MICREOS

Application for Salmonelex™ as a Processing Aid against Salmonella on Fresh Meat and Poultry Products

Micreos Food Safety

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3.1.1 Executive Summary

This application is for Salmonelex™, a bacteriophage based solution, aimed at killing *Salmonella* during post-slaughter processing of fresh meat and poultry products.

OzFoodNet, the food-borne disease surveillance network operating in Australia, undertook a study to estimate the amount of food-borne gastroenteritis in a typical year. This study estimated that in a typical year (around the year 2000) there were approximately 92,000 total cases of salmonellosis (95% credible interval 26,000-158,000) (Hall et al, 2005). The report also estimated that the proportion of the total cases that could be attributed to food was estimated to be 87% or 81,000 (95% credible interval 23,000-138,000).

Poultry is one of the implicated foods in *Salmonella* outbreaks. In a review of reported salmonellosis outbreaks in Australia during 1995-2000, poultry meat was associated with 13% of the identified salmonellosis outbreaks and 8% of the total outbreak cases (Dalton et al, 2004).

In Australia, raw poultry meat purchased by the consumer is very likely to be contaminated with *Salmonella* (43%, with 13% being non- Sofia *Salmonella* serovars). The higher the prevalence and concentration of this bacteria being present on raw poultry, the greater the likelihood these pathogens could be present at the point of consumption and therefore a greater likelihood of illness occurring.

Raw poultry contaminated with *Salmonella* can cause illness if the poultry meat consumed is undercooked or contamination from the raw poultry is transferred to cooked poultry or other food that is ready-to-eat. Cross contamination between raw and ready-to-eat food is of particular concern, as only small numbers of the bacteria are needed to cause human illness.

Bacteriophages can be regarded as natural enemies of bacteria, and therefore are logical candidates for targeted control of food borne bacterial pathogens like *Salmonella*.

Important attributes of bacteriophages include:

- they kill only *bacterial* target cells (no impact on plant or animal cells);
- they do not cross species or genus boundaries; therefore they will not affect desired bacteria in foods (e.g., starter cultures for cheese and sausages), and commensals in the gastrointestinal tract, or accompanying bacterial flora in the environment;
- they are composed entirely of proteins and DNA, so their breakdown products consist exclusively of amino acids and nucleotides, 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.

With respect to their potential application for the biocontrol of undesired pathogens in foods, feeds, and related environments, it should be considered that phages are the most abundant micro-organisms 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 dairy and meat products, more than 10^8 viable phages per gram are often present (Kennedy and Bitton, 1984). 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 (Breitbart, 2003).

In conclusion, bacteriophages are known to be harmless for all other organisms and are species-specific" (often even specific for only a limited number of strains within this species).

In order to counteract *Salmonella* contamination problems, Micreos has developed a phage product which is highly specific for *Salmonella*; a bacteriophage preparation with the trade name SALMONELEX™.

This dossier contains all available data with regard to this product.

The bacteriophage preparation with its intended use for control of *Salmonella* on fresh poultry and meat product is:

- a liquid culture of two specific bacteriophages
- is effective against *Salmonella* as shown in by efficacy data presented in Appendix 1
- demonstrates a broad spectrum killing of *Salmonella* as presented in Appendix 2
- rapidly disintegrates into amino acids and nucleotides which are naturally present in abundance in food products,
- results in negligible amounts of residuals (amino acids and nucleotides)
- has no technical effect in the finished food

Based on these features we consider SALMONELEX™ to be a processing aid.

Regulation concerning processing aids is laid down in 1.3.3 "Processing Aids" of the Food Standards Code. SALMONELEX™ falls within the scope of a processing aid, as defined by the definitions in Standard 1.3.3 but is not listed in the clauses. To get an approval for a new processing aid and a change in Standard 1.3.3, an application has to be made to the FSANZ.

For that reason this dossier has been prepared along the lines as laid down in the Food Standards Australia New Zealand document of 1 September 2013: "Food Standards Australia New Zealand Application Handbook".

3.1.2 Applicant Details

3.1.2.1 ***Name and address of the applicant and responsible person***

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Responsible persons:

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[redacted], Chief Scientific Officer [redacted]

3.1.2.2 ***Manufacturer***

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Nature of manufacturer's business:

Micreos develops phage-based products against dangerous bacteria and is viewed as global technology leader spearheading this exciting new field. The company focuses on antibacterial solutions for human health and food safety. Micreos has phage-based products on the market and in the pipeline.

3.1.3 Purpose of Application

3.1.3.1 ***Why SALMONELEX™ is considered a processing aid***

To establish whether Salmonelex™ is a processing aid or an additive the definition of both substances according to Australian Standards Code (1.3.1 and 1.3.3) is given below.

Food Additive:

'A food additive is any substance not normally consumed as a food in itself and not normally used as an ingredient of food, but which is intentionally added to a food to achieve one or more of the technological functions specified in schedule 5. It or its by-products may remain in the food. Food additives are distinguishable from processing aids (see standard 1.3.3) and vitamins and minerals for nutritional purposes (see standard 1.3.2).'

Processing aid:

'Processing aid means a substance listed in clauses 3 to 18, where-

a) the substance is used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food; and

b) the substance is used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified.'

Assessing Salmonelex™ based on these qualifications we believe it should be categorized as a processing aid (rather than a food additive) because:

Salmonelex™ does not perform a technological function in the end product. As can be seen in chapter 5.3, phage loses its ability to eradicate *Salmonella* within 6-24 hours after application.

The levels of Salmonelex™ that are used in the manufacturing of a food product are the lowest levels necessary to achieve the reduction of *Salmonella* that is wanted (1log, 2log or more). As an example, the GMP level of Salmonelex™ is already 3,000 times lower than the GMP level of octanoic acid, qualified by FSANZ as an antimicrobial processing aid (see chapter 5.3.1).

The intrinsic characteristics of interaction are similar and in essence generic to an earlier by FSANZ as a processing-aid approved bacteriophage product LISTEX™ that targets *Listeria monocytogenes*.

3.1.3.2 ***Phages***

Bacteriophages are bacterial viruses that infect only bacteria, and have no effect on humans, animals or plants. Vaneechoutte *et al*. (2009) made a review for the Dutch ministry of Agriculture concluding that phages can be generally regarded as safe for the use on humans and foods for human consumption, and are efficient antibacterial agents.

At any time the number of phages on the planet exceeds the number of bacteria. When a virulent phage infects a bacterium the lytic cycle is started as a method for phage replication. Many progeny phage (10-200) are liberated after lysis of the host because in order for phages not to become extinct at least one of these progeny phage needs to find a new bacterial host, often in a three-dimensional matrix, before becoming inactivated by factors such as UV-light, denaturing and proteolytic compounds or simple adsorption to particles rendering them inactive.

Bacteriophages are especially abundant in environments with a large number of bacteria. Marine and freshwater ecosystems are teeming with bacteria and as a consequence phage numbers typically reach 10^7 per ml and sometimes exceed this number 300-fold (Fuhrman 1999; Otawa *et al*., 2007; Filipini *et al*., 2006).

Furthermore many bacteria have temperate phages incorporated in their genome and a small proportion of such populations is lysed and thus sets free bacteriophages.

3.1.3.2.1 Natural presence of phages in food

Very few foodstuffs are completely sterile. This means that most food consumed will contain bacteria and therefore phages are likely to be present. This holds true especially for fermented products as well as unprocessed vegetables. As an example, phages can readily be isolated from Sauerkraut (Yoon *et al*., 2002; Barrangou *et al*., 2002). In one study (Lu *et al*., 2003) 26 different phages were isolated from the product of 4 commercial Sauerkraut fermentation plants.

While in most commercial cheese production settings a lot of effort has been put into ensuring that starter cultures are free of 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 x10^5 pfu/g (Gautier *et al*., 1995). Phages infecting thermophilic lactic acid bacteria have been isolated from Argentinean 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).

Also *Campylobacter* phages have been isolated at levels of 4 x10^6 pfu from chicken (Atterbury *et al.*, 2003) and *Brocothrix thermosphacta* phages from beef (Greer 1983).

In all these cases the researchers were looking for phages infecting one particular organism, but when one considers the myriad of bacteria associated with soil and vegetables it becomes clear that in addition one would be likely to find phages associated with this multitude of other species if one were to look.

A recent article on the presence of *E. coli* and *Campylobacter* phages in New Zealand vegetables and chicken found coliphages in more than 90% of the samples at numbers of 250 per gram (Tsuei *et al.*, 2007). The investigators point out that the indicator organisms employed ensured that both male specific and other phages would be identified. Still, the incidence and numbers are likely to be an underestimate of the total coliphage population. This is because many phages are serovar-specific, recognizing features associated only with specific strains or they recognize surface proteins such as the maltose receptor, whose presence or shape may vary between strains and growth conditions.

3.1.3.3 ***Purpose of the Application***

The purpose of Salmonelex™ is to reduce the levels of *Salmonella* post-harvest on beef, pork and poultry. The intended use of Salmonelex™ is on carcasses, fresh pork cuts, fresh beef cuts and on fresh poultry.

Meat processing is susceptible for Salmonella contamination. Poultry more so than meat. Poultry processing is a highly automated industry in which many points exist for cross-contamination when *Salmonella*-positive birds enter the processing plant. To address the multiple points where birds may be contaminated, several antimicrobial controls are applied at various steps during processing. This multi-hurdle approach generally results in multiple antimicrobial interventions being used. Generally, sites where antimicrobials are applied include online reprocessing (OLR) or inside/outside bird washes (IOBW), the poultry chiller and post-chill applications where carcasses are disassembled.

Applications

Sensitive to contamination in the process are the picker fingers, evisceration and the chillers. Interventions can take place in these areas but also when the bird is cut up and held before further processing like grinding. This is currently the area of industry scrutiny due to recent recalls/outbreaks and risks of undercooking by the consumer. The growth in popularity of ground turkey and chicken as lower fat alternatives to beef requires extra attention and

safeguards. Since *Salmonella* is commonly found in poultry there are at least four interesting areas of application:

Picker fingers/ Plucking
Evisceration
Chillers (pre-chiller)
Carcass or parts treatment (post-chill and pre-packaging)

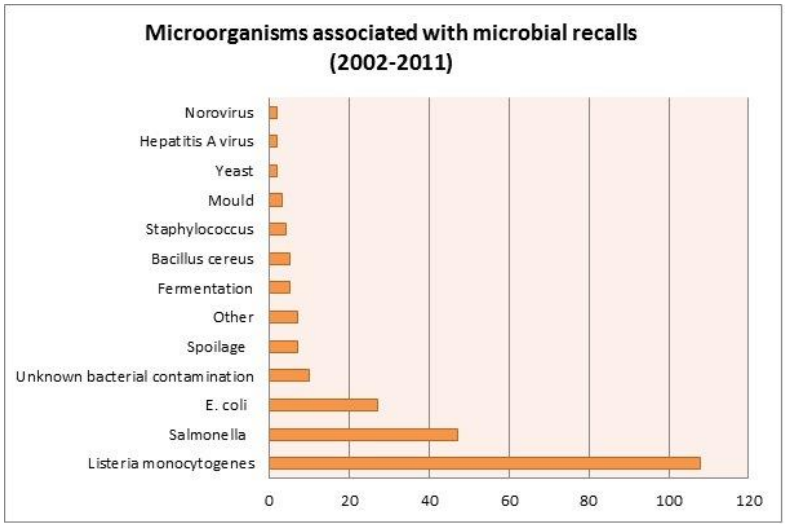
Data have been provided (Appendix II.) on the efficacy of Salmonelex™ on artificially contaminated drumsticks simulating pre-chiller conditions as well as on the efficacy of Salmonelex™ on all types of poultry carcass surfaces (skin, muscle and cut meat) and on cuts of pork and beef.

The data clearly show that the bactericidal effect of Salmonelex™ occurs shortly after application with the largest effect taking place within the first two hours. After this the effect quickly diminishes over a period of 24 hours with no remaining effect beyond this point in time. The data also show that treatment with short contacts time (i.e. 15 min) can already be effective in significantly reducing *Salmonella* numbers. This also confirms that Salmonelex™ can be defined as a *processing aid* as it has a technical effect in the processing but is present in the finished food at insignificant levels and does not have any technical or functional effect in that food.

Intended use will be up to a level of 10^8 pfu/g of treated product.

3.1.3.4 ***Technological Need***

Numerous cases of food infection resulting from the presence of *Salmonella* have been reported. Also *Salmonella* is associated with a great number of recalls. See the table for Australia below.



Despite increased insights into microbial hygiene during manufacturing, incidence of salmonellosis does not seem to decrease. Although the reason for this is not known exactly, it may well be changing eating habits: i.e. a shift towards convenience foods. In addition of course we are seeing an aging population in many developed countries. Since the Australian production of poultry has increased on a year by year basis (+9,3% between 2009-10) the cases are likely to grow, ceteris paribus, congruent to this trend.

Salmonella bacteria can survive for some time without a host; thus, they are frequently found in polluted water, contamination from the excrement of carrier animals being particularly important. The economic impact of production, detection and destruction of *Salmonella*-contaminated food products costs the industry billions per year. In addition, the health care expenses (counting only hospitalizations) are very high and are estimated by the Australian Institute of Health and Welfare (AIHW) to be between $14 - 74 million annually.

For these reasons an effective control of *Salmonella* at all stages of the food production chain is necessary. The application of Salmonelex™ can be seen as an additional tool for control of *Salmonella* in food. It can supplement GMP, HACCP and other measures aimed at the prevention of *Salmonella,* though it should NOT be seen as a replacement of hygiene, but as an integral part of it. On the basis of results obtained in experimental work it can be concluded that Salmonelex™ is effective in controlling or eradicating *Salmonella* on various food products and in different stages of production in a dose-dependent manner.

3.1.3.5 ***3.4 Safety***

Most phages are very specific for only one bacterial species, and therefore cannot affect or influence the natural bacterial flora of a food or raw material used to produce food or feed.

Strictly lytic (i.e., virulent) phages lack the genetic factors required for integration, will always enter the lytic cycle, and eventually kill and lyse the infected cells. In contrast to virulent phages, many other tailed phages may not be suitable for use as natural antimicrobials, since they are temperate and can integrate their genome into the host bacterial genomes, to form a lysogenic cell. This state is sometimes accompanied by undesired phenotypical changes, i.e., the integrated phage (prophage) can potentially carry and express genes encoding properties which increase pathogenicity and/or virulence of the host bacteria. In several cases, temperate phages have been identified as the carriers of toxins or regulators needed for development of full virulence of the host (reviewed by Boyd, 2005).

Salmonelex™ phages are virulent (non-temperate) and the genetic structure of the genome excludes any possible presence of a lysogeny module. It is preferable to select phages which

are not capable of transduction, i.e., packing of host genetic material instead of phage-encoded DNA.

3.1.3.6 ***Phage identity***

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 bio control agent for *Salmonella* in food.

Name: FO1a

Order: Caudavirales

Family: Myoviridae

Genus: FelixO1-like phages

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; 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.

The full genomic sequences of both phages are in the public domain. They are available under Genbank accession numbers HQ331142 and JF461087 respectively.

