FINAL REPORT
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Salmonella-specific bacteriophages: characterization and optimization of production for rendering applications

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Lay Summary:

The goals of this project were to determine the optimal conditions for propagating bacteriophages in broth media and to develop a model for large-scale production of *Salmonella*-specific bacteriophages using a bioreactor in combination with purification steps involving liquid chromatography.

At the beginning of this study, the selection of avirulent *Salmonella* host strain and lytic bacteriophages was made based on host range, electron microscopy observation, and restriction enzyme analysis. All five bacteriophages [JC1, MbE2, H3353S6p2, H4717S5p2, Felix O1(FO)] were able to lyse the avirulent *Salmonella* Typhimurium strain 8243, which was used for bioreactor study later on. Using a model phage-host system for producing *Salmonella*-specific bacteriophages, phage MbE2 was selected for optimization of bacteriophage purification using fast protein liquid chromatography (FPLC). Phage MbE2 was shown to be stable at NaCl concentrations ranging from 0.1 to 2M and pH ranging from 5 to 10, which is important for the optimization of FPLC using anion exchange columns.

We also evaluated the effect of low level of antibiotics and $MgSO_4$ on increasing phage titer. Several concentrations of $MgSO_4$ were tested, and 10 mM of this salt was able to increase bacteriophage titer by ca.1 log (10 fold increase of phage population). Our results also demonstrated that 0.003~0.03 µg/ml of ampicillin or nalidixic acid was the most effective, achieving ca.1 log increase of bacteriophage titer over the control. These conditions have been used for growing *Salmonella* bacteriophages in a scale-up bioreactor. Scale-up of phage production can be achieved in a bioreactor with capacity of 4~8 liters. With multiplicity of infection (MOI) of 0.1, each liter of TSB broth can produce about ca. 3~8 x 10^{10} PFU of bacteriophages within 6 h of incubation when a low level of antibiotic or $MgSO_4$ was added.

We have successfully utilized FPLC to purify phage T4 with 91% recovery rate as well as phage MbE2 at a 72% recovery rate. Additionally, fractions containing the most phage titer had a greater than 91 and 90% reduction in host DNA for T4 and MbE2, respectively. A step-wise approach was also developed by testing several combinations of elution gradients. Bacteriophage MbE2 was consistently recovered from the disc in amounts between 60 and 75% of injected amounts. Therefore, the method produces a phage solution in a low salt buffer that is free from most of the host strain's DNA.

Introduction:

Rendered animal products are frequently contaminated with *Salmonella.* The presence of *Salmonella* in the finished by-products indicates either a portion of the *Salmonella* is surviving the heat processing or that the *Salmonella* contamination is a result of post-processing contamination. Assuming the later reason to be at fault, the finished products require additional treatment prior to shipment.

The primary control methods have included the addition of chemical preservatives such as acids, alcohols, and other compounds to animal feeds in an attempt to reduce if not completely eliminate fungi and *Salmonella* spp. contamination in feeds or prevent colonization of the pathogens in food-producing animals. However, a recent study indicates that treating animal feeds with organic acids/formaldehyde may mask the presence of *Salmonella*, when assessed by standard cultural methods. Consequently, the false negative results for *Salmonella* and other pathogens may occur. Therefore, alternative biological treatments need to be investigated.

Recent years, bacteriophages have gained renewed interest as biological control agent for pathogen controls in human medicines, food products, and environmental applications.

Bacteriophages are bacterial viruses that infect and replicate in bacteria. Bacteriophages are very specific to the target pathogens, and the phage treatment is considered as a very safe and cheap technique to use (Doyle 2007). FDA has approved the use of bacteriophages to control *Listeria monocytogenes* in ready-to-eat food products. Many studies have shown that bacteriophages can reduce *Salmonella* contamination on poultry carcasses and skins, and fresh produce as well (Fiorentin et al., 2005; Higgins et al. 2005; Joerger 2003; Leverentz et al. 2001). Results from our previous studies have demonstrated that the bacteriophage mixture was effective to reduce *Salmonella* contamination level on various surface materials (steel, plastic, and rubber), raw animal meat, and finished animal meals.

