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14. ABSTRACT
During the studies supported by our Phase I STTR grant from the US Army, we began developing and testing a novel concept for a probiotic preparation – based on naturally occurring bacteriophages – as a way to condition the GI tract's microflora gently and favorably. The preparation (designated "ShigActive") is a bacteriophage cocktail that specifically targets Shigella spp. (significant diarrhea-causing pathogens that sicken ca. 165 million people/year, ca. 1.1 million of whom die). We have successfully isolated and rigorously characterized a large number of phages lytic for Shigella, and we have developed a murine model in which the in vivo efficacy of our

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Report Title

Final Report: Bacteriophage-based Probiotic Preparation for Managing Shigella Infections

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During the studies supported by our Phase I STTR grant from the US Army, we began developing and testing a novel concept for a probiotic preparation – based on naturally occurring bacteriophages – as a way to condition the GI tract's microflora gently and favorably. The preparation (designated "ShigActive") is a bacteriophage cocktail that specifically targets Shigella spp. (significant diarrhea-causing pathogens that sicken ca. 165 million people/year, ca. 1.1 million of whom die). We have successfully isolated and rigorously characterized a large number of phages lytic for Shigella, and we have developed a murine model in which the in vivo efficacy of our phage preparation can be evaluated. During this Phase II project, we propose to perform studies required to obtain regulatory approval of ShigActive and to generate data critical for subsequent clinical trials (performance of actual clinical trials is not stipulated during this Phase II grant). During these studies, we will also perform metagenomic analyses of the gut microbiota (using DGGE and/or state-of-the-art 454-pyrosequencing of 16S rRNA genes) of mice before and after their treatment with ShigActive, in order to (i) obtain additional safety data for the FDA, and (ii) compare rigorously the effect of ShigActive and ampicillin (an antibiotic commonly used to treat shigellosis) on the intestinal microflora of mice.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Manuscripts:
Ukhanova, M., M. Li, A. Sulakvelidze, M. Reinhard and V. Mai. Bacteriophage administration significantly reduces Shigella colonization and shedding by Shigella-challenged mice without deleterious side effects and altering the gut microbiota. Bacteriophage (Submitted, 2015)

Meeting presentations:
1. Oral presentation, 3rd International Conference and Exhibition on Probiotics, Functional & Baby Foods. September 23-25, 2014 Hotel Royal Continental, Naples, Italy. Bacteriophage-based probiotic preparations for maintaining healthy gut microflora.
2. Oral presentation, Kosice, Slovakia, 2013. Bacteriophages as a novel class of probiotics: Phagebiotics.
3. Oral presentation, 7th Prebiotics, Probiotics, and New Foods Meeting, Rome, Italy, September 8 – 10, 2013. Phagebiotics: bacteriophages as a new class of probiotics for managing gut microflora.
4. Oral presentation, Lisbon, Portugal, October 4 – 7, 2013. "How Will the EU Health System Handle the Age Wave?" conference, Buck Institute of Aging. Bacteriophages for healthy living.

Number of Presentations: 6.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Faculty Supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

Bacteriophage-based Probiotic Preparation for Managing *Shigella* Infections, Phase II

Contract # W911NF-11-C0074

Project #A090-T012

Final report to US Army Research Office
Research Triangle Park, NC

04/11/11 – 01/22/14

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1 PROJECT OVERVIEW

1.1 General overview

Intralytix, Inc., has developed and is commercializing a novel antibacterial technology that may become an important method for improving food safety. The technology utilizes bacteriophages, naturally occurring predators of bacteria, to reduce contamination of various foods with specific pathogenic bacteria. Intralytix's first commercial, bacteriophage-based product for improving food safety (ListShield™) targets *Listeria monocytogenes* and is the first phage product approved by the FDA (21 CFR §172.785) for food safety applications. Intralytix's 2nd phage-based food safety product (EcoShield™) was recently FDA-cleared as "Food Contact Notification" or FCN, for the use on red meat parts and trim intended to be ground (FCN No. 1018). EcoShield™ specifically targets *Escherichia coli* O157:H7 and is effective in eliminating or significantly reducing that bacterium's presence in ground beef, fruits, and vegetables (the above-mentioned FCN approval is pertinent for ground beef only).

The goal of the current project #A090-T012 is to continue the development of a phage cocktail (tentatively designated ShigActive™) possessing strong, broad-spectrum lytic activity against selected, highly virulent strains of *Shigella* spp. Our Phase I project was an early-stage proof-of-concept study that showed that our approach had merit. This Phase II of the project will include performing *in vivo* efficacy studies as well as other optimization measures to meet regulatory guidelines for the product's commercial release. The project's main milestone is to assemble a Notification for a New Dietary Ingredient (NNDI) application package and to submit it to the Food and Drug Administration (FDA).

1.2 Project goals

The ultimate goal of this project is to develop a novel "probiotic-like" approach for using naturally occurring lytic bacteriophages to help prevent or treat illnesses caused by various bacterial pathogens. Our approach for these phage-based probiotics is to use them prophylactically rather than therapeutically. The approach of using phages prophylactically is similar to that used for bacteria-based probiotic preparations, which are administered over a period of time and act by favorably conditioning the gut's microflora. The specific aims of the project, as indicated in the initial proposal, are as follows:

Specific Aim # 1. Characterize the *in vitro* properties of, and perform nucleotide sequencing and bioinformatics analyses of, our *Shigella* phages in order to determine the optimal formulation for ShigActive and to obtain the data required by the FDA.

Specific Aim #2. Develop and validate the optimal manufacturing protocol for ShigActive, and prepare experimental lots of ShigActive needed to perform *in vivo* efficacy and toxicity studies.

Specific Aim #3. Perform *in vivo* efficacy and dosing optimization studies, and characterize the effect of ShigActive on the intestinal microbiota of mice.

Specific Aim #4. Prepare an NNDI application package and submit it to the FDA.

1.3 Research Design - General approach

The proposed study focuses on the development of a phage preparation lytic for *Shigella* spp. and the generation of data required for its regulatory approval. During the proposed studies, Intralytix will (i) characterize the genomic variability of our ten most promising *Shigella* phages, (ii) optimize the phages' propagation in a WAVE Bioreactor, (iii) determine the minimal effective dose (MED) and the time and frequency of administration required to eliminate or significantly reduce *Shigella* colonization in experimentally infected mice, and (iv) develop an NNDI application package for ShigActive™.

During Year 1 of the proposed project, our studies will focus on characterizing the *Shigella* phages and optimizing the product manufacturing process. During Year 2 of the project, our focus will shift to *in vivo* testing of ShigActive™ and assembly of the NNDI application data. The project's estimated schedule, which covers both years described in the proposal, is included below:

Table 1. Estimated project schedule

Tasks	**Quarters** 1 2 3 4 5 6 7 8
In vitro characterization of *Shigella* phages	x x x
Nucleotide sequencing and bioinformatics analyses	x x x x x
Formulate the final candidate phage cocktail (ShigActive)	x x x x
In vitro specificity studies of ShigActive; Identity Test	x x x x x
In vitro efficacy studies of ShigActive	x x x
Develop manufacturing protocol for ShigActive; produce and QC test ≥ 3 lots of ShigActive	x x x x
In vivo efficacy and dosing optimization studies	x x x x x x x x
In vivo toxicity and effect on the GI microflora studies	x x x x x x x x x x
Ongoing data analysis, preparing reports, etc.	x x x x x x x x x x x x x x x x x x x x
Assemble and submit an NNDI application to the FDA	x x x x

2 PHASE I SUMMARY

The current project #A090-T012 is a continuation of the Phase I project under the same title. The Phase I project was successfully completed, and its brief summary is

outlines below, followed by description of tasks and accomplishments for the present Phase II project.

2.1 Bacterial strains

The starting point of the project was to establish *Shigella* strains collection. Thus, we have obtained 50 strains of *Shigella* from Dr. Karen Kotloff's laboratory at the University of Maryland in Baltimore. This is a diverse strain collection of *Shigella* strains isolated from patients in various countries around the world, including Chile, Mali, Pakistan, and Peru (Table 2).

Table 2. *Shigella* strain collection

#	Strain	Country of origin	Original ID #	#	Strain	Country of origin	Original ID #
1	SH.d 1	Chile	514	27	SH.f 27	Pakistan	996
2	SH.d 2	Chile	601	28	SH.f 28	Pakistan	428
3	SH.d 3	Chile	608	29	SH.f 29	Pakistan	504
4	SH.d 4	Pakistan	505	30	SH.f 30	Pakistan	418
5	SH.d 5	Peru	137-031283	31	SH.f 31	Chile	428
6	SH.f 6	Peru	045-311082	32	SH.f 32	Chile	466
7	SH.f 7	Peru	045-291182	33	SH.f 33	Chile	469
8	SH.f 8	Peru	093-300483	34	SH.f 34	Pakistan	93
9	SH.f 9	Peru	041-020384	35	SH.f 35	Pakistan	133
10	SH.f 10	Peru	128-090484	36	SH.s 36	Chile	423
11	SH.f 11	Pakistan	212	37	SH.s 37	Chile	433
#	Strain	Country of origin	Original ID #	#	Strain	Country of origin	Original ID #
12	SH.f 12	Pakistan	389	38	SH.s 38	Peru	055-110483
13	SH.f 13	Chile	536	39	SH.s 39	Peru	090-020583
14	SH.f 14	Chile	628	40	SH.s 40	Peru	137-190883
15	SH.f 15	Chile	450	41	SH.s 41	Peru	107-260983
16	SH.f 16	Chile	462	42	SH.s 42	Pakistan	81
17	SH.f 17	Chile	531	43	SH.s 43	Pakistan	90
18	SH.f 18	Chile	576	44	SH.s 44	Mali	200308
19	SH.f 19	Mali	200200	45	SH.s 45	Mali	200470
20	SH.f 20	Pakistan	300	46	SH.s 46	Mali	200527
21	SH.f 21	Pakistan	311	47	SH.b 47	Chile	425
22	SH.f 22	Pakistan	340	48	SH.b 48	Chile	482
23	SH.f 23	Chile	440	49	SH.b 49	Chile	559
24	SH.f 24	Chile	441	50	SH.b 50	Pakistan	248
25	SH.f 25	Chile	533	51	SH.f 51	Tokyo	2457T
26	SH.f 26	Chile	200294	52	SH.s 52	ATCC	9290

All *Shigella* strains were propagated and screened for purity as per Intralytix standard procedure described in Method M002-30 ("Method for preparing frozen bacterial cultures for general storage," effective date: 6/5/2006). The method is summarized immediately below:

- The original strain is received from the outside donor.
- The original strain is streaked, for well-isolated colonies, on LB agar and incubated (37°C, 16-48 hr).
- A single colony is streaked, for well-isolated colonies, on LB agar and incubated (37°C, 16-24 h).
- A single colony is used to inoculate LB broth (usually 3-5 ml) and the inoculated broth is incubated with shaking (37°C, 50-150 rpm, 16-24 h).
- 0.1 ml of the broth culture is added to fresh LB broth (usually 5-10 ml), and the newly-inoculated broth is incubated with shaking (37°C, 50-150 rpm, 3-4 h)."
- Sterile glycerol is gently but thoroughly mixed with the bacterial culture, to yield a final concentration of ca. 30% glycerol:70% broth culture (vol/vol).
- Aliquots (0.5-1.5 ml) of the mixture are aseptically added to 1.8- to 2.5-ml capacity cryoprotectant tubes (e.g., Nunc CryoTube Vials [Nalge Nunc International, Rochester, NY]) and stored frozen at -70° to -85°C. Prepare at least 2 vials for each strain; 10 vials per strain are recommended. Label the vials with:

  Original strain ID, and
  Intralytix ID # if different.

- The next day (or as soon thereafter as technically feasible), one frozen vial is opened and its contents are tested for viability and purity, by plating on LB agar and incubating at 37°C for 16-48 h. The remaining vials are stored frozen and are used as needed.

2.2 Bacterial host strain

In an effort to streamline subsequent phage production, a single host strain (SH.s 43) was identified and shown to be capable of generating high titers of all *Shigella* phages in our candidate phage preparation. The PFGE profile of the strain is shown in Figure 1.

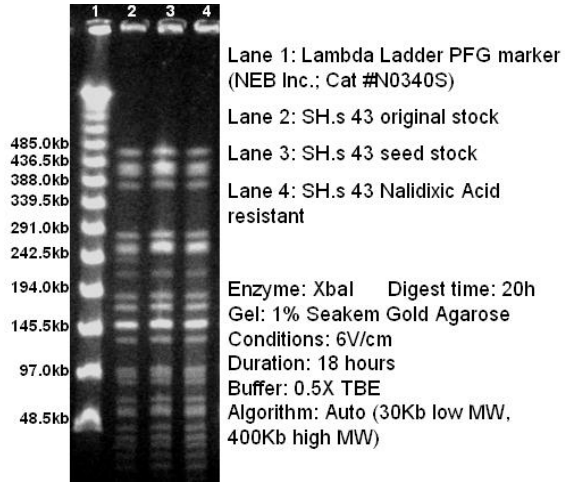

Figure 1. DNA PFGE profiles of host *Shigella* strain SH.s43

Host strain SH.s43 was plated onto LB agar to characterize its plaque morphology. Colonies were grown with established inoculum procedures up to approximately 0.2 OD_{600} and plated with top agar. Figure 2 shows an image of the colonies while a description follows below.

> Colonies are **large**, **shiny**, and **cream-colored**. They are **round** with **erose edges**. Each is **slightly raised**. Open plate has a **pungent smell** at room temperature.

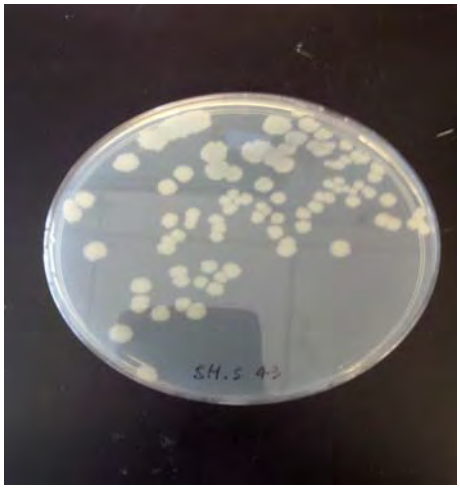

Figure 2. Host strain SH.s43 on LB agar

The strain was confirmed to be *Shigella sonnei* (98% likelihood) by the API analysis (**Table 3**).

Table 3. Biochemistry / API analysis of SH.s 43

Strip	Taxonomy	% ID	T index	Test against	Value	Test against	Value
API 20 E V4.1	Shigella sonnei	98.4	0.59	RHA	75	McC	100
API 20 E V4.1	Escherichia coli 2	1.4	0.49	ODC	20	McC	100

Host strain SH.s43 was determined to not contain any endogenous phage. Filtrate of the strain culture was applied to lawns of bacteria from other *Shigella sonnei* strains as described in Intralytix method M002-40 Method to Perform Testing for Endogenous Phage. None of the treated plates exhibited zones of lysis.

2.3 Phages

2.3.1 Introduction/Overview

To date, we have isolated several phages from various lots of commercial phage preparations produced by the Eliava Institute of Bacteriophage in Tbilisi, Georgia, and from various environmental sources in Maryland, USA. The preliminary information about the phages is shown in Table 4. The table will be updated as additional phages are identified and additional information about them is generated. One previously

identified bacteriophage (SHDML-1) increasingly exhibited properties of a lysogenic phage (small and turbid plaques, inability to grow in high titers in the host bacterial strain, etc.) and we have removed this monophages as a candidate in an optimal ShigActive™ formulation.

Table 4. *Shigella* phage collection

#	Intralytix Designation	Source	Lot #	Taxonomy	Genome size	Host Strain
1	SHFML-6	Intesti phage	010504	*Siphoviridae*	ca. 48Kb	SH.f6
2	SHFML-35	Intesti phage	010706	*Podoviridae*	ca. 30Kb	SH.f35
3	SHFML-20	Encophagum D-90	140704	*Siphoviridae*	ca. 48Kb	SH.f20
4	SHFML-31	Encophagum D-90	140704	*Podoviridae*	ca. 30Kb	SH.f31
5	SHFML-26	SES D-90	010104	*Myoviridae*	ca. 180Kb	SH.f26
	~~SHDML-1~~	~~Inner Harbor~~			~~ca. 80Kb~~	~~SH.d5~~
6	SHFML-27	Inner Harbor		*Myoviridae*	ca. 50Kb	SH.f27
7	SHSML-36	Inner Harbor		*Myoviridae*	ca. 180Kb	SH.d1
8	SHFML-21	Inner Harbor		*Myoviridae*	ca. 190Kb	SH.f21
9	SHDML-4	Inner Harbor		*Podoviridae*	ca. 30Kb	SH.d4
10	SHFML-11	Intesti phage	010504	*Myoviridae*	ca. 190Kb	SH.f11
11	SHSML-45	Encophagum D-90	140704	*Siphoviridae*	ca. 130Kb	SH.s45
12	SHSML-46	Intesti phage	010706	*Podoviridae*	ca. 30Kb	SH.s46
13	SHBML-47	Park water		*Podoviridae*	ca. 60Kb	SH.b47
14	SHBML-50-1	Park water		*Myoviridae*	ca. 190Kb	SH.b50
15	SHBML-50-2	Park water		*Myoviridae*	ca. 180Kb	SH.b50
16	SHSML-52-1	Park water		*Myoviridae*	ca. 190Kb	SH.s43
17	SHSML-52-2	Park water		*Myoviridae*	ca. 90Kb	SH.s43

2.3.2 Susceptibility screening

Seventeen phages were used to perform screening of our collection of *Shigella* strains. Results are summarized in Table 5. The final column in the table illustrates the screening results for a candidate ShigActive™ cocktail made of SHFML-26, SHSML-45, SHFML-11, SHBML-50-1, and SHSML-52-1. All screening was conducted on host strain SH.s43.

We have an excellent combination/collection of *Shigella* phages that can be used to design a potent, wide target range *Shigella* phage cocktail. Selected phages will be thoroughly characterized, including determination of their taxonomic classification with electron microscopy.

2.3.3 PFGE profiles

The phages were purified via sequential plague purification process, as per standard procedure used by Intralytix for all its bacteriophages. After the plague homogeneity was established (suggesting that single monophage clone was selected), additional genomic analyses were performed for purity and identity verification. These analyses included Pulsed Field Gel Electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP), respectively.

Shigella monophages were examined for purity by PFGE (uncut DNA). Observation of a single DNA band is the metric and acceptability criterion for purity. Results of the PFGE analysis are shown in Figure 3.

