ORIGINAL SUBMISSION

The Columbus Center
701 East Pratt Street
Baltimore, MD 21202
T 877-ITX-PHAGE
F 410-625-2506
E info@intralytix.com
W intralytix.com

intralytix
SAFETY BY NATURE

GRN 000528

14 July 2014

Dr. Paulette Gaynor
Senior Regulatory Health Project Manager
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
U.S. Food & Drug Administration
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

RECEIVED
JUL 15 2014
OFFICE OF
FOOD ADDITIVE SAFETY

Reference: Intralytix GRAS Notification for ListShield™

Dear Dr. Gaynor:

In accordance with the Federal Register [62 FR 18938 (17 April 1997)] proposed regulations for GRAS notifications (21CFR§170.36), Intralytix is pleased to submit a notice that the bacteriophage cocktail, ListShield™, has been determined by us to be generally recognized as safe, through scientific procedures, and is exempt from the pre-market approval requirements for the use in foods as an antimicrobial to control *Listeria monocytogenes* when applied at up to 10^8 PFU/g of food.

If there are any questions or concerns, please contact us.

Sincerely,

(b) (6)

Alexander Sulakvelidze
Vice President & Chief Scientist
Intralytix, Inc

The Columbus Center
701 East Pratt Street
Baltimore, MD 21202
T 877-ITX-PHAGE
F 410-625-2506
E info@intralytix.com
W intralytix.com

intralytix
SAFETY BY NATURE™

14 July 2014

Dr. Paulette Gaynor
Senior Regulatory Health Project Manager
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
U.S. Food & Drug Administration
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

Reference: Intralytix GRAS Notification for ListShield™

Dear Dr. Gaynor:

In accordance with the Federal Register [62 FR 18938 (17 April 1997)] proposed regulations for GRAS notifications (21CFR§170.36), Intralytix is pleased to submit a notice that the bacteriophage cocktail, ListShield™, has been determined by us to be generally recognized as safe, through scientific procedures, and is exempt from the pre-market approval requirements for the use in foods as an antimicrobial to control *Listeria monocytogenes* when applied at up to 10^8 PFU/g of food.

If there are any questions or concerns, please contact us.

Sincerely,

(b) (6)

Alexander Sulakvelidze
Vice President & Chief Scientist
Intralytix, Inc.

Intralytix

GRAS Notification:

ListShield™

TABLE OF CONTENTS

1 GRAS EXEMPTION CLAIM 4
1.1 NAME & ADDRESS OF NOTIFIER 5
1.2 COMMON OR USUAL NAME 5
1.3 CONDITIONS OF USE 5
1.4 BASIS FOR THE GRAS DETERMINATION 5
1.5 AVAILABILITY OF INFORMATION 6
2 IDENTITY AND SPECIFICATIONS OF LISTSHIELD™ 7
2.1 IDENTITY 7
2.2 METHOD OF MANUFACTURE 7
2.3 SPECIFICATIONS 8
2.4 CHARACTERISTIC PROPERTIES 8
2.5 PHAGE CLASSIFICATION 10
2.6 POTENTIAL HUMAN TOXICANTS 11
2.7 STABILITY 11
3 SELF-LIMITING LEVELS OF USE 11
4 BASIS OF DETERMINATION GRAS: GRAS THROUGH SCIENTIFIC PROCEDURES 11
4.1 PREVIOUS REGULATORY APPROVALS 11
4.2 COMPONENTS OF LISTSHIELD™ 12
4.2.1 Phages 12
4.2.2 Sodium chloride 18
4.2.3 By-products 18
4.3 MANUFACTURING OF LISTSHIELD™ 18
4.3.2 Quality Control 21
4.4 APPLICATION RATES AND DIETARY INTAKE 23
4.4.1 Application rates 23
4.4.2 Dietary intakes 23
4.5 SUBSTANTIAL EQUIVALENCE TO PREVIOUSLY APPROVED BACTERIOPHAGE PRODUCTS 27
4.5.1 Previously approved bacteriophage cocktails 27
4.6 SUMMARY AND BASIS FOR GRAS 28
5 REFERENCES 31
6 APPENDICES 34
6.1 EFFICACY DATA 34

LIST OF TABLES

Table 1 Product specifications for individual monophage lots..... 8
Table 2 Product specifications for ListShield™..... 8
Table 3 Composition of ListShield™ 9
Table 4 Genome size and composition of phages contained in ListShield™ 16
Table 5 Lytic activity of ListShield™ against non-*L. monocytogenes* strains of bacteria.....17
Table 6 Summary of *L. monocytogenes* host strain specifications.....20
Table 7 Volume of ListShield™ consumed per day when applied at $1x10^8$ PFU/g food.....24

LIST OF FIGURES

Figure 1 Overview of ListShield™ manufacturing process 36

1 GRAS EXEMPTION CLAIM

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

The bacteriophage cocktail, ListShield™, has been previously FDA-cleared for direct applications onto meat and poultry products that comply with the ready-to-eat definition (21 CFR §172.785). It has been also EPA-registered for application on non-food contact surfaces in food processing facilities and other food-handling establishments (EPA Reg. Number 74234-1). ListShield™ has been determined by Intralytix, Inc., to be generally recognized as safe, through scientific procedures, and is exempt from the pre-market approval requirements under the intended use conditions described within this notification.

(b) (6)

July 11, 2014

Alexander Sulakvelidze, Ph.D.

VP Research and Development

asulakvelidze@intralytix.com

Date

1.1 NAME & ADDRESS OF NOTIFIER

Intralytix, Inc.
701 E Pratt St.
Baltimore, MD 21202
Tel: 877-489-7424
Fax: 410-625-2506

1.2 COMMON OR USUAL NAME

Intralytix produces a lytic bacteriophage cocktail with potent lytic activity against Gram-positive bacterium *Listeria monocytogenes* under the trade name ListShield™.

1.3 CONDITIONS OF USE

ListShield™ is intended for use as an antimicrobial to control *L. monocytogenes* on food when applied to food surfaces up to $1x10^8$ plaque forming units (PFU) / gram of food, including the following food categories:

- Fish and shellfish (including smoked varieties; e.g., smoked salmon)
- Fresh and processed fruits
- Fresh and processed vegetables
- Dairy products (including cheese)

1.4 BASIS FOR THE GRAS DETERMINATION

Pursuant to the GRAS proposed rule, Intralytix has determined that ListShield™ is GRAS through scientific procedures.

1.5 AVAILABILITY OF INFORMATION

The data and information that are the basis for Intralytix's determination of GRAS for ListShield™ are available for review and copying by FDA or will be sent to FDA upon request, made to:

Intralytix
Joelle Woolston
The Columbus Center
701 E Pratt St.
Baltimore, MD 21202
jwoolston@intralytix.com

2 IDENTITY AND SPECIFICATIONS OF LISTSHIELD™

2.1 IDENTITY

ListShield™ consists of a mixture of equal proportions of six individually purified lytic bacteriophages (hereinafter referred to as component phage(s) or component monophage(s)). Each of these monophages is specifically effective against *Listeria monocytogenes* serotypes. The component phages in ListShield™ were isolated from the environment and have not been genetically manipulated in any way (i.e., not GMO).

The current ListShield™ article of commerce is a liquid made up of equal parts of six monophages that combined have a minimum lytic titer of 10.0 ± 0.33 $\log_{10}$ PFU per mL. This article of commerce is a concentrate that is normally diluted with water at the application site to form the ListShield™ working solution, typically with a lytic titer of ca. 9.0 ± 0.33 $\log_{10}$ PFU/mL. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food.

2.2 METHOD OF MANUFACTURE

The component monophages of ListShield™ are prepared using an aerobic fermentation process in animal-product free media. For each monophage, its host *L. monocytogenes* strain is grown to a target OD_{600}, at which point the culture is infected with the monophage at a previously determined MOI (multiplicity of infection; the ratio of phage to bacteria) and the combination is incubated with aeration and mixing. The suspension is clarified by removal of bacteria by filtration. Following the initial filtration, the monophage is concentrated, washed with 0.1M sodium chloride, then sterilized using 0.22μm filtration. After all six component monophages have each passed quality control specifications, proper volumes of each monophage, and 0.1M sodium chloride as necessary, are combined to form the ListShield™ article of commerce so that:

Each monophage is equally represented

AND

The mean lytic titer is ≥10.0 ± 0.33 $\log_{10}$ PFU/mL

The ListShield™ article of commerce is typically diluted with water at the application site, to form the “working solution” or “working concentration” of ListShield™ with a mean lytic titer of 9.0 ± 0.33 $\log_{10}$ PFU/mL. Figure 1 provides an overall schematic of the process.

2.3 SPECIFICATIONS

Due to the two-step manufacturing process, there are two levels of quality control. First, each individual monophage lot is analyzed to ensure it meets the release specifications listed in Table 1 before it can be used to prepare a lot of ListShield™.

Table 1 Product specifications for individual monophage lots

Parameter	Specification
Potency (PFU/mL)	≥10.0 ± 0.33 $\log_{10}$ PFU/mL
Microbial sterility	No growth
RFLP	Identical to reference

Only after all component monophages have met the release specifications can a lot of ListShield™ be produced. Each lot of ListShield™ is analyzed to ensure it meets the following release specifications listed in Table 2.

Table 2 Product specifications for ListShield™

Parameter	Specification	Limit of detection
Potency (mean titer)	≥10.0 ± 0.33 $\log_{10}$ PFU/mL	n/a
Listeriolysin O	None detected (at ca. 10.0 ± 0.33 $\log_{10}$ PFU/mL)	5 HU/mL
Microbial sterility	No growth	100 CFU/lot

2.4 CHARACTERISTIC PROPERTIES

ListShield™ is a clear to opalescent odorless liquid with a specific gravity of approximately 1.01. The phage component of ListShield™ (typical working solution of 1 x 10^9 PFU/mL) is roughly estimated to be 0.0000292% by weight and the remainder is 0.1M sodium chloride. Typical composition of ListShield™ (at the typical working concentration of ca 1 x 10^9 PFU/mL) is shown below. The values shown are derived (averages) from the chemical analysis of three separate ListShield™ lots.

Table 3 Composition of ListShield™
(at typical working concentration of 1x10^9 PFU/mL, unless noted)

Property/analysis/composition	Lot# 0113E300142	Lot# 0113F200116	Lot# 0113F210146	ListShield™ typical
Total nitrogen (mg/L)	1.9	1.7	1.9	1.8
Specific gravity (at 25°C)	1.01	1.01	1.01	1.01
Ash (inorganic solids) %	ND	ND	ND	ND
Arsenic (mg/L)	ND	ND	ND	ND
Barium (mg/L)	ND	ND	ND	ND
Cadmium (mg/L)	ND	ND	ND	ND
Calcium (mg/L)	0.195	0.187	0.171	0.184
Chromium (mg/L)	ND	ND	ND	ND
Cobalt (mg/L)	ND	ND	ND	ND
Copper (mg/L))	ND	ND	ND	ND
Iron (mg/L)	ND	ND	ND	ND
Lead (mg/L)	0.005	ND	ND	≤0.005
Magnesium (mg/L)	ND	ND	ND	ND
Manganese (mg/L)	ND	ND	ND	ND
Molybdenum (mg/L)	ND	ND	ND	ND
Nickel (mg/L)	ND	ND	ND	ND
Potassium (mg/L)	ND	ND	ND	ND
Silicon (mg/L)	0.1	ND	ND	≤0.1
Sodium (mg/L)	205	160	191	185
Tin (mg/L)	ND	ND	ND	ND
Zinc (mg/L)	ND	ND	ND	ND
Chloride (mg/L)	399	300	319	339
Nitrate (as N) (mg/L)	0.23	ND	ND	≤0.23
Nitrite (as N) (mg/L)	ND	ND	ND	ND
Total Organic Carbon (mg/L)	16.1	15.3	21.6	17.7
Total Kjeldahl Nitrogen (mg/L)	1.67	1.72	1.9	1.8
Total Dissolved Solids (mg/L)	598	456	478	511
Total Phosphorous (mg/L)	0.94	0.84	0.91	0.90
Silica (mg/L)	ND	ND	ND	ND
LLO (HU/mL)*	ND	ND	ND	ND

ND = none detected

* Measured in undiluted “article of commerce” (ca. 1 x 10^{10} PFU/ml) preparation

2.5 PHAGE CLASSIFICATION

The current component phages in ListShield™ were isolated by Intralytix's scientists from the environment. Each monophage was fully characterized by a variety of methods, including pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), electron microscopy (EM), full-genome sequence analysis, lytic activity against *L. monocytogenes* strains, and lytic activity against non-*L. monocytogenes* strains.

The six component bacteriophages currently included in ListShield™ are listed below:

Name:	LIST-36
ATCC #:	PTA-5376
Order:	Caudovirales
Family:	*Siphoviridae*
Properties:	Double-stranded DNA, Lytic
Name:	LMSP-25
ATCC #:	PTA-8353
Order:	Caudovirales
Family:	*Myoviridae*
Properties:	Double-stranded DNA, Lytic
Name:	LMTA-34
ATCC #:	PTA-8354
Order:	Caudovirales
Family:	*Myoviridae*
Properties:	Double-stranded DNA, Lytic
Name:	LMTA-57
ATCC #:	PTA-8355
Order:	Caudovirales
Family:	*Myoviridae*
Properties:	Double-stranded DNA, Lytic
Name:	LMTA-94
ATCC #:	PTA-8356
Order:	Caudovirales
Family:	*Myoviridae*
Properties:	Double-stranded DNA, Lytic
Name:	LMTA-148
ATCC #:	PTA-8357
Order:	Caudovirales
Family:	*Myoviridae*
Properties:	Double-stranded DNA, Lytic

2.6 POTENTIAL HUMAN TOXICANTS

The host strains are *Listeria monocytogenes*, Gram-positive bacteria that can produce listeriolysin O, a toxin associated with *L. monocytogenes* infectivity. Intralytix tests every lot of ListShield™ for LLO to ensure it meets the release criteria, that no LLO is detectable (in ca. $1x10^{10}$ PFU/mL). LLO is further discussed below, in Sections 4.2.3, 4.3.1.4, and 4.3.2.2.

2.7 STABILITY

The proposed shelf life of ListShield™ article of commerce is one year when stored at 2–6°C in a dark, UV-protected area.

3 SELF-LIMITING LEVELS OF USE

The proposed use for ListShield™ is as an antimicrobial processing aid for foods that are at high risk to be contaminated with *L. monocytogenes*. The purpose of ListShield™ is to significantly reduce or eliminate *L. monocytogenes* in the final product, thus making it safer for human consumption.

The self-limiting levels of use are:

- Due to the cost of the product, the end-user would use the minimum dose required to achieve a significant reduction or elimination of *L. monocytogenes*.
- Once the *L. monocytogenes* contamination is depleted, the phage will slowly decrease in number due to a lack of host.
- Phages are susceptible to many environmental factors, including sunlight, heat, and UV light. Exposure to these will cause the number of phage to decrease.

4 BASIS OF DETERMINATION GRAS: GRAS THROUGH SCIENTIFIC PROCEDURES

4.1 PREVIOUS REGULATORY APPROVALS

ListShield™ (formerly LMP-102) is FDA-listed as a food additive permitted for direct addition to food for human consumption in 21 CFR §172.785; to be used in accordance with current good

manufacturing practice to control *L. monocytogenes* by direct application to meat and poultry products that comply with the ready-to-eat definition in 9 CFR §430.1.

ListShield™ is listed on the USDA FSIS Directive 7120.1 as a safe and suitable ingredient for use production of various RTE meat and poultry products.

ListShield™ is also EPA registered (EPA registration 74234-1) for application on non-food contact surfaces in food facilities and other establishments.

