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Edible bacteriophage based antimicrobial coating on fish feed for enhanced treatment of bacterial infections in aquaculture industry

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ABSTRACT

As the fastest growing food-producing industry in the world, aquaculture industries frequently suffer from the outbreaks of various bacterial diseases. There is a need to develop alternatives to antibiotics to address the challenge of bacterial infections without using conventional antibiotics. This study developed a bacteriophage based edible antimicrobial coatings on fish feed, providing an effective biological control strategy for treating fish and human pathogens in an aquaculture system. The edible whey protein isolate (WPI) coatings were effective in enhancing loading of phages on fish feed pellets and reducing the loss of phage activity during storage of feed. The feasibility of both dip coating and air-brush spray coating methods were demonstrated in this study. The edible coatings decreased the release of phages in salt water by > 1 log PFU/pellet after 2-h exposure as compared to control coatings on feed without the biopolymer coatings. The simulated gastric-intestinal digestion assay demonstrates that this coating provides enhanced stability of phages in a simulated gastric environment and a significant bacterial reduction (3–5 logs of target bacteria) in a simulated intestinal digestion. These antimicrobial activity results were demonstrated using a model bacterium (*E. coli*) and a fish pathogen (*Vibrio spp.*). Overall, the results show that phage incorporated WPI coatings on fish feed can be an effective approach for enhancing the treatment fish infections, reducing the risk of antibiotic resistance in aquaculture environment, and improving the safety and sustainability of aquaculture industry.

1. Introduction

Aquaculture is emerging as the fastest growing food-producing industry in the world due to the increasing demand for fish and seafood. Worldwide, the aquaculture industry has grown at an average rate of 8.9% per year since 1970, compared with a 1.2% growth rate of capture fisheries and 2.8% growth rate of terrestrial farmed meat production over the same period (Subasinghe, 2005, pp. 117–124). However, aquaculture industries frequently suffer heavy financial losses (estimated at billions of dollars every year) that threaten their growth and sustainability, mainly due to the outbreaks of various bacterial diseases. Among several factors contributing to disease outbreaks, bacterial infections have been recognized as a major impediment to aquaculture production (Sommerset et al., 2005, pp. 89–101). According to the data from Food and Agriculture Organization (FAO), the global economic impact of bacterial diseases on aquaculture production would be within the range of hundreds of millions to billions of dollars annually (FAO, 2016). Thus, there is an unmet need to effectively treat bacterial infections in aquaculture industry.

The conventional treatment approach based on antibiotics may have limitations especially due to emergence of antibiotic resistance in aquaculture facilities (Santos and Ramos, 2018,; Watts et al., 2017, pp. 158). Apart from the risk of antibiotic resistance in fish, the use of antibiotics also leads to a direct threat to human health and to the environment. It has been reported that antimicrobial resistance genes and antimicrobial-resistant bacteria from the aquaculture environments can be transferred to terrestrial environment and humans (Defoirdt et al., 2007, pp. 472–479; O'Neill, 2015, pp. 44). In summary, there is a need to develop alternatives to antibiotics to address the challenge of bacterial infections without using conventional antibiotics.

Bacteriophage based approaches have a significant potential to specifically prevent and treat bacterial infections in aquaculture environment. With the increasing incidence of antibiotic resistance, bacteriophage based therapeutics are being evaluated for potential to treat diversity of bacterial infections in animals (Doss et al., 2017, pp. 50; Lourenço et al., 2018,; Richards, 2014, pp. e975540). The unique advantages of lytic bacteriophages are their specificity for the host pathogen and ability to lyse bacteria and rapidly multiply upon successful