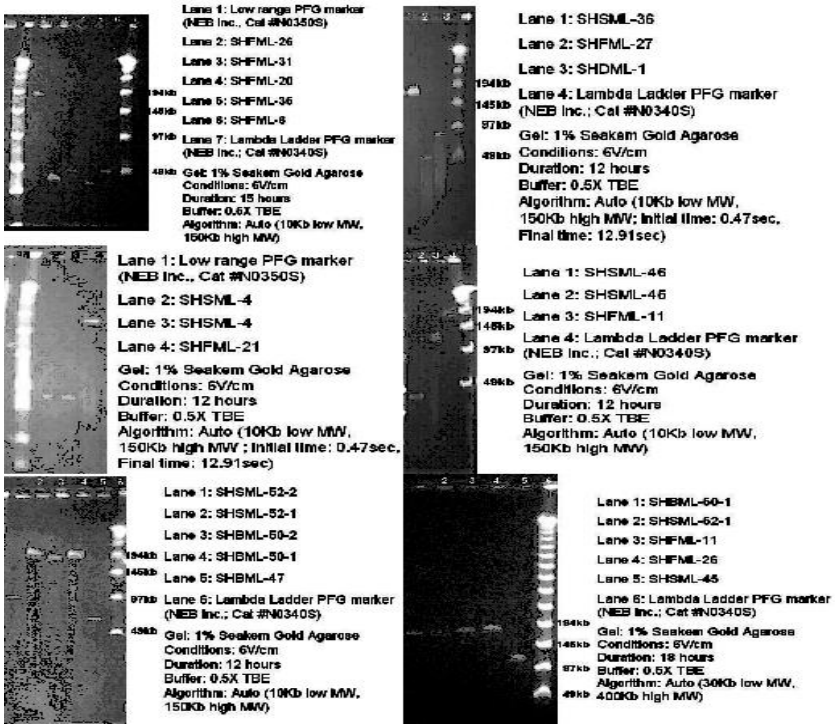

Figure 3. PFGE profiles for *Shigella* phages

Table 5. Susceptibility of *Shigella* strains to bacteriophages at $2x10^4$ PFU/mL

#	Itx ID	Provider	Species	SHFML-6	SHFML-35	SHFML-20	SHFML-31	SHFML-26	SHFML-27	SHSML-36	SHFML-21	SHDML-4	SHFML-11	SHSML-45	SHSML-46	SHBML-47	SHBML-50-1	SHBML-50-2	SHSML-52-1	SHSML-52-2	ShigActive
1	SH.d 1	Univ.of Maryland-chile	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+
2	SH.d 2	Univ.of Maryland-chile	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+
3	SH.d 3	Univ.of Maryland-chile	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+
4	SH.d 4	Univ.of Maryland-pakistan	dysenteriae	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+
5	SH.d 5	Univ.of Maryland-peru	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
6	SH.f 6	Univ.of Maryland-peru	flexneri	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+
7	SH.f 7	Univ.of Maryland-peru	flexneri	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+
8	SH.f 8	Univ.of Maryland-peru	flexneri	-	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	+
9	SH.f 9	Univ.of Maryland-peru	flexneri	+	-	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+
10	SH.f 10	Univ.of Maryland-peru	flexneri	+	-	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+
11	SH.f 11	Univ.of Maryland-pakistan	flexneri 1	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
12	SH.f 12	Univ.of Maryland-pakistan	flexneri 1	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
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15	SH.f 15	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
16	SH.f 16	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
17	SH.f 17	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
18	SH.f 18	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
19	SH.f 19	Univ.of Maryland-mali	flexneri 1b	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+
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21	SH.f 21	Univ.of Maryland-pakistan	flexneri 2	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+
22	SH.f 22	Univ.of Maryland-pakistan	flexneri 2	+	-	+	-	+	-	-	+	-	+	+	-	-	-	-	-	+	+
23	SH.f 23	Univ.of Maryland-chile	flexneri 2a	+	-	+	-	+	-	-	+	-	+	+	-	-	-	-	-	+	+
24	SH.f 24	Univ.of Maryland-chile	flexneri 2a	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+
25	SH.f 25	Univ.of Maryland-chile	flexneri 2a	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+
26	SH.f 26	Univ.of Maryland-mali	flexneri 2b	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+
27	SH.f 27	Univ.of Maryland-pakistan	flexneri 3	+	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	-	+

#	Itx ID	Provider	Species	SHFML-6	SHFML-35	SHFML-20	SHFML-31	SHFML-26	SHFML-27	SHSML-36	SHFML-21	SHDML-4	SHFML-11	SHSML-45	SHSML-46	SHBML-47	SHBML-50-1	SHBML-50-2	SHSML-52-1	SHSML-52-2	ShigActive
28	SH.f 28	Univ.of Maryland-pakistan	flexneri 4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	SH.f 29	Univ.of Maryland-pakistan	flexneri 4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	SH.f 30	Univ.of Maryland-pakistan	flexneri 5	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+
31	SH.f 31	Univ.of Maryland-chile	flexneri 6	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	SH.f 32	Univ.of Maryland-chile	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
33	SH.f 33	Univ.of Maryland-chile	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
34	SH.f 34	Univ.of Maryland-pakistan	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
35	SH.f 35	Univ.of Maryland-pakistan	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
36	SH.s 36	Univ.of Maryland-chile	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
37	SH.s 37	Univ.of Maryland-chile	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
38	SH.s 38	Univ.of Maryland-peru	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
39	SH.s 39	Univ.of Maryland-peru	sonnei	-	-	-	-	+	-	+	-	-	+	-	-	-	+	+	+	+	+
40	SH.s 40	Univ.of Maryland-peru	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
41	SH.s 41	Univ.of Maryland-peru	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
42	SH.s 42	Univ.of Maryland-pakistan	sonnei	-	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
43	SH.s 43	Univ.of Maryland-pakistan	sonnei	-	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
44	SH.s 44	Univ.of Maryland-mali	sonnei	-	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
45	SH.s 45	Univ.of Maryland-mali	sonnei	-	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
46	SH.s 46	Univ.of Maryland-mali	sonnei	-	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
47	SH.b 47	Univ.of Maryland-chile	boydii	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+
48	SH.b 48	Univ.of Maryland-chile	boydii	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
49	SH.b 49	Univ.of Maryland-chile	boydii	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
50	SH.b 50	Univ.of Maryland-pakistan	boydii	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
51	SH.f 51	Univ.of Maryland-Tokyo	flexneri 2a	+	-	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	+
52	SH.s 52	ATCC	sonnei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

2.3.4 RFLP profiles of *Shigella* phages

Phage DNA was isolated using the AquaRNA kit (MultiTarget Pharmaceuticals, LLC; Salt Lake City, Utah). After extraction, DNA was digested with *EcoR*V, and the resulting bands were electrophoresed in a 0.8% agarose gel.

Shigella monophages were examined for identity and homogeneity by RFLP. Observation of a unique DNA pattern that is identical to the known DNA pattern is the metric and acceptability criterion for homogeneity. Results of the PFGE analysis are shown in Figure 4.

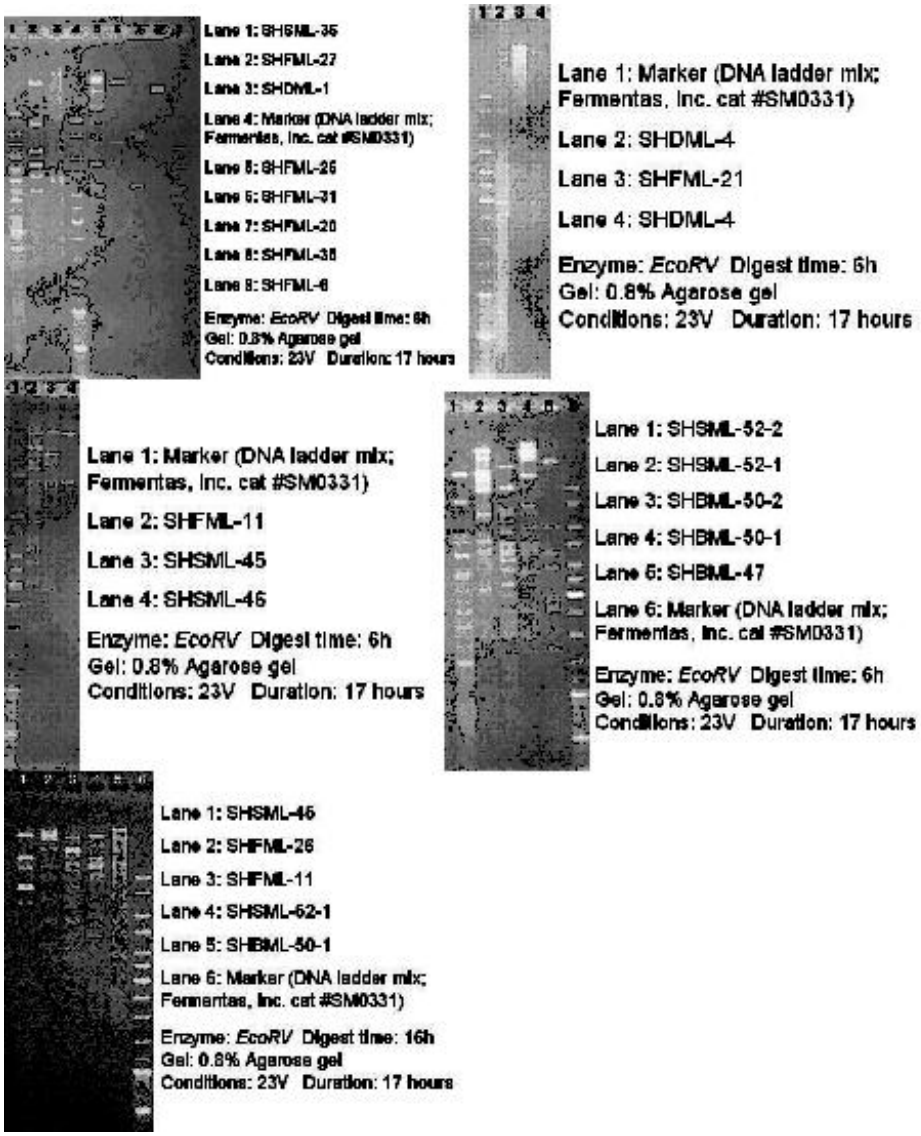


Figure 4. RFLP profiles for Shigella phages

2.4 Electron microscopy characterization

The photographs obtained by EM of our most potent eight *Shigella* phages to date are shown in Figure 5. The studies were performed by Professor Hans Ackermann at Laval University, and the taxonomic assignments were made by Prof. Ackermann according to the phage classification scheme developed by Ackermann and Berthiaume (Ackermann and Berthiaume 1995).

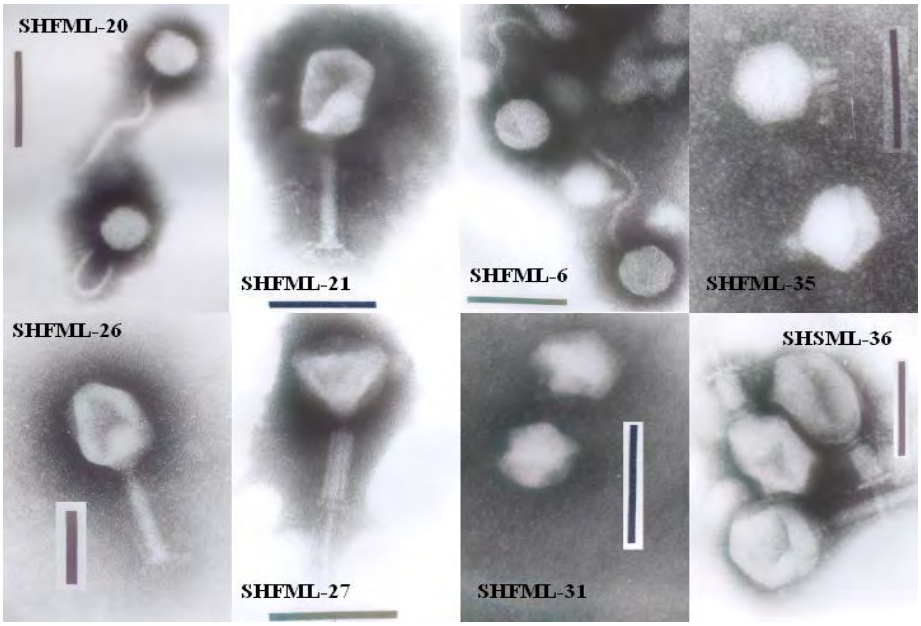


Figure 5. Electron microscopy photos of selected *Shigella* phages (bar = 100nm)

The photographs of eight additional phages are shown below in Figure 6.

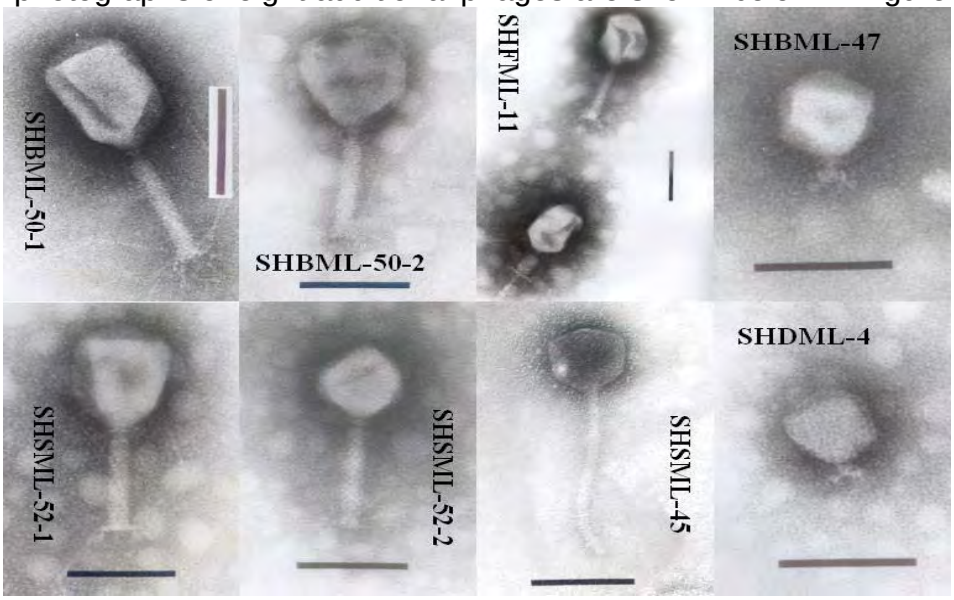


Figure 6. Electron microscopy photos of selected Shigella phages (bar = 100nm)

3 PHASE II EFFORTS

3.1 Overview

The ultimate goal of this Phase II project is to develop a novel approach for using naturally occurring lytic bacteriophages to help prevent or treat illnesses caused by various bacterial pathogens, in this case *Shigella* spp. Our approach to this development is to (i) characterize the phages used in an optimal formulation of ShigActive™, an anti-*Shigella* phage-based probiotic, (ii) develop the optimal manufacturing protocol to prepare ShigActive™, (iii) conduct *in vivo* efficacy and dosing studies, and (iv) submit an NNDI application for ShigActive™ to the FDA. Our plan for meeting these objectives, along with a rudimentary/tentative timetable for completion, is described below.

Table 6. Gantt Chart for *Shigella* Phase II Project

Task	Description	Start Date	End Date
Task 1	Characterize the *in vitro* properties of, and perform nucleotide sequencing and bioinformatics analyses of, our *Shigella* phages in order to determine the optimal formulation for ShigActive and to obtain data required by the FDA	04/11/11	10/10/11
Task 1.1	Prepare project Gantt chart		
Task 1.2	Tabulate (i) plaque morphology, (ii) EM, (iii) host range, (iv) burst size, (v) genomic profile (RFLP and PFGE), and (vi) protein composition for 17 *Shigella* phages		
Task 1.3	Obtain nucleotide sequencing and bioinformatics for ShigActive phages		
Task 1.4	Update PhageSelector™ program to incorporate burst size and genomic composition		
Task 1.5	Develop a PCR-based identity test		
Task 1.6	Conduct additional *in vitro* efficacy tests using HeLa cell invasion assay		
Task 1.7	Repeat sensitivity and specificity testing for formulation		
Task 2	Develop and validate the optimal manufacturing protocol for ShigActive, and prepare experimental lots of ShigActive needed to perform *in vivo* efficacy and toxicity studies	10/11/11	04/10/12
Task 2.1	Prepare cGMP manufacturing protocol for ShigActive™		
Task 2.2	Optimize ShigActive production at small scale		
Task 2.3	Scale up optimized ShigActive production in a WAVE Bioreactor		
Task 2.4	Prepare three lots of ShigActive with full QC testing		
Task 2.5	Repeat sensitivity and specificity testing		
Task 2.6	Investigate drug delivery options		
Task 3	Perform *in vivo* efficacy and dosing optimization studies, and characterize the effect of ShigActive on the intestinal microbiota of mice	04/11/12	10/10/12
Task 3.1	Characterize and compare ShigActive with ampicililn in mice		
Task 3.2	Determine the MED and time and frequency of administration to reduce *Shigella* colonization in mice		
Task 3.3	Administer toxicity studies		
Task 3.4	Process specimen for microbiota analysis		
Task 3.5	Repeat sensitivity and specificity testing		
Task 4	Prepare an NNDI application package and submit it to the FDA	10/11/12	04/10/13
Task 4.1	Confirm that NNDI app is submitted >75 days before ShigActive is introduced for interstate commerce		
Task 4.2	Assemble supporting documents for application		
Task 4.3	Submit efficacy and safety data for publication		
Task 4.4	Begin IND application		
Task 4.5	Begin Phase I and Phase II trials		

3.2 Monophage characterization efforts

Section 2.3 recaps many of the characterization efforts used in investigating the candidate ShigActive™ monophages. RFLP and PFGE profiles illustrate the purity and homogeneity of the phages isolated and banked as monophage stocks. EM photos show the capsid and tail morphologies of the individual monophages. Additional characterization studies are underway.

Plague morphology was determined by plating 50-100 colonies of monophage culture in tog agar (0.7% suspension of agar-agar in LB medium) onto a lawn of SH.s43 on LB plates. The figure and table below depict and describe the plaque morphologies of five monophages.

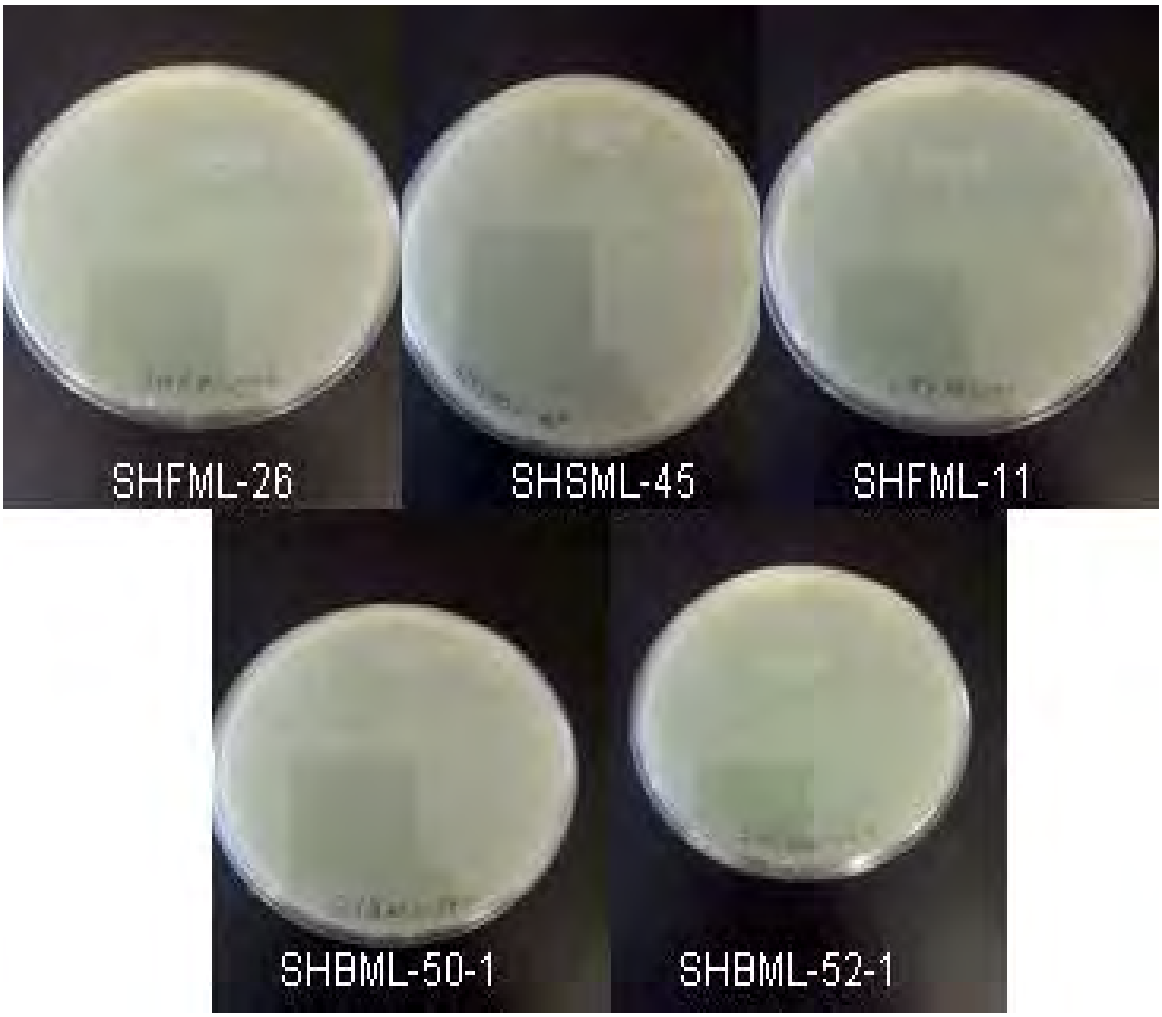


Figure 7. Plaque morphologies of SHFML-26, SHSML-45, SHFML-11, SHBML-50-1, and SHSML-52-1.

Table 7. Description of plaque morphologies

Phage	Size	Shape	Appearance	Uniformity
SHFML-26	Small-medium	Round, entire	Clear, no surround	Uniform to type
SHSML-45	Medium-large	Round, entire	Clear, no surround	Uniform to type
SHFML-11	Small-medium	Round, entire	Clear, no surround	Uniform to type
SHBML-50-1	Medium	Round, entire	Clear, no surround	Uniform to type
SHBML-52-1	Large	Round, entire	Clear, no surround	Uniform to type

The SDS-Page profile of the five *Shigella* monophages is displayed below in Figure 8. This analysis is an additional test for homogeneity along with PFGE and RFLP. It shows the protein pattern of each monophage along with a marker to determine the sizes. Each monophage has a unique identity profile as shown in the figure.

