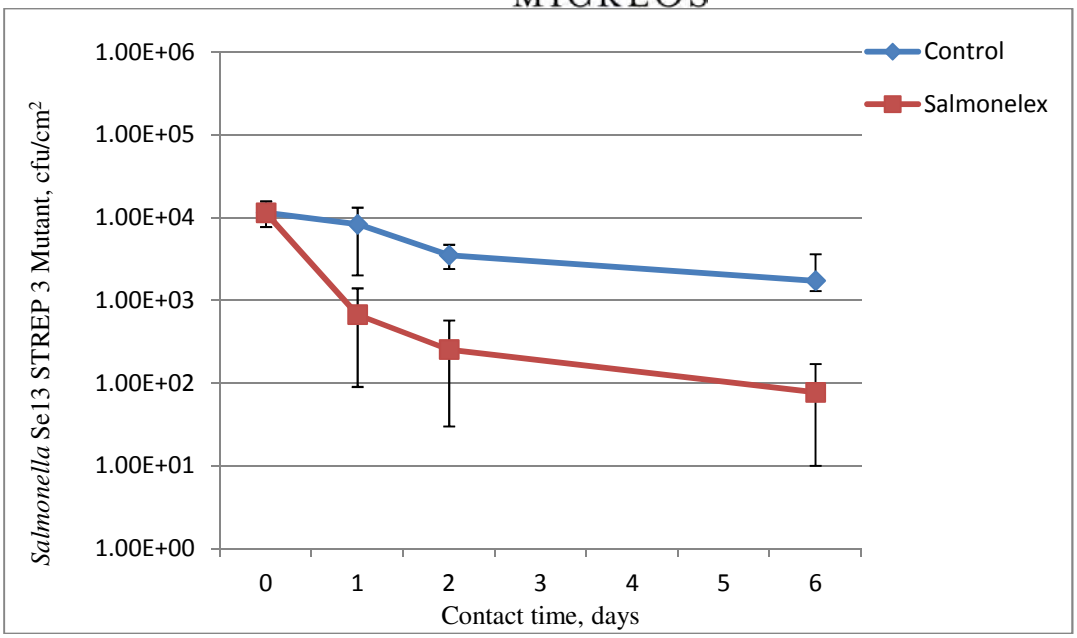


Figure 4: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on CUCUMBERS at 4 °C over 6 days. Contact times of 24 hours, 48 hours and 6 days; phage concentrations or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

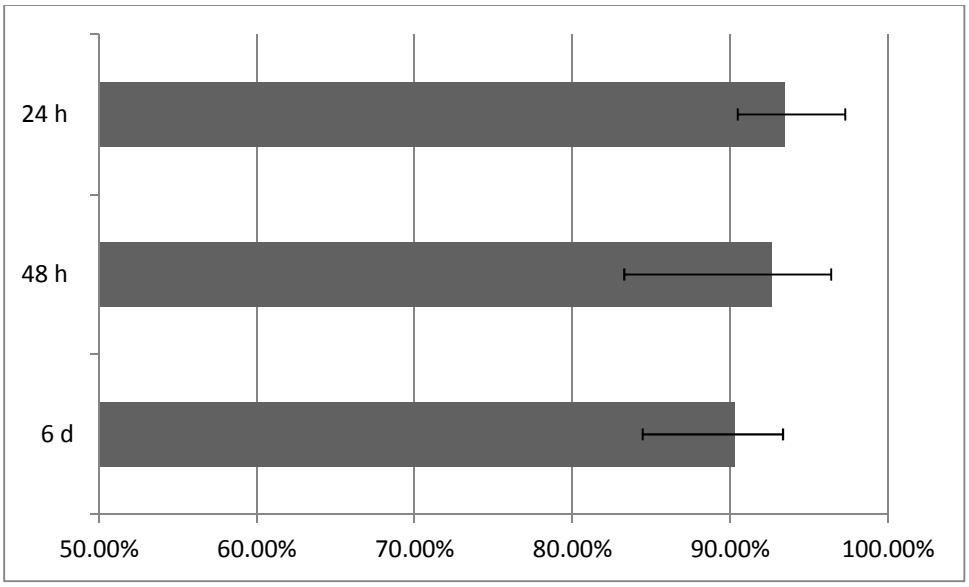


Figure 5: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated ICEBERG LETTUCE samples