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infection (Kutateladze and Adamia, 2010, pp. 591–595; Se and Nakai, 2003, pp. 33–39; Vonasek et al., 2014, pp. 7–13). Despite significant potential, there are challenges in translating these solutions to industrial practice. Firstly, antibacterial phage therapy is more effective with MOI (multiplicity of infection) in the range of 10–1000 fold (Abedon, 2016, pp. e1220348; Shabram and Aguilar-Cordova, 2000, pp. 420–421). This implies that often high titer of phages (10^4–10^7 PFU/ml) is required for controlling bacterial infection. Ingestion of phages from bulk water by fish is limited. Thus, it is difficult to achieve the target MOI in the fish gut by simply adding phages to a fish tank. Second, due to their protein capsid structure and fragile tail fibers, phages are highly susceptible to loss in activity upon desiccation and environmental conditions such as heat (Jończyk et al., 2011, pp. 191–200). Thus, direct addition of phages to animal feed may result in a rapid loss of phage activity. Also, prior studies have observed significant decrease in phage activity in low pH environment (Abuladze et al., 2008, pp. 6230–6238; Leverentz et al., 2003, pp. 4519–4526). It has been reported that the pH values in the gastric environment of fish varies from pH 2 to 7 (Kihara et al., 2012, pp. 285–286), therefore, degradation of phages in the gastric tract can be a significant limitation for achieving efficacious antimicrobial therapy (Colom et al., 2015, pp. 4841–4849; Nobrega et al., 2016, pp. 39235). In addition, one of the concerns with introducing large number of phage concentration in aquatic environments is the ecological imbalance that could result due to spread of phages in the environment (Chibani-Chennoufi et al., 2004, pp. 3677–3686; Sime-Ngando, 2014, pp. 355).

The overall goal of this study was to develop a bacteriophage based edible antimicrobial coatings on fish feed, providing an effective biological control strategy for treating fish and human pathogens in an aquaculture system. Whey protein isolate coating was selected as a model edible coating material because of its excellent mechanical properties and oxygen barrier properties (Janjarasskul and Krochta, 2010, pp. 415–448). T7 bacteriophage and *E. coli* BL21 were used in this study as a model phage and a model bacteria respectively for the demonstration of this concept; while a *Vibrio* specific phage and *Vibrio* spp. were used for demonstrating this concept for a model fish pathogen, respectively. Also, two widely used industrial coating processes (Scriven, 1988, pp. 717 (13 pages); Susanna et al., 2011, pp. 1775–1778), dip coating and air-brush spray coating were used in this study, in order to evaluate the loading efficiency of phages on fish feed. The key innovations and the unique aspects of this study are: a) to assess the influence of biopolymer coatings on phage stabilization: storage stability, release of phage in a simulated seawater, and phage survivability in a simulated gastric digestion environment; b) to evaluate the feasibility of two coating methods, i.e. dip coating and air-brush spray coating; and c) to demonstrate the in-vitro antimicrobial efficacy of phages and biopolymer coatings to reduce target bacteria in a simulated intestinal digestion. Success in developing these phage based antimicrobial coatings on fish feed will significantly enhance the treatment of fish diseases, reduce the risk of antibiotic resistance in aquaculture environment and improve efficiency and sustainability in aquaculture industry.

2. Materials and methods

2.1. Chemicals and reagents

Whey protein isolate (WPI) was purchased from Davisco Foods International, Inc. (Eden Prairie, MN, USA) and glycerol was purchased from Fisher Scientific (Waltham, MA, USA). Fluorescein isothiocyanate (FITC) dye were purchased from Sigma-Aldrich (St. Louis, MO). Luria Bertani (LB) broth, LB agar, tryptic Soy broth (TSB) and tryptic soy agar (TSA) were purchased from Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of bacteriophage

T7 phages (#BAA-1025-B2) and host strain *E. coli* BL21 (#BAA-1025) were purchased from the American Type Culture Collection (ATCC) and propagated according to recommended methods by the ATCC. Briefly, 10 mL of Luria Bertani (LB) broth was inoculated with a colony of *E. coli* BL21 and grown at 37 °C at 150 rpm. The following day, 100 μL of the overnight culture was inoculated in 10 mL of pre-warmed LB broth and incubated until exponential phase was reached. 0.1 mL of T7 phages were cultured using an exponential-phase *E. coli* BL21 cells in the LB medium, and allowed to incubate in a shaking incubator at 37 °C and 250 rpm for 4 h to allow for several phage life-cycles. Phage propagation was blocked by adding 20% *v*/v chloroform and incubating for 15 min at 37 °C to lyse bacterial cells. The culture with chloroform was centrifuged at 10000 × *g* for 15 min. The phage in the supernatant was extracted and passes through a 0.22-μm filter (Huang and Nitin, 2017, pp. 207–217; Vonasek et al., 2018, pp. 1871–1879).