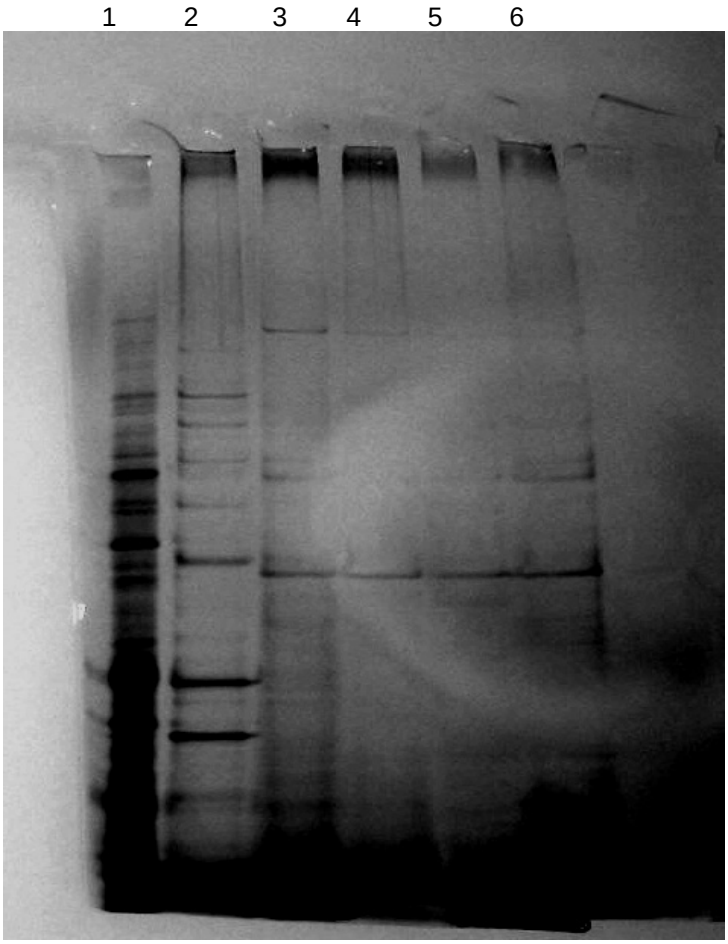


Figure 8. SDS-Page of *Shigella* monophages
Lanes: 1. Mark 12 Standard; 2. SHSML-45; 3. SHFML-26; 4. SHFML-11; 5. SHSML-52-1; 6. SHBML-50-1

3.3 Nucleotide sequencing and bioinformatics

Twelve anti-*Shigella* phages were sent to SeqWright DNA Technology Services (Houston, TX) for *de novo* sequencing. Preliminary results are as follows:

Table 8. Summary of sequencing results

Phage	A260/280 ratio	Read number	Total base	Average read length
SHFML-6	1.85	109,697	44,627,165	407
SHFML-20	1.90	208,379	86,089,087	413
SHFML-31	1.88	99,368	41,745,769	420
SHFML-45	1.85	91,590	38,081,000	416
SHSML-36	1.80	89,800	37,071,501	413
SHFML-11	1.85	31,572	12,584,225	399
SHBML-50-1	1.82	82,155	32,247,232	393
SHBML-50-2	1.81	132,022	53,377,302	404
SHSML-52-1	1.87	52,019	20,901,667	402
SHSML-52-2	1.85	93,017	37,206,962	400
SHFML-26	1.83	72,889	29,552,010	405
SHFML-21	1.80	44,288	17,075,203	386

Complete analysis is pending and will include number of contigs, number of bases assembled, largest contig size, and guanine-cytosine content percentage.

The sequencing of 12 *Shigella* phages was completed in August 2011. DNA from the samples were used to perform 454 sequencing (see Table 10), and the raw sequences were assembled with the Roche Newbler software (see Table 8).

Total gap length was estimated with Poisson statistics as a function of fold-coverage. As each phage had a very high fold-coverage, it is highly likely, if not guaranteed, that no gene was unsequenced (see Table 9).

Candidate ORF sequences were taken from the contig files using coordinates defined by the NCBI ORF finder and analyzed by NCBI BLASTX. The numbers of ORFs identified are listed in Table 9.

Table 9. Summary of assembly results

Phage	Number of contigs	Number of bases assembled	Largest contig size	GC%	Total gap length	ORFs identified
SHFML-6	42	92,298	50,218	43.93	5.95E-60	930
SHFML-20	23	69,124	40,320	44.71	0	736
SHFML-31	6	39,883	12,911	50.32	0	385
SHFML-45	24	290,434	108,050	40.03	3.08E-52	2704
SHSML-36	3	171,697	109,378	40.44	2.66E-89	1731
SHFML-11	1	170,429	170,429	35.24	1.35E-27	1505
SHBML-50-1	1	166,634	166,634	35.37	1.18E-79	1501
SHBML-50-2	12	172,540	99,952	40.96	9.6E-130	854
SHSML-52-1	106	215,227	25,809	37.60	1.37E-37	2139
SHSML-52-2	4	91,200	63,890	39.02	6E-173	792
SHFML-26	104	214,272	36,597	35.51	3.15E-55	2109
SHFML-21	11	179,562	73,411	38.72	8.08E-37	1714

Summary files from the BLASTX results were reviewed for sequences similar to those of undesirable genes listed in 40 CFR §725.421. Results of the searches were reviewed manually and yielded two possible ORF protein matches: gi|330858509 in SHFML-6 and SHFML-20 and gi|38707815 in SHSML-52-2. On further review, however, neither of the genes found has activities similar to the gene products listed in 40 CFR §725.421. The former is not only a protein common to phage but "integrally involved in their ability to release newly formed viral products", according to the sequencing report from SeqWright. The latter was previously curated by NCBI and is known to "use endolysins or muralytic enzymes (lysozyme) in conjunction with hollin, a small membrane protein, to degrade the peptidoglycan found in bacterial cell[s]". As quoted, neither of these proteins is an unusual or unexpected feature of a phage.

The primer pairs were designed to be unique to each phage based on ther genome sequences. Detailed information about the selected primers is included in Table 10.

Table 10. Summary of selected primers

Phage	Sequences	Primer length (bp)	Primer melting temperature (°C)	Product size
SHFML-6	62	18 – 23	57.8 – 62.7	100 - 499
SHFML-20	9	18 - 22	57.5 -62.7	100 - 496
SHFML-31	9	19 – 23	58.0 – 62.7	100 – 494
SHFML-45	9	20 - 25	57.3 – 60.8	100 - 500
SHSML-36	1	20	59.6 – 60.3	109 - 160
SHFML-11	4	20	59.1 – 60.7	104 – 482
SHBML-50-1	1	20 - 23	58.5 – 60.5	100 - 483
SHBML-50-2	99	18 – 23	57.1 – 61.8	100 – 491
SHSML-52-1	99	18 – 25	57.1 - 62.8	100 – 494
SHSML-52-2	9	20 – 22	58.5 – 60.9	100 - 475
SHFML-26	9	19 -22	59.2 – 61.0	100 – 420
SHFML-21	99	18 - 25	57.2 – 61.5	100 – 497

The primers were used to develop the "Identity Test" for *Shigella* phages; i.e., test that helps identify each *Shigella* phage specifically.

With a specific set of primers received from the contracted sequencing lab and a candidate formulation for ShigActive™, Intralytix operators were able to draft a PCR-based identity protocol. The method is captured in document number M016-31 and titled "ShigActive™ Identity Test – PCR based". The primers required for the test are listed below in Table 11.

Table 11. Primers for PCR based Identity Test

Phage	Primers 5' → 3'	Primer Sequence	Amplicon size
SHFML-11	SHFML-11 F	TCG CAA CGA TAT AAG GAC CC	116
	SHFML-11 R	CGC TTT GCA GCT TTA ATT CC	

Phage	Primers 5' → 3'	Primer Sequence	Amplicon size
SHFML-26	SHFML-26 F	TTG TGG CGA TGT TCA AGT GT	252
	SHFML-26 R	TGC GCA TAG CAC CGA TAA TA	
SHSML-45	SHSML-45 F	ACA AGG CTA GAA TGC GCC TA	232
	SHSML-45 R	AAT ACA GTTTCA CCG GAC GC	
SHBML-50-1	SHBML-50-1 F	TTG ACG CGA TTA TAA GGG TTG	103
	SHBML-50-1 R	ACC AAC TGA ACT ACG GCC AC	
SHSML-52-1	SHSML-52-1 F	GGA GAC TTC GGT CTC CCA TT	115
	SHSML-52-1 R	CAA AGA CGG TTC TCC AGC AT	

3.4 Candidate formulation

We have identified a tentative candidate phage preparation composition based on the 17 well-defined phages in our collection. Intralytix has developed proprietary software (the PhageSelector™ program) that facilitates designing optimally effective phage preparations. The program (designed by Intralytix's Bradley Anderson) uses various algorithms to analyze the database of our bacterial strains in conjunction with phage sensitivity and other data, in order to suggest which phages should be included in the preparations. For example, the program can examine the data for phage potency variation; i.e., it determines the total number of bacterial strains each phage is effective against. This method promotes redundancy by identifying phages that have high-kill counts and, therefore, are most likely to lyse the same bacterial strains lysed by other high-kill count phages. Also, the program can perform analyses to determine the bacterial lyse efficiency, which orders the candidate phages according to their ability to lyse the maximum number of bacterial strains in the bacterial database. That is to say, it identifies phages that are most diverse in their lytic activity against the strains in the bacterial database. A snapshot of the basic analysis performed by the PhageSelector program on the 17 *Shigella* monophages is shown Table 12. As shown on the table ("*Sorted by Efficiency*" section), we can lyse 100% of the 52 *Shigella* strains in our collection by including seven bacteriophages in the ShigActive™ preparation.

Table 12. Snapshot of the basic Phage Selector™ analysis of *Shigella* phages

Sorted by kills

Index	Phage Name	Kills	Resisted by	# Untested	Unique Kills	Cocktail Kills	% killed
1	SHFML-26	26	26	0	26	26	50%
2	SHFML-21	22	30	0	4	30	58%
3	SHFML-11	21	31	0	1	31	60%
4	SHSML-45	20	32	0	0	31	60%
5	SHSML-52-1	20	32	0	9	40	77%
6	SHBML-50-2	19	33	0	7	47	90%
7	SHBML-50-1	19	33	0	1	48	92%
8	SHSML-52-2	19	33	0	0	48	92%
9	SHFML-6	16	36	0	0	48	92%
10	SHFML-20	16	36	0	0	48	92%
11	SHSML-36	15	37	0	1	49	94%
12	SHFML-31	11	41	0	3	52	100%
13	SHSML-46	10	42	0	0	52	100%
14	SHFML-27	5	47	0	0	52	100%
15	SHFML-35	4	48	0	0	52	100%
16	SHDML-4	1	51	0	0	52	100%
17	SHBML-47	1	51	0	0	52	100%

Sorted by efficiency

Index	Phage Name	Kills	Resisted by	# Untested	Unique Kills	Cocktail Kills	% killed
1	SHFML-26	26	26	0	26	26	50%
2	SHBML-50-2	19	33	0	13	39	75%
3	SHSML-52-1	20	32	0	6	45	87%
4	SHFML-31	11	41	0	3	48	92%
5	SHFML-21	22	30	0	2	50	96%
6	SHBML-50-1	19	33	0	1	51	98%
7	SHSML-36	15	37	0	1	52	100%
8	SHBML-47	1	51	0	0	52	100%
9	SHDML-4	1	51	0	0	52	100%
10	SHFML-35	4	48	0	0	52	100%
11	SHFML-27	5	47	0	0	52	100%
12	SHSML-46	10	42	0	0	52	100%
13	SHFML-20	16	36	0	0	52	100%
14	SHFML-6	16	36	0	0	52	100%
15	SHSML-52-2	19	33	0	0	52	100%
16	SHSML-45	20	32	0	0	52	100%
17	SHFML-11	21	31	0	0	52	100%

Another option for the cocktail is to use a somewhat scaled-down version of it (containing 5 phages instead of 7). Such cocktail still kills 48 of the 52 *Shigella* strains in our collection (ca. 92% target range) – and the majority of non-lysed strains belong to

the *S. boydii* spp. known to be of reduced virulence. An advantage of the smaller cocktail is that all phages in it can be grown on a single host strain, SH.s43, which simplifies production and data assembly for regulatory agencies. A five phage alternative of ShigActive™, made of monophages with some known characterization details, is described below.

Table 13. Five phage candidate ShigActive™ formulation

Sorted by kills

Index	Phage Name	Kills	Resisted by	# Untested	Unique Kills	Cocktail Kills	% killed
1	SHFML-26	26	26	0	26	26	50%
2	SHFML-11	21	31	0	1	27	52%
3	SHSML-45	20	32	0	3	30	58%
4	SHSML-52-1	20	32	0	10	40	77%
5	SHBML-50-1	19	33	0	8	48	92%

Sorted by efficiency

Index	Phage Name	Kills	Resisted by	# Untested	Unique Kills	Cocktail Kills	% killed
1	**SHFML-26**	**26**	**26**	**0**	**26**	**26**	**50%**
2	**SHBML-50-1**	**19**	**33**	**0**	**13**	**39**	**75%**
3	**SHSML-52-1**	**20**	**32**	**0**	**6**	**45**	**87%**
4	**SHSML-45**	**20**	**32**	**0**	**2**	**47**	**90%**
5	**SHFML-11**	**21**	**31**	**0**	**1**	**48**	**92%**

Upon completion of sequencing and screening studies, the formulation depicted in Table 13 is the final formulation of the candidate ShigActive™ preparation.

3.5 Susceptibility screening

Seventeen phages were used to perform screening of our collection of *Shigella* strains. Results are summarized in Table 5 and Table 14 with treatment at $2x10^4$ PFU/mL in the former and $1x10^9$ PFU/mL in the latter. The final column in each table illustrates the screening results for a candidate ShigActive™ cocktail made of SHFML-26, SHSML-45, SHFML-11, SHBML-50-1, and SHSML-52-1. All screening was conducted on host strain SH.s43.

Table 14. Susceptibility of Shigella strains to bacteriophages at $1x10^9$ PFU/mL

#	Itx ID	Provider	Species	SHFML-6	SHFML-35	SHFML-20	SHFML-31	SHFML-26	SHFML-27	SHSML-36	SHFML-21	SHDML-4	SHFML-11	SHSML-45	SHSML-46	SHBML-47	SHBML-50-1	SHBML-50-2	SHSML-52-1	SHSML-52-2	ShigActive
1	SH.d 1	Univ.of Maryland-chile	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+
2	SH.d 2	Univ.of Maryland-chile	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+
3	SH.d 3	Univ.of Maryland-chile	dysenteriae	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+
4	SH.d 4	Univ.of Maryland-pakistan	dysenteriae	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	+	+
5	SH.d 5	Univ.of Maryland-peru	dysenteriae	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	+	-	+
6	SH.f 6	Univ.of Maryland-peru	flexneri	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	+	+	+
7	SH.f 7	Univ.of Maryland-peru	flexneri	+	-	+	+	-	-	-	+	-	+	+	-	-	-	-	+	+	+
8	SH.f 8	Univ.of Maryland-peru	flexneri	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
9	SH.f 9	Univ.of Maryland-peru	flexneri	+	-	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	+
10	SH.f 10	Univ.of Maryland-peru	flexneri	+	-	+	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+
11	SH.f 11	Univ.of Maryland-pakistan	flexneri 1	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
12	SH.f 12	Univ.of Maryland-pakistan	flexneri 1	-	-	-	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+
13	SH.f 13	Univ.of Maryland-chile	flexneri 1a	+	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+
14	SH.f 14	Univ.of Maryland-chile	flexneri 1a	+	-	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+
15	SH.f 15	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
16	SH.f 16	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
17	SH.f 17	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
18	SH.f 18	Univ.of Maryland-chile	flexneri 1b	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+
19	SH.f 19	Univ.of Maryland-mali	flexneri 1b	+	-	+	+	+	-	+	+	-	-	+	+	-	-	-	+	-	+
20	SH.f 20	Univ.of Maryland-pakistan	flexneri 2	+	+	+	+	+	-	-	+	-	+	+	-	-	-	-	-	+	+
21	SH.f 21	Univ.of Maryland-pakistan	flexneri 2	+	+	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	+
22	SH.f 22	Univ.of Maryland-pakistan	flexneri 2	+	-	+	+	+	-	-	+	-	+	+	-	-	-	-	-	+	+
23	SH.f 23	Univ.of Maryland-chile	flexneri 2a	+	-	+	+	+	-	-	+	-	+	+	-	-	-	-	-	+	+
24	SH.f 24	Univ.of Maryland-chile	flexneri 2a	+	-	+	+	+	-	-	+	-	+	+	-	-	-	-	-	+	+
25	SH.f 25	Univ.of Maryland-chile	flexneri 2a	+	-	+	+	+	-	+	+	-	+	+	-	-	-	-	-	+	+
26	SH.f 26	Univ.of Maryland-mali	flexneri 2b	+	-	+	+	+	-	-	+	-	-	+	+	-	-	-	+	+	+
27	SH.f 27	Univ.of Maryland-pakistan	flexneri 3	+	-	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+

#	Itx ID	Provider	Species	SHFML-6	SHFML-35	SHFML-20	SHFML-31	SHFML-26	SHFML-27	SHSML-36	SHFML-21	SHDML-4	SHFML-11	SHSML-45	SHSML-46	SHBML-47	SHBML-50-1	SHBML-50-2	SHSML-52-1	SHSML-52-2	ShigActive
28	SH.f 28	Univ.of Maryland-pakistan	flexneri 4	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	+
29	SH.f 29	Univ.of Maryland-pakistan	flexneri 4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	SH.f 30	Univ.of Maryland-pakistan	flexneri 5	-	-	+	-	+	-	+	+	-	+	+	-	-	-	-	-	-	+
31	SH.f 31	Univ.of Maryland-chile	flexneri 6	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	SH.f 32	Univ.of Maryland-chile	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
33	SH.f 33	Univ.of Maryland-chile	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
34	SH.f 34	Univ.of Maryland-pakistan	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
35	SH.f 35	Univ.of Maryland-pakistan	flexneri 6	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
36	SH.s 36	Univ.of Maryland-chile	sonnei	-	-	-	-	+	-	+	-	-	-	-	-	-	+	+	+	+	+
37	SH.s 37	Univ.of Maryland-chile	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
38	SH.s 38	Univ.of Maryland-peru	sonnei	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
39	SH.s 39	Univ.of Maryland-peru	sonnei	-	-	-	-	+	-	+	-	-	+	-	+	-	+	+	+	+	+
40	SH.s 40	Univ.of Maryland-peru	sonnei	-	-	-	-	+	-	+	-	-	+	-	+	-	+	+	+	+	+
41	SH.s 41	Univ.of Maryland-peru	sonnei	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+
42	SH.s 42	Univ.of Maryland-pakistan	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
43	SH.s 43	Univ.of Maryland-pakistan	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
44	SH.s 44	Univ.of Maryland-mali	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
45	SH.s 45	Univ.of Maryland-mali	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
46	SH.s 46	Univ.of Maryland-mali	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
47	SH.b 47	Univ.of Maryland-chile	boydii	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+
48	SH.b 48	Univ.of Maryland-chile	boydii	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
49	SH.b 49	Univ.of Maryland-chile	boydii	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
50	SH.b 50	Univ.of Maryland-pakistan	boydii	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
51	SH.f 51	Univ.of Maryland-Tokyo	flexneri 2a	+	-	+	+	+	-	+	+	-	+	+	+	-	-	-	-	+	+
52	SH.s 52	ATCC	sonnei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
53	SH.s 53	SH.s43 Nal^R25 intrlytix	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+
54	SH.s 54	SH.s43 Nal^R50 intrlytix	sonnei	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+

For specificity screenings, the candidate formulation was prepared in concentrations of $1x10^4$ PFU/mL, $1x10^7$ PFU/mL, and $1x10^9$ PFU/mL to determine whether the phages would lyse strains of non-*Shigella* species. Lawns of each bacterial strain are prepared and infected with droplets of the candidate ShigActive formulation at various concentrations, similar to the method used for identity testing. Results for seven other non-*Shigella* species are shown below in Table 15. Positive results indicate a clearly visible zone of lysis and are captured with a plus sign, +. Negative results indicate no sign of lysis and are recorded by '-'.