Similarities between phage genomes and undesirable genes

All plausible open reading frames of phage S16 encoding proteins of 29 amino acids or more were analyzed for possible functions by using blastx (translated DNA sequence, standard genetic code) against the non-redundant protein sequence database of all organisms at the NCBI (expect: 10.0, word size: 3, matrix: BLOSUM62, gap cost: existence 11, extension 1). This analysis did not reveal any similarities of S16 genes or gene products to any genes or proteins or other factors known or believed to play a direct or indirect role in the pathogenicity or virulence of *Salmonella*, or of any other infectious, toxin-producing or otherwise harmful microorganism.

All plausible open reading frames of phage FO1a encoding proteins of 29 amino acids or more were analyzed for possible functions by using blastx (translated DNA sequence, standard genetic code) against the non-redundant protein sequence database of all organisms at the NCBI (expect: 10.0, word size: 3, matrix: BLOSUM62, gap cost: existence 11, extension 1). This analysis did not reveal any similarities of FO1a genes or gene products to any genes or proteins or other factors known or believed to play a direct or indirect role in the pathogenicity or virulence of *Salmonella*, or of any other infectious, toxin-producing or otherwise harmful microorganism.

Phage FO1a is highly similar to the well-known phage FelixO1 (Whichard et al. 2010). The genome is ~2500 bp shorter, owing to the deletion of 5 ORFs present in FelixO1. Otherwise the sequences are almost 100% identical. Whichard et al. also did not find undesirable genes in their analysis of FelixO1.

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 bio control, 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.

The host range *sensu strictu* (plaque formation) of phage S16 has been reported (Marti et al. 2013). The *Salmonella* collection of Micreos includes but is not limited to the strains used in this study. The same experiments were performed using phage FO1a (data not published). The host ranges of the two phages largely overlap but a number of strains allow only plaque formation of one of the two phages. Lysis was observed in all strains with both phages when spotting 10^8 pfu in 10µl on soft agar overlays. To investigate the 6 strains where no plaques were observed with either phage but killing was observed at high phage density about 300 cfu of the strain were plated on LB plates followed by plating 2 x10^7 phages/cm^2. In fully susceptible strains (plaque formation) this treatment leads to ~95% reduction. The same level of reduction was observed when performing this experiment with the 6 strains in question. It was concluded that cells infected by the phages were killed in a 1:1 ratio and that while infection did not lead to progeny phage the desired effect of phage application was achieved. Complete lists of the Salmonella strains used as well as the other bacteria investigated are attached and can be found in Appendix 2.

3.1.3.7 ***Bacterial resistance***

One might wonder whether employing phages will lead to the development of resistant bacterial strains. The answer to this is simply: “No this will not happen”. In 1969 Salvador E. Luria and Max Delbrück received the Nobel Prize for medicine for their work on phage replication and interaction between phages and bacteria. In part this prize was based on the famous Luria-Delbrück experiment which unequivocally showed that resistance to bacteriophages develops independently of the presence of phages, underlining an evolutionary principle: mutations in nature occur randomly at a certain frequency (Luria and Delbrück, 1943). Therefore a mutation, for example in a molecule which a particular phage recognizes will occur once every so often. In those cases where this mutation is not fatal to the bacterium the phage may no longer be able to infect or it might infect at a lower efficiency. A realistic frequency of mutation of such a receptor molecule would be in the range of 10^{-7} (Carlton, 1999). Taking this number, in an eradication scenario one would need 10 million bacteria in order for one non-sensitive to escape treatment. For a product which aims at a 2-4 $\log_{10}$ reduction of the target bacterial numbers, this occurrence is irrelevant.

Inducing resistance and selecting for it are two very different things. On a food item with an accidental and low-number contamination, the occurrence of a non-sensitive mutant would be extremely unlikely and in any case an isolated event.

Bacteria have developed strategies to counter phage infection, including restriction modification (R/M) systems, abortive infection mechanisms and super-infection exclusion mechanisms. In a treatment situation or pertaining to these mechanisms are not relevant. Abortive infection mechanisms are not relevant in a treatment situation because they depend on host-cell suicide after infection. Ecologically this is a disaster for bacteriophages but in a treatment situation it is inconsequential because the target cell dies. Super-infection exclusion works *only for temperate phages* (see chapter 3.4, Safety-Introduction) in keeping related phages outside the host. As a strictly virulent (non-temperate) phage, is not affected. R/M systems are never 100% effective, since phage DNA may be modified by host enzymes before other host enzymes degrade the DNA rendering the phage and progeny completely immune to this system. One of the two phages in Salmonelex™ has highly modified DNA rendering it resistant to many restriction enzymes (Marti et al. 2013)**.**

With hundreds of isolates tested, no insensitive strains have been found to date. While strains with a reduced sensitivity are likely to exist their occurrence on a particular food item again will be an extremely rare and isolated event.

3.1.3.8 ***Safety assessment***

There are more individual bacteriophages in the biosphere than there are of any other group of organisms, including all the prokaryotes. The shape of the best studied group of phages, the tailed phages, is so distinctive that their numbers in aquatic environments were estimated simply by centrifuging them onto an electron microscope sample grid and counting them. In coastal seawater, there are typically as many as 10^7 tailed phages per millilitre. In some fresh water sources, there are up to 10^9 phages per millilitre.

Numerous papers attest to the fact that humans are exposed to huge numbers of phages daily, through food and water, without evidence of any harm. Gorski and Weber-Dabrowska (2005) have also presented evidence that phages are helpful to humans by exerting immunosuppressive activity in the gut to control local inflammatory and autoimmune reactions and act in concert with the immune system in immunosurveillance against bacteria and viruses. These reviewers cited thousands of cases where phages have been used in treatment of patients suffering from diseases caused by antibiotic resistant bacteria, either by injection or oral administration, resulting in an 80% success rate. No negative side effects of phage administration were observed. While these studies and therefore conclusions about the beneficial treatment effects did not follow western standards (there were no double blind trials) this massive body of evidence shows conclusively that phage administration, either orally or by injection, has no ill effects.

Virulent bacteriophages have been used as prevention or treatment for many bacterial diseases including sepsis for years. Although much of the literature comes from studies in Eastern Europe and the Soviet Union, Western nations are becoming more aware of the possibilities of phage treatment of bacteria that have become resistant to antibiotics (Sulakvelidze, 2005). No allergic reactions in humans have been reported despite evidence that phage enter circulation (Matsuzaki *et al.*, 2005).

Human volunteers have been fed *E. coli* phage T4 phage with no harmful effects noted in a controlled study; and no phage or phage-specific antibodies could be detected in the serum of the human subjects (Bruttin and Brussow, 2005). The authors propose that use of such phages may be a useful therapy for acute diarrhoea caused by *E. coli* worldwide (Brussow, 2005).

Bacteriophages have been purposefully placed in the food chain, particularly used as treatment or prevention of gastrointestinal diseases of poultry (Carillo *et al.,* 2005; Berchieri *et al.*, 1991). These phages obviously will be present on the food following slaughter.

Other studies on the application of phages to animals also reported no adverse or unexpected effects of bacterial phages in animals (Biswas *et al.*, 2002; Cerveny *et al.*, 2002; Chibani-Chenouffi, 2004b; Merril *et al.*, 1996).

Further evidence that treating food products with phage is not likely to cause harm to humans who consume such foods is the abundance of bacteriophages of many genera and species in the human intestine. Given that the intestines are colonized by vast numbers of bacteria and that bacteria are often infected with phages; it is therefore estimated that humans have billions of phages in their intestines at any one time.

Thus, if a small number of phage continues to be present on the surface of a food product after application at the time of product making, and is ingested by a consumer, it is impossible that these phages pose any hazard because:

Large numbers would not be hazardous
Salmonella phages both like and unlike to Salmonelex™ phages are ingested regularly
A large proportion of any phage will be destroyed in the stomach
Ingestion of the number of phages is relatively small compared to the billions of phage particles of other species already present
Salmonelex-treated food will be cooked destroying almost if not all phages. The numbers that escape would be negligible compared to phage numbers ingested daily.
Phage does not contain genetic elements harmful to humans nor does it have any other undesirable properties such as the ability to lysogenize and or transduce host DNA
The *Salmonella* phages in Salmonelex™ are not able to infect and kill bacteria from belonging to other genera of bacteria, and therefore will not disturb the intestinal flora.

3.1.3.9 ***Allergenicity***

Phage components

A full-length alignment of the entire proteomes of phages S16 and FO1a was performed at the website (http://www.allergenonline.org/) of the University of Nebraska. The database is updated regularly and contains known allergens and proteins with more than 67% identity with known allergens. No significant matches to any food-allergens were found.

A precautionary search using a sliding window of 80 amino acid segments of each protein to find identities greater than 35% (according to CODEX Alimentarius guidelines, 2003) was performed with the phages structural proteins (i.e. the proteins that make up the mature phage particle). No matches were found with food allergens in the database.
Together with the data on the components of the process we believe that this shows that the preparation has no allergenic potential.

Relevant Medium Components

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 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 allergenicity 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.

3.1.3.10 ***Efficacy***

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 Appendix 1 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 Appendix 1, 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 this 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 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.

3.1.4 Justification for the Application

3.1.4.1 *Cost/Benefits*

Salmonella and Campylobacter are two of the most commonly reported causes of food-borne illness in

Australia, according to FSANZ. As mentioned above, apart from health damage (USDA estimates costs of *Salmonella* infections in the US at $2.65 billion per year*)* and the suffering for those involved, high economic damages are likely to originate from a salmonellosis outbreak. Economic damages such as production, detection and destruction of contaminated products, as well as product recalls, reputational damage and anxiety for those concerned, including those responsible for quality control, all add to the costs for society.

Much higher cost estimates were offered in March 2010 by the Produce Safety Project, a group at US Georgetown University that works for mandatory safety standards for produce.

The group estimated the annual cost of salmonellosis cases in the US at $14.6 billion. The group came up with an overall estimate of almost $152 billion a year for all foodborne diseases. The estimates included medical costs, lost life expectancy, pain and suffering, and functional disability but not costs to government or the food industry.

The actual benefits might be even higher because a salmonellosis outbreak causes reputational damage not only for the company involved, but often for an entire country or industry concerned. As such, measures against *Salmonella* should be considered ‘pre-competitive’, and a comprehensive part of good corporate citizenship.

3.1.4.2 *Impact on international trade*

There will no impact on international trade. The target of the Salmonelex™ product for which we ask admission is *Salmonella*. The users will be Australian and New Zealand food producers help reduce the occurrence of *Salmonella* on domestically produced food.

3.1.5 Information to Support the Application

3.1.5.1 ***Type of processing aid***

Bacteriophage preparation Salmonelex™ does not fall within the categories mentioned in Standard 1.3.3 – Processing Aids, but in our opinion can be considered an antimicrobial against *Salmonella* which fits in the category L, *miscellaneous functions*.

3.1.5.2 ***Identity***

The final product is an opaque liquid containing: 2 x10^{11} plaque forming units per ml product in buffered saline.
The product consists of a purified strictly virulent (lacking lysogenic activity) bacteriophage (phage) specific against *Salmonella.*
The phages are deposited at, and assigned an identifying code by, a scientifically recognized culture collection centre.
The agent is produced from cell cultures of *Salmonella bongori* in a safe and suitable nutrient medium.

3.1.5.3 ***Physical and chemical properties***

Phages comprise a number of proteins enveloping nucleic acids. pI's (isoelectric point) of phage structural proteins are generally low, and all structural proteins exhibit pI's of around 4. Addition of phages during treatment occurs at such low levels that food properties are not changed at all. Salmonelex™ has an effect only when *Salmonella* is actually present: it has no effect on the foodstuff itself.

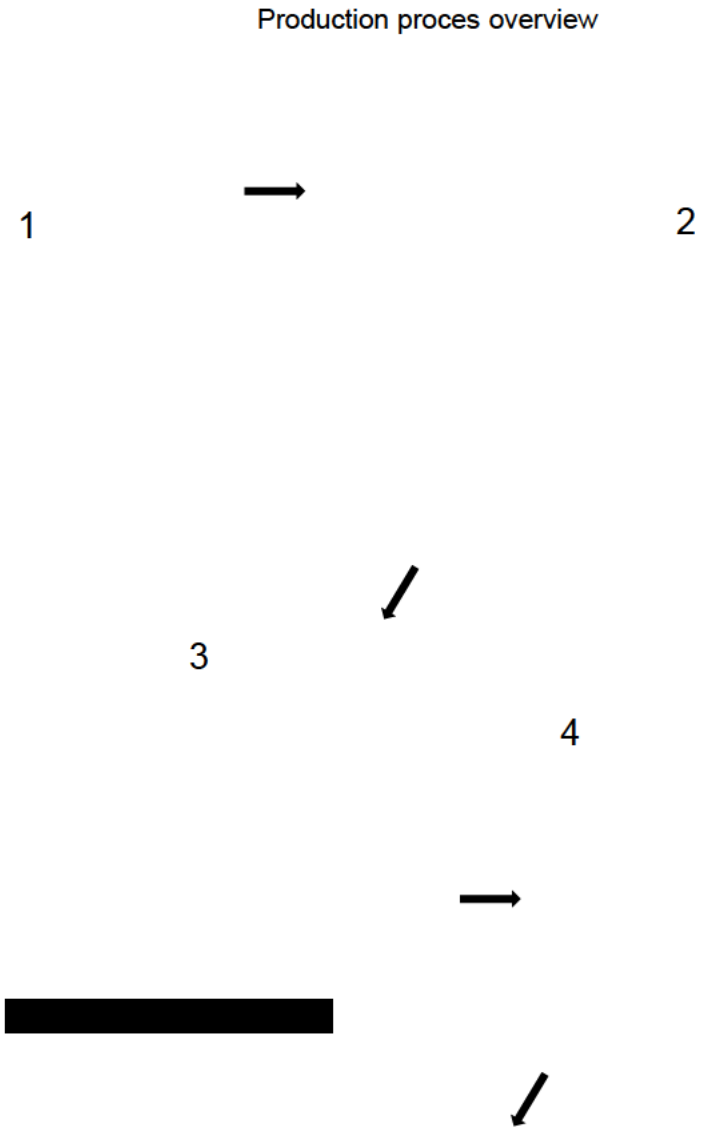
The amount of proteins and nucleic acids present in the added dose of bacteriophages is negligible compared to the level of proteins and nucleic acids already existing in the foodstuff. In addition, bacteriophage residues are is essence identical to food compounds.

3.1.5.4 ***Manufacturing process***

Both phages are grown separately on the same *S. bongori* production strain in a fermenter 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.

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



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3.1.5.5 ***Specification for Identity and Purity***

3.1.5.5.1 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.

3.1.5.5.2 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.

3.1.5.5.3 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

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

analysis and average values are depicted in Table 1.

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).

3.1.5.5.4 Loss of function

Processing aids are not allowed to have a technical function in the final product. Salmonelex™ is inactivated within 24 hours after addition to the food. This inactivation is caused by various factors such as adsorption of phages to particles, proteolytic degradation of the phage particle by chemicals and enzymes, temperature, salts and light (Suttle and Chen 1992; Garza and Suttle 1998; Hurst *et al*., 1980). Eventually, phages will fall apart into amino acids and nucleotides.