As a surrogate for a fish pathogen, *Vibrio* phages (#11985-B1) and host strain *Vibrio* spp. (#11985) were also purchased from ATCC and propagated according to the recommended methods by the ATCC. Similarly, 10 mL of Tryptic Soy Broth (TSB) supplemented with 3% NaCl was inoculated with a colony of Vibrio spp. and grown at 37 °C at 150 rpm. Overnight culture is firstly re-diluted (100 μL culture in 10 mL broth) to allow the bacteria to multiply into the exponential phase. 0.1 mL of Vibrio phages were cultured using an exponential phase *Vibrio* spp. cells in TSB supplemented with 3% NaCl and allowed to incubate in a shaking incubator at 37 °C for 6–8 h. Phage propagation was blocked with a chloroform treatment as described above and the recovered phages were stored at 4 °C until further use.

2.3. Bacteriophage activity assay – plaque forming unit counting

Phage activity was measured with a top agarose overlay plating method and reported in plaque forming units per milliliter (PFU/mL). Briefly, top agar (0.7% agar) was melted, and 3 mL aliquots were kept at 45 °C in a water bath until used. Phage samples were serially diluted into PBS buffer, and 100 μL of each sample was combined with 250 μL of fresh exponential phase bacteria culture. The samples were incubated for 10 min and then combined with an aliquot of molten agar. The molten agar mixture was poured onto a pre-warmed agar plate and allowed to solidify. Once the plates solidified, they were allowed to incubate at 37 °C and then counted.

2.4. Preparation of phage incorporated biopolymer coatings

The modified edible biopolymer coating formulation utilized in this study is based on our prior studies (Nitin and Vonasek, 2013,; Vonasek et al., 2018, pp. 1871–1879; Vonasek et al., 2014, pp. 7–13). Briefly, 5% *w/v* WPI was added to deionized (DI) water and stirred vigorously for 20 min. Glycerol at a 2:1 WPI to glycerol weight ratio was added to the solution and then stirred for an additional 20 min. The solution was placed in a water bath at 90 °C for 30 min to denature the whey protein and promote cross-linking of the protein. After cooling to room temperature in an ice bath, the WPI solution was then degassed with vacuum pump. Phages in a 1 mL aliquot were then added to the solution and gently mixed with the WPI coating solution. The final concentration of phages in the WPI coating solution was approximately 1×10^7 PFU/mL.

2.5. Loading efficiency of phages on WPI-coated fish feed

To compare the coating efficiency of two different deposition methods (dip coating and air-brush spray coating), the phage loading in WPI coatings were evaluated. Fish feed pellets were purchased from LaBudde Group, Inc. (Grafton, WI). The shape of pellets was close to

cylindrical with a length of 5 mm and a diameter of 5 mm. In the case of dip coating, the feed pellets were dip coated by full immersion into the coating solution and slowly pulled out of the coating. The air-brush spray coating was performed by using an air-brush sprayer (Master, San Diego, CA). The monolayer of feed pellets was placed in a pan and exposed to spraying for 15 s. The distance between spray nozzle and pellets were maintained at 10 cm. After coating, the pellets were dried in air for 2 h. Once dried, the mass of fish feed pellet was measured using an analytical balance (AE 160, Mettler, Columbus, OH, USA) with a measuring accuracy of 0.0001 g. To determine the phage loading efficiency, the pellets were transferred to a sterile test tube containing 1 mL of maximum recovery diluent (MRD, Sigma, St. Louis, MO), crumbled into small pieces using a spatula, and allowed to stand for 2 min. After vortexing at full speed for 1 min, the active phage concentration in the homogenized liquid was measured using the standard plaque counting assay as described above.

To demonstrate the uniformity of WPI coating on fish feed, the fluorescence imaging was performed on uncoated and fluorescently labeled WPI coated feed samples. WPI was chemical conjugated to a fluorescein isothiocyanate (FITC) dye following a conjugation procedure described in the prior publications (de Belder and Granath, 1973, pp. 375–378; Heilig et al., 2009, pp. 646–653). The dip-coated and spray-coated fish feed samples were prepared as described above. Fluorescent images were acquired with a TCS SP8 confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) using the 20× objective lens with the excitation and emission wavelengths of 494 nm and 522 nm, respectively.

