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October 4, 2016

Dr. Paulette Gaynor
Division of Biotechnology and GRAS Notice Review
Center for Food Safety & Applied Nutrition (HFS-255)
U.S. Food & Drug Administration
5100 Campus Drive
College Park, MD 20740

Reference: Intralytix GRAS Notification for ShigaShield™

Dear Dr. Gaynor:

In accordance with the Federal Register [81 Fed. Reg. 159 (17 August 2016)] issuance on GRAS notifications (21 CFR Part 170), Intralytix is pleased to submit a notice that we have concluded, through scientific procedures, the bacteriophage cocktail, ShigaShield™, is generally recognized as safe and is not subject to the pre-market approval requirements for the use in foods, generally, as a processing aid to control *Shigella*.

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety and Inspection Service, regarding the use of ShigaShield™ as a safe and suitable antimicrobial used in the production of meat and poultry products as a processing aid. ShigaShield™ is substantially equivalent to the several other bacteriophage products also listed in FSIS Directive 7120.1 as processing aids.

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

Sincerely,

Alexander Sulakvelidze
Vice President & Chief Scientist
Intralytix, Inc.

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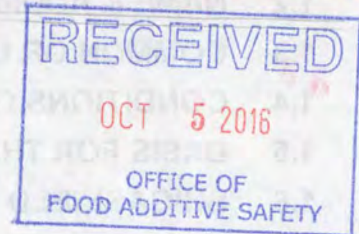
GRN 000672



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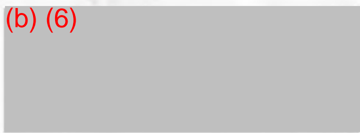

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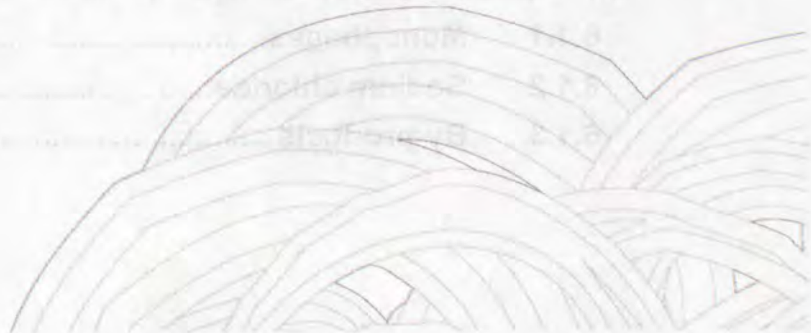

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1 SIGNED STATEMENTS AND CERTIFICATION

1.1 STATEMENT OF INTENT

In accordance with the 21 CFR 170 Subpart E, regulations for GRAS notifications, Intralytix is pleased to submit a notice that we have concluded, through scientific procedures, the bacteriophage preparation, ShigaShield™, is generally recognized as safe and is not subject to the premarket approval requirements for the use in foods, generally, as a processing aid to control *Shigella* under the intended use conditions described within this notification.

1.2 NAME & ADDRESS OF NOTIFIER

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

1.3 COMMON OR USUAL NAME

Intralytix produces a lytic bacteriophage preparation with potent lytic activity against the Gram-negative bacterium *Shigella* under the trade name ShigaShield™.

1.4 CONDITIONS OF USE

ShigaShield™ is intended for use as an antimicrobial to control *Shigella* spp. on food when applied to food surfaces up to $1x10^8$ PFU / gram of food, including the following food categories:

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

1.5 BASIS FOR THE GRAS CONCLUSION

Pursuant to the GRAS rule, Intralytix has concluded that ShigaShield™ is GRAS through scientific procedures, in accordance with 21 CFR 170.30 (a) and (b).

1.6 SHIGASHIELD IS NOT SUBJECT TO PREMARKET APPROVAL

Because Intralytix has concluded that ShigaShield™ is GRAS, it is not subject to the premarket approval requirements for the use in foods, generally, as a processing aid to control *Shigella* under the intended use conditions described within this notification.

1.7 AVAILABILITY OF INFORMATION

The data and information that are the basis for Intralytix's conclusion that ShigaShield™ is GRAS are available for review and copying by FDA during customary business hours, at the location below, or will be sent to FDA upon request, made to:

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

1.8 FREEDOM OF INFORMATION ACT

It is our view that the information contained in this notification are not exempt from disclosure under the Freedom of Information Act.

1.9 CERTIFICATION

To the best of our knowledge, this GRAS notification is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of ShigShield™.

1.10 SIGNATURE

(b) (6)

10/04/2016

Alexander Sulakvelidze
VP Research and Development
asulakvelidze@intralytix.com

Date

1.11 FSIS AUTHORIZATION

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety and Inspection Service, regarding the use of ShigaShield™ as a safe and suitable antimicrobial used in the production of meat and poultry products as a processing aid. ShigaShield™ is substantially equivalent to the several other bacteriophage products also listed in FSIS Directive 7120.1 as processing aids.

2 IDENTITY AND SPECIFICATIONS OF SHIGASHIELD™

2.1 IDENTITY

ShigaShield™ consists of a mixture of approximately equal proportions of five individually purified lytic bacteriophages (hereinafter referred to as component phage(s) or component monophage(s)). Each of these monophages is specifically effective against *Shigella.* The component phages in ShigaShield™ were isolated by Intralytix's scientists and have not been genetically manipulated (i.e., not GMO).

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

2.2 METHOD OF MANUFACTURE

The component monophages of ShigaShield™ are prepared using an aerobic fermentation process in animal-product free media. For each monophage, the host *Shigella sonnei* 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 tangential-flow filtration. Following the initial filtration, the monophage is concentrated, washed with 0.1M sodium chloride, then sterilized using filtration. After all five component monophages have each passed quality control specifications, proper volumes of each monophage, and sterile 0.1M sodium chloride as necessary, are combined, and final filtration is carried out using a sterilizing grade filter. The ShigaShield™ article of commerce is prepared so that:

Each monophage is approximately equally represented

AND

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

The ShigaShield™ article of commerce is diluted with clean water at the application site, to form the "working solution" or "working concentration" of ShigaShield™ with a lytic titer of 9.0 $\log_{10}$ 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 ShigaShield™.

Table 1 Product specifications for individual monophage lots

Parameter	Specification
Potency (PFU/mL)	≥10.0 $\log_{10}$ PFU/mL
Microbial purity	No growth
Identity	Matches reference

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

Table 2 Product specifications for ShigaShield™

Parameter	Specification
Potency (PFU/mL)	≥10.0 $\log_{10}$ PFU/mL
Microbial purity	No growth
Endotoxin Content (EU/mL)	≤25,000 EU/mL (at ca. 9.0 $\log_{10}$ PFU/mL)
Identity Test	All component phages are present

2.4 CHARACTERISTIC PROPERTIES

ShigaShield™ is a clear to opalescent odorless liquid with a specific gravity of approximately 1.01. The phage component of ShigaShield™ (typical working concentration of ca. 1 x 10^9 PFU/mL) is roughly estimated to be 0.0000381% by weight and the remainder is 0.1M sodium chloride. Typical chemical analysis of ShigaShield™ (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 ShigaShield™ lots.

Table 3 Typical chemical analysis of ShigaShield™ (at standard working concentration of $1x10^9$ PFU/mL)

Property/analysis/composition	Reporting Detection Limit	ShigaShield™ Lot# (b) (6)	ShigaShield™ Lot#	ShigaShield™ Lot#	ShigaShield™ average
Total Nitrogen (mg/L)	0.5	3.8	3.4	3.3	3.5
pH	n/a	6.16	6.03	6.04	6.08
Specific gravity (at 25°C)	n/a	1.01	1.01	1.01	1.01
Ash (inorganic solids) (%)	0.01	ND	ND	ND	ND
Arsenic (mg/L)	0.005	ND	ND	ND	ND
Barium (mg/L)	0.01	ND	ND	ND	ND
Cadmium (mg/L)	0.005	ND	ND	ND	ND
Calcium (mg/L)	0.1	0.275	0.284	0.294	0.284
Chromium (mg/L)	0.01	ND	ND	ND	ND
Cobalt (mg/L)	0.005	ND	ND	ND	ND
Copper (mg/L))	0.01	0.031	0.030	0.027	0.029
Iron (mg/L)	0.02	0.022	0.022	0.020	0.021
Lead (mg/L)	0.005	0.005	ND	ND	≤0.005
Magnesium (mg/L)	0.1	ND	ND	ND	ND
Manganese (mg/L)	0.01	ND	ND	ND	ND
Molybdenum (mg/L)	0.01	ND	ND	ND	ND
Nickel (mg/L)	0.01	ND	ND	0.01	≤0.01
Phosphorus (mg/L)	2	ND	ND	ND	ND
Potassium (mg/L)	0.5	0.71	0.64	0.58	0.64
Silicon (mg/L)	0.1	ND	ND	ND	ND
Sodium (mg/L)	0.5	240	242	250	244
Tin (mg/L)	0.02	0.023	0.022	0.020	0.022
Zinc (mg/L)	0.01	0.019	0.018	0.015	0.017
Chloride (mg/L)	5	352	373	389	371
Nitrate (as N) (mg/L)	0.1	0.13	ND	ND	≤0.13
Nitrite (as N) (mg/L)	0.1	ND	ND	ND	ND
Total Organic Carbon (mg/L)	1	20.2	17.8	18.2	18.7
Total Kjeldahl Nitrogen (mg/L)	0.4	3.77	3.37	3.27	3.47
Total Dissolved Solids (mg/L)	10	538	578	592	569
Total Phosphorous (mg/L)	0.02	0.41	0.40	0.41	0.41
Silica (mg/L)	1	1.73	1.38	1.39	1.50
Protein (%)	0.1	ND	ND	ND	ND
Non-Volatile Solids (105°C) (mg/L)	10	604	636	668	636
Non-Volatile Solids (180°C) (mg/L)	10	520	564	600	561

ND = none detected

2.5 PHAGE CLASSIFICATION

The current component phages in ShigaShield™ were 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 *Shigella* strains, and lytic activity against non-*Shigella* strains.

The five component bacteriophages currently included in ShigaShield™ are listed below:

Name:	SHFML-11
ATCC #:	PTA-121234
Order:	Caudovirales
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

Name:	SHFML-26
ATCC #:	PTA-121236
Order:	Caudovirales
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

Name:	SHSML-45
ATCC #:	PTA-121238
Order:	Caudovirales
Family:	Siphoviridae
Properties:	Double-stranded DNA, Lytic

Name:	SHBML-50-1
ATCC #:	PTA-121239
Order:	Caudovirales
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

Name:	SHSML-52-1
ATCC #:	PTA-121241
Order:	Caudovirales
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

2.6 POTENTIAL HUMAN TOXICANTS

The *Shigella* host strain is a Gram-negative bacterium. As with all Gram-negative bacteria, it produces bacterial endotoxin or lipopolysaccharide (LPS). Intralytix tests every lot of ShigaShield™ for LPS to ensure it meets the release criteria. Endotoxins are further discussed below, in Sections 3.1.2.3, 6.1.3, and 6.2.1.3.

Shigella strains are often known to carry enterotoxins. Even though great care is taken to remove media products, processing enzymes, and host material - including nucleic acids - from phage

lysates, bacterial strains that may be used for phage propagation are routinely screened for enterotoxins. The Sh.s43 host strain, used for propagation of all ShigaShield™ component phages, does not contain the genes for any of the known enterotoxins. *Shigella* toxins are further discussed in Section 6.2.1.3 and Appendix 2.

2.7 STABILITY

The proposed shelf life of ShigaShield™ article of commerce is one year when stored at 2–8°C in a dark, UV-protected area.

3 DIETARY EXPOSURE

3.1 APPLICATION RATES AND DIETARY INTAKE

3.1.1 Application rates

The current ShigaShield™ article of commerce is a concentrate that is typically diluted with water at the application site to form the ShigaShield™ working solution. 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. Future preparations may be sold in more concentrated form, but the accompanying instructions for dilution and application rate will be appropriately adjusted to ensure the final concentration of phage on the food articles is always at or below $1x10^8$ PFU/g of food.

3.1.2 Dietary intakes

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

- Ready-to-eat meats
- 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 ShigaShield™ 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 was used.[56] 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 ShigaShield™. This worst-case scenario assumes 100% market saturation (i.e. that the entire

food supply is treated with ShigaShield™), 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 ShigaShield™, and its constituents, that would be consumed via the five food categories are very small, as shown in the following calculations.

3.1.2.1 Dietary intakes for ShigaShield™

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

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

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

Using 0.1mL ShigaShield™ applied per gram of food, the volume of ShigaShield™ that would be consumed per day via each food category can be calculated and is presented in Table 4. 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 ShigaShield™ consumed per day would be about 189 mL or the equivalent of about ¾ cup.

