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GRAS Notice Review

Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Attn. Mrs. Moraima J. Ramos Valle, M.S.
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740
USA

April 2, 2013

Dear Mrs. Ramos,

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 a GRAS notification of the bacteriophage cocktail Salmonelex™ for bio-control of *Salmonella* in food.

A CD-rom free of viruses with an electronic version of the GRAS notification is included, we would appreciate if you can ensure a copy is forwarded to USDA FSIS review for the intended use of Salmonelex™ as a suitable processing aid in pork and poultry products.

Please let me know if you have any questions,

Sincerely, (b) (6)

(b) (6)

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

SALMONELEX™ NOTIFICATION

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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 to be generally recognized as safe by Micreos B.V. 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)
(b) (6)

02 - 04 - 2013
Date

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

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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
Nieuwe Kanaal 7P
6709 PA Wageningen
The Netherlands

s.hagens@micreos.com
Tel: + 31 317 421414

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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.

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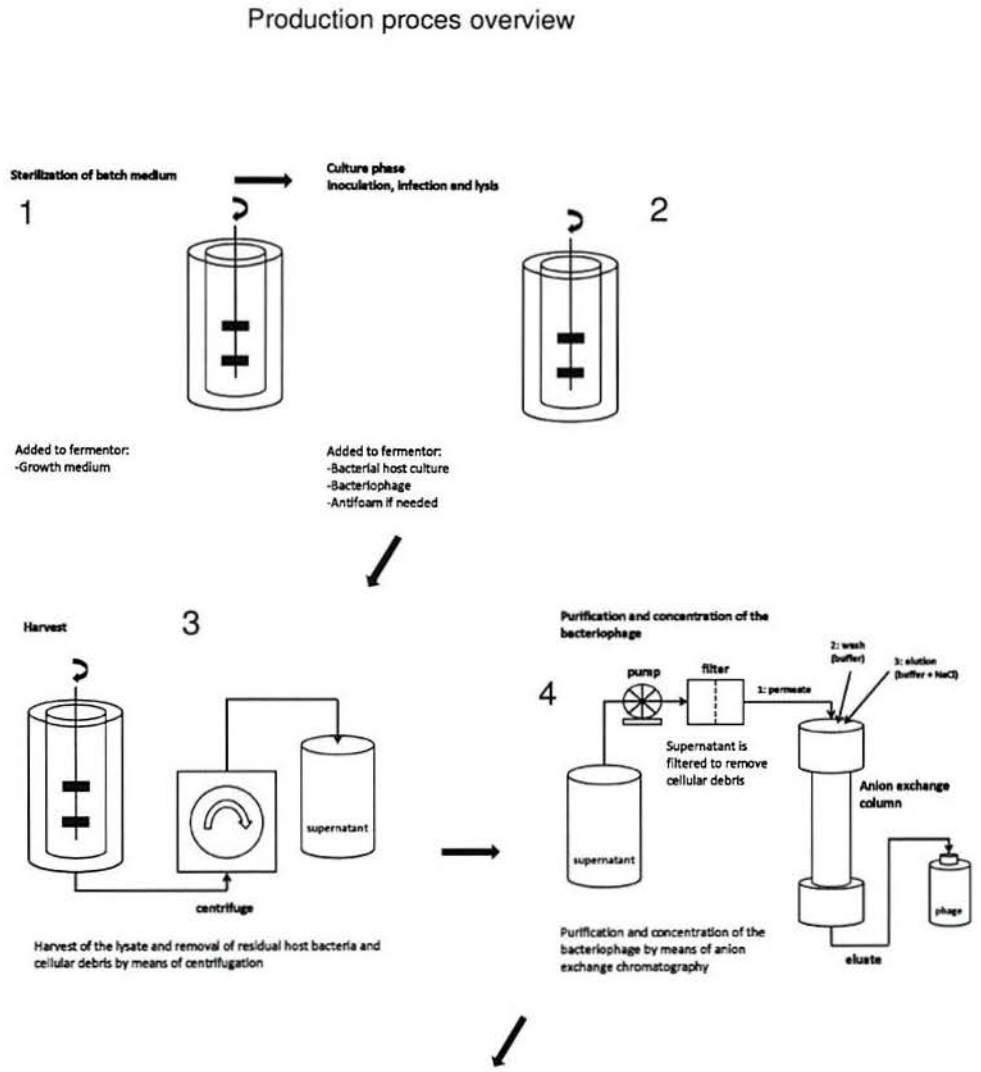
Figure 1. Schematic representation of the production process of Salmonelex™



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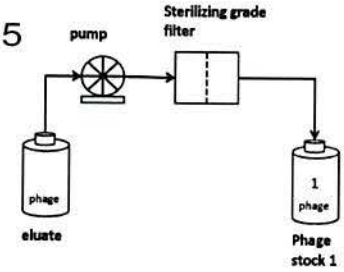
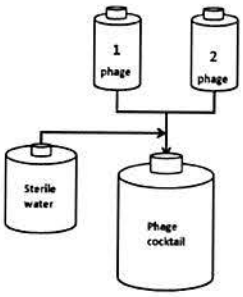
Sterile filtration

5



Phage stock tests:
-Bacterial sterility
-Phage titer

Blending of the 2 bacteriophages

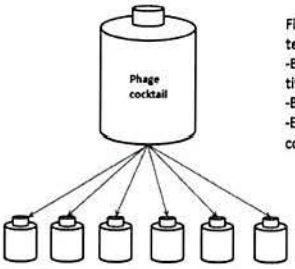


6

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

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C. Specifications

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

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

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 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.