2.6. Phage stability in WPI coated fish feed

The storage stability of phages coated on fish feed was determined by storing fish feed pellets over a period of 1 month. Fish feed were prepared and coated in WPI coating and water coating with phages as previously described. Samples were placed in petri dishes and placed in an incubator at 30 °C with humidity of 40–45%. After 0, 7, 14, 21, and 28 days, phage survivability was measured as described in the previous section. Stability measurements were completed in triplicate and reported as log PFU/pellet.

2.7. Release of phages in salt water

To determine the release profile of phages from the WPI coatings in salt water, the coated fish feeds were exposed to 3.5% NaCl solution without any mechanical shaking to measure passive release of phages from WPI coatings. The feed samples with phages without biopolymer coatings were tested as a control group. The fish feed was recovered from a salt solution at 0.5, 1, and 2 h, and the phage activity was measured with PFU counting to determine the number of phages remaining on the fish feed. Each sample was done in triplicate.

2.8. Phage stability in simulated gastric digestion

Phage survivability in a simulated gastric environment was measured. Simulated gastric fluid (SGF) was prepared according to the procedure described by (Yasumaru and Lemos, 2014, pp. 74–84). 1 L of SGF was prepared by adding 5 g sodium chloride and 5 mL of HCl. The pH was adjusted to 1.2. 0.018 g NaCl and 0.032 g pepsin was dissolved in 10 mL SGF. This digestion mixture was placed in a dialysis cassette and a fish feed pellet was put into the dialysis cassette. The dialysis cassette was placed in a 1 L SGF maintained at temperature of 37 °C. The fish feed was sampled after every 30 min and phage activity was measured using the method described earlier.

2.9. In-vitro challenge assay in simulated intestinal environment

The antimicrobial efficacy of WPI-phage coating was tested by in-vitro challenge assay in a simulated intestinal environment. Simulated intestinal fluid (SIF) consisted of 0.05 M potassium dihydrogen phosphate solution (pH 7.5). One gram of fish feed was mixed with 10 mL of SIF containing 0.6 g of lipase, 0.2 g calcium chloride and 1.5 g bile salts. The final concentrations of potassium dihydrogen phosphate and lipase were 0.025 M and 10 mg/mL, respectively. Fresh bacterial culture in the exponential phase of growth was diluted and added in digestion mixture to obtain a final concentration of 6 and 8 log CFU/mL, respectively. The digestion mixtures were incubated at 37 °C. The aqueous samples were collected after incubation for 0.5, 1, and 2 h, respectively, then serially diluted and plated on agar plates. The plates were incubated at 37 °C for 24 h. After incubation, the plates were counted. Each measurement was measured in triplicate.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism software V.5.04 (Graphpad Software, Inc., La Jolla, CA). To analyze differences between multiple groups on each data set, one-way analysis of variance ($p < .05$) were performed in this study.

3. Results and discussion

3.1. Loading efficiency of phages in WPI coatings

To compare the deposition efficiency of dip coating and air-brush spray coating methods, the mass of biopolymer materials deposited onto fish feed was measured. The results in Fig. 1 show that the dip coating was able to deposit significantly higher amount of coating materials on the fish feed pellets as compared to air-brush spray coating method ($P < .05$). The average mass of WPI deposited using a dip coating method was 0.0056 ± 0.0016 g/pellet, while 0.0031 ± 0.0007 g/pellet of WPI was deposited using a spray coating methods. Similar observation was reported in the prior work (Garcia et al., 2017, pp. 020017) focusing on the preparation of Silicon/Zirconium sol-gel film for corrosion protection of aluminum alloy, the average mass and thickness of coating achieved by dip coating method was 2–3 times more than spray coating method. This observation was further supported by the fluorescence imaging of the deposited WPI on fish feed using dip coating or air-brush spray coating (Fig. 2). The thickness of dip-coated WPI layer was higher as compared to air-brush spraying, which indicates a higher mass of coating materials deposited on fish feed. In addition, the WPI coating obtained by the air-brush spraying was more uniform than that prepared by the dip coating method.