4.2 COMPONENTS OF LISTSHIELD™

ListShield™ is a mixture of component bacteriophages together with added sodium chloride and small amounts of residual production by-products. The primary active ingredient is not a single chemical substance but a mixture of naturally-occurring bacteriophages. In the appropriate sections below, we consider separately the safety of the:

- Phages (active component)
- Added salts
- Manufacturing by-products

4.2.1 Phages

The safety and ubiquity of bacteriophages have been well established. The pertinent safety data on bacteriophages is reviewed below. The published literature on phages, and other information developed by Intralytix, shows that:

- Bacteriophages are the most ubiquitous organisms on earth. For example, one milliliter of non-polluted stream water has been reported (Bergh et al., 1989) to contain approximately 2 x 10^8 PFU of phages/mL, and the total number of phages on this planet has been estimated to be in the range of 10^{30} – 10^{32} (see http://www.asm.org/division/m/M.html and Brussow & Hendrix, 2002). This abundance of phages in the environment, and the continuous exposure of animals to them, explains the extremely good tolerance of mammalian organisms to phages.

- Phages have been used therapeutically in humans for more than 80 years, without any serious side effects. During the long history of using phages as therapeutic agents in Eastern Europe and the former Soviet Union (and, before the antibiotic era, in the United States, France, Australia, and other countries), phages have been administered to humans:
    - orally, in tablet or liquid formulations,

- rectally,
- locally (skin, eye, ear, nasal mucosa, etc.); in tampons, rinses and creams,
- as aerosols or intrapleural injections, and
- intravenously

- There have been virtually no reports of serious complications associated with their use. Recent reviews summarize the results of some of the human therapy studies involving bacteriophages (Alisky et al., 1998; Sulakvelidze et al., 2001; Summers, 2001).

- Phages have also been administered to humans for non-therapeutic purposes without any recorded illness or death. To give just a few examples, phage preparations have been used extensively to monitor humoral immune function in humans in the United States in the 1970s-1990s, including in patients with Down's syndrome, the Wiskott-Aldrich syndrome, and immunodeficient patients (Lopez et al., 1975; Ochs et al., 1992; Ochs et al., 1982; Ochs et al., 1993a). In some of the studies (including several studies performed by the FDA), the purified phages were injected intravenously into HIV-infected patients or other immunodeficient individuals without any apparent side effects (Fogelman et al., 2000; Ochs et al., 1971; Ochs et al., 1993b).

- Phages have also been administered to humans via various sera and FDA-approved vaccines commercially available in the United States (Merril et al., 1972; Milch & Fornosi, 1975; Moody et al., 1975).

- The biology of phages has been exhaustively studied. These studies have clearly shown that phages are obligate intracellular parasites of bacteria and are not infectious in humans or other mammals.

- Bacteriophages are common commensals of the human gut, and they are likely to play an important role in regulating the diversity and population structure of various bacteria in human GI tracts. For example, phages capable of infecting *E. coli, Bacteroides fragilis* and various *Salmonella* serotypes have been isolated from human fecal specimens in concentrations as high as 10^5 PFU/100 g of feces (Calci et al., 1998; Furuse et al., 1983; Havelaar et al., 1986). The recent data based on metagenomic analyses (using partial shotgun sequencing) of an uncultured viral community from human feces suggested that bacteriophages are the second most abundant category after bacteria in the uncultured fecal library (Breitbart et al., 2003).

- No adverse immunologic or allergic sequelae have ever been reported because of human or animal exposure to phages (Alisky et al., 1998; Sulakvelidze et al., 2001).

- Bacteriophages are commonly consumed via drinking water (Armon et al., 1997; Armon & Kott, 1993; Grabow & Coubrough, 1986; Lucena et al., 1995).

- Bacteriophages are natural components of all fresh, unprocessed foods and are commonly consumed via various foods. For example, bacteriophages have been

commonly isolated from a wide range of food products, including ground beef, pork sausage, chicken, farmed freshwater fish, common carp and marine fish, oil sardine, raw skim milk, and cheese (Atterbury et al., 2003; Eller et al., 2012; Gautier et al., 1995; Greer, 2005; Greer et al., 2007; Hsu et al., 2002; Kennedy et al., 1984; Whitman & Marshall, 1971). Several studies have suggested that 100% of the ground beef and chicken meat sold at retail contain various levels of various bacteriophages. To give just a few examples, bacteriophages were recovered from 100% of examined fresh chicken and pork sausage samples and from 33% of delicatessen meat samples analyzed by Kennedy et al (1984). The levels ranged from 3.3 to 4.4 x 10^{10} PFU/100 g of fresh chicken, up to 3.5 x 10^{10} PFU/100 g of fresh pork, and up to 2.7 x 10^{10} PFU/100 g of roast turkey breast samples.

- Because of the (1) highly specific nature of bacteriophages and (2) extremely common exposure of humans and animals to bacteriophages (including daily consumption of bacteriophages with various foods and drinking water), bacteriophages do not deleteriously affect the GI microflora. For example, when *E. coli*-specific phage T4 was administered orally to 15 healthy adult volunteers, it did not cause a decrease in total fecal *E. coli* counts. In addition, no substantial phage T4 replication on the commensal *E. coli* population was identified, and no adverse events related to phage application were observed in any of the volunteers (Bruttin & Brussow, 2005).

- Bacteriophages are commonly consumed by animals (including agriculturally-important species) via various foods. For example, in a recent study from Texas A&M University (Maciorowski et al., 2001), male-specific and somatic coliphages were detected in all animal feeds, feed ingredients, and poultry diets examined, even after the samples were stored at -20°C for 14 months.

4.2.1.1 Lytic phages are GRAS

All lytic phages are, by nature, GRAS. There are two major types of phages: “virulent” (also called “lytic”) and “temperate” (often mistakenly called “lysogenic”). Lytic phages lyse host bacteria without integrating into the host genome. In contrast, temperate phages may integrate into the host genome and a small subset of these may theoretically transduce undesirable bacterial genes, such as those encoding toxins or antibiotic resistance. Both lytic and temperate phages are extremely common in the environment, the human and animal gut, the human oral cavity, foods sold at retail, sewage, and many other places that we encounter daily. Humans shed large numbers of both lytic and temperate phages into the environment every day – estimated to be on the order of 4 x 10^9 single phage daily per person (Sulakvelidze & Barrow, 2005). Temperate phages are found in almost all bacterial genera, including *Staphylococcus*, *Vibrio*, *Pseudomonas*, *Salmonella*, *Shigella*, *Bacillus*, *Corynebacterium*, *Listeria,* and *Streptococcus* (Jacob & Wollman, 1959; Schicklmaier & Schmieger, 1995; Eggers et al., 2001; Langley et al., 2003). Indeed, some strains can release as many as five different types of temperate phages. Although the possibility of added gene transfer events is highly unlikely to bring danger to any individual consuming temperate phages, the use of such phages on an industrial scale could increase the overall risk of potentially harmful genes being acquired by new bacterial strains. Therefore, Intralytix identifies and uses only lytic phages in its phage preparations (including ListShield™).

4.2.1.2 ListShield™ monophages are GRAS

The current component phages in ListShield™ were isolated by Intralytix's scientists from the water of the Inner Harbor of Baltimore. Each was characterized by various approaches, including electron microscopy, genotypic fingerprinting, and full genome sequence analysis. The component phages in ListShield™ are members of the *Siphoviridae* and *Myoviridae* double-stranded DNA phage families, as defined by the International Committee on the Taxonomy of Viruses (ICTV) and by Ackermann and Berthiaume (1995).

Intralytix has fully sequenced all component monophages included in ListShield™. This approach is used to exclude bacteriophages carrying sequences encoding undesirable genes (e.g., bacterial toxin genes), and phages displaying prior evidence of transduction (e.g., bacterial 16s RNA genes).

Intralytix excludes all bacteriophages carrying sequences encoding any undesirable genes. Undesirable genes include genes encoding bacterial toxins (including genes listed in 40 CFR § 725.421) or genes associated with drug resistance. Undesirable genes are identified by comparing a complete bacteriophage sequence to all sequences contained in GenBank and other databases available through the National Center for Biotechnology Information website of the National Library of Medicine using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/).

The cut-off *e*-value level for the latter analysis was 1×10^{-4}, which detects virtually all undesirable genes in the phages' genomes. In practice, significant matches are considered to be those with *e*-values of $\leq 10^{-5}$ (Miller et al., 2003). Therefore, our proposed cut-off value provides a very strong (10-fold higher than the proposed 10^{-5} cut-off) assurance that undesirable genes are not missed during the analysis.

Intralytix has sequenced the complete genome of each phage incorporated into ListShield™. Table 4 summarizes their genome properties. Analysis of the sequences yielded the following results:

- No toxin genes have been identified among the open reading frames of the annotated genomes of any of the six monophages.
- No 16S ribosomal RNA genes have been identified among annotated genomes of any of the six monophages.
- No antibiotic resistance genes have been identified among annotated genomes of any of the six monophages.

Summary: The approach of obtaining the full nucleotide sequence for each commercialized phage and complete bioinformatics analysis of all open reading frames insures that no detrimental genes

are present in any of the phages used. This provides the fullest assurance of the phage safety as can presently be obtained by any method.

Table 4 Genome size and composition of phages contained in ListShield™

Phage	ATCC #	GenBank Accession #	GC%	Size (bp)	Number of open reading frames (ORFs)	Undesirable genes
LIST-36	PTA-5376	KJ535721	36.0	131,952	191	None
LMSP-25	PTA-8353	KJ535722	35.9	138,038	201	None
LMTA-34	PTA-8354	KJ668715	36.4	113,482	159	None
		KJ668716	34.4	11,210	27	
		KJ668717	34.1	8,458	13	
		KJ668718	31.6	2,341	5	
LMTA-57	PTA-8355	KJ591605	35.8	136,589	210	None
LMTA-94	PTA-8356	KJ586795	35.8	136,470	203	None
LMTA-148	PTA-8357	KJ591604	36.0	132,541	187	None

4.2.1.3 ListShield™ is specific to *L. monocytogenes*

Lytic activity of ListShield™ is targeted against *L. monocytogenes* strains. ListShield™ has been screened for its lytic activity against 275 *L. monocytogenes* strains in the Intralytix collection. ListShield™ is effective against our collection, lysing 96% of the strains in the collection at ca. $1x10^9$ PFU/mL.

ListShield™ is also highly specific. Table 5 shows that ListShield™ does not lyse any of the non-*L. monocytogenes* Gram-positive strains examined. These strains include strains of *Staphylococcus aureus* and *Enterococcus* species. ListShield™ also does not lyse strains of several Gram-negative bacteria examined, including 5 strains each of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, and various *Shigella* species. Bruttin & Brussow (2005) demonstrated orally administration of *E. coli*-specific phage T4 did not affect fecal *E. coli* counts and had no adverse effects in any volunteers. Therefore, ListShield™ would also have no deleterious effect upon the natural gut flora – as recently substantiated by the in vivo data by Mai et al (2010).

Table 5 Lytic activity of ListShield™ against non-*L. monocytogenes* strains of bacteria

Non-L. monocytogenes strains		Species	Susceptibility to ListShield™ ($1x10^9$ PFU/mL)
Intralytix ID	Original ID		
Sa36	ATCC25923	*Staphylococcus aureus*	-
Sa37	ATCC29213	*Staphylococcus aureus*	-
Sa211	ATCC700699	*Staphylococcus aureus*	-
Sa298	ATCC49775	*Staphylococcus aureus*	-
Sa299	ATCC14458	*Staphylococcus aureus*	-
E102	WCC188	*Enterococcus* spp.	-
E402	ATCC11823	*Enterococcus faecalis*	-
E403	ATCC19433	*Enterococcus faecalis*	-
E404	1133455	*Enterococcus avium*	-
E405	1126611	*Enterococcus faecalis*	-
Ab3	ATCC19606	*Acinetobacter baumannii*	-
Ab4	HER1401	*Acinetobacter baumannii*	-
Ab5	4308-2	*Acinetobacter baumannii*	-
Ab6	3247-1	*Acinetobacter baumannii*	-
Ab7	1673-2	*Acinetobacter baumannii*	-
Pa76	ATCC10145	*Pseudomonas aeruginosa*	-
Pa161	ATCC15692	*Pseudomonas aeruginosa*	-
Pa162	ATCC51674	*Pseudomonas aeruginosa*	-
Pa163	ATCC43390	*Pseudomonas aeruginosa*	-
Pa164	ATCC39324	*Pseudomonas aeruginosa*	-
S.E566	ATCC13076	*Salmonella enterica* serovar Enteritidis	-
S.T567	ATCC13311	*Salmonella enterica* serovar Typhimurium	-
S.H568	ATCC51956	*Salmonella enterica* serovar Hadar	-
S.T795	ATCC19585	*Salmonella enterica* serovar Typhimurium	-
S.He899	ATCC8326	*Salmonella enterica* serovar Heidelberg	-
SH.d1	514	*Shigella dysenteriae*	-
SH.f6	045-311082	*Shigella flexneri*	-
SH.f20	300	*Shigella flexneri 2*	-
SH.s43	90	*Shigella sonnei*	-
SH.s52	ATCC9290	*Shigella sonnei*	-
Ec147	ATCC43895	*Escherichia coli* O157:H7	-
Ec148	ATCC35401	*Escherichia coli* O78:H11	-
Ec150	ATCC700728	*Escherichia coli* O157:H7	-
Ec154	ATCC11303	*Escherichia coli*	-
Ec155	ATCC12435	*Escherichia coli*	-

+ Lysed by phage cocktail - Not lysed by phage cocktail

4.2.2 Sodium chloride

Sodium chloride "salt" is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

4.2.3 By-products

The *L. monocytogenes* host strains are Gram-positive bacteria. LLO are further discussed below, in Sections 4.3.1.4 and 4.3.2.2. Otherwise, there are no known potentially noxious by-products in ListShield™.

4.3 MANUFACTURING OF LISTSHIELD™

ListShield™ is prepared by cultivation of individual host *L. monocytogenes* strain/phage combinations followed by filtration, concentration, wash, and final sterile filtration. After each monophage passes quality control, the monophages are combined with 0.1M sodium chloride to form the ListShield™ concentrate. Final filtration is then carried out with a sterilizing grade filter.

4.3.1.1 Starting components

There are four starting components for manufacture of ListShield™ components monophages:

- Animal-product free media
- Antifoam
- Host strains
- Monophages

The safety of each is considered separately below.

4.3.1.2 Animal-product free media

The animal-product free media is a vegan custom blend. The main components are described here and have an existing regulatory status as regulated GRAS ingredients or additives.

Phytone Peptone and *Soytone:* Peptones are GRAS affirmed at 21 CFR § 184.1553 for use as processing aids, among other uses, at levels not to exceed good manufacturing practice. Peptones are protein hydrolysates consisting of free amino acids and short peptides in an aqueous salt solution.

Yeast Extract: Yeast extract is a commonly used food ingredient. For example, baker's yeast extract is GRAS affirmed as a flavoring agent or adjuvant at up to 5% in foods generally. 21 CFR § 184.1983.

Sodium Chloride: Sodium chloride "salt" is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

Magnesium Sulfate: Magnesium sulfate salt is GRAS affirmed at 21 CFR § 184.1443 for use as a processing aid, among other uses, at levels not to exceed good manufacturing practice. Magnesium sulfate is a component of the animal-product free growth media used in ListShield™ production.

4.3.1.3 Antifoaming agent

P2000 antifoam is polypropylene glycol-based, Kosher-certified product, approved for a variety of food additive uses, both direct and indirect (The Dow Chemical Company, Midland, Michigan; http://www.dow.com). Small amounts of the P2000 antifoam are used in the initial fermentation of the individual monophages.

