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July 19, 2019

GRN 888
RECEIVED
JUL 22 2019
OFFICE OF
FOOD ADDITIVE SAFETY

BY FEDERAL EXPRESS
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 Notification for GPI Biotech VAM-S**

Dear Sirs:

This follows our GRAS submission made on behalf of Gum Products International, dated July 18, 2019, which was received by the agency on July 19, 2019. We note that our July 18 submission was an old version and did not include all information as intended by Gum Products International. We ask that The Office of Food Additive Safety accept the enclosed submission, to replace and supersede our submission dated July 18, 2019.

The enclosed GRAS Notification is for GPI Biotech VAM-S to be used as a food ingredient when used in the applications and under the conditions of use described herein. In compliance with 21 C.F.R. §170.210(b), we are enclosing one original paper version of this notice.

Should you have any questions regarding this Notice, please do not hesitate to contact me.

Sincerely,

J. Mason Weeda

OFW:ap
Enclosure: VAM-S GRAS Submission

VAM-S GRAS APPLICATION

PART I

GRAS exemption claim

A. Claim of Exemption From The Requirement for Premarket Approval Requirements Pursuant to 21 CFR§170.36(c)(1).

GPI Biotech VAM-S was determined by GPI to be generally recognized as safe through scientific procedures, and therefore exempt from the requirement of premarket approval, under the conditions of intended use as described below. The basis for this finding is described in the following sections.

Signed

Dated

July 15, 2019

Henry Liu
Technical Director
Gum Products International

B. Name and address of Notifier

Gum Products International
1255 Journey's End Circle
Newmarket, Ontario L3Y 837
CANADA

C. Common or Usual Name of the Notified Substance

GPI Biotech VAM-S

D. Conditions of Use

The intended use of GPI Biotech VAM-S is as an antimicrobial on food to control Salmonella at an application rate of up to 1×10^{10} PFU/mL (plaque forming units) per gram of food.

E. Basis for the GRAS Determination

Pursuant to 21 CFR§170.30, GPI Biotech has determined that GPI Biotech VAM-S is GRAS through scientific procedures. To the best of our knowledge, this GRAS notice is complete, representative, and balanced that includes favorable and unfavorable information pertinent to the evaluation of the safety and GRAS status of the use of GPI Biotech VAM-S.

F. Freedom of Information Act

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

G. Availability of Information

All data and information that serve as basis for this GRAS determination is available for the Food and Drug Administration review or will be sent to the agency upon request, made to:

Henry Liu
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1 (905) 853-8828 (Work)
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PART II

A) Phage Identity and Host Range

GPI Biotech VAM-S consists of a mixture of 3 bacteriophages (phages) that were isolated from chicken cloacae and pig rectal swabs from farms in different geographical areas of Spain. All 3 phages are *Salmonella*-specific virulent phages that has the ability to lyse a wide range of *Salmonella enterica* serovars.

The phages are named Phi_16, Phi_78, and Phi_87. Electron microscopy reveals all 3 phages belong to the order of *Caudovirales*. The morphology of Phi_16 is compatible with the *Siphoviridae* family, with a non-contractile flexible tail. Phi_78 has an icosahedral head and non-contractile short tail, indicating it belongs in the *Podoviridae* family. Electron micrographs of Phi_87 revealed an icosahedral head with long, ridged, and contractile tail, which are particular characteristics of the *Myoviridae* family. The Phi_16 capsid has a diameter of approximately 60.17 ± 4.07 nm and a tail length of 130 ± 1.41 nm. The Phi_78 capsid is 66 ± 1.7 nm with a tail length of 14 ± 0.7 nm. Phi_87 has a capsid diameter of 68 ± 2.7 nm with a tail length of 114 ± 4.3 nm.

The genome sizes of Phi_16, Phi_78, and Phi_87 range from 45.5 kilo-base pairs (kb) and 88.0 kb. Each of the 3 genomes do not include any known genes encoding virulence, toxins, and allergens. Genes required for lysogenic activity are also not detected in any of the 3 genomes.

Host range studies were conducted by Universitat Autònoma De Barcelona. Lytic activity was demonstrated in 73 clonally unrelated strains of *S. enterica* serovars Enteritidis, Hadar, Infantis, Newport, Typhimurium, and Virchow.