Table 4 Volume of ShigaShield™ consumed per day when applied at $1x10^8$ PFU/g food

	Consumed per American per year (lbs)	Consumed per American per day* (g)	ShigaShield™ consumed per person per day (mL)
Poultry / Red meat	252.7	315	31.5
Fish/Shellfish	14.3	18	1.8
Fruits	261.1	325	32.5
Vegetables	384.4	479	47.9
Dairy	605.8	754	75.4
Total of all categories	1518.3	1891	189

*The ERS per capita usage data is given as lbs/year.[56] This column simply converts lbs/year to grams/day (lbs/year x 1000g ÷ 2.2lbs ÷ 365days).

The majority of the 189 mL of ShigaShield™ 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.

3.1.2.2 Dietary intakes for ShigaShield™ phages

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

Total phages (PFU) consumed per day:

$$\frac{1x10^8 \text{ PFU}}{\text{g food}} \times \frac{1891 \text{ g food}}{\text{day}} = \frac{1.9x10^{11} \text{ PFU}}{\text{day}}$$

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

$$\frac{1.9x10^{11} \text{ PFU}}{\text{day}} \times \frac{3.83x10^{-16} \text{ g}}{\text{phage}} \times \frac{1x10^6 \text{ µg}}{\text{g}} = \frac{72.4 \text{ µg}}{\text{day}}$$

Where $3.83x10^{-16}$ g = mass of one phage

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

$$\frac{72.4 \text{ µg}}{\text{day}} \times \frac{\text{day}}{3 \text{ kg}} = 24.1 \text{ ppb}$$

The weight of phages consumed per day via ShigaShield™ would be 72.4 µg, or 24.1 ppb in a 3 kg diet. This is insignificant.

3.1.2.3 Dietary intake of endotoxin

Normal saliva contains approximately 1 mg endotoxin per mL. [55] For endotoxin, 1 EU/mL is approximately equal to 1 ng/mL. This means that the 1 mg/mL of endotoxin in saliva is equivalent to approximately 1 x 10^6 EU/mL. Specification for ShigaShield™ lots for endotoxin is ≤ 25,000 EU/mL at $1x10^9$ PFU/mL.

The approximate daily volume of ShigaShield™ consumed is 189 mL (see Section 3.1.2.1). Again using the worst case scenario (maximum allowable endotoxin level by specification), the maximum amount of endotoxin consumed via ShigaShield™ is thus:

$$\frac{189 \text{ mL ShigaShield™}}{\text{day}} * \frac{2.5 \times 10^4 \text{ EU}}{\text{mL ShigaShield™}} = \frac{4.7 \times 10^6 \text{ EU}}{\text{day}}$$

Humans produce approximately 500 to 750 mL of saliva per day. Using the lower, more conservative number, healthy humans consume from saliva:

$$\frac{500\text{ mL saliva}}{\text{day}} * \frac{1 \times 10^{6}\text{ EU}}{\text{mL saliva}} = \frac{5 \times 10^{8}\text{ EU}}{\text{day}}$$

The maximal amount contributed by ShigaShield™ would thus constitute 0.95% of the daily load of endotoxin from saliva. The level of endotoxin found in ShigaShield™ is therefore considered safe.

3.1.2.4 Sodium and potassium content

From Section 2.4, the highest value obtained for sodium content in a ShigaShield™ lot was 250 mg/L. From this value and using the worst-case scenario value from Table 4 (all foods from each food category are treated with ShigaShield™), the amount of sodium contributed to the daily diet via ShigaShield™ can be calculated as follows:

$$\frac{250\text{ mg sodium}}{1000\text{ mL ShigaShield™}} \times \frac{189\text{ mL ShigaShield™}}{\text{day}} = \frac{47.3\text{ mg sodium}}{\text{day}}$$

The recommended daily allowance of sodium is 2,400 mg (21 CFR § 101.9(c)(9)). The amount of sodium per day contributed by ShigaShield™ thus represents 1.97 % of the RDA and is negligible. The amount of sodium per day contributed by ShigaShield™, 47.3 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, highest value obtained for potassium content a ShigaShield™ lot was 0.71 mg/L. From this value, the amount of potassium contributed to the daily diet via ShigaShield™ on the five food categories can be calculated as follows:

$$\frac{0.71\text{ mg potassium}}{1000\text{ mL ShigaShield™}} \times \frac{189\text{ mL ShigaShield™}}{\text{day}} = \frac{0.13\text{ mg potassium}}{\text{day}}$$

Assuming the potassium levels of ShigaShield™ are just below the detection limit, then the amount of potassium per day contributed by ShigaShield™, 0.13 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 ShigaShield™ thus represents 0.004% of the RDA and is negligible.

4 SELF-LIMITING LEVELS OF USE

The proposed use for ShigaShield™ is as an antimicrobial processing aid for foods that are at high risk to be contaminated with *Shigella*.

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 *Shigella*.
- Once the *Shigella* 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.

5 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This section is not applicable to this notification.

6 NARRATIVE

In the following sections, the data and information providing the basis for our conclusion that ShigaShield™ is GRAS, through scientific procedures, under the conditions of its intended use is presented. The information provided below, and elsewhere in this document, that is generally available has been properly cited. The list of references is presented in Part 7.

6.1 COMPONENTS OF SHIGASHIELD™

ShigaShield™ is a mixture of component bacteriophages together with added sodium chloride; due to the method of production, there may also be 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

6.1.1 Monophages

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 show that:

- Bacteriophages are the most ubiquitous organisms on earth. For example, one milliliter of non-polluted stream water has been reported [1] 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 [2]). 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 almost 100 years, without any serious side effects.[3, 4] 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. [5-9]

- 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. [10, 11] 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. [12-14]

- Phages have also been administered to humans via various sera and FDA-approved vaccines commercially available in the United States. [15-17]

- 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 populace/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 gastrointestinal (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. [18-20] 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. [21, 22] There are an estimated 10^{15} phages [23] typically present in the human gastrointestinal tract.

- No serious adverse immunologic or allergic sequelae have ever been reported because of human or animal exposure to phages. [3, 6]

- Bacteriophages are commonly consumed via drinking water. [24-26]

- Bacteriophages are natural components of all fresh, unprocessed foods and are commonly consumed via various foods. For example, bacteriophages have been readily 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. [27-36] 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, Oblinger [36]. 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. Additionally, *E. coli*- and *Shigella*-specific bacteriophages were recently isolated from 100% of beef and 68% of mixed salad purchased in a variety of markets.[37]

- 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. [38]
  - A pharmacokinetic and toxicological study using mice and guinea pigs did not show any signs of acute toxicity or histological changes, even when the dose administered was 3500-fold higher than the human dose projected in the course of the study. [39]
  - High doses of *Listeria* phage preparations (i.e. ListShield™ and P100) were administered to laboratory animals (mice and rats) without any adverse effects.[40, 41]
  - **Most relevant, a long-term toxicity study with ShigaShield™ (under the tradename ShigActive™) in mice, showed no significant effect on any health or toxicity markers in the mice. Additionally, the phage preparation did not significantly affect the microbiota of the treated mice.[42]**
- Bacteriophages are commonly consumed by animals (including agriculturally-important species) via various foods. For example, in a recent study from Texas A&M University, 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. [43]

6.1.1.1 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. [4] Temperate phages are found in almost all bacterial genera, including *Staphylococcus*, *Vibrio*, *Pseudomonas*, *Salmonella*, *Shigella*, *Bacillus*, *Corynebacterium*, *Listeria,* and *Streptococcus.* [44-47] 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 ShigaShield™).

6.1.1.2 ShigaShield™ monophages are GRAS

The component phages in ShigaShield™ were isolated by Intralytix's scientists. Each was characterized by various approaches, including electron microscopy, genotypic fingerprinting, and full genome sequence analysis. The component phages in ShigaShield™ are members of the *Myoviridae* and S*iphoviridae* double-stranded DNA phage families, as defined by the International Committee on the Taxonomy of Viruses (ICTV) and by Ackermann and Berthiaume [48].

Intralytix has fully sequenced all component monophages included in ShigaShield™. This approach is used to exclude bacteriophages carrying sequences encoding undesirable 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), other known toxin genes, and 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 is 1 x 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 $\leq 10^{-5}$. [49] 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 ShigaShield™. Table 5 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 five monophages.
- No 16S ribosomal RNA genes have been identified among annotated genomes of any of the five monophages.
- No antibiotic resistance genes have been identified among annotated genomes of any of the five 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 5 Genome size and composition of phages contained in ShigaShield™

Phage	ATCC #	GenBank Accession #	GC%	Size (bp)	Number of Open Reading Frames (ORFs)	Undesirable genes
SHFML-11	PTA-121234	KX130861	35.2	170,650	270	None
SHFML-26	PTA-121236	KX130862	35.4	168,993	269	None
SHSML-45	PTA-121238	KX130863	38.7	108,050	139	None
SHBML-50-1	PTA-121239	KX130864	35.4	166,634	265	None
SHSML-52-1	PTA-121241	KX130865	37.6	169,621	269	None

6.1.1.3 ShigaShield™ is specific to *Shigella*

Lytic activity of ShigaShield™ is targeted against *Shigella* strains. ShigaShield™ has been screened for its lytic activity against just over 100 *Shigella* isolates in the Intralytix collection, representing all 4 species. As shown in Table 6, ShigaShield™ is very effective against the collection.

Table 6 *Shigella* species in Intralytix's collection and the percent of each susceptible to ShigaShield™ at 1x10^9 PFU/mL.

Species	# isolates in Intralytix collection	Percent kill (1x10^9 PFU/mL ShigaShield™)
S. dysenteriae	9	100%
S. flexneri	46	93%
S. sonnei	39	100%
S. boydii	9	100%
All isolates	103	97%

ShigaShield™ is also highly specific. Table 7 shows that ShigaShield™ does not lyse any of the non-targeted gram positive isolates examined. These strains include 5 strains each of *Staphylococcus aureus*, *Enterococcus*, and *Listeria species*. ShigaShield™ also does not lyse several non-*Shigella* gram negative strains, including 5 strains each of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Of the 5 strains of *E. coli* tested, ShigaShield™ was able to lyse all at 1x10^9 PFU/mL. This is not unexpected, as both *Shigella* and *E. coli* are members of the Enterobacteriaceae family. In fact, the two are so closely related that genetic analysis shows *Shigella* are genetically a sister species of *E. coli* within the genus *Escherichia*. [50] Bruttin and Brüssow [38] demonstrated oral administration of *E. coli*-specific phage T4 did not affect fecal *E. coli* counts and had no adverse effects in any volunteers. Therefore, ShigaShield™ would also be expected not to have a deleterious effect upon the natural gut flora (and would be much more specific compared to commonly available antibiotics).

Table 7 Lytic activity of ShigaShield™ against non-*Shigella* strains of bacteria

Non-*Shigella* isolates		Species	Susceptibility to ShigaShield™ ($1x10^9$ 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*	-
Lm 314	ATCC19117	*Listeria monocytogenes*	-
Lm 315	ATCC19118	*Listeria monocytogenes*	-
L. innocua 316	ATCC51724	*Listeria innocua*	-
Lm 317	ATCC19116	*Listeria monocytogenes*	-
L. innocua 318	ATCC33090	*Listeria innocua*	-
Ab3	ATCC19606	*Acinetobacter baumannii*	-
Ab4	HER1401	*Acinetobacter baumannii*	-
Ab5	4308-2	*Acinetobacter baumannii*	-
Ab6	3247-1	*Acinetobacter baumannii*	-
Ab7	1673-2	*Acinetobacter baumannii*	-
E102	WCC188	*Enterococcus* spp.	-
E402	ATCC11823	*Enterococcus* spp.	-
E403	ATCC19433	*Enterococcus* spp.	-
E404	1133455	*Enterococcus* spp.	-
E405	1126611	*Enterococcus* spp.	-
Pa76	ATCC10145	*Pseudomonas aeruginosa*	-
Pa161	ATCC15692	*Pseudomonas aeruginosa*	-
Pa162	ATCC51674	*Pseudomonas aeruginosa*	-
Pa163	ATCC43390	*Pseudomonas aeruginosa*	-
Pa164	ATCC39324	*Pseudomonas aeruginosa*	-
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 preparation - Not lysed by phage preparation

6.1.1.4 ShigaShield™ toxicity study

As mentioned above, a long-term toxicity study was performed using ShigaShield™ (under the trade name ShigActive™) in mice. [42] In this study, mice received either ShigaShield™ or phosphate buffered saline (PBS) for a period of up to 28 days. ShigaShield treated mice received $1x10^9$ PFU two times a day for the first seven days. After the first week, the same dose was administered every other day for the remaining 21 days. Several health and toxicity markers were examined. Comparisons between the two groups showed no significant difference at one or four weeks for body weight and weight gain, total and differential white blood cell counts, and ketone

content, specific gravity, pH, or protein content of urine samples. Additionally, neither the phage-treated nor control (PBS) group showed abnormal levels of leukocytes, nitrites, urobilinogen, bilirubin, or glucose in urine samples. There were no significant histopathological differences between the groups nor were there any pathologic lesions in the kidney, GI tract, liver, spleen, heart, lung, or brain. Finally, the microbiota composition of the GI tract did not differ in diversity measures between the two groups. This extensive study provides further evidence of the safety of oral ShigaShield™ / ShigActive™ administration.

