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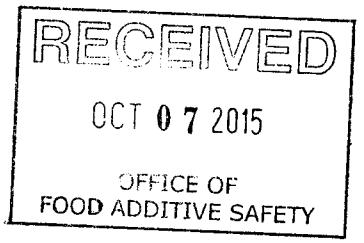
Phagelux GRN 000603



September 28, 2015

Office of Food Additive Safety
Attn: Mr. Richard Bonnette, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Reference: Phagelux GRAS Application for SalmoPro®

Dear Mr. Bonnette

In accordance with proposed 21 CFR 170 36 (Notice of a claim of for exemption based on GRAS determination) published in the Federal Register (62 FR 18938), Phagelux is submitting, in triplicate, a GRAS notification for the bacteriophage cocktail SalmoPro® for bio-control of *Salmonella enterica* in food.

Please let me know if you have any questions.

Best regards

(b) (6)

Rosemonde Mandeville, M.B., Ch.B.; PhD
Chief Scientific Officer
Email: mandeville@phagelux.com
Cell: 514-246-5329

PhageLux

GRAS Notification:

SalmoPro®

Phagelux (Canada) Inc.
6100 Royalmount, Montreal, Quebec, H4P 2R2, Canada
Tel: 514-496-7722; Fax: 514-496-1521

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Phagelux (Canada) Inc.
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SECTION 1

GRAS EXEMPTION CLAIM

Claim of Exemption from the Premarket Approval Requirements Pursuant to Proposed 21CFR§170.36 (c) (1).

The bacteriophage cocktail, SalmoPro® containing bacteriophages BP-63 and BP-12, was determined by Phagelux Inc., to be generally recognized as safe, through scientific procedures, and is exempt from premarket approval requirements under the intended use conditions described within this notification. The following sections are describing the basis for this finding.

(b) (6)

Sept 28, 2015

Phagelux Inc.
Name and Address of Notifier

Date

Rosemonde Mandeville
Chief Scientific Officer
Phagelux (Canada) Inc.
1600 Royalmount, Montreal,
Quebec, H4P 2R2, Canada
Email: *mandeville@phagelux.com*
Phone: 514-246-5329

1.2 Common or Usual Name

Phagelux Inc. produces a Salmonella-specific bacteriophage cocktail under the trade name SalmoPro®.

1.3 Conditions of use

SalmoPro® is intended for use as an antimicrobial processing aid to control Salmonella on food, when applied to food surfaces up to $1x10^8$ PFU (Plaque Forming Units) per gram of food.

1.4 Basis of the GRAS Determination

Pursuant to 21CFR§170.36 (c) (1) proposed rule, Phagelux (Canada) Inc. has determined that SalmoPro® is GRAS through scientific procedures.

1.5 Availability of Information

The data and information that are the basis for Phagelux (Canada) Inc. determination of GRAS for SalmoPro® are available for review and copying by FDA or will be send to FDA upon request, made to:

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SECTION 2.

IDENTITY AND SPECIFICATIONS OF SALMOPRO®

2.1. IDENTITY AND HOST RANGE

SalmoPro® consists of a mixture of equal concentrations of two *Salmonella*-specific lytic bacteriophages (hereinafter referred to as "monophage(s)". Each of these monophages is specifically effective against a wide host range of *Salmonella enterica* serotypes. These phages were isolated by Phagelux (Canada) scientists from farms in the US and Canada.

The current SalmoPro® is a liquid made up of equal parts of two monophages (BP-63 and BP-12 Triumvirate), which are produced and purified separately and mixed in equal concentrations. The commercial product SalmoPro® has a minimal titer of $1x10^9$ PFU/mL. This solution is concentrated and will be diluted with water at application sites by a factor of 10 to ensure application rate at a maximum of 1×10^8 PFU/g of food.

Host range studies were conducted by Phagelux scientists (on 150 strains of *Salmonella*) and by an independent 3rd party, Dr. Ran Wang (Nanjing, China), on more than 310 strains of *Salmonella*. SalmoPro® was shown to be *Salmonella* specific and has a broad host range. Lytic activity was demonstrated on over 95% of the tested *Salmonella enterica* strains and non-serotyped strains. The tested *S. enterica* strains included (but not limited to) strains of Typhimurium, Enteritidis, Hadar, Heidelberg, Infantis, Ohio, Kentucky, Derby, Newport, Indiana, California, San Diego, Minnesota, Agona, Anatum, Brandenburg, Shwartzengrund, Krefeld, Mbandaka, Putten, Tennessee, Thompson, Urbana, Senftenberg, Worthington, Panama, Houtenae, Wandsworth, Abortusequi, Pullorum, Javiana, Braenderup, Muenchen, Paratyphi A, Paratyphi C, Newington, Oranienburg, Nchanga, Dublin, Hartford, Montevideo, Chester, Bareilly, Haardt, Galliinarum, Diarizonae, Pomona, Altona, Brendeny, London, Virchow, Lille, Litchfield, Poona, and Saintpaul.

SalmoPro® was also tested over more than 50 non-*Salmonella enterica* strains and did not show any lytic activity, except for a few non-pathogenic *E. coli*. *Salmonella* is known to be phylogenetically closely related to *E .coli* bacteria (Bern & Goldberg, 2005). The tested non-Salmonella strains included (but not limited to) *Staphylococcus aureus. Pseudomonas aeruginosa, E. coli, Burkholderia cepacia, Citrobacter freundii, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumonia, Listeria monocytogenes, Staphylococcus epidermidis, Proteus mirabilis, Serratia marcescens, and Streptococcus agalactiae.*

2.2. PHAGE IDENTITY

Both monophages were isolated by Phagelux (Canada) scientists from farms in the US and Canada. Each phage was fully characterized by a variety of methods, including electron microscopy, polymerase chain reaction (PCR), full-genome analysis, lytic activity against a large number of *Salmonella* strains, lytic activity against non-*Salmonella*-related bacteria strains (pathogenic and non-pathogenic).

Name:	BP-63
Order:	Caudovirales
Family:	*Myoviridae*
Properties:	Double-stranded DNA, lytic

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Name: BP-12 (triumvirate BP-12A, BP-12B, BP12C)
Order: Caudovirales
Family: *Podoviridae/Siphoviridae**
Properties: Double-stranded DNA, lytic

BP-12A and BP-12B are Caudovirales from *Podoviridae* family, whereas BP-12C is Caudovirales from *Siphoviridae* (Flagella-specific) family.

2.3. HOST IDENTITY

Both monophages are produced in non-pathogenic *E. coli:* BP-63 in 8N2 and BP-12 in BL-21. Underlying the choice of using non-pathogenic *E.coli* strains than *Salmonella enterica* strains is the lack of enterotoxins and virulence plasmids in these *E. coli* strains. The other fact is the significant reduction of risks for personnel in the production facility.

As tested by an independent 3rd party (National Research Council of Canada, Montreal, Quebec), microarrays hybridization confirmed that both non-pathogenic *E.coli* strains (8N2 and BL-21) do not contain any virulence genes associated with the most abundant pathotypes of *E. coli*. **Data generated clearly demonstrated that both host bacteria do not contain any virulent plasmids** associated with E_EC (STEC, EPEC (Typical and Atypical Pathogenic E. coli), EHEC, EAEC, and ETEC), ExPEX (MNTEC, UOEC, SPEC, and UPEC SAMPLES) or Incomplete EXPEC.

Undesirable host-derived components including host DNA and proteins (LPS or endotoxins) are removed by ion-exchange chromatography and will be described in Section 2.4.

2.4. METHOD OF MANUFACTURING

Batches of the two monophages are produced separately by aerobic fermentation using a broth media which is animal-product free. Initially, each of the host bacterium (non-pathogenic *E. coli*) is grown from a working bank sample (itself derived from the master bank) to a pre-determined optical density (OD) in an animal-product free medium. Each monophage (from a working bank sample) is then added at a pre-determined multiplicity of infection (MOI; phage to bacteria ratio). The culture is incubated under specific aeration and agitation conditions.

After a determined time of incubation, the culture is clarified by filtration to remove bacteria and the filtrate is washed with 0.1M sodium chloride buffer (TMN), and concentrated by tangential flow filtration (TFF). The concentrated product is then passed through an ion exchange column to remove endotoxin proteins.

Finally, the monophage solution is filter-sterilized and diluted with TMN buffer to reach the required product concentration of $1x10^9$ PFU/mL. After each monophage solution has passed the Quality Control (QC) specification steps (Table 1), they are blended (1:1) to form SalmoPro® for commercialization, with a final product titer of $1x10^9$ PFU/mL. SalmoPro® is sterile filtered and packaged into sterile packaging components and placed in refrigerated storage (2-6 °C).

SalmoPro® is diluted with water at the application site, to form the "working solution" with a maximum lytic activity of $1x10^8$ PFU/mL. Figure 1 is an overview of the manufacturing process.

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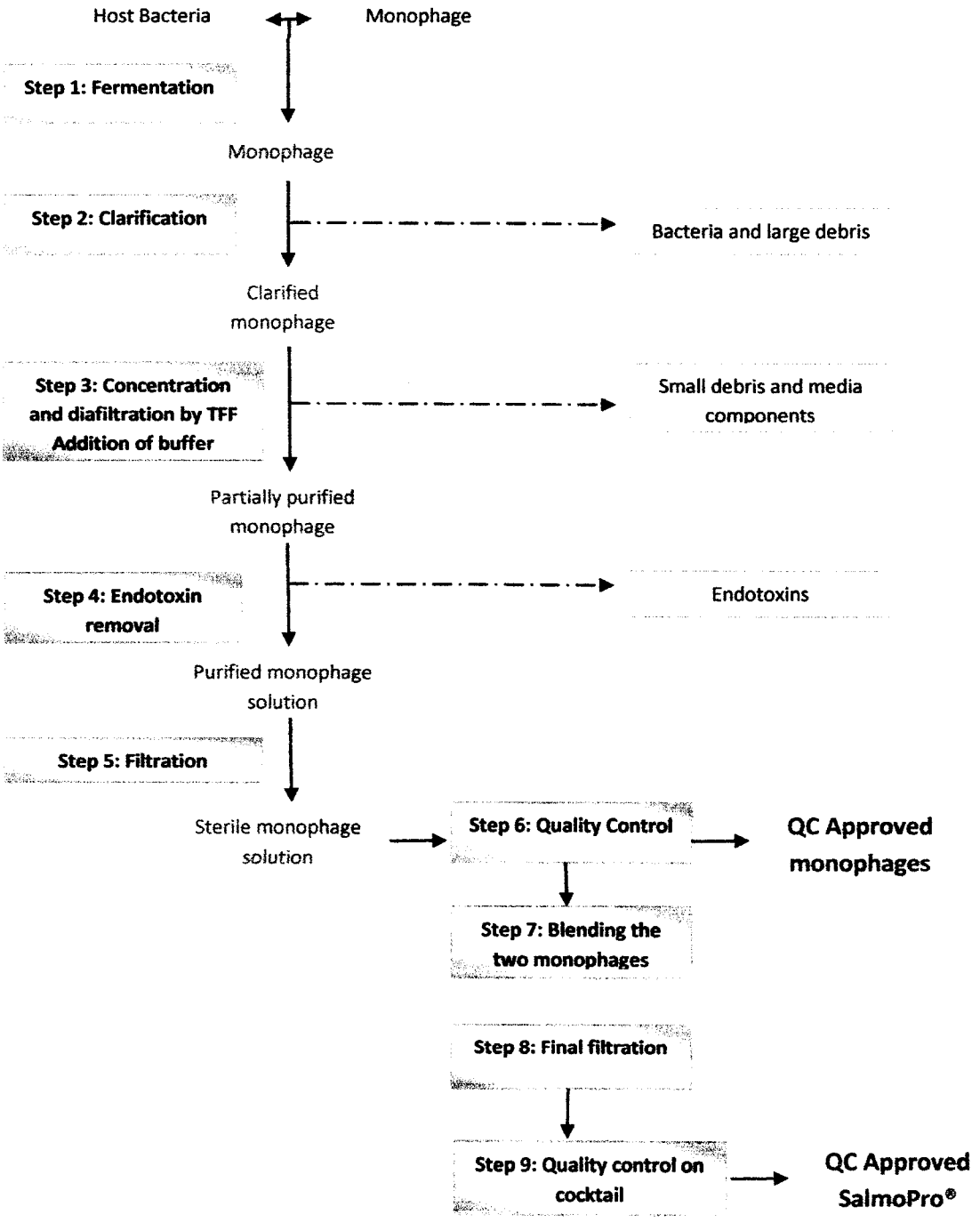
Figure 1: Overview of SalmoPro® Method of Manufacturing



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2.5. SPECIFICATIONS

Quality control consists of 2 steps: initially, each monophage batch needs to pass the specification tests (Table 1), and each batch of the final cocktail of SalmoPro® also needs to pass the specification tests. The Quality Control tests consist of analyzing:

a) The Potency: Standard phage titration protocols are used to insure lytic activity. Method QC-101.1 is used for lytic activity determination.

b) The Identity: Identity is tested by specific potency using 3 different *Salmonella* strains (including exclusive strains) and by specific PCR with predetermined reference profiles. Methods QC-101.1 and QC-103.1 are respectively used for identity determination. The strains are: S. Typhimurium (DT-104O), Hadar (SHA), and Enteritidis (SE-1), negative control is Mbandaka (SM).

c) The Endotoxin Content: Endotoxin content is tested by using FDA-approved endpoint quantitative LAL assay (QCL-1000 TM Endpoint gel clot LAL type assay).

d) The Bacterial sterility: Sterility is tested by enrichment of 1% of each batch in growth medium, followed by plating of the enrichment on elective agar plates (TSA agar). Absence of growth is the required result. Method QC-105.1 is used for sterility determination.