4.3.1.4 Host strains

The component monophages are produced on *L. monocytogenes*. Strains from Intralytix's collection of *L. monocytogenes* strains were selected as the production hosts for ListShield™ component monophages (Table 6). The *L. monocytogenes* host strains were characterized at Intralytix for their biochemical properties, background genomic composition/type, the presence of endogenous phage(s), and their susceptibility to five commonly prescribed antibiotics (ampicillin, penicillin, gentamicin, meropenem, and sulfamethoxazole/trimethoprim).

Table 6 Summary of *L. monocytogenes* host strain specifications

Current *L. monocytogenes* host strain	Phage	Serotype	Biochemistry	PFGE	Endogenous phage	Antibiotic susceptibility
Lm117	LIST-36	4b	*Listeria monocytogenes*	Itx type P-28	-	4/5*
Lm57	LMSP-25, LMTA-34	3b	*Listeria monocytogenes*	Itx type P-16	-	4/5**
Lm94	LMTA-57, LMTA-94	4b	*Listeria monocytogenes*	Itx type P-6	-	4/5**
Lm114	LMTA-148	4b	*Listeria monocytogenes*	Itx type P-9	-	4/5*

* Resistant to penicillin

** Intermediate susceptibility to penicillin

Listeriolysin O, is a toxic protein produced by *L. monocytogenes* and associated with its infectivity (Geoffroy, Gaillard et al. 1987). LLO is a sulfhydryl-activated (SH-activated) toxin with inflammatory activity in animals and humans produced by its capacity to lyse membranes and cellular organelles. It is cytolytic for many eukaryotic cells and cardiotoxic and lethal to mice at levels similar to other SH-activated protein toxins. The LD_{50} of purified LLO was determined by i.v. injection in mice to be 0.8 µg per mouse or 0.8 µg/0.03 kg body weight (bw) = 26.7 µg/kg bw. Matar et al. have shown that *L. monocytogenes* strains of serotype 1/2a and 4b may produce two variants of LLO (1/2a and 4b variants) (Matar et al., 1992). The possible existence of another variant of LLO (β-listeriolysin) was reported by Parrisius et al., who suggested that 55–60 kDa toxin was immunologically distinct from the previously-recognized LLO (the gene encoding β-LLO has not yet been identified) (Parrisius et al., 1986). The three LLO variants are slightly different in size (LLO of 1/2a is ca. 55.7 kDa, LLO of 4b is ca. 54.9 kDa, and β-LLO is ca. 55-60 kDa), but they all have similar hemolytic activities, which can be measured by the same, standard LLO assay (Matar et al., 1992; Parrisius et al., 1986). The scientific literature on human *L. monocytogenes* infection identifies this single substance, listeriolysin O, as toxic in humans.

The potency of SH-activated toxins is typically determined by their ability to lyse human and animal erythrocytes. One hemolytic unit (HU) is the amount of toxin needed to release half the hemoglobin (50% lysis) of the erythrocytes in a standard assay (Appendix H32). Since the specific activity of LLO is 10^6 HU/mg, the LD_{50} of LLO in the mouse is therefore approximately 26,700 HU/kg body weight.

The stability of LLO as a function of pH and temperature has been investigated (Matar et al., 1992). Matar et al. have shown that 1/2a and 4b variants of LLO are maximally stable at pH 8 and irreversibly degrade at low pH. More specifically, when incubated at pH 5 and 4°C, LLO from strain 4b lost all of its hemolytic activity in 1 h; LLO from strain 1/2a lost half of its activity under these conditions. LLO 1/2a was completely inactivated when stored for 24 h in the elution buffer from an affinity purification column (0.1 M acetic acid, 0.85% NaCl, pH 2.8). The irreversibility of this inactivation at low pH may be inferred by the fact that activity was not regained upon serial

dilution and incubation in 0.01 M PBS at pH 6, an optimum pH for hemolytic activity. Thus, LLO is expected to rapidly and irreversibly degrade (i.e. denature) when subjected to 0.1 M HCl in the stomach. It is also likely that this protein when administered orally will be hydrolyzed by the acid and enzymes in the stomach and will not pose a toxic threat to humans.

Although LLO variants have slightly different molecular sizes, they are all ≤ 60 kDa in size, and it is likely that a significant fraction of all LLO molecules that may be present in the crude production medium will pass through the filtration during the 0.1M NaCl exchange step (100-kDa filter). Typical levels of listeriolysin released in conventional culture media average from 20–200 HU/ml (Geoffroy et al., 1987). The production process eliminates most of the LLO; the concentration in ListShield™ is undetectable with a method sensitive to approximately 5 HU/mL. While LLO would not be expected to survive its passage in the stomach, to exclude even this possibility, as a standard quality control protocol, Intralytix analyzes every ListShield™ batch for the presence and levels of the LLO in the final product. All product lots must have no detectable LLO with a limit of detection of 5 HU/mL at $1x10^{10}$ PFU/mL level in order to pass the release criteria for LLO. See Section 4.4.2.3 for discussion of dietary intake.

4.3.1.5 Monophages

The safety of monophages is discussed in Section 4.2.1.2.

4.3.2 Quality Control

4.3.2.1 Monophages

The following tests are performed upon each monophage lot:

Lytic titer test

The test measures the lytic titer of each monophage lot, by determining the number of plaque forming units per milliliter (PFU/mL). The specification for each monophage lot is a titer of ≥10.0 ± 0.33 $\log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Bacterial sterility

Bacterial sterility is a determination of the viable microbial contamination in a phage solution. Briefly, a 1% representative sample of each monophage solution is tested by combining with a concentrated growth media and incubating for 14 days. Growth is monitored visually and by plating, if growth is not visually detectable. Both positive and negative controls are included. The

specification is that each monophage lot must be bacteriologically sterile. Lots failing the sterility test may be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Genotypic fingerprinting

Genotypic fingerprinting, through restriction fragment length polymorphism (RFLP), is used to confirm the identity of each monophage lot. The specification for RFLP is that all major bands in the reference pattern must be present. Lots failing the RFLP test will be discarded.

4.3.2.2 ListShield™

The following tests are performed upon each batch of ListShield™:

Lytic titer test

The lytic titer test method is based on determining the mean titer (PFU/mL) of the ListShield™ preparation. The specification for this test is ListShield™ has a mean titer of ≥10.0 ± 0.33 $\log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Bacterial sterility

Bacterial sterility is a determination of the viable microbial contamination in a phage solution. Briefly, a 1% representative sample of each lot of ListShield™ is tested by combining with a concentrated growth media and incubating for 14 days. Growth is monitored visually and by plating, if growth is not visually detectable. Both positive and negative controls are included. The specification for this test is that ListShield™ must be bacteriologically sterile. Lots failing the sterility test will be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Listerolysin O content test

Listeriolysin O (LLO) is a toxin associated with the host bacteria that are removed from phage preparations. Intralytix tests all lots of ListShield™ to ensure they are free of LLO. The specification for this test is each lot of ListShield™ must have no detectable LLO with a limit of detection of 5 HU/mL in ListShield™ (ca. 10.0 ± 0.33 $\log_{10}$ PFU/mL). Lots failing to meet the specification can be washed with sterile 0.1M NaCl and retested.

4.4 APPLICATION RATES AND DIETARY INTAKE

4.4.1 Application rates

The current ListShield™ article of commerce is a 10X concentrate (although more concentrated solutions may be available in the future; e.g., 20X or 50X) that is typically diluted with water at the application site to form the ListShield™ working solution. In all instances, irrespective of the original concentration of the article of commerce, working solution is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food.

4.4.2 Dietary intakes

ListShield™ is envisioned to be used upon foods, including those in the following food categories:

- Fish and shellfish (including smoked varieties; e.g., smoked salmon)
- Fresh and processed fruits
- Fresh and processed vegetables
- Dairy products (including cheese)

The calculations described in the subsequent sections were performed to estimate the dietary intake of ListShield™ when used at the maximum application of $1x10^8$ PFU/g for each of the above food categories.

To determine the daily intake of each of the food categories for the US population as a whole, the Food Availability (Per Capita) Data System, provided by the United States Department of Agriculture's Economic Research Services (USDA ERS, 2014a-c) was used. The per capita usage is a measure of food disappearance that is calculated by dividing the total supply available by the US population and does not account for spoilage and waste. Because losses are not taken into consideration, the per capita estimations are most likely higher than actual consumption.

All calculations below are based on a maximum (worst-case scenario) consumption of ListShield™. This worst-case scenario assumes 100% market saturation (i.e. that the entire food supply is treated with ListShield™), there are no losses from the food supply, and that the maximum application rate of $1x10^8$ PFU/g is used. Even with the added margin of safety added by these overestimations, the amounts of ListShield™, and its constituents, that would be consumed via the five food categories are very small, as shown in the following calculations.

4.4.2.1 Dietary intakes for ListShield™

The following calculation to determine the maximum (worst-case scenario) consumption of ListShield™ by the average American uses the highest rate of ListShield™ application ($1x10^8$ PFU/g):

The maximum concentration recommended for the working solution of ListShield™ is $1x10^9$ PFU/mL. Using that maximum, the volume of ListShield™ that would be applied per gram treated food can be calculated as follows:

$$\frac{1x10^8 \text{ PFU}}{\text{g food}} \times \frac{1 \text{ mL ListShield™}}{1x10^9 \text{ PFU}} = \frac{0.1 \text{ mL ListShield™}}{\text{g food}}$$

Using 0.1mL ListShield™ applied per gram of food, the volume of ListShield™ that would be consumed per day via each food category can be calculated and is presented in Table 7. Assuming the worst case scenario, where 100% of the foods in the five food groups were treated at the maximum application ($1x10^8$ PFU/g), the combined total amount of ListShield™ consumed per day would be about 158 mL or the equivalent of less than ¾ cup.

Table 7 Volume of ListShield™ consumed per day when applied at $1x10^8$ PFU/g food

	Consumed per American per year (lbs)	Consumed per American per day* (g)	ListShield™ consumed per person per day (mL)
Fish/Shellfish	14.2	18	1.8
Fruits	244.7	305	30.5
Vegetables	394.8	492	49.2
Dairy	611.0	761	76.1
Total of 4 categories	1265	1575	157.5

*The ERS per capita usage data (USDA ERS, 2013a-c) is given as lbs/year. This column simply converts lbs/year to grams/day (lbs/year x 1000g ÷ 2.2lbs ÷ 365days).

The majority of the 158 mL of ListShield™ would constitute water; the phages, sodium, and potassium contained within that approximate ¾ cup would be negligible, as evidenced by the dietary calculations presented below.

4.4.2.2 Dietary intakes for ListShield™ phages

The following calculation determines the approximate weight of phages consumed per day, again assuming the maximum rate ($1x10^8$ PFU/g) of ListShield™ application:

Total phages (PFU) consumed per day:

$$\frac{157.5 \text{ mL ListShield™}}{\text{day}} \times \frac{1x10^9 \text{ PFU}}{\text{mL}} = \frac{1.6x10^{11} \text{ PFU}}{\text{day}}$$

Weight of total phages consumed/day (in micrograms):

$$\frac{1.6x10^{11} \text{ PFU}}{\text{day}} \times \frac{2.94x10^{-16} \text{ g}}{\text{phage}} \times \frac{1x10^6 \ \mu\text{g}}{\text{g}} = \frac{46.2 \ \mu\text{g}}{\text{day}}$$

Where:

$2.94x10^{-16}$ g = mass of one phage

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

$$\frac{46.2 \ \mu\text{g}}{\text{day}} \times \frac{\text{day}}{3 \text{ kg}} = 15.4 \text{ ppb}$$

The weight of phages consumed per day via ListShield™ would be 46.2 μg, or 15.4 ppb in a 3 kg diet. This is insignificant.

4.4.2.3 Dietary intake of Listeriolysin O (LLO)

Specification for ListShield™ lots for LLO is no detectable LLO with a limit of detection of 5 HU/mL in the concentrate ($1x10^{10}$ PFU/mL). Assuming a worst case scenario, where the level of LLO is just below the detectable limit then the maximum LLO content possible in the working solution, which is at least a 10-fold dilution of the concentrate, would be 0.5 HU/mL.

The approximate daily volume of ListShield™ consumed is 157.5 mL (see Section 4.4.2.1). Again using the worst-case scenario (maximum allowable LLO level by specification), the maximum amount of LLO consumed via ListShield™ daily is thus:

$$\frac{157.5 \text{ mL ListShield™}}{\text{day}} * \frac{0.5 \text{ HU}}{\text{mL ListShield™}} = \frac{78.7 \text{ HU}}{\text{day}}$$

Therefore the LLO concentration will be approximately 78.7 HU/person/day or 1.1 HU/kg body weight/day for a 70 kg adult. This theoretical adult human oral dose is 23,000-fold lower than the reported LD_{50} of injected LLO in mice (26,700 HU/kg mouse). For a child, assuming a 15kg child with the consumption levels equivalent to that of an adult, the theoretical child oral dose is still 5,000-fold lower than the LD_{50}.

Adult calculation:

$$\frac{26{,}700 \text{ HU}}{\text{kg}} \div \frac{78.3 \text{ HU}}{70 \text{ kg adult}} = 23{,}000\text{X}$$

Child calculation:

$$\frac{26{,}700 \text{ HU}}{\text{kg}} \div \frac{78.3 \text{ HU}}{15 \text{ kg child}} = 5{,}000\text{X}$$

These worst-case calculations give assurance of the safety of the LLO component of the product even without taking into account the denaturation and hydrolysis of LLO by the proteolytic enzymes and stomach acid. As noted above (last paragraph of section 4.3.1.4), such denaturation is likely to occur in the event LLO is ingested, which will provide yet additional safety assurance.

4.4.2.4 Sodium and potassium content

From Section 2.4, the highest value obtained for sodium content in a ListShield™ lot was 205 mg/L. From this value and using the worst-case scenario value from Table 7 (all foods from each food category are treated with ListShield™), the amount of sodium contributed to the daily diet via ListShield™ can be calculated as follows:

$$\frac{205 \text{ mg sodium}}{1000 \text{ mL ListShield™}} \text{ x } \frac{157.5 \text{ mL ListShield™}}{\text{Day}} = \frac{32.3 \text{ mg sodium}}{\text{day}}$$

The recommended daily allowance of sodium is 2,400 mg (21 CFR § 101.9(c)(9)). The amount of sodium per day contributed by ListShield™ thus represents 1.4 % of the RDA and is negligible. The amount of sodium per day contributed by ListShield™, 32.3 mg, would be spread across several servings and meals. The amount of sodium consumed per serving would be below the level that would change nutritional content labeling by the end-user.

From Section 2.4, potassium content in ListShield™ was undetectable. Because any levels of potassium were below the detection limit of 0.5mg/L, the detection limit was used for these calculations. From this value, the amount of potassium contributed to the daily diet via ListShield™ on the four food categories can be calculated as follows:

$$\frac{0.5 \text{ mg potassium}}{1000 \text{ mL ListShield™}} \times \frac{157.5 \text{ mL ListShield™}}{\text{day}} = \frac{0.08 \text{ mg potassium}}{\text{day}}$$

Assuming the potassium levels of ListShield™ are just below the detection limit, then the amount of potassium per day contributed by ListShield™, 0.08 mg, is well below the level that would change nutritional content labeling by the end-user. The recommended daily allowance of potassium is 3,500 mg (21 CFR § 101.9(c)(9)). The amount of potassium per day contributed by ListShield™ thus represents 0.002% of the RDA and is negligible.