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

Chemical Property	**Salmonelex™ batch12N21-S(1)**	**Salmonelex™ batch12N21-S(2)**	**Salmonelex™ batch12N21-S(3)**	**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

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

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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, 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

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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 which 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 analysed 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.

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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 *Staphylococcus aureus* bacteriophages can be effectively recovered using anion exchange

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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).

Complete removal of endotoxin during the production process of Salmonelex™ is not feasible but after removal of cellular debris and anion exchange chromatography endotoxin levels are extremely low and will not significantly contribute to the daily dietary intake of endotoxin by consumers. This will be discussed in detail in the section discussing estimated dietary intake of Salmonelex™ phages and by-products.

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.

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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:

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

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- 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 a lot of effort has been put

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into ensuring that starter cultures are free from phages and to some extent resistant to phage infection, this is certainly not the case for artisanal cheeses and one might even argue that as long as timing is correct, host lysis by phages and thus liberation of the proteolytic enzymes may even be desirable. Phages infecting *Propionibacterium freudenreichii* have been isolated from Swiss cheese at levels of up to 7 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

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on the other hand has to date been shown to carry undesirable genes and most virulent phages do not transduce host genes. Some virulent phages have shown ability for generalized transduction and safety data should include not only genome sequence data but experimental and/or theoretical proof that the candidate phages cannot transduce.

If 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

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.

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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 is used to adjust pH of the medium only at the start of fermentation.

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

D) Allergenicity

I. Phage components

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

II. Relevant Medium Components

Soy Pepton

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

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producer ELISA and PCR testing point out that the main allergens are absent in this soy pepton, within the limits of detection . Micreos also confirms negative allegenicity on incoming product using the Reveal 3 D (Soy Test), NEOGEN.

The anion exchange chromatography step used to purify the phages will furthermore remove >99% of all proteins including medium components.

E) 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 ~62 lbs consisted of poultry (chicken and turkey) and 47.7 lbs of pork. On a daily basis this translates to 82 g of poultry and 60 g of pork.
In the case of these products *Salmonella* contaminations are on the surface. Therefore, it is surfaces that are treated. This means the earlier Salmonelex™ is used in meat-processing the smaller the surface to weight ratio becomes, resulting in lower usage of Salmonelex™ and thus in lower costs to the food producer. However, if we consider use on final products such as chicken breast filets and cuts and assuming a 2:1 surface to weight ratio (2 cm^2 per gram of product) and assuming maximum use levels of $1x10^8$ pfu/cm^2 (is equal to the maximum requested use level of $2x10^8$ pfu/gram) we can perform the following calculations.

Phage intake

142 grams/meat x $2cm^2/g$ x $1x10^8$ pfu/cm^2 = 2.84×10^{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:

$2.84 \times 10^{10} \times 10^8 \times 1.66 \times 10^{-27}$ kg = 0.0000000047144 kg/day = 4.7ug/day.

Or in terms of treated product:

33.5 ppb or 0.0335 ppm (parts per billion/parts per million). This level is insignificant.

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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. However the dilution factor varies slightly with phage titers and therefore we will consider the amount of salt deposited if there is no dilution.

At a phage level of 2x 10^8 pfu/g and treatment of 142 grams of meat and a salt concentration of 0.029g/mL of phage solution the following calculation can be made:

0.029g/Salt x 142g/meat/day x 0.001 mL phage solution = 0.00412 g sodium chloride/day/serving.

The sodium content per serving (Molecular weight Chloride = 35.45 and Sodium = 22.9) would amount to 1.6 mg. This amount represents 0.064% of the recommended daily intake levels and thus would not change nutritional content labeling by the end user.

Endotoxin levels

At the maximum level of endotoxin allowed for product release (250,000 EU per mL/ 2x 10^{11} pfu/mL = 250 EU per 2 x10^8 pfu) levels of endotoxin consumed on daily basis can be calculated as follows:

142g/meat/day x 250 EU = 35,500 EU/day.

This value corresponds to 250 EU/g of food and as such is lower than background EU-levels found in common foodstuffs. In comparison 1 mL of saliva contains 1 mg of EU corresponding to 1000 EU/ml. Saliva is produced at levels exceeding 500 ml/day. The total amount of endotoxin consumed because of Salmonelex™ use is insignificant when compared with background levels found in foods and endotoxin consumed via other routes and no adverse effects on health can be expected.