To characterize the effect of WPI coatings on the loading efficiency of phages on fish feed, the phage loading for both the dip- and spray-WPI coatings was evaluated. Control samples were coated with phages

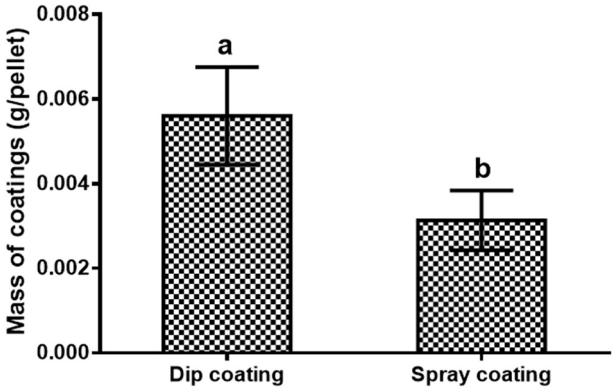

Fig. 1. Mass of deposited materials based on two industrial coating methods: dip coating and air-brush spray coating.

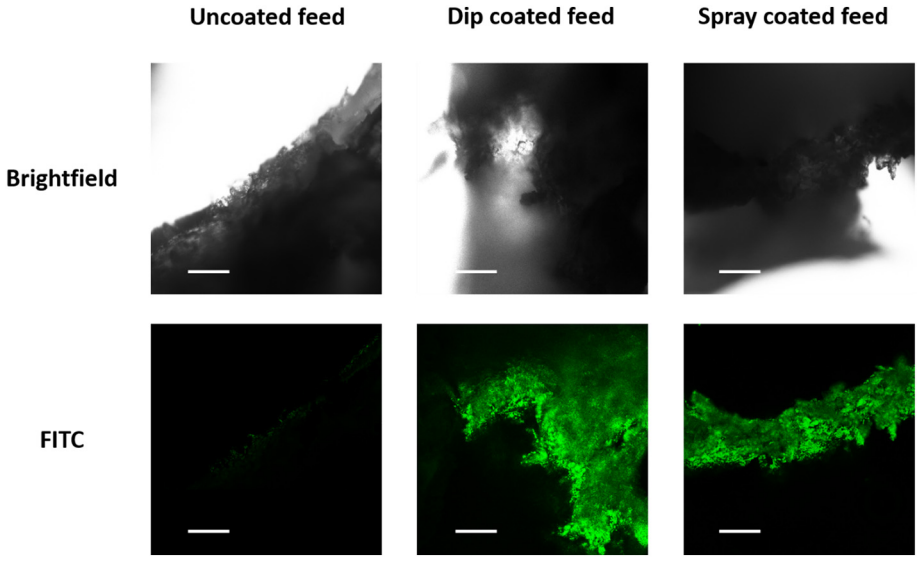

Fig. 2. Fluorescent images of fish feed pellets with and without WPI coatings. From left to right, top row: White light images of control feed without coatings, dip coated feed, and spray coated feed; From left to right, bottom row: FITC images of control feed without coatings, dip coated feed, and spray coated feed. The scale bars in the images represent the length of 100 μm.

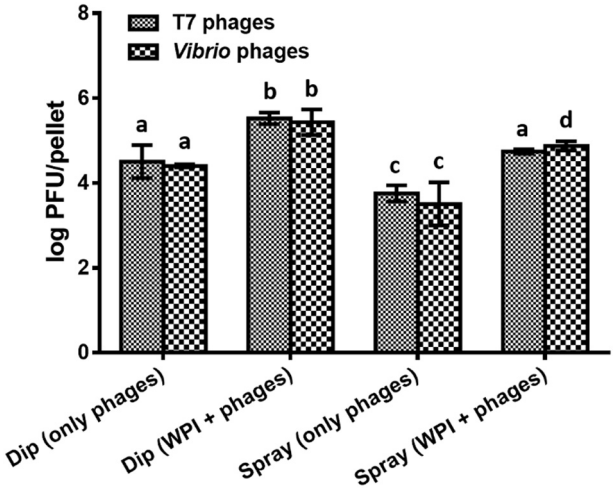

Fig. 3. Loading efficiency of T7 and Vibrio phages in WPI coating and water control on fish feed using both dip coating and air-brush spray coating methods.