B) Host Identity

All 3 phages are amplified in a non-virulent strain of *Salmonella typhimurium*. Derivation of the non-virulent strain is from another strain of *S. typhimurium* named LT2 (Bullas and Ryu 1983). It is developed to have all three *hsd* genes that regulates the restriction-modification system mutated. The attenuated LT2 strain has been widely used in laboratories since its isolation. This strain is mutated in the *rpoS* gene, which is important for the bacteria to display an acid-tolerance response. Several studies have established that the *rpoS* mutation is the cause of the attenuation of the LT2 strain (Swords, Cannon, and Benjamin 1997; Wilmes-Riesenberg, Foster, and Iii 1997). Virulence of the LT2 strain can be recovered by inserting a functional *rpoS* gene by genetic recombination. The mutation in the non-virulent strain does not contribute any virulence to the otherwise avirulent strain.

C) Method of Manufacture

The 3 phages are amplified separately with the non-virulent host into high concentrations for commercial distribution. A suitable volume of host bacteria is grown to a pre-determined optical

density (OD) to use as inoculum for the large volume of aerobic fermentation used for phage amplification. For each phage, an optimized amount of phage to host bacteria ratio, or multiplicity of infection, have been worked out to yield the highest possible concentration of phage after a specific amount of time for infection. This amount of phage is added to the fermenter when the host bacteria has reached a pre-determined OD, and then incubated to allow for repeated cycles of phage infection, amplification, and release. The entire content of the fermenter is then qualified for concentration, purity, and sterility (**Error! Reference source not found.**). VAM-S is then created by blending these 3 phages to a final phage concentration of approximately 1×10^{10} PFU/mL. After blending, VAM-S is qualified again for concentration, level of endotoxin, and sterility. It is then stored in refrigerated conditions. Before application, VAM-S can then be diluted as desired to achieve the desired level of *Salmonella* reduction.

D) Specifications

Each phage production batch is quality controlled for concentration, purity, endotoxin level and sterility. After the final product of VAM-S is blended, the concentration and sterility is again measured to ensure conformity to pre-determined specifications (**Error! Reference source not found.**).

The phage concentration is measured using the double agar overlay plaque assay (Kropinski et al. 2009). Briefly, a titration of phage is mixed with an amount of host bacteria actively growing at early log phase in a tube containing a diluted agar medium. This is then poured onto an agar plate where the host bacteria will grow a confluent lawn. During the course of incubation at suitable conditions, phages will repeatedly infect, lyse, and then released to infect surrounding cells until a visible clearing can be viewed by the naked eye. Therefore, this process of enumeration also qualifies the infectivity of the phages. For a detailed protocol, see "Quantitative determination of *Salmonella* bacteriophage via plaque assay" in the Appendix.

For its sensitivity and specificity, the quantitative PCR (qPCR, also known as real-time PCR) is used to measure the purity of each production. The Phage DNA Isolation Kit (Norgen Biotek Corp.) was used to isolate and purify the phage DNA from each production. This kit utilizes spin-column chromatography, so the use of harsh chemicals usually required for nucleic acid isolation can be avoided. The manufacturer's protocol was followed. A nanodrop was used to measure the concentration of extracted DNA.

By creating a standard curve with known amounts of a specific phage DNA to correlate it to the number of PCR cycles to achieve a certain threshold of signal (C_t value), absolute quantification can be extrapolated. In addition, by extending the lower range of concentrations to close to undetectable levels, the limit of detection, where the lowest amount of DNA copy number that can be reliably detected, can be estimated (Armbruster and Pry 2008). By taking into consideration both the limit of detection and the final concentration of each phage in VAM-S, it is determined that contaminant signals of less than 10^5 copies (10^5 phages) will be approved for blending into the final product. For a detailed protocol, see "Molecular detection of VAM-S bacteriophages" in the Appendix.