3.1.5.5.5 Phage adsorption

Rapid phage inactivation is caused largely by adsorption of the pages to the food matrix. It is commonly known that proteins adsorb to surfaces (Ruggiero *et al.*, 2005) and since phages consist of a protein hull containing DNA also phages are likely to adsorb. There are several interactions between the phage and the food surface that contribute to the strong binding:

Hydrophobic interactions: the side chain of several amino acids is non-polar and hence interacts poorly with polar molecules like water. When non-polar residues are exposed at the surface of two different molecules, it is energetically more favourable for their non-polar surfaces to approach each other closely, displacing the water from between them.
Ionic interaction: proteins contain both positively and negatively charged amino acids. These interact with and bind to other, oppositely charged groups.
Hydrogen bonds: a strongly electronegative atom (e.g., oxygen, nitrogen) approaches a hydrogen atom which is covalently attached to a second strongly electronegative atom. These can be formed in the case of phages and foodstuffs between the –C=O group and the H-N- groups, and between –C=O groups and H-O- groups proteins and sugars.

Individually these bonds are much weaker than covalent bonds (typically about 20 times), but many of them together can have formidable strength. The first bond to occur brings the phage closer and holds it to the food surface, increasing the likelihood of additional bonds to form. This is the reason why adsorption only becomes stronger over time. Any one bond can be broken with relative ease, but for phages to desorb, all bonds must be broken simultaneously which is impossible in the commercial setting.

3.1.5.6 ***Analytical Method***

While phages will adsorb quickly to the surface of a treated foodstuff, rendering them ineffective *in situ*, they may be recovered from the food in a laboratory for some time after application before they degrade completely. 25 g of the foodstuff in question should be stomachered in 225 mL of SM buffer (100 mM NaCl, 10 mM MgSO4,50 mM Tris-HCl, pH 7.5). This fluid should be sterile filtrated and a 10 fold dilution series made. 4 ml of LB soft agar (0.4%) kept at 42°C should be mixed with 100 µl of an overnight culture of indicator bacteria (I.e *Salmonella enterica*) and 100 µl of the dilution series and poured on a standard LB plate. Overnight incubation at 30°C will result in plaque formation. The number of plaques x the dilution factor (including stomachering step) reflects the number of phages present.

3.1.5.7 ***Dietary Exposure to the Processing Aid***

3.1.5.7.1 Food or food groups to be treated

Many different food categories are susceptible to *Salmonella* contamination. *Salmonella* is mainly spread to humans when they eat under-cooked food made from contaminated animals (that is, meat, poultry, eggs, and their by-products).

Products like processed foods, produce and meat & poultry could be made safer by using a *Salmonella* intervention. We would include the availability of Salmonelex™ as an intervention tool for all susceptible categories.

An obvious target market is the chicken meat industry. Sensitive areas in this value chain is the picking, evisceration, chilling and dressing of the bird. In Australia approximately 75% of

chicken carcasses are chilled using water only, with the remainder being chilled using a combination of water and air and a very small percentage air only. There is ample opportunity for interventions against cross-contamination.

3.1.5.7.2 Application dosages

Application doses are discussed under 3.1.3 Efficacy

3.1.5.7.3 Average Daily Intake

*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. We will use these numbers and correct this for the numbers as available from the Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES). They indicate consumption of 24,6 kg pork and 35,6 kg chicken over 2010/11 which is 22% higher than in the US.

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 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:

2.84 $x10^{10}$ x 10^8 x 1.66 x 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). Even with a 22% upward correction to reflect the Australian consumption 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. 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.

3.1.5.7.4 Percentage of market likely to use the processing aid

Although there are no salmonella standards in Australia and New Zealand poultry processors will continue to be required to identify and control food safety hazards associated with poultry processing and verify the effectiveness of the control measures. Processors of foods which have a heightened risk of *Salmonella* presence or foods with a heightened public health risk are the designated part of the market to use Salmonelex™. It is predicted that innovative companies, which are looking for a more natural solution to control *Salmonella* , will be the first to use Salmonelex™. In the future it is expected that bacteriophage applications like

Salmonelex™ will become a standard in food production. It is difficult to predict percentages of the market likely to use Salmonelex™ but companies representing about 50% of the poultry market have already expressed their interest in Salmonelex™.

3.1.6 Assessment Procedure

In assessing the application of Salmonelex™, we believe the appropriate procedure to be adopted is the General Procedure, level 2, because the application handbook listed under General Procedure, level 2 includes ‘a new microorganism’.

3.1.8 Exclusive Capturable Commercial Benefit

The application of Salmonelex™ is expected to confer an ECCB to Micreos because:

Micreos has a financial gain from the coming into effect of the draft standard or draft variation: When Salmonelex™ is approved as a processing aid; sales in Australia and New Zealand can commence for this product meaning a financial gain for Micreos.
Any other unrelated person or body would require the agreement of Micreos in order to benefit financially from the approval of Salmonelex™: one of the bacteriophages is patented.

There will no impact on international trade. The target of the Salmonelex™ product for which we ask admission is *Salmonella*. The users will be Australian and New Zealand food producers help reduce the occurrence of *Salmonella* on domestically produced food.

3.1.9 International and Other National Standards

LISTEX™ in Australia & New Zealand

FSANZ has concluded that it was appropriate to permit LISTEX™ P100 as a processing aid to treat specific solid RTE foods and it therefore amended Standard 1.3.3. Permission was granted to use LISTEX™ P100, under conditions of GMP, in appropriate processed foods in the Table to clause 14 – Permitted processing aids with miscellaneous function, for which its use has been assessed to be both safe and efficacious. The specific food groups permitted are meat (including poultry) and meat products, fish and fish products, and fruits and vegetables and their products and cheese.

A new definition of "approved food for use of phage" has been included in Standard 1.3.3, incorporating the existing definition for RTE food in Chapter 3.2.2. Submitters questioned an earlier approach to relocate the definition for RTE food to Standard 1.1.1 – Preliminary Provisions – Application, Interpretation and General Prohibitions. They argued this would mean the definition would apply too broadly across the Code and that any unintended consequences would not have been fully evaluated. FSANZ agreed and for the purposes of this Application decided to limit the use of the definition to Standard 1.3.3 only. FSANZ initially proposed that solid RTE foods fully or partially covered in a liquid were to be excluded from the permission. After further consideration and discussions with jurisdictional submitters, whose role is to ensure compliance with the Code, it was agreed that simplified drafting was more appropriate. Because the permission for using LISTEX™vP100 for the proposed purpose is as a processing aid, then any use in foods where it had the potential to function as a food additive is not permitted. Therefore, simplified drafting was written stating only that specific types of solid foods can be treated with P100.

LISTEX™ Overseas approvals

The European Food Safety Authority (EFSA) issued a scientific opinion in 2009 on the general use of bacteriophages in food products and concluded that each phage/food application should be considered on a case-by-case basis taking into consideration the biology and safety aspects of each bacteriophage and the food matrix to which it is applied (EFSA 2009). EFSA subsequently released an opinion on the safety and efficacy of using LISTEX™ P100 to treat raw fish (EFSA, 2012). This opinion was requested by the European Commission to evaluate an application dossier, submitted by the same Applicant (Micreos B.V.), to treat raw fish with LISTEX™ P100 to reduce L. monocytogenes contamination. Only two efficacy studies were considered as part of the analysis. EFSA raised a number of issues relating to ensuring efficacy of treatment in raw fish, but concluded that P100 should not present any human safety concerns.

On 14 July 2009, the Dutch Ministry of Public Health permitted the use of LISTEX™ P100 as a processing aid for use on all foods in The Netherlands.

In 2006, LISTEX™ P100 was granted Generally Recognized as Safe (GRAS) status by the United States Food and Drug Administration (USFDA) for use as a processing aid in cheese and in 2007, its use was extended to all food products susceptible to L. monocytogenes. Ingredient labeling requirements were initially specified for LISTEX™ P100-treated meat and

poultry products by the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA). However, in 2011, the USDA permitted its use as a processing aid on the surface of RTE meat and poultry products to achieve a level of 107 to 109 plaque forming units (pfu) per gram, without the need for labeling. The letter of permission requires that the treatment is integrated into the HACCP (Hazard Analysis and Critical Control Points) programs of the industry.

On 3 September 2010, Health Canada issued a “letter of no objection” for the use of LISTEX™ P100 as a processing aid in several foods; “mainly deli meat and poultry products (e.g. wieners, sliced ham), cold-smoked fish, vegetable prepared dishes, soft cheeses and/or other dairy foods”. A recommendation was made to provide clear instructions on the conditions of application to potential users. A proposed level of use within the range of 107–109 pfu/g was also specified.

Other USDA approvals for bacteriophages products in the USA

Bacteriophage preparation (*Salmonella* targeted)	On the hides of live animals in the holding pens prior to slaughter	Applied as a spray mist or wash
Bacteriophage preparation (*E. coli* O157:H7 targeted)	On the hides of live animals (cattle) in the holding pens prior to slaughter and hide removal	Applied as a spray, mist, rinse or wash to the hides of live animals (cattle) within lairage, restraining areas, stunning areas, and other stations immediately prior to hide removal.
Bacteriophage preparation (*Salmonella* targeted)	On the feathers of live poultry prior to slaughter	Applied as a spray mist or wash
Bacteriophage preparation (*Salmonella* targeted)	Raw poultry prior to and after grinding and ready-to-eat (RTE) poultry products prior to slicing	Applied as a spray at 10^{6} to 10^{7} plaque forming units (pfu) per gram of food product

SALMONELEX™

The Dutch Medicine Evaluation Board (CBG) has issued three temporary use exemptions for field trials related to chicken and calves. This is without the need to destroy products.

In 2013, SALMONELEX™ was Generally Recognized as Safe (GRAS) status by FDA and approved as a processing aid by the United States Department of Agriculture (USDA) for use as a processing aid in poultry and pork Ingredient labeling requirements were initially specified for SALMONELX™ treated meat and poultry products by the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA). (pfu) per gram, without the need for labeling.

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3.1.10 Statutory Declaration

I, [redacted], Business Development Director of Micreos. B.V., a private company with limited liability (besloten vennootschap met beperkte aansprakelijkheid), organized under the laws of the Netherlands, having its registered office(statutaire zetel) in The Hague, the Netherlands, and with business address: Nieuwe Kanaal 7P, 6709 PA Wageningen, The Netherlands, registered with Dutch trade register under number: 27279042,

Make the following declaration under the Statutory Declarations act 1959:

the information provided in this application fully sets out the matters required,
the information provided in this application is true to the best of my knowledge and belief; and
no information has been withheld that might prejudice this application, to the best of my knowledge and belief.

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

[redacted]

Declared at _____ on _____of _____

MICREOS

The undersigned, [redacted] civil law notary officiating in Wageningen, the Netherlands, herewith declares that the signature on the attached document is the signature of:

[redacted]

This statement explicitly contains no judgement as (i) to the contents of the attached document and (ii) the authority and/or the competence of the signatory of the attached document. The undersigned has not informed the signatory of the document and the consequences which will result from the contents of the attaches document. Any and all liability of the undersigned and [redacted] shall be excluded.

Wageningen, the Netherlands, April 5th, 2013

[redacted]

Civil law notary

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3.1.11 Checklists

3.1.12 Appendix I: [redacted]

Application for Salmonelex™ as a Processing Aid against Salmonella on Fresh Meat and Poultry Products

1. 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.

3. 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 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.

4. Safe and food grade ingredients as of starting material

The growth medium for producing Salmonelex™ contains only suitable ingredients/processing aids. The main components of the medium are Soy peptone, Yeast extract and Sodium chloride.

The antifoaming agent used is organic sunflower oil (organic) 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.

3.1.13 Appendix II: 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.

In addition a challenge study showed that phage activity is highest shortly after application and diminishes over time Treatment for 8 hours is more effective than treatment for 2 hours but only insignificantly so. This study shows that where time is not limited the best effects will be observed, but also shows that effective treatment is possible within short time frames. The experiments were essentially performed as previously carried out. Chicken skin and chicken breast filet was artificially contaminated with approximately $1x10^4$ cfu/cm^2. Treatment with Salmonelex was performed at a level of $2x10^7$ pfu/cm^2. Bacterial cells were retrieved and enumerated after 15 min, 2hours, 4 hours and 8 hours. The experiments were performed in duplicate and duplicate samples were analyzed.

Salmonelex™ efficacy in a pre-chill processing step was mimicked on laboratory scale with chicken drumsticks. Drumsticks were artificially contaminated with a streptomycin resistant mutant of *Salmonella* strain *Se13* (exponentially growing cells and overnight culture) allowing

the cells to attach for 2 hours at room temperature followed by dipping the samples in a water bath (room temperature) containing $1x10^8$ pfu/mL and $1x10^9$ pfu/mL phages for 15 minutes. In addition phage efficacy on *Salmonella* in the pre-chill bath itself was tested after 15 minutes and 1 hour.

2 Materials and methods

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

2.1.1 Materials

Samples

Chicken breast fillet*

Chicken skin*

Pork meat*

Beef*

* Purchased at a local supermarket

Bacteria/bacteriophage

- *Salmonella* serotype Enteritidis *Se13* Streptomycin resistant mutant (500µg/mL

→ titer overnight (ON) culture on selective agar plates + streptomycin: ~$1.13x10^9$ cfu/mL

- Bacteriophage formulation Salmonelex™ (titer: ~$2x10^{11}$ pfu/mL)

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)

2.1.2 Methods

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

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 $1x10^4$ cfu/cm² or $1x10^3$ cfu/cm², for testing with 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 (sample treatment schemes see Table 1). In the fume hood 5µl/cm² were transferred onto the samples. The liquid was distributed with the pipette tip.

The petri dishes were closed and incubated at 4°C for 24 hours, 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.

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

A

Sample	No. samples
t=0	
chicken breast	2
chicken skin	2
pork	2
beef	2
t=24h	
chicken breast	6*
chicken skin	6*
pork	6*
beef	6*
t=48h	
chicken breast	6*
chicken skin	6*
pork	6*
beef	6*
t=6d	
chicken breast	6*
chicken skin	6*
pork	6*
beef	6*
TOTAL No. SAMPLES	80

* 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	No. samples
t=0 pork	2
t=8h pork	4*
t=24h pork	4*
t=48h pork	4*
t=6d pork	4*
TOTAL No. SAMPLES	18

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

Appropriate amounts of the homogenate were plated in duplicate on selective agar plates (+ 200µg streptomycin/mL).

Plates were incubated for 24 to 48 hours at 37°C.

2.2 Simulating Salmonelex™ efficacy in a pre-chill application

2.2.1 Materials

Samples

Chicken drumsticks (skin-on) purchased at a local supermarket

Bacteria and phages

- *Salmonella* Se13 STREP3 MUTANT overnight culture (resistant to 500µg streptomycin/mL)

- *Salmonella* Se13 STREP3 MUTANT exponentially growing cells (resistant to 500µg streptomycin/mL)
- Bacteriophage Salmonelex™

LB broth + 500µg streptomycin/mL
streptomycin stock solution (100mg/mL)
70% EtOH
PBS buffer
Laminar flow hood
stomacher bags
0.1 % peptone water + 5g NaCl/L (retrieval buffer)
sterile tweezers
Salmonella selective XLD + 200µg streptomycin/mL agar plates
37°C incubator

2.2.2 Methods

Bacterial overnight cultures

One colony of the *Salmonella* strain was inoculated in 4ml broth and incubated overnight at 30ºC shaking.