4.5 SUBSTANTIAL EQUIVALENCE TO PREVIOUSLY APPROVED BACTERIOPHAGE PRODUCTS

4.5.1 Previously approved bacteriophage cocktails

Several lytic bacteriophage products (including ListShield™) have already been designated GRAS and/or cleared for food safety usage and other applications by a number of regulatory agencies:

- ListShield™ (formerly known as LMP-102), the phage preparation that is the subject of this notice, containing six lytic *L. monocytogenes*-specific phages, is FDA-cleared as a food additive (21 CFR §172.785) for ready to eat foods not covered by this application.

- ListShield™ is also listed by the FSIS for use on various RTE meats and poultry products (FSIS Directive 7120.1).

- ListShield™ is also EPA-registered for use on non-food surfaces in food processing plants to prevent or significantly reduce contamination of *L. monocytogenes* (EPA registration #74234-1).

- SalmoFresh™, a phage preparation containing six lytic *Salmonella*-specific phages is GRAS (GRAS Notice No. 000435).

- SalmoFresh™ is also listed by the FSIS for use as processing aid when applied at a level of 10^6 to 10^7 PFU/g food product (FSIS Directive 7120.1).

- Listex™, a phage preparation containing a single *L. monocytogenes* lytic phage, P100, is GRAS (GRAS Notice No. 000218).

- Listex™ is also listed by the FSIS for use as processing aid when applied at a level of $1x10^7$ to $1x10^9$ PFU/g food product (FSIS Directive 7120.1).

- EcoShield™ (formerly ECP-100™), a phage preparation containing three lytic *E. coli* O157:H7-specific phages, is FDA-cleared for use as a food contact substance (FCN No. 1018).

- EcoShield™ is also listed by the FSIS for use as processing aid on red meat parts and trim prior to grinding (FSIS Directive 7120.1).

- AgriPhage™, a phage preparation targeting *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *Tomato*, is EPA-registered for use on tomatoes and peppers (EPA Reg. No. 67986-1).

- Two bacteriophage preparations – one *Salmonella* targeting and one *E. coli* O157:H7 targeting – are listed by the FSIS for use as processing aids on the hides and feathers of live animals before slaughter (FSIS Directive 7120.1).

Several regulatory agencies are represented in the preceding list, each of which separately determined that a different bacteriophage preparation was safe. The variety of these previously cleared or registered bacteriophage preparations attests to the general safety of bacteriophages and therefore supports their natural GRAS status. ListShield™ is substantially equivalent to the above bacteriophage cocktails and therefore is also GRAS.

4.6 SUMMARY AND BASIS FOR GRAS

ListShield™ is an all-natural product made of six *L. monocytogenes*-specific lytic bacteriophages from the *Myoviridae* and *Siphoviridae* families. All phages included in ListShield™ are lytic phages that were obtained from the environment and have not been genetically manipulated in any way. The component phages of ListShield™ have been rigorously characterized (including full genome sequencing) and were found not to contain any undesirable genes.

Phages are omnipresent in the environment. Bacteriophages are the oldest, most ubiquitous organisms on earth, with their numbers on Earth are estimated to be between 10^{30} and 10^{32}. Phages are present everywhere – including in our mouths, on our skin, and within our gastrointestinal tracks. They are also common and natural ingredients of all fresh, unprocessed foods. The omnipresence of phages (including in foods) and their daily consumption by humans makes them naturally GRAS.

In further recognition of their safety, several lytic bacteriophage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage and other applications by a number of regulatory agencies.

Although all lytic bacteriophages are, by nature, GRAS, the phages in ListShield™ must be verified to be lytic and to not contain any undesirable genes listed in 40 CFR § 725.421. The monophages included in ListShield™ belong to the *Myoviridae* and *Siphoviridae* families of double-stranded DNA bacteriophages. Many *Myoviridae* and *Siphoviridae* phages are known to be excellent lytic phages and are increasingly being used in various phage cocktails, including several previously FDA and EPA cleared bacteriophage preparations.

The genomes of the six bacteriophages in ListShield™ have been sequenced. Bioinformatic analysis of the component phages' sequences shows none contain any undesirable genes listed in 40 CFR §725.421. Furthermore, no antibiotic resistance gene, no 16S RNA sequences, or other known toxin genes were identified in any of the phage genomes.

ListShield™ is manufactured and QC-tested using Intralytix's standard procedures. The FDA has previously reviewed these procedures for ListShield™ (21 CFR §172.785). Additionally, these procedures are also used in the manufacturing of Intralytix's other previously reviewed bacteriophage food safety products, SalmoFresh™ (GRAS Notice No. 000435) and EcoShield™ (FCN No. 1018), and are currently used to manufacture commercial lots of these products.

The only manufacturing byproduct of potential concern for ListShield™ is LLO. Intralytix tests every lot of ListShield™ for LLO to ensure it meets the release criteria. The LLO levels of the ListShield™ must not be detectable with a limit of detection of 5 HU/mL for the lot to be released.

ListShield™ is produced on animal-product free media. The final ListShield™ product contains no preservatives or additives. ListShield™ has been certified both Kosher and Halal. ListShield™ is also OMRI-listed; i.e., it is suitable for use in the production of organic foods.

ListShield™ has been administered to laboratory animals (mice) without impact on their normal gut microflora and without any detectable side effects over a relatively long period of time (at least 90 days). (Mai et al., 2010)

The proposed application rate for ListShield™ is up to $1x10^8$ PFU per gram of food article. Assuming the maximum application rate of $1x10^8$ PFU/g of food and 100% market saturation, the average daily consumption due to treated foods would be a mere 55.3 μg of phage particles, 38.6 mg of added sodium, and 0.09 mg of added potassium. Both the added sodium and potassium levels are so low as to not require any changes to labeling. The amount of added phage is negligible.

ListShield™ is substantially equivalent to the lytic bacteriophage cocktails that have been previously designated GRAS and/or cleared by other regulatory agencies for various applications, including direct food applications. Furthermore, with the proposed maximum application rate for ListShield™ of up to $1x10^8$ PFU per gram of food article, even in the worst case scenario ($1x10^8$ PFU/g), the rate is similar to or lower than the rates previously cleared for those other cocktails

as safe and effective. For instance, the maximum proposed application rate of ListShield™ is 10 times lower than that of the previously GRAS-listed Listex P100 bacteriophage preparation.

In summary, the data presented in this document fully supports our designation of ListShield™ as GRAS. The basis for our conclusion is five-fold. First, the scientific literature extensively documents that lytic bacteriophages pose no safety concerns to humans. Second, all bacteriophages in ListShield™ are lytic, non-genetically modified, and free of undesirable genes. Third, Intralytix's manufacturing process ensures the safety and quality of the final ListShield™ product. Fourth, the estimated daily intake of the ListShield™ phage preparation is so low as to be negligible. And, fifth, the bacteriophage product is substantially equivalent to several bacteriophage products already receiving regulatory clearance, including GRN000218 and GRN000435. Based on this information, it is evident that ListShield™ is GRAS.

5 REFERENCES

Ackermann, H. W., & Berthiaume, L. (1995). *Atlas of virus diagrams*. Boca Raton, FL.: CRC Press.

Alisky, J., Iczkowski, K., Rapoport, A., & Troitsky, N. (1998). Bacteriophages show promise as antimicrobial agents. *J Infect*, **36,** 5-15.

American Meat Institute. U.S. Meat and Poultry Production & Consumption: An Overview (July 2010). Retrieved from: http://www.meatami.com/ht/a/GetDocumentAction/i/63785

Armon, R., & Kott, Y. (1993). A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. *J Appl Bacteriol*, **74,** 490-496.

Armon, R., Araujo, R., Kott, Y., Lucena, F., & Jofre, J. (1997). Bacteriophages of enteric bacteria in drinking water, comparison of their distribution in two countries. *J Appl Microbiol*, **83,** 627-633.

Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E., & Connerton, I. F. (2003). Isolation and characterization of Campylobacter bacteriophages from retail poultry. Appl Environ Microbiol, **69**, 4511-4518.

Bergh, O., Borsheim, K. Y., Bratbak, G., & Heldal, M. (1989). High abundance of viruses found in aquatic environments. Nature, **340**, 467-468.

Breitbart, M., Hewson, I., Felts, B., Mahaffy, J. M., Nulton, J., Salamon, P., & Rohwer, F. (2003). Metagenomic analyses of an uncultured viral community from human feces. J Bacteriol, **185**, 6220-6223.

Brussow, H., & Hendrix, R. W. (2002). Phage genomics: small is beautiful. Cell, **108**, 13-16.

Bruttin, A., & Brussow, H. (2005). Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. Antimicrob Agents Chemother, **49**, 2874-2878.

Calci, K. R., Burkhardt, W., 3rd, Watkins, W. D., & Rippey, S. R. (1998). Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human-associated wastewaters. *Appl Environ Microbiol*, **64,** 5027-5029.

Centers for Disease Control and Prevention. (2011). Surveillance for foodborne disease outbreaks – US 2008.. *Morbidity & Mortality Weekly Report* **60(35)**, 1197-1206. Retrieved from: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6035a1.htm

Eggers, C.H., Kimmel, B.J., Bono, J.L., Elias, A.F., Rosa, P., Samuels, D.S. (2001). Transduction by BB-1, a bacteriophage of Borrelia burgdorferi. Amer. Soc. Microbiol. 183(16):4771-4778.

Eller MR, Dias RS, De Moraes CA, De Carvalho AF, Oliveira LL, Silva EA, da Silva CC, De Paula SO. (2012). Molecular characterization of a new lytic bacteriophage isolated from cheese whey. *Arch Virol*, **157(12)**, 2265-72.

Fogelman, I., Davey, W., Ochs, H.D., Elashoff, M., Feinberg, M.B., Mican, J., Siegel, J.P., Sneller, M., & Lane, H.C. (2000). Evaluation of $CD4^+$ T cell function *in vivo* in HIV-infected patients as measured by bacteriophage phiX174 immunization. *J. Infect. Dis.* **182**, 435-441.

Furuse, K., Osawa, S., Kawashiro, J., Tanaka, R., Ozawa, A., Sawamura, S., Yanagawa, Y., Nagao, T., & Watanabe, I. (1983). Bacteriophage distribution in human faeces: continuous survey of healthy subjects and patients with internal and leukaemic diseases. *J Gen Virol*, **64 (Pt 9),** 2039-2043.

Gautier, M., Rouault, A., Sommer, P., & Briandet, R. (1995). Occurrence of *Propionibacterium freudenreichii* bacteriophages in swiss cheese. *Appl Environ Microbiol*, **61,** 2572-2576.

Geoffroy C, Gaillard JL, Alouf JE, Berche P. (1987). Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from Listeria monocytogenes. *Infect Immun*, **55(7)**, 1641–1646.

Grabow, W. O., & Coubrough, P. (1986). Practical direct plaque assay for coliphages in 100-ml samples of drinking water. *Appl Environ Microbiol*, **52,** 430-433.

Greer G. G., Dilts B. D., Ackermann H. W. (2007). Characterization of a Leuconostoc gelidum bacteriophage from pork. *Int J Food Microbiol*., **114**, 370–375.

Greer, G. G. (2005). Bacteriophage control of foodborne bacteria. *J Food Prot*, **68,** 1102-1111.

Havelaar, A. H., Furuse, K., & Hogeboom, W. M. (1986). Bacteriophages and indicator bacteria in human and animal faeces. *J Appl Bacteriol*, **60,** 255-262.

Hsu, F. C., Shieh, Y. S., & Sobsey, M. D. (2002). Enteric bacteriophages as potential fecal indicators in ground beef and poultry meat. *J Food Prot*, **65,** 93-99.

Jacob F. & Wollman, E.L. (1959). Lysogeny. In M.H. Adams (Ed.), *Bacteriophages* (pp. 365-380). New York, NY: Interscience Publishers, Inc.

Kennedy, J. E. J., Oblinger, J. L., & Bitton, G. (1984). Recovery of coliphages from chicken, pork sasuage, and delcatessen meats. *J Food Protection*, **47,** 623-626.

Kennedy, J. E., Jr., Wei, C. I., & Oblinger, J. L. (1986). Methodology for enumeration of coliphages in foods. *Appl Environ Microbiol*, **51,** 956-962.

Langley, R., Kenna, D.T., Vandamme, P., Ure, R., Govan, J.R.W. (2003). Lysogeny and bacteriophage host range within the *Burkholderia cepacia* complex. *J. Med. Microbiol.* 52:483-490.

Leenstra, T. S., van Saene, J. J., van Saene, H. K., & Martin, M. V. (1996). Oral endotoxin in healthy adults. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **82,** 637-643.

Lopez, V., Ochs, H.D., Thuline, H.C., Davis, S.D., & Wedgewood, R.J. (1975). Defective antibody response to bacteriophage X174 in Down syndrome. J. Pediatrics, 86, 207-211.

Lucena, F., Muniesa, M., Puig, A., Araujo, R., & Jofre, J. (1995). Simple concentration method for bacteriophages of *Bacteroides fragilis* in drinking water. *J Virol Methods*, **54,** 121-130.

Maciorowski, K. G., Pillai, S. D., & Ricke, S. C. (2001). Presence of bacteriophages in animal feed as indicators of fecal contamination. *J Environ Sci Health B*, **36,** 699-708.

Mai, V., M. Ukhanova, L. Visone, T. Abuladze, & A.S. Sulakvelidze. (2010). Bacteriophage administration reduces the concentration of *Listeria monocytogenes* in the gastrointestinal tract and its translocation to spleen and liver in experimentally infected mice. *Int J Microbiol*. doi:10.1155/2010/624234.

Merril, C.R., Friedman, T.B., Attallah, A.F.M., Geier, M.R., Krell, K., & Yarkin, R. (1972). Isolation of bacteriophages from commercial sera. *In Vitro*, **8(2)**, 91-93.

Milch, H., & Fornosi, F. (1975). Bacteriophage contamination in live poliovirus vaccine. *J. Biol. Standardization*, **3**, 307-310.

Miller, E. S., Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Durkin, A. S., Ciecko, A., Feldblyum, T. V., White, O., Paulsen, I. T., Nierman, W. C., Lee, J., Szczypinski, B., & Fraser, C. M. (2003). Complete genome sequence of the broad-host-range vibriophage KVP40: comparative genomics of a T4-related bacteriophage. *J Bacteriol*, **185,** 5220-5233.

Moody, E.E.M., Trousdale, M.D., Jorgensen, J.H., & Shelokov, A. (1975). Bacteriophages and endotoxin in licensed live-virus vaccines. *J. Infect. Dis.* **131(5),** 588-591.

Ochs, H.D., Buckley, R.H., Kobayashi, R.H., Kobayashi, A.L., Sorensen, R.U., Douglas, S.D., Hamilton, B.L., & Hershfield, M.S. (1992). Antibody responses to bacteriophage patients with adenosine deaminase deficiency. *Blood*, **80(5)**, 1163-1171.

Ochs, H.D., Davis, S.D., & Wedgewood, R.J. (1971). Immunologic responses to bacteriophage ΦX174 in immunodeficiency diseases. *J. Clinical Investigation*, **50**, 2559-2568.

Ochs, H.D., Lum, L.G. , Johnson, L., Schiffman, G., Wedgewood, R.J. & Storb, R. (1982). Bone marrow transplantation in the Wiskott-Aldrich syndrome. *Transplantation.* **34(5)**, 284-288.

Ochs, H.D., Nonoyama, S., Farrington, M.L., Fischer, S.H., & Aruffo, A. (1993a). The role of adhesion molecules in the regulation of antibody responses. *Semin Hematol.* **30(4Suppl4)**, 72-9.