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F) 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

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.

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G) 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.
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. In the appendix data showing the effect of Salmonelex™ on relevant foodstuffs is presented.
In short application of phages at levels of $1x10^7$ pfu/cm^2 and $2 x10^7$ pfu/cm^2, representing the effect of application of Salmonelex™ to meat 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 1 to >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. While *Salmonella* does not grow at refrigeration

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temperatures experiments at room temperature (20°C) clearly show that after initial reduction over the first 8 hours after treatment, any

remaining bacteria will grow out at similar growth rates as in the untreated controls. This shows that Salmonelex™ has no function in the final product and should be considered a processing aid.

H) 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 poultry and pork.
Based on these findings and significant equivalence with the other GRAS-affirmed phage preparation, Salmonelex™ is also considered GRAS.

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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 chicken breast fillet, chicken skin and pork meat. The challenge testing was performed at Micreos, Wageningen, The Netherlands. 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. 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. Samples were incubated at 4°C. Duplicate samples were tested and the challenge studies were performed twice.

As *Salmonella* does not grow at refrigerator temperatures the challenge study as described above does not show that phages only have an initial effect on *Salmonella* cells. After an initial reduction bacterial cells start growing out again at higher temperatures. Therefore, the challenge was repeated with incubation at room temperature treating pork meat samples with a final phage concentration of $2x10^7$ pfu/cm².

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

Chicken breast fillet*

Chicken skin*

Pork meat*

* Purchased at a local supermarket

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Bacteria/bacteriophage

- *Salmonella* serotype Enteritidis *Se13* Streptomycin resistant mutant (500µg/mL) (Se13 STREP3 MUTANT) → titer Overnight (ON) culture on selective agar plates + streptomycin: ~1.13×10^9 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 meat samples

Bacterial overnight cultures

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

Preparation of samples

Sample pieces of 6x3x1cm 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 sterilized with 70% EtOH to get rid of background bacteria and placed in sterile petri dishes.

Artificial contamination

An appropriate dilution of the overnight culture was prepared in PBS to allow the contamination of the samples with a final concentration of approximately 1×10^4 cfu/cm² or 1×10^3 cfu/cm², for testing with

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incubation at room temperature. To control the concentration of the dilution used to contaminate the samples, the titer was determined by plating an appropriate dilution on selective agar plates.
In the laminar flow hood 2µl/cm² of the dilution was transferred to each sample and rubbed in evenly with the pipette tip. Samples were left in the fume hood to dry.

Treatment with Salmonelex™

To allow the treatment of the samples with a final concentration of $1x10^7$ pfu/cm² and/or $2x10^7$ pfu/cm² (incubation at room temperature only $2x10^7$ pfu/cm²), a dilution of Salmonelex™ was prepared in SM buffer. In the fume hood 5µl/cm² were 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. For samples incubated at room temperature cells were also retrieved after 8 hours incubation at 4°C to show the complete initial effect of Salmonelex™ before the re-growth taking place at room temperature.

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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	
chicken breast	2
chicken skin	2
pork	2
t=24h	
chicken breast	6*
chicken skin	6*
pork	6*
t=48h	
chicken breast	6*
chicken skin	6*
pork	6*
t=6d	
chicken breast	6*
chicken skin	6*
pork	6*
TOTAL Nr. SAMPLES	60

* 2 samples not phage-treated (control), 2 samples treated with $1x10^7$ pfu/cm², 2 samples treated with $2x10^7$ pfu/cm² Salmonelex™

B

Sample	Nr.samples
t=0	
pork	2
t=8h	
pork	4*
t=24h	
pork	4*
t=48h	
pork	4*
t=6d	
pork	4*
TOTAL Nr. SAMPLES	2

* 2 samples not phage-treated (control), 2 samples treated with $2x10^7$ pfu/cm² Salmonelex™

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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 37°C.

3 Results

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

3.1.1 Incubation at 4°C

Figures 1 to 5 show the percentage reduction of *Salmonella* on pork meat, chicken breast fillet and chicken skin when treated with two phage concentrations at an incubation temperature of 4°C.

Pork

With a phage concentration of $1x10^7$ pfu/cm², cell numbers could be reduced by 92% (corresponding to 1.1 log) with a contact time of 24 hours. The number of cells did not change significantly after retrieval after 48 hours and 6 days of contact time (see Figure 1 and 2).

On average a 95% reduction (~1.3 log reduction) of cell numbers could be achieved when treating the samples with a phage concentration of $2x10^7$pfu/cm². Also for this phage concentration there was no significant difference comparing the different contact times.

