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By FedEx

November 30, 2022

Office of Food Additive Safety (HFS–200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

Re: GRAS Notice for *Salmonella Enteritidis* Phage Preparation (Strain SP8)

Dear Sir or Madam:

On behalf of my client, Qingdao Phagepharm Bio-Tech Co., Ltd, I hereby submit the enclosed GRAS notice for the use of a *Salmonella*-specific bacteriophage strain under the commercial name *Salmonella Enteritidis* Phage Preparation (Strain SP8) as an antimicrobial on ground chicken to control *Salmonella* at an application rate of up to 2×10^8 PFU (plaque forming units) per gram of food. The statutory basis of the GRAS conclusion is scientific procedures.

The GRAS notice does not contain any designated confidential business information. In accordance with the Agency's guidelines, we have enclosed one original copy of the GRAS notice, and one complete electronic copy of the GRAS notice on a compact disk (CD).

We are committed to cooperating with the Agency and believe an open dialog is one of the most effective ways to accomplish that objective. We greatly appreciate the feedback we have received from the agency during the pre-submission meeting on October 11, 2022. If any questions arise in the course of your review, please contact us, preferably by telephone or e-mail, so that we can provide a prompt response.

Sincerely,

Xin Tao
Counsel
Hogan Lovells US LLP
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GRAS NOTICE FOR *SALMONELLA ENTERITIDIS* PHAGE PREPARATION (STRAIN SP8)

Name of Notifier:

Qingdao Phagepharm Bio-Tech Co., Ltd
Future Science and Technology Industrial Park,
No.106, Xiangyang Road, Chengyang District, Qingdao.
Shandong, China

November 30, 2022

Table of Contents

PART 1 Signed Statements and Certification

1.1 Compliance with 21 CFR §170.225

Qingdao Phagepharm Bio-Tech Co., Ltd (QPB) is hereby submitting a GRAS notice in accordance with 21 CFR §170.225.

1.2 Name and Address of Notifier

QPB
Building 6, Future Science and Technology Industrial Park, No.106, Xiangyang Road, Chengyang District, Qingdao, Shandong, China.

1.3 Name of Notified Substance

QPB manufactures a *Salmonella*-specific bacteriophage strain under the commercial name *Salmonella Enteritidis* Phage Preparation (Strain SP8).

1.4 Intended Use of the Notified Substance

The intended use of Strain SP8 is an antimicrobial on ground chicken to control *Salmonella* at an application rate of up to 2×10^8 PFU (plaque forming units) per gram of food.

1.5 Basis for GRAS Determination

Pursuant to 21 CFR §170.30, QPB has determined the intended use of Strain SP8 is GRAS through scientific procedures.

1.6 Exemption from Premarket Approval

The intended use of Strain SP8 was determined by QPB to be GRAS and thus is exempt from premarket approval requirements when used under the intended use conditions described within this notification.

1.7 Availability of Information

All data and information that serve as basis for this GRAS determination are available for review upon request:

Feiyang Zhao
Building 6, Future Science and Technology Industrial Park,
No.106, Xiangyang Road, Chengyang District, Qingdao.

Shandong, China

1.8 Freedom of Information Act

All information included can be disclosed under the Freedom of information Act, 5 U.S.C. 552.

1.9 Certification

To the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to QPB and pertinent to the evaluation of the safety and GRAS status of the use of Strain SP8.

1.10 Signature of Notifier

Feiyang Zhao
Registration Director
QPB

PART 2 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 General Identity

Salmonella Enteritidis Phage Preparation (Strain SP8) consists of one bacteriophage (phage) that was isolated from chicken manure water samples collected in Shandong, China. It was saved in China General Microbiological Culture Collection Center, CGMCC. This phage is characterized by full-genome sequencing, electron microscopy, and lytic activity against a large number of *Salmonella* strains. Bioinformatic analysis of the phage genome sequence reveals that it is strictly lytic and lacks any virulence, or undesired genes as identified in GenBank.

Phage: SP8
Order: Caudovirales
Family: Siphoviridae
Genome: dsDNA
Type: Lytic phage

The full genome sequence of SP8 is publicly available through GenBank as ON381768.

SP8 is soluble in water. The phage is diluted in sterile water so that SP8 solution has a minimal of total phage concentration of 1×10^{10} PFU/mL. SP8 can then be applied at a rate of 0.5-2% v/w at the discretion of the food manufacturer, which corresponds to a maximum use level of 2×10^{8} PFU/g of food.

2.2 Host Identity

The phage is amplified in a non-virulent strain of *Salmonella Enteritidis* named C1106, which was isolated from chicken farm sewage or soil of Hebei, China. Strain C1106 is non-pathogenic and does not contain any enterotoxin genes. 1/

Salmonella host C1106 is also sensitive to antibiotics such as chloramphenicol, kanamycin, naphthalinic acid, furantoin, penicillin and tetracycline.

2.3 Host Range

A host range study for SP8 was carried out by Jiangsu Zoonosis Laboratory at Yangzhou University. A total of 85 *Salmonella Enteritidis* strains including strains

1/ *See* **Appendix A** "PCR Testing of Production Bacteria C1106."

of Enteritidis, Typhimurium and other types of *Salmonella* were tested. The lytic activity of SP8 was demonstrated for 90% of those 85 strains.

2.4 SP8 Properties

SP8 solution is a clear translucent liquid and is composed of phages and sterile water. Its physical properties are summarized in **Table 1**, below.

Table 1. Physical Properties of SP8				
Name	**Color**	**Odor**	**State**	**Solubility**
SP8	Translucent	None	Liquid	Soluble in water

2.5 Specifications

Each phage production batch of SP8 is quality controlled for its concentration, purity, endotoxin level, and sterility (*see* **Table 2**). Test data from three non-consecutive batches of SP8 demonstrate they are all in compliance with the specifications. Also, each test method referenced in **Table 2** is validated for their intended uses.