Table 1: Quality Control Specifications

Parameters	
Concentration	$>10^{11}$ PFU/mL
Purity	$<10^{5}$ DNA copies from contaminant phages
Endotoxin	<2500 EU/mL
Bacterial sterility	No growth detected >14 days

The endotoxin content for each batch of phage production and in the final VAM-S blend is tested by using a quantitative endpoint limulus amebocyte lysate (LAL) assay. Results of less than 2500 EU/mL will be considered acceptable. Bacterial sterility is confirmed 14 days or after post-production or blending of final product by plating a small sample onto a non-selective growth agar plate (Luria-Bertani) and on a 3M aerobic count plate petri film. Both methods would detect aerobic bacteria including *Salmonella*. The absence of growth after an appropriate incubation time is necessary to pass these criteria.

Each individual phage production is tested for all parameters specified in table 1. If all requirements are satisfied, they are blended together with mulled vinegar for the final product. The final product is again tested for all parameters, except for purity.

E) Chemical Analysis

Table 2: Chemical Composition

	Units	Reportable Detection Limit	LOT # 1806251S	1806252S	1806253S	Method Reference
Arsenic	µg/g	0.01	0.01	0.01	0.01	EPA 6020B mod./200.3
Lead	µg/g	0.01	ND	ND	ND	EPA 6020B mod./200.3
Mercury	µg/g	0.005	ND	ND	ND	Health Canada Method
Sulphur	µg/g	20	120	130	120	AOAC 984.27
Kjeldahl Nitrogen	mg/L	20	820	820	790	OMOE E3516 m
Organic Carbon	mg/L	250	43000	44000	47000	SM 23 5310B m
Sulphate	mg/L	20	ND	ND	ND	EPA 375.4 m
Endotoxin	EU/mL	0.25	3.78	3.81	3.65	LAL Chromogenic Endotoxin Quantitation
ND = Not detected						

Three lots of VAM-S was tested for its chemical composition. The endotoxin test was conducted by GPI following the manufacturer's protocol. All other tests were conducted by Maxxam Analytics (Canada).

F) Undesirable host-derived components

The non-virulent *Salmonella* strain used for phage amplification is Gram-negative bacteria, which have an outer membrane containing lipopolysaccharides (LPS) and may also produce other endotoxins. Each

phage production lot is measured for its endotoxin level and only released for final blending if it is below the threshold level set out in the specification (Table 1).

PART III
Self-Limiting Levels of Use

The functional properties of VAM-S are phage-targeted killing of *Salmonella enterica*. Therefore, the proposed use of VAM-S is as an antimicrobial processing aid for food that is contaminated with *Salmonella*. VAM-S is self-limiting by nature as phages are susceptible to degradation from various environmental factors, such as temperature, acidity, salinity (H. W. Ackermann, D. Tremblay, and S. Moineau 2004). Phage inactivation can also occur by chemicals and enzyme breaking down the virion (Suttle and Chen 1992). Therefore, once the *Salmonella* targets are depleted, the phages will gradually degrade. Phages are composed of proteins and nucleic acids, making them inherently non-toxic (Kutter et al. 2010; Abedon and Thomas-Abedon 2010).

PART IV
Narrative

A) Background on *Salmonella* related illness

Foodborne illnesses remain a public health problem in both industrialized and developing countries (Käferstein and Abdussalam 1999). According to the Centre of Disease Control (CDC), non-typhoidal *Salmonella* is estimated to cause over 1 million illnesses and 450 deaths annually in the United States alone. Infection by *Salmonella* can cause mild to severe gastroenteritis, resulting in abdominal cramps, diarrhea, and fever. Invasive infections can also occur where the *Salmonella* infection spreads from the intestines to the blood stream, leading to a life-threatening illness.

Salmonella can be found in various foods, such as meats, fruits, vegetables, eggs, and processed foods. In addition, contamination can occur anywhere between pre-processing in the farm, to the site of consumption. For these reasons, *Salmonella* infection causes more hospitalizations and deaths compared to other food-borne pathogens ("Salmonella Homepage | CDC" 2019).