TABLE 1

QUALITY CONTROL OF PRODUCED BATCHES

PARAMETER	MONOPHAGE BATCHES	SALMOPRO® BATCHES
Potency	>1 x10^9 PFU/mL	>1 x10^9 PFU/mL
Identity	Potency: matches reference profiles PCR: Matches reference bands	Potency: matches reference profiles PCR: Matches reference bands
Endotoxin Content	< 25,000 EU/mL for concentrated product containing 1x 10^9 PFU/mL	< 25,000 EU/mL for concentrated product containing 1x 10^9 PFU/mL
Bacterial sterility	No growth after 14 days	No growth after 14 days

2.6. CHARACTERISTICS PROPERTIES

SalmoPro® is a clear to opalescent, odorless liquid with an average weight of the phages of 7x10^6 Dalton since BP-12A is 39696 bp; BP-12 B is 43602 bp; BP-12 C is 60606 bp, and BP63 is 52437 bp. This implies that the phages weigh: 196341 bp x 37 = 7264617 Da= 7 x10^6 Da = 1.16 x 10^{-17} g.

For 1 x 10^9 PFU/mL we estimate the weight of phages to be 1.16 x 10^{-8} g/mL, the remainder of the weight being attributed to 0.1M of sodium chloride (5.88 g/L). The monophage is roughly estimated to be 0.000197 % of the total weight of the concentrated liquid.

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Three lots of SalmoPro® (1 x10^9 PFU/mL) were analyzed for typical physical and chemical composition by an independent 3rd party (Quality Compliance Laboratories, Inc.) and results are presented in Tables 2 and 3.

TABLE 2

PHYSICAL PROPERTIES

Physical properties	**SalmoPro® Lot # 130815-S1**	**SalmoPro® Lot # 130815-S2**	**SalmoPro® Lot # 130815-S3**
Odor	Odorless	Odorless	Odorless
Color	Opalescent	Opalescent	Opalescent
Physical State & Appearance	Liquid	Liquid	Liquid
pH	7.69	7.76	7.68
Special Gravity (vs. Water)	1.0079 g/cm^3	1.0081 g/cm^3	1.0083 g/cm^3
Solubility	Soluble in water	Soluble in water	Soluble in water

* All tests were conducted by Phagelux (Canada) Inc. with the exception of Special Gravity, conducted by Quality Compliance Laboratories, Inc. (Ontario, Canada) under method USP38/NF33<841>.

TABLE 3

CHEMICAL COMPOSITION

Property/analysis/ composition	**Method No./Ref.**	**SalmoPro® Lot # 130815-S1**	**SalmoPro® Lot # 130815-S2**	**SalmoPro® Lot # 130815-S3**	**AVERAGE Values ± SD**
Total nitrogen (USP 461) (%)	USP38/NF33 <461>	0.05	0.06	0.06	0.05 ± 0.005
Total organic carbon (mg/L)	USP38/NF33 <643>	34	43	29	35.3 ± 7.1
Sulfur (mg/L)	QCL-11-0134.01	315	339	315	323 ± 14
Arsenic (µg/L)	QCL-15-0295.RD.00	1	1	1	1.0 ± 0.0
Lead (µg/L)		2	2	1	1.7 ± 0.6
Iron (µg/L)		10	2	2	4.7 ± 4.6
Mercury (µg/L)		0	0	0	0 ± 0
Manganese (µg/L)		5	5	6	5.3 ± 0.6
Magnesium (mg/L)	QCL-11-0134.01	230	228	229	229 ± 1
Endotoxin (EU/mL per 1 x10^9 PFU/mL)	QCL-1000	400 EU/mL	1600 EU/mL	6400 EU/mL	2800 ± 3174

* All tests were conducted by Quality Compliance Laboratories, Inc. (Ontario, Canada), with the exception of *Endotoxin, conducted by Phagelux (Canada) Inc. under method QCL-1000.*

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2.7. POTENTIAL HUMAN TOXICANTS

Endotoxin is the only known human toxicant present in SalmoPro® commercial product. The non-pathogenic *E. coli* strains used for manufacturing are Gram-negative bacteria. As with all Gram-negative bacteria, they produce bacterial endotoxins or lipopolysaccharide (LPS). Each batch of SalmoPro® is tested for LPS content to ensure it meets the release criteria. Endotoxins are further discussed below in Section 3.4.1. As tested, using microarray hybridization, the selected non-pathogenic *E. coli* (8N2 and BL-21) used for monophage production do not contain any virulence genes associated with the most abundant pathotypes of *E. coli*.

2.8. SHELF LIFE

The proposed shelf-life of SalmoPro® is one year when stored at 2-6 °C in a dark, UV-protected area.

2.9. SELF LIMITING LEVELS OF USE

The proposed use of SalmoPro® is as an antibacterial processing aid for foods that are at high risk to be contaminated with *Salmonella enterica*. The purpose of SalmoPro® is to significantly reduce or eliminate *Salmonella enterica* in the finished product.

The use of the product and potential intake would be self-limiting levels by several factors:

2.9.1. Due to the cost of the product, the manufacturer would use the minimum dose required to achieve the desired reduction levels of *Salmonella enterica*.

2.9.2. After the host bacteria *Salmonella enterica* contamination is depleted on the food, the phage will stop replicating and would gradually degrade; virions consist of only proteins and DNA.

2.9.3. Phages are susceptible to a variety of environmental factors, including sunlight (Wommack, *et al.*, 1996), heat (Quiberoni, *et al.*, 2003), and UV light (Rigvava, 2012). Exposure to these environmental factors will cause the number of phages to decrease.

SECTION 3

BASIS OF DETERMINING OF GRAS: BY SCIENTIFIC PROCEDURES

The bacteriophage components of SalmoPro®, as well as composition of the final cocktail will be assessed in detail.

3.1. BACKGROUND ON SALMONELLOSIS

Salmonellosis is a common cause of food-borne diseases worldwide, causing diarrhea (sometimes bloody), fever, abdominal cramps and even life-threatening infections. *Salmonella* is considered to be one of the principal causes of zoonotic disease reported worldwide. *Salmonella enterica,* Enteritidis and Typhimurium, are responsible for the majority of the outbreaks, and most events are reported to be due to consumption of contaminated eggs and poultry, pig, and bovine meats, respectively. *Salmonella* is also a known spoilage bacterium in processed foods.

Each year in the United States, *Salmonella* causes approximately 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths. Direct medical costs are estimated to be $2.4 billion annually, which constitutes a substantial economic hardship on national and local economies (Mead *et al.*, 1999).

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Salmonella are also an international health risk, causing an estimates 93.8 million illnesses globally, and approximately 155,000 deaths, each year (Majowicz *et al.*, 2010). *Salmonella* infections are particularly severe in vulnerable persons such as young children, elderly people and immune-suppressed patients. According to the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), about 75% of the annual cases of human Salmonellosis are due to the consumption of contaminated poultry, beef and egg products (*http://www.cdc.gov/vital sign/food safety*). More recently, outbreaks have been associated with contamination of vegetables with fecal pathogens (Jain *et al.*, 2009).

Fluoroquinolones, such as Ciprofloxacin are strongly recommended for the treatment of severe infections. However, there are rising concerns with the increasing occurrence of multidrug-resistant *Salmonella* globally, particularly in Asia. The CDC is seeing resistance to ceftriaxone in about 3% of non-typhoidal *Salmonella* tested, and some level of resistance to ciprofloxacin in about 3%. About 5% of non-typhoidal *Salmonella* tested by CDC are resistant to 5 or more types of drugs (*http://www.cdc.gov/salmonella/index.html*).

Antibiotic-resistance will inevitably increase worldwide as the bacteria they are meant to kill mutate and multiply. Antibiotic-resistant pathogens constitute a worsening global health problem exacerbated by interconnected travel, antibiotic overuse, horizontal gene transfer, and bacterial evolution. New classes of antimicrobials are needed to treat these pathogens but the drug development pipeline is dry (Boucher *et al*, 2008; Friere-Moran *et al.*, 2011). As a result, regulatory agencies worldwide have shown a renewed interest in novel biocontrol measures; (*http://www.niaid.nih.gov/topics/antimicr...nts/arstrategicplan2014.pdf*) and phages, are considered as the single most promising processing aid (Nilsson, 2014).

3.2. LYTIC PHAGES ARE GRAS

3.2.1. *Lytic versus Lysogenic: All lytic phages are by nature* GRAS

Phages can be classified into two broad categories: lytic (virulent) and lysogenic (temperate).

- Lytic phages are viruses that attack and kill specific bacteria, adhering to specific cell-surface proteins. Once attached to the bacterial host, phages inject their genetic material into the cytoplasm of the host cell, hijacking the bacterium's replication machinery via the expression of specific enzymes encoded by the phage genome, which redirects the bacterial synthesis machinery to reproduction of the new phage particles. The production of phage's enzymes in the later stage, such as lysins and holins, induce destruction of the cell membrane, enabling the newly formed virions to burst out from the lysed bacterial host cell into the extracellular environment. The lytic cycle of the virulent phages fit the class of 'natural antimicrobial controlling agents'.

- Temperate phages, in addition to being capable to enter the lytic cycle, possess the ability to persist as a Prophage in the genome of their bacterial host in the lysogenic cycle. The phage genome remains in a repressed state in the host genome and is replicated as part of the bacterial chromosome until lytic cycle is induced. Hence, temperate phages are not suitable for direct therapeutic use as they may mediate transduction by transferring genetic material of one bacterium to the other.

The biology of lytic phages has been exhaustively studied, demonstrating their safety. Development of recent techniques and the power of comparative genomics are moving us towards more satisfying answers about bacteriophages' biology and understanding the bacteria-phage interaction (Koskella & Meaden, 2013). These studies have clearly shown that phages are obligate intracellular parasites of bacteria and are not infectious or toxic to humans or other mammals.

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The host range of a bacteriophage, defined by which bacteria strains can be infected, depends on the host cell surface receptor (proteins, lipopolysaccharide, or other surface components) recognized through functional receptors located on their tail extremity (Brüssow & Kutter, 2005). Many phages are known to be highly specific for their receptors and are therefore characterized by a narrow host range, limiting their infectivity to a single species or to specific bacterial strains within a species (Ackermann & DuBow, 1987). However, some phages show a broader host range allowing them to infect a large number of strains within a bacterial species, the application of such phages may help prevent an incidence of foodborne diseases caused by pathogens like *Salmonella, Campylobacter, Listeria, Escherichia,* and others. For example Micreos' Listex™, a phage preparation containing a single *Listeria monocytogenes* lytic phage, P100, is used for biocontrol of all *Listeria* strains in susceptible foodstuffs.

Bacteriophages serve as the natural counterbalance to bacteria and herewith have become the most abundant and diverse biological entities on Earth (10^{30}-10^{32}). They are approximately 10 times more abundant than bacteria and archaea. Bacteriophages are probably the most diverse micro-organisms identified on Earth, and in theory, all bacteria are susceptible to viral infection, often by several types of phages (Ackermann & DuBow, 1987).

3.2.2. The major advantages of lytic phages

- Lytic phages replicate exponentially and eradicate the bacteria rapidly regardless of their antibiotic-resistance profile.
- Most lytic phages display very limited host range even among specific bacteria and bacteria strains;
- Phages are **self-replicating and self-limiting**: *In situ* activity increases numbers (though only given favorable bacterial densities).
- Lytic phages have a reduced potential for bacterial development of resistance. They constantly evolve as do the bacteria and overcome mutating resistant bacteria strains.
- Antibiotic-resistant bacteria tend to retain phage sensitivity
- Phages are natural products: Potential appeal to natural medicinal market; Public perception of use of phages as antibacterials seemingly is positive
- Phages have low inherent toxicity ; virions consist of only proteins and DNA
- Phages eliminate pathogens more rapidly and effectively than standard antibiotics
- Phages can be grouped in cocktails and can be used with other agents: Versatility in formulation development and combination with other drugs including antibiotics.
- Certain phages, unlike most chemical antibiotics, can be relatively good at biofilm clearance

Phages present a viable alternative and, potentially, the last resort for the treatment of antibiotic-resistant pathogens.

3.2.3. Phages as biocontrol agents of *Salmonella.*

Ensuring food safety is a complex process that depends on the implementation of a wide range of coordinated control measures at all levels of the food production chain (based on the farm-to-fork principle). Among the various approaches of food safety currently under exploration, bacteriophages have emerged as a novel tool for the biocontrol of bacterial contamination in foods. In the following sections, we will focus on the biocontrol of *Salmonella.*

Studies on the Pre-harvest control of *Salmonella spp*:

Several researchers demonstrated the use of bacteriophage as a pre-harvest intervention to decrease *S. enterica* concentration in poultry (Andreatti *et al*, 2007; Atterbury *et al*, 2007; Bardina *et al*, 2012). The administration of

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phages prior to challenge and the continuous dosing of phages were able to achieve a significant reduction of Salmonella in the animals over time. Bardina et al. used a cocktail of three bacteriophages (UAB_Phi20, UAB_Phi78 and UAB_Phi87) against *Salmonella* Enteritidis & Typhimurium in both mouse and chicken. The cocktail treatment achieved a 50% survival of the mouse model when it was administered simultaneously with infection and at 6, 24 and 30 h post-infection. A more significant reduction of *Salmonella* concentration in chicken cecum was observed when the cocktail was administered a day before infection followed by continuous dosing after infection.

These results were supported by other studies (Andreatti *et al*, 2007) in which cocktails of 4 different phages (CB4Ø) and 45 bacteriophages isolated from wastewater plant (WT45Ø) showed a significant reduction of *S.* Enteritidis in cecal tonsils of chicks after 24 h post-infection but persistent reduction was not observed after 48h post infection, suggesting that continuous dosing is required to sustain the efficacy of bacteriophage in reducing *Salmonella* colonization in poultry. Furthermore, several studies based on the combination use of bacteriophage and competitive exclusion products have been performed to reduce *Salmonella* colonization in experimentally induced infection in chickens (Toro *et al.*, 2005; Borie *et al.*, 2009). These studies concluded that the combination of both types of biocontrol agents (bacteriophage and exclusion products) can be an effective approach to reduce *Salmonella* colonization in poultry.