Table 2. Specifications and Test Results of Three Non-consecutive Batches of SP8

	Standard	**Unit**	**Phage Productions**			**Method**
			Batch: 20220305	**Batch: 20220312**	**Batch: 20220403**	
Concentration	$>10^{10}$	PFU/mL	7.2×10^{10}	6.7×10^{10}	7.5×10^{10}	Plaque Assay
Endotoxin	<2,500	EU/mL	2,350	2,300	2,410	Color matrix method
Bacterial sterility	No growth detected after 7 days	-	No growth detected after 7 days	No growth detected after 7 days	No growth detected after 7 days	Luria-Bertani agar plates
Arsenic	<0.02	mg/kg	ND	ND	ND	AOAC 2015.01 Heavy Metals in Food ICP-MS
Lead	<0.02	mg/kg	ND	ND	ND	
Mercury	<0.01	mg/kg	ND	ND	ND	
Kjeldahl	<1000	mg/L	850	810	820	OMOE

Nitrogen						E3516 m
Organic Carbon	<45000	mg/L	39000	41000	42000	Standard Methods: 5310B: Total organic carbon by High-Temperature Combustion
ND = Not detected						

2.6 Method of Manufacture

The phage is produced by aerobic fermentation of production strain with broth media. Phage for infecting the non-pathogenic production strain is added at desired MOIs (multiplicity of infection) when the appropriate OD600 value is reached. After infection, the culture is further incubated under agitation and aeration conditions to reach high concentration.

After incubation, the culture is centrifuged to remove bacterial debris, and then purified through micro-filtration, and further purified with sterile filtration. Ultra-filtration is also used to wash the phages with phosphate-buffer saline (PBS). Any residual endotoxins in the phage are expected to be further removed during clarification and extensive washing.

After each SP8 lot is tested according to the specifications in **Table 2**, the phage is then stored in a refrigerated (2-8 °C) environment and shielded from light exposure.

2.7 Food-grade Material

All raw materials used in the manufacturing of SP8 are food grade and animal-product free.

2.8 Efficacy Data at the Intended Level of Use

Challenge studies were designed to evaluate the efficacy of SP8 in ground chicken. Three different *Salmonella* serovars were mixed equally and applied to pre-ground chicken meats. SP8 was sprayed onto the pre-ground chicken to promote even distribution so that the 2×10^8 PFU/g of phage was applied. It is shown that SP8 reduced *Salmonella* at 1.5 to 1.9 logs in ground chicken (*see* **Appendix B**).

PART 3 Dietary Exposure

3.1 Application Rates

For the dietary exposure estimation, the assumption is that SP8 will be applied at the maximum rate of 2×10^8 PFU/g of food (i.e., ground chicken).

3.2 Dietary Intakes of Ground Chicken and SP8

We conducted an dietary intake assessment to calculate the estimated daily intake (EDI) from the intended use of SP8 on ground chicken. The assessment estimated SP8 intake associated with this proposed use by the U.S. population 2 years and older. The EDI of ground chicken was based on food consumption data from foods reported consumed in the What We Eat in America (WWEIA) dietary component of the National Health and Nutrition Examination Surveys (NHANES) 2017-2018. The NHANES is a continuous survey that uses a complex multistage probability sample designed to be representative of the civilian U.S. population. NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States. Statistical weights are provided by the National Center for Health Statistics (NCHS) to adjust for the differential probabilities of selection and non-response.

As part of the examination, trained dietary interviewers collected detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone three to ten days after the first dietary interview, but not on the same day of the week as the first interview. The dietary component of the survey is conducted as a partnership between the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS). DHHS is responsible for the sample design and data collection, and USDA is responsible for the survey's dietary data collection methodology, maintenance of the databases used to code and process the data, and data review and processing.

The dietary recall portion of the NHANES survey consists of two non-consecutive 24-hr recalls. For each subject with a complete 2-day dietary recall, a 2-day average intake estimate was derived by summing their intakes on day 1 and day 2 of the survey and dividing that sum by 2. A 2-day average typically overestimates chronic daily intake and does not necessarily represent long-term intakes.

The food codes from NHANES 2017-20108 corresponding to the intended uses in ground chicken are provided in **Appendix C**. This approach very conservatively assumes that the ground chicken treated with Strain SP8 will have 100% market share

of these food categories, and 100% of these food products are made from ground chicken. The average and 90th percentile *per user* intake of ground chicken among the general population aged 2 years and older can be summarized in the table below.

Table 3. Ground Chicken Consumption in the U.S. (NHANES 2017-2018)	
Average Consumption	**90th Percentile Consumption**
64.36 g/person	113.51 g/person

The average and 90th percentile dietary exposures for Strain SP8 can be calculated using the following very conservative assumptions:

- Manufacturers add a maximum level of 2×10^{8} PFU/g of Strain SP8 in all of the ground chicken.
- The ground chicken treated with Strain SP8 has a 100% market share in the U.S. in these product categories.
- Weight of Strain SP8 phage is 4.80×10^{-17} g/PFU. 2/

Average estimated daily intake (EDI) of SP8 from the intended use =
2×10^{8} PFU/g * 64.36 g/person/day * 4.80×10^{-17} g/PFU = 6.17×10^{-7} g/person/day.

90th percentile EDI of Strain SP8 from the intended use = 2×10^{8} PFU/g * 113.51 g/person/day * 4.80×10^{-17} g/PFU = 1.09×10^{-6} g/person/day.

Further, at the maximum rate of 2% v/w SP8 application, 1 g of food is treated with 0.02 mL of SP8. The 90th percentile daily consumption of all ground chicken treated with SP8 was calculated to be 113.51 g (*see* **Table 3**). Accordingly, the 90th percentile intake of SP8 by volume can be calculated as:

113.51 g * 0.02 mL =2.27 mL

Therefore, the 90th percentile daily consumption of SP8 by volume is 2.27 mL.

3.3 Estimated Dietary Exposure to Endotoxins

The use of a gram-negative bacteria as host strain to produce SP8 leads to the release of endotoxins. As discussed above in Section 2.6, through multiple filtration steps, most of the endotoxins are removed and are not expected to end up in the SP8. As shown in **Table 2**, the levels of endotoxin in each of the three SP8 lots were less than the specification of 2,500 EU/mL. Using the maximum endotoxin level allowed for product release, we can calculate the theoretical worst-case daily consumption of endotoxins from the use of SP8 as:

2/ *See* **Appendix D** "Weight of Strain SP8 Phage Calculation."