Chicken skin

On chicken skin a *Salmonella* cell reduction of 97% (~1.6 log reduction) could be achieved when Salmonelex™ was applied in a concentration of $1x10^7$pfu/cm². No significant difference between the different contact times was observed. Additionally no big difference was observed when applying the higher phage concentration of $2x10^7$ pfu/cm² (98% reduction, corresponding to a log reduction of 1.8). (Figure 3 and 4)

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Chicken breast fillet

On chicken breast fillet cell counts dropped by approximately 92% (~1.1log) when treating the samples with $1x10^7$ pfu/cm² Salmonelex™. A reduction of 96 to 97% (1.5 to 1.6 log) was observed when applying $2x10^7$ pfu/cm². No significant difference was seen between the different contact times (Figure 5 and 6).

For detailed results see Appendix Tables 2 to 6.

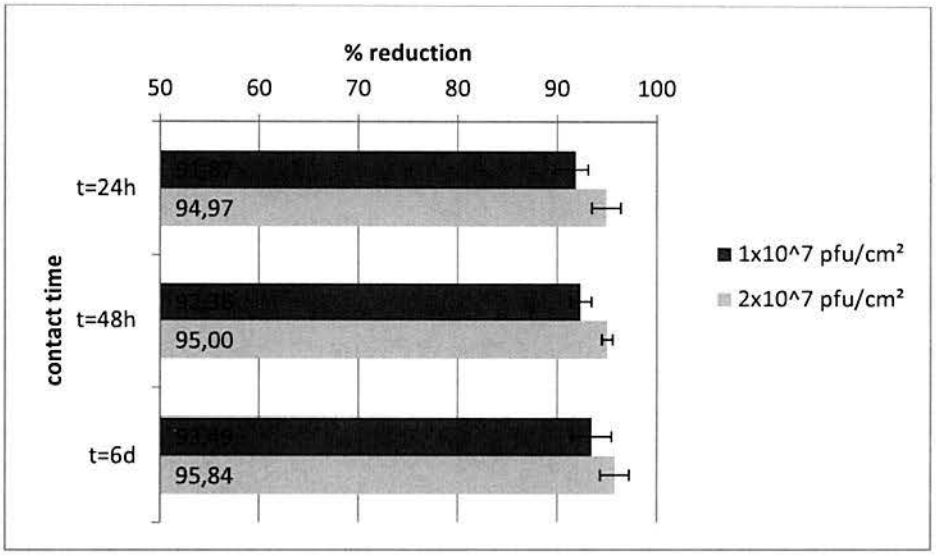

Figure 1: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated PORK samples
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

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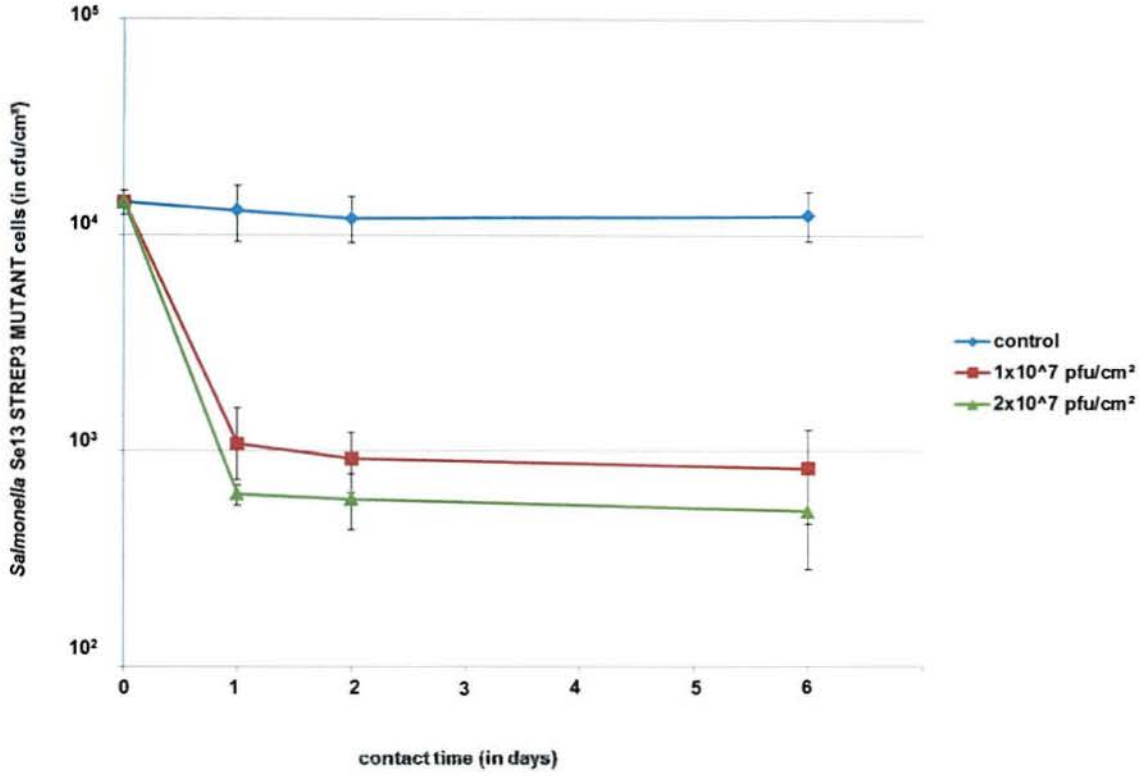

