Temporal distribution of encapsulated bacteriophages during passage through the chick gastrointestinal tract

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ABSTRACT Encapsulation of bacteriophages (“phage”) protects phage against environmental deactivation and provides a product that is easy to handle for storage and application with animal feed as an antibiotic alternative. The objective of this study was to evaluate an orally administered, encapsulated phage for efficient phage release in the gastrointestinal tract (GIT) of young chicks receiving feed. An optimized formulation that consisted of 0.8% low molecular weight (MW) alginate, 2% ultra-low molecular weight alginate and 3% whey protein completely released the encapsulated phage within 60 min under simulated intestinal conditions. This product was given to broiler chicks to determine passage time and distribution of the viable phage within the GIT. Following a single oral dose of 10^6 plaque-forming unit (PFU)/chick, the major portion (peak concentration) of the encapsulated phage passed through the chick’s GIT and was detected in the feces within 4 h, with low levels being continuously excreted for up to 24 h. In comparison, the passage of free phage through the GIT occurred faster as indicated by a peak concentration in feces after 1.5 h. In assessing the temporal phage distribution, both encapsulated and free phage treatments showed no apparent difference, both having low levels of 10^2 to 10^6 PFU/g of contents along the entire GIT after 1, 2 and 4 h. These low concentrations recovered in vivo led us to examine various exposure conditions (with feed, fecal material, and buffer solutions) that were suspected to have affected phage viability/infectivity during oral delivery, sample recovery, and enumeration by plaque assay. Results showed that the exposure conditions examined did not significantly reduce phage viability and could not account for the observed low phage levels following oral administration in chicks that are on feed. In conclusion, an oral encapsulated phage dose can take more than 4 h to completely move through the GIT of young chicks. Thus, repeated or higher doses may be necessary to attain higher phage concentrations in the GIT.

Key words: Bacteriophage, oral phage therapy, broiler chicks, salmonella Typhimurium DT104

INTRODUCTION

Many Salmonella enterica serovars, especially S. Typhimurium DT104 (Hogue et al., 1997; Fedorka-Cray et al., 2001), are prevalent food-borne pathogens commonly associated with poultry and swine products. When present, they pose a high risk of antibiotic-resistant salmonellosis. With the impending ban on general antibiotic use in food animal production, a safe and low-cost bio-control strategy, is highly desirable to reduce the pathogen in livestock and poultry at the pre-harvest production stage in order to reduce not only the risk of pathogen spread to humans but also the potential for food-borne disease outbreaks (Jordan et al., 1999; Soon et al., 2011; Berge and Wierup, 2012). Recently, bacteriophages have re-emerged as a potential alternative to antibiotics for control of pathogens in farm animal production, primarily due to their specificity and potential role in the maintenance of normal gut microbiota (Maura and Debarbieux, 2011; Kim et al., 2013; Billington et al., 2014; Ly-Chatain, 2014). However, apart from reported small experimental trials in various farm animals (Capparelli et al., 2010; Goodridge, 2010; Saez et al., 2011; Ly-Chatain, 2014), their potential use as a bio-control agent in large-scale agricultural settings has yet to be fully exploited. There are also reports that certain factors such as stomach acidity affect phage survival during their passage through the gastrointestinal tract (GIT; Adams, 1959; Dini et al., 2012; Ly-Chatain, 2014). Encapsulation of phage to produce a stable and solid form of...
protection could promote phage application in large scale food animal production by enhancing phage delivery to the lower gut and providing long term storage convenience and stability (Ma et al., 2008; Dini et al., 2012).

Prior to initiating oral encapsulated phage therapy studies, several parameters require investigation to gain a better understanding of the relationships in vivo between phage stability, temporal distribution and potential therapeutic effect. In order to optimize conditions for oral administration of encapsulated phage, a dosing study is necessary to determine the phage passage time and phage concentrations achievable in various sections of the GIT over time. This information is necessary for development of the encapsulation formulations and dosing frequencies for optimal efficacy, i.e., to achieve a desired concentration of phage at the location of high pathogen load to optimize the phage to bacteria ratio or multiplicity of infection (MOI). There are few studies published (Oliveira et al., 2009; Sarker et al., 2012; Jaiswal et al., 2013) that have investigated the temporal distribution of phages in animals and humans following oral delivery in the absence of identified host bacteria in which the phage replicate. Previously we developed an encapsulated phage product made of Ca\(^{2+}\)-alginate-whey protein gel beads that insufficiently released the phage within the body of young chicks after oral administration (Wang et al., 2010). This could be due to the relatively short GIT and immaturity, i.e., low enzymatic activities, of the young chick’s digestive system (Noy and Sklan, 1995; Jin et al., 1998). It is known that proteases contribute to the release of core substances encapsulated in the alginate-whey protein microparticles; achieved mainly via pepsin and trypsin breakdown of the protein component (Chen and Subirade, 2007). Therefore, there is a need to further improve the encapsulation formulation so that release of encapsulated phage can occur rapidly in the absence of mature digestive enzymes (mainly trypsin), and still provide adequate protection to the phage.