In order to apply the bacteriophages to the rendering environment, a large supply of bacteriophages without host cells and toxins is needed. The traditional methods used in purification and concentration of bacteriophages are centrifugation, precipitation with polyethylene glycol (PEG) 6000 and equilibrium centrifugation in CsCl gradients. There is very limited study on bacteriophage production beyond for research purpose. Bujanover (2004) filed a patent application for intermediate to large scale commercial production of bacteriophages for therapeutic uses. In that invention, bacteriophages are propagated within specific semi-solid hydrocolloid media, leading to a high titer (ca. 10^{15} to 10^{16} pfu/l semi-solid medium). Siquet-Descans et al. (1973) reported that the total yield of a 100-liter culture is around 10^{16} infectious particles. However, the final purification step in a CsCl gradient results in a 60 to 80% inactivation of the phage. Therefore, the improvement of current method for phage purification and concentration needs be explored.

When the bacteria die, their cell walls disintegrate and release the toxins. Toxicity is associated with the lipid portion of the lipopolysaccharide (LPS) molecule. Besides LPS endotoxins, *Salmonella* also produce protein toxins (exotoxins) and these exotoxins are largely responsible for their pathogenicity. Both capsids of the phages and toxins produced by *Salmonella* consist of protein subunits. In general, proteins carry a net positive or negative charge, which can be purified and concentrated by ion-exchange chromatography. Recently, CIM monolithic supports were successfully used for purification and concentration of bacteriophage T4 (Smrekar, 2008). In addition, the sizes of bacteriophage particles and toxins are quite different, which a size exclusion chromatography can be used for phage separation.

For practical application of bacteriophages in an industry setting, bacteriophages in a lyophilized form can be easy to store, transport and use. In order to keep active against *Salmonella* when bacteriophages are rehydrated, the bacteriophages should maintain the lytic activities. Lyophilization or freeze-drying method has been widely used for long-term preservation of phages (Clokie and Kronpinski, 2009). For example, 25 *Staphylococcus aureus* phages remained highly infectious after storage for 12-18 years at -20ºC with less than l-log drop in titer (Zierdt, 1959). Depending on phage species and storage condition, the loss of bacteriophage titers can vary. In general, there was a slight loss of phage titers, esp. l-log drop directly post-lyophilization. In order to have a steady supply of bacteriophages for use in rendering processing plants or added into the animal feed, there is a need to produce the bacteriophages in large quantity with low cost and evaluate the stability of bacteriophages during storage.

Therefore, the objectives of this study were to:

1). Characterize bacteriophages by electron microscope, restriction enzyme digestion, and host range analysis.

2). Produce bacteriophages using bioreactor and purify bacteriophages using FPLC chromatography.

3). Evaluate the stability of lyophilized phages during long term storage.

Materials & Methods:

Host range determination: The host range of 13 *Salmonella* bacteriophages [2PA, 2PE, CPA, 8PA, 8PD2, 8PE2, DPC, 9PC, JC1, MbE2, H3353S6p2, H4717S5p2, Felix O1(FO)] were tested on 58 strains of *Salmonella* host strains. Host strains were overlaid in 3 ml of 0.6% soft agar onto TSA at a concentration of ca. 10^6 CFU/plate. Bacterial lysis was determined by spotting 10 µl of bacteriophage suspension onto lawns of *Salmonella*. The plates were inverted after a short adsorption period and incubated at 37°C overnight, and then examined for lysis.

Transmission electron microscopy: Copper grids (400 mesh, EMS, Hatfield, PA) were coated with 0.5% formvar dissolved in dichloroethane (Fischer, Fairlong, NJ). Dried grids were placed on parafilm (American National Can, Menasha, WI) and 5 µl of phage solution (10^{10} PFU/ml) was pipetted on the surface for 1 min. The liquid was drawn off with filter paper and the grid was stained for 30 seconds with 5 µl of 0.5% phosphotungstic acid (PTA; EMS, Hatfield, PA) or 2% uranyl acetate (UA; EMS, PA). Grids were air-dried and viewed on a Hitachi H-7600 electron microscope (Hitachi, Tokyo, Japan) at 120-keV accelerating voltage.

Bacteriophage DNA isolation and restriction endonuclease analysis: Fresh bacteriophage stocks were prepared as described above prior to DNA extraction. Qiagen Lambda mini kit (Qiagen, Valencia,CA), MoBio Ultra Clean Microbial DNA Isolation Kit (MoBio,Carlsbad,CA) and a method using cetyltrimethyl ammonium bromide (CTAB) were compared for producing quality DNA. DNA samples were analyzed by electrophoresis in 1.5% agarose at 50 V and stained with ethidium bromide for 20 min followed by a 15 min de-staining in distilled water.