Table 15. Specificity testing for ShigActive™

				ShigActive™		
Host strain	Stain morphology	Serotype	Intralytix ID	$1x10^4$ PFU/mL	$1x10^7$ PFU/mL	$1x10^9$ PFU/mL
Shigella	Gram-negative bacilli	dysenteriae	SH.d 1	+	+	+
Shigella	Gram-negative bacilli	flexneri	SH.f 6	+	+	+
Shigella	Gram-negative bacilli	flexneri	SH.f 20	+	+	+
Shigella	Gram-negative bacilli	sonnei	SH.s 43	+	+	+
Shigella	Gram-negative bacilli	sonnei	SH.s 52	+	+	+
Salmonella	Gram-negative bacilli	Typhimurium	S.T567	+	+	+
Salmonella	Gram-negative bacilli	Heidelberg	S.He899	+	+	+
Salmonella	Gram-negative bacilli	Enteritidis	S.E566	+	+	+
Salmonella	Gram-negative bacilli	Typhimurium	S.T563	+	+	+
Salmonella	Gram-negative bacilli	Hadar	S.H568	+	+	+
E. coli	Gram-negative bacilli	O157:H7	Ec 147	+	+	+
E. coli	Gram-negative bacilli	O78:H11	Ec 148	-	+	+
E. coli	Gram-negative bacilli	O157:H7	Ec 150	-	+	+
E. coli	Gram-negative bacilli	undetermined	Ec 154	+	+	+
E. coli	Gram-negative bacilli	undetermined	Ec 155	+	+	+
Listeria	Gram-positive bacilli	monocytogenes	Lm 314	-	-	-
Listeria	Gram-positive bacilli	monocytogenes	Lm 315	-	-	-
Listeria	Gram-positive bacilli	innocua	L. innocua 316	-	-	-
Listeria	Gram-positive bacilli	monocytogenes	Lm 317	-	-	-
Listeria	Gram-positive bacilli	innocua	L. innocua 318	-	-	-
Staphyloccus	Gram-positive cocci	aureus	SA-36	-	-	-
Staphyloccus	Gram-positive cocci	aureus	SA-37	-	-	-
Staphyloccus	Gram-positive cocci	aureus	SA-211	-	-	-
Staphyloccus	Gram-positive cocci	aureus	SA-298	-	-	-
Staphyloccus	Gram-positive cocci	aureus	SA-299	-	-	-
Enterococcus	Gram-positive cocci	undetermined	E102	-	-	-
Enterococcus	Gram-positive cocci	faecalis	E103	-	-	-
Enterococcus	Gram-positive cocci	faecalis	E104	-	-	-
Enterococcus	Gram-positive cocci	avium	E105	-	-	-
Enterococcus	Gram-positive cocci	Faecalis	E106	-	-	-
Acinetobacter	Gram-negative bacilli	baumannii	*Ab* 3	-	-	-
Acinetobacter	Gram-negative bacilli	baumannii	*Ab* 4	-	-	-
Acinetobacter	Gram-negative bacilli	baumannii	*Ab* 5	-	-	-
Acinetobacter	Gram-negative bacilli	baumannii	*Ab* 6	-	-	-
Acinetobacter	Gram-negative bacilli	baumannii	*Ab* 7	-	-	-
Pseudomonas	Gram-negative bacilli	aeruginosa	Pa76	-	-	-
Pseudomonas	Gram-negative bacilli	aeruginosa	Pa161	-	-	-
Pseudomonas	Gram-negative bacilli	aeruginosa	Pa162	-	-	-
Pseudomonas	Gram-negative bacilli	aeruginosa	Pa163	-	-	-
Pseudomonas	Gram-negative bacilli	aeruginosa	Pa164	-	-	-

The results of the specificity tests confirm the host range of ShigActive. They indicate that the candidate ShigActive™ formulation is effective against at least three serotypes of *Shigella* and is lytic at higher concentrations against some other Gram-negative species. This is observed with some bacteriophages, particularly those targeting *E. coli* and closely related spp; a particular phage may lyse bacteria of different – but closely related - species while not lysing some strains of the same species. The susceptibility studies described in Section 3.5 indicate a broad range screening of phages against *Shigella* strains from the four most prevalent serogroups.

As an additional screening measure, the antibiotic susceptibility of the host strain – SH.s43 - was determined. Information obtained from the CDC and other medical reference sites provided a list of antibiotics commonly prescribed for *Shigella* infections. Table 16 describes the results of the SH.s43 antibiotic screening study. Antibiotic susceptibility of a candidate host strain is a precautionary measure often required by regulatory agencies. The screening denotes possible treatment options in case of unexpected infection of laboratory personnel or contamination of a production facility.

Table 16. Antibiotic susceptibility of SH.s43

Antibiotic	Zone diameter	Response
Azithromycin	10 mm	Resistant
Ceftriaxone	30 mm	Susceptible
Ciprofloxacin	28 mm	Susceptible
Levofloxacin	26 mm	Susceptible
Sulfamethoxazole trimethoprim	18 mm	Susceptible

3.6 ShigActive™ optimization

The candidate formulation of ShigActive™ will be prepared as five separate monophages grown to optimal titers and blended before aseptic transfer and final packaging. Optimization efforts include but are not limited to studies of infection stage and OD, MOI (multiplicity of infection), harvest age, and growth media.

Before monophage production can be optimized, the growth kinetics of the host strain must be explored. Bacterial cultures grow in three phases – lag, linear, and latent – that result in a unique growth curve. Viability, or concentration of living viable cells, differs at each point on the growth curve. Figure 9 shows the logarithmic growth curve
of SH.s43. An overnight culture was grown to an OD_{600} 1.74. A shake flask of 75mL growth media was inoculated with 0.375mL of this overnight culture and monitored at 30 minute intervals.

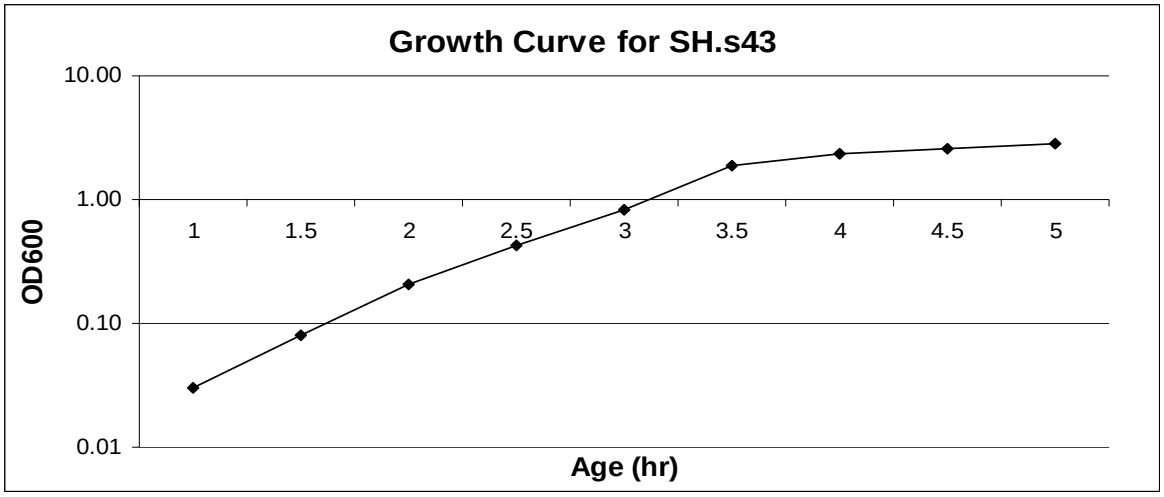


Figure 9. Growth curve of *Shigella* host SH.s43.

Samples of this monitored growth culture were tested for viability. Table 17 shows the results of this testing. Viable counts corresponding to OD are critical for subsequent optimization trials, particularly the MOI studies. The values will become constants in the MOI calculator for all bench scale and 10L optimization studies.

Table 17. Viability at growth phages of SH.s43

OD_{600}	Viability (CFU/mL)
0.08	2.35E+07
0.831	5.40E+08
2.60	1.32E+09

The first round of optimization studies explored potential infection OD. The growth curve in Figure 9 shows the latent, linear and lag growth phases for SH.s43. Just as viability differs at each phase, the titers do as well (see Table 18).

Table 18. ShigActive™ Optimization Study 1

Phage	Sample	Titer	Titer Increase (logs)*
SHFML-11	Parent	1.30E+08	-
	Latent	1.63E+10	2.10
	Linear	5.90E+10	2.66
	Lag	2.36E+11	3.26
SHFML-26	Parent	1.30E+07	-
	Latent	2.62E+10	3.30
	Linear	3.19E+10	3.39
	Lag	1.01E+11	3.89
SHSML-45	Parent	7.00E+06	-
	Latent	1.37E+10	3.29
	Linear	2.88E+10	3.61
	Lag	1.18E+10	3.23

Phage	Sample	Titer	Titer Increase (logs)*
SHBML-50-1	Parent	4.00E+06	-
	Latent	3.30E+08	1.92
	Linear	4.10E+10	4.01
	Lag	5.00E+09	3.10
SHSML-52-1	Parent	1.60E+08	-
	Latent	3.70E+10	2.36
	Linear	4.50E+10	2.45
	Lag	6.10E+10	2.58

* Increase in logs from parent lot

All prepared samples showed at least 2-log increases but linear and lag phases consistently showed the highest increases in titers from the parent lot for each monophage. However, infecting the host at later stages of growth - higher ODs – increases the time for producing harvest material and also increases the final OD of this harvest material. Even with high titers, harvest material with high optical density can be more difficult to clarify rendering the phage unavailable.

A second optimization study was administered at four different points of the latent growth phase. Host cultures were grown to OD 0.05, 0.1, 0.2, and 0.4 before infections with phage. Table 19 shows the results of this second optimization study for ShigActive™.

Table 19. ShigActive™ Optimization Study 2

Phage	Sample	Titer	Titer Increase (logs)*
SHFML-11	OD 0.05	8.40E+09	1.81
	OD 0.1	2.16E+10	2.22
	OD 0.2	3.30E+10	2.40
	OD 0.4	3.80E+10	2.47
SHFML-26	OD 0.05	6.50E+09	2.70
	OD 0.1	1.01E+10	2.89
	OD 0.2	1.19E+10	2.96
	OD 0.4	4.30E+10	3.52
SHSML-45	OD 0.05	4.40E+09	2.80
	OD 0.1	5.60E+09	2.90
	OD 0.2	1.02E+10	3.16
	OD 0.4	2.09E+10	3.48
SHBML-50-1	OD 0.05	1.71E+09	2.63
	OD 0.1	1.75E+08	1.64
	OD 0.2	9.70E+08	2.38
	OD 0.4	4.00E+08	2.00
SHSML-52-1	OD 0.05	1.60E+10	2.00
	OD 0.1	2.13E+10	2.12
	OD 0.2	1.48E+10	1.97
	OD 0.4	9.50E+09	1.77

* Increase in logs from parent lot in Table 18

In nearly all cases, the titer increases from these latent phase infections were lower than the increases in the linear and lag phases for the same monophages. Subsequent experiments will likely be performed with host cultures in linear and lag phases. Scale up activities to 10L batches and eventually 1000L batches may involve revisiting infection OD studies.

The third optimization study explored four different MOIs for each *Shigella* phage. Host cultures were grown to the optimal ODs (as determined from ShigActive™ optimization study 2) before being infected with phage at four different MOIs. The range of MOIs was chosen from the conditions in studies 1 and 2 that yielded highest titers. Table 20 shows the results of the third optimization study for ShigActive™.

Table 20. ShigActive™ Optimization Study 3

Phage	Sample	Titer	Titer Increase (logs)*
SHFML-11	MOI 0.001	1.90E+11	3.16
	MOI 0.005	2.30E+11	3.25
	MOI 0.01	2.89E+10	2.35
	MOI 0.05	2.26E+10	2.24
SHFML-26	MOI 0.0005	1.40E+10	3.03
	MOI 0.001	2.18E+10	3.22
	MOI 0.005	2.30E+11	4.25
	MOI 0.01	3.30E+11	4.40
SHSML-45	MOI 0.0005	1.80E+10	3.41
	MOI 0.001	1.37E+11	4.29
	MOI 0.005	3.50E+10	3.70
	MOI 0.01	3.90E+10	3.75
SHBML-50-1	MOI 0.001	1.40E+10	3.54
	MOI 0.005	2.90E+09	2.86
	MOI 0.01	1.40E+09	2.54
	MOI 0.05	9.00E+08	2.35
SHSML-52-1	MOI 0.0001	2.30E+10	3.76
	MOI 0.0005	3.20E+10	3.90
	MOI 0.001	3.00E+11	4.88
	MOI 0.005	4.00E+10	4.00

* Increase in logs from parent lot in Table 18

An in-process goal of the optimization studies is to reach titers of at least 1.0E+10 at bench-scale before scaling the process to 500mL volumes using the WAVE bioreactor. Four of the five ShigActive™ monophages have successfully met and exceeded this mark by more than one log; however, SHBML-50-1 has not yielded titers as high as the others. Since, the titer of the SHBML-50-1 parent lot is considerably lower than the other titers of the other monophages, an additional MOI optimization study (Study 3) was performed to evaluate four additional MOIs for that particular monophage. Results for the continuation of ShigActive™ Optimization Study 3 part 2 are shown in Table 21.

Table 21. ShigActive™ Optimization Study 3, Part 2

Phage	Sample	Titer	Titer Increase (logs)*
SHBML-50-1	MOI 1E-03	1.7E+10	3.63
	MOI 5E-03	2.6E+09	2.81
	MOI 1E-04	1.2E+09	2.48
	MOI 5E-04	2.5E+09	2.80
	MOI 1E-05	9.0E+09	3.35
	MOI 5E-05	3.0E+09	1.88
	MOI 1E-06	9.0E+09	2.35
	MOI 5E-06	2.6E+09	2.81

* Increase in logs from parent lot in Table 18

ShigActive™ Optimization Study 3 Part 2 evaluated MOIs that repeated the two most successful outcomes of the previous study while accessing others that were lower. The repeat samples were nearly identical in yield to those recovered beforehand but the lower MOIs produced lower titers. At this point, starting material must be made for ShigActive™ proof-of-concept evaluation using the parameters illustrated in Table 22. Further optimization will be explored in scale-up to the WAVE bioreactor.

Table 22. Optimal Production Parameters for ShigActive™

Phage	Inoculum ratio	Target Infection OD_{600}	MOI	Target Harvest OD_{600}	Expected Harvest Titer
SHFML-11	0.04	2.00	1.00E-02	0.269	2.36E+11
SHFML-26	0.04	2.00	1.00E-02	0.240	3.30E+11
SHSML-45	0.04	2.00	1.00E-03	0.051	1.40E+11
SHBML-50-1	0.04	1.00	1.00E-03	0.167	4.0E+10
SHSML-52-1	0.04	2.00	1.00E-02	0.039	3.0E+11

Using the optimization parameters in Table 22, working stocks of the monophages were produced. Each was produced in volumes of 75 mL and clarified with a Nalgene 0.22 µm filter. None required concentration or diafiltration with a wash buffer. The assigned lot numbers and titers are displayed below in Table 23.

Table 23. Working stocks of ShigActive™ monophages

Phage	Harvest OD_{600}	Titer, Nanosight PFU/ml	Particle size, Nanosight	Titer, QC PFU/ml	Lot Number
SHFML-11	0.667	2.03E+11	128	5E+10	020712SHFML11A
SHFML-26	0.423	3.19E+11	139	6E+10	020712SHFML26A
SHSML-45	0.058	1.57E+11	138	2E+10	020712SHSML45A
SHBML-50-1	0.052	1.57E+10	95	2E+08	020712SHBML50-1A
SHSML-52-1	0.042	2.61E+11	139	5E+10	020712SHSML52-1A

The SHBML-50-1 lot, 020712SHBML50-1A, cannot be used as a working stock for production due to its low titer but can be used to produce a working stock. It requires further optimization or production in larger volumes to be concentrated. The

remaining four lots passed all qualifying assays, particularly bacterial sterility, minimum phage titer, plaque homogeneity, and RFLP profile. Each can be used in subsequent production efforts.

Additional studies were administered with monophage SHBML-50-1 that explored various inoculum ODs, inoculum volumes, and MOIs. Table 24 displays these results along with the conditions used for each. Again, the low yields suggest that none of these particular combinations are the optimal production parameters for SHBML-50-1. They also suggest that SHBML-50-1 may be an especially fastidious phage as very small differences in operations can result in great differences in yield. For further optimization efforts, only the parameters that appear to give the most reproducible outcome will be considered for further evaluation.

Table 24. ShigActive™ Optimization Study 3, Part 3

Inoc OD	Inoc volume	MOI	Harvest OD	Titer, PD plate assay
1.10	1.5	1.0E-03	0.022	2.0E+08
1.10	1.5	5.0E-04	0.043	4.0E+08
1.10	3.0	1.0E-03	0.051	1.0E+08
1.10	3.0	5.0E-04	0.061	3.0E+08
1.10	4.5	1.0E-03	0.120	1.0E+09
1.10	4.5	5.0E-04	0.164	1.0E+09
1.50	1.5	1.0E-03	0.064	3.0E+08
1.50	1.5	5.0E-04	0.046	<1.0E+08
1.50	3.0	1.0E-03	0.070	2.0E+08
1.50	3.0	5.0E-04	0.173	4.0E+08
1.50	4.5	1.0E-03	0.296	1.2E+09
1.50	4.5	5.0E-04	0.132	2.0E+08

In order to produce a small aliquot of ShigActive™ for *in vivo* studies, the most reproducible parameters were used to prepare five liters of SHBML-50-1 that were then concentrated down to 250mL (a 20X concentration). Unfortunately, the concentrated solution only yielded 1.0E+09 PFU/ml and was unusable for a ShigActive™ cocktail.

An additional optimization study was performed with the intention of producing a high titer aliquot that would be used for a working stock as well as determining a benchmark for the optimal conditions in WAVE production. These results are displayed below in Table 25.

Table 25. ShigActive™ Optimization Study 3, Part 4

Flask	Inoc OD	Inoc volume	MOI	Harvest OD	Titer, PD plate assay
A	1.02	1.5	1.0E-02	0.048	1.0E+10
B	1.02	1.5	5.0E-02	0.050	1.8E+09
C	1.02	3.0	1.0E-02	0.141	9.5E+09
D	1.02	3.0	5.0E-02	0.080	5.8E+09
E	1.02	4.5	1.0E-02	0.307	2.7E+10

Flask	Inoc OD	Inoc volume	MOI	Harvest OD	Titer, PD plate assay
F	1.02	4.5	5.0E-02	0.124	4.0E+09
G	1.56	1.5	1.0E-02	0.079	6.9E+09
H	1.56	1.5	5.0E-02	0.034	5.7E+09
I	1.56	3.0	1.0E-02	0.188	6.0E+09
J	1.56	3.0	5.0E-02	0.145	1.8E+09
K	1.56	4.5	1.0E-02	0.456	6.0E+09
L	1.56	4.5	5.0E-02	0.151	3.7E+09

The titers determined by plate assay appear to be misleading and not reproducible. When tittered by QC personnel, the result for flask E was only 7.0E+08. The titer determined by nanoparticle analysis for the same sample was 1.46E+10. This result is typically 1.0 log higher than an accurate plate assay. Additional efforts must be taken to stabilize the sample before titration to determine a true titer as both PD and QC plate assay titers appear to be incorrect.

Optimization efforts continued with a study that determined if a lower temperature would improve yield of SHBML-50-1. Six of the most promising propagation parameters were selected for growth at both 37°C and 30°C. Titer was determined by nanoparticle analysis minus one log. Table 26 shows the results of that study.

Table 26. ShigActive™ Optimization Study 3, Part 5

Flask	Inoc OD	Inoc volume	MOI	Temperature	Harvest OD	Titer, adjusted Nanosight
A	1.46	1.5	5.0E-02	30	0.023	4.86E+08
B	1.46	3.0	1.0E-03	30	0.128	1.19E+09
C	1.46	3.0	5.0E-04	30	0.133	1.01E+09
D	1.46	3.0	1.0E-04	30	0.232	1.63E+09
E	1.46	3.0	5.0E-02	30	0.137	1.69E+09
F	1.46	4.5	5.0E-02	30	0.060	1.53E+09
G	1.46	1.5	5.0E-02	37	0.032	4.70E+08
H	1.46	3.0	1.0E-03	37	0.693	5.34E+08
I	1.46	3.0	5.0E-04	37	0.770	5.50E+08
J	1.46	3.0	1.0E-04	37	0.759	9.46E+08
K	1.46	3.0	5.0E-02	37	0.066	1.02E+09
L	1.46	4.5	5.0E-02	37	0.048	1.24E+09

At each metric, yields at 37°C were higher than those at 30°C. This study shows a lower temperature of 30°C is not an optimal growth condition for SHBML-50-1.

To produce material for *in vivo* testing, a 5000mL volume of SHBML-50-1 was prepared and concentrated down to 250mL. Five flasks of material were grown in 1L volumes. Each flask was inoculated with 60mL bacterial culture infected at an MOI of 1.0E-02 and grown for 4 hours. The initial harvest titer was 3.80E+09 PFU/mL and the final concentrated titer was 2.88E+10 PFU/mL. This concentrate was used to prepare ShigActive™ lot 1112D300146 for animal studies.

An additional optimization study was performed to determine the ranges of some of the previously more successful optimization attempts. These results are displayed below in Table 27.