Ochs, H.D., Nonoyama, S., Zhu, Q., Farrington, M., & Wedgewood, R.J. (1993b). Regulation of antibody responses: the role of complement adhesion molecules. *Clinical Immunology & Immunopathology* **67(3)**, S33-S40.

Schicklmaier, P. & Schmieger, H. (1995). Frequency of generalized transducing phages in natural isolates of the *Salmonella typhimuium* complex. *Appl Environ. Microbiol*, **61(4),** 1637.

Sulakvelidze, A., & Barrow, P. (2005). Phage therapy in animals and agribusiness. In E. Kutter & A. Sulakvelidze (Eds.), *Bacteriophage: Biology and Applications* (pp. 335-380). Boca Raton, FL: CRC Press.

Sulakvelidze, A., Alavidze, Z., & Morris, J. G., Jr. (2001). Bacteriophage therapy. *Antimicrob Agents Chemother*, **45,** 649-659.

Summers, W.C. (2001). Bacteriophage therapy. *Ann. Rev. Microbiol.* **55**, 437-451.

USDA ERS. (2014a). Dairy products data set. *Food Availability (Per Capita) Data System*. Retrieved from: http://www.ers.usda.gov/datafiles/Food_Availabily_Per_Capita_Data_System/Food_Availability/dymfg.xls (June 24, 2014).

USDA ERS. (2014b). Fish and shellfish data set. *Food Availability (Per Capita) Data System*. Retrieved from: http://www.ers.usda.gov/datafiles/Food_Availabily_Per_Capita_Data_System/Food_Availability/mtfish.xls (June 24, 2014).

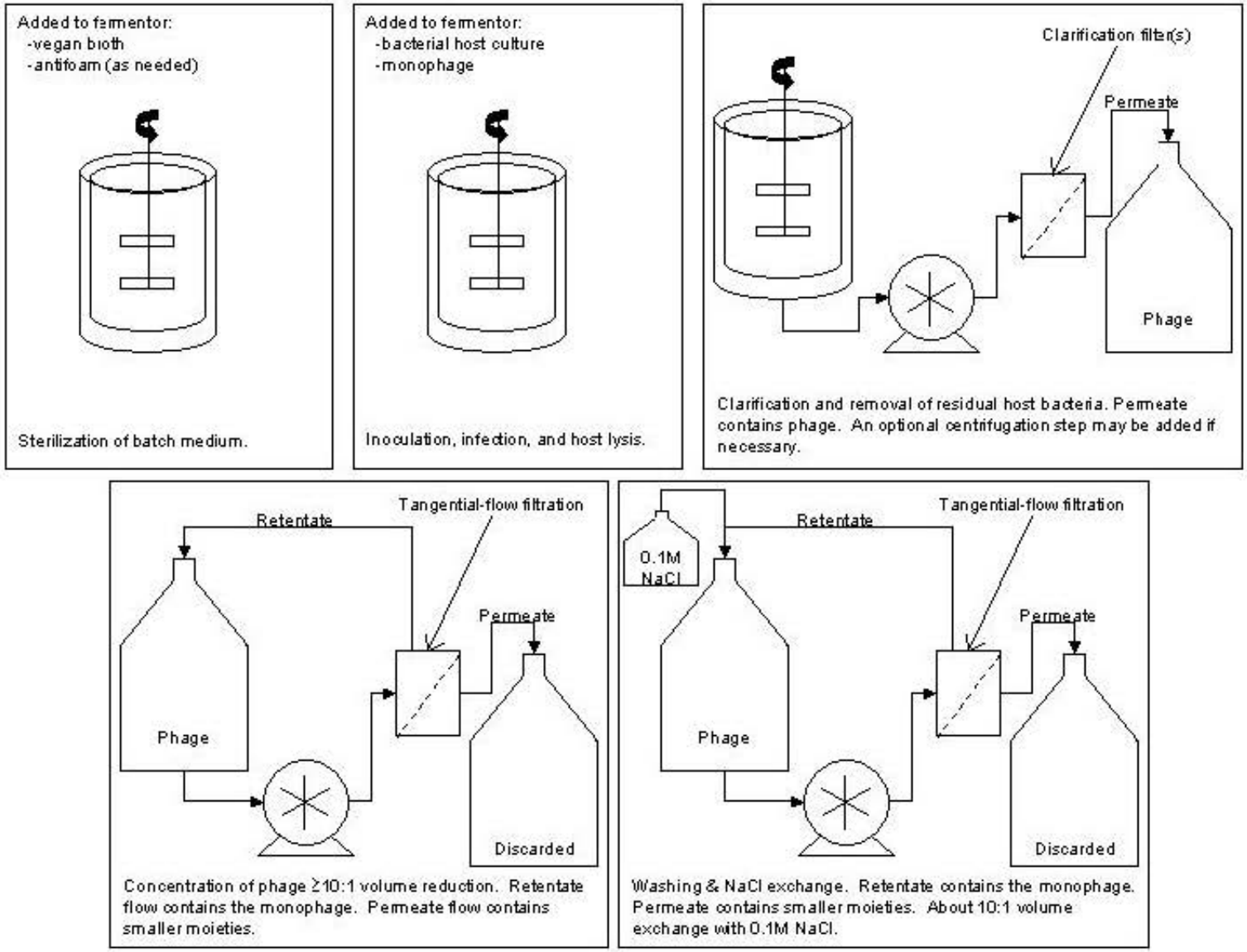
USDA ERS. (2014c). Fruit and vegetables data set. *Food Availability (Per Capita) Data System*. Retrieved from: http://www.ers.usda.gov/datafiles/Food_Availabily_Per_Capita_Data_System/Food_Availability/fruitveg.xls (June 24, 2014).

Wang, X. and P.J. Quinn. 2010. Lipopolysaccharide: Biosynthetic pathway and structure modification. Progress in Lipid Research. **49**, 97-107.

Whitman, P. A., & Marshall, R. T. (1971). Isolation of psychrophilic bacteriophage-host systems from refrigerated food products. *Appl Microbiol*, **22,** 220-223.

6 APPENDICES

6.1 EFFICACY DATA


Added to fermentor:
-vegan broth
-antifoam (as needed)
Sterilization of batch medium.
Added to fermentor:
-bacterial host culture
-monophage
Inoculation, infection, and host lysis.
Clarification filter(s)
Permeate
Phage
Clarification and removal of residual host bacteria. Permeate contains phage. An optional centrifugation step may be added if necessary.
Retentate
Tangential-flow filtration
Permeate
Phage
Discarded
Concentration of phage ≥10:1 volume reduction. Retentate flow contains the monophage. Permeate flow contains smaller moieties.
0.1M NaCl
Retentate
Tangential-flow filtration
Permeate
Phage
Discarded
Washing & NaCl exchange. Retentate contains the monophage. Permeate contains smaller moieties. About 10:1 volume exchange with 0.1M NaCl.

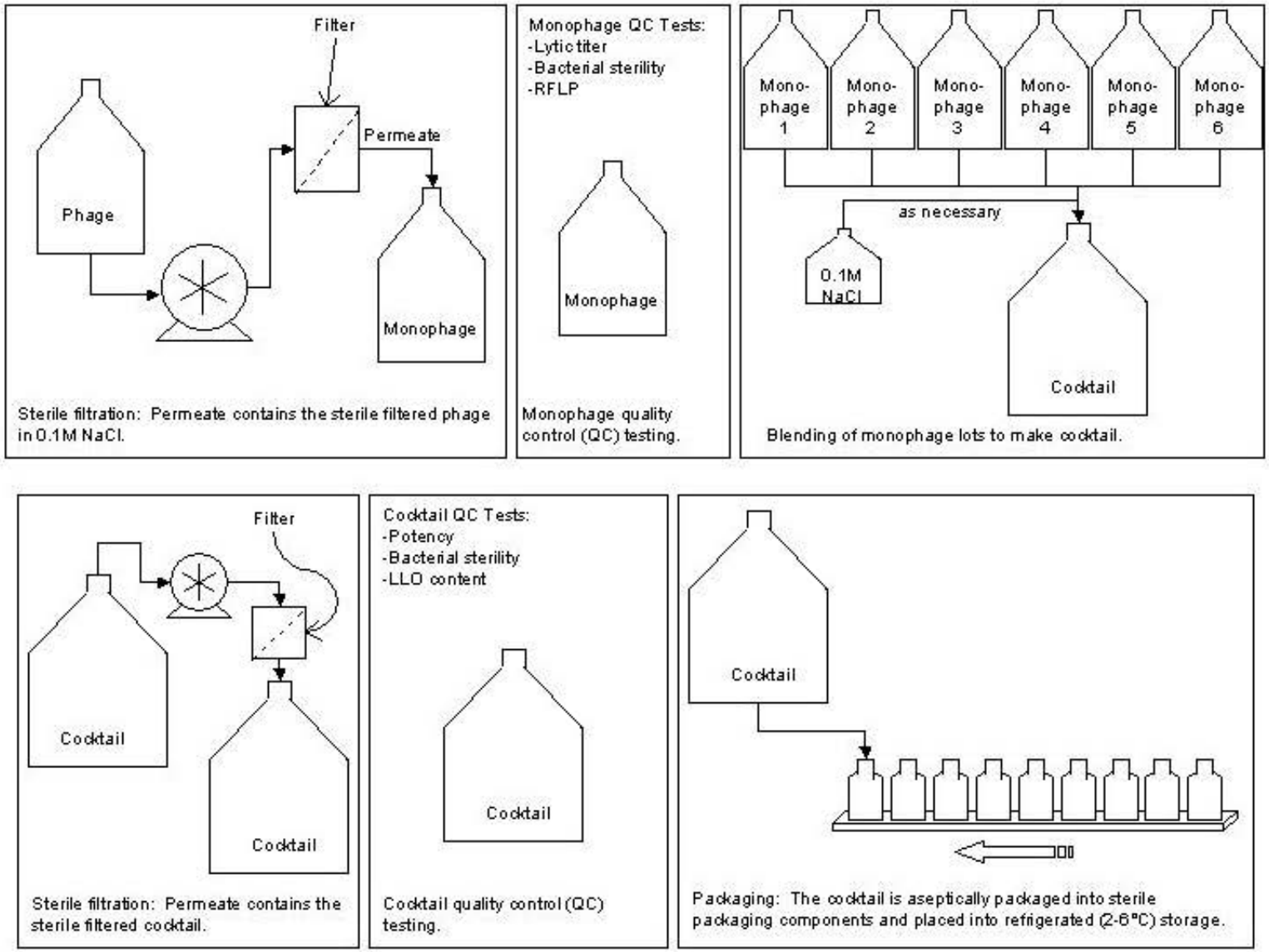


Figure 1 Overview of ListShield™ manufacturing process

APPENDIX 1: EFFICACY STUDIES

Substance: Bacteriophage preparation (*Listeria monocytogenes* targeted)

Product:

- Fish and shellfish
- Fresh and processed fruits
- Fresh and processed vegetables
- Dairy

Amount: Applied as a spray to the surface of the product at a level of ca. $9x10^5$ - $1x10^8$ plaque forming units (PFU) per gram of product

Reference: Acceptability determination

Labeling Requirements: None under the accepted conditions of use

ListShield™ is an all-natural product made of six *L. monocytogenes*-specific lytic bacteriophages from the *Myoviridae* and *Siphoviridae* families. All phages included in ListShield™ are lytic phages that were obtained from the environment in the USA and have not been genetically manipulated in any way. The component phages of ListShield™ have been rigorously characterized, including full genome sequencing.

The ListShield™ preparation is intended for use in food products as a processing aid to control *L. monocytogenes* when added at ca. $9x10^5$ - $1x10^8$ PFU per gram of food. ListShield™ has been determined by Intralytix, Inc. to be generally recognized as safe (GRAS), and therefore, we believe it to be exempt from the requirement of pre-market approval, under the conditions of its intended use.

LISTSHIELD™ APPLICATION RATES AND DIETARY INTAKE

The proposed application of ListShield™ is ca. $9x10^5$ - $1x10^8$ PFU/g of food. Based on this application, the estimated daily intake of ListShield™ is also very safe and similar to the intakes of other already cleared bacteriophage products. For example, assuming the worst case scenario where *all* food (of the food categories listed above) consumed in the US is treated with the *maximum amount* of ListShield™, the daily intake of one person would be approximately 55.3 µg of phage per day, an insignificant amount.

Table 1 Comparison of the proposed application rate of ListShield™ and application rates of FDA-cleared Listex™, SalmoFresh™ and EcoShield™

Product	Test article	Reference	Phage concentration on food article (PFU/g)
ListShield™	Dairy, fruits, vegetables, fish	This document	9E+05 - 1E+08
ListShield™ (formerly LMP-102)	RTE meats	21 CFR §172.785	2E+06 – 4E+06
Listex™ P100	Cheese and RTE meats	GRN 000218	3E+08 – 6E+08
SalmoFresh™	Poultry, fruits, vegetables, fish	GRN 000435	≤ 1E+07
EcoShield™	Beef slices	FCN No. 1018	3E+06 – 1E+07

LISTSHIELD™ IS EFFECTIVE.

Target range

ListShield™ has been screened for its lytic activity against 275 *L. monocytogenes* strains. At ca. $1x10^9$ PFU/mL, it kills 265 (96%) of the Lm strains in our collection.

Effect on *L. monocytogenes* levels in foods

Studies done by Intralytix demonstrate that ListShield™ application at the proposed level of up to $9x10^5$ - $1x10^8$ PFU/g significantly reduces *L. monocytogenes* levels in a variety of experimentally contaminated foods. The studies are briefly summarized below:

- Study #LS11F09TA: ListShield™, applied at $9x10^5$ - $2x10^6$ PFU/g of smoked salmon prior to slicing, significantly reduced *L. monocytogenes* levels by 65-90%.

- Study #LS12C05TA: ListShield™, applied at $1x10^6$ PFU/g of sliced apples, significantly reduced *L. monocytogenes* levels by 79-89%.

- Study #LS14A22ML: ListShield™, applied at $1x10^7$ - $1x10^8$ PFU/g of lettuce, significantly reduced *L. monocytogenes* levels by 68-91%.

- Study #LS14A16TA: ListShield™, applied at $1x10^8$ PFU/g of cheese, significantly reduced *L. monocytogenes* levels by 68-82%.

Description of intended technical effect

ListShield™ is intended to produce a statistically significant reduction of *L. monocytogenes* contamination vs. a water or carrier control when applied as directed to food products.

Efficacy study summary

ListShield™ was examined for its ability to reduce *L. monocytogenes* contamination when applied to various foods. Detailed reports of the studies are included in Appendix 1.1 - Appendix 1.4 and a summary of the results is given below.

Description of test systems

Fish and shellfish

Smoked salmon was inoculated with a mix of three *L. monocytogenes* strains then treated with water or ListShield applied at $9x10^5$ or $2x10^6$ PFU/g of fish. The ListShield™ contact time was 24 hours at 4°C, after which the samples were analyzed for populations of *L. monocytogenes*.

Fresh and processed fruits

Freshly cored and sliced apples were inoculated with one *L. monocytogenes* strain then treated with water or ListShield applied at ca. $1x10^7$ PFU/g of fruit. The ListShield™ contact time was 24 - 72 hours at 4°C, after which the samples were analyzed for populations of *L. monocytogenes*.

Fresh and processed vegetables

Long-leafed lettuce leaves were inoculated with a mix of three *L. monocytogenes* strains then treated with water or ListShield™ applied at $1x10^7$ or $1x10^8$ PFU/g of lettuce. The ListShield™ contact time was 5 minutes at room temperature after which the samples were analyzed for populations of *L. monocytogenes*.