Figure 2: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on PORK meat at 4°C over 6 days.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

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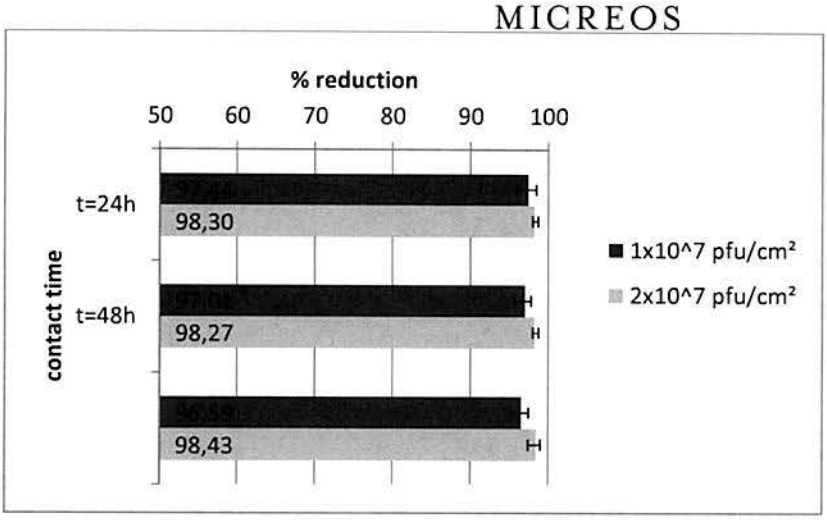

Figure 3: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated CHICKEN SKIN samples
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

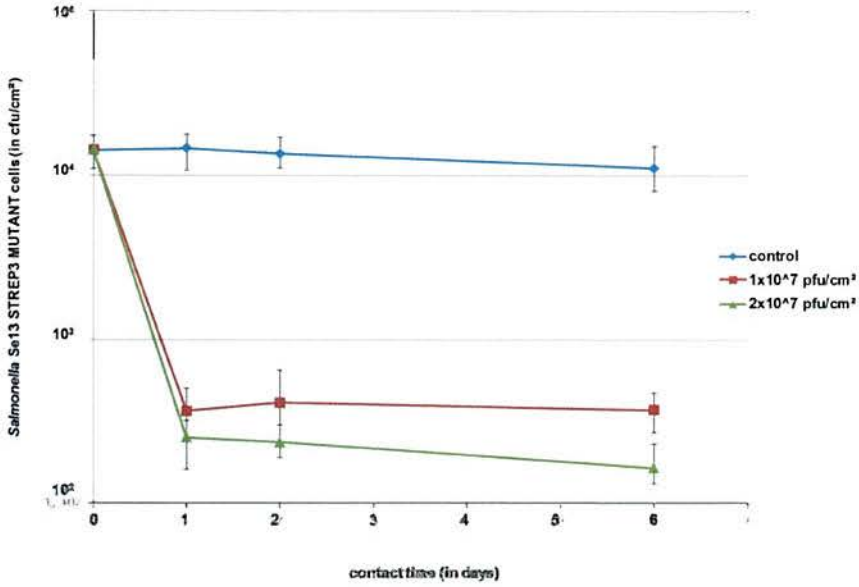

Figure 4: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on CHICKEN SKIN at 4°C over 6 days.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

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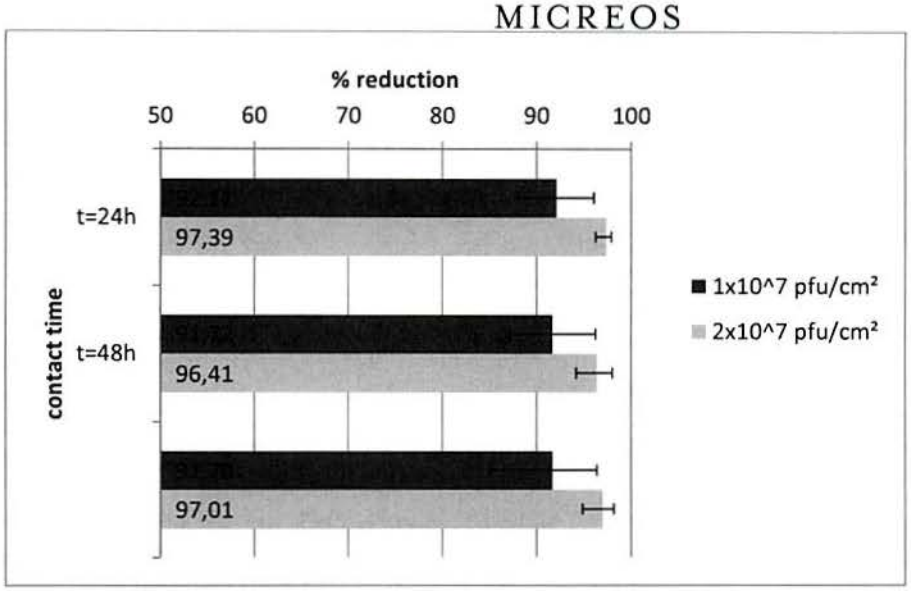