In the present study, we used newly hatched broiler chicks sourced from a Salmonella-free commercial poultry producer. We aimed to identify any potential exposure conditions during oral delivery that may have a detrimental effect on phage survival or during sample recovery for enumeration by plaque assay, e.g., to feed and simulated digestive conditions, as phages are known to be sensitive to many conditions (Adams, 1959; Atterbury et al., 2007b; Ly-Chatain, 2014). Thus, the main objectives of the present research were: 1) to modify the calcium alginate-whey protein formulation to produce a faster release rate, 2) to identify any potential exposure conditions during oral delivery that may have a detrimental effect on phage survival during sample recovery from the GIT for enumeration, and finally, 3) to determine the passage and excretion times and in vivo distribution of orally administered phage (single dose). Such information could provide invaluable guidance for subsequent challenge trials to test the efficacy of phage therapy for bio-control of Salmonella in broiler chicks.

**MATERIAL AND METHODS**

**Chicks Used**

All animal maintenance, manipulations and experimental procedures were performed according to the Animal Utilization Protocol (#09R117) approved by the University of Guelph Animal Care Committee. Thirty-six day-of-hatch broiler chicks were obtained from a commercial poultry producer (Maple Leaf Farms, New Hamburg, Ontario, Canada) and transferred to isolation units at the Central Animal Facility, University of Guelph. Chicks receiving the same treatment were housed in individual isolation units (up to 12 chicks) and allowed ad libitum access to non-medicated starter feed crumbles (Floradale Feed Mill Ltd., Floradale, Ontario; Table A1) and water for the entire study length. The wire floors of the units with pans beneath permitted feces collection and testing. Bacteria and phage manipulations and collection of GIT contents were performed within a bio-safety cabinet to avoid contamination. Surgical instruments and exterior of the euthanized chicks were disinfected with 70% ethanol and allowed to dry before dissection of each animal.

**Bacterial Strain Used for Phage Enumeration**

Salmonella Typhimurium DT104 (ATCC # 700408) was grown and selected for resistance to nalidixic acid sodium salt (STDT104 Nal\(^{B}\)) (Sigma-Aldrich) for studies involving digestive material to enable visualization/isolation of the target strain colonies among the background bacterial flora that could overgrow on non-selective media. This selection was done by first streaking onto TSA (tryptic-soy agar, Becton-Dickinson) plates supplemented with 20-40 \(\mu\)g/mL Nal and incubating at 37°C overnight; following which, cells were subcultured on TSA-Nal or BGS-Nal plates. A single colony was picked for overnight propagation in liquid TSB when a fresh culture was needed.

**Bacteriophages Used**

Bacteriophage Felix O1 (FO1) was obtained from the Felix d’Herelle Reference Center (Université Laval, Quebec, Canada). Phage FO1 was individually batch-propagated as previously reported (Ma et al., 2008; Tang et al., 2013) and was stored and diluted in sterile SM buffer made up of: 5.8 g/L NaCl, 2 g/L MgSO\(_4\cdot6\)H\(_2\)O, 0.1 g/L gelatin (bovine type, Sigma-Aldrich), and adjusted to pH 7.5 with Tris-HCl (50 mM), 1 M NaOH and Millipore filtered water.
Table 1. Compositions of the different calcium alginate-whey protein formulations used for phage encapsulation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Low MW alginate (w/v%)</th>
<th>Ultra-low MW alginate (w/v%)</th>
<th>Whey protein (w/v%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F2</td>
<td>0.5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>F3</td>
<td>0.5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>F4</td>
<td>0.8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F5</td>
<td>0.8</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>F6</td>
<td>0.8</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