Table 27. ShigActive™Optimization Study 3, Part 6

Flask	Inoc OD	Inoc volume	MOI	Harvest OD	Titer, adjusted Nanosight
A	1.79	1.5	1.0E-02	0.059	8.0E+09
B	1.79	1.5	5.0E-02	0.050	7.9E+09
C	0.552	3.0	1.0E-05	0.359	7.8E+10
D	1.79	3.0	1.0E-05	0.697	1.1E+11
E	1.79	3.0	5.0E-04	0.129	3.6E+09
F	1.79	3.0	1.0E-04	0.131	1.3E+10
G	1.79	3.0	5.0E-05	0.166	4.2E+10
H	1.79	3.0	1.0E-03	0.108	1.3E+10

This study showed general consistency and reproducibility with many of the higher MOIs but suggested that the lower MOIs required additional investigation. Those on the order of E-04 and E-05 yielded most of the highest titers.

Another optimization study was performed to explore lower MOIs and to compare a 4 hour harvest time to a 6 hour harvest time. These results are displayed below in Table 28.

Table 28. ShigActive™Optimization Study 3, Part 7

Flask	Inoc OD	Inoc volume	MOI	Harvest OD, 4 hr	Titer, adjusted Nanosight, 4 hr	Harvest OD, 6 hr	Titer, adjusted Nanosight, 6 hr
A	1.13	1.5	5.0E-03	0.040	9.3E+09	0.067	7.6E+09
B	1.55	1.5	5.0E-03	0.039	1.0E+10	0.071	8.5E+09
C	1.13	1.5	1.0E-04	0.071	1.5E+10	0.108	1.2E+10
D	1.55	1.5	1.0E-04	0.077	1.4E+10	0.092	1.3E+10
E	1.13	4.5	1.0E-04	0.304	1.7E+10	0.176	1.6E+10
F	1.55	4.5	1.0E-04	0.387	1.6E+10	0.210	1.8E+10
G	1.55	3.0	1.0E-04	0.262	1.5E+10	0.165	1.3E+10
H	1.55	3.0	1.0E-05	0.527	2.0E+10	0.588	2.9E+10

Like the previous study, Part 7 showed that MOIs on the order of E-04 and E-05 yielded most of the highest titers. However, not all of these results mimicked previous findings. These parameters and some of those close to them will be repeated for reproducibility. Additionally, titers at 4 hours post-infection varied very little from those at 6 hours post-infection. The additional two hours of incubation does not appear to optimize growth.

A final optimization study was performed to demonstrate reproducibility of the more successful parameters and to again compare a 4 hour harvest time to a 6 hour harvest time. These results are displayed below in Table 29.

Table 29. ShigActive™Optimization Study 3, Part 8

Flask	Inoc OD	Inoc volume	MOI	Harvest OD, 4 hr	Titer, adjusted Nanosight, 4 hr	Harvest OD, 6 hr	Titer, adjusted Nanosight, 6 hr
A	0.565	3.0	1.0E-05	0.376	8.7E+09	0.266	4.1E+10
B	0.678	4.5	1.0E-02	0.071	1.3E+10	0.103	1.4E+10
C	1.13	3.0	1.0E-05	0.647	5.5E+10	0.693	9.1E+10
D	1.58	3.0	1.0E-03	0.129	3.9E+10	0.101	3.1E+10
E	1.77	1.5	1.0E-02	0.047	9.1E+09	0.028	9.8E+09
F	1.77	1.5	5.0E-02	0.031	8.1E+09	0.064	6.4E+09
G	1.77	4.5	5.0E-02	0.055	4.2E+10	0.106	1.1E+10
H	1.77	3.0	1.0E-03	0.157	2.8E+10	0.115	4.1E+10
I	1.77	3.0	5.0E-02	0.069	1.1E+10	0.133	1.1E+10

This study confirmed some of the previous findings and yielded optimal parameters for large scale production. Monophage SHBML-50-1, along with all other ShigActive™ are optimized for scale-up activities.

Complications with the optimization of SHBML-50-1 prompted the exploration of additional efforts, particularly those that have been successful with other fastidious bacteriophages. Below are the optimized results using a second set of parameters for SHBML-50-1.

Table 30. ShigActive™Optimization Study 4

Inoc OD	Inoc Volume (mL)	Media Volume (mL)	MOI	Harvest OD	Titer (PFU/mL)
0.223	135	990	5.0E-07	0.464 - 0.554	1.0E+11 – 3.0E+11

The conditions used in these studies were vastly different than those used to optimize the other four monophages of ShigActive™. The medium used for these trials was NZCYM at half strength, i.e. prepared at a 1:1 dilution with sterile water. Also, the flasks were incubated at 26°C and 200 rpm for 16 - 17 hours instead of at 37°C and 200 rpm for 4 – 6 hours. These conditions may be revisited for production in the WAVE bioreactor if needed.

As each of the monophages yields in excess of 1.0E+10 PFU/mL at harvest, additional experiments to investigate ideal post-infection age and optimal growth medium are not necessary. The optimization studies will conclude with the preceding results unless successful technical transfer to the WAVE bioreactor requires further investigation.

3.7 Draft cGMP manufacturing protocol

Production of ShigActive™ under cGMP conditions was not stipulated by this Phase II project. For food safety applications, cGMP manufacturing is not required. It is also not required for Phase I and Phase II human clinical trials. However, it is required for eventual commercial production of ShigActive™ either as a drug or as a new dietary ingredient. Thus, efforts will be made to scale up the manufacturing in WAVE bioreactor, ideally suited for cGMP manufacturing of biologics. The diagram in Figure

10 provides an overall schematic of the process. ShigActive™ is prepared by cultivation of individual host *Shigella* strain/ phage combinations followed by filtration.

3.7.1 Production of component monophages

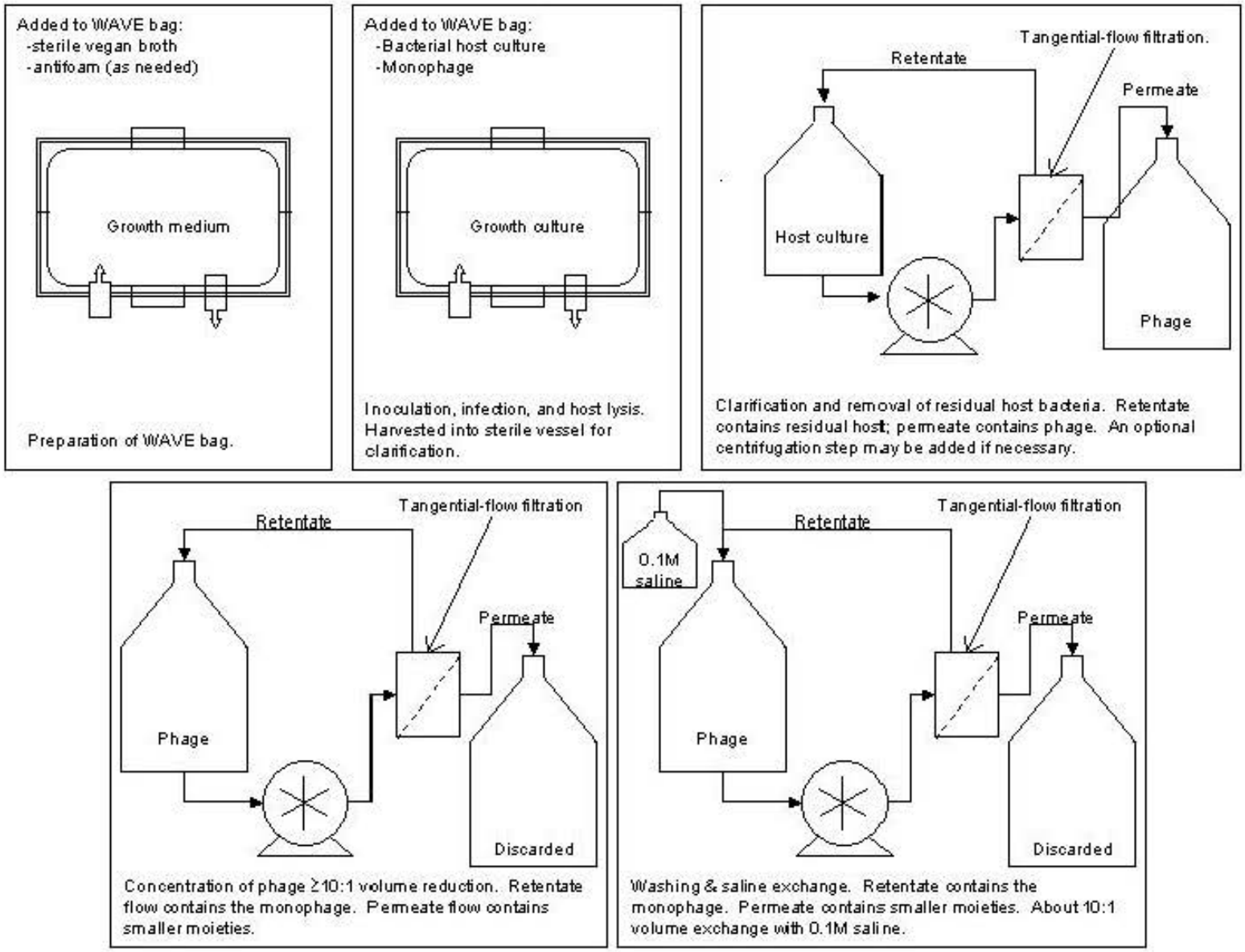
For small-scale production, shake flask batches of each monophage were produced in 2L flasks rotated at 200 rpm in an incubator-shaker. *Shigella* host strains were grown in a custom, animal-product-free NZCYM broth at 37°C to an OD_{600} of approximately 0.2 absorbance units. Cultures were then infected at a multiplicity of infection (MOI; the ratio of phage to bacteria) previously determined to be optimal for each phage-host strain combination. Growth was monitored visually (and verified by spectrophotometer) until lysis occurred (at lysis the culture "crashes" or drops rapidly in optical density). Specific OD information for infecting with phage and for harvesting phage and the MOI data for the five component monophages contained in ShigActive™ were determined in optimization studies and are shown below in Table 31.

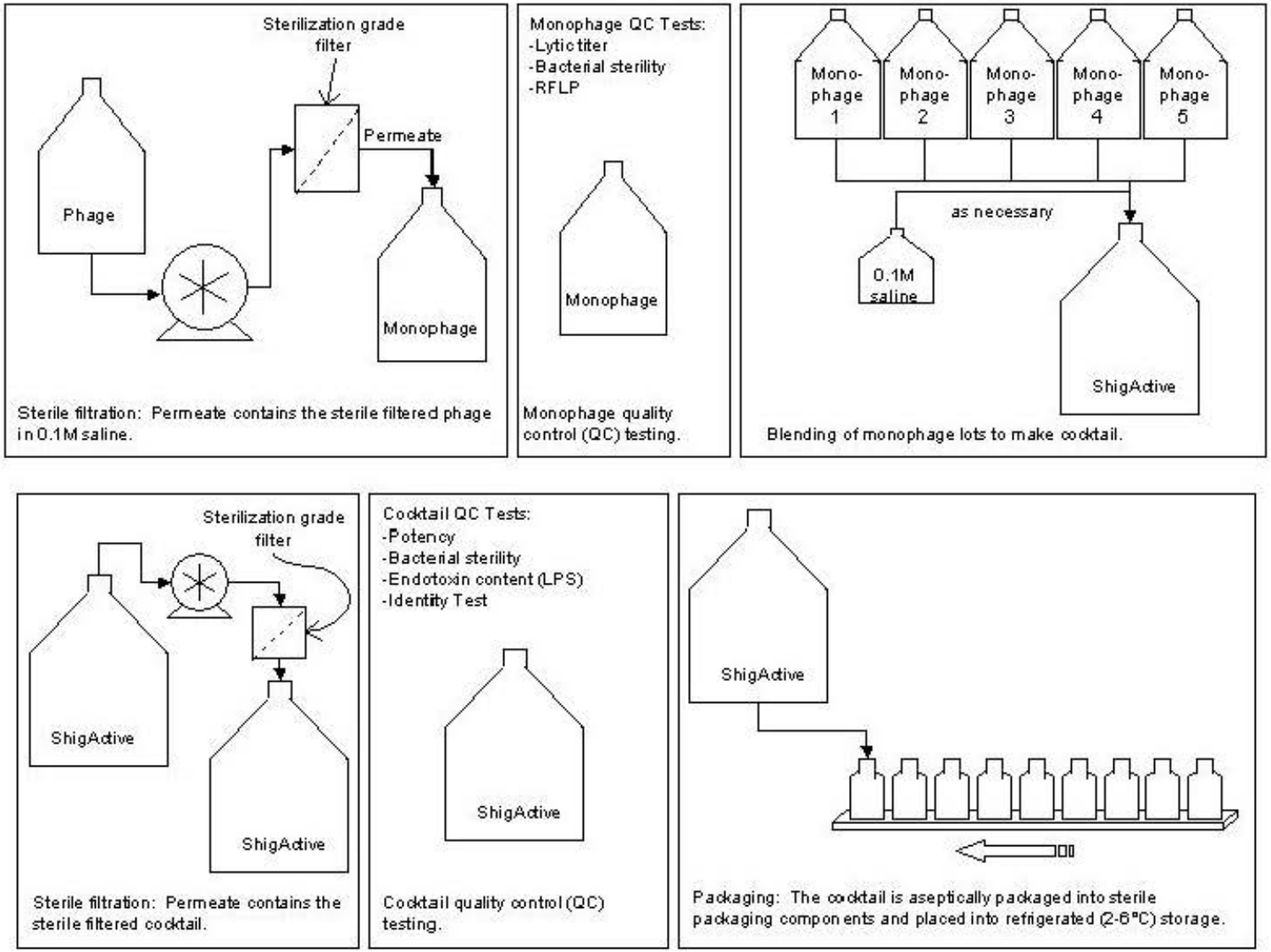
Table 31. Optical density information used in the production of ShigActive™

Phage	Host *Shigella* strain	Multiplicity of Infection (MOI)	OD_{600} for Infection	OD_{600} for Harvest
SHFML-11	SH.s43	0.01	2.0	0.15 – 0.25
SHFML-26	SH.s43	0.01	2.0	0.15 – 0.25
SHSML-45	SH.s43	0.001	2.0	0.05 – 0.15
SHBML-50-1	SH.s43	0.00001	1.0	0.5 – 0.7
SHSML-52-1	SH.s43	0.01	2.0	0.03 – 0.10

We anticipate scaling up our flasks studies into WAVE bioreactor, in 5-10L bags containing NZCYM broth. The medium will be supplemented with P2000 antifoam as needed, up to a maximum of 200 µl per 10L bag. The 10L bag will be inoculated with 50 - 500 ml of an infected bacterial seed culture, prepared as described in small-scale production. The dissolved oxygen and the pH maybe controlled if needed. Foaming can be controlled by addition of antifoam through an aseptic addition port. Growth will be monitored visually (and verified by spectrophotometer) until lysis occurs. The material will be then harvested for downstream processing.

Figure 10. Schematic of manufacturing process




Sterilization grade filter
Permeate
Phage
Monophage
Sterile filtration: Permeate contains the sterile filtered phage in 0.1M saline.
Monophage QC Tests:
-Lytic titer
-Bacterial sterility
-RFLP
Monophage
Monophage quality control (QC) testing.
Mono-phage 1
Mono-phage 2
Mono-phage 3
Mono-phage 4
Mono-phage 5
as necessary
0.1M saline
ShigActive
Blending of monophage lots to make cocktail.
Sterilization grade filter
ShigActive
ShigActive
Sterile filtration: Permeate contains the sterile filtered cocktail.
Cocktail QC Tests:
-Potency
-Bacterial sterility
-Endotoxin content (LPS)
-Identity Test
ShigActive
Cocktail quality control (QC) testing.
ShigActive
Packaging: The cocktail is aseptically packaged into sterile packaging components and placed into refrigerated (2-6°C) storage.

3.7.2 Clarification of monophage lysates

At the end production, i.e. when lysis is observed and the culture is believed to be at its optimal titer, harvest and downstream processing is initiated. First, the suspension is clarified by filtration for removal of bacterial. From small cultures (<5L), this is done by depth filtration using a filter unit with a transfer vessel, a sterile holding vessel, a 0.22 µm filter, and a port for vacuum aspiration. The lysate is pulled through the filter by vacuum and collected in the sterile bottle. For fermentor cultures, filtration is accomplished by tangential-flow filtration, a process in the fluid is pumped tangentially along the surface of the membrane with 0.22 µm filters. An applied pressure serves to force a portion of the fluid through the membrane to the permeate side. The particulates and macromolecules that are too large to pass through the membrane pores are retained on the upstream side. In the TFF system, the retained components do not build up at the surface of the membrane as they are swept along by the tangential flow.

The phage lysate is continuously passed across the surface of the membrane in TFF processing. Materials that pass through the membrane are referred to as the permeate. The materials that do not pass through the membrane are referred to as the retentate. The retentate is recycled to the feed reservoir to be re-filtered. For clarification of monophage lysates, the permeate – the medium that contains phage – is collected in a sterile vessel. The retentate – the medium that contains bacteria and lysis debris – is held in the harvest container and decontaminated.

3.7.3 Nuclease treatment

Bacterially derived nucleic acids in the solution are removed by nuclease treatment. For this, the volume of the collected permeate is determined and DNase I and RNase A are each added at a final concentration of 4 mg/L. The solution is continuously mixed and incubated at 25 ± 2°C for 60 minutes.

3.7.4 Concentration and diafiltration

Following enzyme treatment, the monophages are concentrated by up to 10 times their original volume using tangential-flow filtration with a 50-kDa filter. In the case of concentration, the permeate – containing water, enzymes, and media components – is discarded while the retentate is salvaged. Phages are then washed via buffer exchange with a sterile saline solution of 10 times the retentate volume. This diafiltration, which involves adding sterile saline at the same rate that the permeate is removed, washes the phage solution further removing media components so that the final solution consists of monophages suspended in saline.

3.7.5 Sterile filtration

Final sterile filtration of the concentrated phages is then carried out by depth filtration through a filter unit using a 0.2 µm cartridge filter. Sterile monophage solution is then sampled for quality testing and stored at 2-6°C until use in a cocktail.

3.7.6 Quality control test

Each monophage must pass thorough quality testing to be considered for inclusion in a cocktail batch. A production batch must meet each pre-determined specification of four QC assays to be assigned a determination of PASS.

Lytic titer is determined using the traditional plaque assay, sometimes by more than one operator, and in some cases is confirmed by an automated nanoparticle count. The specification for inclusion is a minimum of 1.0E+10 PFU/mL. If processing volumes are sufficient, monophage lots that fall below this metric can be further concentrated. Lots that meet or exceed this specification are stored in their initial post-processing state and diluted with sterile saline during cocktail formulation.

The bacterial sterility assay is administered to determine if there is any viable microbial contamination in the phage solution. The specification for this assay is less than 1 CFU/mL as a measure of 0 cannot be determined without exhausting the entire lot. Monophage lots that are above this metric can be sterile filtered and re-assayed. Lots that meet this specification are stored in their initial post-processing state.

Pulsed-field gel electrophoresis (PFGE) of uncut DNA is administered to confirm the identity of each monophage. The specification for this assay is homogeneity indicated by a single band on the gel. Monophage lots that do not meet this specification are discarded; the lot is contaminated. Lots that meet this specification are stored in their initial post-processing state.

Restriction fragment length polymorphism (RFLP) is also administered to confirm the identity of each monophage. The specification for this assay is identity to the pattern in a reference material, a sample known to be pure. Monophage lots that do not meet this specification are discarded; the lot is either contaminated or a different material. Lots that meet this specification are stored in their initial post-processing state.

Each monophage of a phage product is prepared as described in this section. When all component monophages of a product are prepared and pass quality control testing, they are combined aseptically and mixed for a single lot of phage material. This new cocktail lot is bottled, labeled, sampled as a single formulation for final quality testing, confirmed to have passed all testing, and shipped to a customer. All records of the cocktail lot from its host seed production to final quality testing are maintained electronically and archived in both logbooks and development reports.

3.8 WAVE Production

3.8.1 Determination of growth parameters

For development-scale production, WAVE batches of each monophage were produced in 2L bags at 37°C on the WAVE bioreactor 20/50EH platform. *Shigella* host strains were grown in shake flasks of a custom, animal-product-free NZCYM broth at 37°C to a target OD_{600}. Aliquots of the cultures were then infected at a previously determined MOI, allowed to incubate for 10 minutes at room temperature, and transferred to the batched WAVE bags. Growth was monitored visually (and verified by spectrophotometer) until lysis occurred while pH and dissolved oxygen (DO) were

recorded for observation. The results for SHFML-11 are shown below in Table 32. All experiments were conducted at 37°C and with 0.1L/minute aeration. No agents were added for pH or DO control.