Interestingly, bacteriophage therapy can play a role in preventing horizontal transmission of the *Salmonella* between livestock, Lim *et al.* (2012) showed that ϕCJ07, a virulent bacteriophage, resulted in significant decrease in intestinal colonization of *Salmonella* Enteritidis in both infected chicks and the uninfected cohabitating chicks.

Studies on the Post-harvest control of *Salmonella* spp:

In the post-harvest control of *Salmonella*, promising results were obtained when bacteriophages were used to control the growth of *Salmonella* on ready-to-eat foods, such as chicken skin, pig skin, egg products, and cheese (Guenther *et al.*, 2009; Hooton *et al.*, 2011) and fresh produce. For example, Guenther *et al.* (2012) described the application of the bacteriophage FO1-E2, to control the growth of *Salmonella* Typhimurium in some ready-to-eat foods such as cooked and sliced turkey breast and egg yolk. Hungaro *et al.* (2013), used a bacteriophage cocktail and chemical agents such as dichloroisocyanurate, peroxy acetic acid and lactic acid to control *Salmonella* Enteritidis on chicken skin under simulation of an industrial condition. The authors concluded that bacteriophages may be employed as an alternative biocontrol agent for *Salmonella* in poultry industrial setting due to the similar efficacy of the bacteriophage demonstrated in reducing the *Salmonella* Enteritidis on chicken skin when compared to other chemical agents.

Magnone *et al.* (2013) found that combined treatment of fresh vegetables (phage application before storage at 10°C and levulinic acid produce wash after storage at 10°C) was more successful in reduction in bacterial count (*E. coli O157:H7, Shigella* spp. *and Salmonella*) in cases where one-step treatment did not bring satisfactory results.

As in previously described trials based on reduction in *E. coli* O157:H7 in lettuce, similar attempts with *Salmonella* Enteritidis and Typhimurium serovars were made (Spricigo *et al.*, 2013). Fresh-cut romaine lettuce were contaminated by *Salmonella* Enteritidis or by *Salmonella* Typhimurium (10^5 CFU/mL for 5 min). During three-phage cocktail treatment at room temperature (10^9 PFU/mL), the number of bacterial cells was evaluated after 30 and 60 min. In all examples, the phage cocktail significantly ($P < 0.05$) reduced *Salmonella* concentration.

Cutting fresh fruits for commercial purposes deprives them of peel and rind, which constitute a natural barrier against bacterial pathogens. Leverentz *et al.* (2001) state that *Salmonella* Enteritidis populations can survive on fresh-cut melons and apples, showing increased growth with increasing temperature. The fruit slices after

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contamination with 25 µl of *Salmonella* suspension (10^6 CFU/mL) were treated with 25 µl of a phage mixture ($2x10^{10}$ PFU/ mL, diluted before application to 10^8 PFU/mL) consisting of four lytic phages obtained from Intralytix, Inc. Slices were incubated at 5, 10 and 20°C, and the number of Salmonella cells was measured at 0, 3, 24, 48, 120 and 168 h after phage inoculation. During examination, phage persistence was much higher on melon slices and decreased to a non-detectable level after 24 h on apple slices. Further investigation showed that low pH of apples (4.2) was a possible factor inhibiting phage survivability. In contrast, the *Salmonella* strain survived at all pH and temperature regimes. Moreover, at 20°C, its population started increasing 3 h after inoculation on both melon and apple slices. Phages were able to significantly reduce *Salmonella* populations only on melon slices (greater reduction than the use of chemical sanitizers), but no significant reduction of *Salmonella* was observed on the contaminated apple slices. Overall, phages seemed to be pH sensitive during treatment. More recently Zinno *et al.* (2014) demonstrated appreciable *Salmonella* Typhimurium inactivation, in the order of 2 log cycles, using phage P22 in different food matrices: liquid eggs, energy drinks, whole and skimmed milk, apple juice, chicken breast and chicken mince.

In conclusion, most studies point to the fact that various factors determine the efficacy of the phage application such as lytic as opposed to lysogenic, stability of the phage(s) under physicochemical conditions of the food (pH, aW), under its storage conditions (temperature) and the ratio of phages to host cells (MOI). While efficacy of specific phages of *Salmonella and Campylobacter spp.* at refrigerated temperatures has been investigated, the required dose of phage is relatively high and may incur regulatory difficulties. The majority of studies examine the lytic activity of the phage at the optimum growth temperature of the host pathogen. The phage broad host range is also another important issue to consider in the design of phage applications. To avoid emergence of resistance, a phage cocktail is also important.

3.3. GRAS STATUS OF STARTING MATERIAL

All ingredients used in the manufacturing process are animal-product free, GRAS substances or food ingredients.

Select Phytone UF, ultra-filtered papaic digest of soybean meal *http://www.bdbiosciences.com/documents/Phytone_Soytone.pdf)*

NaCl: Sodium Chloride is a GRAS substance according 21 CFR regulation #182-70

MgSO4: Magnesium Sulfate is a GRAS substance according 21 CFR regulation #184-1443

Industrial production may require addition of glucose (dextrose, α-D-glucose), and glycerol. Glycerin is currently listed as GRAS in the Code of Federal Regulations (CFR) as a multiple purpose GRAS food substance (21CFR 182.1320).

Antifoam emulsion Xiameter AFE-1510: water, polydimethyl siloxane, polyethylene glycol, sorbitan tristearate

Host strains: Non-pathogenic *E. coli*, see section 2.1 for details.

Monophages: Lytic phages are generally recognized as being safe and numerous phage solutions are already approved either as GRAS product or by other regulatory authorities (see section 3.5 for details). In particular, SalmoPro® was determined to be generally recognized as safe by Phagelux (Canada) Inc. through scientific procedures (the present document).

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3.4. SAFETY

SalmoPro® is a mixture of 2 monophages (active ingredients), added salts and residual fermentation by-products. Sections below are presenting the safety of these ingredients or residuals.

3.4.1 LPS (only toxic by-products known)

Within the manufacturing process, the only known toxic ingredient is the LPS released from the non-pathogenic *E. coli* host bacteria (LPS is a component of the outer membrane of Gram-negative bacteria). As presented in section 2.3, the non-pathogenic *E.coli* host bacteria was tested for absence of undesirable genes.

During the manufacturing process, a specific step (ion exchange chromatography) was added to remove endotoxins, to ensure a final concentration of less than 25,000 EU/mL in a $1x10^9$ PFU/mL phage preparation, as assessed by QC procedure for each monophage lot and SalmoPro® lot (Table 3).

3.4.2. Phages are Non-toxic

All available data indicate that the oral consumption of phages (even at high levels) is entirely harmless to humans. Safety studies have been performed for example with the Listeria-phage P100, in which rats were fed high doses of phages with no measurable effects compared to the control group (Carlton *et al.*, 2005). A study with *E. coli* phages, both in mice and in human volunteers, also showed no significant effects on the test subjects (Chibani-Chenoufi *et al.*, 2004; Bruttin & Brussow, 2005). In our hands (Murthy *et al.*, 2002; Mandeville *et al*, 2003), pre-treatment of piglets with bacteriophages three hours prior to bacterial challenge, or treatment at the onset of diarrhea, demonstrated a statistically significant reduction in the severity of diarrhea in phage-treated animals. No adverse effects such as fever or any other adverse reactions were observed with these treatments. In these studies, and in contrast to antibiotics, phages seemed to have little effect on the *E. coli* occurring in the animals' intestinal flora.

3.4.3 Phages are ubiquitous in the environment

Whether found in the soil (Gomez & Buckling, 2011; Griffiths *et al.*, 2011), the ocean (Marston *et al.*, 2012) or the human body (Smillie *et al.* 2011), bacteriophages play a key role in shaping bacterial population dynamics, serving as the natural counterbalance to bacteria. Phages have been or can be isolated from virtually any aquatic or terrestrial habitat where bacteria exist. A single drop of seawater can hold literally millions of phages (Wommack & Cowell, 2000). The abundance of phages in the environment and the continuous exposure of humans to them, explains the extremely good tolerance of the human organism to phages.

The human gut contains approximately 10^5 bacteriophages (the phageome) (Dalmasso, 2014) having been consumed by humans via various foods. In this context, bacteriophages have been commonly isolated from a wide variety of foods and food products; including carrots (Endley *et al*, 2003); cheese (Gautier *et al.*, 1995), meat (Atterbury *et al.*, 2003; Hsu *et al.*, 2002), with fermented foods like wine (Poblet-Icart *et al.*, 1998), yogurt (Kilic *et al.*, 1996) and Sauerkraut (Lu *et al.*, 2003) having especially high number of these phages. In one study (Lu *et al.*, 2003) 26 different phages were isolated from the product of 4 different Sauerkraut fermentation plants. Phages infecting *Propionibacterium freudenreichii* have been isolated from Swiss cheese at levels of up to 7×10^5 PFU/g (Gautier *et al.*, 1995). In Argentina, phages infecting thermophilic lactic acid bacteria have been isolated from dairy plant samples at numbers up to 10^9 PFU/mL (Suarez *et al.*, 2002). Also Campylobacter phages have been isolated at levels of 4×10^6 PFU/g from chickens (Atterbury *et al.*, 2003) and *Brochothrix thermosphacia* phages from beef (Greer, 1983).

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In humans, phages have been isolated from dental plaques (Delisle & Donkersloot, 1995), feces (Gantzer *et al.*, 2002; Grabow *et al.*, 1995), saliva (Bachrach *et al.*, 2003) and vagina (Kilic *et al.*, 2001). Phages were shown to be present in municipal water supplies of large European cities, indicating resistance to physico-chemical methods of purification of drinking water (Weber-Dabrowska *et al.*, 2014). This example clearly shows the continuous direct contact of humans with phages. Such widespread and frequent consumption of phages every day, supports the view that phages can safely be consumed and therefore deserve the GRAS status *(http://www.cfsan.fda.gov/rdb/opa-g218.htmL)*.

3.4.4. Circumventing phage resistance mechanisms

The prospect of using phages to combat bacterial infection in food has rendered the understating of the interactions between phages and their hosts crucial. Effectively controlling bacterial populations in bio-industries implicates a better understanding of phage resistance barriers and the evolutionary strategies that phages employ to circumvent them. Many bacterial antiviral mechanisms have been reported in the literature (reviewed by Labrie *et al.*, 2010), and can be classified in 4 categories depending on which step is targeted in the phage replication cycle. Interestingly, for every antiviral mechanism reported, a counter-mechanism has been uncovered, allowing the phages to overcome and persist. Table 4 summarizes the co-evolutionary host-phage mechanisms.

Bacteria can alter their cell surface to limit phage propagation by blocking phage receptors. In the case of *Salmonella*, phages can use a number of cell surface moieties as receptors, including glycolipids (O-and Vi-antigens), integral membrane proteins (e.g. *OmpF, BtuB, and TolC*), and flagella proteins (FliC, FljB, and FliK) (Ho *et al.*, 2011; Chaturongakul & Ounjai, 2014). This variety in host receptors leads to wider possibilities in successful host-phage adsorption when using a cocktail of different phages. Moreover, phages have been shown to evolve to target new receptors by acquiring mutations in the genes encoding the receptor binding proteins or tail fibers. For example, *OmpC* porin is used as a receptor by *Salmonella Gifsy* and T4-like phages (Ho & Slach, 2011), while vitamin B_{12} uptake protein BtuB is used by T5-like phages (Kim & Ryu, 2011). Although resistance to BtuB-targeting phages have been shown to develop in *Salmonella*, the trait is not heritable and progeny bacteria can revert and become susceptible to these phages again.

Bacteria can prevent phage adsorption by producing an extracellular matrix; the expression of surface molecules at the receptor site can limit or prevent phage access. However, many phages have been shown to possess a depolymerase which degrades secreted substances and unmask the receptors. In *Salmonella*, tail spike proteins of *Siphophages* and *Podophages* recognize and hydrolyze the O-antigen of LPS. *Siphophage* SSU5 can also use core oligosaccharides of LPS as receptors (Kim *et al.*, 2014) making it a beneficial part of a cocktail against insensitive *Salmonella* populations capable of O-antigen glycosylation. It is thus important to note that phage-host interactions are not exclusive to single types of protein-receptor recognition and that bacterial hosts resistant to flagellatropic phages are sensitive to phages targeting BtuB and LPS. **Cross-infection by different types of phages naturally limits the development and abundance of resistant strains.**

Preventing phage DNA entry is another tactic used by both bacteria and phages to ensure their environmental fitness. Superinfection exclusion systems are used by prophages to confer immunity to their host again secondary infection by other incoming phages. In lysogenic *S. enterica*, expression of SieA and SieB proteins encoded by lysogenic Podophage P22, induces lysis of superinfected host cells and degradation of superinfecting phage genome.

When a phage manages to inject its DNA in its host, a restriction endonuclease can cut the invading foreign DNA at specific recognition sites. Moreover, **restriction modification (RM) systems** cluster with other antivirus defense

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systems (toxin-antitoxin, abortive infection) and operate synergistically in order to increase the overall resistance to phage infection (Oliveira *et al.*, 2014). It has recently been shown that a majority of novel motifs observed in *Salmonella enterica* serovars were modified by Type I RM systems (Pirone-Davies *et al.*, 2015). Phages employ diverse strategies to escape these systems: (a) Some phage have few restriction sites in their genomes, or these sites are too far apart to be recognized by the restriction endonuclease; (b) the phage can be modified by the host metlytransferase (MTase) or acquire its own MTase, and thus be protected during replication of its DNA; (c) the phage can co-inject proteins that directly bind to the DNA and mask the restriction sites; (d) a phage protein can mimic the target DNA and sequester the restriction enzyme, or (e) a phage protein can activate the activity of the MTase or inhibit it by perturbing the REase-MTase complex (Samson *et al.*, 2013).