The use of antibiotics have played a vital role in agriculture to prevent or control pathogenic bacterial infections such as *Salmonella* to improve growth and production in the past 60 years (Economou and Gousia 2015). However, there is mounting evidence that the use of antibiotic use in food-producing animals is concomitant to the increase of antibiotic resistance in bacteria that cause human infections. Antibiotic-resistant bacteria can be transmitted to humans by the consumption of infected foods treated with antibiotics, transmitting resistant bacteria to the human chain. This can lead to illnesses that are difficult to treat, thereby increasing morbidity and mortality rates. In addition, fecal waste from animals treated with antibiotics can be composted and spread as fertilizer, allowing antibiotic resistant bacteria to spread into the environment (Economou and Gousia 2015).

B) Phage background

Phages are natural predators of bacteria in nature, found anywhere bacteria are present (R Young 1992; Ryland Young 2014). Each phage often targets only specific species of bacteria, while having no effect on other bacteria, human, animal, or plant cells. In natural environments, phages and their bacterial hosts are involved in continuous cycles of co-evolution. The bacteria can adopt various changes biochemically or structurally to resist phage infection, but unlike antibiotics, phages also have mechanisms to counter bacterial resistance (Samson et al. 2013; Hyman and Abedon 2010).

Since its discovery, phages have been used to treat human diseases such as dysentery, food poisoning, typhoid fever, and various other infections in the former Soviet Union, Poland, France, and Georgia (Chanishvili 2012). Therefore, the utilization of phages as a natural solution to reduce harmful bacteria that can be found in foods can be considered. By directly applying virulent phages in the food, the level of target pathogenic bacteria present can be significantly reduced, making the food safer to consume (Zhang et al. 2015; Sillankorva, Oliveira, and Azeredo 2012; Wong et al. 2014; Sulakvelidze 2013; Endersen et al. 2014).

Virulent phages can be an effective tool to control pathogens due to their rapid and specific killing nature. Lysis of the bacterial host cell can occur by either the enzymatic actions of the phage to release newly created progenies, or by the loss of membrane potential due to the attachment of a large number of phage particles onto the cell wall (Abedon 2011). The latter method leads to a quicker bacterial cell death, as it does not involve the internalization of the phage genome and completion of the phage replication cycle. In contrast, the other class of phages called temperate phages enter the lysogenic cycle where their genetic information is injected into the host cell, which can exist either as a plasmid or inserted into the host genome, staying in this quiescent state until an induction event that triggers it into the lytic cycle. Therefore, temperate phages should not be used as antimicrobial agents due to the possibility of horizontal gene transfer of toxic or antibiotic resistance genes (Shousha et al. 2015).

C) GRAS status of starting material

The growth medium for producing GPI Biotech VAM-S contains only GRAS ingredients/processing aids. The main components of the medium are GRAS affirmed peptones, yeast extracts (GRAS affirmed), dextrose, sodium chloride, phosphates, and sulfate. Hydroxides and/or acids are used to adjust pH of the medium only during fermentation. These components are removed during anion exchange chromatography during down-stream processing.

The host strain of *Salmonella* used for amplification of phages is non-virulent.

Virulent (or lytic) phages are inherently generally recognized as being safe. They are obligate intracellular parasites that target only specific species of bacteria, while having no effect on other

bacteria, human, animal, or plant cells (Loc-Carrillo and Abedon 2011). Numerous studies have demonstrated that consumption of phages is harmless to humans (Bruttin and Brüssow 2005; Carlton et al. 2005)

Allergenicity

I. Phage components

Bacteriophages consist of proteins and nucleic acids. Assuming the unlikely scenario that all phage proteins (capsid proteins, tail proteins, tail fibers and tail spike proteins and base plate components) of the three phages would be equally allergenic as the peanut allergen, estimated daily intake (see below) indicate that approximately 18 lbs of treated food would need to be consumed in a single sitting in order to ingest 100 μ g of phage proteins (approximately half the weight of a phage is made up of proteins). We therefore consider the allergenicity potential of GPI Biotech VAM-S application due to the phage components negligible.

II. Relevant Medium Components

Soy Peptone

The only medium component with allergenicity potential is soy peptone. However, the level of soy was not detected in 3 lots of the final blended product where the reportable detection limit is 2.5ppm (Maxxam Analytics, Canada). The level of soy will be continuously monitored in each production lot.