Figure 5: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated CHICKEN BREAST FILLET samples
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

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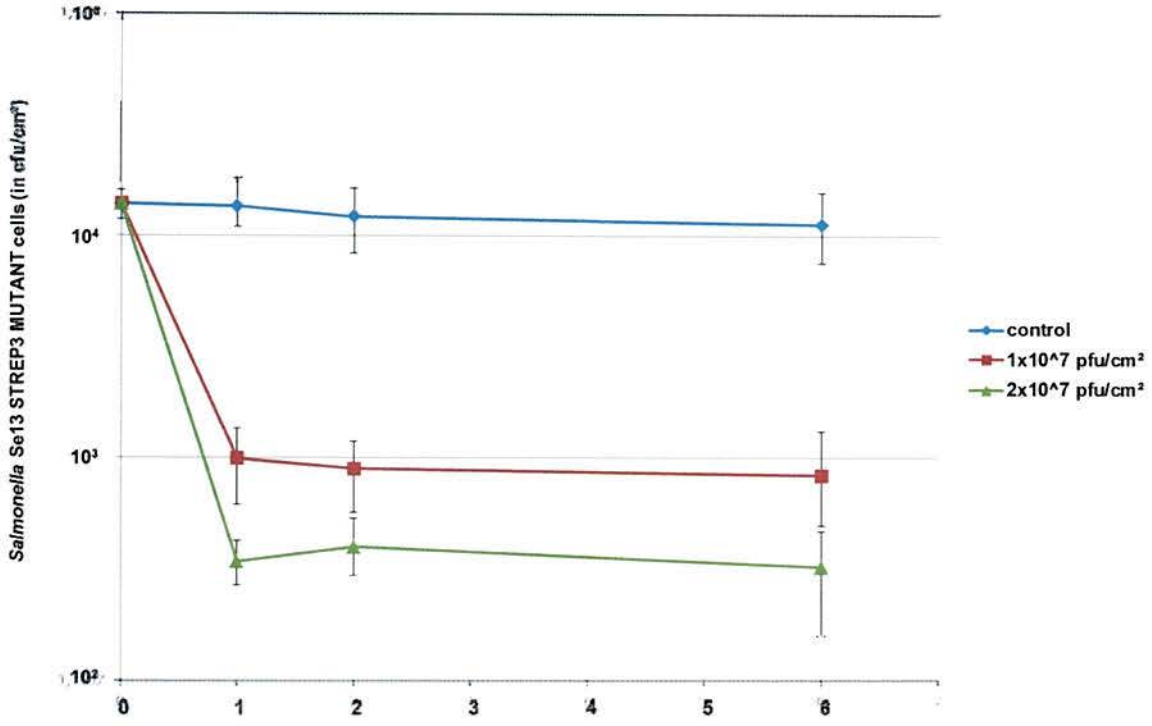

Figure 6: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on CHICKEN BREAST FILLET at 4°C over 6 days.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

3.1.2 Incubation at room temperature

After an initial cell reduction of 91% (corresponding to 1.05 log) on treated pork samples after an 8 hour incubation at 4°C, *Salmonella* cells started growing out again. Cells on treated samples followed the growth pattern of untreated control samples, while reaching the same cell numbers after 6 days of incubation (see Figure 7) .