**Bacteriophage Encapsulation into Ca\(^{2+}\)-alginate-whey Protein Gel Beads**

Encapsulation materials used were: low molecular weight (MW) (4–12 cP of a 1% aqueous solution at 25°C) sodium alginate (#A1112, Sigma-Aldrich, Canada), ultra-low molecular weight (0.3–0.7 cP of a 10% aqueous solution at 25°C) sodium alginate (Manugel LBA, FMC Biopolymer, Ireland), whey protein isolate (Bipro, Davisco Foods International Inc., USA) and calcium chloride hexahydrate (Sigma-Aldrich, Canada). Solutions were prepared in distilled de-ionized water (Millipore) and sterilized by autoclave or filtration using 0.2 μm membrane filters (Millipore). The procedure for phage encapsulation was based on previous work and an encapsulation formulation (1.5% low MW alginate + 3% whey protein) developed previously by our group (Ma et al., 2008; Wang et al., 2010). Various modified formulations were prepared by introducing an ultra-low MW alginate and modifying the low MW alginate and whey protein concentrations as shown in Table 1. The first step involved making stock solutions of 3% low MW alginate, 10% ultra-low MW alginate, and 10% denatured whey protein, respectively. The pre-encapsulation solutions were made by combining the stock solutions with purified, concentrated bacteriophage suspended in SM buffer to desired concentrations as listed in Table 1. The solutions were mixed gently for 10 min with a magnetic stir bar and then extruded by air pressure on an Inotech IE-50R encapsulator (Inotech Biosystems International) equipped with a 300 μm nozzle into the 0.1 M CaCl\(_2\) hardening bath (250 to 1000 mL) vessel below, rapidly forming cloudy-white gel beads. The beads were further stirred for 30 min to complete gelation and then were filtered, washed lightly with distilled water and after excess water was dabbed away, they were weighed and placed into closed 50 mL sterile polypropylene tubes and stored at 4°C. Phage loading was determined by weighing (200 to 250 mg) wet beads into a glass vial, then by adding sterile microbeads breaking solution (MBS) (50 mM of sodium citrate tribasic and Tris-HCl pH 7.5, 200 mM sodium bicarbonate) to dissolve the beads into a final volume corresponding to a 2 to 10× initial dilution, with gentle shaking at 37°C. The average phage loading was determined to be 2.93 × 10\(^{10}\) ± 2.02 × 10\(^9\) PFU/g beads.

**Bacteriophage Enumeration Assay**

Viable phages were enumerated by a plaque counting technique. After appropriate serial (10×) dilutions with SM buffer, samples (10 μL) were spotted in triplicate onto phage agar plates supplemented with Nal (Fisherbrand square disposable petri dish with grid, Fisher Scientific, Canada) and seeded on the surface with fresh S. Typhimurium lawns at a cell density of ~10\(^8\) CFU/mL. Viable counts were enumerated after plaque formation on the bacterial lawn following 8 to 12 h aerobic incubation at 37°C.

**In vitro Release of Encapsulated Phage from Ca\(^{2+}\)-alginate-whey Protein Gel Beads**

Initial phage concentrations used were between 6.0 × 10\(^5\) to 1.4 × 10\(^6\) PFU/mL. Phage viability was assessed after 2 h incubation in simulated gastric fluid (SGF) (34 mM NaCl adjusted to pH 2.5 with 0.2 N HCl). Afterwards, the acid-treated beads were subjected to simulated intestinal fluid (SIF) (50 mM monobasic potassium phosphate, adjusted to pH 6.8 with 0.2 N HCl or NaOH) conditions to determine the release of viable phage after 30, 45, 60, 90, and 120 min to determine the percentage of phage released relative to the initial dose.

**In vitro Phage Incubation with Chick Feed Under Simulated Gastric and Intestinal Conditions**

Chick starter feed crumbles were incubated with phage FO1 for 2 h in SGF (pH 2.5) with or without porcine pepsin (3.2 g/L) then for another 2 h in SIF (pH 6.8) with or without pancreatin (10 g/L) added (Sigma-Aldrich, Oakville, Canada). Using 1 g of feed, an initial inoculum of ~10\(^8\) PFU phage in SM buffer (200 μL) or encapsulated form (200 mg) were added followed by 4 mL of SGF and subsequent incubation at 41°C with gentle mixing on a platform rocker (Roto-Shake Genie, Cole-Palmer Canada) placed within an incubator. At the end of 2 h incubation in SGF, 0.5 mL of supernatant was taken for phage enumeration, then additionally 4 mL of SIF were added and incubation continued for another 2 h. For the free phage (FP) incubation there was complete loss of viability after incubation in SGF, so a second dose of FP was added after the addition of SIF for the second part of incubation. One hundred microliters were removed at each time point (30, 45, 60, 90 or 120 min) for viable phage enumeration.
In vivo Phage Distribution Following Oral Administration to Chicks