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

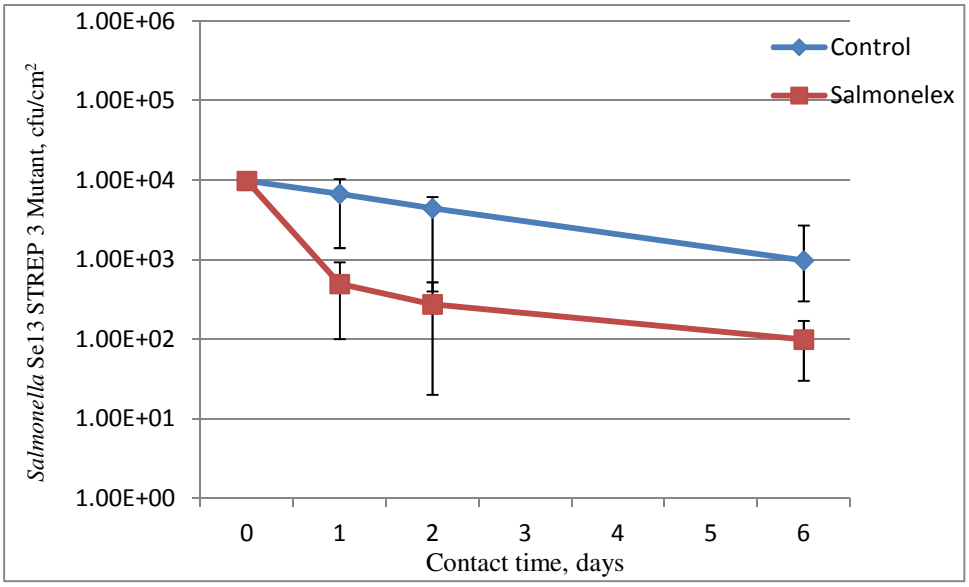


Figure 6: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on ICEBERG LETTUCE at 4°C over 6 days.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

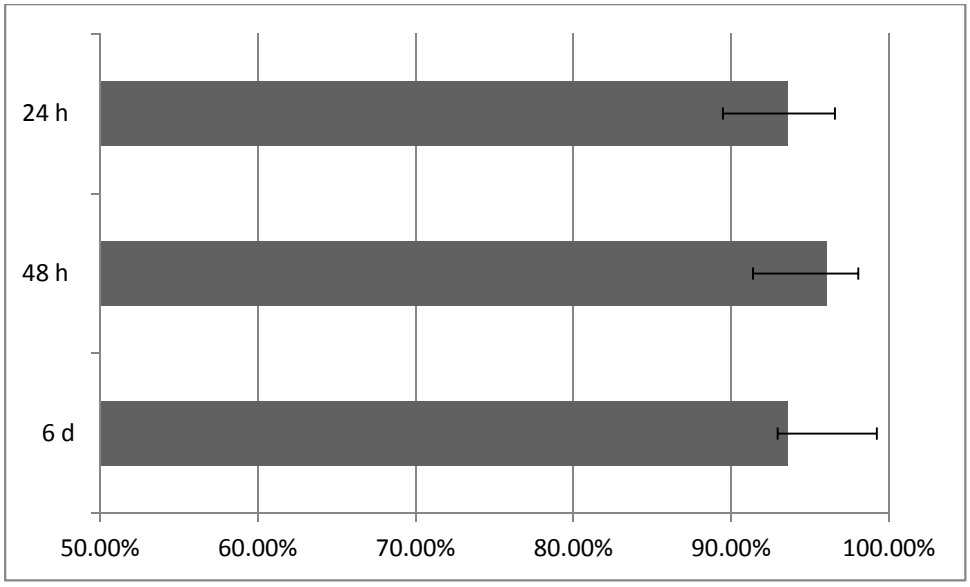


Figure 7: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated SPINACH samples