DNA samples from 5 selected *Salmonella* phages were digested with the restriction endonucleases DraI and NdeI (Promega, Madison, WI) according to the supplier's recommendations. DNA fragments were separated by electrophoresis in 1.5% agarose gel in 1X Tris-borate-EDTA buffer at 50 V with a wide Mini-Cell® Sub GT agarose gel electrophoresis system (Bio-Rad Laboratories Hercules, CA). The BioRad Gel Doc System was used to capture the image of ethidium bromide stained gels.

Preparation of bacteriophages in a bioreactor: For phage production in large scale, two liters of Tryptic Soy Broth medium were prepared either in a 4-liter flask or 8-liter carboy. An overnight culture of avirulent *Salmonella* Typhimurium 8243 Rif^r was centrifuged at 5,000 x rpm for 10 min, washed with 0.85% saline and adjusted to optical density of 0.5 (ca. 9 logs CFU/ml). Twenty milliliter of prepared bacterial suspension was inoculated into above containers at a final concentration of ca. 7 logs CFU/ml. Oxygen level in production system was balanced through filtered tubing installed on the top of each system. To increase the yield of phage production, either Mg^{2+} (50 mM), ampicillin (0.003 or 0.06 µg/ml) or nalidixic acid (0.003 µg/ml) were added to the phage production system to slightly inhibit the growth of bacterial host. These chemicals were added into phage production system immediately after the inoculation of bacterial host culture.

The entire phage production system was incubated at 37°C for 30 min, then 2 ml of bacteriophage strain FO, which has been tested having a broad host range against predominant *Salmonella* spp. found in finished animal meals, was inoculated into phage production system at MOI of 0.1 and 0.01. To determine the growth curve of *Salmonella* and titer of bacteriophages during incubation, a sample of 5 ml was aseptically collected from the culture container every 2 h until 16 h. Optical density was measured using a spectrometer at a wavelength of 600 nm. Phage particles and bacterial host cells were separated by centrifugation at 5,000 x rpm for 10 min. Phage titer was determined using soft agar overlay method and population of host cells was enumerated by spirally plating onto Tryptic Soy Agar plates followed by overnight incubation at 37 °C.

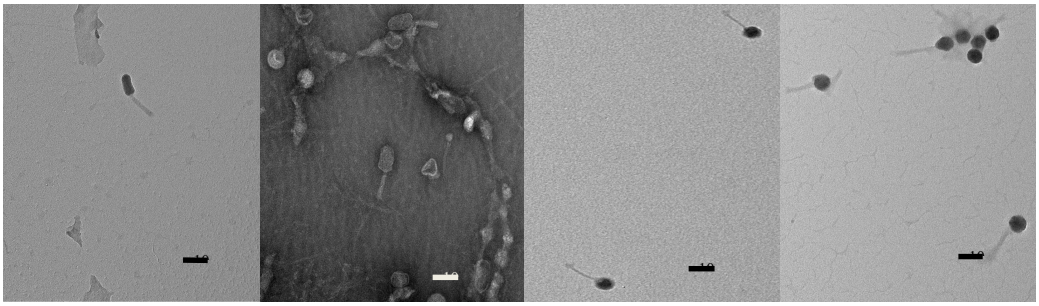
A New Brunswick bioreactor (Model BF-115) was also employed for optimizing phage production since parameters such as temperature and agitation speed can be better controlled and monitored as compared to using flask and carboy systems. To a 5-liter bioreactor, four liters of TSB medium were added and the whole system was sterilized. After cooling down to room temperature, pH and dissolved oxygen probes were calibrated using standard buffers and gases, respectively. Agitation speed was set to 200 rpm, and temperature was controlled to 37°C. At pre-determined intervals, samples were collected and tested using the same methods as described above.