6.1.2 Sodium chloride

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

6.1.3 By-products

Even though great care is taken to remove media products, processing enzymes, and host material - including nucleic acids - from phage lysates, bacterial strains that may be used for phage propagation are routinely screened for enterotoxins. The most commonly known *Shigella* enterotoxins are (1) Shiga toxin, (2) *Shigella* enterotoxin 1 (ShET1), and (3) *Shigella* enterotoxin 2 (ShET2).[51, 52] The host strain, Sh.s43, has been determined not to carry the genes for any three of these enterotoxins. The *Shigella* enterotoxins are further discussed in Section 6.2.1.3 and Appendix 2.

The *Shigella* host strains are Gram-negative bacteria. As with all Gram-negative bacteria, they produce bacterial endotoxin or LPS. Intralytix tests every lot of ShigaShield™ to ensure its LPS levels fall below the established release criteria. Endotoxins are further discussed in Sections 6.2.1.3 and 6.2.2.2.

6.2 MANUFACTURING OF SHIGASHIELD™

ShigaShield™ is manufactured using Intralytix's standard procedures. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, ListShield™ (21 CFR §172.785), EcoShield™ (FCN No. 1018) and SalmoFresh™ (GRAS Notice No. 435) and are currently used to manufacture commercial lots of these products.

ShigaShield™ is prepared by cultivation of individual host *Shigella* 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 ShigaShield™ concentrate. Final filtration is then carried out with a sterilizing grade filter.

6.2.1 Starting materials

There are four starting materials for manufacture of ShigaShield™ component monophages:

Animal-product free media

Antifoam

Host strain

Monophages

The safety of each is considered separately below.

6.2.1.1 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 "table 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.

6.2.1.2 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 may be used in the initial fermentation of the individual monophages.

6.2.1.3 Host strains

The component monophages are all produced on the same *Shigella sonnei* isolate, Sh.s43, from Intralytix's collection of *Shigella* strains (Table 8). This *Shigella sonnei* host strain was characterized at Intralytix. Its biochemical properties were examined using the bioMérieux API testing kit. Its background genomic composition/type was examined through the standard PFGE protocol for bacteria. Sh.s43 was also examined for the presence of endogenous phage(s) and its susceptibility to five commonly prescribed antibiotics (azithromycin, ceftriaxone, ciprofloxacin, levofloxacin, and sulfamethoxazole / trimethoprim).

Table 8 Summary of *Shigella* host strain specifications

Current *Shigella* host strain	Species	Biochemistry	PFGE	Endogenous phage	Antibiotic susceptibility
Sh.s43	sonnei	*Shigella* spp	+	-	4/5*

* Resistant to azithromycin

The *Shigella* host strain is not known to produce any enterotoxins that could compromise the safety of the final product. As discussed in Appendix 2, while *Shigella* strains are known to produce enterotoxins, the host strain, Sh.s43, has been determined to be free of the enterotoxin genes: *stxA/B*, *set1A*, *set1B*, and *senA*.

The only production host strain-related toxin that is relevant for ShigaShield™ safety is endotoxin or LPS. ShigaShield™ phages are propagated in a *Shigella sonnei* host strain. As with all Gram-negative bacteria, the outer membrane of *Shigella* contains lipopolysaccharide or LPS.[53] Due to the lysis of host cells during the fermentation process (as the result of phage lytic cycle), *Shigella* LPS is present in the resulting phage lysates. Most of the endotoxin is expected to be removed during phage purification process.

LPS is of concern if sufficiently high amounts enter the human bloodstream, where it can trigger the signaling cascade for macrophage/endothelial cells to secrete pro-inflammatory cytokines and nitric oxide that may lead to "endotoxic shock." However, LPS has not been shown to cross the intestinal mucosa and oral administration of LPS shows no negative effects and may even elicit beneficial responses in the GI system.[54] Additionally, there is no FDA specification for levels of endotoxin in oral products. Still, as a standard quality control protocol, Intralytix analyzes every ShigaShield™ batch for the presence and levels of the LPS endotoxin in the final product. All product lots must be at or below 25,000 endotoxin unit (EU)/mL at $1x10^9$ PFU/mL level in order to pass the release criteria for LPS. This level is very safe and is based upon the levels of endotoxins that are found naturally in healthy human saliva.[55] See Section 3.1.2.3 for discussion of dietary intake.

6.2.1.4 Monophages

The safety of monophages is discussed in Section 6.1.1.2.

6.2.2 Quality Control

6.2.2.1 Monophages

The following tests are performed upon each monophage lot:

Lytic titer

The lytic titer 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 the titer is ≥10.0 $\log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Microbial purity

The microbial purity test confirms that the monophage solution does not contain viable microbes. Briefly, samples of each monophage solution are tested by a) direct plating onto non-selective agar and b) after enrichment. The specification is that each monophage lot must be bacteriologically sterile. Lots failing the test may be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Identity

Currently, genotypic fingerprinting, through restriction fragment length polymorphism (RFLP,) is used to confirm the identity of each monophage lot. The specification for RFLP is that the bands should visually match those in the reference pattern. Lots repeatedly failing the RFLP test will be discarded.

6.2.2.2 ShigaShield™

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

Lytic titer test

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

Microbial purity

The microbial purity test is a determination of the viable microbial contamination in a phage solution. Briefly, a 1% representative sample of each lot of ShigaShield™ 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 ShigaShield™ must be bacteriologically sterile. Lots failing the test may be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Endotoxin content test

Endotoxins are toxins associated with host bacteria, of which a residual amount could be present in the phage preparations. A commercially available quantitative LAL-based test specifically for measurement of endotoxin is currently used by Intralytix. The specification for this test is each lot of ShigaShield™ must contain ≤ 25,000 EU/mL (at standard working concentration ca. 9.0 $\log_{10}$ PFU/mL). Lots failing to meet the specification may be washed with sterile 0.1M saline and subjected to the full panel of quality control tests.

Identity test

The identity test verifies that all phages claimed to be present in ShigaShield™ are actually present. There are currently three methods available to confirm this; any one can be used alone or in combination with the others. The first method uses RT-PCR to confirm the presence of each monophage. In this case, five sets of primer pairs, each specific to a single ShigaShield™ component monophage, are screened against ShigaShield™. The specification is that all expected amplicons are present. The second method uses the spot test method. Briefly, five *Shigella* strains, each of which is susceptible to only one component monophage, are screened for lysis by ShigaShield™. The specification for this test is that all reference bacterial strains are lysed by the preparation (e.g., if one of the strains is not lysed, it is because the phage specifically

lytic for that strain was not included in the phage preparation). The third method uses visual, signature-based confirmation that all five monophages were included in the ShigaShield™ lot during manufacturing. Briefly, as the lot is mixed, a second employee must be present to observe and confirm that each and every of the five component monophages is actually added. At least two employees must sign the preparation mixing worksheet, which is archived by the QC department for a minimum of 2 years. Lots that fail to meet the specification may be retested. Lots repeatedly failing the specification may be supplemented with the missing component monophage and retested for all QC tests.

6.3 SUBSTANTIAL EQUIVALENCE TO APPROVED PRODUCTS

6.3.1 Previously approved bacteriophage preparations

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:

- ListShield™ (formerly known as LMP-102,) a phage preparation containing six lytic *Listeria monocytogenes*-specific phages, is FDA-cleared as a food additive (21 CFR §172.785).
- ListShield™ is also GRAS (GRAS Notice No. 000528).
- 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 *Listeria monocytogenes* (EPA registration #74234-1).
- Listex™, a phage preparation containing a single *Listeria 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).
- SalmoFresh™, a phage preparation containing six *Salmonella*-specific lytic phages is GRAS (GRAS Notice No. 435)
- SalmoFresh™ is also listed by the FSIS for use on various poultry products (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 concluded that a different bacteriophage preparation was safe and effective. The variety of these previously cleared or registered bacteriophage preparations attests to the general safety of bacteriophages and therefore supports their natural GRAS status. ShigaShield™ is substantially equivalent to the above bacteriophage preparations and therefore is also GRAS.

6.4 SUMMARY AND BASIS FOR GRAS

ShigaShield™ is an all-natural product made of five *Shigella*-specific lytic bacteriophages from the *Myoviridae* and *Siphoviridae* families. All phages included in ShigaShield™ are lytic phages and they have been rigorously characterized (including full genome sequencing).

Phages are omnipresent in the environment. Bacteriophages are the oldest, most ubiquitous organisms on earth, with their numbers on Earth 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 ShigaShield™ must be verified to be lytic and to not contain any undesirable genes listed in 40 CFR § 725.421. All monophages included in ShigaShield™ belong to the *Myoviridae* and *Siphoviridae* families of

double-stranded DNA bacteriophages. Many of these phages are known to be excellent lytic phages and are increasingly being used in various phage preparations, including several previously FDA and EPA cleared bacteriophage preparations.

The genomes of the five bacteriophages in ShigaShield™ 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.

ShigaShield™ is manufactured using Intralytix's standard procedures. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, ListShield™ (21 CFR §172.785), EcoShield™ (FCN No. 1018) and SalmoFresh™ (GRAS Notice No. 435) and are currently used to manufacture commercial lots of these products.

The only manufacturing byproduct of potential concern during ShigaShield™ manufacturing is LPS. Intralytix tests every lot of ShigaShield™ for LPS to ensure it meets the release criteria. The LPS levels of the ShigaShield™ (at maximum working concentration ca. $1x10^9$ PFU/mL) must be below 25,000 EU/mL for the lot to be released. This standard is the same as the maximum LPS level previously cleared by the FDA for EcoShield™ (per FCN 1018).

ShigaShield™ is produced on animal-product free media. The final ShigaShield™ product contains no preservatives, known allergenic substances, or additives. ShigaShield™ is eligible for certification as both Kosher and Halal, as the manufacturing process has previously been certified for both ListShield™ and SalmoFresh™. ShigaShield™ is also eligible for OMRI-listing, to certify it is suitable for use in organic production. These approvals will be pursued dependent upon market demands.

The proposed application rate for ShigaShield™ is up to $1x10^8$ PFU per gram of food article. Assuming the maximum application rate of $1x10^8$ PFU/g of all five target food groups, the average daily consumption of these foods would contain a mere 72.4 μg of phage particles, 47.3 mg of added sodium, and 0.13 mg of added potassium. This consumption would be spread out across several servings and meals, so the added sodium and potassium levels per serving would be so low as to not require any changes to labeling. The weight of added phage is negligible.

ShigaShield™ is substantially equivalent to the lytic bacteriophage preparations that have been previously designated GRAS and/or cleared by other regulatory agencies. Furthermore, with the proposed maximum application rate for ShigaShield™ of up to $1x10^8$ PFU per gram of food article, even in the worst case scenario ($1x10^8$ PFU/g) the rate is equal to or lower than the rates previously cleared for those other preparations as safe and effective. For instance, the maximum

proposed application rate of ShigaShield™ 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 ShigaShield™ 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 ShigaShield™ are lytic, non-genetically modified, and free of any and all undesirable genes. Third, Intralytix's manufacturing process ensures the safety and quality of the final ShigaShield™ product. Fourth, the estimated daily intake of the ShigaShield™ phage preparation is so low it is negligible. And, fifth, the bacteriophage product is substantially equivalent to several bacteriophage products already receiving regulatory clearance. Based on this information, it is evident that ShigaShield™ is GRAS.

Added to fermentor:
-vegan broth
-antifoam (as needed)

Sterilization of batch medium.

Added to fermentor:
-Bacterial host culture
-Monophage

Inoculation, infection, and host lysis.

Tangential-flow filtration.
Retentate
Permeate
Phage

Clarification and removal of residual host bacteria. Rententate contains residual host; permeate contains phage. An optional centrifugation step may be added if necessary.

Tangential-flow filtration
Retentate
Permeate
Phage
Discarded

Concentration of phage ≥10:1 volume reduction. Retentate flow contains the monophage. Permeate flow contains smaller moieties.

0.1M saline
Tangential-flow filtration
Retentate
Permeate
Phage
Discarded

Washing & saline exchange. Retentate contains the monophage. Permeate contains smaller moieties. About 10:1 volume exchange with 0.1M saline.