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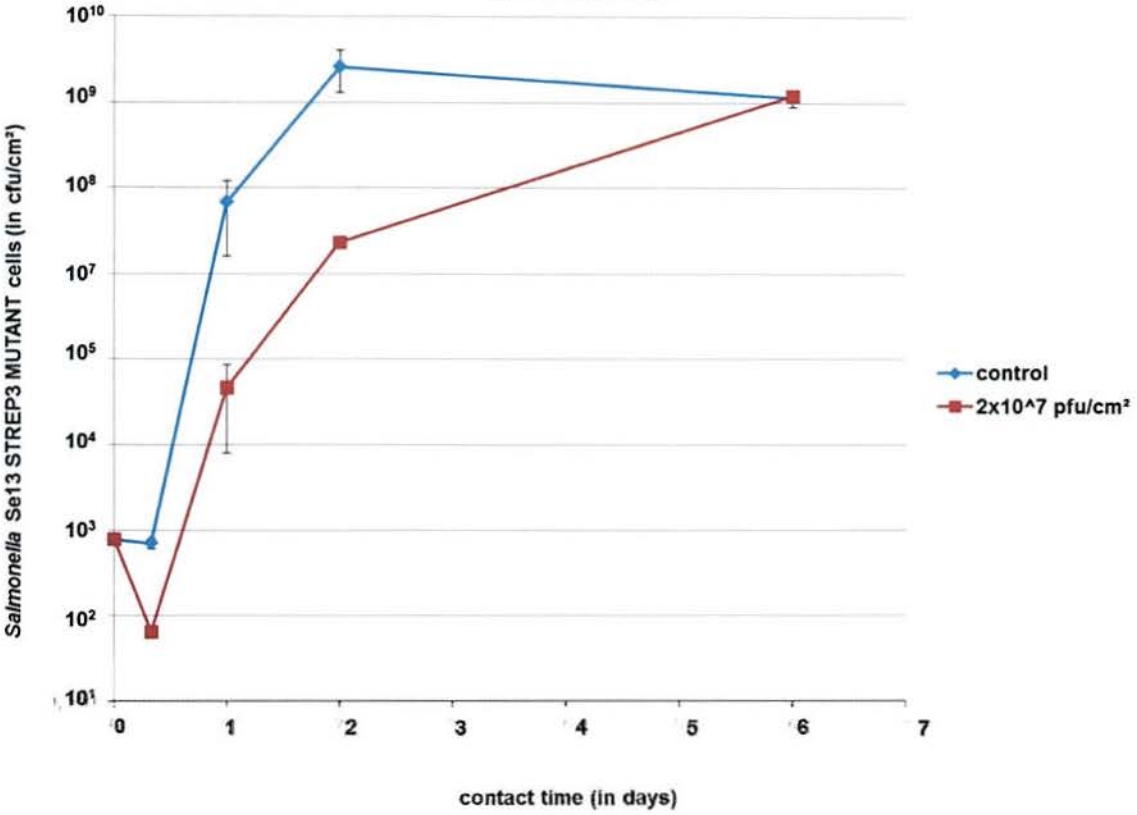

Figure 7: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on PORK MEAT at room temperature over 6 days (initial 8 hour incubation at 4°C)
Contact times of 8 hours, 24 hours, 48 hours and 6 days; phage concentration $2x10^7$ pfu/cm²; contamination with ~$1x10^3$ cfu/cm²

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APPENDIX

Table 2: *Salmonella* in cfu/plate and cfu/cm² retrieved from **PORK MEAT** 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

		control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	169 158 147 146	158 132 123 129	66 65 60 56
	average cfu/cm²	$1.55x10^4$	1355	617.5
	% reduction		**91.26**	**96.02**
	log reduction		**1.06**	**1.4**
48h	cfu/plate*	141 151 141 136	112 122 100 102	66 67 78 74
	average cfu/cm²	$1.42x10^4$	1090	712.5
	% reduction		**92.34**	**94.99**
	log reduction		**1.12**	**1.3**
6d	cfu/plate*	159 159 130 138	88 96 125 124	53 56 80 83
	average cfu/cm²	$1.47x10^4$	1083	680
	% reduction		**92.61**	**95.36**
	log reduction		**1.13**	**1.33**

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

B

		control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	117 118 96 93	73 77 83 86	61 60 68 69
	average cfu/cm²	$1.06x10^4$	797.5	645
	% reduction		**92.38**	**95.01**
	log reduction		**1.12**	**1.3**
48h	cfu/plate*	92 97 101 101	69 64 80 85	43 49 49 54
	average cfu/cm²	$9.78x10^3$	745	487.5
	% reduction		**92.38**	**95.01**
	log reduction		**1.12**	**1.3**
6d	cfu/plate*	97 94 105 109	46 47 68 67	34 28 42 45
	average cfu/cm²	$1.01x10^4$	570	372.5
	% reduction		**94.37**	**96.32**
	log reduction		**1.25**	**1.43**

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

Table 3: *Salmonella* in cfu/plate and cfu/cm² retrieved from **CHICKEN SKIN** 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)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	180 177 162 160	46 38 32 26	32 30 28 30
	average cfu/cm²	$1.69x10^4$	355	300
	% reduction		**97.91**	**98.23**
	log reduction		**1.68**	**1.75**
48h	cfu/plate*	160 171 141 134	65 56 34 33	25 19 30 29
	average cfu/cm²	$1.52x10^4$	470	257.5
	% reduction		**96.9**	**98.3**
	log reduction		**1.51**	**1.77**
6d	cfu/plate*	115 116 152 143	33 34 43 47	13 13 13 15
	average cfu/cm²	$1.32x10^4$	393	135
	% reduction		**97.02**	**98.97**
	log reduction		**1.53**	**1.99**