D) Estimated daily dietary intake of Phages and by-products

According to USDA information (www.usda.gov/factbook/chapter2.pdf) Americans consume a total of approximately 195.2 lbs. of meat, poultry and egg products per capita per annum. Of the 113.5 lbs. of red meat consumed, 64.4 lbs. consist of beef, 47.7 lbs. of pork, 1.4 lbs. of lamb are consumed per capita per annum. Of the 66.5 lbs. of poultry consumed, 52.9 lbs. of chicken and 13.6 lbs. of turkey is consumed.

Phage intake 80 grams of beef x 3×10^9 PFU/g = 2.4×10^{11} phages/day.
Phage intake 59 grams of pork x 3×10^9 PFU/g =1.8×10^{11} phages/day.
Phage intake 66 grams of chicken x 3×10^9 PFU/g = 2×10^{11} phages/day.
Phage intake 17 grams of turkey x 3×10^9 PFU/g = 5.1 x $\times 10^{11}$ phages/day.

Assuming an average weight of 1 x10^8 Da/phage the following calculation gives the total weight of phages consumed on a daily basis:

$1.13 \times 10^{12} \times 10^8 \times 1.66 \times 10^{-27}$ kg =0.00000018758 kg/day = 187ug/day

Or in terms of treated product:

33 ppb or 0.033 ppm (parts per billion/parts per million). This level is insignificant.

The specifications as outlined in Table 1 are measured for both individual phage productions and for the final VAM-S blend. Each individual phage production is confirmed to have a titer of at least 1×10^{11} PFU/mL with no more than 1×10^{5} PFU/mL of contaminant phages. The endotoxin levels of each phage production are measured and must be less than 2500 EU/mL to proceed for final product blending. Bacterial sterility is confirmed for both individual phage productions and after final blending of VAM-S.

E) Efficacy data at the intended levels of use

Study 1: Determine the effectiveness of VAM-S on skin-on chicken experimentally contaminated with *S. enterica* serovars Tyhpimurium, Heidelberg, and Newport

Objective: Compare the levels of *Salmonella* between untreated or VAM-S treated chicken

Materials:

- skin-on chicken drumsticks
- LB broth
- Buffered peptone water (BPW)
- XLD agar
- electrostatic sprayer
- VAM-S
- *Salmonella* cocktail (1:1:1 ratio) consisting of *Salmonella* enterica subsp. enterica serovars Typhimurium, Heidelberg, and Newport

General procedure:

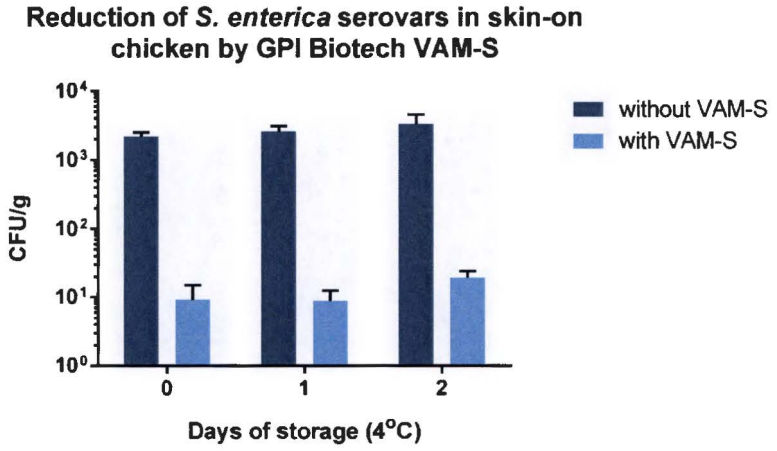
1. *Salmonella* cocktail was diluted to 10^{5} CFU/mL, and 2mL was applied on the chicken drumstick surface evenly. For non-inoculated chicken drumstick, 2mL of PBS was applied instead.
2. Chicken drumsticks were left for 15 min to allow for bacterial attachment.
3. A sprayer was used to apply BPW or VAM-S onto chicken drumsticks.
4. After incubation, the surface of the chicken drumstick was washed with BPW by massaging, followed by 1 min in the stomacher.
5. Viable *Salmonella* was determined by standard plating the appropriate dilutions on XLD agar plates.