2,500 EU/mL * 2.27 mL = 5,675 EU

Endotoxins, also called lipopolysaccharides (LPS), are a major component of the outer membrane of gram-negative bacteria. It is reported that endotoxin, when consumed at very high levels, may elicit a wide variety of pathophysiological effects, such as endotoxin shock, tissue injury, and even death. 3/ Endotoxins do not act directly against cells or organs but through activation of immune system, especially the monocytes and macrophages, thereby enhancing immune responses. 4/ Gram-negative bacteria, which contain endotoxin, are found at very high levels in the mammalian gut, especially the lower intestine. They are also commonly found in saliva, dental plaque, skin, lungs, respiratory tract and urinary tract. In particular, as gram-negative bacteria normally residing in human mouths produces endotoxin, it is reported that human saliva contains approximately 1 mg of endotoxin per mL of saliva, equating to 1×10^6 EU/mL. 5/ Saliva is produced at levels exceeding 500 mL/day, which amounts to 5×10^8 EU/day. The maximum amount of theoretical endotoxin intake from the use of SP8 only constitutes around 0.001% of the daily endotoxin load from saliva, and, as such, can be considered safe. 6/

3/ Magalhães PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TC, Pessoa A Jr. Methods of endotoxin removal from biological preparations: a review. *J Pharm Pharm Sci.* 2007;10(3):388-404. PMID: 17727802.

4/ Ogikubo Y, Norimatsu M, Noda K, Takahashi J, Inotsume M, Tsuchiya M, Tamura Y. Evaluation of the bacterial endotoxin test for quantification of endotoxin contamination of porcine vaccines. *Biologicals* 32:88-93. 2004.

5/ Leenstra, Thomas S., et al. "Oral endotoxin in healthy adults." *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 82.6 (1996): 637-643.

6/ 5,675 EU ÷ 5×10^8 EU/day $\approx$ 0.001%.

PART 4 Self-Limiting Levels of Use

The amount of SP8 that can be added to food is self-limiting because due to the cost of the phage product, we expect the manufacturer to use the minimum dose required to achieve the desired reduction of *Salmonella enterica*. Further, once the *Salmonella* targets are depleted in foods, the phages will stop replicating.

PART 5 Experience Based on Common Use in Food Before 1958

This section is not applicable to this GRAS notification, which is based on scientific procedures.

PART 6 Narrative

The basis of QPB's determination of the intended use of SP8 as GRAS is provided below.

6.1 Background on *Salmonella*-Related Illnesses and Usage of Phage

Salmonellosis is a common cause of food-borne diseases worldwide, causing diarrhea, fever, abdominal cramps, and even life-threatening infections. *Salmonella enterica* serotype Enteritidis and *Salmonella enterica* serotype Typhimurium are responsible for the majority of the outbreaks, and most events relate to the consumption of contaminated eggs, meat and poultry products.

Phages are naturally occurring viruses that infect both gram-positive and gram-negative bacteria. 7/ Phages are very specific, meaning they only attack their targeted bacterial hosts, and they cannot infect human or other eukaryotic cells. They are generally unaffected by antibiotic resistance and, unlike most antibiotics, are able to target bacteria encased in biofilms. 8/

Several commercially available phages specific for various bacterial pathogens including *Salmonella* have been favorably reviewed by FDA in the past, including, but are not limited to the following:

Table 4. Summary of Previous FDA Review of Phage Food Applications

Reference	Product Name	Intended Use
GRN No. 917	GPI Biotech VAM-S.	a phage product for control of *Salmonella* on poultry, eggs, red meat, fruits, vegetables, fish, and shellfish
GRN No. 603	SalmoPro®	a phage product for control of *Salmonella* on poultry products
GRN No. 435	SalmoFreshTM	a phage product for control of *S. enterica* on poultry, fish and shellfish, and fresh and processed fruits and vegetables
GRN No. 468	SalmonelexTM	a phage product for control of *Salmonella* in pork and poultry products
GRN No. 528	ListShieldTM	a phage product for control of *L. monocytogenes* in fish and shellfish, fresh

7/ Luong T, Salabarria AC, Roach DR. Phage Therapy in the Resistance Era: Where Do We Stand and Where Are We Going? *Clin Ther.* 2020 Sep;42(9):1659-1680. doi: 10.1016/j.clinthera.2020.07.014. Epub 2020 Aug 31. PMID: 32883528.

8/ Forti F.Roach D.R.Cafora M.et al.Design of a broad-range bacteriophage cocktail that reduces Pseudomonas aeruginosa biofilms and treats acute infections in two animal models. *Antimicrob Agent Chemother.* 2018; 62 (e02573-17).

		and processed fruits and vegetables, and dairy products
GRN No. 218	ListexTM	a phage product for control of *L. monocytogenes* in poultry products

6.2 Lytic Phages Are Inherently Safe

There are two major types of phages: "virulent" (also called "lytic") and "temperate" (also called "lysogenic"). Lytic phages generally do not cross species or genus boundaries, and will therefore not affect desired bacteria in foods, commensals in the gastrointestinal tract, or accompanying bacterial flora in the environment. Lytic phages are normal commensals of humans and animals. 9/ Lytic phages can be used to lyse specific pathogens without disturbing normal bacterial flora and phages pose no risk to anything other than their specific bacterial host. 10/ 11/ As such, all lytic phages (including SP8) are, by nature, safe for human.

6.3 Phages Are Ubiquitous

Phages are ubiquitous, humans not only come into contact with them, but constantly consume and release them. The estimated phage abundance ranges from 10^6 to 10^8 mL^{-1}. 12/ 13/ Phages are found from human faecal and oral samples, faecal samples from other animals, freshwater lakes and rivers, marine ecosystems, sediments, hot springs, soils, deep subsurface habitats and the built environment. 14/ Phages are extremely common in environment and regularly consumed in foods. 15/ In all environments, phages exist as part of a complex microbial ecosystem which may be either a free-living environment such as the ocean, or a microbial environment within

9/ Carlton R M, Noordman W H, Biswas B, et al. Bacteriophage P100 for control of Listeria monocytogenes in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology & Pharmacology*, 2005, 43(3):301-312.

10/ Guo Z, Lin H, Ji X, et al. Therapeutic applications of lytic phages in human medicine. *Microbial Pathogenesis*, 2020, 142:104048.

11/ Schenk M. [Bacteriophages: an alternative to antibiotics?]. *Deutsche Medizinische Wochenschrift*, 2014, 139(4):124-5.

12/ BREITBARTL M, ROHWER F. Here a virus, there a virus, everywhere the same virus? *Trends Microbiol*, 2005, 13(6):278-284.