Targeting and cleaving foreign DNA: CRISPR–Cas can target and cleave invading foreign phage DNA. Phages can circumvent this system by acquiring mutations in the phage protospacers or in the protospacer-adjacent motif (PAM). Some phages, such as *Pseudomonas aeruginosa* lysogens, encode an anti-CRISPR protein that prevents the formation or blocks the action of the CRISPR–Cas complexes (Samson *et al.*, 2013). Interestingly, new research shows that in *Salmonella*, the CRISPR-Cas locus has ceased undergoing adaptive events suggesting that the *Salmonella* CRISPR-Cas systems are no longer immunogenic (Shariat *et al.*, 2015).

Abortive infection systems consist of two proteins, a toxin and an antitoxin. During phage infection, an imbalance in the toxin–antitoxin ratio or inactivation of the antitoxin results in liberation of the toxin, which is free to act on its target and inhibits bacterial growth, thus aborting phage infection. Phages can by-pass abortive-infection (Abi) systems, by acquiring certain mutations of genes involved in nucleotide metabolism or by encoding a molecule that replaces the bacterial antitoxin, thereby counteracting toxin activity and avoiding host death.

TABLE 4

CIRCUMVENTING PHAGE RESISTANCE MECHANISMS

Antiviral mechanisms		**Phage evasion tactics**
Preventing phage adsorption	Blocking phage receptors	Diversity generating retroelement systems
	Production of extracellular matrix	Extracellular polymer degradation mechanisms (i.e. lyases, hydrolases, and hasluronidases)
	Production of competitive inhibitors	Recognition of multiple receptors
Preventing phage DNA entry	Superinfection exclusion systems	
Cutting phage nucleic acid	*Restriction-modification systems*	*Anti-restriction strategies* (e.g. absence of endonuclease recognition sites by point mutations, acquisition of the cognate methylase gene, acquisition of a gene encoding internal proteins, acquisition of restriction alleviation mechanism encoded by *ral*, etc.)
	CRISPR-Cas systems	Acquisition of simple point mutation (or deletion) in the targeted proto-spacer, or mutation in the conserved PAM of the phage genome.
Abortive infection systems		Acquisition of point mutations (e.g. mutation in gene 1.2 and/or 10 in T7 to bypass PifA resistance mechanisms).

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3.4.5. Immune interactions

Treatment with phages can give rise to immunological reactions, depending on where the location of the infection is, and how the phages are administered. It is important to mention that each phage is unique; phage surfaces are covered with peptides that the body does not recognize. Moreover, phage titers fall rapidly after intravenous administration, mainly due to innate immunity and phagocytosis in the blood and liver, and less due to the adaptive immune system (Sokoloff *et al.*, 2000).

A number of studies reported that consumption of large amounts of phages did not lead to any immunological complications (Sarker *et al.*, 2012; McCallin *et al.*, 2013), and topical application has not shown any adverse effects (Wright *et al.*, 2009; Merabishvili *et al.*, 2009). Other internal organs, including the bloodstream, are however not natural environments for phages, and it has been suggested that phages may modulate both the innate and the adaptive immune system when administered intravenously (Merrill *et al.*, 2006). They could affect free-radical production and phagocytosis (Przerwa *et al.*, 2006).

Phages may inhibit interleukin (IL-2), tumor necrosis factor and, to some extent, Interferon-gamma (Gorski *et al.*, 2012; Dabrowska *et al.*, 2014). Phages were also shown to increase non-neutralizing antibodies, IgM and later IgG, and enhance the immune response (Biswas *et al.*, 2002). Previous clinical and animal trials have, however, not resulted in serious immunologic reactions (Merrill *et al.*, 2006; Skurnik *et al.*, 2007), but the risk after intravenous phage therapy cannot be completely ruled out since all phages are different. It is therefore very important to test the immunological response of every single phage, particularly if intravenous therapy is being considered.

Despite these intriguing findings, virtually nothing is known about whether phages can influence innate and adaptive immunity during natural associations with mammals. Although there have been no reports of adverse effects or incidents resulting from the direct exposure to naturally occurring bacteriophage, in treating patients with phage there is reason for caution regarding potential immunological reactions perhaps associated with the lack of formulation purification. Phage preparations for therapy must, however, be purified and free from any toxic or allergenic substances emanating from the bacteria used for the propagation of the phage.

3.4.6. Determination of absence of undesirable genes from sequence

The Complete DNA genome sequences of phages BP-63 and BP-12 was performed by the A.A.C Genomic facility (University of Guelph, Guelph, Ontario, Canada) and deposited in the GenBank under accession number: KM366099 for BP-63. For the triumvirate BP-12, (BP-12A, BP-12B, and BP-12C); the accession numbers for these genes are KM366096; KM366097 and KM366098, respectively.

The size of the DNA and comparative studies of the complete DNA sequences demonstrates the uniqueness of these phages. Bioinformatic analysis of data generated on the genomic analysis of BP-63 and BP-12 sequences demonstrated the lack of harmful or undesired genes against a panel of virulence or transduction genes identified in GenBank.

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3.4.7. Estimated daily dietary intake of Phages and by-products

The Economic Research Services of the USDA (2015) estimates that, in 2016, the per capita consumption of chicken will reach 90.1 lbs., and that of turkey will reach 16.2 lbs. accordingly, people will thus consume, on average, 106.3 lbs. of poultry per year. SalmoPro® is manufactured in a 10X concentrate to be diluted with water at the application site, to form a working solution containing a maximal phage concentration of $1x10^8$ PFU/mL. Supplementary instructions regarding dilution and application rate will be provided to ensure that the final concentration of phages in food produce is always equal to, or below $1x10^8$ PFU/g of food. SalmoPro® is intended to treat *Salmonella* contaminations, which are usually on the surface of the products.

3.4.7.1. Phages

Assuming that:

1. all chicken and turkey are treated with SalmoPro®,
2. the product will be applied on the final product, such as chicken breast filets, for a maximal surface to weigh ratio of 2 cm^2 per gram of poultry
3. The highest rate of application ($1x10^8$ PFU/g of poultry) is used.

Weight of poultry consumed per day:

(106.3 lbs. /1 year) * (1 year/365 days) * (1000 g/2.2 lbs.) = 132.4 g of poultry/day

Number of phages consumed per day:

(132.4 g /day) * ($1*10^8$ PFU/cm^2) *($2cm^2$/g) = 2.64×10^{10} phages/day

Furthermore, SalmoPro® contains a mixture of BP-12 (143,904 bp) and BP-63 (52,437 bp) for a total weight of 7264617 Da

The total weight of phages consumed daily:

(2.64×10^{10} phages/day) * ($7x10^6$ Da) x ($1.66 x10^{-27}$ kg) = 3.07×10^{-10} kg/day = 0.3 µg/day

Assuming an average diet is 3kg/day, the dietary concentration of phages is

(0.3×10^{-6} g / day) / (3×10^3 g/day) = 0.1 ppb

This level is therefore, insignificant.

3.4.7.2 By-products

Phages are eluted from the anion exchange column using a solution containing 100 mM of NaCl for a total of 0.0058 g/mL. The eluted phage product is generally at concentrations higher than 10^{10} PFU/mL and is further

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diluted to reach the required concentration of 1 x 10^9 PFU/mL. For 132.4 g of poultry a day and at a phage level of 1 x 10^8 PFU/g we can calculate the daily amount of NaCl consumed:

0.0058 g of NaCl/ mL x 132.4 g of poultry x 0.01 mL of SalmoPro® /g of poultry = 0.0077 g of NaCl

This amounts to 0.00003 g of sodium per day.

The recommended daily allowance of sodium is 2400 mg (21 CFR § 101.9 (c) (9)). The amount of sodium contributed by SalmoPro® represents 0.00013% of the recommended daily allowance, is negligible, and would not change nutritional content labeling by the end-user.

3.4.7.3. Endotoxins

Bacterial endotoxins, found in the outer membrane of Gram-negative bacteria are members of a class of phospholipids called lipopolysaccharides (LPS). As a consequence, endotoxins are 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, endotoxins can lead to toxic shock syndrome and regulations exist for medicinal reparations that are injected.

No regulations exist for food, moreover foodstuffs could contain high levels of endotoxins. For example, Jay *et al.*, (1979) found endotoxin levels in ground beef in ranges of 500-75,000 EU/g. Townsend *et al.*, (2007) investigated the presence of endotoxins in infant formula in 75 samples collected from seven countries (representing 31 brands). The endotoxin levels ranged from 40 to 55,000 EU/g and did not correlate with the number of viable bacteria. Gehring *et al.*, (2008) measured endotoxin in approximately 400 farm milk and shop milk samples and found levels ranging from 100,000 to 1,000,000 EU/mL of milk samples in Switzerland and Germany.

Additionally, Gram-negative organisms living in the oral cavity also produce endotoxin and Leenstra *et al.* (1966) showed that saliva contains approximately 1 mg of endotoxin/mL. In a nationwide study, Thorne *et al.* (2009) assayed 2,552 house dust samples, the weighted geometric mean endotoxin concentration ranged from 18.7 to 80.5 EU/mg for 5 sampling locations in the houses, and endotoxin load ranged from 4,160 to 95,000 EU/m^2.

Complete removal of endotoxin during the production process of SalmoPro® is not feasible. However, following removal of cellular debris and anion exchange chromatography, endotoxin levels are extremely low, and will not significantly contribute to the daily dietary intake of endotoxins by consumers.

The Limulus Amoebocyte Lysate (LAL) method was used to detect and quantify Gram-negative bacteria endotoxins (aka: lipopolysaccharides [LPS], or endogenous pyrogens) that may be present in biotechnological product.

The LAL method (*Cape Cod pyrotell T*) was used to detect endotoxin levels in each lot of SalmoPro® purified lots produced. The level of Endotoxin in the purified stocks was *150-25000 EU/mL* for one treatment dose (10^9 PFU/mL). Using the worst case scenario, the maximum amount of endotoxin allowed for product release would be **2500 EU per 10^8 PFU/mL**. We can calculate the daily consumption of endotoxins:

132.4g of poultry x 0.01 mL SalmoPro® /g of poultry x 2500 EU/mL of SalmoPro
= 3310 EU of endotoxin per day.

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Human saliva contains approximately 1 mg of endotoxins/mL (Leenstra et al., 1996) which is equivalent to $1x10^6$ EU/mL. Saliva is produced at levels exceeding 500 mL/day, which amounts to $5x10^8$ EU/day. The maximum amount of SalmoPro® only constitutes 0.00066 % of the daily endotoxin load from saliva and is thus considered safe.

3.5. SUBSTANTIAL EQUIVALENCE TO PREVIOUSLY APPROVED PRODUCTS

The USFDA (1996) approval of Listeria-specific phage preparations as food additives several lytic phage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage by a number of regulatory agencies, including two GRAS approved phage products against *Salmonella*, as the presented SalmoPro®:

Listex™

- Listex™ a phage preparation containing a single *Listeria monocytogenes* lytic phage, P100, used for biocontrol of Listeria in susceptible foodstuffs, is GRAS (GRAS Notice No.000218.)
- Listex™ is also listed by the USDA FSIS for use as processing aid for use on RTE meat products (FSIS Directive 7120.1).
- Listex™ is also approved as a processing aid for susceptible foodstuffs in many countries, including approval in Canada by Health Canada and FSANZ in Australia and New Zealand. The Dutch Ministry of Health has issued a formal statement confirming that Listex™ can be used as a processing aid. Additionally, Listex™ has been approved for use in Switzerland in cheese-making and also as processing aids in keeping with European legislation on food safety
- Listex™ is listed by the Organic Materials Review Institute (OMRI). This means that Listex™ may be used in the certified organic production of food processing and handling according to the USDA National Organic Program Rule

ListShield™

- ListShield™ (formerly known as LMP-102), a phage preparation containing six lytic *Listeria monocytogenes*-specific phages, is FDA-cleared as food additive (21 CFRF§172.785);
- ListShield™ is also listed by the USDA FSIS for use as processing aid with no labeling requirements when applied to various RTE meats and poultry products (FSIS Directive 7120.1).
- ListShield™, is GRAS for direct application to fish and shellfish (including smoked varieties; e.g., smoked salmon), fresh and processed fruits, fresh and processed vegetables, and dairy products (including cheese) (GRN No. 528).
- ListShield™ is also EPA-registered for use on non-food surfaces in food processing plants to prevent or significantly reduce contamination of *Listeria monocytogenes* (EPA registration #74234-1.)
- ListShield™ is Health Canada approved for use on ready-to-eat meat and poultry, smoked salmon, fresh-cut apples, and long leaf lettuce (iLONO).
- ListShield™ is National Food Service of Israel approved as a food processing aid for the treatment of ready-to-eat meat and poultry products (Ref: 70275202).

EcoShield™

- EcoShield™ (formerly ECP-100™), a phage preparation containing three lytic phages *E. coli* 0157:H7-specific phages, is FDA-cleared, through a "Food Contact Notification" or FCN, for use on red meat parts and trim intended to be ground (FCN No. 1018).for use as a food contact substance (FCN No. 1018).

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- EcoShield™ is also listed by the USDA FSIS as safe and suitable for use in the production of red meat parts and trim prior to grinding as processing aid with no labeling requirements (FSIS Directive 7120.1).
- EcoShield™, is Health Canada approved for use on red meat parts and trim prior to grinding (iLONO).
- EcoShield™, is National Food Service of Israel approved as food processing aid for the treatment of meat *immediately before grinding* (Ref: 70275202).

AgriPhage™

- AgriPhage™, a phage preparation targeting *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*, is EPA-registered for use on tomatoes and peppers. AgriPhage can be applied directly as a foliar spray and can be used as a curative on symptomatic plants or preventively prior to visual signs of damage. (EPA Reg. No.67986-1)
- AgriPhage has been amended to now include organic usage on tomato and pepper plants as governed by the USDA National Organic Program (NOP).

AgriPhage-CMM™

- AgriPhage-CMM™, a phage preparation targeting *Clavibacter michiganensis* pv. *michiganensis*, is EPA-registered for use on tomatoes. AgriPhage-CMM can be applied directly as a foliar spray and can be used as a curative on symptomatic plants or preventively prior to visual signs of damage (EPA Reg. No.67986-6).
- The Canadian Pest Management Regulatory Agency (PMRA) has approved bio-pesticide AgriPhage-CMM for bacterial stem canker in tomato caused by *Clavibacter michiganensis* pv. *michiganensis* (30301).