After one day of acclimatization, chicks were gavaged with FO1 in two forms: free phage in liquid suspension of SM buffer (3 × 10^10 PFU/chick, 12 per group) and in encapsulated form (4.6 to 9 × 10^9 PFU/chick, 12 per group), using a 1 mL syringe (Eppendorf Combitips), while control chicks (n = 3) received SM buffer containing no phage. After the time points of 1, 2, or 4 h, four chicks (as replicates) were dissected immediately following cervical dislocation and the GIT contents collected and weighed individually for FO1 enumeration from the following sections: crop (0.25 to 5.1 g), gizzard (1.35 to 2.4 g), ileum (0.28 to 1.1 g), and cecum (0.13 to 0.84 g) from each animal. After obtaining the weight of individual contents, sterile suspension buffer (0.1% peptone + 0.1% Tween 80) was added at a 1:2 (weight:volume) ratio to loosen and disperse the contents following vortexing. Afterwards, ~500 μL of supernatant were filtered through 0.45 μm filter centrifuge tubes (Costar Spin-X, Fisher-Scientific, Canada) by centrifuging at 11,000 × g for 5 min, and then were serially diluted using sterile dilution buffer (0.1% peptone + 0.1% Tween 80) at a 1:2 (weight:volume) ratio to dilute and disperse the contents following vortexing. Supernatants from each animal were then spotted (10 μL) onto freshly seeded plates for phage enumeration after plaque formation.

Fecal Phage Excretion Profile Following Oral Phage Administration to Chicks

The fecal excretion of phage from chicks was determined in a separate animal trial using 6 chicks per group serving as replicates. Two groups of chicks were given: 1) free phage in liquid suspension and 2) encapsulated phage FO1 by gavage (10^9 PFU/chick), then all the feces after each time point were collected (pooled) from the floor pan of the isolation unit and SM buffer added in an amount equal to 1 to 9× the weight of collected fecal material, corresponding to a 2 to 10× initial dilution. Lower dilutions were used when a low phage concentration was expected at the earliest and latest time points. Control chicks were given 200 μL of SM buffer and feces were collected at the same interval. Samples were mixed by vortexing and then centrifuged to settle solid materials; the supernatant was used to spot in triplicate onto freshly seeded STDT104 NaI^2- lawns to determine plaque counts post incubation. Supernatant from control feces were spotted onto STDT104 NaI^2- agar plates to confirm the absence of endogenous plaque formation against our selected strain overnight, prior to the fecal phage excretion study.

In vitro Phage Incubation with Chick Fecal Material and Buffer Solutions

Fecal material excreted by control chicks was quickly collected into sterile screw-cap tubes for use in incubation studies to determine its effects on phage FO1 viability. Free phage suspension (0.1 mL) or encapsulated phage (0.1 g beads) was mixed with feces (0.5 g) in a 10 mL screw cap vial and a 10× volume (6 mL) of SM buffer or MBS solution was added to disintegrate the beads, exposing phage to the mixture. The control did not contain added fecal material. Incubations were carried out at 0.5, 1, 2, 4, 8 or 24 h at 37°C with gentle shaking. The viable phages were determined at the end of each incubation time point using the bacteriophage enumeration assay previously described above to assess the percentage of viable phage compared to the initial titer. The combinations of MBS or SM buffer solutions (10 mL) individually and with fecal material (0.5 g) were tested as well for any negative effects on phage survival.

Microscopic Observation

To monitor the movement and state of degradation of the beads along the chicks’ GIT over time, samples were collected during dissection to obtain the GIT contents. Pictures were taken prior to collection of samples into tubes, to determine if any beads were present. Beads were made visible by incorporation of trypan blue (0.1% w/v) prior to extrusion of the alginate-whey protein mixture into a calcium bath.

Statistical Analysis

Concentrations (PFU/mL) of phage were converted to log_{10} (PFU/mL) and then averaged. Data are presented as means ± standard deviation (STDEV)/standard error (SEM). Differences between means were evaluated using SAS 9.0 software for Windows with a P-value < 0.05 considered statistically significant. Multiple comparisons between means were done with PROC GLM with LSMEANS and Tukey’s correction to perform 2-way analysis of variance (ANOVA).

RESULTS

Phage release from different formulations was tested in the absence of added digestive enzymes. The results indicate that substitution of low MW alginate partially with ultra-low MW alginate substantially accelerated the release of phage from the beads compared to a formulation without ultra low MW alginate, as shown in Figure 1. After exposure to SIF, there was close to 80% release by 30 min and around 100% after 60 min from all formulations. There was little difference between the 6 formulations in terms of the phage release rate. As such, formulation F5 was chosen for further study because the beads from this formulation were more uniform in size distribution and contained more high-MW alginate for better mechanical strength.
In vitro Incubation of Phage FO1 with Chick Feed and Digestive Enzymes