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

B

		control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	107 113 143 137	31 25 45 50	16 18 23 25
	average cfu/cm²	$1.25x10^4$	377.5	205
	% reduction		**96.98**	**98.36**
	log reduction		**1.52**	**1.79**
48h	cfu/plate*	121 111 132 122	31 30 47 32	20 22 23 21
	average cfu/cm²	$1.22x10^4$	350	215
	% reduction		**97.12**	**98.36**
	log reduction		**1.54**	**1.75**
6d	cfu/plate*	81 86 99 97	27 28 41 43	17 17 20 23
	average cfu/cm²	$9.08x10^4$	347.5	192.5
	% reduction		**96.17**	**97.88**
	log reduction		**1.41**	**1.67**

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

Table 4: *Salmonella* in cfu/plate and cfu/cm² retrieved from **CHICKEN BREAST FILLET** 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)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	159 147 182 141	62 62 63 70	33 33 36 37
	average cfu/cm²	$1.57x10^4$	642.5	347.5
	% reduction		**95.91**	**97.79**
	log reduction		**1.39**	**1.66**
48h	cfu/plate*	148 163 149 147	59 57 68 71	41 38 32 30
	average cfu/cm²	$1.52x10^4$	637.5	352.5
	% reduction		**95.8**	**97.68**
	log reduction		**1.38**	**1.63**
6d	cfu/plate*	129 130 137 157	57 50 63 69	47 45 37 30
	average cfu/cm²	$1.38x10^4$	598	398
	% reduction		**95.68**	**97.47**
	log reduction		**1.36**	**1.53**

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

B

		control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	121	134	40
		116	131	43
		110	135	27
		111	136	28
	average cfu/cm²	1,15E+04	1340	345
	% reduction		**88.30**	**96.99**
	log reduction		**0.93**	**1.52**
48h	cfu/plate*	99	115	39
		100	109	35
		89	119	54
		84	117	53
	average cfu/cm²	9,30E+03	1150,00	452.5
	% reduction		**87.76**	**95.13**
	log reduction		**0.91**	**1.31**
6d	cfu/plate*	90	83	24
		98	94	16
		76	123	45
		87	131	36
	average cfu/cm²	8775	1078	302.5
	% reduction		**87.72**	**96.55**
	log reduction		**0.91**	**1.46**

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

Table 5: Overview *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from meat samples (in cfu/cm² inclusive 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

PORK										
	t=0	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)	14200	13050	1076.25	631.25	12000	917.5	600	12387.5	826.25	526.25
Max (cfu/cm²)	16000	16900	1580	690	15100	1220	780	15900	1250	830
Min (cfu/cm²)	12400	9300	730	560	9200	640	430	9400	460	280
Plus	1800	3850	503.75	58.75	3100	302.5	180	3512.5	423.75	303.75
Minus	1800	3750	346.25	71.25	2800	277.5	170	2987.5	366.25	246.25
CHICKEN SKIN										
		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)	14300	14737.5	366.25	252.5	13650	410	236.25	11112.5	370	163.75
Max (cfu/cm²)	17600	18000	500	320	17100	650	300	15200	470	230
Min (cfu/cm²)	11000	10700	250	160	11100	300	190	8100	270	130
Plus	3300	3262.50	133.75	67.50	3450	240	63.75	4087.50	100	66.25
Minus	3300	4037.50	116.25	92.50	2550	110	46.25	3012.50	100	33.75
CHICKEN BREAST										
		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)	13950	13587.5	991.25	346.25	12237.5	893.75	402.5	11300	837.5	326.25
Max (cfu/cm²)	16000	18200	1360	430	16300	1190	540	15700	1310	470
Min (cfu/cm²)	11900	11000	620	270	8400	570	300	7600	500	160
Plus	2050	4612.50	368.75	83.75	4062.50	296.25	137.50	4400	472.50	143.75
Minus	2050	2587.50	371.25	76.25	3837.50	323.75	102.50	3700	337.50	166.25

PORK						
	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)	91.87	94.97	92.36	95	93.49	95.84
Max (% red)	93.11	96.39	93.45	95.60	95.46	97.23
Min (% red)	89.81	93.49	91.30	94.48	91.47	94.33
Plus	1.25	1.42	1.09	0.60	1.97	1.39
Minus	2.06	1.48	1.05	0.53	2.02	1.51
CHICKEN SKIN						
	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)	97.44	98.30	97.01	98.27	96.59	98.43
Max (% red)	98.47	98.72	97.82	98.75	97.49	99.01
Min (% red)	96.00	98.00	95.71	98.02	95.26	97.47
Plus	1.02	0.42	0.81	0.48	0.90	0.59
Minus	1.44	0.30	1.30	0.25	1.33	0.96
CHICKEN BREAST						
	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)	92.11	97.39	91.72	96.41	91.70	97.01
Max (% red)	96.06	97.90	96.24	98.02	96.38	98.18
Min (% red)	88.12	96.24	87.20	94.19	85.07	94.87
Plus	3.95	0.51	4.53	1.62	4.68	1.17
Minus	3.98	1.14	4.51	2.21	6.63	2.14

Table 6: Overview percentage reduction of *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from meat samples (inclusive 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

SUBMISSION END