suspended in a water solution. For the dip coating method, the WPI coating achieved almost 5.5 log PFU/pellet loading for both T7 and *Vibrio* phages, while the control coating without the biopolymer formulation at the same initial phage concentration only achieved a loading of approximately 4.5 log PFU/pellet for both the selected phages (Fig. 3). The enhanced loading of phages by edible WPI coatings may be attributed to the higher viscosity in the WPI solution compared to water, which can increase the material amount that can be deposited on a surface (Cisneros-Zevallos and Krochta, 2003, pp. 503–510; Vonasek et al., 2018, pp. 1871–1879). For the air-brush spray coating, WPI coating loaded approximately 4.8 log PFU/pellet, which was almost 1 log lower than the dip coating method. The higher loading efficiency of phages achieved by the dip coating method could be attributed to: a) higher amount of coating materials deposited on fish feed (Fig. 1); and b) potential inactivation of phages during spray coating by shear (Leung et al., 2016, pp. 1486–1496). Considering the relatively low initial loading levels of phages (< 4 log PFU/pellet) achieved using a spray-coated control samples (without WPI coatings), this group of samples was not included for further analysis of the phage coated pellets.

3.2. Storage stability of phages in WPI coatings

The results in Fig. 4 show the stability test of T7 and *Vibrio* phages coated on fish feed during storage of the coated fish feed at the temperature of 30 °C and humidity of 40–45% over 4 weeks. The fish feed samples dip-coated with phages suspended in water were used as the control group. For the WPI-T7 phage coated feed pellets using the dip coating or the air-brush spray coating methods, a decrease of 1.2 and 0.9 log PFU/pellet were observed after 4 weeks of storage at 30 °C and humidity of 40–45%, respectively. In contrast, control feed-phage samples without WPI formulation lost approx. 3.5 log PFU/pellet after

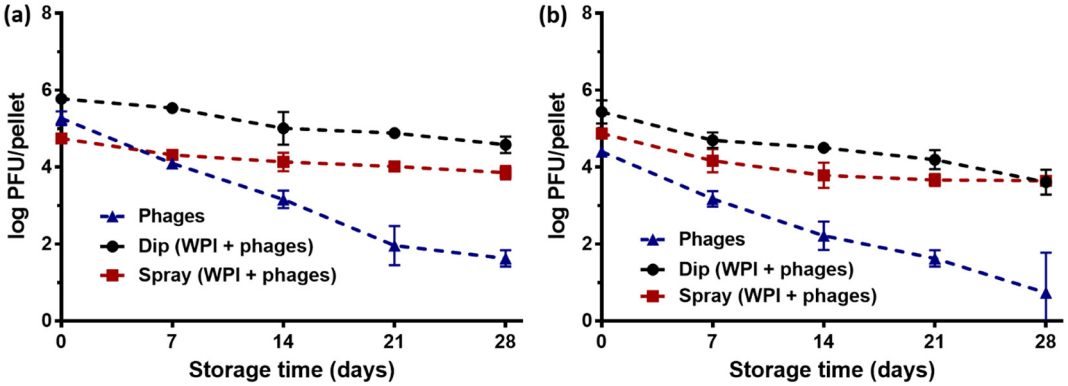

Fig. 4. Stability of phages in WPI coated fish feed as a function of time under 30 °C. The stability of phages in edible coating was also compared against phages deposited directly on fish feed without coating.

4 weeks of storage under the same set of conditions. Similar trend was observed for the *Vibrio* phage -WPI coating as compared to the control. Based on these results, it is concluded that WPI coating provides enhanced stability for phages on fish feed over 4 weeks of storage at 30 °C.

One of the key challenges in developing phage based antimicrobial materials is the stability of phages in material formulation (Ma et al., 2008, pp. 4799). Most of the current understanding of phage stability is based on the analysis of phages in an aqueous environment while there is very limited understanding of the stability of phages in material formulations (Fister et al., 2016, pp. 1152; Jończyk et al., 2011, pp. 191–200). The enhanced stability may result from composition of the edible biopolymer coating. The presence of a significant fraction of the plasticizer- 2.5% *w/v* of glycerol, can potentially prevent desiccation of the coated phages during storage (Mendez et al., 2002, pp. 215–224). On the other hand, the WPI protein environment may help stabilize the viral protein capsid (Mylon et al., 2010, pp. 1035–1042; Puapermpoonsiri et al., 2010, pp. 168–175). The loss in phage activity without edible WPI coatings can be attributed to desiccation induced unfolding of viral capsid as well as proteolytic degradation of phage particles on the fish feed (Vonasek et al., 2018, pp. 1871–1879; Vonasek et al., 2014, pp. 7–13). Prior studies have demonstrated the denaturation of viral capsid by desiccation may lead to a significant loss of phage activity (Iriarte et al., 2007, pp. 1704–1711; Laidler et al., 2013, pp. 13927–13,929). In addition, the proposed WPI coating may also protect phages against oxidation induced by environmental factors including light and oxygen (Atamer et al., 2013, pp. 191).