Finalyse™

- Finalyse™, a phage preparation targeting *E.coli* 0157:H7, received USDA's Food Safety and Inspection Services approval for commercialization and application as a spray mist or wash on live animals prior to slaughter to decrease pathogen transfer to meat.

Armament™

- Armament™, a phage preparation targeting *Salmonella,* received USDA's Food Safety and Inspection Services approval for commercialization and application as a spray mist or wash on the feathers of live poultry prior to slaughter to decrease pathogen transfer to meat.

Salmonelex™

- Salmonelex™, a phage preparation containing two specific phages, S16 and FO1a, for use as antimicrobial to control *Salmonella* serovars in certain pork and poultry products at levels up to 10^8 PFU/g of food was designated as GRAS (GRAS Notice No. GRN 000468).

SalmoFresh™

- SalmoFresh™, a phage preparation for controlling the foodborne bacterial pathogen *Salmonella enterica,* is GRAS for direct application onto poultry, fish and shellfish, and fresh and processed fruits and vegetables (GRN No. 435).
- SalmoFresh™, is also FSIS-listed as safe and suitable antimicrobial for use in the production of poultry products as a processing aid with no labeling requirements (FSIS Directive 7120.1).
- SalmoFresh™, is Health Canada approved as a processing aid for use on fish, shellfish, and fresh and process fruits and vegetables or on ready-to-eat poultry products prior to slicing and on raw poultry prior to grinding or after grinding (iLONO).
- SalmoFresh™, is National Food Service of Israel approved as a as a food processing aid for the treatment of fish, shellfish, fresh and processed fruits and vegetables and poultry immediately before or after grinding, and on ready to eat products before slicing (ref: 70275202).

Biotector®

- BIOTECTOR® S1 phage product from CheilJedang Corporation is developed to replace antibiotics in animal feed. It is particularly efficient to control *Salmonella Galliinarum* (SG) and *S. Pullorum* (SP)

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responsible for fowl typhoid and pullorum disease, respectively. While BIOTECTOR® S4 is the second phage product (additives in swine feed) which could specifically control *Salmonella* Typhimurium (ST).

3.6. EFFICACY DATA AT THE INTENDED LEVELS OF USE

The literature reports on multiple studies concerning the application of bacteriophages on chicken and turkey carcasses for the reduction of *Salmonella* spp. (Higgins et al., 2005; Fiorentin *et al.*, 2005; Zinno. *et al.*, 2014). Goode *et al.*, (2003) studied the efficacy of lytic bacteriophages in reducing *Salmonella spp.* on chicken skin and showed a rapid 2 log reductions after storage for 48h, as well as rapid eradication of *Salmonella* Enteritidis at low levels of contamination. Wichard et al., (2003) reported a significant reduction (1.8-2.1 log) of *Salmonella* Typhimurium contamination of chicken frankfurters when treated with phage Felix-O1. Bigwood et al. (2008) showed that Phage P7 significantly reduced *Salmonella* Typhimurium levels by 2 log units at 5 °C and by 5.9 log units at 24°C in raw and cooked beef. Efficacy increased when the phage: bacteria ratio was increased to 10 000:1 and host density was high. Treatment with phage FO1-E2 (3×10^8 PFU/g of food) reduced viable counts of *S.* Typhimurium from turkey deli meat (Guenther et al., 2012).

Sharma *et al.*, (2015) showed a 1.3 log reduction in Salmonella load when phages were applied to the surface of turkey breast. However, no significant reduction of *Salmonella* Heidelberg ($P > 0.05$) was observed in ground turkey when turkey meat pieces inoculated with *Salmonella* Heidelberg were surface treated with phage preparation (10^7 PFU/g) before grinding. These findings indicate that the bacteriophage preparation was **effective in reducing *Salmonella* on turkey breast cutlets as a surface treatment but did not cause any reduction of *Salmonella* Heidelberg in ground turkey.**

Moreover, the USDA-FSIS has recently approved the use of a *Salmonella* lytic bacteriophage preparation (*SalmoFresh*™) during processing of raw and ready-to-eat poultry products with phage concentration up to 10^7 PFU/g in the finished product. The product was able to achieve > 1 log unit (a maximal reduction of 1.9 log unit) reduction of susceptible strains. Their results agree with previously reported studies, where *Salmonella* did not grow at 4°C (therefore the phage is not amplified), and there was no prolonged phage activity after the initial application (Guenther *et al.*, 2012). They clearly demonstrate that after the initial treatment and initial reduction of bacterial load, any remaining bacteria will grow out at similar growth rates as the untreated controls.

Based on the above results, we designed multiple comprehensive challenge studies to determine whether SalmoPro® would significantly reduce the population of different *Salmonella* strains using chicken parts. Commercial foods, such as chicken parts, are usually stored at 4 °C or remain at room temperature (25°C) with a short contact time. We show that the application of SalmoPro® to working levels of 1 x 10^7 PFU/cm^2 to chicken breast trim contaminated with *Salmonella* strains results in >1 log unit reduction of *Salmonella* at room temperature (Appendix 1). Moreover, our results indicate that the phage cocktail (SalmoPro®) has a wider lytic range than the individual monophages. This is consistent with other published results (Hooton *et al.*, 2011). Our results are also in accord with Guenther *et al.* (2012), and Micreos (Salmonex™) where no amplification of phages (i.e. no significant reduction of bacterial load) was detected at 4 °C, and residual bacteria grew at similar growth rates as the untreated controls after the initial reduction in Salmonella population. This shows that SalmoPro® has no function in the **final product and that it should only be considered as a processing aid.**

3.7. SUMMARY SALMOPRO® AND GRAS

SalmoPro® is a Salmonella specific cocktail of two naturally occurring monophages (BP-63 and BP-12 triumvirale). A number of bacteriophage products for the biocontrol of pathogens have previously been GRAS-

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approved. The current SalmoPro phage product is equivalent to other Salmonella specific phage preparation that were already GRAS-approved.

Based on genetic and biologic/chemical analysis as well as experimental challenges, scientific data are showing that the individual phages contained in SalmoPro® are safe:

- By nature : strict lytic phage devoid of harmful genes
- By manufacturing process controls: QC analysis of each batch ensures that SalmoPro® is effective, devoid of live contaminants (bacterial sterility testing) and has a minimal safe amount of residual LPS.

SalmoPro® is also shown to be effective in reducing *Salmonella* contaminations on chicken parts (Appendix 1).

***Based on these findings and significant equivalence with the other GRAS-approved phage products,* SalmoPro® should also be considered GRAS.**

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Appendices: Challenge Studies

Appendix 1: Study Number # PL15-045

Appendix 2: Study Number # PL15-196

Appendix 3: Study Number # PL15-197

Appendix 4: Study Number # PL15-191

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Appendix 1

Evaluation of the ability of SalmoPro® to reduce *Salmonella* contamination in experimentally contaminated chicken trim.

Study Number # PL15-045

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Table of contents

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1. STUDY TITLE

Evaluation of the ability of SalmoPro® to reduce *Salmonella* contamination in experimentally contaminated chicken trim when applied at a rate $1x10^7$ PFU/g.

2. STUDY DIRECTOR

Nancy Tawil, Ph.D.

3. STUDY PERSONNEL

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

Name:	Title:	Role:
Nancy Tawil, Ph.D.	Chief Scientist	Study director
Annie Martineau	Research Scientist	Hands-on-research
Vitalie Stepanof	Research technician	Hands-on-research

4. PERFORMING LABORATORY

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5. STUDY OBJECTIVE

Determine the load reduction resulting from the application of 10^7 PFU/g SalmoPro® to chicken meat pieces inoculated with artificially high levels of *Salmonella* (10^2 CFU/g).

6. TEST MATRIX

Chicken trims were obtained from a farm located near Montreal, Quebec, Canada.
Samples were not washed or pre-treated prior to studies.

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7. SALMOPRO® LOT AND APPLICATION RATE

- SalmoPro ® lot # 20150813-1
- SalmoPro ® titer: $1x10^9$ PFU/mL
- The application rate was 0,1mL SalmoPro® at 10^8 PFU/mL per gram of poultry for a total applied rate of $1x10^7$PFU/g poultry.

8. BACTERIAL STRAINS USED IN CHALLENGE STUDY

Each chicken sample was challenged with a cocktail containing 10^2 CFU/g *Salmonella* isolates as *indicated below:*

Cocktail (Serovars/Isolates [1:1 ratio])

- *Salmonella* Hadar *(Sha-4)*
- *Salmonella* Heidelberg (SH)

The total reduction in *Salmonella* bacterial load is evaluated.

9. MEDIA AND REAGENTS

- LB (Sigma, St-Louis, MO; catalog # L3022)
- Peptone water (OrganoTechnie; La Courneuve, France; catalog # E110)
- Brilliant green agar modified (BGA) (Sigma, St-Louis, MO; catalog #70134)
- PBS (HyClone, Thermo Scientific, Logan, Utah; catalog #SH30028-02)

10. GENERAL OUTLINE OF STUDY

1. Original trim pieces were cut into three smaller 10 g chicken trim test samples.
2. 1mL of the bacterial culture (cocktail) at 10^3 CFU/mL was applied on the samples surface evenly. One sample was not treated with bacterial culture, as the uncontaminated, untreated control.
3. The bacteria were allowed to colonize the matrix samples surfaces at the tested temperature (RT or 37°C)
4. PBS (control) or SalmoPro® was applied on the samples at the appropriate concentration (10^7 PFU/g).
5. After 30 min of incubation SalmoPro® was applied on the samples at the appropriate concentration (10^7 PFU/g). 10 mL of peptone water was added and samples were vortexed.
6. The number of viable *Salmonella* in the samples was determined by plating aliquots (0.5 mL) of the peptone water mixture on BGA plates.
7. BGA plates were incubated at 37°C for 24-48h and the number of viable *Salmonella* in the samples was determinate by enumerating colonies as follows:

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Total CFU/g of treated chicken = $\frac{\text{CFU/mL plated x mL of peptone water}}{\text{g sample analyzed}}$

8. All tests were done in triplicates

11. RESULTS

11.1 Raw data

Table 1: *Salmonella* plate counts raw data for Study # 15-045 (RT)

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature	Bacteria Cocktail B*	10g samples	CFU/g
1 x10^8 30min	10	RT	Yes	3	27 20 11
1 x10^8 60min	10	RT	Yes	3	130 140 135
PBS 30min	10	RT	Yes	3	275 300 375
PBS 60min	10	RT	Yes	3	350 400 370

Table 2: *Salmonella* plate counts raw data for Study #15-045 (37°C)

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Bacteria Cocktail B*	10g samples	CFU/g
1 x10^8 30min	10	37	Yes	3	50 58 86
1 x10^8 60min	10	37	Yes	3	360 120 270
PBS 30min	10	37	Yes	3	368 414 266
PBS 60min	10	37	Yes	3	450 1060 720

***Cocktail B** = *S.* Heidelberg+ *S.* Hadar

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4.1.2 Tables presentation of results

Table 3: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution ($1x10^7$ PFU/g) at RT

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Replicates	Mean CFU/mL	% reduction	Significant
$1 x10^8$ 30min	10	RT	n = 3	19	94	Yes
PBS	10	RT	n = 3	317		
$1 x10^8$ 60min	10	RT	n = 3	135	64	Yes
PBS	10	RT	n = 3	373		

Table 4: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution ($1x10^7$ PFU/g) at 37°C

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Replicates	Mean CFU/mL	% reduction	Significant?
$1 x10^8$ 30min	10	37	n = 3	65	82	Yes
PBS	10	37	n = 3	349		
$1 x10^8$ 60min	10	37	n = 3	250	66	Yes
PBS	10	37	n = 3	743		

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11.3 Graphical representation of results

Figure 1: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution ($1x10^7$PFU/g) at RT over time

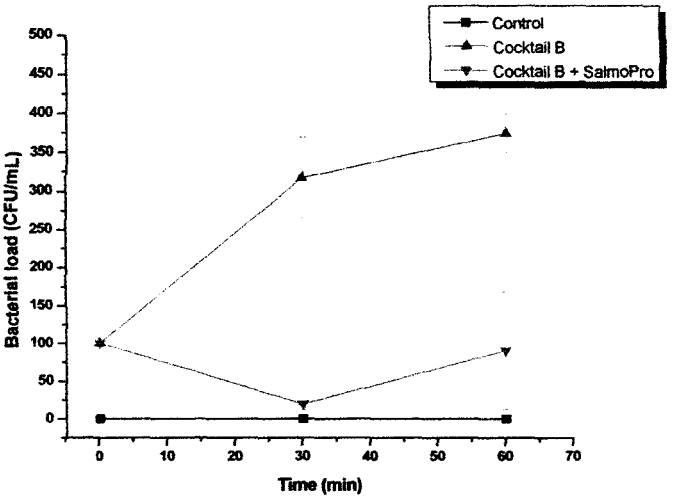
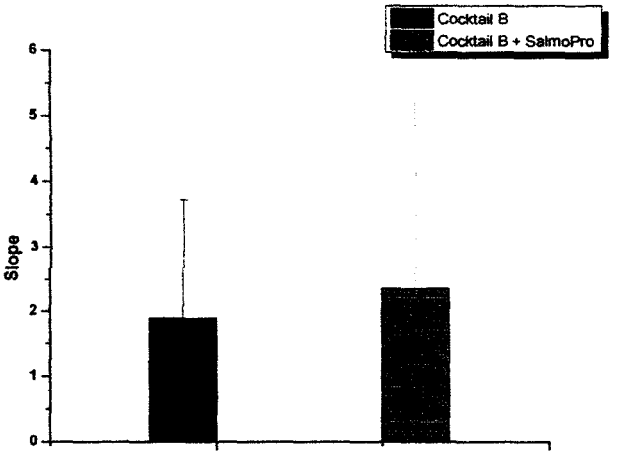


Figure 2: Comparison of growth rates of *Salmonella* after the initial drop due SalmoPro® treatment at RT



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11.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 for Windows (GraphPad software; San Diego, CA; www.graphpad.com)

1- The efficacy of SalmoPro ® treatment in reducing the number of viable *Salmonella* in the experimentally contaminated chicken samples was evaluated by comparing the data obtained with PBS control samples and SalmoPro® treated samples.