Preparation of exponentially growing cells

A *Salmonella* overnight culture (see above) was diluted 1:10 in fresh broth and subsequently grown to an OD_{600} of ~0.5 for 1 ½ to 2 hours at 30°C shaking. To avoid further growth cells were immediately diluted in PBS buffer cooled in ice water.

Preparation of samples

Chicken drumsticks were sterilized with 70% EtOH and placed on sterile aluminum foil in the laminar flow hood.

Artificial contamination

An appropriate dilution of the *Salmonella* overnight culture or the exponentially grown cells was prepared to allow the contamination of the samples with a final concentration of approximately $1x10^4$ cfu/cm². In the laminar flow hood 2µl/cm² (50cm² contamination surface) of the dilution was transferred to each sample and rubbed in evenly with the pipette tip.

To allow *Salmonella* cells to attach to the samples, drumsticks were stored at room temperature (RT) for 2 hours.

Phage treatment

Phages were added to 200mL tap-water to a final concentration of $1x10^8$ pfu/mL and $1x10^9$ pfu/mL. A 200mL water bath without the addition of phages was prepared to serve as control. The drumsticks were then incubated in the bath for 15 minutes at room temperature while gently shaking once in a while.

Retrieval of *Salmonella* cells

After 15 minutes the drumsticks were transferred to new stomacher bags using sterile tweezers. To each bag 100mL of retrieval buffer was added. To allow a high retrieval rate, the shrink bags containing the samples were manually massaged for three minutes. An appropriate amount of the homogenate was plated on selective agar plates in duplicate.

To test the phage efficacy in the pre-chill liquid, an appropriate amount of artificially contaminated liquid was tested after 15 minutes of incubation with and without $1x10^8$ pfu/mL and $1x10^9$ pfu/mL phages. The liquid was additionally tested after 1 hour.
Plates were incubated overnight 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 8 show the percentage reduction as well as the effect on the growth over time of *Salmonella* on pork meat, beef, 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. Cell numbers 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)

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).

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 (Figure 7 and 8).

For detailed results see raw data annex for this appendix.

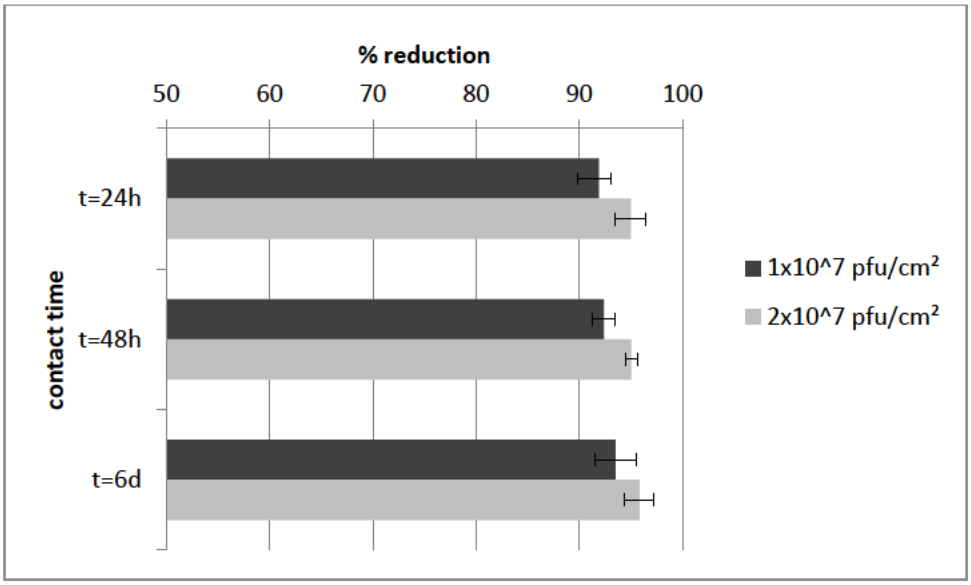

Figure 1: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated PORK samples.
Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

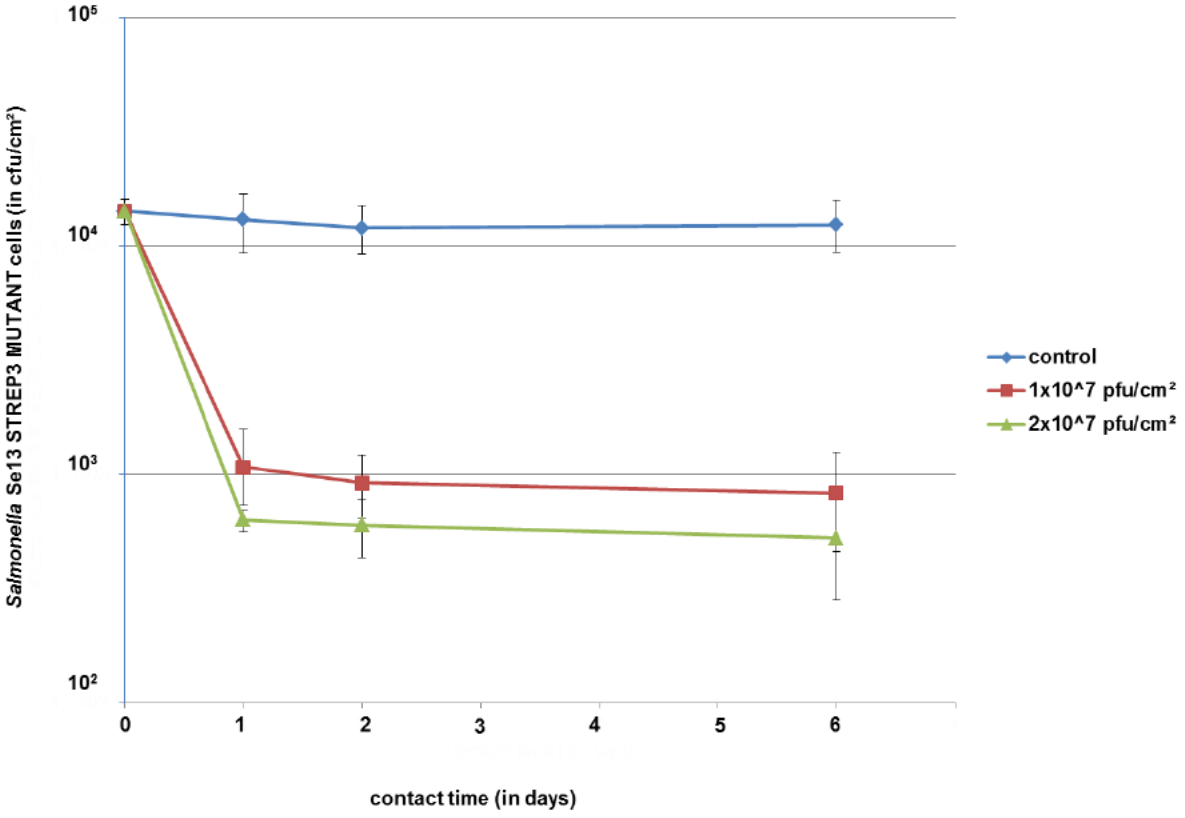

Figure 2: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on PORK meat at 4°C over 6 days

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

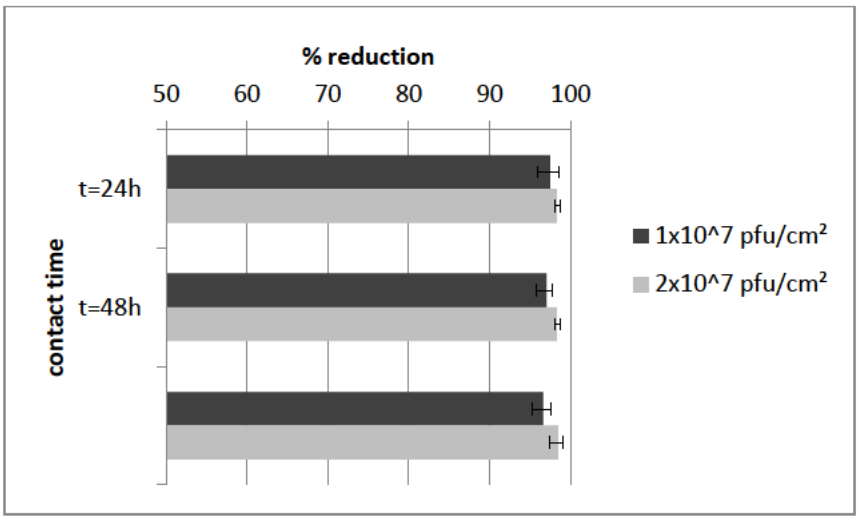

Figure 3: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated CHICKEN SKIN samples. Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

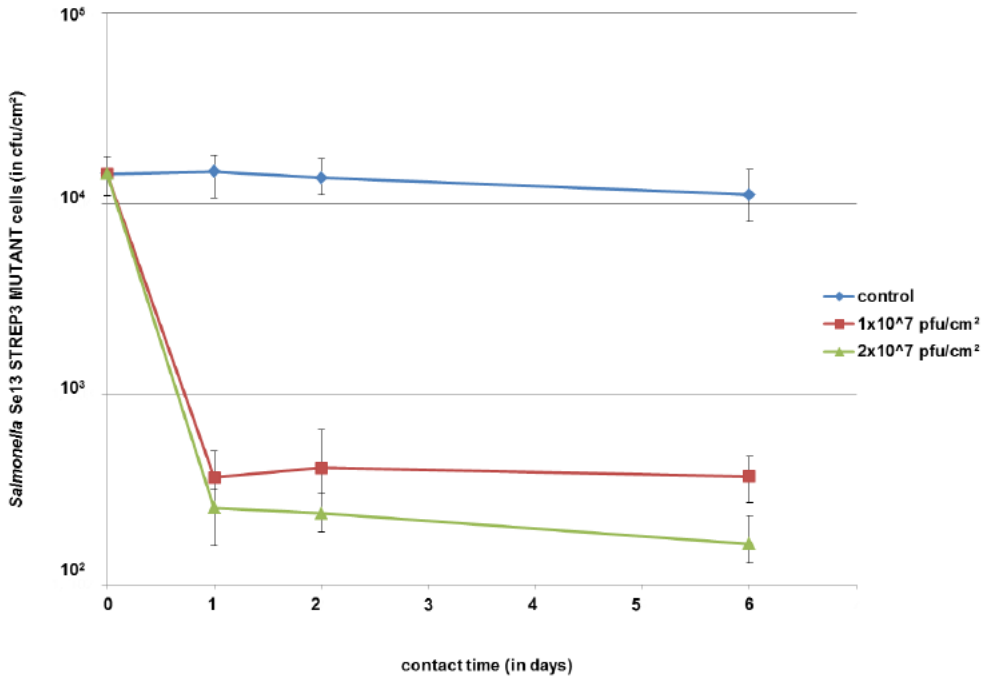

Figure 4: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on CHICKEN SKIN at 4°C over 6 days. Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

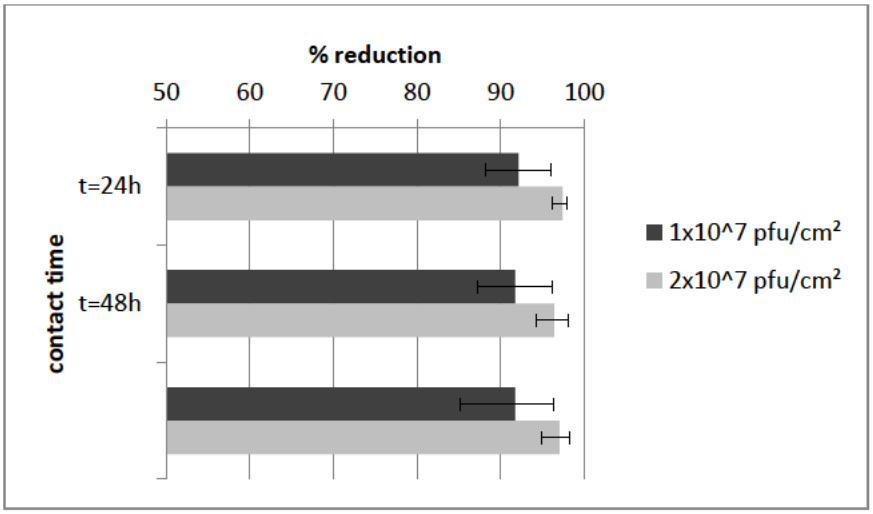

Figure 5: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex™ treated CHICKEN BREAST FILLET samples Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

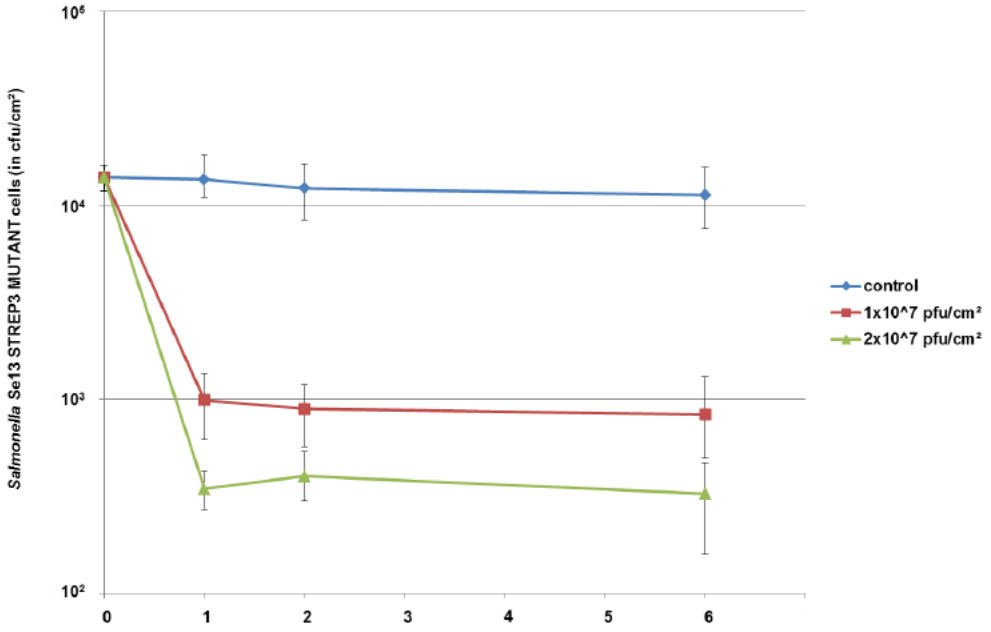

Figure 6: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on CHICKEN BREAST FILLET at 4°C over 6 days. Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