13/ SÄWSTRÖM C, GRANÉLI W, LAYBOURN-PARRY J, et al. High viral infection rates in Antarctic and Arctic bacterioplankton. *Environ Microbiol*, 2007, 9(1):250-255.

14/ Al-Shayeb B, Sachdeva R, Chen L X, et al. Clades of huge phage from across Earth's ecosystems. *Nature*.

15/ Bergh, Børsheim K Y, Bratbak G, et al. High abundance of viruses found in aquatic environments. *Nature*, 1989, 340(6233):467-468.

a macroorganism. 16/ Indeed, phages have been commonly found in human gastrointestinal tract, skin and mouth, where they are harbored in saliva and dental plaques. 17/

6.4 SP8 Is Strictly Lytic, and Lacks any Virulence or Undesired Genes

Phages are made up of relatively simple proteins and DNA. The toxicity is low, and many studies have shown that phages are harmless to humans and animals. SP8 is the most closely related to SHWT1, and its sequence similarity is 97% according to 90% query coverage. No toxin genes, virulence genes, antibiotic resistance genes and integrase genes were found in SHWT1 phage genome, indicating that this phage can be potentially used as a food additive. 18/ Bioinformatic analysis of the phage genome sequence also reveals that SP8 is strictly lytic and lacks any virulence, or undesired genes as identified in GenBank.

6.5 GRAS Status of Starting Materials

The growth medium for producing SP8 contains only ingredients that can be considered GRAS for the intended uses. Examples of these components include peptones (21 CFR §184.1553), yeast extracts (21 CFR §184.1983), dextrose (21 CFR §168.110), sodium chloride (21 CFR §182.70), and phosphates (21 CFR §182.1778). Sodium hydroxide (21 CFR §582.1763) is also used to adjust pH of the medium during fermentation. These components are mostly washed away during down-stream processing with PBS.

6.6 Undesirable Host-Derived Components

The host strain of *Salmonella* used for amplification of phages is non-virulent and does not encode any enterotoxin genes. They are also removed post-fermentation by filtration and SP8 is verified to be devoid of live bacterial during quality control as specified in section 2.6.

The host strain used for phage amplification is a gram-negative bacteria, which has an

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16/ Martha R.J. Clokie, Andrew D. Millard, Andrey V. Letarov & Shaun Heaphy (2011) *Phages in nature, Bacteriophage*, 1:1, 31-45, DOI: 10.4161/bact.1.1.14942.

17/ Bachrach G, Leizerovici-Zigmond M, Zlotkin A, et al. Bacteriophage isolation from human saliva. *Letters in Applied Microbiology*, 2010, 36(1):50-53.

18/ Tao C, Yi Z, Zhang Y, Wang Y, Zhu H, Afayibo DJA, Li T, Tian M, Qi J, Ding C, Gao S, Wang S and Yu S(2021) Characterization of a Broad-Host-Range Lytic Phage SHWT1 Against Multidrug-Resistant Salmonella and Evaluation of Its Therapeutic Efficacy *in vitro* and *in vivo*. *Front. Vet. Sci.* 8:683853.doi: 10.3389/fvets.2021.683853.

outer membrane containing LPS and may also produce other endotoxins. During manufacturing in the filtration phase, the culture media is washed with PBS to remove most of the endotoxins. Endotoxins levels are also measured before release, with the maximum limit set as 2,500 EU/mL. The maximum amount of theoretical endotoxin intake from the use of SP8 only constitutes around 0.001% of the daily endotoxin load from human saliva.

6.7 Summary and Basis for GRAS

SP8 consists of one naturally occurring lytic phage that has specificity to lyse various serovars of *Salmonella enterica*. The phage is strictly lytic and does not contain any virulence or undesired genes. Each phage production is also required to pass specifications to ensure the safety of the final product. FDA in the past favorably reviewed other bacteriophage products for pathogen reduction, and SP8 is similar to these products. The 90th percentile EDI of SP8 from the intended use is 1.09×10^{-6} g/person/day or 1.09 µg/person/day, which is even lower than the "threshold of regulation" dietary exposure level of 1.5 µg/person/day under 21 CFR §170.39, and can be considered presenting no health or safety concern at this *de minimis* level. Further, the host strain of *Salmonella* used for amplification of phages is non-virulent and does not encode any enterotoxin genes. The maximum amount of theoretical endotoxin intake from the use of SP8 only constitutes around 0.001% of the daily endotoxin load from human saliva and is thus considered safe.

Based on genetic, biological, and chemical analysis, SP8 is considered safe as it is a strictly lytic phage absent of undesirable genes, has low endotoxin levels, and devoid of bacterial contamination according to the specifications. SP8 is also demonstrated to be effective in reducing *Salmonella* in ground chicken.

QPB has reviewed the available data and information, and is not aware of any data and information that are, or may appear to be, inconsistent with our conclusion of GRAS status.

Part 7: List of Supporting Data and Information

- Magalhães PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TC, Pessoa A Jr. Methods of endotoxin removal from biological preparations: a review. *J Pharm Pharm Sci.* 2007;10(3):388-404. PMID: 17727802.

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- H. W. Ackermann, D. Tremblay, and S. Moineau.Long-term bacteriophage preservation. *World Fed Cult Collect Newsl*. 2004;38:35-40.

- Suttle CA, Chen F. Mechanisms and rates of decayof marine viruses in seawater. *Appl Environ Microbiol*. 1992;58(11):3721-3729.

- Luong T, Salabarria AC, Roach DR. Phage Therapy in the Resistance Era: Where Do We Stand and Where Are We Going? *Clin Ther.* 2020 Sep;42(9):1659-1680. doi: 10.1016/j.clinthera.2020.07.014. Epub 2020 Aug 31. PMID: 32883528.

- Forti F.Roach D.R.Cafora M. et al. Design of a broad-range bacteriophage cocktail that reduces Pseudomonas aeruginosa biofilms and treats acute infections in two animal models. *Antimicrob Agent Chemother.* 2018; 62 (e02573-17).

- Carlton R M, Noordman W H, Biswas B, et al. Bacteriophage P100 for control of Listeria monocytogenes in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology & Pharmacology*, 2005, 43(3):301-312.

- Guo Z, Lin H, Ji X, et al. Therapeutic applications of lytic phages in human medicine. *Microbial Pathogenesis*, 2020, 142:104048.

- Schenk M. [Bacteriophages: an alternative to antibiotics?] *Deutsche Medizinische Wochenschrift*, 2014, 139(4):124-5.