Purification of bacteriophages using fast protein liquid chromatography (FPLC):
The FPLC can purify the bacteriophages by removing host DNA and most other cellular components and toxins, and also exchanging the TSB medium that the phages are in with a buffer. Purification of bacteriophages was performed using an AKTA FPLC system. In this method, two solutions, sodium dihydrogen phosphate (NaH_2SO_4, 125 mM) and a combination of NaH_2SO_4 and sodium chloride (NaCl, 1.5 M, pH=7), were used as running buffer and elution buffer, respectively. Ultrasonication was used to remove all air bubbles from buffer stocks to reduce signal interference. The FPLC system was cleaned with distilled water (dH_2O) first, and then NaH_2SO_4 buffer running at 10 ml/min until signal line was stable to replace the dH_2O. A quaternary amine ion exchange chromatography column of either CIM® QA disk monolithic column or Sartobind® Q SingleSep nano was connected into the system. The whole system was rinsed with running buffer at 2 ml/min to clean up the ethanol that kept column sterile. Bacteriophage suspension was injected into the system continuing with wash of running buffer. At this time, phage particles were separated and purified from TSB medium and other contaminants by adsorbing to the column. Then elution buffer was used to wash phage particles off under increasing ionic strength. Washed phages were collected in sterile sample tubes at a titer of 10^9-10^{10} PFU/ml. Further concentration can be performed using PEG precipitation or Centricon concentrators.

Stability of bacteriophages in lyophilized form during storage: The bacteriphage cocktail was suspended in 10% (w/v) non-fat dried milk solution to a titer of 10^{12} PFU/ml, and lyophilized overnight at a temperature < -50ºC in a freeze-dryer unit (Clokie and Kronpinski, 2009). The lyophilized bacteriophages were mixed with the finished animal meal and stored at 30ºC for up to 1 month. A portion of bacteriphage sample was taken out periodically and enumerated by added to a *Salmonella* culture in its exponential growth phase. This culture was then be plated onto TSA and plaques were counted to obtain the bacteriophage titers. The refrigerated bacteriophages in SM buffer was used as control for stability testing.

Results & Discussion:

Host range determination: A total of 102 bacteriophages were isolated and 12 of them plus bacteriophage strain FO were tested for host range against 58 *Salmonella* strains. Among those most lytic bacteriophages, five phages (MbE2, JC1, H3353S6p2, H4717S5p2 and FO) were further analyzed by transmission electron microscope and restriction enzyme digestion. Bacteriophage JC1 and H4717S5p2 had contractile tails and were of the family *Myoviridae* (Fig.1A-B). The presence of a flexible non-contractile tail for MbE2 and H3353S6p2 suggests that they belong to the family of *Siphoviridae.*



A B C D

Fig. 1. Transmission electron microscopic images of individual bacteriophages. JC1 (A), H4717S5p2 (B), MbE2 (C), and H3353S6p2 (D) observed at 100,000X.

Restriction analysis by NdeI of the five *Salmonella* phages revealed that phage MbE2 and JC1 share the same patterns, whereas the rest of the phages appeared to have unique patterns. H3353S6p2 was not clearly digested by NdeI, whereas DraI worked (Fig2).

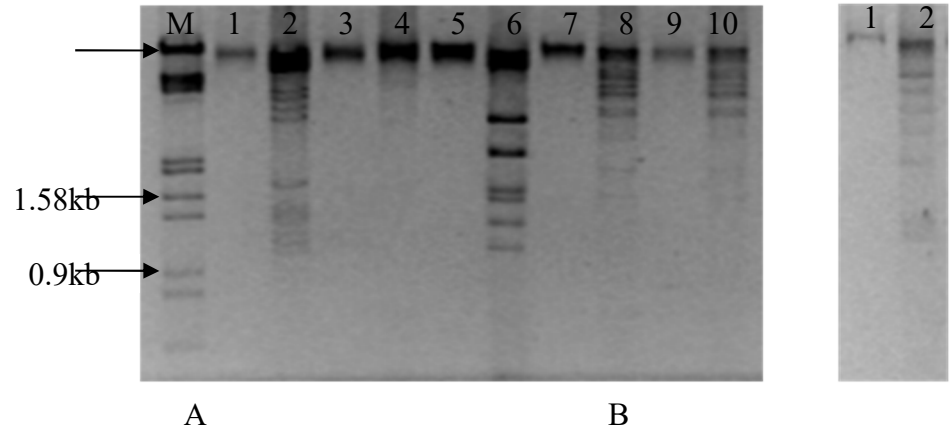
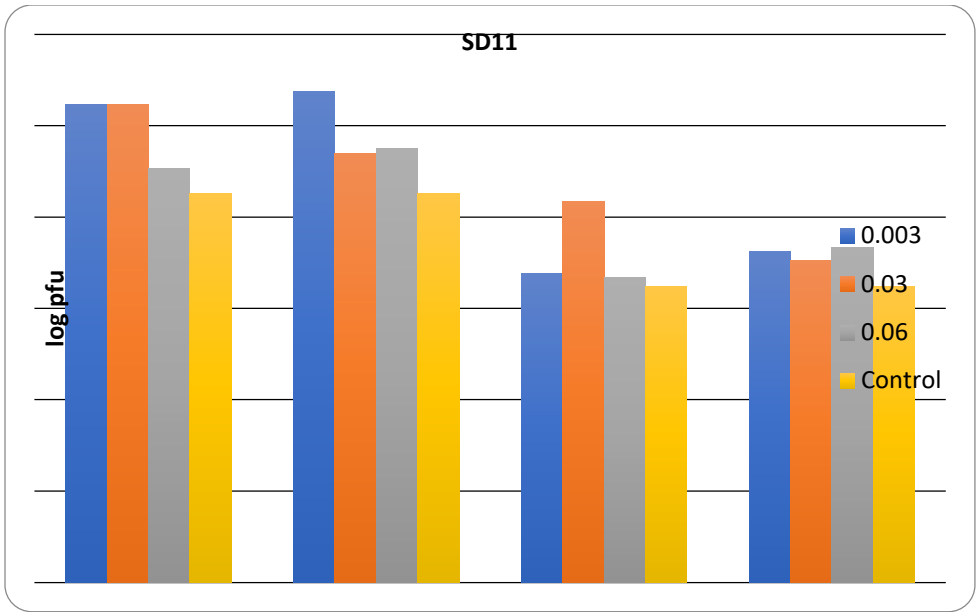