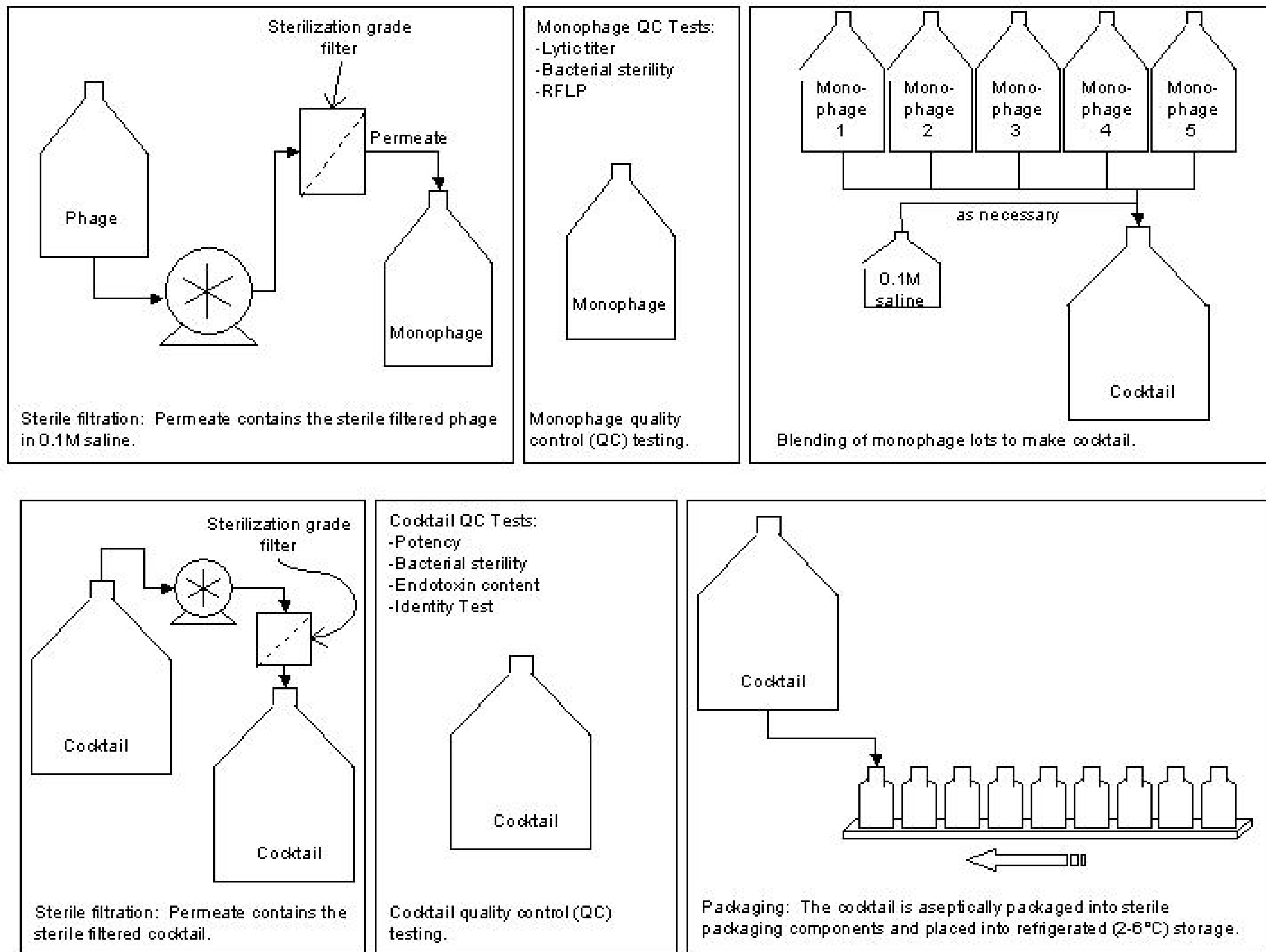

Figure 1. Overview of ShigaShield™ manufacturing process

7 LIST OF SUPPORTING DATA AND INFORMATION

7.1 APPENDICES (INCLUDES NOT GENERALLY AVAILABLE DATA)

Appendix 1 Efficacy of ShigaShield™ on Foods

Appendix 2 Study of *Shigella* Toxins

7.2 REFERENCES (FOR GENERALLY AVAILABLE DATA)

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APPENDIX 1: EFFICACY STUDIES

Substance: Bacteriophage preparation (*Shigella* spp. targeted)

Product:

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

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

Reference: Acceptability determination

Labeling Requirements: None under the accepted conditions of use

ShigaShield™ is an all-natural product made of five *Shigella*-specific lytic bacteriophages from the *Myoviridae* and *Siphoviridae* families. All phages included in ShigaShield™ are lytic phages that have not been genetically manipulated in any way. The component phages of ShigaShield™ have been rigorously characterized, including full genome sequencing.

The ShigaShield™ preparation is intended for use in food products to control *Shigella* when added at ≤ ca. $1x10^8$ PFU per gram of food. Intralytix, Inc. has concluded that ShigaShield™ is generally recognized as safe (GRAS), and therefore, we believe it is not subject to the requirement of pre-market approval, under the conditions of its intended use.

SHIGASHIELD™ IS EFFECTIVE.

Target range

ShigaShield™ has been screened for its lytic activity against 67 *Shigella* strains. At the standard "working concentration" of $1x10^9$ PFU/mL, it lyses 64 (96%) of the *Shigella* strains in our collection.

Effect on *Shigella* levels in foods

ShigaShield™ is intended to produce a statistically significant reduction of *Shigella* contamination vs. a water control when applied as directed to food products.

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Efficacy study summary

ShigaShield™ was examined for its ability to reduce *Shigella* contamination when applied to various foods. Detailed reports of the studies are included in Appendix 1.1 - Appendix 1.6. A summary of the results is given below.

Description of the test system

For each food tested, portions were inoculated with *Shigella sonnei Sh.s*53, a nalidixic acid resistant isolate. After allowing the bacteria to colonize, the food was then treated with water or ShigaShield™ applied at ca. 1mL / 100 g of food. The ShigaShield™ contact time was 5 minutes at room temperature, after which the samples were analyzed for populations of *Shigella*.

Summary of results

Ready-to-eat poultry and red meat

Study ShA12J15ML and Study ShA12J09ML examined the efficacy of ShigaShield™ in reducing *Shigella* levels on deli corned beef and pre-cooked chicken strips, respectively. In both studies, three concentrations of ShigaShield™ ($9x10^5$, $9x10^6$, and $9x10^7$ PFU/g) were applied. After 5 minutes at room temperature, each concentration significantly reduced the number of viable *Shigella*. In the deli corned beef, the reductions were 39%, 74%, and 97%, respectively. In the chicken strip study, the reductions were 49%, 76%, and 98%, respectively. The complete details of these studies can be seen in Appendix 1.1 and Appendix 1.2.

Fish and shellfish

Study ShA12J02ML examined the efficacy of ShigaShield™ on reducing *Shigella* levels on smoked salmon. Three concentrations of ShigaShield™ ($9x10^5$, $9x10^6$, and $9x10^7$ PFU/g) were applied. After 5 minutes at room temperature, each concentration significantly reduced the number of viable *Shigella* by 31%, 68%, and 92%, respectively. The complete details of this study can be seen in Appendix 1.3.

Fresh and processed fruits

Study ShA12J08ML examined the efficacy of ShigaShield™ on reducing *Shigella* levels on pre-cut honeydew. Three concentrations of ShigaShield™ ($9x10^5$, $9x10^6$, and $9x10^7$ PFU/g) were applied. After 5 minutes at room temperature, each concentration significantly reduced the number of viable *Shigella* by 45%, 79%, and 96%, respectively. The complete details of this study can be seen in Appendix 1.4.

Fresh and processed vegetables

Study ShA11F23ML examined the efficacy of ShigaShield™ on reducing *Shigella* levels on lettuce. Two concentrations of ShigaShield™ ($2x10^6$ and $2x10^7$ PFU/g) were applied. After 5

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minutes at room temperature, each concentration significantly reduced the number of viable *Shigella* by ca. 73% and 95%, respectively. The complete details of this study can be seen in Appendix 1.5.

Dairy

Study ShA12J31ML examined the efficacy of ShigaShield™ on reducing *Shigella* levels in yogurt. Three concentrations of ShigaShield™ ($9x10^5$, $9x10^6$, and $9x10^7$ PFU/g) were mixed into the yogurt. After 5 minutes at room temperature, each concentration significantly reduced the number of viable *Shigella* by 16%, 45%, and 90%, respectively. The complete details of this study can be seen in Appendix 1.6.

Summary

We believe the data summarized here fully supports our conclusion that ShigaShield™ is GRAS and our request for ShigaShield™ to be included in FSIS directive 7120.1 as a safe and suitable ingredient used in the production of red meat and poultry products as a processing aid. Its intended use is as a spray applied to significantly reduce levels of *Shigella* when applied at $\leq 1x10^8$ PFU/g. Additionally, no foods treated to product specifications should require ShigaShield™ as a listed ingredient on product labels.

Appendices

Appendix 1.1 **Report ShA12J15ML**

Deli corned beef

Appendix 1.2 **Report ShA12J09ML**

Pre-cut chicken strips

Appendix 1.3 **Report ShA12J02ML**

Smoked salmon

Appendix 1.4 **Report ShA12J08ML**

Pre-cut honeydew

Appendix 1.5 **Report ShA11F23ML**

Lettuce

Appendix 1.6 **Report ShA12J31ML**

Yogurt

Appendix 1.1

Report ShA12J15ML

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated deli beef

Study # ShA12J15ML

Intralytix

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1 Study Title

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated deli beef.

2 Study Director

Alexander Sulakvelidze, Ph.D.

3 Study Personnel

Name:	**Title:**	**Role:**
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, M.D.	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 ShigaShield™ reduces the number of viable *Shigella* on deli corned beef when applied at the rate of $9x10^5$ – $9x10^7$ PFU/g.

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6 TEST MATRIX

Fully cooked corned beef was obtained from a local Baltimore grocery store deli. It was not washed or pre-treated prior to our studies.

7 SHIGASHIELD™ LOT AND APPLICATION

- ShigaShield™ Lot (b) (6)
- Titer: approx. $1x10^{10}$ PFU/mL
- ShigaShield™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.9mL ShigaShield™ per 100g beef.
- ShigaShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED BEEF

The beef test matrix was experimentally contaminated with *Shigella sonnei* strain:

- *Sh.s53*: A nalidixic acid resistant mutant developed from Intralytix strain Sh.s43

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/ml. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Sh.s53). 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 supplemented with nalidixic acid (25 µg/ml.) Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

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

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

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- Peptone water (BD, Sparks, MD cat #218105)
- MacConkey Agar (BD, Sparks, MD; cat # 212123)

10 GENERAL OUTLINE OF STUDY

1) Four 100g portions of beef were each assigned as test groups A, B, C, or D.
2) The challenge dose of bacteria was applied onto the beef's surfaces. Bacterial cultures were evenly spread onto all sides of the beef 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 ShigaShield™ was applied as described in section 7. Treatments were evenly applied to the beef samples' surfaces as follows:
   - Group A = 0.9mL $1x10^{10}$ PFU/mL ShigaShield™ / 100g = $9x10^{7}$ PFU/g
   - Group B = 0.9mL $1x10^{9}$ PFU/mL ShigaShield™ / 100g = $9x10^{6}$ PFU/g
   - Group C = 0.9mL $1x10^{8}$ PFU/mL ShigaShield™ / 100g = $9x10^{5}$ PFU/g
   - Group D = 0.9mL water / 100g = 0 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 ShigaShield™, from each sample group, triplicate ~25g samples of beef 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 *Shigella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached meat/peptone water mixture onto separate MacConkey plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

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

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).