- BREITBARTL M, ROHWER F. Here a virus, there a virus, everywhere the same virus? *Trends Microbiol*, 2005, 13(6):278-284.

- SÄWSTRÖM C, GRANÉLI W, LAYBOURN-PARRY J, et al. High viral infection rates in Antarctic and Arctic bacterioplankton. *Environ Microbiol*, 2007, 9(1):250-255.

- Al-Shayeb B, Sachdeva R, Chen L X, et al. Clades of huge phage from across Earth's ecosystems. *Nature*.

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- Martha R.J. Clokie, Andrew D. Millard, Andrey V. Letarov & Shaun Heaphy (2011) *Phages in nature*, *Bacteriophage*, 1:1, 31-45, DOI: 10.4161/bact.1.1.14942.

- Bachrach G, Leizerovici-Zigmond M, Zlotkin A, et al. Bacteriophage isolation from human saliva.*Lett Appl Microbiol*, 2003,36(1):50-53.

- Tao C, Yi Z, Zhang Y, Wang Y, Zhu H, Afayibo DJA, Li T, Tian M, Qi J,Ding C, Gao S, Wang S and Yu S(2021) Characterization of a Broad-Host-Range Lytic Phage SHWT1 Against Multidrug-Resistant Salmonella and Evaluation of Its Therapeutic Efficacy in vitro and in vivo. *Front. Vet. Sci.* 8:683853.doi: 10.3389/fvets.2021.683853.

Appendix A

PCR Testing of Production Bacteria C1106

Materials:

• C1106（self-made in the laboratory with a concentration of 1×10^{9}CFU/ml）

Methods:

Stn primers (F: 5'-TTG TGT CGC TAT CAC TGG CAA CC-3', R: 5'-ATT CGT AAC CCG CTC TCG TCC-3') were used to detect enterotoxin genes [1].

DNA extraction was carried out through a heat treatment. Amplifications and afterwards electrophoresis was performed in 1% agarose gel electrophoreses by using target genes, amplicons sizes and cycling conditions showed in Table (1).

Table 1 Target genes, amplicons sizes and cycling conditions

Target gene	Amplified segment (bp.)	Primary Denaturation	Amplification (30 cycles)			Final extension
			Secondary denaturation	Annealing	Extension	
Stn	617	95˚C 3 min.	95˚C 15sec.	60˚C 30sec.	72˚C 42sec.	72˚C 5min.

Results:

The test results showed that strain C1106 for production had no enterotoxin genes.

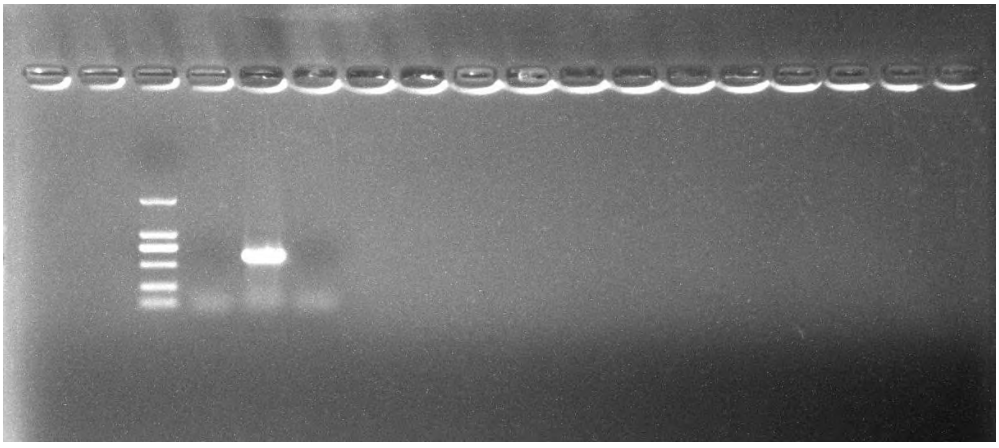


Fig 1: Results of PCR identification of C1106 strain

M：BM2000 DNA Marker；1：C1106；+：positive control；—：negative control.

Conclusion:

PCR results showed that strain C1106 had no enterotoxin genes.

Reference：

[1] Abdelaziz I, El-Tawab A A, Maarouf A ,et al. Bacteriological and molecular studies on Salmonella isolatedfrom duckling farmsatKaliobia, Egypt. Benha Veterinary Medical Journal, 2020(1).

Appendix B

Study: Determination of the effectiveness of SP8 on ground chicken experimentally contaminated with Tyhpimurium, Enteritidis, Infantis

Objective: Compare the levels of *Salmonella* between untreated or SP8 treated ground chicken

Materials:

• chicken breast

• LB broth

• Buffered peptone water (BPW)

• XLD agar

• electrostatic sprayer

• SP8

• *Salmonella* cocktail (1:1:1 ratio) consisting of *Salmonella* enterica subsp. enterica serovars Typhimurium, Enteritidis, and Infantis

General Procedures:

1.Skinless chicken breasts were aseptically cut into 100 g pieces.

2. The *Salmonella* cocktail was diluted to 10^7 CFU/mL, and 1 mL was applied onto the chicken surface evenly. For non-inoculated chicken, 1 mL of BPW was applied instead.

3. Chicken breast pieces were left for 10 min to allow for bacterial attachment.

4. An electrostatic sprayer was used to apply BPW or SP8 onto chicken breast pieces.

5. After a 5 min incubation, chicken breast pieces were grounded with a meat grinder. Grinder equipment parts that were in contact with the meat were cleaned thoroughly between samples, and separate parts were used for untreated and SP8 treated samples to minimize cross-contamination.

6. 10 g of ground chicken breast was put into a sterile stomacher bag with filter.

7. 10 mL of BPW was added into the stomacher bag, and homogenized for 1 min.