Incubation of viable phage FO1 was performed with chick starter feed to evaluate any negative effects under simulated gastric (low pH) and intestinal (neutral pH) conditions. Table 2 summarizes the effects of incubating free and encapsulated phage FO1 with chick feed and digestive enzymes over time. The results showed that there was no time-dependent detrimental effect on phage viability (i.e., >1 log10 reduction) observed during incubation with the chick feed. In addition, the presence of added digestive enzymes for 2 h in SGF (pepsin) followed by 2 h in SIF (pancreatin) did not affect phage viability over the time course tested. Free phage lost viability completely in the simulated acidic environment in the absence of feed, but was resilient to the acidic conditions in the presence of feed. This was caused by the observed buffering effect of feed, which raised the pH of the feed–phage mixtures to between 4.5 to 5.2 after mixing with SGF of pH 2.5. Phage FO1 survived in the encapsulated form without feed due to the Ca2+-alginate-whey protein matrix preventing direct exposure to acid. Although there were some statistically significant differences observed against some controls at the initial time points (within rows and within columns) in Table 2, they were likely caused by the use of a more concentrated phage stock during the preparation of controls for each treatment time point. These differences were minor (<0.7 log) and did not occur against all controls and the level of phage remained steady across the incubation times (within column).

Table 2. Incubation of phage FO1 with and without chick feed and digestive enzymes in simulated gastrointestinal conditions. Values are presented in log10 (PFU/mL) (AVE ± SE, n = 3). FP = free phage, EP = encapsulated phage. Means with significant difference (at P < 0.05 level) as determined by a 2-way ANOVA and Tukey’s correction, are indicated by different letters (a, b) across row and by different subscript numbers (1,2) within column.

<table>
<thead>
<tr>
<th>Initial log10(PFU/mL)</th>
<th>(Feed, enzymes)</th>
<th>(Feed, no enzymes)</th>
<th>(No feed, enzymes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FP</td>
<td>EP</td>
<td>FP</td>
</tr>
<tr>
<td>0</td>
<td>FP</td>
<td>9.59</td>
<td>EP</td>
</tr>
<tr>
<td>120</td>
<td>FP</td>
<td>9.17±0.22</td>
<td>EP</td>
</tr>
<tr>
<td>17</td>
<td>FP</td>
<td>9.50±0.09</td>
<td>EP</td>
</tr>
<tr>
<td>30</td>
<td>FP</td>
<td>9.50±0.09</td>
<td>EP</td>
</tr>
<tr>
<td>45</td>
<td>FP</td>
<td>9.80±0.08</td>
<td>EP</td>
</tr>
<tr>
<td>45</td>
<td>FP</td>
<td>9.80±0.08</td>
<td>EP</td>
</tr>
<tr>
<td>60</td>
<td>FP</td>
<td>9.70±0.09</td>
<td>EP</td>
</tr>
<tr>
<td>90</td>
<td>FP</td>
<td>9.62±0.08</td>
<td>EP</td>
</tr>
<tr>
<td>120</td>
<td>FP</td>
<td>9.66±0.06</td>
<td>EP</td>
</tr>
</tbody>
</table>

Figure 1. Release profiles of the 6 encapsulation formulations compared to the 1.5% Alginate-3% whey protein formulation, in simulated intestinal fluid without digestive enzymes. F1–F6 refer to the different encapsulation formulations found in Table 1 (AVE ± STDEV, n = 3).

Phage Distribution Within the Chicken Digestive Tract Following Single Oral Dose in Absence of Host Bacteria

The distribution of phage FO1 within the GIT contents of chicks administered as a single oral dose of FP or EP was determined after 1, 2 or 4 h (Figure 2a, b, and c, respectively), in the absence of host Salmonella. Figure 2a shows that phage from EP and EP were present in the gizzard, ileum and cecum by 1 h but the highest concentrations remained in the crop. After 2 h, both encapsulated and free phage were present in the ileum and cecum at between 3–4 log10 PFU/g contents, which was about ~3 log10 PFU lower than in the crop. After 4 h, numerous beads were still present in digestion of the crop and gizzard as seen in Figure 3; seemingly functioning as a reservoir for many h post-gavage. Secondary treatment of these crop contents with MBS solution yielded phage levels between 6–8 log10 PFU/g, which indicated that high titers of phage were protected within the beads still in the crop but were disintegrated in the other regions as comparable levels were found pre- vs. post-treatment with MBS (data not shown). The distribution of FP in the GIT was similar to that for EP in that significantly higher phage concentrations were found in the crop contents even after 4 h, compared to the levels observed in the gizzard, ileum and cecum. There were no significant differences between the formulations within each time point. Nonetheless, phage FO1 was able to reach all parts of the chick digestive tract within 1 h, appearing at concentrations similar to the beads of around 3 to 5 log10 PFU/g, which was fairly low relative to the initial dose of 9.77 log10...
Figure 2. The in-vivo distribution of free/released FO1 after single oral doses of encapsulated phage (EP) and free phage (FP) in SM buffer after (a) 1 h, (b) 2 h, and (c) 4 h. (AVE ± STDEV, n = 4). *indicates significant difference between means at p < 0.05 level after 2-way ANOVA and Tukey’s correction. Chicks were given a dose of (FP) $3 \times 10^{10}$ PFU/chick and (EP) $4.6\times9 \times 10^{9}$ PFU/chick.