Dairy

Semi-soft cheese was inoculated with a mix of three *L. monocytogenes* strains then treated with PBS or ListShield™ applied at $1x10^8$ PFU/g of cheese. The ListShield™ contact time was 5 minutes at room temperature and 24 and 96 hours at 4°C, after which the samples were analyzed for populations of *L. monocytogenes*.

Summary of results

Fish and shellfish

Study LS11F09TA examined the efficacy of ListShield™ on reducing *L. monocytogenes* levels in smoked salmon. Two concentrations of ListShield™ ($9x10^5$ and $2x10^6$ PFU/g) were applied prior to storage at 4°C for 24 hours and significantly reduced the number of viable *L. monocytogenes* by ca. 65% and 95%, respectively. The complete details of this study can be seen in Appendix 1.1.

Fresh and processed fruits

Study LS12C05TA examined the efficacy of ListShield™ on reducing *L. monocytogenes* levels in sliced apples. ListShield™ was applied at ca. $1x10^7$ PFU/g prior to storage at 4°C for 24, 48, and 72 hours and significantly reduced the number of viable *L. monocytogenes* by ca. 79-93%. The study also showed no continued technical effect, after the initial reduction, no further decrease in *L. monocytogenes* occurred during the storage. The complete details of this study can be seen in Appendix 1.2.

Fresh and processed vegetables

Study LS14A22ML examined the efficacy of ListShield™ on reducing *L. monocytogenes* levels in lettuce. Two concentrations of ListShield™ ($1x10^7$ and $1x10^8$ PFU/g) were applied for 5 minutes at room temperature and significantly reduced the number of viable *L. monocytogenes* by ca. 68% and 91%, respectively. The complete details of this study can be seen in Appendix 1.3.

Dairy

Study LS14A16TA examined the efficacy of ListShield™ on reducing *L. monocytogenes* levels in sliced apples. ListShield™ was applied at ca. $1x10^8$ PFU/g for 5 minutes at room temperature. The samples were analyzed immediately or stored at 4°C for 24 and 96 hours and significantly reduced the number of viable *L. monocytogenes* by ca. 68-82%. The study also showed no continued technical effect, after the initial reduction, no further decrease in *L. monocytogenes* occurred during the storage. The complete details of this study can be seen in Appendix 1.4.

Summary

We believe the data summarized here fully supports that ListShield™ is an effective antimicrobial processing aid when used to reduce or eliminate *L. monocytogenes* contamination on foods. Its intended use is as a spray applied to significantly reduce levels of *L. monocytogenes* when applied at ca. $9x10^5$ - $1x10^8$ PFU/g. Additionally, no foods treated to product specifications should require ListShield™ as a listed ingredient on product labels.

Appendices

Appendix 1.1	**Report LS11F09TA**
Appendix 1.2	**Report LS12C05TA**
Appendix 1.3	**Report LS14A22ML**
Appendix 1.4	**Report LS14A16TA**

Table 2 Summary of efficacy data presented in Appendix 1.1 - Appendix 1.4

Study #	Matrix	Application rate (PFU/g)	Storage at 4°C (hrs)	Log_{10} reduction	% reduction	Significant?	Continued residual effect?
LS11F09TA	Salmon	$9x10^5$	0	0.4	65	Yes	N/A
		$2x10^6$	0	1.0	90	Yes	N/A
LS12C05TA	Apple	$1x10^6$	24	1.0	89	Yes	N/A
			48	1.0	89	Yes	No
			72	0.7	79	Yes	No
LS14A22ML	Lettuce	$1x10^7$	N/A	0.5	68	Yes	N/A
		$1x10^8$	N/A	1.1	91	Yes	N/A
LS14A16TA	Cheese	$1x10^8$	0	0.7	82	Yes	N/A
			24	0.7	78	Yes	No
			96	0.5	68	Yes	No

Appendix 1.1:
Report LS11F09TA

Evaluation of the ability of ListShield™ to reduce *L. monocytogenes* contamination in experimentally contaminated smoked salmon

Study # LS11F09TA

Intralytix

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

www.intralytix.com

Table of Contents

1 Study Title 3
2 Study Director 3
3 Study Personnel 3
4 Performing Laboratory 3
5 Study Objective 4
6 Test Matrix 4
7 ListShield™ Lot and Application 4
8 Bacterial Strains Used to Experimentally Contaminated smoked salmon 4
9 Media and Reagents 5
10 General Outline of Study 5
11 Results 6
11.1 Raw Data 6
11.2 Tabular presentation of results 6
11.3 Graphical presentation of results 7
11.4 Statistical analysis 7
11.5 Brief discussion of results and study's conclusions 8
12 Summary Conclusion of the Study 8
13 Signatures 9

1 STUDY TITLE

Evaluation of the ability of ListShield™ to reduce *L. monocytogenes* contamination in experimentally contaminated smoked salmon.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Tamar Abuladze	Research Scientist	Hands-on-research
Joelle Woolston, MS	Research Scientist / Laboratory Manager	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of ListShield™ reduces the number of viable *L. monocytogenes* on smoked salmon when applied at the rate of $9x10^5$ - $2x10^6$ PFU/g.

6 TEST MATRIX

Smoked salmon was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies.

7 LISTSHIELD™ LOT AND APPLICATION

- ListShield™ Lot 0111E040222
- Titer: approx. $1x10^{10}$ PFU/mL
- ListShield™ was diluted, when necessary, with PBS just prior to application.
- The application rate was ca. 0.20 mL ListShield™ per 100 g smoked salmon.
- ListShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED SMOKED SALMON

The smoked salmon test matrix was experimentally contaminated with a 1:1:1 mixture of three *L. monocytogenes* strains:

- *Lm*68: A *Listeria monocytogenes* strain
- *Lm*82: A *Listeria monocytogenes* strain
- *Lm*320: A *Listeria monocytogenes* strain obtained from ATCC (also known as ATCC 19115).

Upon receipt, the above-noted Intralytix strain designation was assigned (i.e., *Lm*320). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 µg of nalidixic acid/ml.

Shortly before performing the study, the strains were thawed and grown (37 ± 2°C, 16-24 h) in LB broth. Overnight growth corresponds to ca. $1x10^9$ CFU/mL. Equal volumes of the three strains were combined for a 1:1:1 mixture and the mixed culture was diluted 1,000-fold just prior to performing the study.

The smoked salmon was experimentally contaminated with ca. $1x10^3$ CFU / g of smoked salmon.

9 MEDIA AND REAGENTS

- LB broth, Miller (Neogen, Lansing, MI; catalog # 7279C)
- Oxford Agar (Fluka Biochemica, catalog # 75805)
- Oxford *Listeria* Selective Supplement (Fluka Biochemica, catalog # 75806)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)

10 GENERAL OUTLINE OF STUDY

1) Three ca. 240g portions of smoked salmon were each assigned as test group A, B, or C.
2) The challenge dose of bacteria was applied onto the top surface of the smoked salmon. Bacterial cultures were evenly spread onto each sample using hockey sticks.
3) The samples were covered loosely and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
4) PBS (control) or ListShield™ was applied as described in section 7. Treatments were evenly applied to the smoked salmon samples' surfaces as follows:
    - Group A = 0.20mL water / 100g = 0 PFU/g
    - Group B = 0.20mL $5x10^8$ PFU/mL ListShield™ / 100g = $9x10^5$PFU/g
    - Group C = 0.20mL $1x10^9$ PFU/mL ListShield™ / 100g = $2x10^6$PFU/g
5) The samples were covered and stored at 4°C for ca. 24 hours.
6) At 24hr post-treatment with PBS or ListShield™, from each sample group, triplicate ~25g samples of smoked salmon were cut, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were stomached for a minimum of 60 seconds at 230rpm.

7) The number of viable *L. monocytogenes* in the samples was determined by plating a 0.1mL aliquot of the stomached smoked salmon / peptone water mixture onto separate Oxford agar plates supplemented with Oxford *Listeria* Selective Supplement. The plates were incubated (37 ± 1°C, 48±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated smoked salmon}} = \frac{\textit{CFU}}{\textit{0.1mL plating}} \times \frac{\textit{225 mL peptone}}{\textit{25 g sample}}$$

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #LS11F09TA

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.1 mL	CFU/g
A (Control)	Yes	222	PBS	3	12,11,8	1080,990,720
B ($5x10^8$ PFU/mL)	Yes	259	$9x10^5$ PFU/g ListShield™	3	3,7,1	270,630,90
C ($1x10^9$ PFU/mL)	Yes	230	$2x10^6$ PFU/g ListShield™	3	2,1,0	180,90,0

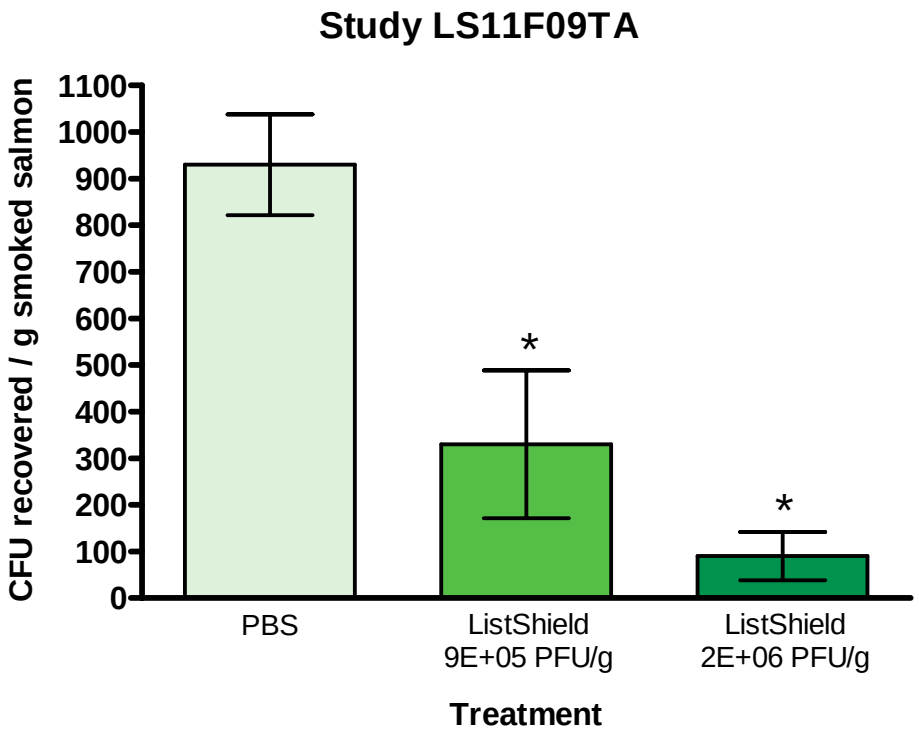
11.2 Tabular presentation of results

Table 2 Reduction of *L. monocytogenes* counts on smoked salmon treated with ListShield™ when applied at ca. $9x10^5$ – $2x10^6$ PFU/g (0.20mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A (Control)	Yes	PBS	*n =3*	930	-	-	-
B ($5x10^8$ PFU/mL)	Yes	$9x10^5$ PFU/g ListShield™	*n = 3*	330	65	0.4	Yes
C ($1x10^9$ PFU/mL)	Yes	$2x10^6$ PFU/g ListShield™	*n =3*	90	90	1.0	Yes

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)



*** indicates statistically different from the PBS control (P<0.05)**

11.4 Statistical analysis

The efficacy of the ListShield™ treatment in reducing the number of viable *L. monocytogenes* in the experimentally contaminated smoked salmon was evaluated by comparing the data obtained with the water-treated control samples and the ListShield™-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is 0.0053, considered very significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
PBS vs $9x10^5$ PFU/g	600.00	5.222	* P<0.05
PBS vs. $2x10^6$ PFU/g	840.00	7.311	** P<0.01
$9x10^5$ PFU/g vs. $2x10^6$ PFU/g	240.00	2.089	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying ListShield™ at ca. $9x10^5$ PFU/g smoked salmon reduced the number of viable *L. monocytogenes* by ca. 65% (0.4 log) after 24h storage at 4°C. The observed reduction was statistically significant (P<0.05).

- Applying ListShield™ at ca. $2x10^6$ PFU/g smoked salmon reduced the number of viable *L. monocytogenes* by ca. 90% (1.0 log) after 24h storage at 4°C. The observed reduction was statistically significant (P<0.01).

- Reduction in *L. monocytogenes* levels achieved by using more concentrated ListShield™ was higher compared to those obtained with more dilute ListShield™ (90% vs. 65% when using ca. $2x10^6$ PFU/g and $9x10^5$ PFU/g, respectively).

- The difference in *L. monocytogenes* recovered when ListShield™ was applied in the more concentrated form (application rates $2x10^6$ PFU/g) vs. the less concentrated form ($9x10^5$ PFU/g) was not statistically significant (P>0.05).

12 SUMMARY CONCLUSION OF THE STUDY

ListShield™ can significantly reduce viable *L. monocytogenes* levels in experimentally contaminated smoked salmon by ca. 65-90% after 24 hour storage at 4°C, when applied at ca. $9x10^5$ – $2x10^6$ PFU/g.

Using the higher ListShield™ application rate (ca. $2x10^6$ PFU/g) did not result in statistically significantly better reduction of *L. monocytogenes* levels compared to lower ListShield™ application rate (ca. $9x10^5$ PFU/g).

13 SIGNATURES

(b) (6)

Tamar Abuladze

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.2:
Report LS12C05TA

Evaluation of the ability of ListShield™ and/or ListShield™/AS mixture to significantly reduce or eliminate *L. monocytogenes* contamination in fresh cut apples

Study # LS12C05TA

Intralytix

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

www.intralytix.com

Table of Contents

1 Study Title 3
2 Study Director 3
3 Study Personnel 3
4 Performing Laboratory 3
5 Study Objective 3
6 Test Matrix 4
7 ListShield™ Lot and Application Rate 4
8 Bacterial Strains Used to Experimentally Contaminate Apples 4
9 Media and Reagents 5
10 General Outline of Study 5
11 Results 6
11.1 Raw Data 6
11.2 Tabular presentation of results 7
11.3 Graphical presentation of results 8
11.4 Statistical analysis 8
11.5 Brief discussion of results and study's conclusions 10
12 Summary Conclusion of the Study 11
13 Signatures 12

1 STUDY TITLE

Evaluation of the ability of ListShield™ and/or ListShield™/AS mixture to significantly reduce or eliminate *L. monocytogenes* contamination in fresh cut apples

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Tamar Abuladze, B.S.	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, M.S.	Research Scientist / Laboratory Manager	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.

Research and Development

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of AS, ListShield™, or ListShield™/AS reduces the number of viable *L. monocytogenes* on experimentally contaminated fresh cut apples when applied at the rate 0.106 mL / 10 g (the equivalent of 1.1×10^6 PFU/g for ListShield).

To determine whether the dilution of ListShield™ with AS affects the efficacy of ListShield in reducing the number of viable *L. monocytogenes* on experimentally contaminated fresh cut apples.

6 TEST MATRIX

Whole apples were obtained from a fresh-cut apple producer. They were washed with running tap water prior to our studies.

7 LISTSHIELD™ LOT AND APPLICATION RATE

- ListShield™ Lot # 0112B240183
- ListShield™ Titer: approx. $1x10^{10}$ PFU/mL
- AS (special antioxidant powder / antibrowning solution) solution concentration: 80g / L
- ListShield™ was diluted, when necessary, with water or AS solution just prior to application.
- The application rate was ca. 0.106 mL ListShield™ per 10 g apple.
- Treatments were applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE APPLES

The apple test matrix was experimentally contaminated with *L. monocytogenes* strain:

- *Lm*320: A *Listeria monocytogenes* strain obtained from ATCC (also known as ATCC 19115).