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11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #ShA12J15ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A ($1x10^{10}$ PFU/mL)	Yes	100	$9x10^7$ PFU/g ShigaShield	3	1,2,1	18,36,18
B ($1x10^9$ PFU/mL)	Yes	100	$9x10^6$ PFU/g ShigaShield	3	10,15,17	180,270,306
C ($1x10^8$ PFU/mL)	Yes	100	$9x10^5$ PFU/g ShigaShield	3	35,34,28	630,612,504
D (Control)	Yes	100	Water	3	61,56,42	1098,1008,756

11.2 Tabular presentation of results

Table 2 Reduction of *Shigella* counts on beef treated with ShigaShield when applied at ca. $9x10^5$ – $9x10^7$ PFU/g (0.9mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^{10}$ PFU/mL)	Yes	$9x10^7$ PFU/g ShigaShield	*n* =3	24	97%	1.6	Yes
B ($1x10^9$ PFU/mL)	Yes	$9x10^6$ PFU/g ShigaShield	*n* = 3	252	74%	0.6	Yes
C ($1x10^8$ PFU/mL)	Yes	$9x10^5$ PFU/g ShigaShield	*n* =3	582	39%	0.2	Yes
D (Control)	Yes	Water	*n* =3	954	-	-	-

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

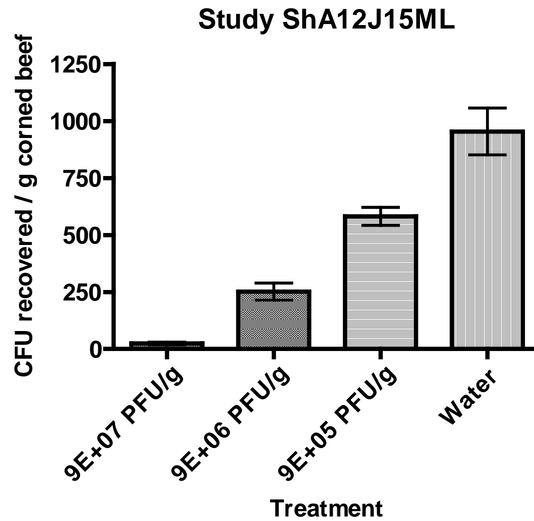
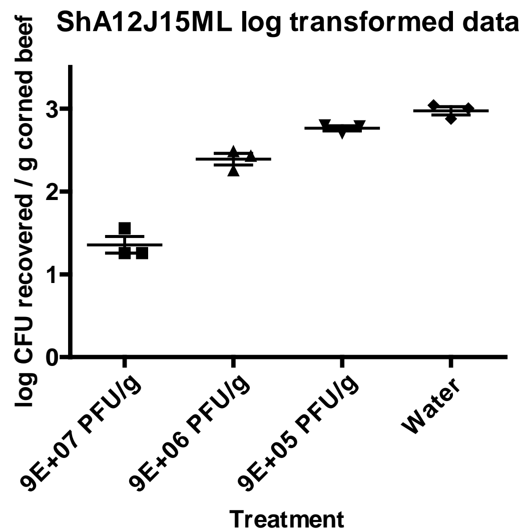


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the ShigaShield™ treatment in reducing the number of viable *Shigella* in the experimentally contaminated beef was evaluated by comparing the data obtained with the water-treated control samples and the ShigaShield™-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 <0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
9E+07 PFU/g vs Water	-930.00	16.030	*** P<0.001
9E+06 PFU/g vs Water	-702.00	12.100	*** P<0.001
9E+05 PFU/g vs Water	-372.00	6.412	** P<0.01
9E+07 PFU/g vs 9E+06 PFU/g	-228.00	3.930	ns P>0.05
9E+07 PFU/g vs 9E+05 PFU/g	-558.00	9.618	*** P<0.001
9E+06 PFU/g vs 9E+05 PFU/g	-330.00	5.688	* P<0.05

11.5 Brief discussion of results and study's conclusions

- Applying ShigaShield™ at ca. 9x10^{7} PFU/g beef reduced the number of viable *Shigella* by ca. 97% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. 9x10^{6} PFU/g beef reduced the number of viable *Shigella* by ca. 74% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. 9x10^{5} PFU/g beef reduced the number of viable *Shigella* by ca. 39% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).

- Reduction in *Shigella* levels achieved by using more concentrated ShigaShield™ was higher compared to those obtained with more dilute ShigaShield™ (97% vs. 74% vs. 39% when using ca. 9x10^{7} PFU/g, 9x10^{6} PFU/g, and 9x10^{5} PFU/g, respectively).

- The difference in *Shigella* recovered when ShigaShield™ was applied in the two most concentrated forms (application rates 9x10^{7} PFU/g vs. 9x10^{6} PFU/g) was not statistically significant (P>0.05).

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- The differences in *Shigella* recovered when ShigaShield™ was applied in the two most concentrated forms vs the least concentrated (application rates $9x10^7$ PFU/g vs. $9x10^5$ PFU/g OR $9x10^6$ PFU/g vs. $9x10^5$ PFU/g) were statistically significant ($P<0.05$).

12 SUMMARY CONCLUSION OF THE STUDY

ShigaShield™ can significantly reduce viable *Shigella* levels in experimentally contaminated beef by ca. 39-97% in 5 minute contact time, when applied at ca. $9x10^5$ – $9x10^7$ PFU/g.

Using the higher ShigaShield™ application rates (ca. $9x10^7$ PFU/g or $9x10^6$ PFU/g) resulted in statistically significantly better reduction of *Shigella* levels compared to lower ShigaShield™ application rate (ca. $9x10^5$ PFU/g).

13 SIGNATURES

(b) (6)

Manrong Li, M.D.

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.2

Report ShA12J09ML

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated pre-cooked chicken

Study # ShA12J09ML

Intralytix

The Columbus Center

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Baltimore, MD 21202

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1 STUDY TITLE

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated pre-cooked chicken.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, M.D.	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 ShigaShield™ reduces the number of viable *Shigella* on pre-cooked chicken when applied at the rate of $9x10^5$ – $9x10^7$ PFU/g.

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6 TEST MATRIX

Fully cooked chicken breast strips were obtained from a local Baltimore grocery store. They were not washed or pre-treated prior to our studies.

7 SHIGASHIELD™ LOT AND APPLICATION

- ShigaShield™ Lot (b) (6)
- Titer: approx. $1x10^{10}$ PFU/mL
- ShigaShield™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.9mL ShigaShield™ per 100g chicken.
- ShigaShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED CHICKEN

The chicken test matrix was experimentally contaminated with *Shigella sonnei* strain:

- *Sh.s53*: A nalidixic acid resistant mutant developed from Intralytix strain Sh.s43

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/ml. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Sh.s53). 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 supplemented with nalidixic acid (25 µg/ml.) Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

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

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- Peptone water (BD, Sparks, MD cat #218105)
- MacConkey Agar (BD, Sparks, MD; cat # 212123)

10 General Outline of Study

1) Four 100g portions of chicken were each assigned as test groups A, B, C, or D.

2) The challenge dose of bacteria was applied onto the chicken's surfaces. Bacterial cultures were evenly spread onto all sides of the chicken 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 ShigaShield™ was applied as described in section 7. Treatments were evenly applied to the chicken samples' surfaces as follows:
   - Group A = 0.9mL $1x10^{10}$ PFU/mL ShigaShield™ / 100g = $9x10^{7}$ PFU/g
   - Group B = 0.9mL $1x10^{9}$ PFU/mL ShigaShield™ / 100g = $9x10^{6}$ PFU/g
   - Group C = 0.9mL $1x10^{8}$ PFU/mL ShigaShield™ / 100g = $9x10^{5}$ PFU/g
   - Group D = 0.9mL water / 100g = 0 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 ShigaShield™, from each sample group, triplicate ~25g samples of chicken were removed, 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 *Shigella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached meat/peptone water mixture onto separate MacConkey plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

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

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).

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11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #ShA12J09ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A ($1x10^{10}$ PFU/mL)	Yes	100	$9x10^7$ PFU/g ShigaShield	3	1,1,2	18,18,36
B ($1x10^9$ PFU/mL)	Yes	100	$9x10^6$ PFU/g ShigaShield	3	19,13,6	342,234,108
C ($1x10^8$ PFU/mL)	Yes	100	$9x10^5$ PFU/g ShigaShield	3	19,37,26	342,666,468
D (Control)	Yes	100	Water	3	60,47,54	1080,846,972

11.2 Tabular presentation of results

Table 2 Reduction of *Shigella* counts on chicken treated with ShigaShield when applied at ca. $9x10^5$ – $9x10^7$ PFU/g (0.9mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^{10}$ PFU/mL)	Yes	$9x10^7$ PFU/g ShigaShield	*n =3*	24	98%	1.6	Yes
B ($1x10^9$ PFU/mL)	Yes	$9x10^6$ PFU/g ShigaShield	*n = 3*	228	76%	0.7	Yes
C ($1x10^8$ PFU/mL)	Yes	$9x10^5$ PFU/g ShigaShield	*n =3*	492	49%	0.3	Yes
D (Control)	Yes	Water	*n =3*	966	-	-	-

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

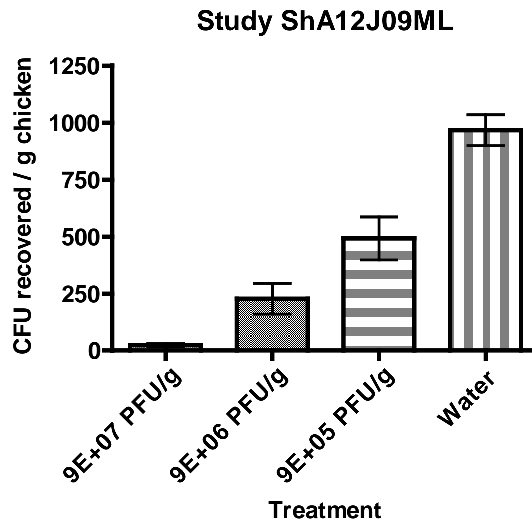
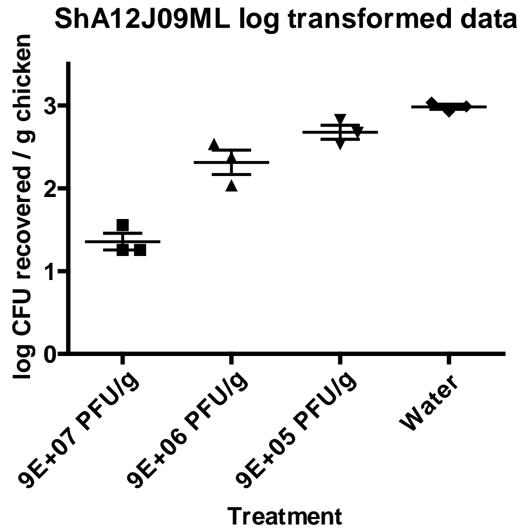


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the ShigaShield™ treatment in reducing the number of viable *Shigella* in the experimentally contaminated chicken was evaluated by comparing the data obtained with the water-treated control samples and the ShigaShield™-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 <0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
9E+07 PFU/g vs Water	-942.00	14.015	*** P<0.001
9E+06 PFU/g vs Water	-738.00	10.980	*** P<0.001
9E+05 PFU/g vs Water	-474.00	7.052	** P<0.01
9E+07 PFU/g vs 9E+06 PFU/g	-204.00	3.035	ns P>0.05
9E+07 PFU/g vs 9E+05 PFU/g	-468.00	6.963	** P<0.01
9E+06 PFU/g vs 9E+05 PFU/g	-264.00	3.928	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying ShigaShield™ at ca. $9x10^7$ PFU/g chicken reduced the number of viable *Shigella* by ca. 98% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^6$ PFU/g chicken reduced the number of viable *Shigella* by ca. 76% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^5$ PFU/g chicken reduced the number of viable *Shigella* by ca. 49% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).

- Reduction in *Shigella* levels achieved by using more concentrated ShigaShield™ was higher compared to those obtained with more dilute ShigaShield™ (98% vs. 76% vs. 49% when using ca. $9x10^7$ PFU/g, $9x10^6$ PFU/g, and $9x10^5$ PFU/g, respectively).

- The differences in *Shigella* recovered when ShigaShield™ was diluted 10-fold (application rates $9x10^7$ PFU/g vs. $9x10^6$ PFU/g OR $9x10^6$ PFU/g vs.$9x10^5$ PFU/g) were not statistically significant (P>0.05).

- The difference in *Shigella* recovered when ShigaShield™ was diluted 100-fold (application rates $9x10^7$ PFU/g vs. $9x10^5$ PFU/g) was statistically significant ($P<0.01$).

12 Summary Conclusion of the Study

ShigaShield™ can significantly reduce viable *Shigella* levels in experimentally contaminated chicken by ca. 49-98% in 5 minute contact time, when applied at ca. $9x10^5$ – $9x10^7$ PFU/g.

Using a 100-fold higher ShigaShield™ application rate (ca. $9x10^7$ PFU/g) resulted in statistically significantly better reduction of *Shigella* levels compared to lower ShigaShield™ application rate (ca. $9x10^5$ PFU/g).

13 Signatures

(b) (6)

____

Manrong Li, M.D.

Research Scientist

(b) (6)

____

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

____

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.3

Report ShA12J02ML

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated salmon

Study # ShA12J02ML

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1 Study Title

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated salmon

2 Study Director

Alexander Sulakvelidze, Ph.D.