Table 32. WAVE studies with phage SHFML-11

Run	Settings	Age	pH	DO (%)	OD	Titer
WAVE022513	32.0% speed Angle 5.5 Inoculum 40mL	0	6.66	84.4	-	2.6E+10 PFU/mL
		1	6.47	67.8	0.368	
		2	6.29	60.4	0.599	
		3	6.24	58.4	0.508	
		4	6.20	55.9	0.553	
WAVE022713	45.0% speed Angle 5.5 Inoculum 40mL	0	7.21	83.8	-	2.3E+10 PFU/mL
		1	7.00	76.0	0.427	
		2	6.81	70.6	0.660	
		3	6.71	68.1	0.604	
		4	6.74	66.3	0.653	
		5	6.71	64.3	0.695	
		5.5	6.69	62.7	0.736	
WAVE030413	45% speed Angle 5.5 Inoculum 20mL	0	7.34	86.1	-	1.2E+11 PFU/mL
		1	7.19	81.0	0.249	
		2	7.06	78.2	0.434	
		3	6.96	75.1	0.538	
		4	6.91	73.0	0.597	

While the first experiment seemed promising, it was repeated with a higher rocking speed because the host culture did not crash. In the second experiment with higher rocking speed, the optical density still did not substantially drop so a third trial was conducted with an inoculum of lower volume. This third experiment also failed to crash to any large extent but the yield of >1.0E+11 PFU/mL deemed the conditions acceptable. For the remaining four monophages, WAVE studies were administered with 20mL inoculums at 45% rocking speed and platform angle 5.5. Like the SHFML-11 study, all experiments were conducted at 37°C and with 0.1L/minute aeration. No agents were added for pH or DO control. See Table 33 for results of the other trials.

Table 33. WAVE studies for remaining *Shigella* monophages

Run	Phage	Age	pH	DO (%)	OD
WAVE041513	SHFML-26	0	7.17	94.0	-
		2	6.92	84.1	0.550
		3.25	6.77	79.5	0.409
		3.75	6.64	71.7	0.653
WAVE041713	SHSML-45	0	6.99	95.1	-
		1	6.92	87.9	0.281
		2	6.61	69.1	0.556
		3	6.52	64.4	0.170
WAVE051613	SHBML-50-1	0	-	-	-
		1	-	-	0.060
		2	-	-	0.188
		3	-		0.138
WAVE042213	SHSML-52-1	0	6.97	97.7	-
		1	6.88	93.9	0.145
		2	6.88	90.3	0.033

3.8.2 Quality control results

With all production monophage lots, each monophage must pass quality testing to be considered for inclusion in a cocktail batch. Results for the WAVE production lots are shown below in Table 34.

Lytic titer is determined using the traditional plaque assay and in some cases is confirmed by an automated nanoparticle count. The specification for inclusion is a minimum of 1.0E+10 PFU/mL. The bacterial sterility assay is administered to determine if there is any viable microbial contamination in the phage solution. The specification for this assay is less than 1 CFU/mL as a measure of 0 cannot be determined without exhausting the entire lot.

Restriction fragment length polymorphism (RFLP) is also administered to confirm the identity of each monophage. A minimum titer of 1.0E+10 PFU/mL is necessary to perform this assay, as that measure provides the requisite amount of DNA. The specification for this assay is identity to the pattern in a reference material, a sample known to be pure. Pulsed-field gel electrophoresis (PFGE) is no longer a required assay for passing quality control testing as RFLP is a satisfactory determinant of homogeneity.

Table 34. Quality control results for WAVE studies

Phage	Lot number	Titer (PFU/mL)	Sterility	RFLP
SHFML-11	041713SHFML11A	3.0E+11	PASS	PASS
SHFML-26	041913SHFML26A	2.0E+10	PASS	PASS
SHSML-45	042213SHSML45A	5.0E+08	PASS	ND
SHBML-50-1	051713SHBML501A	1.0E+09	PASS	ND
SHSML-52-1	042613SHSML521A	1.0E+10	PASS	PASS

*ND – Not determined. Titer of the sample was too low for RFLP analysis.

Three of the WAVE lots of monophages prepared in this study met quality control standards and can be used for subsequent lots of ShigActive™. The failing lots - 042213SHSML45A and 051713SHBML501A – will require additional processing. In cases of low titer, many monophages simply require further concentrating to meet the required metrics for potency and identity through RFLP. This material will be set aside while the scale-up process for WAVE production is re-evaluated.

3.9 Study of *Shigella* toxins

All five monophages – SHSML-52-1, SHFML-11, SHSML-45, SHFML-26, and SHBML-50-1 – contained in ShigActive™ can all be propagated in the same *Shigella* host strain, SH.s43. *Shigella* strains are often known to carry enterotoxins, or cytotoxic proteins emitted by microorganisms. 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).

(1) Shiga toxin consists of two subunits that are encoded by the chromosomal genes, *stxA* and *stxB*. This toxin has been found in *Shigella dysenteriae* only, and it has 99% homology with the *E. coli* shiga toxin-1 gene, *stx1*. There is an ELISA test for detection of this toxin.

(2) Shigella enterotoxin 1 consists of two subunits that are encoded by chromosomal genes *set1A* and *set1B*. This enterotoxin has been found almost exclusively in *Shigella flexneri* serotype 2. There is no commonly accepted test for detection of this toxin.

(3) Shigella enterotoxin 2 is a plasmid-encoded toxin that has been found in all four *Shigella* species. It is encoded by the gene *senA* and there is a possible homolog, *senB*. There is no commonly accepted test for detection of this toxin. *Shigella* spp. are known to lose plasmids upon storage; thus, plasmid encoded toxins may not be an issue in host strain selection (but host strains still need to be examined for the presence of this toxin/plasmid).

Shigella spp. also produce several serine protease autotransporters (SPATEs). SPATEs are a group of highly similar proteins that contain three regions: (1) a signal sequence, (2) a passenger domain, and (3) a translocator domain. Each SPATE protein can translocate itself across the inner and outer bacterial membranes to a cell surface where the passenger domain is cleaved and released to the extracellular environment. There are two groups of SPATEs, Class I and Class II. Class I SPATEs exhibit cytotoxic behavior typically aimed at intracellular structures. Class II SPATEs are not known to be particularly cytotoxic but may have an effect on extracellular targets. See Table 35 for a list of known Shigella SPATES.

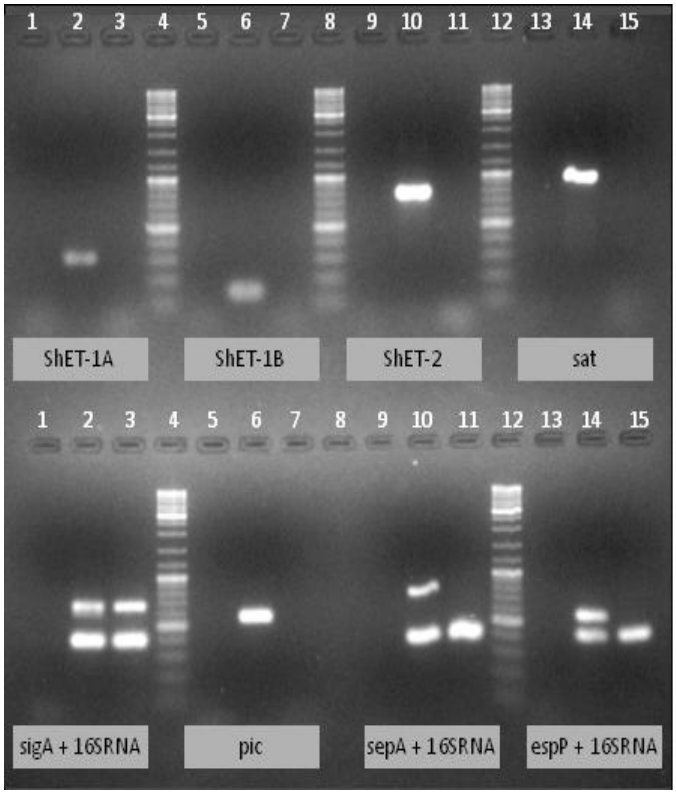
Table 35. List of common SPATEs.

SPATE	Class	Gene	Gene location	Strains
Extracellular serine protease plasmid-encoded	I	**EspP**	plasmid	EHEC (enterohaemorrhagic EC)
Protease involved in intestinal colonization	II	**Pic**	Chromosomal pathogenicity island	UPEC (uropathogenic EC), *Shigella* flexneri
Secreted autotransporter protein	I	**Sat**	Chromosomal pathogenicity island	UPEC, DAEC (diffusely adherent EC), *Shigella* spp
Shigella extracellular protein A	II	**SepA**	plasmid	*Shigella* flexneri
Shigella (Shigella IgA-like protease homolog)	I	**SigA**	Chromosomal pathogenicity island	*Shigella* flexneri

Colony PCR was performed on five bacterial strains, namely Sh.s43 (candidate production host strain), Sh.d2, Sh.d7, Sh.f51, and Ec129 (the latter is an *E. coli* strain which was used as positive control). 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 boiled mixture was spun at 13,000 rpm for 5

minutes; then 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. Each PCR primer pair was analyzed using a water blank (all ingredients except bacterial supernatant), a positive control, and the ShigActive™ host strain, Sh.s43. The samples were then electrophoresed in 2% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator.

Figure 11 shows that Sh.s43 host strain does not contain the genes for any of the known enterotoxins. The host strain does not contain genes for known SPATEs (see Table 36 for list of expected product sizes) either, except for *sigA*. The sigA protein is a temperature-regulated serine protease the can degrade both casein and fodrin. It is a 140 kDa protein that is autonomously processed to a 103 kDa protein which is secreted from the cell. The mechanism of sigA entry into the host cell is unknown.



Top	Bottom
Lane 1: water control	Lane 1: water control
Lane 2: Sh.f51	Lane 2: Sh.d7
Lane 3: Sh.s43	Lane 3: Sh.s43
Lane 4: Ladder	Lane 4: Ladder
Lane 5: water control	Lane 5: water control
Lane 6: Sh.f51	Lane 6: Sh.f51
Lane 7: Sh.s43	Lane 7: Sh.s43
Lane 8: Ladder	Lane 8:
Lane 9: water control	Lane 9: water control
Lane 10: Sh.f51	Lane 10: Sh.f51
Lane 11: Sh.s43	Lane 11: Sh.s43
Lane 12: Ladder	Lane 12: Ladder
Lane 13: water control	Lane 13: water control
Lane 14: Sh.d2	Lane 14: Ec129
Lane 15: Sh.s43	Lane 15: Sh.s43

Figure 11. Identification of enterotoxins and serine protease autotransporter (SPATE) sequences

Intralytix's *S. sonnei* strain 43 contains none of the three known *Shigella* enterotoxins. Thus, the strain should be suitable for producing ShigActive component phages. However, it does contain the genetic sequence for one SPATE, sigA. This protein is not well studied, but it has putatively been linked to virulence, most likely playing a role in host cell cytoskeletal rearrangement. Its mechanism for entry into the host cell has not been determined. While sigA is not a potent toxin (in fact, it is not a

well-recognized virulence factor), Intralytix will examine the options for testing the presence and/or levels of sigA in its final product, in order to conduct a safety analysis.

Table 36. List of enterotoxin primers

Gene	Primer name	Product size (bp)
stxA/B	Stx-Shig-F, Stx-Shig-R	895
set1A	ShET-1A-F, ShET-1A-R	309
set1B	ShET-1B-F, ShET-1B-R	147
senA	ShET-2-F, ShET-2-R	799
sat	sat-Shig-F, sat-Shig-R	930
sigA	sigA-Shig-F, sigA-Shig-R	674
pic	pic-Shig-F, pic-Shig-R	572
sepA	sepA-Shig-F, sepA-Shig-R	794
espP	espP-EHEC-F, espP-EHEC-R	547

3.10 Analysis of experimental ShigActive™ lots

ShigActive™ is a clear to opalescent odorless liquid with a specific gravity of approximately 1.01. The phage component of ShigActive™ (maximum working solution at 1 x 10^9 PFU/mL) is roughly estimated to be 0.0000342% by weight and the remainder is 0.1M sodium chloride. Typical composition of ShigActive™ (at the maximum working concentration of ca 1 x 10^9 PFU/mL) is shown below.

Table 37. Typical composition of ShigActive™ (maximum working concentration of 1x10^9 PFU/mL)

Property/ analysis/ composition	ShigActive™ Lot #1112L210199	ShigActive™ Lot #1113A080197	ShigActive™ Lot #1113A080213	Detection Limit
Total nitrogen (mg/L)	3.8	3.4	3.3	0.5
pH	6.16	6.03	6.04	N/A
Specific gravity (at 25°C)	1.01	1.01	1.01	N/A
Arsenic (mg/L)	ND	ND	ND	0.005
Barium (mg/L)	ND	ND	ND	0.01
Cadmium (mg/L)	ND	ND	ND	0.005
Calcium (mg/L)	0.275	0.284	0.294	0.1
Chromium (mg/L)	ND	ND	ND	0.01
Cobalt (mg/L)	ND	ND	ND	0.005
Copper (mg/L))	0.031	0.030	0.027	0.01
Iron (mg/L)	0.022	0.022	0.020	0.02
Lead (mg/L)	0.005	ND	ND	0.005
Magnesium (mg/L)	ND	ND	ND	0.1
Manganese (mg/L)	ND	ND	ND	0.01
Molybdenum (mg/L)	ND	ND	ND	0.01
Nickel (mg/L)	ND	ND	0.010	0.01
Phosphorus (mg/L)	ND	ND	ND	2
Potassium (mg/L)	0.71	0.64	0.58	0.5
Silicon (mg/L)	ND	ND	ND	0.1

Property/ analysis/ composition	ShigActive™ Lot #1112L210199	ShigActive™ Lot #1113A080197	ShigActive™ Lot #1113A080213	Detection Limit
Sodium (mg/L)	240	242	250	0.5
Tin (mg/L)	0.023	0.022	0.020	0.02
Zinc (mg/L)	0.019	0.018	0.015	0.01
Chloride (mg/L)	352	373	389	5
Nitrate (as N) (mg/L)	0.13	ND	ND	0.1
Nitrite (as N) (mg/L)	ND	ND	ND	0.1
Total Organic Carbon (mg/L)	20.2	17.8	18.2	1
Total Kjeldahl Nitrogen (mg/L)	3.77	3.37	3.27	0.4
Total Dissolved Solids (mg/L)	538	578	592	10
Total Suspended Solids (mg/L)	ND	ND	ND	0.01
Total Phosphorous (mg/L)	0.41	0.40	0.41	0.02
Silica (mg/L)	1.73	1.38	1.39	1
Endotoxin (EU/mL)	2117	1441	1823	N/A

3.11 Ready-to-eat food studies

Investigators at Intralytix performed studies of ShigActive™ efficacy on a number of ready-to-eat foods. Models were selected from the Standard American Diet and purchased at a local grocery. All of the models selected have been reported as known cases of contamination with various foodborne enteric bacterial pathogens, particularly *Shigella*. All studies used *Shigella* strain SH.s43 as the contaminant.

The first set of trials was conducted on long leaf romaine lettuce to determine the efficacy of ShigActive™ at 2 $x10^9$ PFU/mL and at 2 $x10^8$ PFU/mL. Results are displayed in Table 38.

Table 38. RTE food study, vegetable model

	average CFU/g recovered	% reduction vs PBS
PBS	1638	
2E+09 PFU/mL ShigActive™	84	95%
2E+08 PFU/mL ShigActive™	444	73%

Lettuce samples were weighed and measured in triplicate then cut into 100g samples with a sterile knife. Each sample was contaminated with $3x10^5$ CFU/g of *Shigella* strain SH.s43; challenge culture was spread to evenly cover the surface. Samples were then covered with plastic wrap and incubated at room temperature for one hour to allow the bacteria to adhere/attach to lettuce leafs. After this incubation period, samples were sprayed with one mL of either ShigActive™ at $2x10^8$ PFU/mL, ShigActive™ at $2x10^9$ PFU/mL, or phosphate-buffered sterile saline (PBS). Spray was applied evenly to one side of each lettuce surface. Samples were again covered with plastic wrap and this time incubated at room temperature for five minutes. Aliquots of

25g were cut from each sample with a sterile knife. Aliquots were placed in a sterile bag and doused with 225mL of peptone water. Test culture was agitated by hand before stomaching for 30 seconds. Samples of 0.1mL and 0.5mL were plated onto MacConkey agar with 25mg/L Nalidixic Acid and incubated at 35°C. After 24-hour incubation, colonies were counted to determine remaining bacterial concentration (CFU/g). Total phage remaining in the sample culture was also determined.

As the data in Table 38 illustrates, ShigActive™ application at $2x10^9$ PFU/mL reduced the number of viable *Shigella* cells in artificially contaminated lettuce by approximately 95% versus a reduction of 73% with $2x10^8$ PFU/mL, as compared to PBS treatment.

Statistical analysis was performed using both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests. Table 39 shows the results of an ANOVA test with Tukey-Kramer method.

Table 39. Parametric Statistical Analysis of ShigActive™ for lettuce treatment study

Comparison	Mean Difference	q value	P value
ShigActive (H) vs. ShigActive (L)*	-360.00	6.134	P<0.05
ShigActive (H) vs. PBS	-1554.0	26.480	P<0.001
ShigActive (L) vs. PBS	-1194.0	20.346	P<0.001

* ShigActive (H), or high, is the $2x10^9$ PFU/ml concentration. ShigActive (L), or low, is the $2x10^8$ PFU/ml concentration.

At a 95% confidence level, the responses of ShigActive at high concentration and at low concentration are significant (the P value is < 0.0001, considered extremely significant). This analysis supports the conclusion that treating vegetable samples with ShigActive at either high concentration ($2x10^9$ PFU/ml) or low concentration ($2x10^8$ PFU/ml) will significantly reduce *Shigella* contamination; i.e., the variation among column means (ShigActive H vs. PBS control, and ShigActive L vs. PBS control) is significantly greater than expected by chance.

The second set of trials was conducted on smoked salmon to determine the efficacy of ShigActive™ at 1 $x10^6$ PFU/g, 1 $x10^7$ PFU/g, and 1 $x10^8$ PFU/g. Results are displayed in Table 40.

Table 40. RTE food study, seafood model

	average CFU/g recovered	% reduction vs PBS
Water	1940	
1E+08 PFU/g ShigActive™	160	92%
1E+07 PFU/g ShigActive™	620	68%
1E+06 PFU/g ShigActive™	1340	31%

The experiment was conducted nearly identically to the vegetable model. The salmon portions were inoculated with $4x10^3$ PFU/g.

ShigActive™ application to smoked salmon at $1x10^8$ PFU/mL reduced the number of viable *Shigella* cells in artificially contaminated salmon by 92% versus reductions of 68% and 31% with $1x10^7$ PFU/g and $1x10^6$ PFU/g, respectively. All results are in comparisons to the control water treatment.

Statistical analysis was performed using both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests. Table 41 shows the results of an ANOVA test with Tukey-Kramer method. In the table, ShigActive (H) refers to $1x10^8$ PFU/g, ShigActive (M) refers to $1x10^7$ PFU/g, and ShigActive (L) refers to $1x10^6$ PFU/g.

Table 41. Parametric Statistical Analysis of ShigActive™ for salmon treatment study

Comparison	Mean Difference	q value	P value
ShigActive (H) vs. water	-1602.0	42.754	P<0.001
ShigActive (M) vs. water	-1188.0	31.705	P<0.001
ShigActive (L) vs. water	-540.00	14.412	P<0.001
ShigActive (H) vs. ShigActive (M)	-414.00	11.049	P<0.001
ShigActive (H) vs. ShigActive (L)	-1062.0	28.343	P<0.001
ShigActive (M) vs. ShigActive (L)	-648.00	17.294	P<0.001

At a 95% confidence level, the responses of ShigActive at all concentrations are significant (the P value is < 0.001 is considered extremely significant). This analysis supports the conclusion that treating salmon with ShigActive at any of the three concentrations examined will significantly reduce *Shigella* contamination as compared to treating it with water.

A third set of trials was conducted on diced honeydew melon to determine the efficacy of ShigActive™ at 1 $x10^6$ PFU/g, 1 $x10^7$ PFU/g, and 1 $x10^8$ PFU/g. See the table below for results.

Table 42. RTE food study, fruit model

	average CFU/g recovered	% reduction vs PBS
Water	1027	
1E+08 PFU/g ShigActive™	40	96%
1E+07 PFU/g ShigActive™	213	79%
1E+06 PFU/g ShigActive™	567	45%

In this experiment, the honeydew portions were inoculated with $2x10^3$ PFU/g.

ShigActive™ application to honeydew at $1x10^8$ PFU/g reduced the number of viable *Shigella* cells in artificially contaminated honeydew by 96% versus reductions of 79% and 45% with $1x10^7$ PFU/g and $1x10^6$ PFU/g, respectively. All results are in comparisons to the control water treatment.