2- The efficacy of SalmoPro ® treatment in reducing the number of viable *Salmonella* at different temperatures in the experimentally contaminated chicken samples was evaluated by comparing the number of viable *Salmonella* in samples incubated at RT with the number of viable *Salmonella* in samples incubated at 37°C.

3- The efficacy of SalmoPro ® for different incubation times was evaluated by comparing the number of viable *Salmonella* in samples incubated for 30 min with samples incubated for 60 min.

Table 5: Tukey's multiple comparison test (α = 0.05): Effect of temperature on the reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^{7}PFU/g)

Treatment (PFU/mL SalmoPro®)	Group 1	Group 2	Significant?	Adjusted P value
PBS control	30 min RT	30 min 37°C	No	> 0.9999
	60 min RT	60 min 37°C	No	> 0.9999
Salmonella	30 min RT	30 min 37°C	No	0.9788
	60 min RT	60 min 37°C	Yes	0.0009
SalmoPro® treated samples	30 min RT	30 min 37°C	No	0.9465
	60 min RT	60 min 37°C	No	0.2426

Table 6: Tukey's multiple comparison test (α = 0.05): Effect of incubation time on the reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^{7}PFU/g)

Treatment (PFU/mL SalmoPro®)	Group 1	Group 2	Significant?	Adjusted P value
PBS control	30 min RT	60 min RT	No	> 0.9999
	30 min 37°C	60 min 37°C	No	> 0.9999
Salmonella	30 min RT	60 min RT	No	0.9024
	30 min 37°C	60 min 37°C	Yes	0.0004
SalmoPro® treated samples	30 min RT	60 min RT	No	0.8289
	30 min 37°C	60 min 37°C	No	0.1424

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Figure 3: Tukey's multiple comparison test (α = 0.05): Effect of incubation time on the reduction of *Salmonella* counts in chicken trims treated with SalmoPro ®

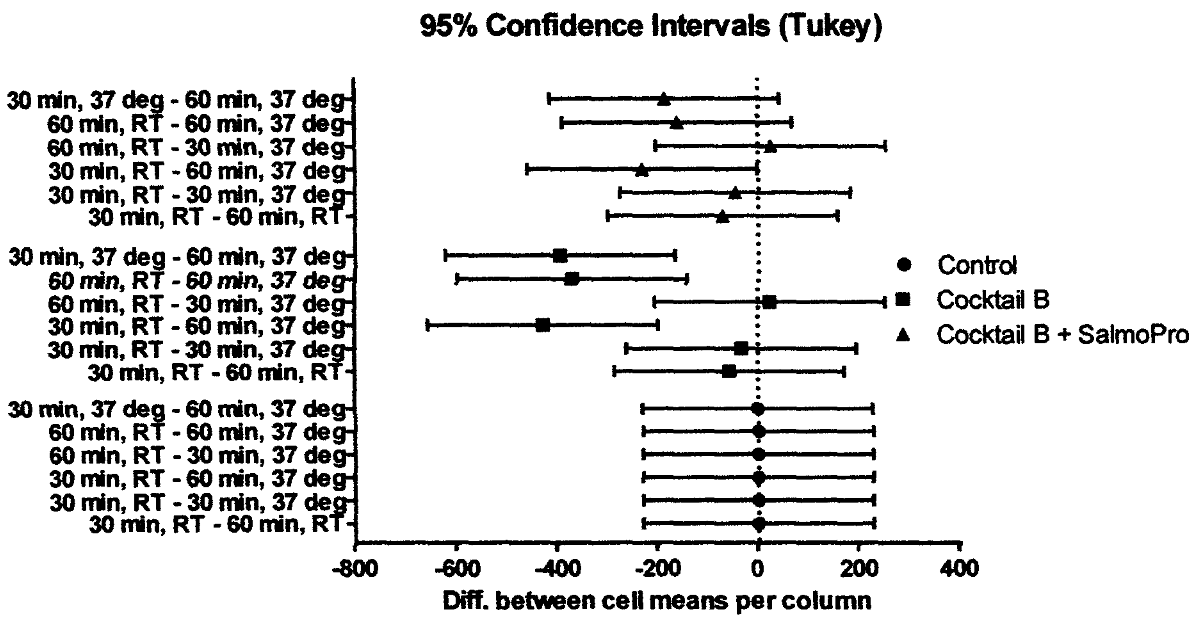
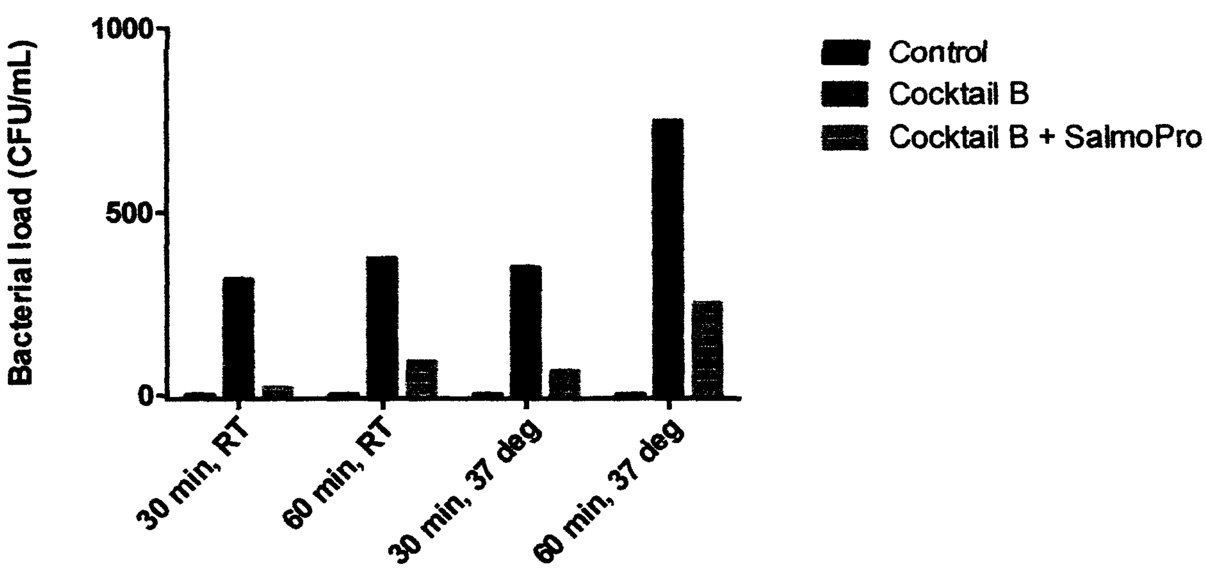


Figure 4: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ®



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11.5 Brief discussion of results and study conclusions

- Applying $1x10^7$ PFU/g of SalmoPro® to chicken trims reduced the number of viable *Salmonella* by 94 % after 30 min of incubation at room temperature and 64 % after 60 min of incubation at room temperature. The observed reduction was statistically significant (P = <0.001) (Table 3 and Figure 4).

- Applying $1x10^7$ PFU/g of SalmoPro® to chicken trims reduced the number of viable *Salmonella* by 82 % after 30 min of incubation at 37°C and 66 % after 60 min of incubation at 37°C. The observed reduction was statistically significant (P = <0.001) (Table 4 and Figure 4).

- The challenge study shows that SalmoPro® only has an initial effect during the first 30 minutes of contact with the *Salmonella*-contaminated chicken trims. Residual *Salmonella* proceeded to grow at the same rate as the *Salmonella* present in the untreated samples (Figure 1 and Figure 2).

- Temperature (RT vs 37°C) did not have a significant effect on the efficacy of SalmoPro®.

- Time of incubation did not have a significant effect on the efficacy of SalmoPro® (Figure 3)

12. SUMMARY CONCLUSION OF THE STUDY

SalmoPro® can significantly reduce viable *Salmonella* (Hadar, Heidelberg) levels in experimentally contaminated chicken trim by 82-94% in 30 min contact time, when used at $1x10^7$ PFU/g. Using a 60 min contact time was not statistically significant, implying that SalmoPro ® acts in the initial 30 min of contact time and does not have any residual effect.

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13. SIGNATURES

(b) (6)

Sept. 28, 2015

Nancy Tawil, Ph.D.
Study Director

Date

(b) (6)

2015/09/28

Annie Martineau, M.Sc.
Research scientist

Date

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Appendix 2

Evaluation of the ability of SalmoPro® to reduce *Salmonella* contamination in experimentally contaminated chicken trim.

Study Number # PL15-196

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Table of contents

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1. STUDY TITLE

Evaluation of the ability of SalmoPro® to reduce *Salmonella* contamination in experimentally contaminated chicken trim when applied at a rate $1x10^7$ PFU/g.

2. STUDY DIRECTOR

Nancy Tawil, Ph.D.

3. STUDY PERSONNEL

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

Name:	**Title:**	**Role:**
Nancy Tawil, Ph.D.	Chief Scientist	Study director
Annie Martineau	Research Scientist	Hands-on-research

4. PERFORMING LABORATORY

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5. STUDY OBJECTIVE

Determine the load reduction resulting from the application of 10^7 PFU/g SalmoPro® to chicken meat pieces inoculated with artificially high levels of *Salmonella*.

6. TEST MATRIX

Chicken trims were obtained from a farm located near Montreal, Quebec, Canada.
Samples were not washed or pre-treated prior to studies.

7. SALMOPRO® LOT AND APPLICATION RATE

- SalmoPro ® lot # 20150813-2
- SalmoPro ® titer: $1x10^9$ PFU/mL
- The application rate was 0.1 mL SalmoPro® at 10^8 PFU/mL per gram of poultry for a total applied rate of $1x10^7$ PFU/g poultry.

8. BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED POULTRY

Each chicken sample was challenged with a cocktail containing 10^2 CFU/g *Salmonella* isolates as indicated below:

Cocktail C (Serovars/Isolates [1:1:1 ratio])

- *Salmonella Hadar (Sha-4)*
- *Salmonella Enteritidis* (Se-1) ATCC13076
- *Salmonella enterica Newport (INSPQ2348)*

The total reduction in *Salmonella* bacterial load is evaluated.

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9. MEDIA AND REAGENTS

- LB (Sigma, St-Louis, MO; catalog # L3022)
- Peptone water (OrganoTechnie; La Courneuve, France; catalog # E110)
- Brilliant green agar modified (BGA) (Sigma, St-Louis, MO; catalog #70134)
- PBS (HyClone, Thermo Scientific, Logan, Utah; catalog #SH30028-02)

10. GENERAL OUTLINE OF STUDY

9. Original trim pieces were cut into three smaller 10 g chicken trim test samples.
10. 1mL of the bacterial culture (cocktail) at 10^3 CFU/mL was applied on the samples surface evenly. One sample was not treated with bacterial culture as uncontaminated untreated control.
11. The bacteria were allowed to colonize the matrix samples surfaces at room temperature (RT).
12. PBS (control) or SalmoPro ® was applied on the samples at the appropriate concentration (10^7 PFU/g).
13. After 30 min of incubation, 10mL of peptone water was added and samples were vortexed.
14. The number of viable *Salmonella* in the samples was determined by plating aliquots (0,5mL) of the peptone water mixture on BGA plates.
15. BGA plates were incubated at 37°C for 24-48h and the number of viable *Salmonella* in the samples was determinate by enumerating colonies as follows:

$$\text{Total CFU/g of treated chicken} = \frac{\text{CFU/mL plated x mL of peptone water}}{\text{gram sample analyzed}}$$

16. All tests were done in sextuplicates.

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11. RESULTS

11.1 Raw data

Table 1: *Salmonella* plate counts raw data for Study #15-196

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (RT)	Bacteria	10 g samples	CFU/g
1 x10^8	10	RT	Yes	6	206 184 138 180 162 148
PBS	10	RT	Yes	6	2000 1320 1480 1880 1280 2040
PBS (chicken control)	10	RT	No	3	0 0 0

11.2 Tabular presentation of results

Table 2: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^7 PFU/g) at RT

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Replicates	Mean CFU/mL	% reduction	Significant?
1 x10^8	10	RT	n = 6	170	90	Yes
PBS	10	RT	n = 6	1667		
PBS (chicken control)	10	RT	n = 6	0		

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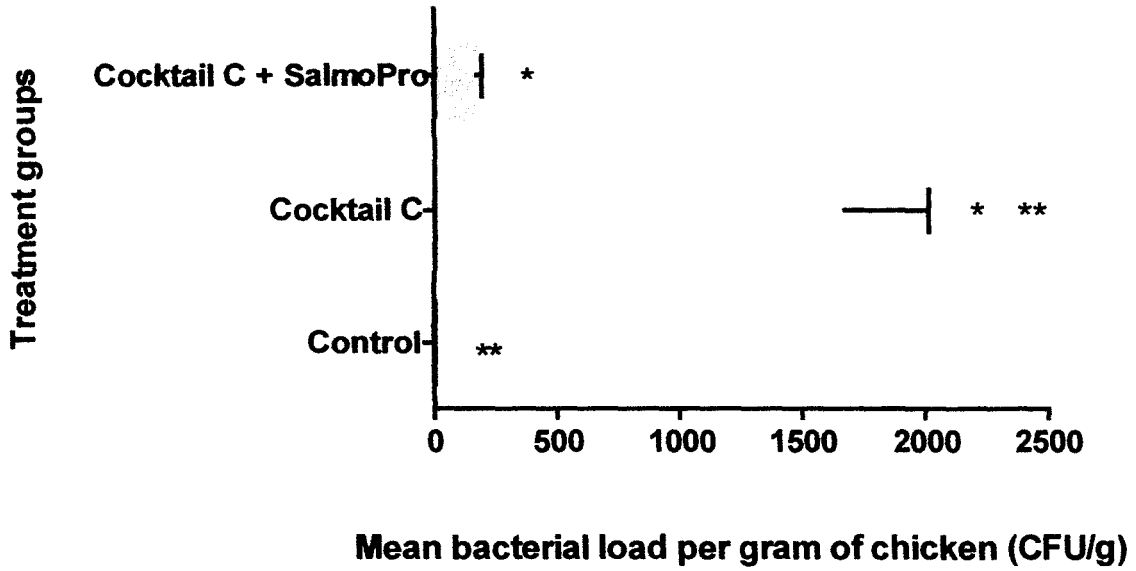
11.3 Graphical presentation of results

Figure 1: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution ($1x10^7$ PFU/g)



11.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 for Windows (GraphPad software; San Diego, CA; www.graphpad.com)

The efficacy of SalmoPro® treatment in reducing the number of viable *Salmonella* in the experimentally contaminated chicken samples was evaluated by comparing the data obtained with PBS control samples and SalmoPro® treated samples.