Results:

Table 3: Level of *S. enterica* in experimentally contaminated chicken drumsticks not-treated or treated with VAM-S. Triplicate samples were stored for 0, 1, or 2 days before surface bacterial extraction.

Day(s) of incubation at 4°C	Concentration of *S. enterica* (CFU/g)	After VAM-S treatment (CFU/g)	Log reduction
0	2.17E+03	9.06E+00	2.38
1	2.61E+03	8.64E+00	2.48
2	3.33E+03	1.92E+01	2.24

Figure 1: Graphical representation of Table 3. Error bars indicate the SEM of 3 samples.



Conclusions:

There was an initial *Salmonella* reduction of 2.38 logs in chicken drumsticks treated with VAM-S compared to those that were not treated. This level of reduction was similarly seen after 1 and 2 days of storage at refrigeration temperatures, which suggests that the initial *Salmonella* reduction was a result of irreversible killing by the phage cocktail. The data shown here demonstrates that 5 minutes of contact time with VAM-S is effective in reducing viable *Salmonella* in skin-on chicken drumsticks.

Study 2: Determine the effectiveness of VAM-S on ground chicken experimentally contaminated with *S. enterica* serovars Tyhpimurium, Heidelberg, and Newport

Objective: Compare the levels of *Salmonella* between untreated or VAM-S treated ground chicken

Materials:

- skinless chicken breast

- LB broth
- Buffered peptone water (BPW)
- XLD agar
- sprayer
- VAM-S
- meat grinder
- *Salmonella* cocktail (1:1:1 ratio) consisting of *Salmonella* enterica subsp. enterica serovars Typhimurium, Heidelberg, and Newport

General procedure:

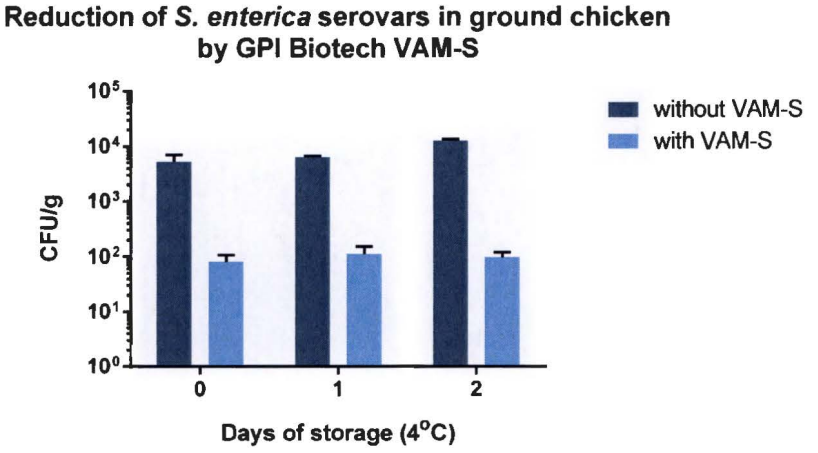
1. Skinless chicken breasts were aseptically cut into 100g pieces.
2. The *Salmonella* cocktail was diluted to 10^5 CFU/mL, and 2mL was applied onto the chicken surface evenly. For non-inoculated chicken, 2mL of BPW was applied instead.
3. Chicken breast pieces were left for 15 min to allow for bacterial attachment.
4. An electrostatic sprayer was used to apply BPW or VAM-S onto chicken breast pieces.
5. After 5 min incubation, chicken breast pieces were ground with a meat grinder. Parts that were in contact with meat was cleaned thoroughly between samples, and separate parts were used for untreated and VAM-S treated samples to minimize cross-contamination.
6. 10g of ground chicken breast was put into a sterile stomacher bag with filter.
7. 40mL 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 4: Level of *S. enterica* in experimentally contaminated ground chicken breast not-treated or treated with VAM-S. Triplicate samples were stored for 0, 1, or 2 days before bacterial extraction.

Day(s) of incubation at 4°C	Concentration of *S. enterica* (CFU/g)	After VAM-S treatment (CFU/g)	Log reduction
0	5.21E+03	8.00E+01	1.81
1	6.26E+03	1.12E+02	1.75
2	1.26E+04	9.63E+01	2.12

Figure 2: Graphical representation of Table 4. Error bars indicate the SEM of 3 samples.