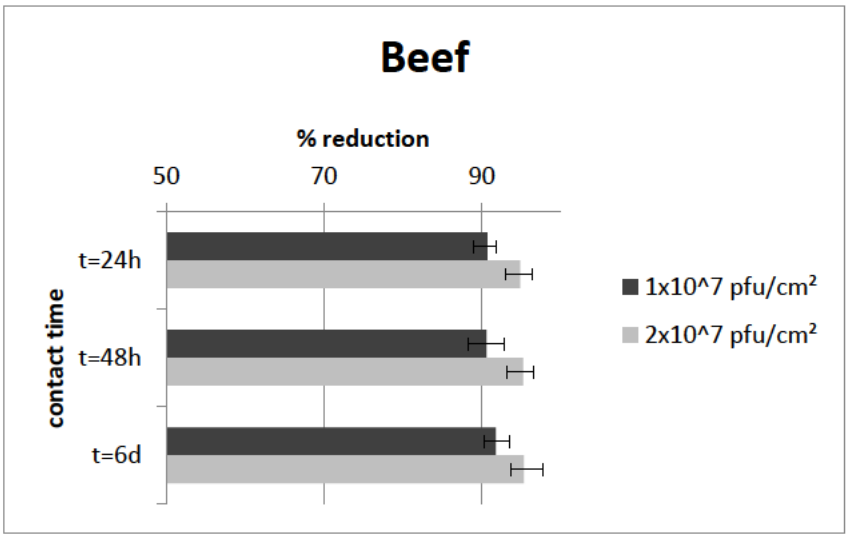

Figure 7: Percentage reduction of *Salmonella* Se13 STREP3 MUTANT cells on Salmonelex treated BEEF samples. Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

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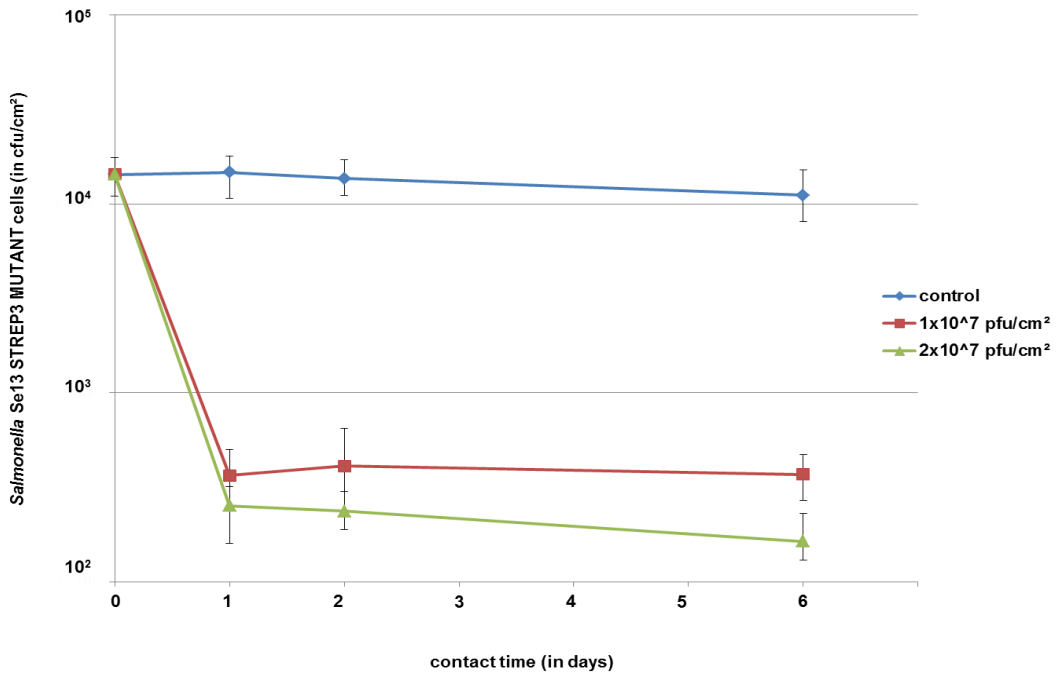

Figure 8: Effect of Salmonelex on growth of *Salmonella* strain Se13 STREP3 MUTANT on BEEF at 4°C over 6 days Contact times of 24 hours, 48 hours and 6 days; phage concentrations $1x10^7$ pfu/cm² or $2x10^7$ pfu/cm²; contamination with ~$1x10^4$ cfu/cm²

3.1.2 Incubation at room temperature

After an initial cell reduction of 91% (corresponding to 1.03 log) on treated pork samples after an 8 hour incubation at 4°C, *Salmonella* cells started growing out again when incubated at room temperature. 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 9).

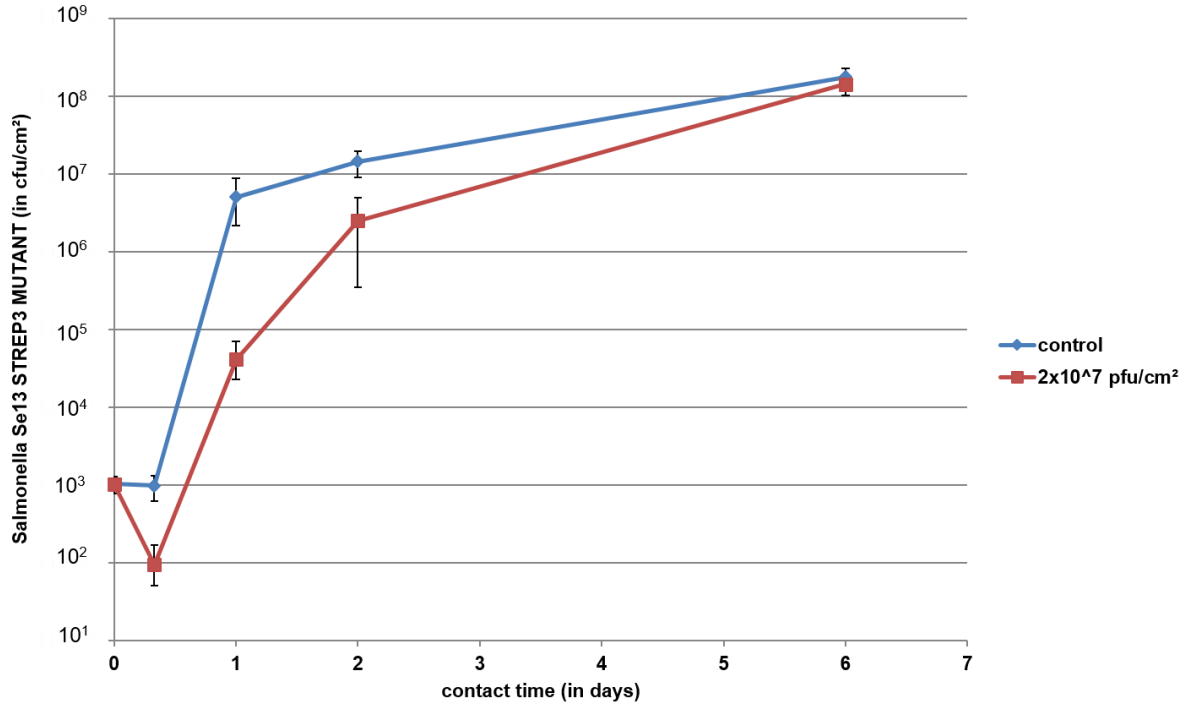

Figure 9: Effect of Salmonelex™ on growth of *Salmonella* strain Se13 STREP3 MUTANT on PORK MEAT at room temperature over 6 days (initial 8 hour incubation at 4°C). Contact times of 8 hours, 24 hours, 48 hours and 6 days; phage concentration $2x10^7$ pfu/cm²; contamination with ~$1x10^3$ cfu/cm²

3.1.3 Challenge study 15 min to 8 hours after treatment

This challenge study shows that phage activity is highest shortly after application and diminishes over time. Treatment for 8 hours is more effective than treatment for 2 hours but only insignificantly so (Figure 10+11). This study shows that where time is not limited the best effects will be observed, but also shows that effective treatment is possible within short time frames.

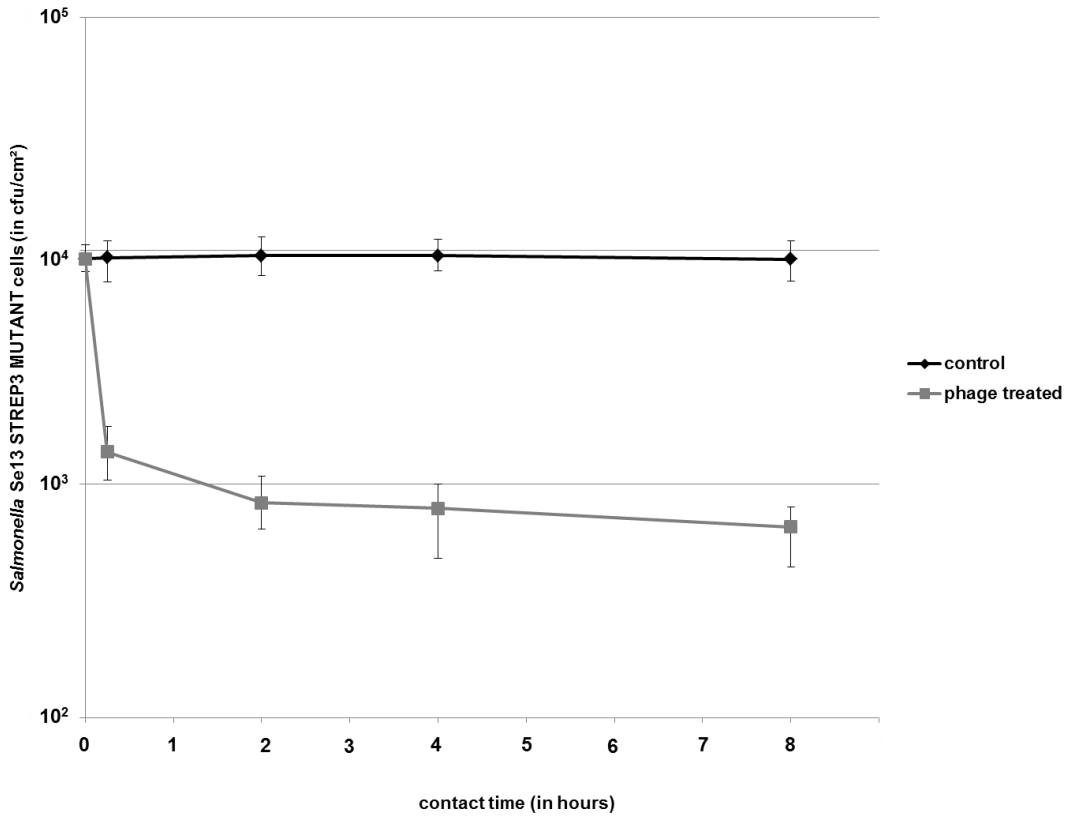

Figure 10: Effect of Salmonelex on growth of *Salmonella* strain Se13 STREP3 MUTANT on CHICKEN BREAST FILLET at 4°C over a period of 8 hours Contact times of 15 minutes, 2 hours, 4 hours and 8 hours; phage concentrations $2x10^7$ pfu/cm² ; contamination with ~$1x10^4$ cfu/cm²

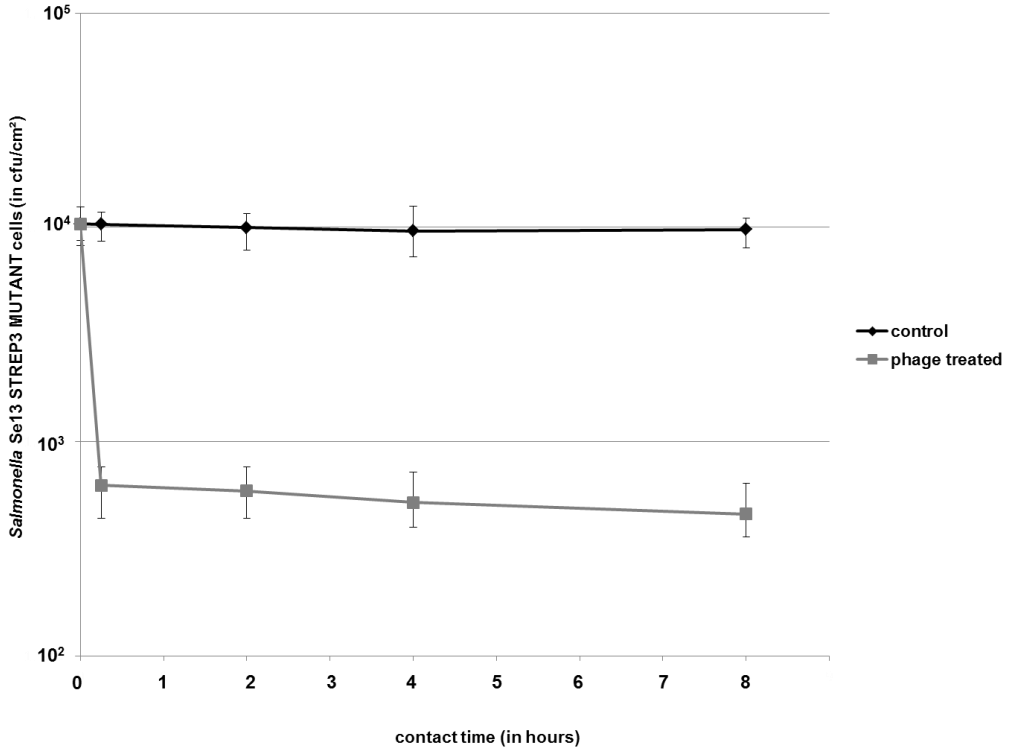

Figure 11: Effect of Salmonelex on growth of *Salmonella* strain Se13 STREP3 MUTANT on CHICKEN SKIN at 4°C over a period of 8 hours. Contact times of 15 minutes, 2 hours, 4 hours and 8 hours; phage concentrations $2x10^7$ pfu/cm² ; contamination with ~$1x10^4$ cfu/cm²

3.1.4 Simulating Salmonelex™ efficacy in a pre-chill application

On the samples itself a reduction of around 39% (with *Salmonella* overnight culture) to 46% (with exponentially growing cells) could be achieved when applying phages by dipping samples in a simulated pre-chill bath at a concentration of $1x10^8$ pfu/mL. With a phage concentration of $1x10^9$ pfu/mL *Salmonella* cell numbers could be reduced by 58 to 69%, respectively (Figure 12). Contact time in this trial was limited to 15 minutes here as it was tested for a specific application were phage activity will be limited to 15 minutes due to a subsequent dipping in a PAA high concentration bath. Figure 10 and 11 show that longer contact times lead to higher reduction levels.