PFU/chick. These distribution patterns strongly suggested that a single phage dose can take much longer than 4 h for complete passage through the GIT.

Fecal Excretion of an Oral Phage Dose Over Time

Another study was carried out to monitor the passage time of phage FO1 through the chick GIT and excretion into the feces following oral doses of EP and FP (5.86 $\times 10^9$ or 9.77 log$_{10}$ PFU/chick) with the results illustrated in Figures 4 and 5, respectively. As shown in Figure 4, it took only 0.5 h for viable phage to be released from encapsulated beads and detected in the chick’s feces. This suggests that a portion of the dose moved rapidly through the chick’s GIT, possibly driven by water consumption and feed intake immediately following gavage; however, the rapid movement could also be due to the immature GIT of young chicks. A peak concentration ($\sim 4.5$ log$_{10}$ PFU/g) was found excreted in feces by 4 h, followed by lower levels ($\sim 3.5$ log$_{10}$ PFU/g) being continuously detected for up to 24 h post gavage. Comparing Figures 4 and 5, the movement of the liquid phage dose, FP, through the GIT of the chicks was more rapid, indicated by the higher fecal concentration after 1.5 h compared to 4 h for EP, then forming a plateau with sustained phage excretion at levels of between 3–4 log$_{10}$ PFU/g for more than 6 h following the initial dose. Data were not collected past 6 h because it was initially estimated that all phage from a liquid dose will have passed out within this timeframe. However, these results suggest phage excretion occurred for a longer period. The concentrations observed in the feces were comparable to EP levels and this meant that the free phage in liquid form survived the journey through the chick’s GIT. The results obtained thus far reveal that phage levels found in the GIT and feces were consistently lower by many orders of magnitude than the high initial dose given.

In vitro Incubation of FO1 in Chick Feces, MBS, and SM Buffer

In order to rule out the possibility that short-term storage of phage in the sample matrices had an effect on phage viability, phages were incubated in the presence of chick fecal material in combination with MBS or SM buffer for up to 24 h. Table 3 illustrates there were no detrimental effects on phage viability during the incubation (storage) with the chick fecal material in combination with the encapsulation materials and the buffer solutions used to dissolve the beads.

DISCUSSION

The main objectives of the current work were to assess specific concerns surrounding the oral delivery of encapsulated phage and free phage to young chicks for eventual bio-control of pathogenic Salmonella. Firstly, we aim to achieve more rapid phage release under intestinal pH conditions considering the specific characteristics of the GI of young chicks. The current result (Figure 4) indeed demonstrated that encapsulated phage appeared in the feces in less than 1 h after oral administration. Therefore, a fast release formulation would minimize the likelihood of phage being excreted from the chicks within the beads. Based on a phage encapsulation formulation from earlier work (Ma et al.,
Figure 3. Images of chicken GIT contents after oral gavage of encapsulated phages. In crop contents (a) some blue beads (encapsulated phage) are observed after 2 h and (b) beads are still visible after 4 h. Numerous blue beads are seen among ingested feed in gizzard contents after (c) 2 h and (d) 4 h following gavage.

Figure 4. Fecal excretion of FO1 over time following gavage of a single oral dose of 200 mg beads delivering $6 \times 10^9$ PFU/chick (AVE ± SEM, n = 6).

Figure 5. Fecal excretion levels of phage FO1 given orally in liquid suspension containing $\sim 6 \times 10^9$ PFU/chick (AVE ± SEM, n = 6).

2008; Tang et al., 2013), rapid release was achieved by incorporation of an ultra-low MW alginate into the formulation and with only slight modifications to the other components, low-MW alginate and whey protein, the 6 formulations showed similar fast phage release kinetics resulting in close to 100% release by 45 min (Figure 1). In addition, the improved formulation did not compromise its protective effect in simulated gastric conditions (Table 2).

Phages are known to have widely differing sensitivities to environmental conditions such as chemicals, pH, heat, UV light and other agents (Adams, 1959; Ly-Chatain, 2014). In oral application, phages are directly exposed to the feed, digestive tract contents and enzyme secretions, most notably, the acidic pH, which can deactivate phages. To combat this, some authors have added acid-neutralizing agents (calcium carbonate) before oral administration of phages to animals (Smith et al., 1987; Atterbury et al., 2007a). Therefore, we investigated the effect of incubating phage together with feed and digestive enzymes on FO1 survival. We found that incubation of encapsulated phage FO1 with feed

| Table 3. Incubation of encapsulated and free phage with buffer in chick feces and its effect on phage survival over time. Data shown are in log$_{10}$ PFU, (AVE ± SE, n = 3). |
|---|---|---|---|
| Time EP + MBS EP + MBS EP + SM (h) (control) + feces buffer + feces | 0.5 9.43 ± 0.03 8.97 ± 0.04 8.90 ± 0.01 | 9.91 ± 0.08 8.92 ± 0.05 8.92 ± 0.06 | 9.39 ± 0.04 9.00 ± 0.10 8.89 ± 0.03 |
| 1 9.43 ± 0.02 8.92 ± 0.04 8.68 ± 0.17 | 9.35 ± 0.02 8.92 ± 0.04 8.28 ± 0.57 | 9.28 ± 0.03 8.94 ± 0.03 7.14 ± 2.28$^*$ |