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

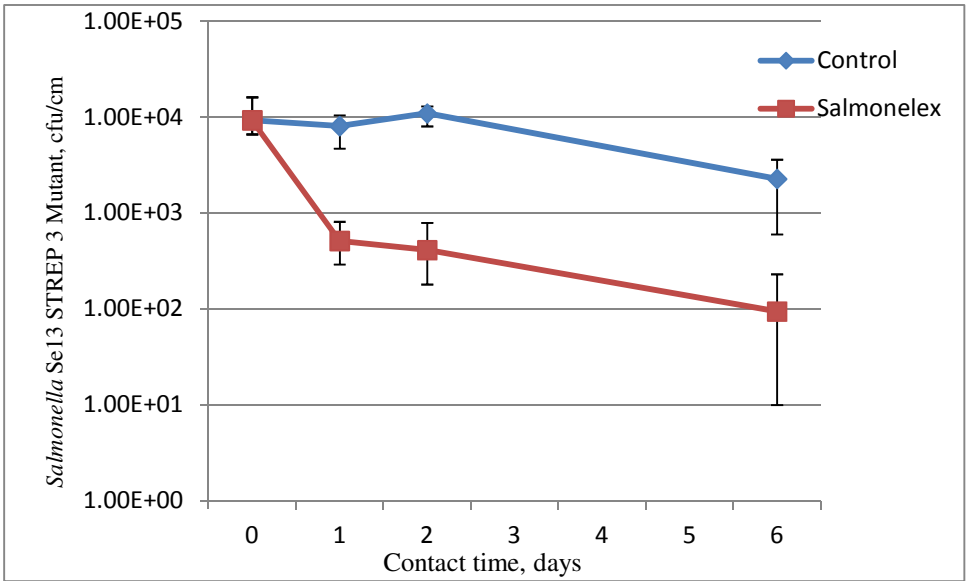


Figure 8: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on SPINACH at 4°C over 6 days.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$2x10^4$ cfu/cm²

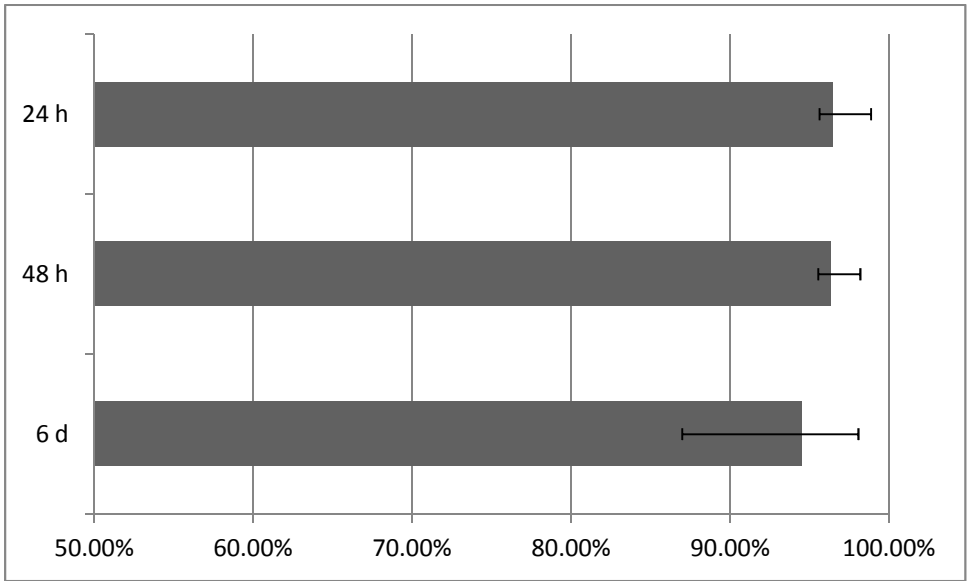


Figure 8: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated ZUCCHINI samples