Fig. 2. **A.** Restriction analysis of five *Salmonella* phage islolates using enzyme NdeI. **Lane M**, Lambda DNA/EcoRI+HindIII marker**; Lane 1 and 2**,FO DNA and digest; **Lane 3 and 4**, H3353S6p2 DNA and digest**; Lane 5 and 6**, H4717S5p2 DNA and digest; **Lane 7 and 8**, MbE2 DNA and digest; **Lane 9 and 10**, JC1 DNA and digest. **B.** Restriction anaylsis of H3353S6p2 using enzyme Dra**I. Lane 1 and 2**, H3353S6p2 DNA and digest.

Selecting bacteriophage for FPLC optimization: Five different bacteriophage candidates (H4717S5P2, FO, Mbe2, JC1, and H535356p2) were tested against two avirulent *Salmonella* strains 8243 and SD11 using agar overlay assay. Selected phage-host systems were then tested for the ability of phages to propagate in broth at several multiplicity of infections (MOIs). Phage MbE2 was the most effective at lysing *Salmonella* strain SD 11 in broth trials at an MOI of 1 and was chosen for following FPLC optimization. To verify if phage MbE2 would retain infectivity at varying salt concentrations or varying pH, an experiment was performed over a range of salinity (0.1 to 2M NaCl) and pH (4 to 10) for 4 h, followed by phage enumeration using standard agar overlay techniques. The results showed no difference between control and all salt concentrations, however a pH of 4 resulted in a >3 log decrease in phage titer, while other pHs tested had no effect.

Evaluating the impact of antibiotics and $MgSO_4$ on bacteriophage yield: In order to determine if low levels of antibiotics can increase bacteriophage titer, 3 concentrations (0.003, 0.03, and 0.06 µg/ml) of 2 antibiotics (ampicillin and nalidixic acid) were tested on two *Salmonella* phages (JC1 and MbE2). Each phage and antibiotic concentration was also done using two avirulent *Salmonella* strains 8243 and SD11. Our results demonstrated that 0.003~0.03 µg/ml of ampicillin or nalidixic acid was the most effective for bacteriophage MbE2, achieving ca.1 log increase of bacteriophage titer over the control (Fig. 3).