* indicates significant difference between means ($P < 0.05$) within row.
in simulated acidic and intestinal conditions showed no consistent negative effects on phage viability. The high initial titer of phage was maintained to the end of incubation in SGF in the case of EP. The chick feed was found to buffer FP against deactivation by acid, whereas FO1 was easily deactivated at pH 2.5 in absence of feed (Ma et al., 2008). Although other studies have found more significant reductions (∼1 to 2 log10 units) in viable phage following addition to feed (Sklar and Joerger, 2001), this was not evident with our tested feed. Incubations in the current study were performed with liquid surrounding the feed particles. Had phage been added directly to a dry substrate (e.g., dry feed pellets), it would have lost viability from desiccation (Adams, 1959). Similarly the incubation of FO1 (both FP and EP) with chick feces showed no negative effects on phage viability (Table 3). Samples incubated in chick feces appeared slightly lower compared to the control (no feces) and this was consistent over all time points, suggesting that it was likely due to surface adsorption of phage (Sklar and Joerger, 2001) onto solid particulates with no significant loss in viability. Overall, our results indicated that phage FO1 was not deactivated under these simulated exposures and corroborates phage being found in diverse environments (Berchieri et al., 1991; Andreatti Filho et al., 2007) such as sewage and fecal material (Anany et al., 2011) and being able to pass through the human digestive tract as well (Sarker et al., 2012). Some deactivation of the free phage occurred after 24 h in the fecal material, which suggests that an assay for viable phage is best performed before this storage time is reached.

We assessed the temporal distribution of phage in young chicks after oral phage administration in encapsulated and liquid forms, and with ad libitum access to feed and water, as our goal was for phage to be delivered along with animal feed. In the current study, host bacteria were excluded in order to avoid phage amplification, which could confound phage counts yielded by passive, oral delivery. Overall, the FP had a similar distribution profile as EP following gavage of a single dose (∼9.8 log10 PFU) of FO1 per chick. Results obtained from fecal phage excretion (Figures 4 and 5) and in vivo distribution measurements (Figure 2) agreed well. There were around 3 log10 PFU of phage present shortly after administration and for a sustained period in the lower intestines, which suggested they had potential to attack pathogenic bacteria. The presence of significantly higher phage concentrations retained in the crop after 4 h suggests longer time is needed for complete passage of a gavaged dose. On the other hand, due to the potential of the crop to act as a reservoir and its neutral pH, the prolonged retention of phage in this site could provide sustained exposure to any new susceptible pathogen being ingested orally, allowing phage to interact and/or propagate to continuously supply the gut with more phage. The fecal phage excretion profiles showed that maximum concentrations were observed after 1.5 and 4 h for free and encapsulated phage, respectively, which indicated that a faster-moving portion of the dose passed through the chicks into feces, yet after 4 h the highest concentrations (between 5 to 8 log PFU) still persisted in the upper GIT (crop and gizzard) following both forms of phage administration, suggesting that non-uniform distribution (separation) of the dose occurred. This was possible since EP allowed portions of the dose to move and distribute separately from the feed along the GIT, similar to a liquid mixed with feed, but because of their size, were thus retained longer as evidenced in the slower drop in phage concentration in the crop. Passage of the FP dose was faster than EP, evidenced by earlier appearance of peak phage concentration in the feces as well as a more rapid drop in phage concentration in the crop between 1 and 4 h. The apparent dissimilar fecal excretion profiles between EP and FP was most likely due to the fact that EP beads required extra time to be broken down and release phage from the gizzard, whereas FO1 behaved more like a liquid, distributing among the GIT digesta while being carried at the rate of feed passage. FP survived the journey through the acidic proventriculus and gizzard, which can be explained by the buffering effect of feed, in addition to the young age of chicks, which do not yet have mature digestive and enzyme secretion capabilities (Noy and Sklan, 1995). These results suggested that upon administration of a phage FO1 dose, the observed low concentrations of viable phage were not due to phage deactivation. Rather, the high initial phage dose experienced segmented movement along the GIT from peristalsis in addition to dilution by continued feeding/drinking, and one cannot rule out absorption of phage into the chick’s organs or other compartments, since phage have been observed to pass into the blood stream following oral administration in chicks (Oliveira et al., 2009), mice and humans (Bruttin and Brussow, 2005), with the spleen and liver being the primary organs responsible for removing phage from the circulation. Further studies should be done to examine intestinal phage distribution after longer periods of time and in the organs in order to observe more complete passage of the dose to the lower GIT. Although neutralizing antibodies to phage have been observed by some authors they require longer times to develop and depended strongly on the route of phage administration (Smith et al., 1987; Capparelli et al., 2010).