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

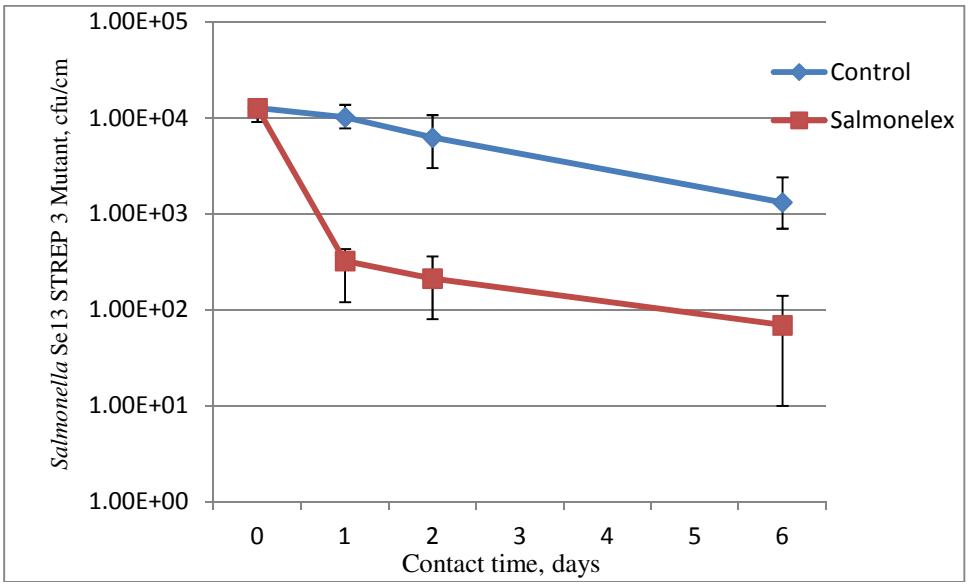


Figure 9: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on ZUCCHINI at 4°C over 6 days.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

APPENDIX

Table 2: **Salmonella** ***in cfu/plate and cfu/cm² retrieved from BEEF and reduction of cell numbers in % as well as log reduction; Artificial contamination of samples with 1x10⁴ cfu/cm²; phage treatment with 1x10⁷ pfu/cm² and 2x10⁷ pfu/cm²; reaction time: 24 hours, 48 hours and 6 days***

A: 1st round with duplicates; **B:** 2nd round with duplicates

A

		t=0	control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	202 193 177 193	114 108 98 97	92 106 84 86	49 47 44 50
	cfu/cm²	$1.91x10^4$	$1.04x10^4$	920	475
	% reduction			**91.18**	**95.44**
	log reduction			**1.05**	**1.34**
48h	cfu/plate*	202 193 177 193	99 87 75 72	90 68 62 60	33 28 40 57

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

	cfu/cm²	$1.53x10^4$	$8.33x10^3$	700	395
	% reduction			**91.59**	**95.26**
	log reduction			**1.08**	**1.32**
6d	cfu/plate*	202 193 177 193	69 72 102 77	65 62 70 52	50 40 32 18
	cfu/cm²	$1.53x10^4$	$8x10^3$	622.5	350
	% reduction			**92.22**	**95.63**
	log reduction			**1.11**	**1.36**

B

		t=0	**control (not treated)**	**$1x10^7$ pfu/cm²**	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	107 103 91 91	62 68 62 61	53 70 60 62	44 40 23 37
	cfu/cm²	$9.8x10^3$	$6.33x10^3$	613	360
	% reduction			**90.32**	**94.31**

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

	log reduction			**1.01**	**1.24**
48h	cfu/plate*	107 103 91 91	70 71 68 73	65 82 74 72	32 31 35 37
	cfu/cm²	$9.8x10^3$	$7.05x10^3$	733	338
	% reduction			**89.61**	**95.21**
	log reduction			**0.98**	**1.32**
6d	cfu/plate*	107 103 91 91	52 59 66 60	46 49 52 57	22 28 34 33
	cfu/cm²	$9.8x10^3$	$5.93x10^3$	510	292.5
	% reduction			**91.39**	**95.06**
	log reduction			**1.07**	**1.31**

Table 3: *Salmonella* in cfu/plate and cfu/cm² retrieved from **CUCUMBERS** and reduction of cell numbers in % as well as log reduction
Artificial contamination of samples with $1x10^4$ cfu/cm²; phage treatment with $1x10^7$ pfu/cm² and $2x10^7$ pfu/cm²; reaction time: 24 hours, 48 hours and 6 days
A: 1st round with duplicates; **B:** 2nd round with duplicates