Fig. 3. Effect of different concentrations of antibiotics (Amp and NA) on the yield of bacteriophages (MbE2 and JC1) using avirulent *Salmonella* SD11 as a host



It is known that some bacteriophages need either Mg^{2+} or Ca^{2+} ions in order to properly attach to bacterial cells and to assist in growth functions. To determine the effect of adding $MgSO_4$, three concentrations (1, 10 and 50 mM) of $MgSO_4$ were added to 9 ml TSB prior to inoculation with host bacteria and bacteriophages. It was found that 10 mM was the most effective obtaining a 1 log increase (10 folds) in titer above the control, whereas both 1 and 50 mM were not found to significantly increase titer (results not shown). Further experiments were performed to determine if antibiotics and $MgSO_4$ together would have a synergistic impact on bacteriophage titer than either separately. In the experiments conducted a control was measured against treatments either with 10 mM $MgSO_4$, 0.003 µg/ml of ampicillin, or 10 mM $MgSO_4$+0.003 µg/ml Amp (Table 1). Each treatment obtained an increased titer of ca. 1 log greater than the control, indicating that while they are all effective at increasing bacteriophage titer the combination of Amp and $MgSO_4$ is not significant ($p>0.05$) more effective than either $MgSO_4$ or Amp alone.

Table 1. Effect of ampicillin and $MgSO_4$ on the yield of bacteriophage MbE2 using avirulent *Salmonella* SD11 as a host

Treatment	***Bacteriophage titer (PFU/ml)***
Control	1.4×10^7
10 mM $MgSO_4$	1.0×10^8
0.003 µg/ml Amp	1.2×10^8
10 mM $MgSO_4$ + 0.003 µg/ml Amp	1.8×10^8

In addition, we also determined if longer incubation time for the host of the bacteriophages would affect phage titer. An experiment was done with a control of 30 min incubation against 2 h and 4 h incubation. We found that the 2 h and 4 h incubation resulted in lower phage titer than 30 min of host incubation. This may be due to the effect that the higher

Salmonella population at 2 and 4 h would have lower the MOI ratio that the phages were inoculated at (results not shown).

Scale-up production of bacteriophages: During the phage production, phage titer increased rapidly from initial titer of 6 logs PFU/ml to ca. 10 logs PFU/ml at 4 h, and then kept relatively stable until 16 h. Further incubation up to 24 h resulted in a reduction of phage titer by approximately 1 log PFU/ml. Population of bacterial hosts slightly increased in the first 2 h of incubation, but decreased to the lowest density at 6 h. Due to the presence of bacteriophage-insensitive mutants, bacterial population increased after 8 h and reached higher number as compared to initial population (data not shown). Therefore, the incubation time of phage production for 6 h is considered as optimal for phage production in large scale.

For the phage production using 8-liter carboy containing 50% TSB medium, phage titer reached to 3 x 10^{10} PFU/ml with an initial MOI of 0.1, whereas phage titer increased to 3.5 x 10^{10} PFU/ml in 100% medium. With a decreased MOI of 0.01 in a 100% TSB medium, phage titer was 3 x 10^{10} PFU/ml. Addition of 0.06 µg/ml of ampicillin increased the phage titer to 4.5 x 10^{10} PFU/ml, while 0.003 µg/ml of ampicillin did not show any effect on yield of phage production (Table 2). Apparently, low level (0.06 µg/ml) of ampicillin has a positive effect on phage production resulting in a 1.5X increase of phage population. Although the phage titer in the production using 50% TSB was lower than 100% TSB, it reduced the medium cost of phage production by 50% that will be a significant saving when phage production is applied to pilot scale.

In the 4-liter flask production system, phage titer was 2.5 x 10^{10} PFU/ml with a MOI of 0.1 in 100% medium, and increased to 3.5 x 10^{10} PFU/ml and 8 x 10^{10} PFU/ml by adding a combination of 0.06 µg/ml ampicillin and 50 mM Mg^{2+}, and nalidixic acid of 0.003 µg/ml, respectively. It suggested that addition of nalidixic acid might have the better effect on improving phage production as compared to ampicillin and Mg^{2+}.

For 5-liter bioreactor, the volume of medium was changed to 4 liters according to the structural design of agitation part in bioreactor. Under the same parameter settings, phage titer reached to 1 x 10^{10} PFU/ml that was relatively low as compared with phage production performed in above two systems. The increased medium volume might be the reason for lower phage yield in bioreactor. Although the same incubation parameters were used, double volume of media may still affect nutrient flow and the level of dissolved oxygen in the system which are important for bacterial growth.