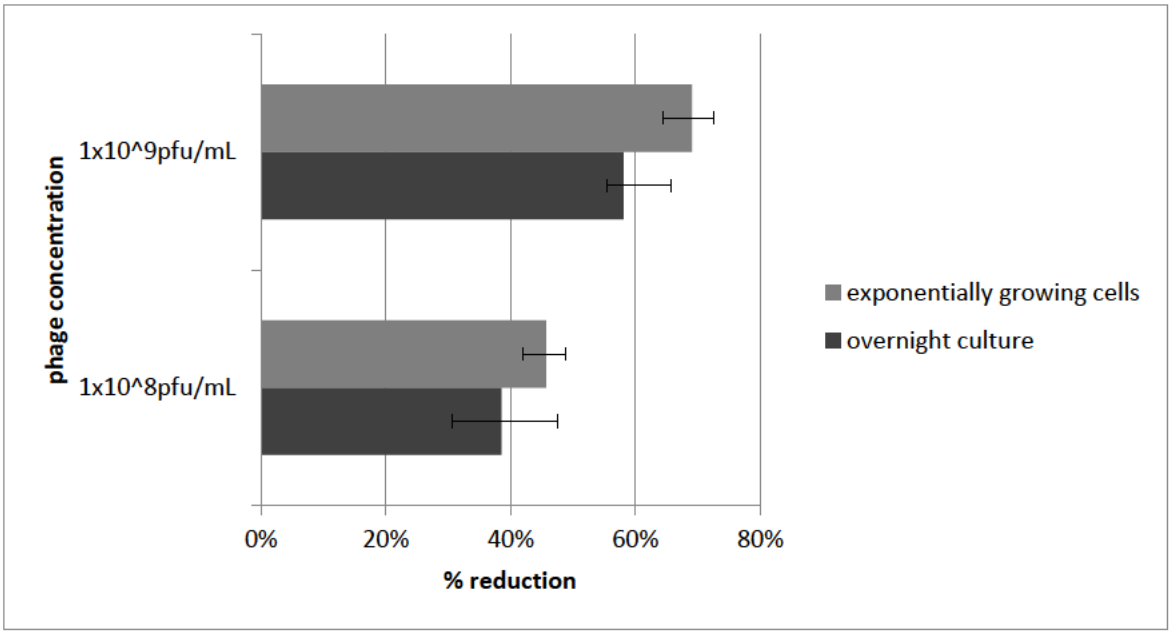

Figure 16: Salmonelex™ efficacy on exponentially growing cells or an overnight culture of Salmonella strain Se13 STREP3 MUTANT on drumsticks
Artificial contamination of samples with $1x10^4$ cfu/cm²; phage treatment with $1x10^8$ pfu/mL and $1x10^9$ pfu/mL; contact time: 15 minutes in 20°C pre-chill bath

In the liquid, exponentially growing *Salmonella* cells could almost be eradicated completely after 15 minutes (~99.1%) and 1 hour (~99.8%) when using a phage concentration of $1x10^9$ pfu/mL in the application. With a phage concentration of $1x10^8$ pfu/mL a cell reduction of 95% after 15 minutes and 97% after 1 hour could be achieved (Figure 13).

Working with *Salmonella* overnight cultures, the phage efficacy on cells in the bath is reduced. With $1x10^9$ pfu/mL 88% of cells was killed after 15 minutes and ~95% after one hour incubation. With a concentration of $1x10^8$ pfu/mL a reduction of cells of ~58% and ~81% could be achieved, respectively (Figure 14).

For raw data see raw data annex for this appendix.

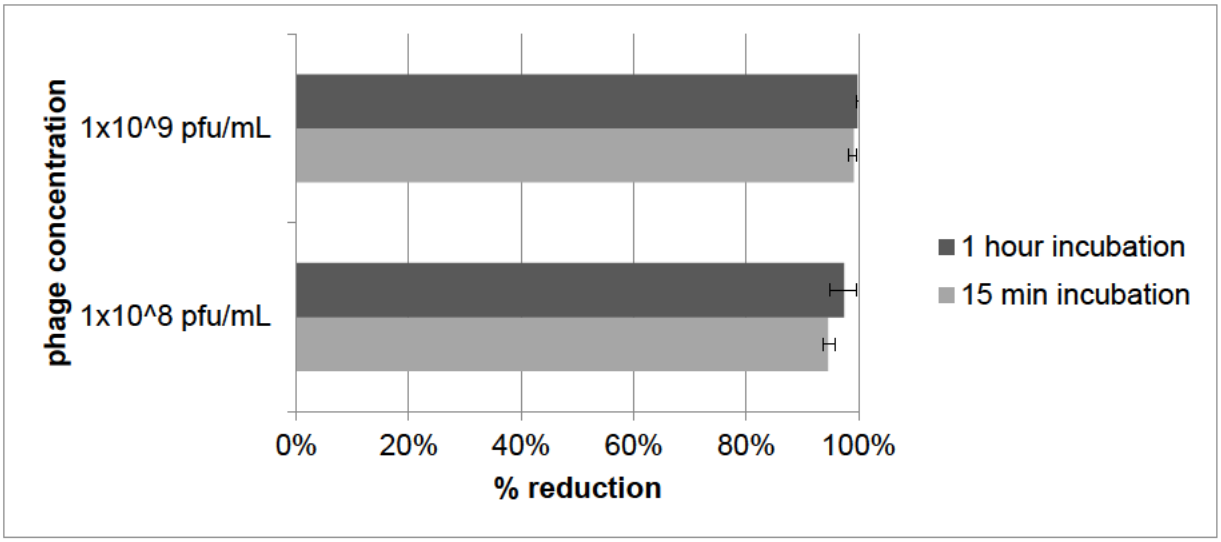

Figure 13: Salmonelex™ efficacy on exponentially growing *Salmonella* strain Se13 STREP3 MUTANT cells in 20°C pre-chill bath
Artificial contamination of liquid with ~2 x 10^4 cfu/10mL; phage treatment with 1x10^8 pfu/mL and 1x10^9 cfu/mL; contact time: 15 minutes and 1 hour

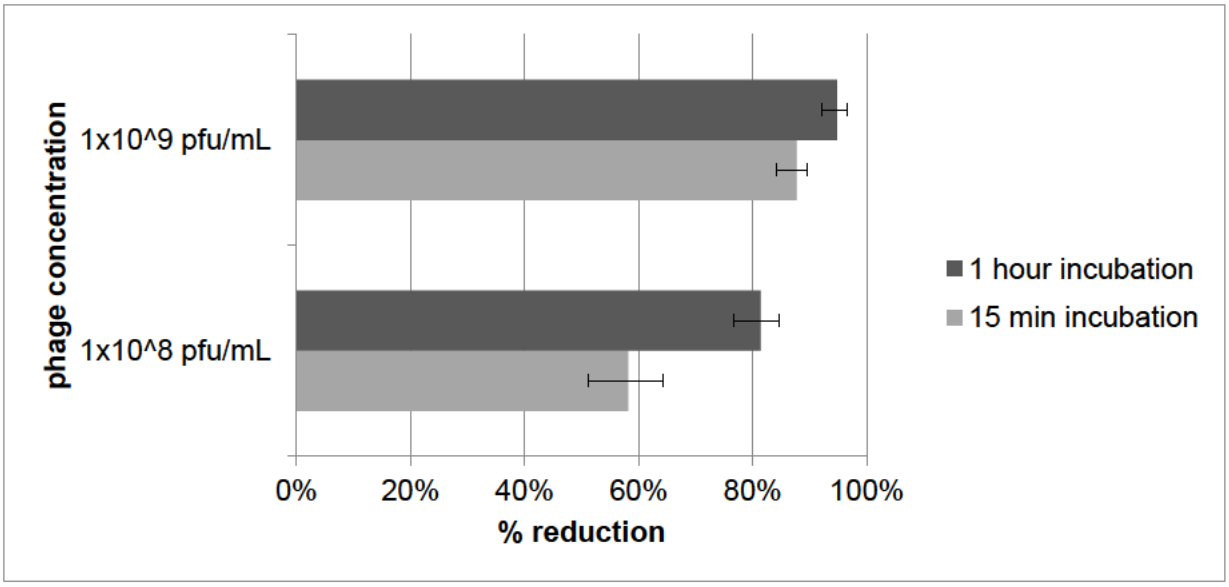

Figure 14: Salmonelex™ efficacy on *Salmonella* strain Se13 STREP3 MUTANT overnight culture in 20°C pre-chill bath
Artificial contamination of liquid with ~2 x 10^4 cfu/10mL; phage treatment with 1x10^8 pfu/mL and 1x10^9 cfu/mL; contact time: 15 minutes and 1 hour

Raw Data Annex to Appendix II

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

		t=0	control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	160	169	158	66
		156	158	132	65
		152	147	123	60
		158	146	129	56
	average cfu/cm²	$1.57x10^4$	$1.55x10^4$	1355	617.5
	% reduction			**91.26**	**96.02**
	log reduction			**1.06**	**1.4**
48h	cfu/plate*	160	141	112	66
		156	151	122	67
		152	141	100	78
		158	136	102	74
	average cfu/cm²	$1.57x10^4$	$1.42x10^4$	1090	712.5
	% reduction			**92.34**	**94.99**
	log			**1.12**	**1.3**

	reduction				
6d	cfu/plate*	160	159	88	53
		156	159	96	56
		152	130	125	80
		158	138	124	83
	average cfu/cm²	$1.57x10^4$	$1.47x10^4$	1080	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

		t=0	**control (not treated)**	**$1x10^7$ pfu/cm²**	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	124	117	73	61
		128	118	77	60
		131	96	83	68
		126	93	86	69
	average cfu/cm²	$1.27x10^4$	$1.06x10^4$	797.5	645
	% reduction			**92.38**	**95.01**
	log reduction			**1.12**	**1.3**
48h	cfu/plate*	124	92	69	43
		128	97	64	49
		131	101	80	49
		126	101	85	54
	average cfu/cm²	$1.27x10^4$	$9\ 78x10^3$	745	487.5

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	% reduction			**92.38**	**95.01**
	log reduction			**1.12**	**1.3**
6d	cfu/plate*	124 128 131 126	97 94 105 109	46 47 68 67	34 28 42 45
	average cfu/cm²	$1.27x10^4$	$1.01x10^4$	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

		t=0	control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	176 169 152 160	180 177 162 160	46 38 32 26	32 30 28 30
	average cfu/cm²	$1.64x10^4$	$1.7x10^4$	355	300
	% reduction			**97.91**	**98.23**
	log reduction			**1.68**	**1.75**
48h	cfu/plate*	176 169	160 171	65 56	25 19

		152	141	34	30
		160	134	33	29
	average cfu/cm²	$1.64x10^4$	$1.52x10^4$	470	257.5
	% reduction			**96.9**	**98.3**
	log reduction			**1.51**	**1.77**
6d	cfu/plate*	176	115	33	13
		169	116	34	13
		152	152	43	13
		160	143	47	15
	average cfu/cm²	$1.64x10^4$	$1.32x10^4$	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

		t=0	control (not treated)	$1x10^7$ pfu/cm²	$2x10^7$ pfu/cm²
24h	cfu/plate*	110	107	31	16
		116	113	25	18
		132	143	45	23
		125	137	50	25
	average cfu/cm²	$1.21x10^4$	$1.25x10^4$	377.5	205
	% reduction			**96.98**	**98.36**
	log reduction			**1.52**	**1.79**
48h	cfu/plate*	110	121	31	20
		116	111	30	22

		132 125	132 122	47 32	23 21
	average cfu/cm²	$1.21x10^4$	$1.22x10^4$	350	215
	% reduction			**97.12**	**98.36**
	log reduction			**1.54**	**1.75**
6d	cfu/plate*	110 116 132 125	81 86 99 97	27 28 41 43	17 17 20 23
	average cfu/cm²	$1.21x10^4$	$9.08x10^3$	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

		t=0	**control (not treated)**	**$1x10^7$ pfu/cm²**	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	160 151 142 140	159 147 182 141	62 62 63 70	33 33 36 37
	average cfu/cm²	$1.48x10^4$	$1.57x10^4$	642.5	347.5
	% reduction			**95.91**	**97.79**

	log reduction			**1.39**	**1.66**
48h	cfu/plate*	160	148	59	41
		151	163	57	38
		142	149	68	32
		140	147	71	30
	average cfu/cm²	1.48x104	$1.52x10^4$	637.5	352.5
	% reduction			**95.8**	**97.68**
	log reduction			**1.38**	**1.63**
6d	cfu/plate*	160	129	57	47
		151	130	50	45
		142	137	63	37
		140	157	69	30
	average cfu/cm²	$1.48x10^4$	$1.38x10^4$	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

		t=0	**control (not treated)**	**$1x10^7$ pfu/cm²**	**$2x10^7$ pfu/cm²**
24h	cfu/plate*	119	121	134	40
		128	116	131	43
		139	110	135	27
		137	111	136	28
	average cfu/cm²	$1.31x10^4$	$1.15x10^4$	1340	345
	% reduction			**88.30**	**96.99**

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	log reduction			**0.93**	**1.52**
48h	cfu/plate*	119	99	115	39
		128	100	109	35
		139	89	119	54
		137	84	117	53
	average cfu/cm²	$1.31x10^4$	$9.30x10^3$	1150	452.5
	% reduction			**87.76**	**95.13**
	log reduction			**0.91**	**1.31**
6d	cfu/plate*	119	90	83	24
		128	98	94	16
		139	76	123	45
		137	87	131	36
	average cfu/cm²	$1.31x10^4$	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

Tabel 5: *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 $1x10^4$ 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

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		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	1.53x10^4	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
	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**
	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 6: *Salmonella* in cfu/plate and average cfu/cm² retrieved from PORK MEAT (re-growth at room temperature)
Artificial contamination of samples with ~$1x10^3$ cfu/cm²; phage treatment with $2x10^7$ pfu/cm²; reaction time: 8hours (at 4°C), 24 hours, 48 hours and 6 days at room temperature
A: 1st round with duplicates; **B:** 2nd round with duplicates

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A

t=0	
cfu/plate	→200µL homogenate
	78
	83
	88
	81
cfu/cm^2	825

		control (not treated)	2x10^7 pfu/cm²
8h	cfu/plate*	→200µL homogenate	→200µL homogenate
		79	6
		75	5
		68	8
		61	6
	cfu/cm²	708	63
24h	cfu/plate*	→40µL 10^{-3}	→40µL 10^{-1}
		175	77
		169	76
		102	100
		103	111
	cfu/cm²	6.86x10^6	4.55x10^4
48h	cfu/plate*	→200µL 10^{-4}	→200µL 10^{-3}
		100	52
		108	50
		93	25
		90	28
	cfu/cm²	9.78x10^6	3.88x10^5
6d	cfu/plate*	→100µL 10^{-5}	→200µL 10^{-5}
		91	140
		92	132
		80	107
		67	101
	cfu/cm²	1.65x10^8	1.2x10^8

B

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t=0	
cfu/plate	→200µL homogenate
	126
	117
	120
	124
cfu/cm^2	1218

		control (not treated)	2x10^7 pfu/cm²
8h	cfu/plate*	→200µL homogenate	→200µL homogenate
		130	17
		131	13
		125	11
		114	9
	cfu/cm²	1250	125
24h	cfu/plate*	→50µL 10^{-3}	→40µL 10^{-1}
		93	97
		104	99
		54	49
		68	45
	cfu/cm²	3.19x10^6	3.63x10^4
48h	cfu/plate*	→200µL 10^{-4}	→20µL 10^{-3}
		190	43
		184	49
		194	20
		192	22
	cfu/cm²	1.9x10^7	3.35x10^6
6d	cfu/plate*	→100µL 10^{-5}	→200µL 10^{-5}
		90	147
		92	149
		111	123
		113	120
	cfu/cm²	2.03x10^8	1.35x10^8

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Table 7: *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 $2x10^7$ pfu/cm²; reaction time: 15 minutes, 2 hours, 4 hours and 8 hours
A: 1st round with duplicates; **B:** 2nd round with duplicates