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Table 3: Tukey's multiple comparison test (α = 0.05): Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^{7}PFU/g)

Tuckey's comparison test	Significant ?	Summary	Adjusted P value	Groups
Control vs. Cocktail C	Yes	****	< 0.0001	A-B
Control vs. Cocktail C + SalmoPro®	No	ns	0.5496	A-C
Cocktail C vs. Cocktail C + SalmoPro®	Yes	****	< 0.0001	B-C

11.5 Brief discussion of results and study conclusions

Applying 1x10^7 PFU/g of SalmoPro® to chicken trims reduced the number of viable Salmonella by 90% after 30 min of incubation at room temperature. The observed reduction was statistically significant (P = <0.0001)

12. SUMMARY CONCLUSION OF THE STUDY

SalmoPro® can significantly reduce viable *Salmonella* levels in experimentally contaminated chicken trim by 90% in 30min contact time, when used at 1x10^7 PFU/g.

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13. SIGNATURES

(b) (6)

Nancy Tawil, Ph.D.
Study Director

Sept. 28, 2015
Date

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Annie Martineau, M.Sc.
Research scientist

2015/09/28
Date

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Appendix 3

Evaluation of the ability of SalmoPro® to reduce *Salmonella* contamination in experimentally contaminated chicken trim.

Study Number # PL15-197

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1. STUDY TITLE

Evaluation of the ability of SalmoPro® to reduce *Salmonella* contamination in experimentally contaminated chicken trim when applied at a rate $1x10^7$ PFU/g.

2. STUDY DIRECTOR

Nancy Tawil, Ph.D.

3. STUDY PERSONNEL

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

Name:	**Title:**	**Role:**
Nancy Tawil, Ph.D.	Chief Scientist	Study director
Annie Martineau	Research Scientist	Hands-on-research

4. PERFORMING LABORATORY

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5. STUDY OBJECTIVE

Determine the load reduction resulting from the application of 10^7 PFU/g SalmoPro® to chicken meat trims inoculated with artificially high levels of *Salmonella*.

6. TEST MATRIX

Chicken trims were obtained from a farm located near Montreal, Quebec, Canada.

Samples were not washed or pre-treated prior to studies.

7. SALMOPRO® LOT AND APPLICATION RATE

- SalmoPro ® lot # 20150813-3
- SalmoPro ® titer: $1x10^9$ PFU/mL
- The application rate was 0.1 mL SalmoPro® at 10^8 PFU/mL per gram of poultry for a total applied rate of $1x10^7$ PFU/g poultry.

8. BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

Each chicken sample was challenged with a cocktail containing 10^3 CFU/g *Salmonella* isolates as indicated below:

Cocktail C (Serovars/Isolates [1:1:1 ratio])

- *Salmonella* Hadar *(Sha-4)*
- *Salmonella* Enteritidis (Se-1) ATCC13076
- *Salmonella enterica Newport (INSPQ2348)*

The total reduction in *Salmonella* bacterial load is evaluated.

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9. MEDIA AND REAGENTS

- LB (Sigma, St-Louis, MO; catalog # L3022)
- Peptone water (OrganoTechnie; La Courneuve, France; catalog # E110)
- Brilliant green agar modified (BGA) (Sigma, St-Louis, MO; catalog #70134)
- PBS (HyClone, Thermo Scientific, Logan, Utah; catalog #SH30028-02)

10. GENERAL OUTLINE OF STUDY

17. Original trim pieces were cut into three smaller 10 g chicken trim test samples.
18. 1mL of the bacterial culture (cocktail) at 10^3 CFU/mL was applied on the samples surface evenly. One sample was not treated with bacterial culture as the uncontaminated, untreated control.
19. The bacteria were allowed to colonize the matrix samples surfaces at 37°C for 20 min.
20. PBS (control) or SalmoPro ® was applied on the samples at the appropriate concentration (10^7 PFU/g).
21. After 30 min of incubation, 10 mL of peptone water was added, and samples were vortexed.
22. The number of viable *Salmonella* in the samples was determined by plating aliquots (0,5mL) of the peptone water mixture on BGA plates.
23. BGA plates were incubated at 37°C for 24-48h and the number of viable *Salmonella* in the samples was determined by enumerating colonies as follows:

$$\text{Total CFU/g of treated chicken} = \frac{\text{CFU/mL plated x mL of peptone water}}{\text{gram sample analyzed}}$$

24. All tests were done in sextuplicates.

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11. RESULTS

11.1 Raw data

Table 1: Salmonella plate counts Raw data for Study #15-197

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Bacteria	10g samples	CFU/g
1 x10^8	10	37	Yes	6	86 106 38 76 106 150
PBS	10	37	Yes	6	1340 1240 1840 1480 980 1300
PBS (chicken control)	10	37	No	3	0 0 0

11.2 Tabular presentation of results

Table 2: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^7 PFU/g) at 37°C

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Replicates	Mean CFU/mL	% reduction	Significant?
1 x10^8	10	37	n = 6	94	93	Yes
PBS	10	37	n = 6	1363		
PBS (chicken control)	10	37	n = 6	0		

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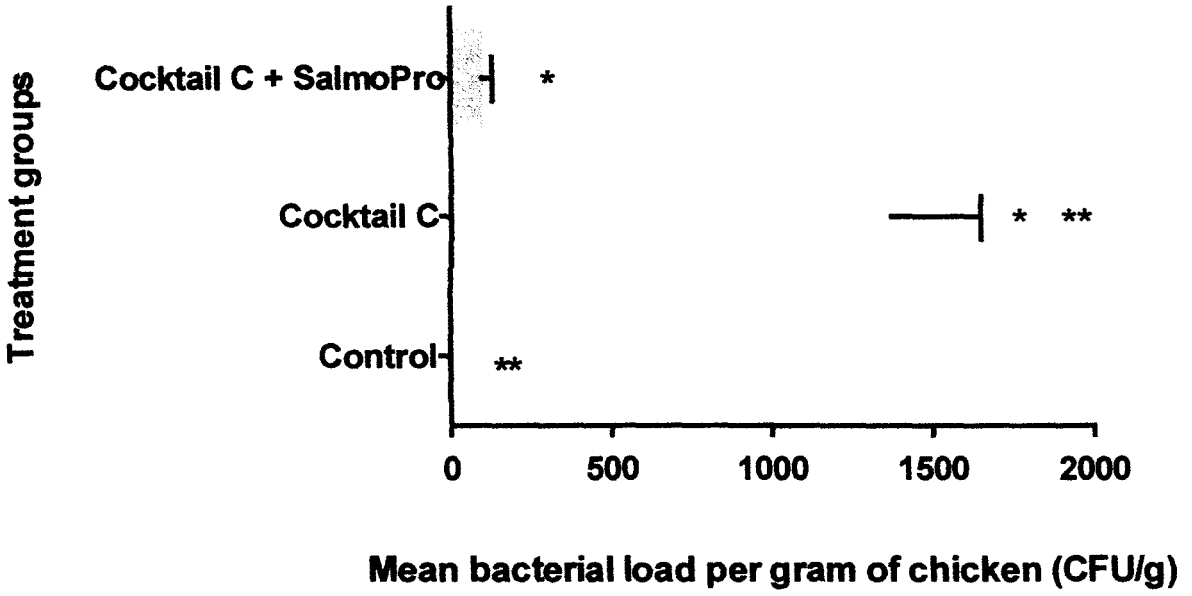
11.3 Graphical presentation of results

Figure 1: One way ANOVA: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution ($1x10^7$ PFU/g)



11.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 for Windows (GraphPad software; San Diego, CA; www.graphpad.com)

The efficacy of SalmoPro ® treatment in reducing the number of viable *Salmonella* in the experimentally contaminated chicken samples was evaluated by comparing the data obtained with PBS control samples and SalmoPro® treated samples.

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Table 3: Tukey's multiple comparison test (α = 0.05): Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^{7}PFU/g)

Tuckey's comparison test	Significant ?	Summary	Adjusted P value	Groups
Control vs. Cocktail C	Yes	****	< 0.0001	A-B
Control vs. Cocktail C + SalmoPro®	No	ns	0.7608	A-C
Cocktail C vs. Cocktail C + SalmoPro®	Yes	****	< 0.0001	B-C

11.3 Prior discussion of results and study conclusions

Applying 1x10^7 PFU/g of SalmoPro® to chicken trims reduced the number of viable Salmonella by 93% after 30 min of incubation at 37°C. The observed reduction was statistically significant (P = <0.001)

12. SUMMARY CONCLUSION OF THE STUDY

SalmoPro® can significantly reduce viable *Salmonella* levels in experimentally contaminated chicken trim by 93% in 30 min contact time at 37°C, when used at 1x10^{7}PFU/g.

13. SIGNATURES

(b) (6)

Nancy Tawil, Ph.D.
Study Director

Sept 28, 2015
Date

(b) (6)

Annie Martineau, M.Sc.
Research scientist

2015/09/28
Date

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Appendix 4

Evaluation of the continued effect of SalmoPro® on Salmonella contaminated chicken trim.

Study Number # PL15-191

Phagelux Canada Inc.

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Table of contents

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1. STUDY TITLE

Evaluation of the continued effect at 4°C of SalmoPro® on *Salmonella* contaminated chicken trim when applied at a rate $1x10^7$ PFU/g.

2. STUDY DIRECTOR

Nancy Tawil, Ph.D.

3. STUDY PERSONNEL

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

Name:	**Title:**	**Role:**
Nancy Tawil, Ph.D.	Chief Scientist	Study director
Annie Martineau	Research Scientist	Hands-on-research

4. PERFORMING LABORATORY

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5. STUDY OBJECTIVE

Determine the bacterial load resulting from the application of 10^7 PFU/g SalmoPro® to chicken meat pieces inoculated with artificially high levels of *Salmonella* and stored at 4°C for 5 days to evaluate the continued effect of the phage preparation.

6. TEST MATRIX

Chicken trims were obtained from a farm located near Montreal, Quebec, Canada.
Samples were not washed or pre-treated prior to studies.

7. SALMOPRO® LOT AND APPLICATION RATE

- SalmoPro ® lot # 20150813-1
- SalmoPro ® titer: $1x10^9$ PFU/mL
- The application rate was 0.1 mL SalmoPro® at 10^8 PFU/mL per gram of poultry for a total applied rate of $1x10^7$ PFU/g poultry.

8. BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

Each chicken sample was challenged with a cocktail containing 10^2 CFU/g *Salmonella* isolates as indicated below:

Cocktail (Serovars/Isolates [1:1:1 ratio])

- *Salmonella* Hadar *(Sha-4)*
- *Salmonella* Enteritidis (Se-1) ATCC13076
- *Salmonella enterica Newport (INSPQ2348)*

The total reduction in *Salmonella* bacterial load is evaluated.

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9. MEDIA AND REAGENTS

- LB (Sigma, St-Louis, MO; catalog # L3022)
- Peptone water (OrganoTechnie; La Courneuve, France; catalog # E110)
- Brilliant green agar modified (BGA) (Sigma, St-Louis, MO; catalog #70134)
- PBS (HyClone, Thermo Scientific, Logan, Utah; catalog #SH30028-02)

10. GENERAL OUTLINE OF STUDY

25. Original trim pieces were cut into three smaller 10 g chicken trim test samples.
26. 1mL of the bacterial culture (cocktail) at 10^2 CFU/g was applied on the samples surface evenly. One sample was not treated with bacterial culture as uncontaminated untreated control.
27. The bacteria were allowed to colonize the matrix samples surfaces at 37°C.
28. PBS (control) or SalmoPro ® was applied on the samples at the appropriate concentration (10^7 PFU/g).
29. After 30min of incubation, 10 mL of peptone water was added and samples were vortexed.
30. The number of viable *Salmonella* in the samples was determined by plating aliquots of the peptone water mixture on BGA plates.
31. BGA plates were incubated at 37°C for 24-48h and the number of viable *Salmonella* in the samples was determinate by enumerating colonies as follows:

    $$\text{Total CFU/g of treated chicken} = \frac{\text{CFU/mL plated x mL of peptone water}}{\text{gram sample analyzed}}$$
32. All samples were stored at 4°C for 8 days and the number of viable Salmonella was determined as in steps 6 and 7 at different times.
33. All tests were done in triplicates.