Table 2: Bacteriophage yields in different phage production systems

Production System	Culture vol. (L)	Medium conc. (%)	MOI	Ampicillin (μg/ml)	Nalidixic acid (μg/ml)	Mg^{2+} (mM)	Phage titer (x 10^{10} PFU/ml)
8-Liter Carboy	2	50	0.1	0	0	0	3
		100	0.01	0	0	0	3
				0	0	0	3.5
		100	0.1	0.003	0	0	3.5
				0.06	0	0	4.5
4-Liter Flask	2	100	0.1	0	0	0	2.5
				0	0.003	0	**8**
				0.06	0	50	3.5
5-Liter Bioreactor	4	100	0.1	0	0	0	1

Purification of bacteriophages using fast protein liquid chromatography: Numerous optimization experiments were performed to purify phage MbE2 using FPLC. The column used for phage purification is a quaternary amine disc, with a bed volume of 0.34 ml, which binds phage under low salt conditions and subsequently elutes the phage as the salt concentration increases. This is possible using the Akta FPLC system which has two pumps that can mix and pump two different buffers to create any combination of salt gradient. As phage elutes, it runs through a UV detector which is set at a wavelength of 280 nm, and then collected in fractions.

During initial experiments, the concentrations of phage in each fraction were determined and percent recovery calculated as well as the concentration of DNA present in each fraction. There were about 37 ~ 54% of loaded phages being recovered with over a 90% reduction in host DNA levels as measured by the Nanodrop® (data not shown). As a validation of the method, bacteriophage T4 was tested using the same experimental parameters. Two of the three fractions collected contained over 90% of the injected phage samples. Apparently, bacteriophage T4 was recovered easier than *Salmonella* phage under these experimental conditions.

To maintain high percentage recovery in elutions but limit the amount of non-phage particles being collected during elution, we tested a series of step-wise NaCl gradients. Initially, a four step process increasing the NaCl concentration from 0 to 1 M at 25%, 50%, 75% and 100% was tested. This method was found to be uneconomical due to the amount of fractions that needed to be collected. A three step method increased NaCl from 0% to 33%, 67% and 100% resulting in a total recovery of 75% in a 30 ml volume (Table 3). In an attempt to decrease the total collection volume a two-step method was attempted using 0, 50% and 100% NaCl, resulting in a 61% recovery in a 15 ml volume (Table 4). Chromatograms all show an increase in the

quality of the peaks recovered when compared with linear gradient style elutions (Figure 4a and b).

Figure 4a. **Figure 4b.**

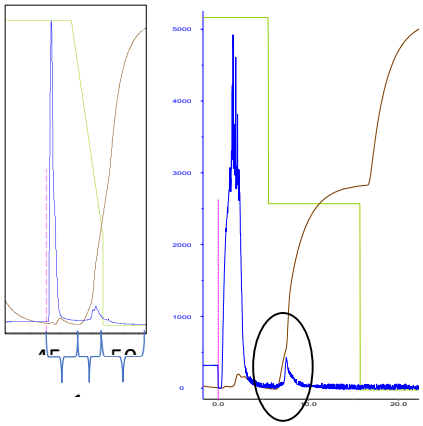


Table 3. Phage recovery for 3 step elution

	Volume (ml)	Total pfu	Recovery (%)
Load (MbE2)	2	4E+09	
Step 1 (33% NaCl)			
Fraction 1	2	1.00E+06	
Fraction 2	2	1.00E+09	25.03
Fraction 3	1.9	1.00E+09	50.03
Fraction 4	2	4.00E+08	60.03
Fraction 5	2.5	1.25E+08	63.16
Step 2 (67% NaCl)			
Fraction 1	2.1	4.00E+07	64.16
Fraction 2	1.7	1.70E+08	68.41
Fraction 3	2	2.00E+08	73.41
Fraction 4	2.2	2.20E+07	73.96
Fraction 5	2.7	1.35E+07	74.3
Step 3 (100% Flush)			
Fraction	10	2.00E+07	74.8

Table 4. Phage recovery for 2 step elution

	Volume (ml)	Total pfu	Recovery (%)
Load (MbE2)	2	4.72E+09	
Step 1 (50% NaCl)			
Fraction 1	2	2.84E+07	
Fraction 2	2	1.37E+09	29.63
Fraction 3	2	8.40E+08	47.42
Fraction 4	2	2.52E+08	52.76
Fraction 5	2.5	1.42E+08	55.77
Step 2 (100% Flush)			
Fraction	5	2.50E+08	61.07

Our results have demonstrated that a broth medium containing *Salmonella*-specific bacteriophages can be purified using anion exchange chromatography. The method produces a phage solution in a low salt buffer that is free from most of the host strain's DNA. The optimal step for eluting phage seems to lie in between 37% NaCl and 50% NaCl to obtain the maximum phage recovery in the smallest volume of buffer. Minor changes to the protocol will allow for optimization followed by scale-up of the column. Once the method is optimized, larger volumes of phage solution will be purified using the 1ml and 8 ml columns, allowing for phage production using the bioreactor.