A

		t=0	control (not treated)	$2x10^7$ pfu/cm²
15min	cfu/plate*	203	204	28
		210	200	28
		209	184	30
		207	185	26
	cfu/cm²	8290	7730	1120
	% reduction			**86.14**
	log reduction			**0.86**
2h	cfu/plate*	203	211	19
		210	207	17
		209	195	16
		207	204	18
	cfu/cm²	8290	8170	700
	% reduction			**91.34**
	log reduction			**1.06**
4h	cfu/plate*	203	207	18
		210	204	19
		209	228	16
		207	227	12
	cfu/cm²	8290	8660	650
	% reduction			**92.49**

	log reduction			**1.12**
8h	cfu/plate*	203	185	15
		210	188	16
		209	204	14
		207	208	11
	cfu/cm²	8290	7850	560
	% reduction			**93.07**
	log reduction			**1.16**

* 2mL retrieval buffer/cm²; 50µL homogenate of untreated samples plated; 50µL homogenate of phage-treated samples plated

B

		t=0	**control (not treated)**	**$2x10^7$ pfu/cm²**
15min	cfu/plate*	255	274	38
		264	275	37
		252	272	44
		254	272	43
	cfu/cm²	$1.03x10^4$	$1.09x10^4$	1620
	% reduction			**84.39**
	log reduction			**0.83**
2h	cfu/plate*	255	286	25
		264	283	27
		252	256	21
		254	260	23
	cfu/cm²	$1.03x10^4$	$1.09X10^4$	960
	% reduction			**90.75**
	log reduction			**1.05**

4h	cfu/plate*	255	272	25
		264	269	23
		252	281	22
		254	274	22
	cfu/cm²	$1.03x10^4$	$1.1x10^4$	920
	% reduction			**91.61**
	log reduction			**1.08**
8h	cfu/plate*	255	259	18
		264	254	19
		252	263	20
		254	274	18
	cfu/cm²	$1.03x10^4$	$1.05x10^4$	750
	% reduction			**92.77**
	log reduction			**1.15**

* 2mL retrieval buffer/cm²; 50µL homogenate of untreated samples plated; 50µL homogenate of phage-treated samples plated

Table 8: *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 $2x10^7$ pfu/cm²; reaction time: 15 minutes, 2 hours, 4 hours and 8 hours

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

A

		t=0	control (not treated)	$2x10^7$ pfu/cm²
15min	cfu/plate*	206	228	13
		217	233	14

		215	215	16
		214	220	11
	cfu/cm²	8520	8960	540
	% reduction			**93.62**
	log reduction			**1.19**
2h	cfu/plate*	206	195	14
		217	205	13
		215	231	11
		214	230	11
	cfu/cm²	8520	8610	490
	% reduction			**93.38**
	log reduction			**1.24**
4h	cfu/plate*	206	196	11
		217	199	13
		215	203	10
		214	182	10
	cfu/cm²	8520	7900	440
	% reduction			**94.8**
	log reduction			**1.28**
8h	cfu/plate*	206	228	11
		217	229	10
		215	218	10
		214	200	9
	cfu/cm²	8520	8750	400
	% reduction			**94.8**

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	log reduction	**1.33**

* 2mL retrieval buffer/cm²; 50µL homogenate of untreated samples plated; 50µL homogenate of phage-treated samples plated

B

		t=0	control (not treated)	$2x10^7$ pfu/cm²
15min	cfu/plate*	309	293	19
		304	295	18
		306	293	17
		305	292	17
	cfu/cm²	$1.22x10^4$	$1.17X10^4$	710
	% reduction			**94.21**
	log reduction			**1.22**
2h	cfu/plate*	309	290	19
		304	289	17
		306	278	17
		305	274	16
	cfu/cm²	$1.22x10^4$	$1.13x10^4$	690
	% reduction			**93.8**
	log reduction			**1.21**
4h	cfu/plate*	309	287	16
		304	280	14
		306	312	18
		305	306	12
	cfu/cm²	$1.22x10^4$	$1.19x10^4$	600
	% reduction			**94.6**

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	log reduction			**1.3**
8h	cfu/plate*	309	264	16
		304	270	13
		306	270	11
		305	278	12
	cfu/cm²	$1.22x10^4$	$1.08X10^4$	520
	% reduction			**94.78**
	log reduction			**1.32**

* 2mL retrieval buffer/cm²; 50µL homogenate of untreated samples plated; 50µL homogenate of phage-treated samples plated

Table 9: *Salmonella* in cfu/plate and cfu/cm² retrieved from **CHICKEN DRUMSTICKS** after a **simulated pre-chill application** and reduction of cell numbers in % as well as log reduction; Artificial contamination of samples with ~$1x10^4$ cfu/cm² **exponentially growing cells**; phage treatment with $1x10^8$ pfu/mL and $1x10^9$ pfu/mL; reaction time: 15 minutes

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

A

Exponentially growing cells	**control (not treated)**	**$1x10^8$ pfu/mL**	**$1x10^9$ pfu/mL**
cfu/plate*	179	190	103
	169	185	108
	170	183	95
	178	178	100
cfu/cm²	6960	3680	2030
% reduction		**47.13**	**70.83**
log reduction		**0.3**	**0.5**

B

Exponentially growing cells	**control (not treated)**	**$1x10^8$ pfu/mL**	**$1x10^9$ pfu/mL**
cfu/plate*	91	102	62

	82 100 92	106 101 107	60 65 63
cfu/cm^2	3650	2080	1250
% reduction		**43.01**	**65.75**
log reduction		**0.24**	**0.5**

* 2mL retrieval buffer/cm²; 50µL homogenate of untreated samples plated; 100µL homogenate of phage-treated samples plated

Table 10: *Salmonella* in cfu/plate and cfu/cm² retrieved from **CHICKEN DRUMSTICKS** after **simulated pre-chill application** and reduction of cell numbers in % as well as log reduction; Artificial contamination of samples with ~1x10^4 cfu/cm² **overnight culture**; phage treatment with 1x10^8 pfu/mL and 1x10^9 pfu/mL; reaction time: 15 minutes

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

A

Overnight culture	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	258 245 232 233	166 168 133 127	106 103 112 109
cfu/cm^2	9680	5940	4300
% reduction		**38.64**	**57.26**
log reduction		**0.2**	**0.35**

B

Overnight culture	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	218 216 223 231	143 142 127 135	95 89 86 86

cfu/cm^2	8880	5470	3560
% reduction		**38.4**	**59**
log reduction		**0.2**	**0.4**

* 2mL retrieval buffer/cm²; 50µL homogenate of untreated samples plated; 50µL homogenate of phage-treated samples plated

Table 11: *Salmonella* in cfu/plate and cfu/cm² retrieved from **liquid** in a **simulated pre-chill application** and reduction of cell numbers in % as well as log reduction; Artificial contamination of **liquid** with ~2x10^3 cfu/mL **exponentially growing cells**; phage treatment with 1x10^8 pfu/mL and 1x10^9 pfu/mL; reaction time: 15 minutes and 1 hour

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

A

15 minutes	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	289	28	8
	282	24	5
	209	24	6
	201	20	4
cfu/mL	2453	120	29
% reduction		**95.11**	**98.83**
log reduction		**1.31**	**1.91**
1 hour	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	289	26	2
	282	22	0
	209	17	1
	201	19	0
cfu/mL	2453	105	5
% reduction		**95.72**	**99.80**
log reduction		**1.37**	**2.69**

B

15 minutes	control (not treated)	$1x10^8$ pfu/mL	$1x10^9$ pfu/mL
cfu/plate*	282	32	5
	285	37	8
	293	31	2
	289	36	1
cfu/mL	2870	170	20
% reduction		**94.08**	**99.30**
log reduction		**1.23**	**2.16**
1 hour	**control (not treated)**	**$1x10^8$ pfu/mL**	**$1x10^9$ pfu/mL**
cfu/plate*	282	10	1
	285	7	1
	293	6	2
	289	3	0
cfu/mL	2873	33	5
% reduction		**98.87**	**99.83**
log reduction		**1.95**	**2.76**

100µL homogenate of untreated samples plated; 200µL homogenate of phage-treated samples plated (artificial contamination with ~$2x10^3$ cfu/mL)

Table 12: *Salmonella* in cfu/plate and cfu/cm² retrieved from **liquid** in a **simulated pre-chill application** and reduction of cell numbers in % as well as log reduction; Artificial contamination of **liquid** with ~$2x10^3$ cfu/mL **overnight culture**; phage treatment with $1x10^8$ pfu/mL and $1x10^9$ pfu/mL; reaction time: 15 minutes and 1 hour

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

A

15 minutes	control (not treated)	$1x10^8$ pfu/mL	$1x10^9$ pfu/mL
cfu/plate*	258	110	27
	246	96	30
	235	91	38
	221	86	28

cfu/mL	2400	958	308
% reduction		**60.1**	**87.15**
log reduction		**0.4**	**0.89**
1 hour	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	258	37	13
	246	38	15
	235	48	8
	221	40	8
cfu/mL	2400	408	110
% reduction		**83.02**	**95.42**
log reduction		**0.77**	**1.34**

B

15 minutes	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	218	98	21
	209	92	21
	195	81	22
	181	80	30
cfu/mL	2008	878	235
% reduction		**56.29**	**88.29**
log reduction		**0.36**	**0.93**
1 hour	**control (not treated)**	**1x10^8 pfu/mL**	**1x10^9 pfu/mL**
cfu/plate*	218	47	16
	209	41	10
	195	32	12
	181	42	9
cfu/mL	2008	405	118
% reduction		**79.83**	**94.15**
log reduction		**0.7**	**1.23**

100µL homogenate of untreated samples plated; 100µL homogenate of phage-treated samples plated (artificial contamination with ~2x10^3 cfu/mL)

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Table 13: Overview *Salmonella* serovare Enteritidis Se13 STREP3 MUTANT cells retrieved from meat and poultry 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	631	12000	918	600	12388	826	526
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	504	59	3100	303	180	3513	424	304
Minus	1800	3750	346	71	2800	278	170	2988	366	246
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	14738	366	253	13650	410	236	11113	370	164
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	3263	134	68	3450	240	63	4088	100	66

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Minus	3300	4038	116	93	2550	110	46	3013	100	34
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	13588	991	346	12238	894	403	11300	838	326
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	4613	369	84	4063	296	138	4400	473	144
Minus	2050	2588	371	76	3838	324	103	3700	338	166

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)	12545	8375	766	418	7688	716	367	6965	566	321
Max (cfu/cm²)	16160	11400	1060	500	9900	900	570	7200	700	500
Min (cfu/cm²)	9100	6100	530	230	6800	600	280	5200	460	180
Plus	3615	3025	294	83	2213	184	205	240	134	179
Minus	3445	2275	236	188	888	116	85	1760	106	141

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Table 14: Overview percentage reduction of *Salmonella* serovare Enteritidis Se13 STREP3 MUTANT cells retrieved from meat and poultry 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

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						

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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
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

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Table 15: Overview *Salmonella* serovare Enteritidis Se13 STREP3 MUTANT cells retrieved from pork samples (in cfu/cm² inclusive error amounts based on maximum and minimum cells retrieved) treated with $2x10^7$ pfu/cm² incubated for 8 hours (at 4°C), 24 hours, 48 hours and 6 days at room temperature; artificial contamination with ~$1x10^3$ cfu/cm²

PORK									
	t=0	t=8h		t=24h		t=48h		t=6d	
		control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²
Average (cfu/cm²)	1021.25	978.75	93.75	$5.03 x10^6$	$4.09 x10^4$	$1.44 x10^7$	$1.87 x10^6$	$1.84 x10^8$	$1.27 x10^8$
Max (cfu/cm²)	1260	1310	170	$8.75 x10^6$	$5.50 x10^4$	$1.94 x10^7$	$4.90 x10^6$	$2.26 x10^8$	$1.47 x10^8$
Min (cfu/cm²)	780	610	50	$2.16 x10^6$	$2.25 x10^4$	$9.00 x10^6$	$2.50 x10^5$	$1.34 x10^8$	$1.01 x10^8$
Plus	238.75	331.25	76.25	$3.72 x10^6$	$1.41 x10^4$	$5.01 x10^6$	$3.03 x10^6$	$4.20 x10^7$	$1.96 x10^7$
Minus	241.25	368.75	43.75	$2.87 x10^6$	$1.84 x10^4$	$5.39 x10^6$	$1.62 x10^6$	$5.00 x10^7$	$2.64 x10^7$

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Table 16: Overview *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from poultry samples (in cfu/cm² inclusive error amounts based on maximum and minimum cells retrieved) treated with $2x10^7$ pfu/cm² incubated for 15 minutes, 2 hours, 4 hours and 8hours at 4°C; artificial contamination with ~$1x10^4$ cfu/cm²

CHICKEN BREAST									
	t=0	t=15min		t=2h		t=4h		t=8h	
		control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²
Average (cfu/cm²)	9230	9330	1370	9510	830	9830	785	9175	655
Max (cfu/cm²)	10600	11000	1760	11400	1080	11200	1000	11000	800
Min (cfu/cm²)	8120	7360	1040	7800	640	8160	480	7400	440
Plus	1370	1670	390	1890	250	1680	215	1825	145
Minus	1110	1970	330	1710	190	1360	305	1775	215
CHICKEN SKIN									
	t=0	t=15min		t=2h		t=4h		t=8h	
		control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²	control	$2x10^7$ pfu/cm²
Average (cfu/cm²)	10360	10345	625	9960	590	9900	520	9785	460
Max (cfu/cm²)	12400	11800	760	11600	760	12500	720	11000	640
Min (cfu/cm²)	8240	8600	440	7800	440	7280	400	8000	360
Plus	2040	1455	135	1640	170	2880	200	1215	180
Minus	2120	1745	185	2160	150	2340	120	1785	100

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Table 17: Overview *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from chicken drumsticks (in cfu/cm² inclusive error amounts based on maximum and minimum cells retrieved) treated with $1x10^8$ pfu/mL and $1x10^9$ pfu/mL incubated for 15 minutes in a **simulated pre-chill bath**; artificial contamination with ~$1x10^4$ cfu/cm² of exponentially growing cells and overnight culture

	exponentially growing cells			**overnight culture**		
	control (not treated)	$1x10^8$pfu/mL	$1x10^9$pfu/mL	control (not treated)	$1x10^8$pfu/mL	$1x10^9$pfu/mL
Average (cfu/cm^2)	5305	2880	1640	9280	5705	3930
Max (cfu/cm^2)	7160	3800	2160	10320	6720	4480
Min (cfu/cm^2)	3280	2020	1200	8640	5080	3440
Plus	1855	920	520	1040	1015	550
Minus	2025	860	440	640	625	490

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Table 18: Overview percentage reduction of *Salmonella* serovare Enteritidis Se13 STREP3 MUTANT cells retrieved from chicken drumsticks (inclusive error amounts based on maximum and minimum percentage reduction) treated with $1x10^8$ pfu/mL or $1x10^9$ pfu/mL incubated for 15 minutes in a simulated **pre-chill application**; artificial contamination with ~$1x10^4$ cfu/cm² of exponentially growing cells or overnight culture

	exponentially growing cells		**overnight culture**	
	$1x10^8$pfu/mL	$1x10^9$pfu/mL	$1x10^8$pfu/mL	$1x10^9$pfu/mL
average (% reduction)	45,71%	69,09%	38,52%	58,12%
max (% reduction)	48,85%	72,70%	47,52%	65,81%
min (% reduction)	41,92%	64,38%	30,58%	55,47%
plus	3,14%	3,61%	9,00%	7,69%
min	3,79%	4,71%	7,94%	2,65%