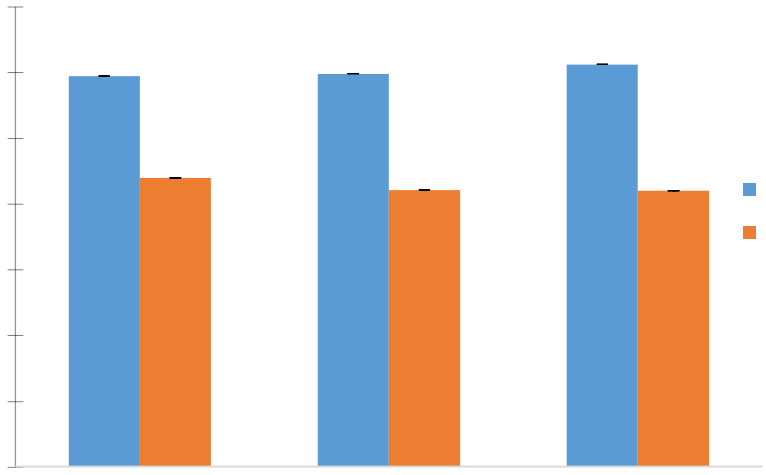
8. Viable *Salmonella* was determined by standard plating the appropriate dilutions of the homogenate on XLD agar plates.

Results:

Table 1: Level of *S. enterica* in experimentally contaminated chicken breast not-treated or treated with SP8. Triplicate samples were stored for 0.5, 1, or 2 hours before surface bacterial extraction.

Hour(s) of incubation at 37°C	Concentration of *S. enterica* (CFU/g)	After SP8 treatment (CFU/g)	Log reduction
0.5	8.91×10^5	2.51×10^4	1.55
1	9.75×10^5	1.64×10^4	1.77
2	1.35×10^6	1.61×10^4	1.92

Reduction of *S.enterica* serovars in chicken breast by SP8



Conclusions:

The reduction of *Salmonella* in experimentally contaminated ground chicken breast was assessed. SP8 was applied on the chicken breast trim prior to grinding, leading to a reduction of 1.5 to 1.9 logs reduction throughout 2 hours of storage at 37℃. In addition, there was no increase in the level of *Salmonella* in SP8 treated samples during the storage time, which suggests that the initial *Salmonella* reduction at "hour 0.5" was a result of irreversible killing by the phage. These results suggest that it is possible to apply SP8 on meat trims prior to grinding to reduce the *Salmonella* load in the grounded product.

Appendix C

Food Code	Food Name
24198671	Chicken patty, breaded
24198677	Chicken fillet, breaded
24198683	Chicken fillet, grilled
24198729	Chicken nuggets, NFS
24198731	Chicken nuggets, from fast food
24198732	Chicken nuggets, from restaurant
24198735	Chicken nuggets, from school lunch
24198736	Chicken nuggets, from frozen
24198737	Chicken nuggets, from other sources
24198739	Chicken tenders or strips, NFS
24198741	Chicken tenders or strips, breaded, from fast food
24198742	Chicken tenders or strips, breaded, from restaurant
24198745	Chicken tenders or strips, breaded, from school lunch
24198746	Chicken tenders or strips, breaded, from frozen
24198747	Chicken tenders or strips, breaded, from other sources

Appendix D

Weight of Strain SP8 Phage Calculation

	kilo-basepairs (k-bp)	weight of phage bp x660 (Da)	weight of phage (g)
SP8	43.823	28923180	4.80×10^{-17}

43823×660 = 28923180 Da

28923180 ÷（6.02214076×10^{23}）=4.80×10^{-17} g

Response to GRN 1134 FDA Questions

For your easy reference, we have copied FDA questions below, followed by Qingdao Phagepharm Bio-Tech Co., Ltd (QPB)'s response.

1. *Question #1: Please indicate if the Salmonella phage preparation is intended to be used in infant formula.*

QPB Response：The *Salmonella* phage preparation is not intended to be used in infant formula.

2. *Question #2: On page 8, you discuss a challenge study where Salmonella serovars were mixed equally and applied to pre-ground chicken meat. You then state that the phage prep was sprayed onto the pre-ground chicken. It is not clear if the description of pre- ground on page 8 means that the chicken was ground and then the ingredient was applied or if it means that the ingredient was applied to the chicken prior to being ground. Please clarify whether the Salmonella phage preparation is intended for use on chicken prior to being ground or after the chicken is ground.*

QPB Response：We hereby clarify that the *Salmonella* phage preparation is intended for use on chicken prior to being ground. In the challenge study, we also applied the phage preparation on chicken prior to being ground.

3. *Question #3: On page 8 of the notice, you state that the Salmonella phage preparation is manufactured using food grade and animal-product free raw materials. Please confirm that the ingredient is manufactured according to current good manufacturing practices and that all materials used in the manufacturing processes are used in accordance with current U.S. regulations, are GRAS for their intended use, or are the subject of an effective food contact notification.*

QPB Response：We hereby confirm that the ingredients of our phage preparation are manufactured according to current good manufacturing practices and that all materials used in the manufacturing processes are used in accordance with the current U.S. regulations, are GRAS for their intended use, or are the subject of an effective food contact notification.

4. *Question #4: You also state on page 8 that residual endotoxins are expected to be removed during clarification and extensive washing after micro- and sterile filtration. Please provide a brief description of the clarification and washing steps.*

QPB Response：The clarification steps noted on page 8 involve the removal of the culture by centrifugation at 12,000 r/min to remove bacterial debris and further purification with sterile filtration through a 0.22 μm filter membrane. The washing steps involve the ultrafiltration by washing the phage with phosphate buffered saline (PBS) buffer.

5. *Question #5: In Table 2, you list the specifications for the ingredient and provide results from the analyses of three non-consecutive batches. The results for arsenic, lead and mercury are listed as "Not detected (ND)." Please indicate the limit of detection for the method used to analyze for these heavy metals and confirm that ND represents values below the LOD. We also note that specifications for heavy metals should be as low as possible and representative of the results of your batch analyses to align with FDA's Closer to Zero initiative of reducing dietary exposure to heavy metals from food.*

QPB Response：The detection limits of the methods used to analyze arsenic, lead, and mercury are 0.02 mg/kg, 0.02 mg/kg, and 0.01 mg/kg, respectively. We hereby also confirm that ND represents values below the LOD. The specifications are established based on these detection limits to keep the heavy metals levels as low as possible.