Bacteriophage stability: Analysis of the stability of the lyophilized bacteriophage cocktail within the dry finished meals at 30°C, revealed that the phage concentration decreased by ca. 1.5 log PFU/g over the 4-week test period (Fig. 5).

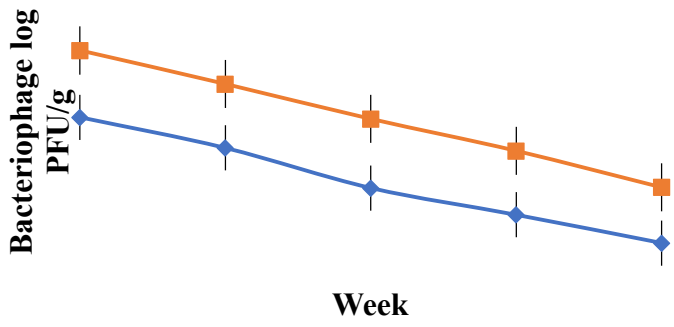


Fig. 5. Stability of lyophilized bacteriophage with MOI of 10 and 100 in finished rendered meal at 30°C. Symbols were MOI of 10 (■) and MOI of 100 (♦).

Conclusions:

In this study, the selection of avirulent *Salmonella* host and lytic bacteriophages has been made based on host range, electron microscopy observation, and restriction enzyme analysis. By adding 0.003~0.06 μg/ml of ampicillin or nalidixic acid to the bacteriophage production medium, the yield of bacteriophages was increased ca.1 log PFU/ml over the control. Scale-up of phage production can be achieved in a bioreactor with capacity of 4~8 liters. With MOI of 0.1, each liter of TSB broth can produce about ca. 3~8 x 10^{10} PFU of bacteriophages within 6 h of incubation when a low level of antibiotic or $MgSO_4$ were added. Additionally, using 50% of TSB broth may be a cost-effective way for production of bacteriophages in large scale. Using anion exchange chromatography, a broth medium containing *Salmonella*-specific bacteriophages can be purified with phage recovery rate in the range of 60~75% and more than 90% bacterial host DNA removed. These results indicate that the FPLC method produces a phage solution in a low salt buffer that is free from most of the host strain's DNA, but the feasibility for purifying bacteriophages in large volume remains challenging.

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Impacts and Significance:

Salmonella has long been established as a contaminant of rendered animal by-products and animal feed. The biological control method using *Salmonella*-specific bacteriophages being developed in this project will have the potential for reducing bacterial populations within animal meals and rendering processing environment as well as acting as a protective agent against future *Salmonella* contamination. Bacteriophages are superior to chemical disinfectants since they are not corrosive and hazardous to the environment, humans and other animals, and self-replicating meaning lower cost. Elimination of this pathogen by bacteriophage approach will ensure the microbiological safety of these rendered animal meal products. The addition of bacteriophages as a feed additive may have the potential that not only produce a safer product but also be successful in reducing *Salmonella* infection in the animal hosts that consume it. The use of bacteriophages on a large scale however requires volumes of phage that are greater than can be produced in the laboratory conditions. Therefore a method that can produce large quantities of phage, free of growth media and bacterial toxins and LPS is needed.

Publications:

Kinley, B., M., Li, S. Heringa, and X. Jiang. Reduction of *Salmonella* on environmental surfaces with bacteriophage treatment. (in preparation).

Outside funding:

Developing and validating practical strategies to improve microbial safety in composting process control and handing practices. X. Jiang (PI), J. Kim, J. Patel, P. Miller. The Center for Produce Safety at UC Davis, $296,368 (Jan. 1, 2011 – Dec. 30, 2012)

Validating *Salmonella* inactivation during thermal processing of the physically heat-treated chicken litter as soil amendment and organic fertilizer. X. Jiang (PI) and J. Kim. The Center for Produce Safety at UC Davis, $147,344 (Jan. 1, 2012 – Dec. 30, 2013)

Future Work: Research on applying *Salmonella*-specific-bacteriophages to a real rendering processing plant has been funded by ACREC and is currently ongoing.

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