Upon receipt, the above-noted Intralytix strain designation was assigned (i.e., *Lm*320). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37 ± 2°C, 16-24 h) in LB broth. Overnight growth corresponds to ca. $1x10^9$ CFU/mL. The culture was diluted 5000-fold just prior to performing the study.

The apple slices were experimentally contaminated by ca. $1x10^4$ CFU / g of apple.

9 MEDIA AND REAGENTS

- LB broth, Miller (Neogen, Lansing, MI; catalog # 7279C)
- Oxford Agar (Fluka Biochemica, catalog # 75805)
- Oxford *Listeria* Selective Supplement (Fluka Biochemica, catalog # 75806)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Sterile water for irrigation, USP (Baxter; catalog # 2F7114)

10 GENERAL OUTLINE OF STUDY

1) The apples were weighed cored and cut into 8 pieces using a hand food corer / slicer. The average weight of eight slices was 138g or ~17g/slice.

2) Five test groups (A, B, C, D, or E) were created, each with nine apple slices.

3) The challenge dose of bacteria was applied onto the apple samples' cut surfaces. Bacterial cultures were evenly spread onto all cut sides of the apple slices' surfaces (**not** including skin side) using hockey sticks. The three slices in group A were **not** treated with bacterial cultures as the uncontaminated, untreated control.

4) The samples were covered loosely and the bacteria were allowed to colonize the apple samples' surfaces at room temperature (RT) for 10 min.

5) Water (control), AS, ListShield™, or ListShield™/AS was applied as described in section 7. Treatments were evenly applied to the apple slices' cut surfaces as follows:

   - Group A was **not** treated = untreated, uncontaminated control
   - Group B = 0.106mL water / 100g = 0 PFU/g
   - Group C = 0.106mL AS (80g/L) / 100g = AS control
   - Group D = 0.106mL $1x10^8$ PFU/mL ListShield™ / 100g = $1.1x10^6$ PFU/g
   - Group E = 0.106mL $1x10^8$ PFU/mL ListShield™ in AS / 100g = $1.1x10^6$ PFU/g / AS

6) The sample containers were covered and stored at 4°C for up to 72 hours.

7) At three storage time points (24h, 48h, and 72h post treatment), from each sample group, triplicate apple slices were removed, placed into individual sterile bags, and 10 mL of sterile peptone water was added. The bags were stomached for 60 seconds on the medium setting.

8) The number of viable *L. monocytogenes* for each sample was determined by plating triplicate aliquots (0.1 mL) of the stomached apple/peptone water mixture onto separate Oxford agar plates supplemented with Oxford *Listeria* Selective Supplement. The plates were incubated (35 ± 2°C, 48±4 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated apple}} = \frac{\textit{CFU}}{\textit{0.1mL plating}} \times \frac{\textit{10 mL peptone}}{\textit{17 g sample}}$$

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #LS12C05TA

Group	Bacteria	Treatment	Storage time	# of ~17g Samples	# CFU in triplicate 0.1 mL	Average CFU/g
A (Control)	No	None	24	3	0,0,0;0,0,0;0,0,0	0;0;0
			48	3	0,0,0;0,0,0;0,0,0	0;0;0
			72	3	0,0,0;0,0,0;0,0,0	0;0;0
B (Control)	Yes	Water	24	3	24,21,12;31,17,19;12,18,22	112;131;102
			48	3	9,11,7;8,13,6;15,12,3	53;53;59
			72	3	7,6,13;5,9,7;2,4,9	51;41;29
C (AS)	Yes	AS	24	3	17,21,12;9,18,22;35,17,25	98;96;151
			48	3	7,10,9;12,11,7;5,8,13	51;59;51
			72	3	5,3,10;13,11,9;3,8,7	35;65;35
D (ListShield™)	Yes	1.1×10^6 PFU/g	24	3	7,2,1;0,3,5;1,0,0	20;16;2
			48	3	0,3,1;0,0,3;1,0,1	8;6;4
			72	3	1,0,3;3,2,1;0,2,1	8;12;6
E (ListShield™/AS)	Yes	1.1×10^6 PFU/g + AS	24	3	0,0,3;5,1,1;2,0,1	6;14;6
			48	3	0,7,0;1,0,0;1,1,0	14;2;4
			72	3	0,2,1;3,1,3;0,0,1	6;14;2

11.2 Tabular presentation of results

Table 2 Reduction of *L. monocytogenes* counts on apple slices treated with AS and/or ListShield™ when applied at ca. 1.1x10^6 PFU/g (0.106mL per 100g) apple.

Group	Bacteria	Treatment	Storage time	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant, vs water at same time point?
B (Control)	Yes	Water	24	*n = 3*	115	-	-	-
			48	*n = 3*	55	-	-	-
			72	*n = 3*	41	-	-	-
C (AS)	Yes	AS	24	*n = 3*	115	0	0.0	No
			48	*n = 3*	54	2	0.0	No
			72	*n = 3*	45	-11	0.0	No
D (ListShield™)	Yes	1.1x10^6 PFU/g	24	*n = 3*	12	89	1.0	Yes
			48	*n = 3*	6	89	1.0	Yes
			72	*n = 3*	8	79	0.7	Yes
E (ListShield™/AS)	Yes	1.1x10^6 PFU / g / AS	24	*n = 3*	8	93	1.1	Yes
			48	*n = 3*	7	88	0.9	Yes
			72	*n = 3*	7	82	0.8	Yes

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

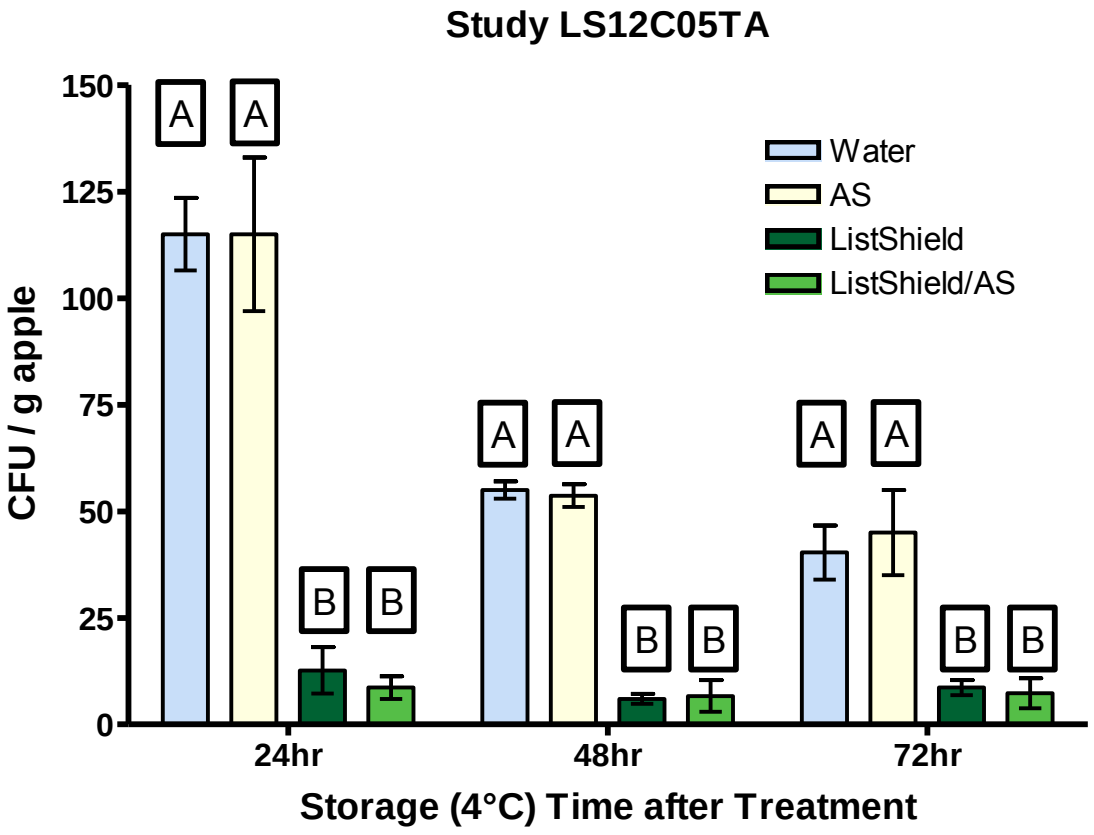


Figure 1 The effect of ListShield or ListShield/AS treatment on the number of *Listeria monocytogenes* recovered from artificially contaminated apples (data are shown as mean values and standard errors of the means (SEM) from triplicates). Bars within the same storage time with different letters are significantly different from one another (P<0.05).

11.4 Statistical analysis

The efficacy of the ListShield™ treatment in reducing the number of viable *L. monocytogenes* in the experimentally contaminated apple slices was evaluated by comparing the data obtained with the water-treated control samples and the AS, ListShield or ListShield/AS-treated samples at each time point.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison-24 hour data	Mean Difference	Q	P value
Sterile water vs AS	0.000	0.000	ns P > 0.05
Sterile water vs ListShield™	102.33	9.829	*** P < 0.001
Sterile water vs ListShield™/AS	106.33	10.214	*** P < 0.001
AS vs ListShield™	102.33	9.829	*** P < 0.001
AS vs ListShield™/AS	106.33	10.214	*** P < 0.001
ListShield™ vs ListShield™/AS	4.000	0.3842	ns P > 0.05

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison-48 hour data	Mean Difference	Q	P value
Sterile water vs AS	1.333	0.5208	ns P > 0.05
Sterile water vs ListShield™	49.000	19.138	*** P < 0.001
Sterile water vs ListShield™/AS	48.333	18.877	*** P < 0.001
AS vs ListShield™	47.667	18.617	*** P < 0.001
AS vs ListShield™/AS	47.000	18.357	*** P < 0.001
ListShield™ vs ListShield™/AS	-0.6667	0.2604	ns P > 0.05

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison-72 hour data	Mean Difference	Q	P value
Sterile water vs AS	-4.667	0.7473	ns P > 0.05
Sterile water vs ListShield™	31.667	5.071	* P < 0.05
Sterile water vs ListShield™/AS	33.000	5.284	* P < 0.05
AS vs ListShield™	36.333	5.818	* P < 0.05
AS vs ListShield™/AS	37.667	6.031	* P < 0.05
ListShield™ vs ListShield™/AS	1.333	0.2135	ns P > 0.05

The continued residual effect of the ListShield™ treatments were evaluated by comparing the data obtained from the two treatments over the storage time.

The P value is 0.7698, considered not significant. Variation among column means is not significantly greater than expected by chance.

Comparison- ListShield™ treated data across all time points	Mean Difference	Q	P value
24hr vs. 48 hr	6.667	1.987	ns P > 0.05
24hr vs. 72hr	4.000	1.192	ns P > 0.05
48 hr vs. 72hr	-2.667	0.7947	ns P > 0.05
Comparison- ListShield™/AS treated data across all time points	Mean Difference	Q	P value
24hr vs. 48 hr	2.000	0.5960	ns P > 0.05
24hr vs. 72hr	1.333	0.3974	ns P > 0.05
48 hr vs. 72hr	-0.6667	0.1987	ns P > 0.05

11.5 Brief discussion of results and study's conclusions

- Applying AS (80g/L) at 0.106mL / 100g apple did not reduce the number of viable *L. monocytogenes*.

- Applying ListShield™ at ca. 1.1×10^6 PFU/g apple reduced the number of viable *L. monocytogenes* by ca. 1.0 log (89%), 1.0 log (89%), and 0.7 log (79%) after 24, 48, and 72 hours (respectively) storage at 4°C. The observed reductions were statistically significant ($P<0.05$).

- Applying ListShield™ diluted in AS at ca. 1.1×10^6 PFU/g apple reduced the number of viable *L. monocytogenes* by ca. 1.1 log (93%), 0.9 log (88%), and 0.8 log (82%) after 24, 48, and 72 hours (respectively) storage at 4°C. The observed reductions were statistically significant ($P<0.05$).

- The difference in *L. monocytogenes* recovered when ListShield™ compared to ListShield™ diluted in AS was applied (application rate of 1.1×10^6 PFU/g) was not statistically significant ($P>0.05$).

- The difference in *L. monocytogenes* recovered when the samples were treated with ListShield™ for longer periods of time (24h vs. 48h vs. 72h) was not statistically significant ($P>0.05$).

- The difference in *L. monocytogenes* recovered when the samples were treated with ListShield™/AS for longer periods of time (24h vs. 48h vs. 72h) was not statistically significant ($P>0.05$).

12 SUMMARY CONCLUSION OF THE STUDY

ListShield™ can significantly reduce viable *Listeria monocytogenes* levels in experimentally contaminated apple slices by ca. 79-93% but it does not provide continued technical effect (i.e. the effect is limited to the initial reduction and does not improve during storage).

Using AS to dilute ListShield™ does not significantly affect the efficacy.

13 SIGNATURES

(b) (6)

Tamar Abuladze

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.3:
Report LS14A22ML

Evaluation of the ability of ListShield™ to reduce *Listeria monocytogenes* contamination in experimentally contaminated lettuce

Study # LS14A22ML

Intralytix

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

www.intralytix.com

Table of Contents

1 Study Title 3
2 Study Director 3
3 Study Personnel 3
4 Performing Laboratory 3
5 Study Objective 3
6 Test Matrix 4
7 ListShield™ Lot and Application 4
8 Bacterial strains used to experimentally contaminate lettuce 4
9 Media and Reagents 5
10 General Outline of Study 5
11 Results 6
11.1 Raw Data 6
11.2 Tabular presentation of results 6
11.3 Graphical presentation of results 7
11.4 Statistical analysis 7
11.5 Brief discussion of results and study's conclusions 8
12 Summary Conclusion of the Study 8
13 Signatures 9

1 STUDY TITLE

Evaluation of the ability of ListShield™ to reduce *Listeria monocytogenes* contamination in experimentally contaminated lettuce.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, MD	Research Scientist	Hands-on-research
Joelle Woolston, MS	Research Scientist / Laboratory Manager	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.

Research and Development

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of ListShield™ reduces the number of viable *Listeria monocytogenes* on lettuce when applied at the rate of $1x10^7$ and $1x10^8$ PFU/g.

6 TEST MATRIX

Long-leaf green lettuce was obtained from a local Baltimore grocery store deli. It was not washed or pre-treated prior to our studies.

7 LISTSHIELD™ LOT AND APPLICATION

- ListShield™ Lot 0113H150142
- Titer: approx. $1x10^{10}$ PFU/mL
- ListShield™ was diluted, when necessary, with water just prior to application.
- The application rate was ca. 1 mL ListShield™ per 100g lettuce.
- ListShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE LETTUCE

The lettuce test matrix was experimentally contaminated with a 1:1:1 mixture of three *L. monocytogenes* strains:

- *Lm*68: A *Listeria monocytogenes* strain
- *Lm*82: A *Listeria monocytogenes* strain
- *Lm*320: A *Listeria monocytogenes* strain obtained from ATCC (also known as ATCC 19115).

Upon receipt, the above-noted Intralytix strain designation was assigned (i.e., *Lm*320). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol.

Shortly before performing the study, the strains were thawed and grown (37 ± 2°C, 16-24 h) in LB broth. Overnight growth corresponds to ca. $4x10^8$ CFU/mL. Equal volumes of the three strains were combined for a 1:1:1 mixture and the mixed culture was diluted 1,000-fold just prior to performing the study.