Statistical analysis was performed using both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests. Table 43 shows the results of an ANOVA test with Tukey-Kramer method. In the table, ShigActive (H) refers to $1x10^8$ PFU/g, ShigActive (M) refers to $1x10^7$ PFU/g, and ShigActive (L) refers to $1x10^6$ PFU/g.

Table 43. Parametric Statistical Analysis of ShigActive™ for honeydew treatment study

Comparison	Mean Difference	q value	P value
ShigActive (H) vs. water	-888.00	11.078	P<0.001
ShigActive (M) vs. water	-732.00	9.131	P<0.001
ShigActive (L) vs. water	-414.00	5.165	P<0.05
ShigActive (H) vs. ShigActive (M)	-156.00	1.946	P>0.05
ShigActive (H) vs. ShigActive (L)	-474.00	5.913	P<0.05
ShigActive (M) vs. ShigActive (L)	-318.00	3.967	P>0.05

At a 95% confidence level, the responses of ShigActive at all concentrations are significant (the P value is < 0.001 is considered extremely significant). Treating honeydew with ShigActive at either concentration significantly reduced *Shigella* contamination as compared to treating the samples with water. The high and low concentrations of ShigActive are significantly different from each other. Comparisons of high to medium concentrations and medium to low concentrations did not yield statistically significant results. The data suggest that using more concentrated ShigActive results in better reduction in *Shigella* contamination in honeydew samples compared to using medium- and low-concentrations of ShigActive.

A fourth set of trials was conducted on cooked chicken breast strips to determine the efficacy of ShigActive™ at 1 $x10^6$ PFU/g, 1 $x10^7$ PFU/g, and 1 $x10^8$ PFU/g. See the table below for results.

Table 44. RTE food study, poultry model

	average CFU/g recovered	% reduction vs PBS
Water	1073	
1E+08 PFU/g ShigActive™	27	98%
1E+07 PFU/g ShigActive™	253	76%
1E+06 PFU/g ShigActive™	547	49%

In this experiment, the chicken strips were inoculated with $2x10^3$ PFU/g.

ShigActive™ application to chicken at $1x10^8$ PFU/g reduced the number of viable *Shigella* cells in artificially contaminated chicken by 98% versus reductions of 76% and 49% with $1x10^7$ PFU/g and $1x10^6$ PFU/g, respectively. All results are in comparisons to the control water treatment.

Statistical analysis was performed using both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests. Table 45 shows the results of an ANOVA test with Tukey-Kramer method. In the table, ShigActive (H) refers to $1x10^8$ PFU/g, ShigActive (M) refers to $1x10^7$ PFU/g, and ShigActive (L) refers to $1x10^6$ PFU/g.

Table 45. Parametric Statistical Analysis of ShigActive™ for chicken treatment study

Comparison	Mean Difference	q value	P value
ShigActive (H) vs. water	-942.00	14.015	P<0.001
ShigActive (M) vs. water	-738.00	10.980	P<0.001
ShigActive (L) vs. water	-474.00	7.052	P<0.01
ShigActive (H) vs. ShigActive (M)	-204.00	3.035	P>0.05
ShigActive (H) vs. ShigActive (L)	-468.00	6.963	P<0.01
ShigActive (M) vs. ShigActive (L)	-264.00	3.928	P>0.05

At a 95% confidence level, the responses of ShigActive at all concentrations are significant (the P value is < 0.001 is considered extremely significant). Like with the honeydew model, only the high and low concentrations of ShigActive are significantly different from each other. Comparisons of high to medium concentrations and medium to low concentrations did not yield statistically significant results. The data suggest that using more concentrated ShigActive results in better reduction in *Shigella* contamination in chicken samples compared to using low-concentration ShigActive.

The fifth set of food study trials was conducted on corned beef. The objective here was the same as in previous studies - to determine the efficacy of ShigActive™ at 1 $x10^6$ PFU/g, 1 $x10^7$ PFU/g, and 1 $x10^8$ PFU/g. See Table 46 below for results.

Table 46. RTE food study, meat model

	average CFU/g recovered	% reduction vs PBS
Water	1060	
1E+08 PFU/g ShigActive™	27	98%
1E+07 PFU/g ShigActive™	280	74%
1E+06 PFU/g ShigActive™	647	39%

In this experiment, the beef slices were inoculated with $2x10^3$ PFU/g.

ShigActive™ application to beef at $1x10^8$ PFU/g reduced the number of viable *Shigella* cells in artificially contaminated lettuce by 98% versus reductions of 74% and

39% with $1x10^7$ PFU/g and $1x10^6$ PFU/g, respectively. All results are in comparisons to the control water treatment.

Statistical analysis was performed using both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests. Table 47 shows the results of an ANOVA test with Tukey-Kramer method. In the table, ShigActive (H) refers to $1x10^8$ PFU/g, ShigActive (M) refers to $1x10^7$ PFU/g, and ShigActive (L) refers to $1x10^6$ PFU/g.

Table 47. Parametric Statistical Analysis of ShigActive™ for beef treatment study

Comparison	Mean Difference	q value	P value
ShigActive (H) vs. water	-930.00	16.030	P<0.001
ShigActive (M) vs. water	-702.00	12.100	P<0.001
ShigActive (L) vs. water	-372.00	6.412	P<0.01
ShigActive (H) vs. ShigActive (M)	-228.00	3.930	P>0.05
ShigActive (H) vs. ShigActive (L)	-558.00	9.618	P<0.001
ShigActive (M) vs. ShigActive (L)	-330.00	5.688	P<0.05

At a 95% confidence level, the responses of ShigActive at all concentrations are significant (the P value is < 0.001 is considered extremely significant). Treatment with the high and low concentrations of ShigActive were significantly different from each other. Comparisons of high to medium concentrations and medium to low concentrations did not yield statistically significant results.

The sixth and final set of food study trials was conducted on yogurt. The objective here was the same as in previous studies - to determine the efficacy of ShigActive™ at 1 $x10^6$ PFU/g, 1 $x10^7$ PFU/g, and 1 $x10^8$ PFU/g. See the table below for results.

Table 48. RTE food study, dairy model

	average CFU/g recovered	% reduction vs PBS
Water	1140	
1E+08 PFU/g ShigActive™	113	90%
1E+07 PFU/g ShigActive™	627	45%
1E+06 PFU/g ShigActive™	960	16%

In this experiment, the yogurt was inoculated with $2x10^3$ PFU/g.

ShigActive™ application to beef at $1x10^8$ PFU/g reduced the number of viable *Shigella* cells in artificially contaminated yogurt by 90% versus reductions of 45% and 16% with $1x10^7$ PFU/mL and $1x10^6$ PFU/mL, respectively. All results are in comparisons to the control water treatment.

Statistical analysis was performed using both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests. shows the results of an ANOVA test with Tukey-Kramer method. In the table, ShigActive (H) refers to $1x10^8$ PFU/g, ShigActive (M) refers to $1x10^7$ PFU/g, and ShigActive (L) refers to $1x10^6$ PFU/g.

Table 49. Parametric Statistical Analysis of ShigActive™ for yogurt treatment study

Comparison	Mean Difference	q value	P value
ShigActive (H) vs. water	-924.00	37.350	P<0.001
ShigActive (M) vs. water	-462.00	18.675	P<0.001
ShigActive (L) vs. water	-163.00	6.548	P<0.01
ShigActive (H) vs. ShigActive (M)	-462.00	18.675	P<0.001
ShigActive (H) vs. ShigActive (L)	-762.00	30.802	P<0.001
ShigActive (M) vs. ShigActive (L)	-300.00	12.127	P<0.001

At a 95% confidence level, the responses of ShigActive at all concentrations are significant (the P value is < 0.001 is considered extremely significant).

3.12 Stability studies

Samples lots prepared in December 2012 and January 2013 were held at 2-8°C for subsequent stability testing. Results are indicated below.

Table 50. Stability testing of ShigActive™ lots

Date	Lot number	Titer (PFU/mL)	Sterility	LPS (EU)
12/21/12	1113A080197	1.0E+10	PASS	1441
01/08/13	1113A080213	1.0E+10	PASS	1873
06/22/13	1113A080197	1.0E+10	PASS	1437
07/03/13	1113A080213	1.0E+10	PASS	1862

ShigActive™ lots 1113A080197 and 1113A080213 passed stability testing and are qualified for use for an additional six months.

3.13 *In vivo* studies

Intralytix currently has a formulation for its ShigActive™ phage product in liquid form. The formulation has been examined *in vivo* (in mice) by our collaborators at the University of Florida.

3.13.1 Recovery study

Investigators at the University of Florida perform studies of ShigActive™ efficacy in mice. The first set of trials was conducted to measure recovery of *Shigella* in the stools of artificially infected mice. Table 51 shows the results of these recovery studies. The counts displayed are the averages from recovered pellets of five mice.

Table 51. *In vivo* Study 1, *Shigella* Recovery

	Day 1	Day 2	Day 3	Day 5
1.2E+07 CFU/mouse	9.83E+04	1.97E+03	1.38E+02	1.00E+01
Percent recovery	81.92%	1.64%	0.115%	0.008%
Log loss	2.09	3.79	4.94	6.08
1.2E+08 CFU/mouse	1.59E+05	3.01E+03	5.53E+02	3.50E+01
Percent recovery	13.23%	0.251%	0.046%	0.029%
Log loss	2.88	4.60	5.34	6.54

On the first day after inoculation, UF investigators recovered ca. 82% of the *Shigella* challenge in the stool of mice in the lower-dosed group. While this is an excellent recovery rate, total counts are more critical in efficacy studies. Therefore, the higher challenge dose of 1.2E+08 CFU/mouse will be used in subsequent efficacy studies.

3.13.2 First efficacy study

For the first pilot phage treatment/ efficacy study, UF investigators challenged the mice with 1.2E+08 CFU of *Shigella* each and treated three groups with different concentrations of ShigActive™: ca. 0E+09 PFU, 1.2E+08 PFU, and 1.2E+07 PFU. This was meant to be a pilot, non-formal efficacy study to gain a very preliminary understanding of whether administration of ShigActive™ was effective in reducing *Shigella* counts and whether the reduction was concentration-dependent. Because of the small number of animals, the PBS control group was not included and bacterial reduction levels were evaluated by comparing bacterial recovery levels with those observed during the challenge study # 1 (from mice challenged with 1.2E+08 CFU of *Shigella*/mouse). The results of this study are displayed in Table 52.

Table 52. *In vivo* Study 2, ShigActive™ Treatment

	Day 1	Day 2
1.0E+09 PFU/mouse	6.70E+03	
Percent reduction	95.8%	
1.2E+08 PFU/mouse	1.39E+04	1.13E+02
Percent reduction	91.2%	96.3%
1.2E+07 PFU/mouse	2.05E+04	2.55E+02
Percent reduction	87.1%	91.5%

After just one day, all treated mice showed *Shigella* reductions of at least 87% in the stool samples. Mice treated with the highest dose of ShigActive™ experienced

reductions of over 96% in stool samples by the second day. While the challenge dose was identical in both studies (1.2E+08 CFU/mouse), the data obtained during Study # 2 must be interpreted with caution due to the lack of PBS control group. Additional dosing studies are currently being performed, and they will include PBS-treated control group for immediate, side-by-side comparison of the data.

3.13.3 Second efficacy study

Another series of experiments were administered with ShigActive™ in mice. All four groups were evaluated concurrently and were treated as follows: group 1 was a PBS control group of 19 specimens; group 2 was treated with ShigActive one hour post-inoculation (19 specimens), group 3 was treated with ShigActive three hours post-inoculation (6 specimens), and group 4 was treated with ShigActive one hour pre-inoculation and one hour post-inoculation (6 specimens). In all experiments, subjects were challenged with *Shigella* at 1.0E+08 CFU/mouse. Phage treated specimen received a dosage at 1.0E+09 PFU/mouse. Stool samples were collected on day 1 and day 2. After sacrifice on day 2, the cecum of each specimen was removed and evaluated. Results are illustrated below in Table 53.

Table 53. *In vivo* Study 3

	Day 1, stool	Day 2, stool	Day 2, cecum
Group 1 – PBS control	2.38E+04 CFU/pellet*	1.76E+03 CFU/pellet**	581 CFU/pellet***
Percent reduction	---	---	---
Log loss	---	---	---
	Day 1, stool	Day 2, stool	Day 2, cecum
Group 2 – treated 1hr post-inoculation	5.78E+03 CFU/pellet	1.33E+03 CFU/pellet	345 CFU/pellet
Percent reduction	74.9%	91.2%	41.22%
Log loss	0.61	1.01	0.07
Group 3 – treated 3hr post-inoculation	6.13E+03 CFU/pellet	7.81E+02 CFU/pellet	192 CFU/pellet
Percent reduction	46.0%	55.6%	63.6%
Log loss	0.27	0.16	0.44
Group 4 – treated 1hr pre- and post-inoculation	3.50E+02 CFU/ pellet	3.75E+01 CFU/ pellet	136 CFU/ pellet
Percent reduction	94.3%	95.2%	83.3 %
Log loss	1.52	1.49	0.78

* An average of four groups: 4.65E+04, 2.56E+04, 1.14E+04, and 1.17E+04
** An average of four groups: 3.40E+03, 1.37E+03, 1.14E+03, and 1.15E+03
*** An average of three groups: 402, 526, and 816

The highest *Shigella* reduction effect was observed in the group 4 day 1 stool samples (94.3%). In this group the amount of recovered *Shigella* cells decreased from

2.38E+04 CFU/pellet for the control animals to 3.50E+02 CFU/pellet for the animals treated with phage. At $p<0.005$, these results are very significant. In the Groups 2 and 3, the effects were 74.9 ($p=0.065$) and 46.0 ($p=0.012$) respectively.

On day 2, the reductions of *Shigella* contamination were similar in both groups treated 1 hour after *Shigella* inoculation: 91.2% in group 2 and 95.2% in the group 4. Again, $p<0.005$, these results are very significant. In group 3, the recovered *Shigella* reduction was 55.7% but still significant ($p<0.015$).

The reduction in cecum samples was significant in all three groups treated with ShigActive™; the highest effect was again in group 4 (83.3%).

Additional *in vivo* studies were conducted to determine phage recovery in the fecal matter of ShigActive™ treated mice. The first experiment began by administering ShigActive™ or PBS (control) by gavage twice per day for 7 days and then every other day for 21 days. Specimens were administered 0.1ml of a 1.0E+10 PFU/mL formulation, or 1.0E+09 PFU per treatment. All mice were monitored for clinical signs.

3.13.4 Third efficacy study

Three days and seven days into the treatment, ten phage treated specimen (group C) were evaluated. Groups A (PBS control) and C were sacrificed on day 8. Twenty-eight days into the study – at the completion of the dosing regimen – group B was evaluated as a PBS control. Group D was evaluated 24 and 48 hours later before being sacrificed. See recovery results in Table 54.

Table 54. Phage recovery for 7 day and 28 day studies

	Day 3	Day 7	Day 28	Day 28 + 24hr	Day 28 + 48 hr
Group A – 7 day PBS control	NA	NA			
Group B – 28 day PBS control			0.0E+00 PFU/g		
Group C – 7 day phage-treated mice	4.7E+06 PFU/g	1.6E+04 PFU/g			
Group D – 28 day phage-treated mice	1.2E+06 PFU/g	0.0E+00 PFU/g		4.2E+04 PFU/g	0.0E+00 PFU/g

Between 3 and 7 days of phage supplementation at a rate of 2 treatments per day, the recoverable phage in pellets of Group C reduced by 99.7% and those of Group D reduced by 100% (values are based on averages of five specimens). Groups A and C were sacrificed at the end of this period.

After seven days of dosing twice a day, the application regimen was lessened to every other day for 21 days. At the end of this phase, Group B was measured as a control and Group D was evaluated 24 and 48 hours later. Between these two time

points, phage recovery reduced by 100% for all five specimens. The PBS control illustrates that there were no traceable levels of residual phage in the specimens' environment.

While the data illustrates that recovery of phage in fecal samples is nearly undetectable at some point after both short-term and long-term treatments, there is an anomaly in this study. The mice treated with ShigActive™ for 28 days (Group D) exhibited undetectable levels of phage (0.0E+00 PFU/g) after 7 days of twice daily treatments. However, testing of the same specimen recovered an average of 4.2E+04 PFU/g after 28 days of treatment (see Table 54).

3.13.5 Phage recovery study

A second experiment was conducted to determine reproducibility of recovery from short-term and long-term treatments of the first experiment. Results from the 7-day treatments are shown in Table 55. Phage recovery for second set of 7 day and 28 day studies. No three day intermediate samples were taken.

Table 55. Phage recovery for second set of 7 day and 28 day studies

	Day 7	Day 28	Day 28 + 24hr	Day 28 + 48 hr
Group A – 7 day PBS control	0.0E+00 PFU/g			
Group B – 28 day PBS control		0.0E+00 PFU/g		
Group C – 28 day phage-treated mice			4.6E+04 PFU/g	8.0E+02 PFU/g
Group D – 7 day phage-treated mice	1.7E+05 PFU/g			

Again, the PBS controls illustrate that there were no traceable levels of residual phage in the specimens' environment. This is critical as it suggests the results for the phage-treated mice were not artificially elevated.

The 7-day phage treated specimen exhibited an average phage recovery of 1.7E+05 PFU/g. The 28-day phage treated specimen exhibited an average phage recovery of 4.6E+04 PFU/g after 24 hours and 8.0E+02 PFU/g after 48 hours. The reductions were substantial. The counts in fecal samples dropped off 72.9% between days 7 and 28 at 24 hours and 99.5% between days 7 and 28 at 48 hours. In the 24 hours post-treatment, phage recovery fell 98.3%.

3.13.6 Antibiotic study

Investigators at the University of Florida also performed studies of ShigActive™ efficacy in mice compared to the antibiotic ampicillin. Mice aged eight weeks old were obtained from the Charles River Laboratory and allowed to acclimate to their new environment for seven days. Subjects were then infected by gavage with ShigActive™

at 1.0E+09 PFU/mouse or ampicillin at 25mg/g in PBS. One hour later, the animals were challenged with *Shigella* strain S43-NalAc^R in 0.1mL PBS. ShigActive™ or ampicillin was administered one hour after inoculation with *Shigella*. Stool samples were collected before treatment, one day after treatment, and two days after treatment. The subjects were sacrificed after second day sampling and the cecum of each specimen was removed for evaluation. Results for the five mice averages are illustrated below in Table 56.

Table 56. *Shigella* recovery for antibiotic study

Sample	*Shigella* recovery, Ampicillin	*Shigella* recovery, ShigActive™	Log difference
Day 1, stool	5.29E+03 CFU/pellet	4.95E+02 CFU/pellet	1.03
Day 2, stool	1.71E+02 CFU/pellet	2.75E+01 CFU/pellet	0.79
Day 2, cecum	3.90E+02 CFU/pellet	1.34E+02 CFU/pellet	0.46

Notably, the mice treated post-gavage with ampicillin had much higher levels of recoverable *Shigella* at each sample point. The difference in mean *Shigella* colony counts between ShigActive™ and ampicillin treatment reached statistical significance at day 2 post-treatment stool samples ($p=0.025$ vs $p=0.088$ and $p=0.058$).

Trials were also conducted to compare the microbiota of ShigActive™ treated mice and those issued ampicillin. Based on DGGE analysis, there was no difference in microbiota diversity between the two groups and no distortion of microbiota after either of the treatments.

3.13.7 Minimum effective dose (MED) study

Investigators also administered a minimum effective dose and frequency study using 8 week-old mice as test subjects. These specimen were obtained from the Jackson Laboratory and allowed to acclimate to their new environment for seven days. Subjects were then infected with ShigActive™ or a PBS control and inoculated with *Shigella Shigella* strain S43-NalAc^R in one of four ways: 1) treatment one hour before inoculation, 2) treatment one hour after inoculation, 3) treatment three hours after inoculation, or 4) treatment one hour before and one hour after inoculation. The dosage of phage treatment was 0.1mL of 1.0E+10 PFU/mL, or 1.0E+09 PFU/mouse. Stool samples were collected before treatment, one day after treatment, and two days after treatment. The subjects were sacrificed after second day sampling and the cecum of each specimen was removed for evaluation. See Table 57 for results.