3 Study Personnel

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, M.D.	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 ShigaShield™ reduces the number of viable *Shigella* on salmon when applied at the rate of $9x10^5$ – $9x10^7$ 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 SHIGASHIELD™ LOT AND APPLICATION

- ShigaShield™ Lot (b) (6)
- Titer: approx. $1x10^{10}$ PFU/mL
- ShigaShield™ was diluted as necessary with water just prior to application.
- The application was ca. 0.9mL ShigaShield™ per 100g salmon.
- ShigaShield™ 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 salmon test matrix was experimentally contaminated with *Shigella sonnei* strain:

- *Sh.s53*: A nalidixic acid resistant mutant developed from Intralytix strain Sh.s43

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/ml. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Sh.s53). 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 supplemented with nalidixic acid (25 µg/ml.) Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The smoked salmon was experimentally contaminated by ca. $4x10^3$ CFU / g of salmon.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- Peptone water (BD, Sparks, MD cat #218105)
- MacConkey Agar (BD, Sparks, MD; cat # 212123)

10 GENERAL OUTLINE OF STUDY

1) Four 100g portions of smoked salmon were each assigned as test groups A, B, C, or D.

2) The challenge dose of bacteria was applied onto the smoked salmon's surfaces. Bacterial cultures were evenly spread onto all sides of the salmon 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 ShigaShield™ was applied as described in section 7. Treatments were evenly applied to the salmon samples' surfaces as follows:

   - Group A = 0.9mL 1x10^{10} PFU/mL ShigaShield™ / 100g = 9x10^{7} PFU/g
   - Group B = 0.9mL 1x10^{9} PFU/mL ShigaShield™ / 100g = 9x10^{6} PFU/g
   - Group C = 0.9mL 1x10^{8} PFU/mL ShigaShield™ / 100g = 9x10^{5} PFU/g
   - Group D = 0.9mL water / 100g = 0 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 ShigaShield™, from each sample group, triplicate ~25g samples of salmon were removed, 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 *Shigella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached salmon/peptone water mixture onto separate MacConkey plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

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

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).

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11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #ShA12J02ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A ($1x10^{10}$ PFU/mL)	Yes	100	$9x10^7$ PFU/g ShigaShield	3	7,11,6	126,198,108
B ($1x10^9$ PFU/mL)	Yes	100	$9x10^6$ PFU/g ShigaShield	3	30,29,34	540,522,612
C ($1x10^8$ PFU/mL)	Yes	100	$9x10^5$ PFU/g ShigaShield	3	62,70,69	1116,1260,1242
D (Control)	Yes	100	Water	3	95,102,94	1710,1836,1692

11.2 Tabular presentation of results

Table 2 Reduction of *Shigella* counts on smoked salmon treated with ShigaShield when applied at ca. $9x10^5$ – $9x10^7$ PFU/g (0.9mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^{10}$ PFU/mL)	Yes	$9x10^7$ PFU/g ShigaShield	*n =3*	144	92%	1.1	Yes
B ($1x10^9$ PFU/mL)	Yes	$9x10^6$ PFU/g ShigaShield	*n = 3*	558	68%	0.5	Yes
C ($1x10^8$ PFU/mL)	Yes	$9x10^5$ PFU/g ShigaShield	*n =3*	1206	31%	0.2	Yes
D (Control)	Yes	Water	*n =3*	1746	-	-	-

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

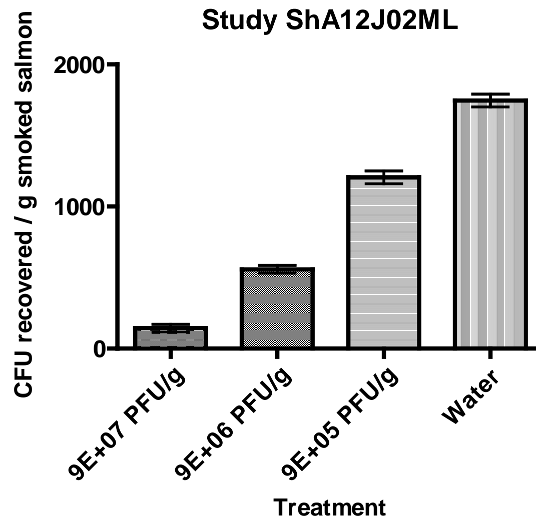
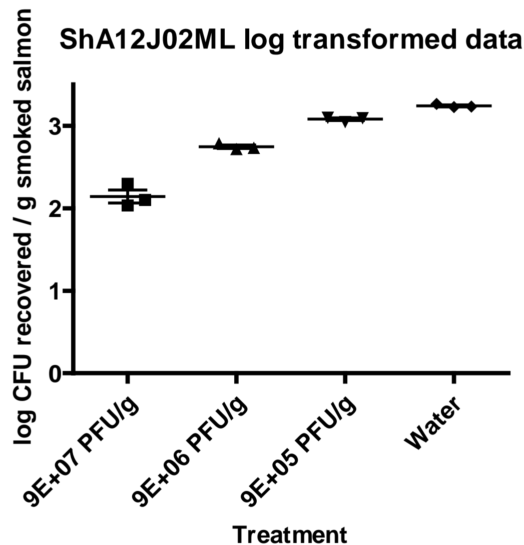


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the ShigaShield™ treatment in reducing the number of viable *Shigella* in the experimentally contaminated salmon was evaluated by comparing the data obtained with the water-treated control samples and the ShigaShield™-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 < 0.0001, considered very significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
9E+07 PFU/g vs Water	-1602.0	42.754	*** P<0.001
9E+06 PFU/g vs Water	-1188.0	31.705	*** P<0.001
9E+05 PFU/g vs Water	-540.00	14.412	*** P<0.001
9E+07 PFU/g vs 9E+06 PFU/g	-414.00	11.049	*** P<0.001
9E+07 PFU/g vs 9E+05 PFU/g	-1062.0	28.343	*** P<0.001
9E+06 PFU/g vs 9E+05 PFU/g	-648.00	17.294	*** P<0.001

11.5 Brief discussion of results and study's conclusions

- Applying ShigaShield™ at ca. $9x10^7$ PFU/g smoked salmon reduced the number of viable *Shigella* by ca. 92% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^6$ PFU/g smoked salmon reduced the number of viable *Shigella* by ca. 68% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^5$ PFU/g smoked salmon reduced the number of viable *Shigella* by ca. 31% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Reduction in *Shigella* levels achieved by using more concentrated ShigaShield™ was higher compared to those obtained with more dilute ShigaShield™ (92% vs. 68% vs. 31% when using ca. $9x10^7$ PFU/g, $9x10^6$ PFU/g, and ca. $9x10^5$ PFU/g, respectively).

- The differences in *Shigella* recovery between various ShigaShield™ application rates ($9x10^7$ PFU/g vs. $9x10^6$ PFU/g vs.$9x10^5$ PFU/g) were statistically significant (P<0.001).

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12 Summary Conclusion of the Study

ShigaShield™ can significantly reduce viable *Shigella* levels in experimentally contaminated smoked salmon by ca. 31-92% in 5 minute contact time, when applied at ca. $9x10^5$ – $9x10^7$ PFU/g.

Using a higher ShigaShield™ application rate (ca. $9x10^7$ PFU/g) resulted in statistically significantly better reduction of *Shigella* levels compared to lower ShigaShield™ application rates (ca. $9x10^6$ PFU/g and $9x10^5$ PFU/g).

13 Signatures

(b) (6)

Manrong Li, M.D.

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.4

Report ShA12J08ML

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated honeydew melon

Study # ShA12J08ML

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1 STUDY TITLE

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated honeydew melon.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, M.D.	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 ShigaShield™ reduces the number of viable *Shigella* on honeydew when applied at the rate of $9x10^5$ – $9x10^7$ PFU/g.

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6 TEST MATRIX

Pre-cut honeydew melon chunks were obtained from a local Baltimore grocery store. They were not washed or pre-treated prior to our studies.

7 SHIGASHIELD™ LOT AND APPLICATION

- ShigaShield™ Lot (b) (6)
- Titer: approx. $1x10^{10}$ PFU/mL
- ShigaShield™ was diluted as necessary with water just prior to application.
- The application was ca. 0.9mL ShigaShield™ per 100g honeydew.
- ShigaShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED HONEYDEW

The honeydew test matrix was experimentally contaminated with *Shigella sonnei* strain:

- *Sh.s53*: A nalidixic acid resistant mutant developed from Intralytix strain Sh.s43

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/ml. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Sh.s53). 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 supplemented with nalidixic acid (25 µg/ml.) Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

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

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- Peptone water (BD, Sparks, MD cat #218105)
- MacConkey Agar (BD, Sparks, MD; cat # 212123)

10 GENERAL OUTLINE OF STUDY

1) Four 100g portions of honeydew were each assigned as test groups A, B, C, or D.

2) The challenge dose of bacteria was applied onto the honeydew's surfaces. Bacterial cultures were evenly spread onto all cut sides of the honeydew 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 ShigaShield™ was applied as described in section 7. Treatments were evenly applied to the honeydew samples' surfaces as follows:

   - Group A = 0.9mL $1x10^{10}$ PFU/mL ShigaShield™ / 100g = $9x10^{7}$ PFU/g
   - Group B = 0.9mL $1x10^{9}$ PFU/mL ShigaShield™ / 100g = $9x10^{6}$ PFU/g
   - Group C = 0.9mL $1x10^{8}$ PFU/mL ShigaShield™ / 100g = $9x10^{5}$ PFU/g
   - Group D = 0.9mL water / 100g = 0 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 ShigaShield™, from each sample group, triplicate ~25g samples of honeydew were removed, 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 *Shigella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached honeydew/peptone water mixture onto separate MacConkey plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

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

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).

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11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #ShA12J08ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A ($1x10^{10}$ PFU/mL)	Yes	100	$9x10^7$ PFU/g ShigaShield	3	1,3,2	18,54,36
B ($1x10^9$ PFU/mL)	Yes	100	$9x10^6$ PFU/g ShigaShield	3	15,11,6	270,198,108
C ($1x10^8$ PFU/mL)	Yes	100	$9x10^5$ PFU/g ShigaShield	3	33,24,28	567,432,504
D (Control)	Yes	100	Water	3	65,52,37	1170,936,666

11.2 Tabular presentation of results

Table 2 Reduction of *Shigella* counts on honeydew treated with ShigaShield when applied at ca. $9x10^5$ – $9x10^7$ PFU/g (0.9mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^{10}$ PFU/mL)	Yes	$9x10^7$ PFU/g ShigaShield	*n =3*	36	96%	1.4	Yes
B ($1x10^9$ PFU/mL)	Yes	$9x10^6$ PFU/g ShigaShield	*n = 3*	192	79%	0.7	Yes
C ($1x10^8$ PFU/mL)	Yes	$9x10^5$ PFU/g ShigaShield	*n =3*	510	45%	0.3	Yes
D (Control)	Yes	Water	*n =3*	924	-	-	-

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

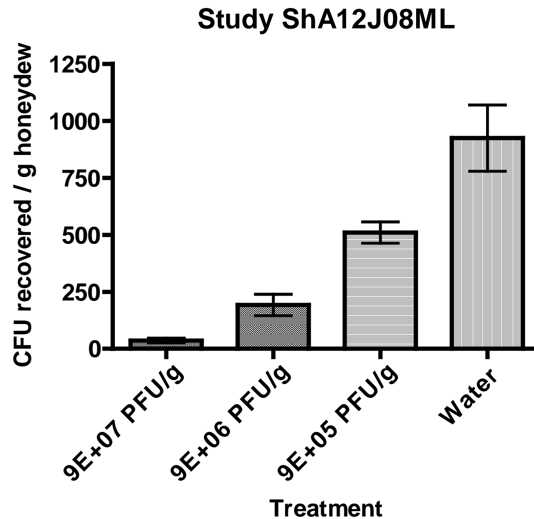
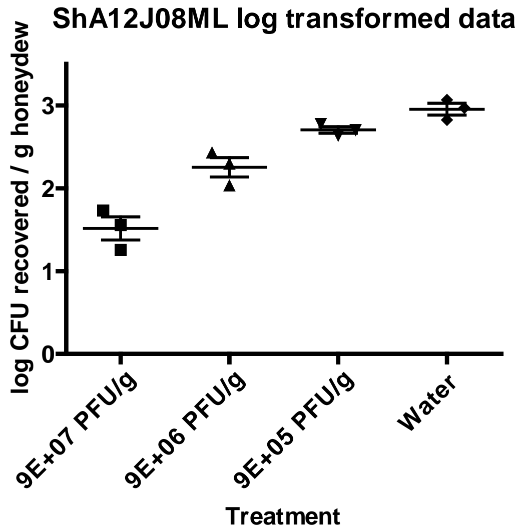


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the ShigaShield™ treatment in reducing the number of viable *Shigella* in the experimentally contaminated honeydew was evaluated by comparing the data obtained with the water-treated control samples and the ShigaShield™-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
9E+07 PFU/g vs Water	-888.00	11.078	*** P<0.001
9E+06 PFU/g vs Water	-732.00	9.131	*** P<0.001
9E+05 PFU/g vs Water	-414.00	5.165	* P<0.05
9E+07 PFU/g vs 9E+06 PFU/g	-156.00	1.946	ns P>0.05
9E+07 PFU/g vs 9E+05 PFU/g	-474.00	5.913	* P<0.05
9E+06 PFU/g vs 9E+05 PFU/g	-318.00	3.967	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying ShigaShield™ at ca. $9x10^7$ PFU/g honeydew reduced the number of viable *Shigella* by ca. 96% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^6$ PFU/g honeydew reduced the number of viable *Shigella* by ca. 79% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^5$ PFU/g honeydew reduced the number of viable *Shigella* by ca. 45% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.05).