6. *Question #6: You state that the intended use of Salmonella Enteritidis phage preparation strain (Strain SP8) is in ground chicken. However, the National Health and Nutrition Examination Survey (NHANES) food codes listed in Appendix C are for chicken patties, fillets, nuggets, tenders, and strips and do not contain the food code for ground chicken. Please indicate why the food code for ground chicken was not included in the dietary exposure estimate and revise the dietary exposure accordingly to reflect all food codes in which Salmonella phage preparation could be used.*

QPB Response: As the NHANES food codes cover various food items consumed by the consumers, instead of using a single food code for "ground chicken" (we also did not identify such food code in the NHANES 2017-2018 dataset), we used the food codes for various food items (e.g., chicken patties, fillets, nuggets) that may contain ground chicken as a main component. This approach very conservatively assumes that 100% of these food items are made from ground chicken, and the 90th percentile consumption we used for ground chicken in GRN 1134 was 113.51 g/person. We note this daily consumption is higher than the poultry daily consumption of 75.1 g/day referenced in GRN 917, which was favorably reviewed by the agency in 2020.

We recognize the food codes referenced in Appendix C may not cover all potential food applications of ground chicken in foods. Another database, Food Commodity Intake Database (*available at*: https://fcid.foodrisk.org/), tracks the food commodity consumption (as opposed to food items). The database translates food consumption as reported eaten in What We Eat in America (WWEIA) (2005-2010 survey cycles) into consumption of U.S. EPA-defined food commodities, including chicken meat. While the database does not track "ground chicken" consumption, the 90th percentile "chicken, meat" consumption for "eaters only" population is reported as 104.9 g/person (*see* **Appendix 3**), smaller than the conservative 113.51 g/person we use for our assessment in GRN 1134.

If we conservatively adopt the 104.9 g/person "chicken, meat" consumption for the purpose of our dietary exposure assessment, and follow the same assumptions we made in GRN 1134, the 90th percentile estimated daily intake (EDI) of SP8 from the intended use can be calculated as:

$$2\times10^{8}\ \text{PFU/g} * 104.9\ \text{g/person/day} * 4.80\times10^{-17}\ \text{g/PFU} = 1\times10^{-6}\ \text{g/person/day}.$$

The updated 90th percentile EDI of 1×10^{-6} g/person/day is slightly lower than the previously calculated 1.09×10^{-6} g/person/day, further confirming the conservativeness of the approach, and would not change our conclusion that the intended use can be considered safe. The updated level is still lower than the "threshold of regulation" dietary exposure level of 1.5 µg/person/day, and can be considered presenting no health or safety concern at this *de minimis* level.

7. *Question #7: You state on page 5 that the phage is diluted in sterile water so that the Salmonella phage preparation has a minimum total phage concentration of 1×10^{10} PFU/mL. This solution is then applied at a rate of 0.5-2% v/w at the discretion of the food manufacturer. You indicate that this corresponds to a maximum use level of 2×10^{8} PFU/g of food. We note that the results of the batch analyses indicate that the concentration of the Salmonella phage preparation ranges from $6.7x10^{10}$ to $7.5x10^{10}$ PFU/mL. Even if this solution was applied at the lowest amount of 0.5%, this would result in a use level greater than 2×10^{8} PFU/g of food. Please explain this inconsistency and indicate the maximum use level for the Salmonella phage preparation. If it is greater than 2×10^{8} PFU/g of food, please revise the dietary exposure accordingly.*

QPB Response: Please note that before the application, the phage preparation is diluted in sterile water so that the *Salmonella* phage preparation has a minimum total phage concentration of 1×10^{10} PFU/mL. This solution is then applied at a rate of 0.5-2% v/w at the discretion of the food manufacturer. If this solution was applied at the highest amount of 2%, this would result in a use level no greater than 2×10^{8} PFU/g of food = 2% v/w × 1×10^{10} PFU/mL.

8. *Question #8: On page 7, you provide a specification for Kjeldahl of <1000 mg/L. Please indicate the purpose of this specification for the Salmonella phage preparation.*

QPB Response: On page 7, we provide a specification for Kjeldahl Nitrogen of <1000 mg/L. This specification is provided to determine whether there is any residual nitrogen in the product. Nitrogen source is generally added to the fermentation medium. A low residual level indicates that the nitrogen source is well utilized during the fermentation process.

9. *Question #9: On page 6, you state that the phage was "saved in China General Microbiological Culture Collection Center, CGMCC." For the administrative record, please confirm whether the phage was deposited in CGMCC and provide the deposit designation number.*

QPB Response: We hereby confirm that the phage was deposited in CGMCC and the deposit designation number is CGMCC NO. 45256.

10. *Question #10: On page 6, you state that the phage is "amplified in a non-virulent strain of Salmonella Enteritidis named C1106…" For the administrative record, please state whether the Salmonella production strain is deposited in a repository and provide the deposit designation number.*

QPB Response: The *Salmonella* production strain C1106 is currently deposited in an internal repository.

11. *Question #11: On page 6, you state that the "phage genome sequence reveals that it is strictly lytic and lacks any virulence, or undesired genes as identified in GenBank." For the administrative record, please clarify what you mean by "undesired genes."*

QPB Response: The term "undesired genes" on page 6 refers to antibiotic resistance genes, integrase genes and lysogenic genes.

12. *Question #12: For the administrative record, please state whether the phage is capable of genetic transfer.*

QPB Response: We hereby confirm that the phage is not capable of genetic transfer.

13. *Question #13: For the administrative record, please confirm that Salmonella production strain is non-pathogenic and non-toxigenic and please briefly discuss (with relevant references, as appropriate) the phenotypic characteristics of the strain (e.g., production of antimicrobials, production of secondary metabolites, antimicrobial resistance), and whether these pose a safety concern. Additionally, please state whether the Salmonella production strain is capable of genetic transfer.*

QPB Response: We hereby confirm that *Salmonella* production strain is non-pathogenic and non-toxigenic. *Salmonella* production strain does not contain SPI-1, SPI-2 virulence genes and enterotoxin genes. The strain is only resistant to erythromycin, polymyxin and rifampicin (*see* **Table 1**). These characteristics do not pose any safety concern. Further, the *Salmonella* production strain is also not capable of genetic transfer.

Table 1. Antimicrobial resistance test results of *Salmonella* production strain		
Antibiotic name	Paper content	Antibiotic resistance
penicillin	10U /piece	S
amoxicillin	20μg /piece	S
cefotaxime	30μg /piece	S
ofloxacin	5μg /piece	S
gentamycin	10μg /piece	S
erythromycin	15μg /piece	R
tetracycline	30μg /piece	S
polymyxin	300IU /piece	R
rifampicin	5μg /piece	R
selectrin	25μg /piece	S
chloramphenicol,	30μg /piece	S
kanamycin	30μg /piece	S
furantoin	300μg /piece	S
naphthalinic acid,	30μg /piece	S

14. *Question #14: For the administrative record, please state whether the phage or the Salmonella production strain are genetically engineered.*

QPB Response: Neither the phage nor the *Salmonella*-producing strains are genetically engineered.