An uneven temporal distribution pattern of ingested material was similarly reported by other researchers who placed soluble and insoluble markers within feed to study the passage rate in 3- to 6-wk-old chickens (Vergara et al., 1989; Svihus et al., 2002). Svihus et al. showed that during feeding the ingested material could be retained in the crop for extended periods of time (after 3 h) before being gradually passed further down the GIT (to the proventriculus) at an average rate of 5 g contents per hour, depending on the chicks’ feed intake level and activity of the gizzard. Yet after 30 min, a notable amount of marker was already distributed into all parts of the small intestine. Vergara et al. (1989)
reported that the typical half-emptying time of the crop of chickens was between 2.3–3.1 h and insoluble materials/particulates were retained by the gizzard longer than soluble ones (eg. Cr-EDTA). The crop and gizzard together regulate downward movement of feed to allow for continuous digestion and absorption, with the crop able to store food for longer periods of time in order to sustain long-term energy needs of the animal. The gizzard regulates passage by withholding material only until particles are reduced to a homogeneously small size before being passed to the intestines for further digestion and absorption. Considering these digestive processes work to regulate movement of material, the complete movement of a phage dose would take much longer than 4 h, consistent with the observed slow and partial movement of both the EP and FP doses. Furthermore, the ceca of chicks only permits very small particles (<0.2 mm) and typically the liquid fraction of the passing digesta to be taken up (where up to 50% of a soluble marker was found accumulated) and can be held for up to 7 h (Vergara et al., 1989). Together, these slow physiological processes along with the absence of host bacteria for phage amplification could explain the low concentrations that we observed in the lower GIT, which can potentially limit high phage concentrations reaching the cecum from an oral (passive) delivery, unless the phage loading density can be increased or multiple dosing of phage is applied to maintain a high ratio of phage to bacteria in vivo.

The studies carried out with the model bacteriophage Felix O1 revealed the temporal distribution of phage in the GI lumen of young broiler chicks consuming feed and water and following oral delivery of a dose of bacteriophage. This information is expected to guide future studies evaluating the ability of bacteriophages to influence pathogen colonization levels in young broiler chicks. Our results showed that the encapsulation matrix successfully allowed delivery of phage to the entire GIT. Viable phage was found distributed along the small intestine by 1 h and appears to be able to persist in the chicks for much longer than 4 h due to gradual emptying of crop contents. Limited phage levels were achieved in the ileum and cecum due to the segmented movement of contents in the digestive system, suggesting that in young chicks multiple dosing may be required with intervals longer than 4 h, or the dosage can be increased. Continuous in-feed phage delivery could present a viable strategy for farmers to protect flocks against incoming Salmonella by maintaining a steady phage supply along the GIT. Further study is necessary to examine the dissemination of phage to various organs.

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REFERENCES


**APPENDIX**

**Table A1.** List of ingredients and major nutrient levels of crumbled chick starter feed.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Nutrients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>corn, chopped</td>
<td>crude protein</td>
<td>≥20%</td>
</tr>
<tr>
<td>wheat, chopped</td>
<td>crude fat</td>
<td>≥3%</td>
</tr>
<tr>
<td>shorts and bakery</td>
<td>crude fibre</td>
<td>≤4%</td>
</tr>
<tr>
<td>canola</td>
<td>calcium</td>
<td>1%</td>
</tr>
<tr>
<td>soybean meal</td>
<td>phosphorus</td>
<td>0.73%</td>
</tr>
<tr>
<td>pork meal</td>
<td>sodium</td>
<td>0.17%</td>
</tr>
<tr>
<td>corn distiller</td>
<td>Vitamin A</td>
<td>10,000 IU/kg</td>
</tr>
<tr>
<td>corn gluten</td>
<td>Vitamin D</td>
<td>2,750 IU/kg</td>
</tr>
<tr>
<td>limestone</td>
<td>Vitamin E</td>
<td>30 IU/kg</td>
</tr>
<tr>
<td>tallow</td>
<td>salt and other macronutrients</td>
<td></td>
</tr>
<tr>
<td>macrominerals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amino acids, part of protein (pelleted at high temperature)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>