- Reduction in *Shigella* levels achieved by using more concentrated ShigaShield™ was higher compared to those obtained with more dilute ShigaShield™ (96% vs. 79% vs. 45% when using ca. $9x10^7$ PFU/g, $9x10^6$ PFU/g, and ca. $9x10^5$ PFU/g, respectively).

- The differences in *Shigella* recovered when ShigaShield™ was diluted 10-fold (application rates $9x10^7$ PFU/g vs. $9x10^6$ PFU/g OR $9x10^6$ PFU/g vs.$9x10^5$ PFU/g) were not statistically significant (P>0.05).

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- The difference in *Shigella* recovered when ShigaShield™ was diluted 100-fold (application rates $9x10^7$ PFU/g vs.$9x10^5$ PFU/g) was statistically significant (P<0.05).

12 Summary Conclusion of the Study

ShigaShield™ can significantly reduce viable *Shigella* levels in experimentally contaminated honeydew by ca. 45-96% in 5 minute contact time, when applied at ca. $9x10^5$ – $9x10^7$ PFU/g.

Using a 100-fold higher ShigaShield™ application rate (ca. $9x10^7$ PFU/g) resulted in statistically significantly better reduction of *Shigella* levels compared to lower ShigaShield™ application rates (ca. $9x10^5$ PFU/g).

13 Signatures

(b) (6)

Manrong Li, M.D.

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.5

Report ShA11F23ML

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated long-leaf lettuce

Study # ShA11F23ML

Intralytix

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1 STUDY TITLE

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated long-leaf lettuce.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

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

4 PERFORMING LABORATORY

Intralytix, Inc.

Research and Development

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5 STUDY OBJECTIVE

To determine whether application of ShigaShield™ reduces the number of viable *Shigella* on lettuce when applied at the rate of $2x10^7$ PFU/g or $2x10^6$ PFU/g.

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6 TEST MATRIX

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

7 SHIGASHIELD™ LOT AND APPLICATION

- ShigaShield™ Lot (b) (6)
- Titer: approx. $2x10^{10}$ PFU/mL
- ShigaShield™ was diluted as necessary with water just prior to application.
- The application was ca. 1.0mL ShigaShield™ per 100g lettuce.
- ShigaShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED LETTUCE

The lettuce test matrix was experimentally contaminated with *Shigella sonnei* strain:

- *Sh.s53*: A nalidixic acid resistant mutant developed from Intralytix strain Sh.s43

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/ml. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Sh.s53). 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 supplemented with nalidixic acid (25 µg/ml.) Overnight growth corresponds to ca. $2.5x10^{8}$ CFU/mL.

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

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- Peptone water (BD, Sparks, MD cat #218105)
- MacConkey Agar (Fisher Scientific; cat # OX CM0115B)

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 surface of the lettuce. Bacterial cultures were evenly spread onto all 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 ShigaShield™ was applied as described in section 7. Treatments were evenly applied to the lettuce samples' surfaces as follows:

   - Group A = 1.0mL $2x10^9$ PFU/mL ShigaShield™ / 100g = $2x10^7$ PFU/g
   - Group B = 1.0mL $2x10^8$ PFU/mL ShigaShield™ / 100g = $2x10^6$ PFU/g
   - Group C = 1.0mL water / 100g = 0 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 ShigaShield™, 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 *Shigella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached lettuce/peptone water mixture onto separate MacConkey plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

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

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).

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11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #ShA11F23ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A ($2x10^9$ PFU/mL)	Yes	100	$2x10^7$ PFU/g ShigaShield	3	4,6,4	72,108,72
B ($2x10^8$ PFU/mL)	Yes	100	$2x10^6$ PFU/g ShigaShield	3	25,31,18	450,558,324
C (Control)	Yes	100	Water	3	99,85,89	1782,1530,1602

11.2 Tabular presentation of results

Table 2 Reduction of *Shigella* counts on lettuce treated with ShigaShield when applied at ca. $2x10^6$ – $2x10^7$ PFU/g (1.0mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($2x10^9$ PFU/mL)	Yes	$2x10^7$ PFU/g ShigaShield	*n* =3	84	95%	1.3	Yes
B ($2x10^8$ PFU/mL)	Yes	$2x10^6$ PFU/g ShigaShield	*n* = 3	444	73%	0.6	Yes
C (Control)	Yes	Water	*n* =3	1638	-	-	-

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

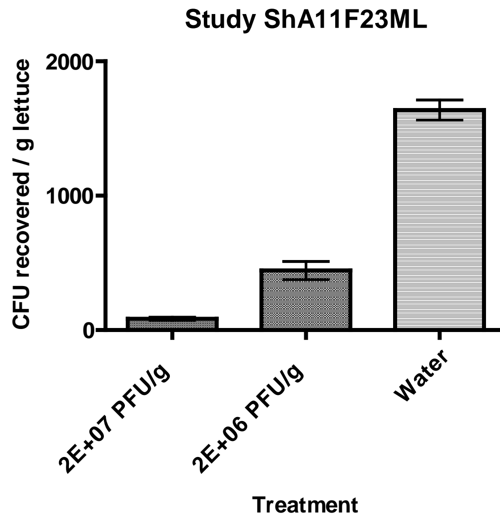
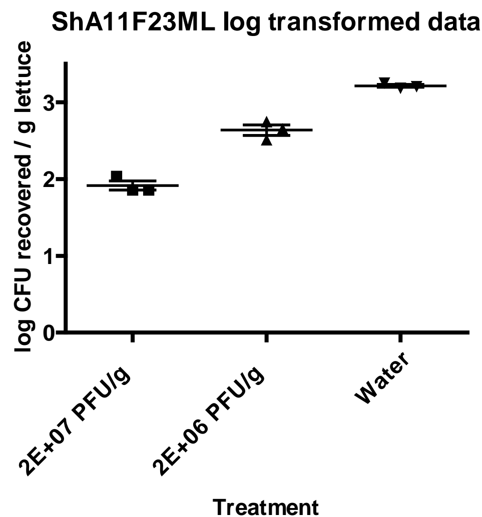


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the ShigaShield™ treatment in reducing the number of viable *Shigella* in the experimentally contaminated lettuce was evaluated by comparing the data obtained with the water-treated control samples and the ShigaShield™-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 <0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
2E+07 PFU/g vs Water	-1554.0	26.480	*** P<0.001
2E+06 PFU/g vs Water	-1194.0	20.346	*** P<0.001
2E+07 PFU/g vs 2E+06 PFU/g	-360.0	6.134	* P<0.05

11.5 Brief discussion of results and study's conclusions

- Applying ShigaShield™ at ca. $2x10^7$ PFU/g lettuce reduced the number of viable *Shigella* by ca. 95% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).
- Applying ShigaShield™ at ca. $2x10^6$ PFU/g lettuce reduced the number of viable *Shigella* by ca. 73% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).
- Reduction in *Shigella* levels achieved by using a higher concentration of ShigaShield™ was higher compared to that obtained with more dilute ShigaShield™ (95% vs. 73% when using ca. $2x10^7$ PFU/g and ca. $2x10^6$ PFU/g, respectively).
- The difference in *Shigella* recovered between samples treated with ShigaShield™ at $2x10^7$ PFU/g vs. $2x10^6$ PFU/g was statistically significant (P<0.05).

12 SUMMARY CONCLUSION OF THE STUDY

ShigaShield™ can significantly reduce viable *Shigella* levels in experimentally contaminated lettuce by ca. 73-95% in 5 minute contact time, when applied at ca. $2x10^6$ – $2x10^7$ PFU/g.

Using more concentrated ShigaShield™ (ca. $2x10^7$ PFU/g) resulted in significantly better reduction of *Shigella* levels compared to 10-fold diluted ShigaShield™ (ca. $2x10^6$ PFU/g).

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13 SIGNATURES

(b) (6)

Manrong Li, M.D.

Research Scientist

(b) (6)

Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 1.6

Report ShA12J31ML

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated yogurt

Study # ShA12J31ML

Intralytix

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1 STUDY TITLE

Evaluation of the ability of ShigaShield™ to reduce *Shigella* contamination in experimentally contaminated yogurt.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

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

4 PERFORMING LABORATORY

Intralytix, Inc.

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5 STUDY OBJECTIVE

To determine whether application of ShigaShield™ reduces the number of viable *Shigella* in yogurt when applied at the rate of $9x10^5$ – $9x10^7$ PFU/g.

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6 Test Matrix

Vanilla yogurt was obtained from a local Baltimore grocery store. It was not pre-treated prior to our studies.

7 ShigaShield™ Lot and Application

- ShigaShield™ Lot (b) (6)
- Titer: approx. 1×10^{10} PFU/mL
- ShigaShield™ was diluted as necessary with water just prior to application.
- The application was ca. 0.9mL ShigaShield™ per 100g yogurt.
- ShigaShield™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).

8 Bacterial Strains Used to Experimentally Contaminated Yogurt

The yogurt test matrix was experimentally contaminated with *Shigella sonnei* strain:

- *Sh.s53*: A nalidixic acid resistant mutant developed from Intralytix strain Sh.s43

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/ml. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Sh.s53). 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 supplemented with nalidixic acid (25 µg/ml.) Overnight growth corresponds to ca. 2×10^8 CFU/mL.

The yogurt was experimentally contaminated with ca. 2×10^3 CFU / g of yogurt.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- Peptone water (BD, Sparks, MD cat #218105)
- MacConkey Agar (BD, Sparks, MD; cat # 212123)

10 General Outline of Study

1) Four 100g portions of yogurt were each assigned as test groups A, B, C, or D.
2) The challenge dose of bacteria was thoroughly mixed into the yogurt using hockey sticks.
3) The samples were covered loosely and the bacteria were allowed to colonize the yogurt matrix at room temperature (RT) for 60 min.
4) Water (control) or ShigaShield™ were thoroughly mixed into the yogurt samples as follows:
   - Group A = 0.9mL $1x10^{10}$ PFU/mL ShigaShield™ / 100g = $9x10^{7}$ PFU/g
   - Group B = 0.9mL $1x10^{9}$ PFU/mL ShigaShield™ / 100g = $9x10^{6}$ PFU/g
   - Group C = 0.9mL $1x10^{8}$ PFU/mL ShigaShield™ / 100g = $9x10^{5}$ PFU/g
   - Group D = 0.9mL water / 100g = 0 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 ShigaShield™, from each sample group, triplicate ~25g samples of yogurt were removed, 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 *Shigella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached yogurt/peptone water mixture onto separate MacConkey plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

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

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).