A

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plated

		control (not treated)	$2x10^7$ pfu/cm²
24h	cfu/plate*	21 20 72 77	12 9 10 15
	average cfu/cm²	$4,75x10^3$	115
	% reduction		**97.58**
	log reduction		**1,6**
48h	cfu/plate*	33 35 24 27	13 5 4 3
	average cfu/cm²	$2,98 \times 10^3$	62,5
	% reduction		**97,90**
	log reduction		**1,67**
6d	cfu/plate*	18 18 15 8	5 7 3 1
	average cfu/cm²	$1,48 \times 10^3$	40
	% reduction		**97.29**

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

	log reduction	

B

* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plated

		control (not treated)	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	132 117 112 115	118 123 140 111
	average cfu/cm²	$1,41 \times 10^4$	$1,32 \times 10^3$
	% reduction		**89,66**
	log reduction		**1,02**
48h	cfu/plate*	34 32 49 47	24 43 57 55
	average cfu/cm²	$4,05 \times 10^3$	$4,47 \times 10^2$
	% reduction		**88,95**
	log reduction		**0,95**
6d	cfu/plate*	13 36 17	9 9 17

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

		13	11
	average cfu/cm²	1,98 x 10^3	1,15 x 10^2
	% reduction		**94,18**
	log reduction		**1,23**

Table 4: *Salmonella* in cfu/plate and cfu/cm² retrieved from **ICEBERG LETTUCE** and reduction of cell numbers in % as well as log reduction ; Artificial contamination of samples with 1x10^4 cfu/cm²; phage treatment with 2x10^7 pfu/cm²; reaction time: 24 hours, 48 hours and 6 days

A

		control (not treated)	**2x10^7 pfu/cm²**
24h	cfu/plate*	59 55 19 14	19 22 10 19
	average cfu/cm²	3,68 x 10^3	1,75 x 10^2
	% reduction		**95,24**
	log reduction		**1,32**
48h	cfu/plate*	15 25 11 4	19 23 2 6
	average cfu/cm²	1,38 x 10^3	1,25 x 10^2

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

	% reduction		**90,91%**
	log reduction		**1,04**
6d	cfu/plate*	3 7 5 3	3 3 3 7
	average cfu/cm²	4,5 x 10^2	40
	% reduction		**91,11**
	log reduction		**1,05**

* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plated

B

		control (not treated)	**2x10^7 pfu/cm²**
24h	cfu/plate*	97 103 94 96	93 81 70 81
	average cfu/cm²	9,75 x 10^3	8,13 x 10^2
	% reduction		**91,67**
	log reduction		**1,07**
48 h	cfu/plate*	80	27

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

		94 59 65	43 47 52
	average cfu/cm²	7,45 x 10^3	4,23 x 10^2
	% reduction		**94,33**
	log reduction		**1,24**
6d	cfu/plate*	17 27 7 9	17 16 16 14
	average cfu/cm²	1,5 x 10^3	1,58 x 10^2
	% reduction		**89,50**
	log reduction		**0,97**

* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plated

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Table 5: *Salmonella* in cfu/plate and cfu/cm² retrieved from **SPINACH** and reduction of cell numbers in % as well as log reduction;
Artificial contamination of samples with $1x10^4$ cfu/cm²; phage treatment with $1x10^7$ pfu/cm² and $2x10^7$ pfu/cm²; reaction time: 24 hours, 48 hours and 6 days

A

		control (not treated)	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	95 104 47 61	81 54 50 73
	average cfu/cm²	$7{,}68 \times 10^3$	$6{,}45 \times 10^2$
	% reduction	0	**91,6%**
	log reduction	0	**1,07**
48h	cfu/plate*	80 80 90 116	79 71 18 23
	average cfu/cm²	$9{,}15 \times 10^3$	$4{,}78 \times 10^2$
	% reduction	0	**94,78%**
	log reduction	0	**1,28**
6d	cfu/plate*	28 32 34	12 11 16

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

		36	23
	average cfu/cm²	3,25 x 10^3	1,55 x 10^2
	% reduction	**0**	**95,23**
	log reduction	**0**	**1,32**

* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plate

B

		control (not treated)	**2x10^7 pfu/cm²**
24h	cfu/plate*	89 82 91 74	31 29 45 46
	average cfu/cm²	8,4 x 10^3	3,78 x 10^2
	% reduction	0	**95,51**
	log reduction	0	**1,34**
48h	cfu/plate*	125 146 108 129	43 34 32 28
	average cfu/cm²	1,27 x 10^4	3,43 x 10^2
	% reduction		**1,56**

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

	log reduction		**1,24**
6d	cfu/plate*	11 17 6 17	3 3 6 1
	average cfu/cm²	1,28 x 10^3	33
	% reduction	**0**	**97,45%**
	log reduction	**0**	**1,58**

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Table 6: *Salmonella* in cfu/plate and cfu/cm² retrieved from **ZUCCHINI** and reduction of cell numbers in % as well as log reduction;
Artificial contamination of samples with $1x10^4$ cfu/cm²; phage treatment with $1x10^7$ pfu/cm² and $2x10^7$ pfu/cm²; reaction time: 24 hours, 48 hours and 6 days

A

		control (not treated)	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	80 81 122 137	27 12 36 23
	average cfu/cm²	$1,05 \times 10^4$	$2,45 \times 10^2$
	% reduction	0	**97,67%**
	log reduction	0	**1,63**
48h	cfu/plate*	45 59 30 42	8 11 15 9
	average cfu/cm²	$4,4 \times 10^3$	$1,08 \times 10^2$
	% reduction	0	**97,56%**
	log reduction	0	**1,61**
6d	cfu/plate*	24	4

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

		17 11 10	3 7 12
	average cfu/cm²	1,55 x 10^3	65
	% reduction	**0**	**95,81%**
	log reduction	**0**	**1,37**

* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plate

B

		control (not treated)	**2x10^7 pfu/cm²**
24h	cfu/plate*	110 104 100 78	39 35 43 43
	average cfu/cm²	9,8 x 10^3	4 x 10^2
	% reduction	0	**95,92%**
	log reduction	0	**1,39**
48h	cfu/plate*	76 85 107 54	33 35 22 36

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

	average cfu/cm²	8,05 x 10^3	3,15 x 10^2
	% reduction	0	**96,09%**
	log reduction	0	**1,4**
6d	cfu/plate*	10 7 12 14	14 12 2 1
	average cfu/cm²	**0**	**93,26%**
	% reduction	**0**	**1,17**
	log reduction		

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

MICREOS

Table 6: Overview *Salmonella* serovare Enteritidis Se13 STREP3 MUTANT cells retrieved beef samples (in cfu/cm² including error amounts based on maximum and minimum cells retrieved) treated with $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm² incubated for 24 hours, 48 hours and 6 days at 4°C; artificial contamination with ~$1x10^4$ cfu/cm²
* 2mL retrieval buffer/cm²; 20µl homogenate of untreated samples plated; 200µl homogenate of phage-treated samples plated

BEEF										
		t=24h			t=48h			t=6d		
		control	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²	control	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²	control	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
Average (cfu/cm²)	14463	8375	766	418	7688	716	367	6965	566	321
Max (cfu/cm²)	20200	11400	1060	500	9900	900	570	7200	700	500
Min (cfu/cm²)	9100	6100	530	230	6800	600	280	5200	460	180
Plus	5738	3025	294	83	2213	184	205	240	134	179
Minus	5363	2275	236	188	888	116	85	1760	106	141

MICREOS B.V.
NIEUWE KANAAL 7P . 6709 PA WAGENINGEN . T +31 (0)317 42 14 14 . F +31 (0)317 41 00 55
CHAMBER OF COMMERCE THE HAGUE: 27279042 . RABOBANK 1039.55.844 . IBAN: NL53 RABO 0103 9558 44 . BIC/SWIFT: RABONL2U
INFO@MICREOS.COM . WWW.MICREOS.COM

Table 7: Overview percentage reduction of *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from meat and poultry samples (including error amounts based on maximum and minimum percentage reduction) treated with $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm² incubated for 24 hours, 48 hours and 6 days at 4°C; artificial contamination with ~$1x10^4$ cfu/cm²