Table 19: Overview *Salmonella* serovar Enteritidis Se13 STREP3 MUTANT cells retrieved from **liquid** in a **simulated pre-chill application** (in cfu/cm² inclusive error amounts based on maximum and minimum cells retrieved) treated with $1x10^8$ pfu/mL and $1x10^9$ pfu/mL incubated for 15 minutes in a **simulated pre-chill bath**; artificial contamination with ~$1x10^4$ cfu/cm² of exponentially growing cells and overnight culture

exponentially growing cells	**15 minutes**			**1 hour**		
	control (not treated)	$1x10^8$pfu/mL	$1x10^9$pfu/mL	control (not treated)	$1x10^8$pfu/mL	$1x10^9$pfu/mL
average (cfu/cm²)	2663	145	24	2663	69	5
max (cfu/cm²)	2930	185	40	2930	130	10
min (cfu/cm²)	2010	100	5	2010	15	0
plus	268	40	16	268	61	5

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min	653	45	19	653	54	5
overnight culture	**15 minutes**			**1 hour**		
	control (not treated)	$1x10^8$pfu/mL	$1x10^9$pfu/mL	control (not treated)	$1x10^8$pfu/mL	$1x10^9$pfu/mL
average (cfu/cm^2)	2204	918	271	2204	406	114
max (cfu/cm^2)	2580	1100	380	2580	480	160
min (cfu/cm^2)	1810	800	210	1810	320	80
plus	376	183	109	376	74	46
min	394	118	61	394	86	34

Table 20: Overview percentage reduction of *Salmonella* serovare Enteritidis Se13 STREP3 MUTANT cells retrieved from **liquid** in a **simulated pre-chill application** (inclusive error amounts based on maximum and minimum percentage reduction) treated with $1x10^8$ pfu/mL or $1x10^9$ pfu/mL incubated for 15 minutes in a simulated **pre-chill application**; artificial contamination with ~$1x10^4$ cfu/cm² of exponentially growing cells or overnight culture

exponentially growing cells	**15 minutes**		**1 hour**	
	$1x10^8$pfu/mL	$1x10^9$pfu/mL	$1x10^8$pfu/mL	$1x10^9$pfu/mL
average (% reduction)	94,55%	99,08%	97,42%	99,81%
max (% reduction)	95,92%	99,83%	99,48%	100,00%
min (% reduction)	93,56%	98,37%	94,70%	99,59%
plus	1,37%	0,75%	2,06%	0,19%
min	0,99%	0,71%	2,72%	0,22%

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overnight culture	15 minutes		1 hour	
	$1x10^8$pfu/mL	$1x10^9$pfu/mL	$1x10^8$pfu/mL	$1x10^9$pfu/mL
average (% reduction)	58,20%	87,74%	81,42%	94,78%
max (% reduction)	64,17%	89,54%	84,58%	96,67%
min (% reduction)	51,18%	84,17%	76,59%	92,03%
plus	5,97%	1,80%	3,16%	1,89%
min	7,02%	3,57%	4,83%	2,75%

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3.1.14 Appendix III: Inclusion (Table 1) and Exclusion (Table 2&3) host range susceptibility data

3.1.14.1

Table 1 *Salmonella* strains used for host range testing All strains were susceptible to one of the two phages tested with most showing sensitivity to both phages The strains include human, animal and food isolates as well as environmental samples as well as some mutant strains with rough phenotypes All strains with an N-designation are human isolates obtained from National Centre for Enteropathogenic Bacteria (NENT), Lucerne, Switzerland Customer refers to poultry producers and processors with whom we have authorized trial as well as potential customers who wanted to ensure their problem strains are sensitive The collection contains strains that have not been sero-typed.

ETH-strains			
Number	**Characterization of the strain**	**Reference numbers**	
142	S Typhimurium DT7155		
1	S Enteritidis H		
2	S Enteritidis I		
4	S Senftenberg		
5	S Typhimurium		
7	S Tennesee		
8	S Blockley		
9	S Derby		
10	S Salmone		
11	S Amherstiana		
12	S Give		
13	S Enteritidis C		
14	S Choleraesuis		
15	S Dublin		
16	S Bredeney		
17	S Hadar A		
18	S Livingston		
19	S Enteritidis B		
20	S Newington		
21	S Typhimurium A		
22	S Panama		
23	S Virchow		
24	S Hadar		
26	S Enteritidis D		
27	S Typhimurium B		

28	S Indiana		
29	S Enteritidis A		
30	S Infantis		
31	S Emec		
32	S Braederup		
33	S Enteritidis F		
34	S Porci		
35	S Wien		
36	S Enteritidis C+		
37	S Enteritidis E		
38	Food Isolate (ILW 15 1 2009)		
40	S Anatum	N1946-08	18040468
41	S Anatum	N2128-08	18043199
42	S Anatum	N2307-08	18047281
43	S Anatum	N1911-08	18039936
44	S Derby	N2236-08	18045760
45	S Derby	N2599-08	18053004
46	S Derby	N2172-08	18044541
47	S Derby	N102-09	19002561
48	S Enteritidis	N58-09	19001198
49	S Enteritidis	N2939-08	18061944
50	S Enteritidis	N2940-08	18061945
51	S Enteritidis	N90-09	19002277
52	S Enteritidis	N2951-08	18061956
53	S Heidelberg	N2743-08	18056461
54	S Heidelberg	N68-09	19001470
55	S Infantis	N63-09	19001203

56	S Infantis	N11-09	19002826
57	S Infantis	N2885-08	18060624
58	S Infantis	N57-09	19001197
59	S Javiana	N2427-08	18050080
60	S Javiana	N1246-08	18027924
61	S Javiana	N2814-08	18058475
62	S Kentucky	N2834-08	18059003
63	S Kentucky	N77-09	19001979
64	S Kentucky	N54-09	19000957
65	S Kentucky	N2892-08	18060643
66	S Muenster	N520-08	18012559
67	S Muenster	N1728-08	18037099
68	S Muenster	N1729-08	18037100
69	S Muenster	N704-08	18017648
70	S Montevideo	N2888-08	18060637
71	S Montevideo	N1689-08	18036458
72	S:Montevideo	N645-08	18016311
73	S Newport	N2821-08	18058703
74	S Newport	N2932-08	18061269
75	S Newport	N93-09	19002282
76	S Newport	N2889-08	18060640
77	S Newport	N2715-08	18055778
78	S Newport	N105-09	19002565
79	S Senftenberg	N1918-08	18040168
80	S Senftenberg	N2313-08	18047287
81	S Senftenberg	N1589-08	18034328
82	S Senftenberg	N2143-08	18043679

83	S Typhimurium	N60-09	19001200
84	S Typhimurium	N59-09	19001199
85	S Typhimurium	N106-09	19002566
86	S Typhimurium	N62-09	19001202
87	S Typhimurium	N75-09	19001977
88	S Virchow	N61-09	19001201
89	S Virchow	N2820-08	18058702
90	S Virchow	N2777-08	18057472
91	S Virchow	N2844-08	18059442
92	S Anatum	N93-07	
93	S Agona	N20-07	
94	S Agona	N160-07	
95	S Enteritidis	N86-07	
96	S Enteritidis	N72-07	
97	S Enteritidis	N253-07	
98	S Enteritidis	N304-07	
99	S Enteritidis	N430-07	
100	S Enteritidis	N482-07	
101	S Enteritidis	N239-07	
102	S Enteritidis	N289-07	
103	S Enteritidis	N507-07	
104	S Enteritidis	N59-07	
105	S Enteritidis	N13-07	
106	S Hadau	N284-07	
107	S Infantis	N142-07	
108	S Infantis	N28-07	
109	S Kentucky	N242-07	

110	S Kentucky	N248-07	
111	S Newport	N34-07	
112	S Newport	N12-07	
113	S Stanley	N29-07	
114	S Stanley	N23-07	
115	S Stanley	N17-07	
116	S Stanley	N21-07	
117	S Typhimurium	N234-07	
118	S Virchow	N90-07	
119	S Virchow	N106-07	
120		N14-07	
121		N15-07	
122		N16-07	
123		N27-07	
124		N30-07	
125		N33-07	
126		N35-07	
127		N57-07	
128		N74-07	
129		N85-07	
130		N121-07	
131		N169-07	
132		N175-07	
133		N184-07	
134		N188-07	
135		N192-07	
136		N213-07	

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137		N219-07	
138		N241-07	
139		N222-07	
140	S Hadar		
141	S Typhimurium LT2 (isolate Thilo Fuchs)		
143	S Cholerasuis SC-B6		
144	S Gallinarum 287/91		
145	S Enteritidis PT4 isolate		
146	*S e* subsp *salamae*		
147	*S e* subsp *arizonae*		
148	*S e subsp diarizonae*		
149	*S e subsp houtenae*		
150	*S bongori*		
151	*S e subsp indica*		
152	*S e rough mutant*		
Mutants	***designation***	**LPS mutation**	
153	SL3770	smooth	
154	SA1355	smooth	
155	SA1627	Ra	
156	SL3749	Ra	
157	SL733	Rb1	
158	SL3750	Rb2	
159	SL3748	Rb3	
160	SL1306	Rc	
161	SL3769	Rd1	
162	SL3789	Rd2	
163	SL1102	Re	

164	SA1377	Re	
165	SL3600	Re	
	Non-ETH strains	**Source/comments**	
166	*S bongori*	Wim Nuboer	
167	*S bongori* 167	Roger Marti	
168	*S enterica*	Customer A	
169	S Ohio	Customer A	
170	S Heidelberg	Customer B	
171	S Heidelberg	Customer B	
172	S Heidelberg	Customer B	
173	S Heidelberg	Customer B	
174	S Heidelberg	Customer B	
175	S Heidelberg	Customer B	
176	S Heidelberg	Customer B	
177	S Heidelberg	Customer B	
178	S Heidelberg	Customer B	
179	S Heidelberg	Customer B	
180	S Heidelberg	Customer B	
181	S Java	Customer A	
182	S Java	Customer C	
183	S Infantis	Customer D	
184	S Infantis	Customer D	
185	S Infantis	Customer D	
186	S Kiambu	Customer D	
187	S Kiambu	Customer D	
188	S Java	Customer C	
189	S Java	Customer C	

190	S Java	Customer C	
191	S Java	Customer C	
192	S Java	Customer C	
193	S Heidelberg	Customer E	
	Chicken isolates analysed by the central veterinary institute (CVI) Lelystad		
194	S Java 1099901286		
195	S Java 1099901283		
196	S Java 7203/98		
197	S Java 675/98		
198	S Java 7916/98		
199	S Typhimurium 281 50		
200	S Typhimurium 281 54		
201	S Typhimurium 282 58		
202	S Typhimurium JEO 3774 WT		
203	S Typhimuirum 7313/98 (human isolate)		

Table 2 Non- E coli strains tested None of the strains listed were infected by either phage

Bacterial Strain	***Strain***	**Misc Info**
1	*C sakazakii*	
2	*C sakazakii*	ATCC29544
3	*C sakazakii*	
4	*C sakazakii*	

5	*C sakazakii*	
6	*C sakazakii*	
7	*C sakazakii*	
8	*C sakazakii*	
12	*C sakazakii*	
13	*C sakazakii*	
15	*C sakazakii*	
16	*C sakazakii*	
17	*C sakazakii*	
18	*C sakazakii*	
19	*C sakazakii*	
20	*C sakazakii*	
21	*C sakazakii*	
22	*C sakazakii*	
23	*C sakazakii*	
24	*C sakazakii*	
25	*C sakazakii*	
26	*C sakazakii*	
27	*C sakazakii*	
28	*C sakazakii*	
29	*C sakazakii*	
30	*C sakazakii*	

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31	*C sakazakii*	
32	*C sakazakii*	
33	*C sakazakii*	
34	*C sakazakii*	
35	*C sakazakii*	
36	*C sakazakii*	
37	*C sakazakii*	
38	*C sakazakii*	
39	*C sakazakii*	
40	*C sakazakii*	
41	*C sakazakii*	
42	*C sakazakii*	
43	Cronobacter genome species 1	
48	*C dublinensis*	
51	*C muytiensii*	
61	*C malonaticus*	
50	*C turicensis*	
62	*C turicensis*	
E3	*E asburiae*	
E12	*E cloacae subsp cloacae*	
E16	*E helveticus*	
610	*Enterobacter aerogenes*	DSM 30053
9	*Escherichia hermannii*	
10	*Escherichia vuneris*	

641	*Klebsiella pneu*	DSM 789
11	*Klebsiella sp 1319*	
14	*Citrobacter sp N0106*	
604	*Citrobacter freundii*	
687	*Ps aerug*	DSM 1117
1848	*Ps aerug PpgI*	
688	*Ps fluo*	
726	*Vibrio natriegens*	DSM 759
1250	*Campylobacter jejuni*	

Table 3 *E coli* isolates tested

Apathogen means non-pathogenic Some of these strains were isolated from cattle (Rind) sheep (Schaf) pig (Schwein) and cheese (Käse) The O157-isolate was obtained from National Centre for Enteropathogenic Bacteria (NENT), Lucerne, Switzerland Only 1 *E coli* isolate (nr 205) was infected by one phage (FO1a)

Strain No	**Strain**	**Misc Infos**
204	E coli F470 (R1)	
205	E coli F576 (R2)	
206	E coli F653 (R3)	
207	E coli F2513 (R4)	
208	E coli K-12 wt	
209	E coli BL21 (DE3) (B)	

269	E coli	Isolat von Rind 1 (apathogen)
270	E coli	Isolat von Rind 2 (apathogen)
271	E coli	Isolat von Rind 3 (apathogen)
272	E coli	Isolat von Rind 4 (apathogen)
273	E coli	Isolat von Rind 5 (apathogen)
274	E coli	Isolat von Rind 6 (apathogen)
275	E coli	Isolat von Schwein 1 (apathogen)
276	E coli	Isolat von Schwein 2 (apathogen)
277	E coli	Isolat von Schwein 3 (apathogen)
278	E coli	Isolat von Schwein 4 (apathogen)
279	E coli	Isolat von Schwein 5 (apathogen)
280	E coli K57/2	"Käsestamm" (apathogen)
281	E coli FAM 19195	Isolat von Käse (apathogen)
282	E coli FAM 19196	Isolat von Käse (apathogen)
283	E coli FAM 19201	Isolat von Käse (apathogen)
284	E coli FAM 21802	Isolat von Käse (apathogen)
285	E coli FAM 21803	Isolat von Käse (apathogen)
286	E coli FAM 21804	Isolat von Käse (apathogen)
287	E coli FAM 21805	Isolat von Käse (apathogen)

288	E coli FAM 21806	Isolat von Käse (apathogen)
289	E coli FAM 21807	Isolat von Käse (apathogen)
290	E coli FAM 21808	Isolat von Käse (apathogen)
291	E coli FAM 21843	Isolat von Käse (apathogen)
292	E coli FAM 21845	Isolat von Käse (apathogen)
293	E coli FAM 21846	Isolat von Käse (apathogen)
	E coli O157:H7 N06-1382	

3.1.15 Appendix IV References

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