Conclusions:

The reduction of *Salmonella* in experimentally contaminated ground chicken breast was assessed. VAM-S was applied on the chicken breast trim prior to grinding, leading to a reduction of 1.7 to 2 logs reduction throughout 2 days of storage at refrigerated temperatures. In addition, there was no increase in the level of *Salmonella* in VAM-S treated samples during the storage time, which suggests that the initial *Salmonella* reduction at "day 0" was a result of irreversible killing by the phage cocktail. These results suggest that it is possible to apply VAM-S on meat trim prior to grinding to reduce the *Salmonella* load in the ground product.

F) Summary *Salmonella* phages and GRAS

VAM-S consists of 3 naturally occurring lytic phages that have specificity to lyse various serovars of *Salmonella enterica*. It is shown here that all 3 phages are strictly lytic and do not contain any genes encoding for virulence, toxins, or allergens. Each phage production is also required to pass specifications to ensure the safety of the final product. Other bacteriophage products for pathogen reduction have previously been GRAS-approved, and VAM-S is equivalent to these products.

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APPENDIX

Objective: To grow isolated plaques of *Salmonella* phage particles and determine sample phage titre.

Equipment:
Heat plate or water bath
Heated shaking incubator
Spectrophotometer
p200 and p1000 pipettes
Biosafety cabinet

Apparatus:
500mL Erlenmeyer flask
Inoculating loop
1.5mL Eppendorf tubes
15mL conical tubes

Materials:
10mM $MgSO_4$
50mL sterilized LB media
Host strain bacteria
3-5mL of soft agar in 15mL conical tubes
LB Agar plates

Recipes:

Prepare LB broth and LB agar by following manufacturer's directions.

To prepare soft agar, mix the following with 1L of ddH_2O and sterilize at 121°C for 30 min. After sterilization, aliquot 5mL into 15mL conical tubes.

	grams
NaCl	5
Tryptone	10
Agar	6

Procedures:

1. Prepare LB agar plates.
2. Aliquot 30-50mL of sterile LB broth into a 500mL Erlenmeyer flask.
3. Inoculate host bacteria into the liquid broth.
4. Incubate in a 37°C shaking incubator @140rpm.
5. Cease incubation once OD_{600} of approximately 1 has reached.
6. Dilute phage sample 10-fold nine-times in 10mM $MgSO_4$.
7. Heat conical tubes containing soft agar until it is fully melted. (e.g. put in boiling water until soft agar is in full solution phase)
8. With the soft agar in full solution, and cooled below 49°C, add 200µL of host bacteria and 100µL of the highest phage dilution.
9. Vortex to mix, then pour onto an agar plate immediately.
10. Repeat steps 8 to 9 with the next two highest phage dilutions.
11. Once the soft agar has solidified, incubate agar plates upside-down in an incubator set at 37°C for 16-24 hours.
12. Count plaques and determine phage titre with the following equation:

$$\text{PFU/mL} = \frac{\#\ plaques}{(100\mu L)(dilution\ factor)}$$

1255 Journey's End Circle, Unit 1A, Newmarket ON L3Y 7V1 Tel: (905) 853 – 8828 Fax: (905) 853 – 8886

Prepared by: Edith Chow	**Standard Operating Procedure**	Revised by: Edith Chow
Effective Date: July 26, 2016	**Molecular detection of VAM-S bacteriophages**	Rev. No. 1
Reference No.:		Revision Date:
Approved by: Henry Liu, Technical Director	DEPARTMENT: R&D	

Objective: To detect for any bacteriophage (phage) impurity in a volume of amplified and purified phages.