BEEF	t=24h		t=48h		t=6d	
	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
Average (% red)	90.75	94.88	90.60	95.26	91.81	95.35
Max (% red)	91.94	96.36	92.79	96.64	93.50	97.75
Min (% red)	88.93	93.04	88.37	93.15	90.37	93.75
Plus	1.19	1.49	2.19	1.39	1.69	2.41
Minus	1.82	1.83	2.23	2.10	1.44	1.60

Table 8: Overview *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from food samples (in cfu/cm² including error amounts based on maximum and minimum cells retrieved) treated with $2x10^7$ pfu/cm² incubated for 24 hours, 48 hours and 6 days at 4°C; artificial contamination with ~$1x10^4$ cfu/cm² (~$2x10^4$ cfu/cm^2 bean sprouts)

CUCUMBERS							
	t=0	t= 24h		t= 48h		t=6d	
		control	$2x10^7$ pfu/cm²	control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²
Average (cfu/cm²)	11500	8325	673	3515	255	1730	78
Max (cfu/cm²)	15700	13200	1400	4700	570	3600	170
Min (cfu/cm²)	7700	2000	90	2400	30	1300	10
Plus	4235	4875	728	1185	315	1870	93

Minus	3765	6325	583	1115	225	430	68
ICEBERG LETTUCE	t=0	t= 24h		t= 48h		t=6d	
		control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²
Average (cfu/cm²)	9765	6715	494	4415	274	975	99
Max (cfu/cm²)	11200	10300	930	9400	520	2700	170
Min (cfu/cm²)	7700	1400	100	400	20	300	30
Plus	1435	3585	436	4985	246	1725	71
Minus	2065	5315	394	4015	254	675	69
SPINACH	t=0	t= 24h		t= 48h		t=6d	
		control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²
Average (cfu/cm²)	9260	8040	512	10900	410	2270	94
Max (cfu/cm²)	16100	10400	810	12900	790	3600	230
Min (cfu/cm²)	6600	4700	290	8000	180	600	10
Plus	6850	2360	299	1980	380	1340	136
Minus	2660	3340	222	2983	230	1670	84
ZUCCHINI	t=0	t= 24h		t= 48h		t=6d	
		control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²	control	$2x10^7$pfu/cm²
Average (cfu/cm²)	12700	10200	323	6230	212	1320	69
Max (cfu/cm²)	14300	13700	430	10700	360	2400	140
Min (cfu/cm²)	9100	7800	120	3000	80	700	10
Plus	1650	3550	108	4480	149	1090	70
Minus	3550	2350	203	3230	132	615	59

Table 9: Overview percentage reduction of *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from vegetable samples (including error amounts based on maximum and minimum percentage reduction) treated with $2x10^7$ pfu/cm² incubated for 24 hours, 48 hours and 6 days at 4°C; artificial contamination with ~$1x10^4$ cfu/cm²

	$2x10^7$ pfu/cm^2		
CUCUMBERS	24 h	48 h	6 d
Reduction (%)	93,62	93,43	95,74
Max(%)	98,11	98,99	99,32
Min(%)	88,64	85,93	91,39
Plus (%)	4,49	5,57	3,59
Minus(%)	4,98	7,50	4,34
ICEBERG LETTUCE	24 h	48 h	6 d
Reduction (%)	93,46	92,62	90,31
Max(%)	97,28	96,38	93,33
Min(%)	90,46	83,27	84,44
Plus (%)	3,83	3,76	3,03
Minus(%)	3,00	9,35	5,87
SPINACH	24 h	48 h	6 d
Reduction (%)	93,55	96,04	93,55
Max(%)	96,55	98,03	99,22
Min(%)	89,45	91,37	92,92
Plus (%)	3,00	1,99	5,67
Minus(%)	4,10	4,67	0,63
ZUCCHINI	24 h	48 h	6 d
Reduction (%)	95,92	96,09	93,26
Max(%)	96,43	97,27	99,07
Min(%)	95,61	95,53	86,98
Plus (%)	0,51	1,18	5,81
Minus(%)	0,31	0,56	6,28

SUBMISSION END