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11. RESULTS

11.1 Raw data

Table 1: Raw data for Study #15-191

Treatment (PFU/mL SalmoPro®)	Weight (g)	Temperature (°C)	Bacteria	10g samples	CFU/g pre-storage	CFU/g After 5 days at 4°C	CFU/g After 8 days at 4°C
1 x10^8	10	RT	Yes	3	32 34 26	32 36 28	220 100 40
PBS	10	RT	Yes	3	138 166 184	1000 1000 600	880 1160 700
PBS (chicken control)	10	RT	No	3	0 0 0	0 0 0	0 0 0

11.2 Tabular presentation of results

Table 2: Residual effect of SalmoPro® on *Salmonella* counts in chicken trims when applied at 1x10^7 PFU/g stored for 5 and 8 days at 4°C

Treatment (pfu/ml SalmoPro®)	4°C Storage time	Weight (g)	Temperature (°C)	Replicates	Mean CFU/g	Standard deviation
1 x10^8	0	10	RT	n = 3	31	4
PBS	0	10	RT	n = 3	163	23
1x10^8	5d	10	4	n = 3	32	4
PBS	5d	10	4	n = 3	867	231
1x10^8	8d	10	4	n = 3	120	91
PBS	8d	10	4	n = 3	913	232

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11.3 Graphical presentation of results

Figure 1: Initial reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^{7}PFU/g) at RT (initial 30 min)

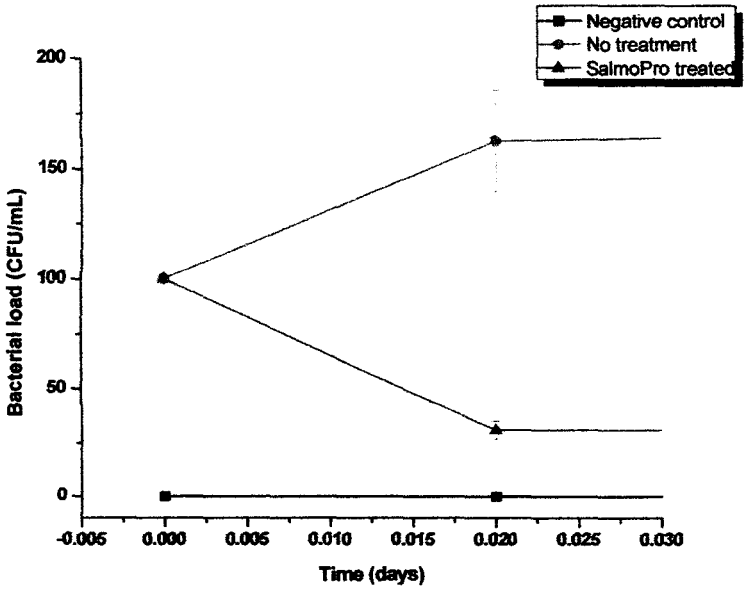

Figure 2: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution (1x10^{7}PFU/g) at RT over time

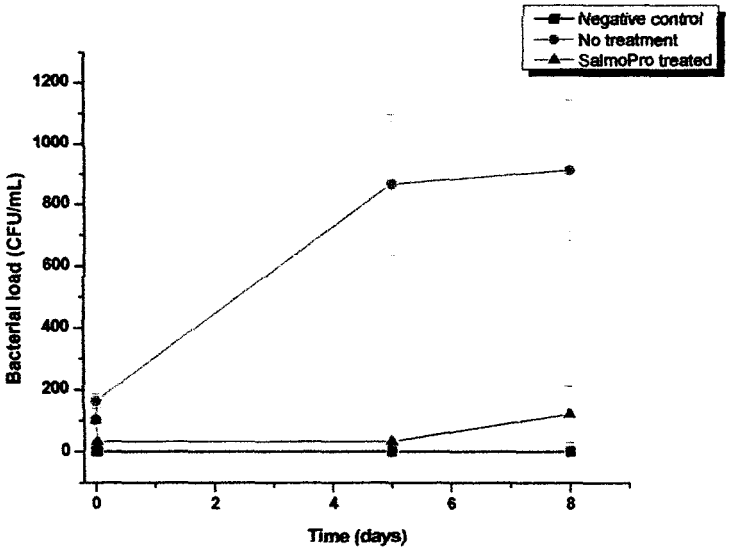

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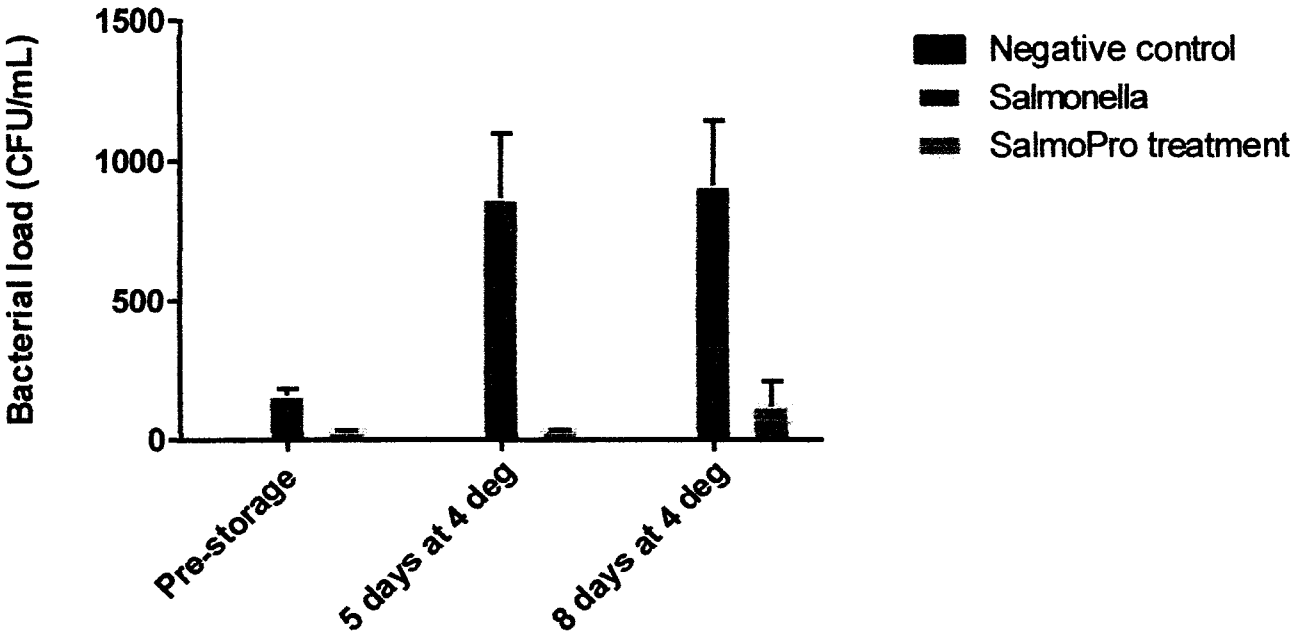
Figure 3: Reduction of *Salmonella* counts in chicken trims treated with SalmoPro ®



11.4 Statistical analysis

Statistical analysis was performed using version GraphPad Prism 6for Windows (GraphPad software; San Diego, CA; www.graphpad.com)

The efficacy of SalmoPro ® treatment in reducing the number of viable *Salmonella* in the experimentally contaminated chicken samples was evaluated by comparing the data obtained with PBS control samples and SalmoPro® treated samples.

The continued effect of SalmoPro ® was evaluated by comparing the data obtained at time 30 min, after 5 days at 4°C, and after 8 days at 4°C.

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Table 3: Sidak's multiple comparison test (α = 0.05): Effect of storage on the reduction of *Salmonella* counts in chicken trims treated with SalmoPro ® phage solution ($1x10^7$ PFU/g)

SalmoPro® vs untreated samples	Significant?	Adjusted P value
Initial reduction (pre-incubation)	Yes	< 0.0001
5 days post-incubation	No	0.9794
8 days post-incubation	No	> 0.9999

Figure 4: Effect of storage on *Salmonella* growth rate. Comparison between untreated chicken trims and chicken trims treated with SalmoPro ®

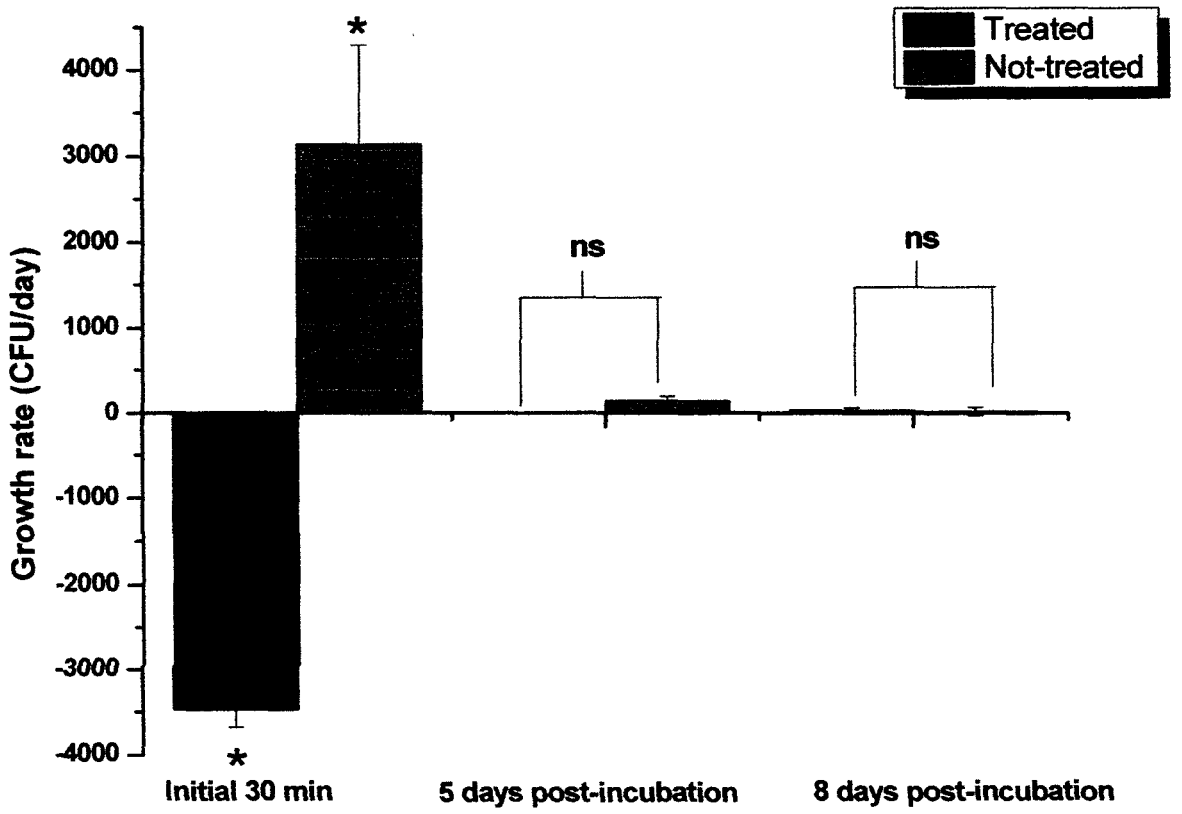

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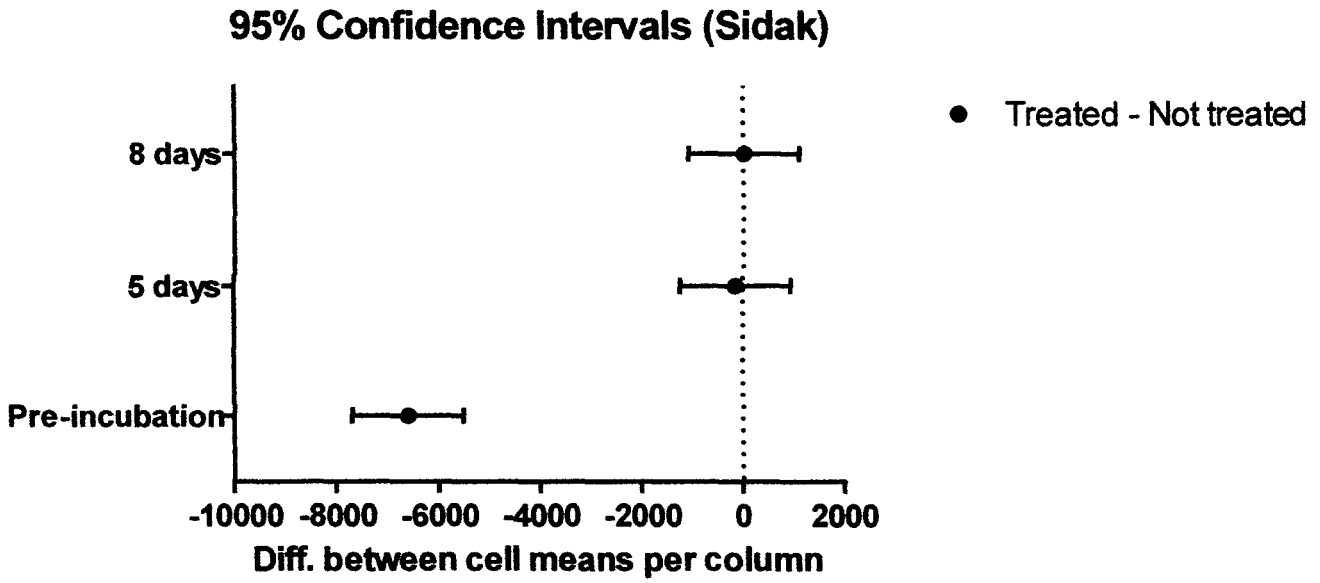
Figure 6: Sidak's multiple comparison test ($\alpha = 0.05$): Effect of storage on the reduction of *Salmonella* counts in chicken trims treated with SalmoPro ®



11.5 Brief discussion of results and study conclusions

The results clearly show that there is a significant initial reduction in the Salmonella counts of chicken trims when treated with SalmoPro®.

SalmoPro® treatment did not have a significant effect on the growth rate of Salmonella after prolonged storage at 4°C (5 days and 8 days).

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12. SUMMARY CONCLUSION OF THE STUDY

SalmoPro® provides an initial, momentary antibacterial effect on treated poultry, and provides no continued technical effect. These results, combined to the fact that SalmoPro® is present in the finished products at insignificant levels, makes SalmoPro® a processing aid, as defined by the FDA in section 21 CFR101.100 (a) (3).

13. SIGNATURES

(b) (6)

Nancy Tawil, Ph.D.
Study Director

Sept. 28, 2015
Date

(b) (6)

Annie Martineau, M.Sc.
Research scientist

2015/09/28
Date

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SUBMISSION END