Equipment: Real-time PCR system
p2, p20, p200, and p1000 pipettes

Materials: Norgen Biotek Corp. Phage DNA Isolation Kit or equivalent
Nanodrop or equivalent to measure DNA concentration
PowerUP SYBR Green Master Mix
Aerosol-resistant pipette tips
Nuclease-free microcentrifuge tubes
Nuclease-free water

Procedures:

1. Following phage production and purification, take 3 random 1mL samples from the production lot.
2. Extract DNA following manufacturer's protocol.
3. Measure concentration of DNA extracted and normalize all samples to 12.5pg/µL.
4. Plan for 9 reactions per sample: triplicate replications for each of the 3 primers specific to the 3 different phages.
5. Include a no-template control (NTC) for each primer mix as a reference for negative signals.
6. Prepare a reaction mix without DNA template according to table 1 for the number of samples and control reactions as required, plus 50% coverage.
7. Mix thoroughly and distribute 12µL to each reaction well.
8. Add 8µL of DNA template or nuclease-free water (NTC) into the appropriate well.
9. Seal the plate and centrifuge briefly to bring the contents to the bottom.
10. Program the thermal cycler as indicated in figure 1.
11. Load the plate into the thermal cycler. Run the thermal cycler program and collect real-time amplification data.

12. If production lots are composed purely of the intended phage amplified, C_T values should be high (17-26 cycles) for each sample with its corresponding primer (E.g. Phi_16 sample would have a high C_T value in reaction wells with the Phi_16 primer). C_T values should be comparable to the NTC signal for the other 2 primers (32 to 40 cycles).

13. If C_T values are found to be significantly higher than NTC (< 30 cycles) for one or both of the non-corresponding primer(s), consult historical data to determine the amount of copy number that correlate with the C_T value. If the contaminant level is greater than 1×10^5 copies, which equates to 1×10^5 phages, then the lot will be rejected for final blending.

Table 1

PowerUp SYBR Master Mix	10 µL
Primer mix (10uM ea)	1 µL
H2O	1 µL
Template	8 µL
Total	20 µL

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Effective Date: July 26, 2016	**Molecular detection of VAM-S bacteriophages**	Rev. No. 1
Reference No.:		Revision Date:
Approved by: Henry Liu, Technical Director	DEPARTMENT: R&D	

Figure 1

1255 Journey's End Circle, Unit 1A, Newmarket ON L3Y 7V1 Tel: (905) 853 – 8828 Fax: (905) 853 – 8886

Prepared by: Edith Chow	**Standard Operating Procedure**	Revised by:
Effective Date: December 12, 2018	**Bacteriophage *In-vivo* Challenge**	Rev. No.
Reference No.: RD-SOP-xxx		Revision Date:
Approved by: Henry Liu, Technical Director	DEPARTMENT: R&D	

Objective: To evaluate the effectivity of bacteriophage on reducing specific bacteria on food products

Equipment: Plate spreader
Aluminum paper
15mL and 50mL conical tubes
Microtubes
Sprayer

Materials: Target bacterial strain
Purified bacteriophages
Buffered peptone water (BPW)

Procedures:

Contamination of food product

1. Dilute bacterial culture to an appropriate concentration
2. Prepare food product as necessary (e.g. cutting into target weigh)
3. Contaminant food product by either spot inoculation, spreading onto the surface with a plate spreader, or by submersion.
4. Allow food product to air-dry after inoculation to allow bacterial attachment.

Bacteriophage treatment

1. Transfer appropriate samples to a tray lined with a clean foil paper.
2. Determine the amount of bacteriophage to be applied onto each food sample.
3. Spray VAM-S onto the surface of the food product.
4. Allow a 5-10 min incubation period after bacteriophage application.
5. If necessary, turn the sample over to spray bacteriophage.
6. If applicable, place sample into the tumbler and set to tumble for 15 min.

Further processing

1. If applicable, use the meat grinder to ground meat samples.

Extraction of bacteria from samples

1. Place samples individually into stomacher bags with filter.
2. Add a pre-determined amount of BPW into the stomacher bag.

3. Place the stomacher bag into the stomacher and homogenize for 1 min.
4. Extract BPW through the filter so to exclude food particles into a 50mL conical tube.

Enumeration of target bacteria

1. From the extracted BPW, make appropriate dilutions as necessary.
2. Spread-plate 100uL of the appropriate dilution on a selective agar.
3. Incubate plates at the appropriate temperature for 24 h.
4. Count colonies and calculate the concentration of bacteria present in each sample.