The lettuce was experimentally contaminated with ca $2x10^3$ CFU / g of lettuce.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Oxford Agar (Fluka Biochemica, catalog # 75805)
- Oxford *Listeria* Selective Supplement (Fluka Biochemica, catalog # 75806)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)

10 GENERAL OUTLINE OF STUDY

1) Three 100g portions of lettuce were each assigned as test groups A, B, or C.
2) The challenge dose of bacteria was applied onto the lettuce surfaces. Bacterial cultures were evenly spread onto all sides of the lettuce sample surfaces using hockey sticks.
3) The samples were covered loosely and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
4) Water (control) or ListShield™ was applied as described in section 7. Treatments were evenly applied to the lettuce samples' surfaces as follows:
    - Group A = 1mL water / 100g = 0 PFU/g
    - Group B = 1mL $1x10^9$ PFU/mL ListShield™ / 100g = $1x10^7$ PFU/g
    - Group C = 1mL $1x10^{10}$ PFU/mL ListShield™ / 100g = $1x10^8$ PFU/g
5) The samples were covered and incubated at room temperature for ca. 5 minutes.
6) At 5 minutes post-treatment with water or ListShield™, from each sample group, triplicate ~25g samples of lettuce were cut, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
7) The number of viable *Listeria monocytogenes* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached lettuce / peptone water mixture onto separate Oxford plates. The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated lettuce}} = \frac{\textit{CFU}}{\textit{0.5mL plating}} \times \frac{\textit{225 mL peptone}}{\textit{25 g sample}}$$

Counts from 0.5 mL plating were used during the analysis, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate).

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # LS14A22ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A (Control)	Yes	100	WATER	3	60, 86, 67	1080, 1548, 1206
B ($1x10^9$ PFU/mL)	Yes	100	$1x10^7$ PFU/g ListShield™	3	16, 29, 23	288, 522, 414
C ($1x10^{10}$ PFU/mL)	Yes	100	$1x10^8$ PFU/g ListShield™	3	8, 5, 6	144, 90, 108

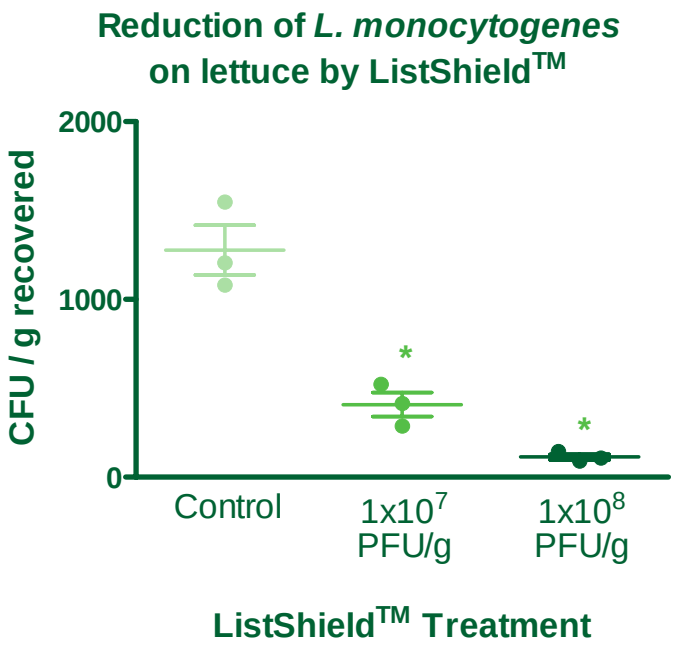
11.2 Tabular presentation of results

Table 2 Reduction of *Listeria monocytogenes* counts on lettuce treated with ListShield™ when applied at ca. $1x10^7$ – $1x10^8$ PFU/g (1mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A (Control)	Yes	WATER	*n =3*	1278	-	-	-
B ($1x10^9$ PFU/mL)	Yes	$1x10^7$ PFU/g ListShield™	*n = 3*	408	68	0.5	Yes
C ($1x10^{10}$ PFU/mL)	Yes	$1x10^8$ PFU/g ListShield™	*n =3*	114	91	1.1	Yes

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)



*** indicates statistically different from the control (P<0.05)**

11.4 Statistical analysis

The efficacy of the ListShield™ treatment in reducing the number of viable *Listeria monocytogenes* in the experimentally contaminated lettuce was evaluated by comparing the data obtained with the water-treated control samples and the ListShield-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0002, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
Water vs $1x10^7$ PFU/g	870.00	9.652	** P<0.01
Water vs $1x10^8$ PFU/g	1164.0	12.914	*** P<0.001
$1x10^7$ PFU/g vs $1x10^8$ PFU/g	294.00	3.262	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying ListShield™ at ca. $1x10^8$ PFU/g lettuce reduced the number of viable *Listeria monocytogenes* by ca. 91% (1.1 log) after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).
- Applying ListShield™ at ca. $1x10^7$ PFU/g lettuce reduced the number of viable *Listeria monocytogenes* by ca. 68% (0.5 log) after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).
- Reduction in *Listeria monocytogenes* levels achieved by using more concentrated ListShield™ was not statistically different from those obtained with more dilute ListShield™ (91% vs. 68% when using ca. $1x10^8$ PFU/g and $1x10^7$ PFU/g, respectively).

12 SUMMARY CONCLUSION OF THE STUDY

ListShield™ can significantly reduce viable *Listeria monocytogenes* levels in experimentally contaminated lettuce by ca. 68-91% in 5 minute contact time, when applied at ca. $1x10^7$ – $1x10^8$ PFU/g.

Using the higher ListShield™ application rate (ca. $1x10^8$ PFU/g) did not result in statistically significant better reduction of *L. monocytogenes* levels compared to lower ListShield™ application rate (ca. $1x10^7$ PFU/g).

13 SIGNATURES

(b) (6)

Manrong Li, MD

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.4:
Report LS14A16TA

Evaluation of the ability of ListShield™ to reduce *Listeria monocytogenes* contamination in experimentally contaminated cheese

Study # LS14A16TA

Intralytix

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

www.intralytix.com

Table of Contents

1 Study title 3
2 Study director 3
3 Study personnel 3
4 Performing laboratory 3
5 Study objective 3
6 Test matrix 4
7 ListShield™ lot and application 4
8 Bacterial strains used to experimentally contaminate cheese 4
9 Media and reagents 4
10 General outline of study 5
11 Results 6
11.1 Raw Data 6
11.2 Tabular presentation of results 6
11.3 Graphical presentation of results 7
11.4 Statistical analysis 7
11.5 Brief discussion of results and study's conclusions 8
12 Summary conclusion of the study 8
13 Signatures 9

1 STUDY TITLE

Evaluation of the ability of ListShield™ to reduce *Listeria monocytogenes* contamination in experimentally contaminated cheese.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Tamar Abuladze, BS	Research Scientist	Hands-on-research
Joelle Woolston, MS	Research Scientist / Laboratory Manager	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.

Research and Development

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of ListShield™ reduces the number of viable *Listeria monocytogenes* on cheese when applied at the rate of $1x10^8$ PFU/g.

6 TEST MATRIX

The cheese was obtained from a dairy industry source. It was not washed or pre-treated prior to our studies.

7 LISTSHIELD™ LOT AND APPLICATION

- ListShield™ Lot #0113H150142
- Titer: approx. $1x10^{10}$ PFU/mL
- ListShield™ was diluted, when necessary, with water just prior to application.
- The application rate was ca. 3 mL ListShield™ per 300 g cheese.
- ListShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE CHEESE

The cheese test matrix was experimentally contaminated with a 1:1:1 mixture of three *L. monocytogenes* strains:

- *Lm*68: A *Listeria monocytogenes* strain
- *Lm*82: A *Listeria monocytogenes* strain
- *Lm*320: A *Listeria monocytogenes* strain obtained from ATCC (also known as ATCC 19115).

Upon receipt, the above-noted Intralytix strain designation was assigned (i.e., *Lm*320). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol.

Shortly before performing the study, the strains were thawed and grown (37 ± 2°C, 16-24 h) in LB broth. Overnight growth corresponds to ca. $8x10^8$ CFU/mL. Equal volumes of the three strains were combined for a 1:1:1 mixture and the mixed culture was diluted 1,000-fold just prior to performing the study.

The cheese was experimentally contaminated with ca. $1x10^4$ CFU / g of cheese.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)

- Oxford Agar (Fluka Biochemica, catalog # 75805)
- Oxford *Listeria* Selective Supplement (Fluka Biochemica, catalog # 75806)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)

10 GENERAL OUTLINE OF STUDY

1) One 600 g portion of cheese was contaminated with bacteria. The challenge dose of bacteria was applied onto the cheese surfaces. Bacterial cultures were evenly spread onto all sides of the cheese sample surfaces using hockey sticks.

2) The sample was covered loosely and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min. The portion was split into two test groups, A and B.

3) PBS (control) or ListShield™ was applied as described in section 7. Treatments were evenly applied to the cheese samples' surfaces as follows:

    - Group A = 3mL PBS / 300g = 0 PFU/g
    - Group B = 3mL $1x10^{10}$ PFU/mL ListShield™ / 300g = $1x10^{8}$ PFU/g

4) The samples were covered and incubated at room temperature for ca. 5 minutes.

5) At 5 minutes post-treatment with PBS or ListShield™, from each sample group, triplicate ~25g samples of cheese were cut, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds. The remaining unsampled cheese was loosely covered and moved to 4°C storage.

6) The number of viable *Listeria monocytogenes* in the samples was determined by plating aliquots (0.1 mL) of the stomached cheese /peptone water mixture onto separate Oxford plates. The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated cheese}} = \frac{\textit{CFU}}{\textit{0.1mL plating}} \times \frac{\textit{225 mL peptone}}{\textit{25 g sample}}$$

7) Steps 5) and 6) were repeated at 24 and 96 hours post-ListShield™ treatment.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # LS14A16TA

Group	Challenged with bacteria	Treatment	~25g Samples	CFU in 0.1 mL	CFU/g
A (Control) – 5'	Yes	PBS	3	110, 110, 122	9900, 9900, 10980
B ($1x10^{10}$ PFU/mL) – 5'	Yes	$1x10^8$ PFU/g ListShield™	3	28, 20, 15	2520, 1800, 1350
A (Control) – 24h	Yes	PBS	3	85, 107, 100	7650, 9630, 9000
B ($1x10^{10}$ PFU/mL) – 24h	Yes	$1x10^8$ PFU/g ListShield™	3	15, 27, 21	1350, 2430, 1890
A (Control) – 96h	Yes	PBS	3	110, 105, 120	9900, 9450, 10800
B ($1x10^{10}$ PFU/mL) – 96h	Yes	$1x10^8$ PFU/g ListShield™	3	16, 42, 50	1440, 3780, 4500

11.2 Tabular presentation of results

Table 2 Reduction of *Listeria monocytogenes* counts on cheese treated with ListShield™ when applied at ca. $1x10^8$ PFU/g.

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. PBS	Log reduction vs. PBS	Significant (compared to control at same time point)?
A (Control) – 5'	Yes	PBS	*n =3*	10260	-	-	-
B ($1x10^{10}$ PFU/mL) – 5'	Yes	$1x10^8$ PFU/g ListShield™	*n =3*	1890	82	0.7	Yes
A (Control) – 24h	Yes	PBS	*n =3*	8760	-	-	-
B ($1x10^{10}$ PFU/mL) – 24h	Yes	$1x10^8$ PFU/g ListShield™	*n =3*	1890	78	0.7	Yes
A (Control) – 96h	Yes	PBS	*n =3*	10050	-	-	-
B ($1x10^{10}$ PFU/mL) – 96h	Yes	$1x10^8$ PFU/g ListShield™	*n =3*	3240	68	0.5	Yes

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

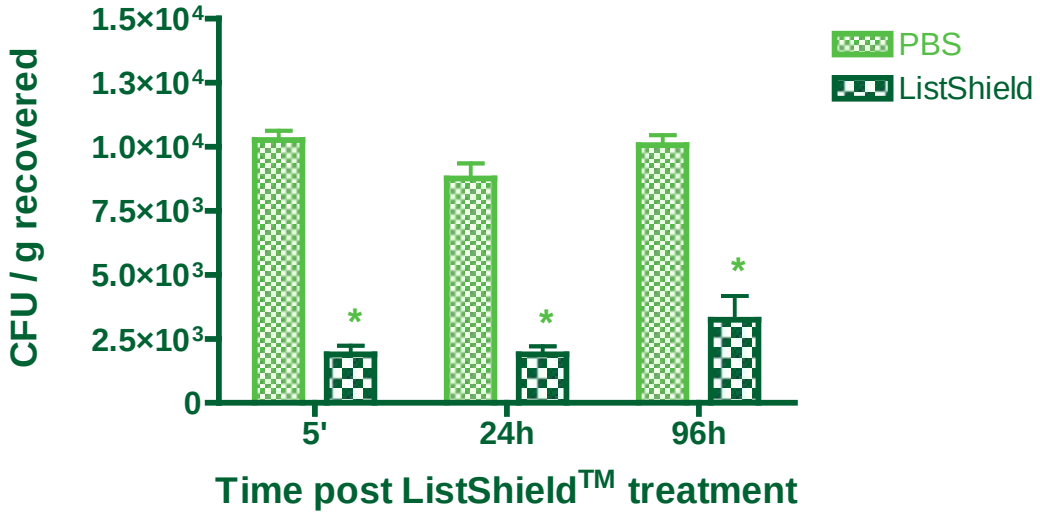


Figure 1 The effect of ListShield treatment on the number of *Listeria monocytogenes* recovered from artificially contaminated cheese (data are shown as mean values and standard errors of the means (SEM) from triplicates). The * indicate bars that are statistically different from the PBS control at the same time point (P<0.05).

11.4 Statistical analysis

The efficacy of the ListShield™ treatment in reducing the number of viable *Listeria monocytogenes* in the cheese experimentally contaminated with bacteria was evaluated by comparing the data obtained with the PBS-treated control samples and the ListShield-treated samples at each time point.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
PBS-5' vs ListShield-5'	8370.0	15.749	*** P<0.001
PBS-24hr vs ListShield-24hr	6870.0	12.927	*** P<0.001

PBS-96hr vs ListShield-96hr	6810.0	12.814	*** P<0.001

The continued residual effect of the ListShield™ treatment was evaluated by comparing the data of the treatment over the storage time.

Comparison	Mean Difference	q	P value
ListShield-5' vs ListShield-24hr	0.000	0.000	ns P>0.05
ListShield-5' vs ListShield-96hr	-1350.0	2.540	ns P>0.05
ListShield-24hr vs ListShield-96hr	-1350.0	2.540	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying ListShield™ at ca. $1x10^8$ PFU/g cheese reduced the number of viable *Listeria monocytogenes* by ca. 82% (0.7 log) after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ListShield™ at ca. $1x10^8$ PFU/g cheese reduced the number of viable *Listeria monocytogenes* by ca. 78% (0.7 log) after 24 hours of incubation at 4°C. The observed reduction was statistically significant (P<0.001).

- Applying ListShield™ at ca. $1x10^8$ PFU/g cheese reduced the number of viable *Listeria monocytogenes* by ca. 68% (0.5 log) after 96 hours of incubation at 4°C. The observed reduction was statistically significant (P<0.001).

- The difference in *Listeria monocytogenes* recovered when the samples were treated with ListShield™ for longer periods of time (5' vs. 24h vs. 96h) was not statistically significant (P>0.05).

12 SUMMARY CONCLUSION OF THE STUDY

ListShield™ can significantly reduce viable *Listeria monocytogenes* levels in experimentally contaminated cheese by ca. 68-82%, but it does not provide continued technical effect (i.e. the effect is limited to the initial reduction and does not improve during storage).

13 SIGNATURES

(b) (6)

Tamar Abuladze, BS

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

SUBMISSION END