Table 57. *Shigella* recovery for MED study

Sample	Control, PBS	ShigActive™, 1 hr post	ShigActive™, 3 hr post	ShigActive™, 1hr pre and post	ShigActive™, 1 hr pre
Day 1, stool	2.08E+04 CFU/pellet	5.78E+03 CFU/pellet	6.13E+03 CFU/pellet	3.50+02 CFU/pellet	2.09E+03 CFU/pellet
Day 2, stool	1.57E+03 CFU/pellet	1.13E+03 CFU/pellet	7.04E+02 CFU/pellet	3.0E+01 CFU/pellet	4.95E+01 CFU/pellet
Day 2, cecum	6.27E+02 CFU/pellet	3.77E+02 CFU/pellet	1.92E+02 CFU/pellet	1.28E+02 CFU/pellet	1.30E+02 CFU/pellet

For all stool samples collected on days 1 and 2 in addition to cecum samples that were collected upon sacrifice, the mice treated with ShigActive™ phage shed significantly less *Shigella* than did the control mice treated with PBS. On day 1 of stool sample collection and plating, ShigActive™ phage administration 1 hour before and after *Shigella* inoculation proved to be most effective with the least amount of Shigella CFU/pellet and phage administration 1 hour before was the second best treatment regimen. Although ShigActive™ phage administration 1 hour after and 3 hours after *Shigella* inoculation showed a reduction in comparison to the control, results were not as drastic as phage administration 1 hour before and after. Day 1 stool results for the phage-treated specimen were all significantly lower than the PBS control-treated specimen ($p<0.05$).

On day 2 of stool sample collection and plating, the number of *Shigella* CFU/pellet decreased in all areas including our control. The greatest reduction however, can be seen when phage was administered 1 hour before inoculation. Phage administration 1 hour before and after inoculation also shows a close effectiveness. Phage administration 1 hour after and 3 hours after again show reduction when compared to controls, but do not show a drastic decrease in the amount of *Shigella* shed when compared to the other treatment regimens. Day 2 stool results for three of the phage-treated specimen – 1 hr post-inoculation, 1hr pre- and post-inoculation, and 1 hr pre-inoculation -were significantly lower than the PBS control-treated specimen ($p<0.05$).

For cecum samples collected and plated, ShigActive™ phage administration 1 hour before and after *Shigella* challenge showed the greatest reduction. Cecum results for the phage-treated specimen were all significantly lower than the PBS control-treated specimen ($p<0.05$). The results of this study suggest that infection 1 hr before inoculation and 1 hr post-inoculation are the optimal frequency conditions for ShigActive™ treatment. ShigActive™ works best when used as both a probiotic and a treatment.

A dosage study evaluated phage treatment at 1.0E+09PFU/mL, 1.2E+08 PFU/mL, and 1.2E+07 PFU/mL. The highest dosage level showed the highest reductions of recoverable *Shigella* in cecum, small intestine, and stool samples from two days.

3.13.8 Toxicity study

Investigators also administered a toxicity study using 8 week-old mice as test subjects. These specimen were obtained from the Jackson Laboratory and allowed to acclimate to their new environment for seven days and weighed. Subjects were infected with ShigActive™ or a PBS control twice each day for 7 days, then once every other day for 3 weeks. The dosage of phage treatment was 0.1mL of 1.0E+10 PFU/mL, or 1.0E+09 PFU/mouse. Stool samples were collected before the regimen, at 7 days, and at 28 days, the end of the experiment. The subjects were sacrificed after either 7 or 28 days and the cecum and tissue samples of each specimen was removed for evaluation. Urine and blood samples were also collected.

Weights of the subjects remained mostly the same and progressed at similar rates for each group and between groups throughout the study, suggesting ShigActive™ treatment did not affect energy balance.

Toxicity results show that ShigActive™ appears to be safe for long-term use. Our DGGE analysis supports this idea as no statistically significant difference was observed in the microbiota diversity profiles of cases and controls. Analysis of microbiota profiles of mice observed on DGGE gel images using Diversity Database software revealed no statistically significant difference of the gut microbiota diversity between controls and phage treated mice (see Table 58).

Table 58. Diversity Indexes based on DGGE microbiota profiles

Treatment	Time	Source	Shannon	Simpson (1/D)
PBS	Baseline	Stool	2.73+/-0.18	14.35+/-2.56
ShigActive™	Baseline	Stool	2.62+/-0.21	12.73+/-2.43
PBS	7 days	Stool	2.62+/-0.22	13.09+/-2.86
ShigActive™	7 days	Stool	2.60+/-0.24	12.83+/-2.97
PBS	28 days	Stool	2.66+/-0.20	13.24+/-2.53
ShigActive™	28 days	Stool	2.64+/-0.19	12.92+/-2.27
PBS	7 and 28 days, avg	Cecum	2.66+/-0.32	13.31+/-3.55
ShigActive™	7 days	Cecum	2.81+/-0.11	14.99+/-1.95
ShigActive™	28 days	Cecum	2.42+/-0.41	11.06+/-4.04

Total and differential white blood cell counts showed no statistically significant difference at 7 and 28 days between controls and cases ($p>0.05$). See results in Table 59.

Table 59. Total and differential white blood cell counts

	Control, 7 days	ShigActive™, 7 days	Control, 28 days	ShigActive™, 28 days
White blood cells (WBC), K/µL	9.82	9.28	10.54	8.36
Neutrophils (NE)	2.67	2.41	2.59	1.69
NE, %	27.03	25.53	25.34	20.67
Lymphocytes (LY)	6.81	6.53	7.62	6.40
LY, %	68.67	70.60	71.56	76.02
Monocytes (MO)	0.25	0.23	0.31	0.25
MO, %	3.26	2.53	2.90	2.95
Eosinophils (EO)	0.068	0.082	0.016	0.016
EO, %	0.70	1.02	0.16	0.17
Basophils (BA)	0.04	0.03	0.00	0.01
BA, %	0.34	0.32	0.03	0.16

Red blood cell counts are higher in phage treated mice after 1 week than controls (10.25 M/µL and 8.99 M/µL respectively as illustrated in Table 60). Hemoglobin counts are also noticeably higher in phage treated mice after 1 week than controls (14.80 g/dL versus 13.58 g/dL respectively). Likewise, hematocrit levels are higher in phage treated mice than control after 1 week (49.60% versus 44.28% respectively).

Table 60. Red blood cell counts

	Control, 7 days	ShigActive™, 7 days	Control, 28 days	ShigActive™, 28 days
Red blood cells (RBC), M/µL	8.99	10.25	9.62	10.41
Hemoglobin (HB), g/dL	13.58	14.80	14.19	16.14
Hematocrit (HCT), %	44.28	49.60	46.94	48.32
Platelets (PLT), K/µL	1230.75	1520.40	1375.58	1466.00
Mean corpuscular volume (MCV), fL	49.25	48.40	48.83	46.42
Mean corpuscular hematocrit (MCH), pg	15.13	14.50	14.81	15.50
MCH concentration (MCHC), g/dL	30.70	29.94	30.32	33.40
Red cell distribution width (RDW), %	19.13	18.48	18.80	16.94
Mean platelet volume (MPV), fL	4.10	4.44	4.27	3.98
Platelet distribution width (PDW), %	27.45	24.92	26.19	22.62

Table 61 illustrates the blood chemistries of the study specimen. After 28 days of treatment, Serum Albumin levels (ALB) were higher in the phage treated group compared to the control (3.8 g/dL versus 3.2 g/dL respectively). Moreover, blood urea nitrogen levels were lower in the phage treated group compared to the control after 28 days (16 mg/dL versus 19 mg/dL respectively). While statistically significantly different levels were observed in some measures, all measures were within physiologically normal range.

Table 61. Blood chemistry results

	Control, 7 days	ShigActive™, 7 days	Control, 28 days	ShigActive™, 28 days
Albumin (ALB), g/dL	3.2	3.3	3.2	3.8
Blood urea nitrogen (BUN), mg/dL	17.0	14.4	19.0	16.0
Cholesterol (CHOL), mg/dL	120.3	134.0	108.8	110.0
Creatinine (CREAT), mg/dL	0.3	0.3	0.3	0.3
Glucose (GLU), mg/dL	261.5	293.4	272.8	248.0
Phosphate (PHOS), mg/dL	8.7	8.2	7.5	4.3
Bilirubin (TBILI), mg/dL	0.4	0.4	0.7	1.3
Total protein (TPROT), g/dL	5.2	5.3	5.6	6.1
Alkaline phosphatase (ALP), U/L	54.3	47.0	35.8	48.6
Alanine aminotransferase (ALT), U/L	135.3	135.2	109.6	143.4
Aspartate aminotransferase (AST), U/L	465.5	579.6	431.2	667.4
Creatinine kinase (CK), U/L	12.0	18.4	0.0	0.0

Although statistical significance ($p<0.05$) was observed when comparing blood test results for controls and phage-treated specimen at 1 week, when the values for red blood cells, hemoglobin, and hematocrit are analyzed individually, they still lie within or are very close in value to the normal, expected range for C57B/6J mice as provided by the Jackson Laboratory. The normal ranges for red blood cells are 7.09 – 9.47 M/µl; hemoglobin 10.9 – 14.3 g/dL; hematocrit 36.6 – 46.7%. Similarly, although statistical significance ($p<0.05$) is observed, when the values for albumin and blood urea nitrogen levels are compared individually, they still lie within the normal, expected range for C57B/6J mice. Normal ranges for albumin levels are 2.8 – 3.8 g/dL and blood urea nitrogen 7 – 28 mg/dL.

This study supports the idea that ShigActive™ bacteriophage is a safe and effective method of targeting *Shigella* in the gastrointestinal tract.

3.14 *In vivo* data: summary with figures and charts

PLEASE NOTE: numbering of figures restarts from this point forward

Our UF collaborators developed a mouse model to study the efficacy of a bacteriophage cocktail, ShigActive™, in reducing fecal *Shigella* counts after oral challenge. In that model, oral challenge with *Shigella* did not induce any signs of clinical shigellosis and no translocation was observed; however, the model could be useful for evaluating transient colonization with *Shigella in vivo*, and the impact of phage treatment (or antibiotic treatment) on that colonization and shedding. Previously, the efficacy of ShigActive™ was evaluated in an *in vitro* system (the HeLA cell invasion assay) where ShigActive™ provided 100% protection with a 1,000:1 phage:bacteria ratio (which would be expected during a typical human infection).

The short-term efficacy study

The short-term *in vivo* efficacy study was performed with a total of 71 mice. ShigActive™ was administrated (i) 1 h after, (ii) 3 h after, (iii) 1 h before and 1 h after, and (iv) 1 h before challenge with *S. sonnei* strain S43 Nal^R (1×10^8 CFU/mouse). Body weight was not affected by treatment. The highest reduction in fecal *Shigella* counts was observed in mice treated with two doses of ShigActive™ (i.e., 1h-prechallenge and 1h-postchallenge), sacrificed 2 days post-challenge and compared to mice treated with sterile PBS. Efficacy was phage concentration-dependent, with complete eradication in stool specimens obtained from mice that received a ShigActive™ dose containing 1×10^9 PFU. ShigActive™ was more effective than ampicillin in reducing fecal Shigella counts (Fig.2).

When we analyzed differences in the fecal microbiota composition bacterial two phyla (*Bacteroidetes* and *Firmicutes*) dominated in all samples. After ampicillin treatment, there was an increase in the proportion of *Actinobacteria* that was not observed in the ShigActive™ group (Figure 3). Furthermore, the number of OTUs, a correlate for the number of bacterial species, decreased after antibiotic treatment more so than with phage-treatment group (366 vs.36 at 98% and 101 vs.40 at 95% similarity) (Table 1A). Thus, while ShigActive™ was more effective in reducing *Shigella* counts it showed less side effects on commensal microbiota.

Table 62 Effect of treatment on OTUs

A		ShigActive		Ampicillin	
98%	ttest	10↑	10↓	10↑	52↓
	ztest	40↑	40↓	25↑	101↓
95%	ttest	5↑	12↓	5↑	36↓
	ztest	20↑	36↓	3↑	366↓

B		PBS		ShigActive	
98%	ttest	14↑	29↓	21↑	11↓
	ztest	53 ↑	163↓	89↑	67↓
95%	ttest	11↑	28↓	14↑	8↓
	ztest	27 ↑	113↓	51↑	42↓

The long-term toxicity study

The long-term toxicity study was performed with a total of 40 mice. The animals in each cage received either PBS or ShigActive™ and were sacrificed on days 7 or 28. We observed no significant difference in body weight or any health and toxicity markers between the two groups. When we analyzed commensal microbiota in both groups, the proportions of bacterial phyla appeared unaffected in either group. Thus, we conclude that long term (28 days) ShigActive™ treatment doesn't affect commensal microbiota composition.

The 16S rRNA based sequencing analysis of the gut microflora

16S rRNA based sequencing provided us a total of 181,922 sequences that represented an average of 3,638 sequences per sample. The average length per sequence was 484 nucleotides. We then clustered sequences using ESPRIT at the 98% and 95% similarity levels to obtain OTUs containing similar sequences for further microbiota analysis. After removal of OTUs containing less than 1 sequence, we retained 6258 and 3411 OTUs at the respective similarity level. No one of QIME package analysis showed any differences between control and ShigActive groups. The proportions of bacterial groups at the phylum level were the same before and after the treatment and no differences were detected between control and ShigActive groups (Fig. 5). Numbers of OTUs changed in the both directions after the treatment were the similar (Table 1B).

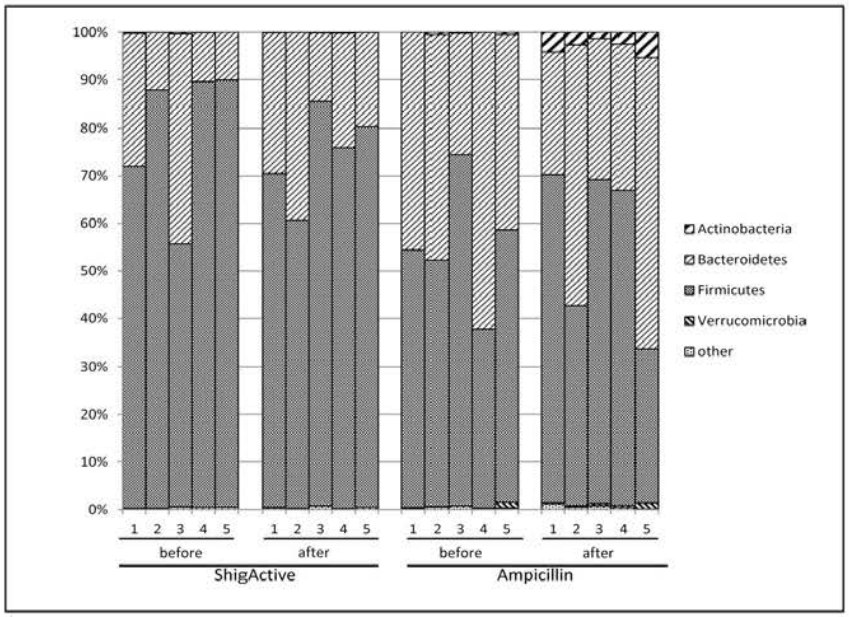


Figure 3

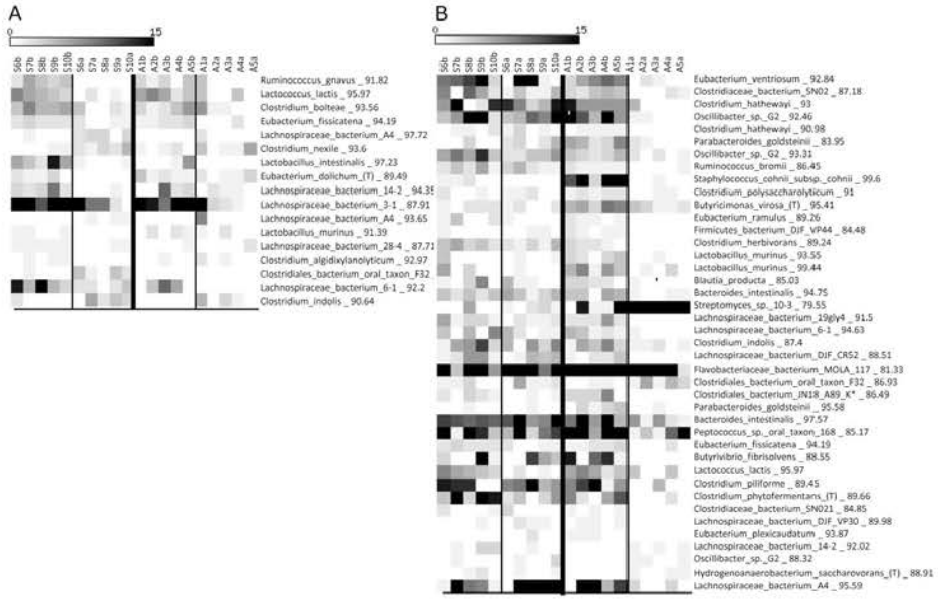


Figure 4

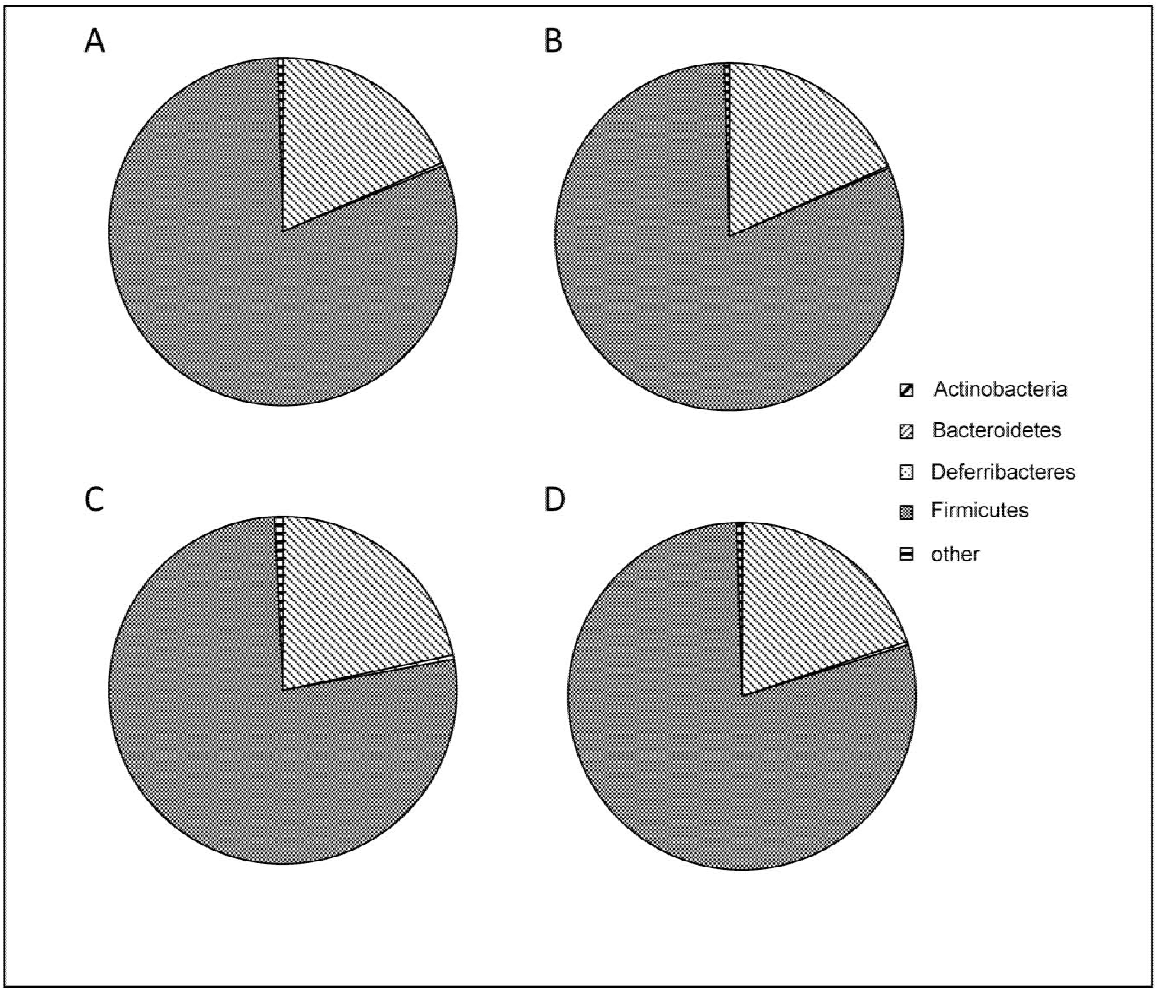


Figure 5

Conclusions

Our animal models studies suggest ShigActive™ treatment is an effective approach for reducing fecal *Shigella* counts and, presumably, transient intestinal colonization with the bacterium as well. Phage administration was completely safe in mice, with all (100%) of "safety" criteria examined (see Section 3.13.8) showing no deleterious impact of the treatment. Moreover, phage administration had no impact on the normal gut microbiota. The data would support the idea that administration of ShigActive to mammals (including humans) would reduce the levels of viable *Shigella* in the gut and, therefore, may reduce the risk of disease after ingestion of *Shigella*, without significant side effects and without disturbing the normal microflora of the gut. Human clinical trials are needed to support the validity of this hypothesis.

4 SIGNATURE

_____ ___January 22, 2014____

Alexander Sulakvelidze, Ph.D. Date

Principal Investigator