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11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #ShA12J31ML

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU in 0.5 mL	CFU/g
A ($1x10^{10}$ PFU/mL)	Yes	100	$9x10^7$ PFU/g ShigaShield	3	4,7,6	72,126,108
B ($1x10^9$ PFU/mL)	Yes	100	$9x10^6$ PFU/g ShigaShield	3	34,32,28	612,576,504
C ($1x10^8$ PFU/mL)	Yes	100	$9x10^5$ PFU/g ShigaShield	3	45,49,50	810,882,900
D (Control)	Yes	100	Water	3	59,57,55	1062,1026,990

11.2 Tabular presentation of results

Table 2 Reduction of *Shigella* counts on yogurt treated with ShigaShield when applied at ca. $9x10^5$ – $9x10^7$ PFU/g (0.9mL per 100g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^{10}$ PFU/mL)	Yes	$9x10^7$ PFU/g ShigaShield	*n =3*	102	90%	1.0	Yes
B ($1x10^9$ PFU/mL)	Yes	$9x10^6$ PFU/g ShigaShield	*n = 3*	564	45%	0.3	Yes
C ($1x10^8$ PFU/mL)	Yes	$9x10^5$ PFU/g ShigaShield	*n =3*	864	16%	0.1	Yes
D (Control)	Yes	Water	*n =3*	1026	-	-	-

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

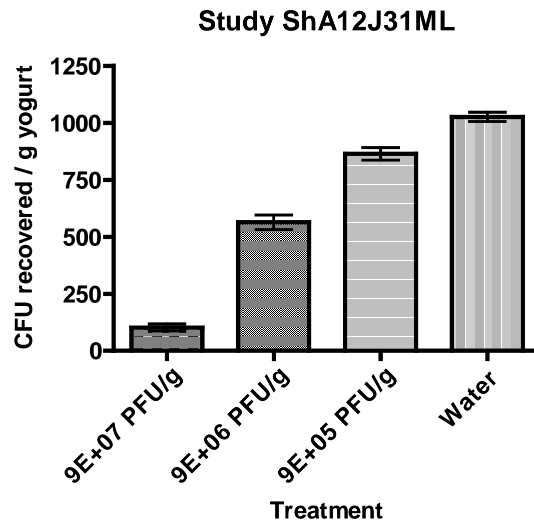
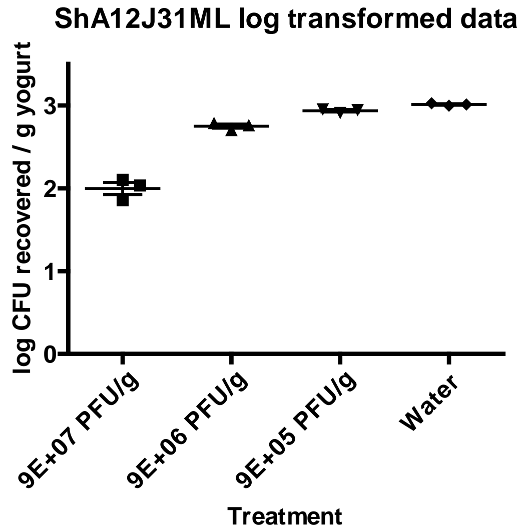


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the ShigaShield™ treatment in reducing the number of viable *Shigella* in the experimentally contaminated yogurt was evaluated by comparing the data obtained with the water-treated control samples and the ShigaShield^{TM}-treated samples.

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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
9E+07 PFU/g vs Water	-924.00	37.350	*** P<0.001
9E+06 PFU/g vs Water	-462.00	18.675	*** P<0.001
9E+05 PFU/g vs Water	-162.00	6.548	** P<0.01
9E+07 PFU/g vs 9E+06 PFU/g	-462.00	18.675	*** P<0.001
9E+07 PFU/g vs 9E+05 PFU/g	-762.00	30.802	*** P<0.001
9E+06 PFU/g vs 9E+05 PFU/g	-300.00	12.127	*** P<0.001

11.5 Brief discussion of results and study's conclusions

- Applying ShigaShield™ at ca. $9x10^7$ PFU/g yogurt reduced the number of viable *Shigella* by ca. 90% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^6$ PFU/g yogurt reduced the number of viable *Shigella* by ca. 45% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying ShigaShield™ at ca. $9x10^5$ PFU/g yogurt reduced the number of viable *Shigella* by ca. 16% after 5 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).

- Reduction in *Shigella* levels achieved by using more concentrated ShigaShield™ was higher compared to those obtained with more dilute ShigaShield™ (90% vs. 45% vs. 16% when using ca. $9x10^7$ PFU/g, $9x10^6$ PFU/g, and ca. $9x10^5$ PFU/g, respectively).

- The differences in *Shigella* recovered when ShigaShield™ was applied at the three application rates(ca. $9x10^7$ PFU/g vs. $9x10^6$ PFU/g vs. $9x10^5$ PFU/g) were statistically significant (P<0.001).

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12 SUMMARY CONCLUSION OF THE STUDY

ShigaShield™ can significantly reduce viable *Shigella* levels in experimentally contaminated yogurt by ca. 16-90% in 5 minute contact time, when applied at ca. $9x10^5$ – $9x10^7$ PFU/g.

Using the higher ShigaShield™ application rate (ca. $9x10^7$ PFU/g) resulted in statistically significantly better reduction of *Shigella* levels compared to lower ShigaShield™ application rates (ca. $9x10^6$ PFU/g or $9x10^5$ PFU/g).

13 SIGNATURES

(b) (6)

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Research Scientist

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Joelle Woolston

Research Scientist / Laboratory Manager

(b) (6)

Alexander Sulakvelidze, Ph.D.

Study Director

Appendix 2: Summary of *Shigella* toxins

The mechanism of shigellosis-induced diarrhea has not been fully defined. While much can be attributed to the actual invasion by the bacteria, three enterotoxins are also suspected to play a role. See Table 1 for the list of possible enterotoxins.

Table 1 List of known *Shigella* enterotoxins.

Toxin	Gene	Location of gene	Strains
Shiga toxin[1] (Stx)	*stxA/B*	chromosome	Found in *Shigella dysenteriae* only
Shigella enterotoxin 1 (ShET1)[2]	*set1A*, *set1B*	chromosome	Found in *Shigella flexneri serotype 2* (almost exclusively)
Shigella enterotoxin 2 (ShET2)[2]	*senA* (possibly 2nd homolog *sen*B)	plasmid	Has been found in all spp.

Shiga toxin (Stx) consists of two subunits, which are encoded by the chromosomal genes, *stxA* and *stxB*. This toxin has been found in *Shigella dysenteriae* only and has 99% homology with the *E. coli* shiga toxin-1 gene, *stx1*.

Shigella enterotoxin 1 (ShET1) consists of two subunits, which are encoded by chromosomal genes *set1A* and *set1B*. This enterotoxin has been found almost exclusively in *Shigella flexneri* serotype 2.[2,3]

Shigella enterotoxin 2 (ShET2) is a plasmid encoded toxin that has been found in all four *Shigella* species.[2] It is encoded by the gene *senA* and there is a possible homolog, *senB*. *Shigella* spp. are known to lose plasmids upon storage.

Colony PCR was performed on the host strain to determine if the genes were present. See Table 2 for a list of primers used and their references. The *E. coli* 16S RNA primer pair was used in multiplex PCR as a positive control to confirm the presence of bacterial DNA.

Primers were purchased from Sigma-Aldrich (St. Louis, MO.) Additionally, Intralytix had in-house primers for 16S RNA sequences of *E. coli*. For colony PCR assays, a single bacterial colony was suspended in 100μL of sterile water and boiled for 5 minutes. The bacterial strains tested were Intralytix strains Sh.s43 (*S. sonnei*) and Sh.f51 (*S. flexneri*). The boiled mixture was

spun (13K, 5') and 2μL of the supernatant was used as the template DNA for each reaction. The PCR amplification reactions were done in 25- or 50μL volumes. Each contained 20mM Tris-HCl, 50mM KCl, 0.2mM dNTPs, 0.4uM of each primer, and 25U/mL Taq DNA polymerase, with varying concentrations of $MgCl_2$. Reactions were performed in an automated thermocycler. See Table 3 for specific conditions. Each PCR primer pair was analyzed using a water control (all ingredients except bacterial supernatant), a positive control (Intralytix *S. flexneri* strain Sh.f51), and the host strain, Sh.s43. The samples were then electrophoresed in 2% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator.

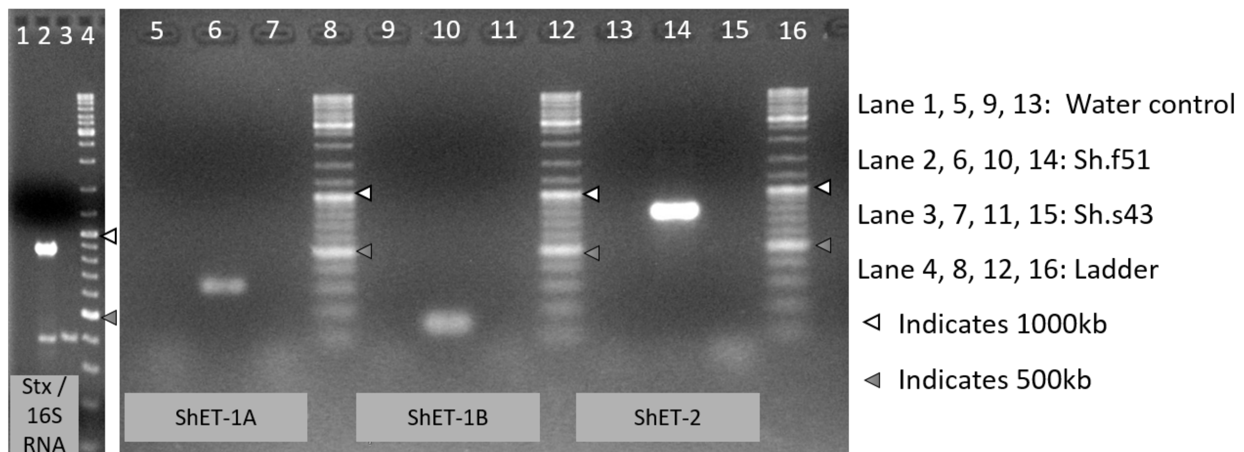

Figure 1 Identification of enterotoxins from *Shigella* isolates by singleplex and multiplex (includes 16S RNA primers) polymerase chain reaction (PCR). The PCR products representing the genes were separated by electrophoresis on 2% agarose gels. The ladder is DNA molecular size markers (Fermentas catalog #SM0333) ranging from 100bp to 10000bp, in 100bp increments from 100bp to 1000bp. See Table 2 for list of expected PCR product sizes.

As seen in Figure 1, the PCR analysis of the host strain Sh.s43 shows that it does not contain the genes for any of the known enterotoxins.

Conclusion

Of the four *Shigella* species, a *S. sonnei* or *S. boydii* isolate that does not contain a virulence plasmid would be optimal choices for host strains. Neither of these species have been found to contain the Shiga toxin and almost none contain the Shigella enterotoxin 1. A virulence plasmid encoding Shigella enterotoxin 2 could be present in these species; however, it is frequently lost during repeated subculturing and prolonged storage and its absence could be confirmed through PCR, using Shigella enterotoxin 2 specific primers. Intralytix's host strain for all five monophages is Sh.s43, a *S. sonnei* strain which contains none of the three known *Shigella* enterotoxins.

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Table 2 List of primers

Enterotoxin	Gene	Tm °C	Primer name	5' - 3'	Reference	Product size (bp)	Notes
Stx	*stxA/B*	60.8	Stx-Shig-F	CAGTTAATGTGGTTGCGAAG	[3]	895	Has 99% homology with *E. coli* shiga-like toxin
		61.5	Stx-Shig-R	CTGCTAATAGTTCTGCGCATC			
ShET1	*set1A*	57.8	ShET-1A-F	TCACGCTACCATCAAAGA	[3]	309	Two subunits combine to make single protein
		60.2	ShET-1A-R	TATCCCCCTTTGGTGGTA			
ShET1	*set1B*	64.5	ShET-1B-F	GTGAACCTGCTGCCGATATC	[3]	147	
		56.5	ShET-1B-R	ATTTGTGGATAAAAATGACG			
ShET2	*senA*	53.0	ShET-2-F	ATGTGCCTGCTATTATTTAT	[3]	799	These primers only identify *senA*
		56.5	ShET-2-R	CATAATAATAAGCGGTCAGC			
n/a	*16S RNA*	59.7	Ec-16S-RNA-F	CCCCCTGGACGAAGACTGAC	ITX in-house	401	16S RNA for *E. coli*
		57.1	Ec-16S-RNA-R	ACCGCTGGCAACAAAGGATA			

Table 3 PCR conditions

Primer pairs	$MgCl_2$ concentration	initial denaturation	# cycles	denaturation	annealing	extension	final extension
Stx-Shig-F & Stx-Shig-R + Ec-16S-RNA-F & Ec-16S-RNA-R	1.5mM	94°, 5'	32	94°, 30sec	58°C, 30sec	72°C, 1'	72°C, 5'
ShET-1A-F & ShET-1A-R	1.5mM	94°, 5'	32	94°, 30sec	58°C, 30sec	72°C, 1'	72°C, 5'
ShET-1B-F & ShET-1B-R	1.5mM	94°, 5'	32	94°, 30sec	58°C, 30sec	72°C, 1'	72°C, 5'
ShET-2-F & ShET-2-R	1.5mM	94°, 5'	30	94°, 30sec	57°C, 30sec	72°C, 1'	72°C, 5'

References

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2 Roy, S., Thanasekaran, K., Dutta Roy, A. R. & Sehgal, S. C. Distribution of Shigella enterotoxin genes and secreted autotransporter toxin gene among diverse species and serotypes of shigella isolated from Andaman Islands, India. *Tropical Medicine & International Health* **11**, 1694-1698, doi:10.1111/j.1365-3156.2006.01723.x (2006).

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SUBMISSION END