15. *Question #15: For the administrative record, please briefly describe the in-process controls you have in place during the fermentation process and clarify how contamination is controlled for during the manufacturing process. Additionally, please state whether the fermentation process is conducted in a contained, sterile environment.*

QPB Response: Growth of the bacteria and subsequent lysis of that bacteria by the phage is also monitored through spectrophotometer as an in-process control. We also hereby confirm that the fermentation process is conducted in a contained, sterile environment. Further, during the manufacturing process, the operators strictly follow the process rules and standard operating procedures (SOPs) for production operation to prevent contamination. In particular, during the process of bacterial and phage seed preparation, inoculation and fermentation, containers, pipelines, tools and culture medium were all treated with high temperature sterilization. Closed pipelines were used for any liquid transfer to reduce the risk of contamination.

16. *Question #16:* Please identify if any materials used in the production of the *Salmonella* phage preparation that are major allergens or derived from major allergens and whether they pose as safety concern. If none of the raw materials used in the manufacturing process are major allergens or are derived from major allergens, please provide a statement of affirmation.

QPB Response: We hereby confirm that none of the raw materials used in the manufacturing process are major allergens or are derived from major allergens.

17. *Question #17: For the administrative record, please briefly describe how the purity of the Salmonella production strain and the phage are ensured.*

QPB Response: To ensure the purity of *Salmonella* production strain, the host bacteria used for production were tested for specificity and purity. The original *Salmonella* production strain was freeze-dried and stored at -80 °C. Freeze-dried host bacteria were produced by multiple serial passages of a single colony, which ensured the purity of the frozen stock. For each production cycle, the strain was streaked out on an agar

plate and a single colony was used to start a pre-culture to be used in the fermentation process. Therefore, the host bacterial inoculum used for the production was always from the original master pool. Further characterization included a sensitivity analysis using a panel of antibiotics, as described in Section 2.2 of GRN 1134. Impurity of the host bacteria can be detected by the different antibiotic resistance.

To ensure the purity of the phage, the seeds and final products of the phage were tested for specificity and sterility. In addition, the fermentation process was conducted in a contained, sterile environment to avoid contamination during the manufacturing process. Stocks of bacteriophage lysates were sterilized by 0.22 μm filtration and stored at 4°C. Immediately before use of the fermentation process, the bacteriophage stocks were sterilized anew by 0.22 μm filtration to minimize the risk of contamination. The concentration, endotoxin levels, and sterility of each phage production batch are measured to control the quality (*see* Table 2 of GRN 1134).

18. *Question #18: On page 8, you provide specifications for a bacterial sterility test. For the administrative record, please clarify whether this test also captures yeast and mold.*

QPB Response：The bacterial sterility test includes the detection of yeast.

FCID Consumption Calculator Reports

Eaters Only Population

Two-Day Average Consumption Commodity Mass (g) per Day (d)

			N	Mean	SE	1%	5%	10%	15%	20%	25%	30%	35%	40%	45%	50%	55%	60%	65%	70%	75%	80%	85%	90%	95%	97.5%	99%	Max
Chicken, meat																												
Age Range	**Gender**	**Race**																										
All ages	All	All	17,645	47.51	0.63	<0.05	0.2	4.3	8.2	12.2	16.3	20.5	23.9	27.6	31.7	35.9	41.0	45.2	50.8	57.8	65.2	75.5	86.9	104.9	133.5	163.2	202.0	461.9†

Notes: '†' indicates estimates are less statistically reliable based on np < 8 * 'Design Effect' guidance published in the Joint Policy on Variance Estimation and Statistical Reporting Standards on NHANES III and CSFII

The **"Two-day average"** results are based on the average of the two days of food consumption reported in the NHANES/WWEIA survey for those "both day" respondents. If the respondent reports zero consumption on one of the two days and non-zero consumption on the other day, his/her average consumption would be the average of zero and nonzero consumption.

Calculation performed on 10/13/2023 using FCID-WWEIA data for years 2005-2010 from https://fcid.foodrisk.org/percentiles

From: Tao, Xin
To: Santos, Marissa
Subject: [EXTERNAL] RE: GRN 1134 - Question Regarding Salmonella Phage Preparation
Date: Wednesday, January 24, 2024 10:34:09 PM
Attachments: image001.png

Dear Marissa -

Please find the response to your question below. Please let me know if you have any follow-up questions.

Regarding the names of *Salmonella* species mentioned on pages 6, 17, and 22: *Salmonella Enterica* is a species of *Salmonella*, and *Salmonella Enteritidis* is a subspecies of *Salmonella Enterica*. On page 22, the specific strain used in the experiment is detailed to demonstrate the materials. Our bacteriophages are broad-spectrum against *Salmonella Enterica* （as stated in page 6 of the note, and the table from the Responses: "A list of the *Salmonella* strains"), hence in page 17, the summary section, "*Salmonella Enterica* " is used.

Best regards,
Xin

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Empowering Global Life Science Innovation through Legal Compliance™

From: Santos, Marissa <Marissa.Santos@fda.hhs.gov>
Sent: Monday, January 22, 2024 3:17 PM
To: Tao, Xin <Xin.Tao@bakermckenzie.com>
Subject: [EXTERNAL] GRN 1134 - Question Regarding Salmonella Phage Preparation

Hi Mr. Tao,

I have one question as we work to finalize our response to GRN 1134.

1. On page 6, the notice states that the *Salmonella* phage preparation was tested for lytic

activity against "85 *Salmonella Enteritidis* strains including strains of Enteritidis, Typhimurium, and other types of *Salmonella* were tested." Additionally, the notice states on page 22 that a *"Salmonella* cocktail (1:1:1 ratio) consisting of *Salmonella* enterica subsp. Enterica serovars Typhimurium, Enteritidis, and Infantis" was used in the efficacy study. Further, on page 17, the notice states that the phage preparation has the "specificity to lyse various serovars of *Salmonella enterica*." For the administrative record, please clarify whether the phage preparation is specific to *Salmonella enterica* or *Salmonella* Enteritidis.

Please let me know if you have any questions.

Regards,
Marissa

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