3.3. Release of phages in simulated sea water

After characterizing the stability of phages on a modified fish feed, the next aim was to characterize the release of phages in simulated sea water mimicking the high salt content of the marine environment. The results demonstrate the edible WPI coatings are able to limit the release of phages from the coated feed in simulated sea water within the first two hours of incubation as compared to the control coating of phages on fish feed (Fig. 5). The results for control coatings indicate a rapid release of phages from the fish feed without WPI coating. During the 2 h of incubation, > 2 log PFU/pellet of phages were released from the control coatings for both the coated T7 and *Vibrio* phages. However, the release of phages from the WPI coatings in a salt rich environment was limited, as only 0.7 log and 0.5 log PFU/pellet of phages were released for T7 and *Vibrio* phages, respectively were released after 2 h of incubation. Dip-coated and spray-coated fish feed pellets did not show significant difference in the number of phages released in a salt water environment ($P > .05$). Thus, the differences in the coating thickness on the fish feed using these two methods did not significantly influence the release of phages in a salt water environment. The release profile of phages in WPI coatings exhibited an initial burst release of 0.3–0.5 logs during the first 30 min followed by a limited release of phages from feed pellets during the subsequent 1.5 h of incubation (P > .05). The observed burst effect was possibly due to dissociation of weakly associated protein molecules from the surface of feed pellet (Amidi et al., 2006, pp. 107–116). The second part of the release profile is related to the slow release of entrapped phages that may result due to swelling of the WPI coatings upon contact with water (Vonasek et al., 2014, pp. 7–13).

Reducing the release of coated phages from fish feed to an aqueous environment using biopolymer coatings is critical for an effective treatment of bacterial infections in aquaculture. It has been reported that effective antibacterial phage therapy usually require high MOI values, i.e. 10–1000 fold higher concentration of phages compared to bacterial concentration (Abedon, 2016, pp. e1220348; Shabram and Aguilar-Cordova, 2000, pp. 420–421). This implies that the scale up cost to achieve the total number of phages required for a large water volume in a commercial scale operation can be cost prohibitive. Thus, deposition of high titer of phages on fish feed enables a localized high concentration of phages. In addition, limited release of phages using the biopolymer coating further improves delivery of high MOI of phages to fish. Furthermore, limited release of phages in aquaculture water also addresses potential ecological concerns associated with the release of large concentration of phages to the environment (Chibani-Chennoufi et al., 2004, pp. 3677–3686; Sime-Ngando, 2014, pp. 355).

3.4. Stability of phages in simulated gastric digestion

The results in Fig. 6 show survivability of phages in the WPI and control coatings on fish feed during a simulated gastric digestion. For T7 phages, the results show that the phages without WPI coating degraded significantly after exposure to a simulated gastric digestion conditions as described in the materials and methods section. After 30 min exposure, phages in the control coating samples presented no detectable phages on the feed pellet as well as in the simulated gastric fluid. Similar decrease in phage activity was observed for the *Vibrio* phages coated control samples. This significant loss of phage activity could result from the acidic condition in the simulated gastric digestion, which caused significant inactivation of phages (Oliveira et al., 2014, pp. 137–142; Vonasek et al., 2018, pp. 1871–1879). For the WPI coatings, no significant difference in phage survivability was observed for both the dip-coating and spray-coating methods. For T7 phages in WPI coating, the dip-coated and spray-coated WPI on fish feed showed a decrease of approximately 1.6 log PFU/pellet after 1-h exposure. In the case of *Vibrio* phages in WPI coating, the decrease in phage activity, upon exposure to simulated gastric conditions was < 1.5 log PFU/pellet. These results are in agreement with the prior studies (Vonasek et al., 2018, pp. 1871–1879), which indicated that the viability of phages in a biopolymer formulation declines about 1 log PFU after 30 min of incubation in a simulated gastric environment. Overall, the WPI coating significantly enhanced the stability of T7 and *Vibrio* phages during the simulated gastric digestion compared to the control coating without biopolymer.

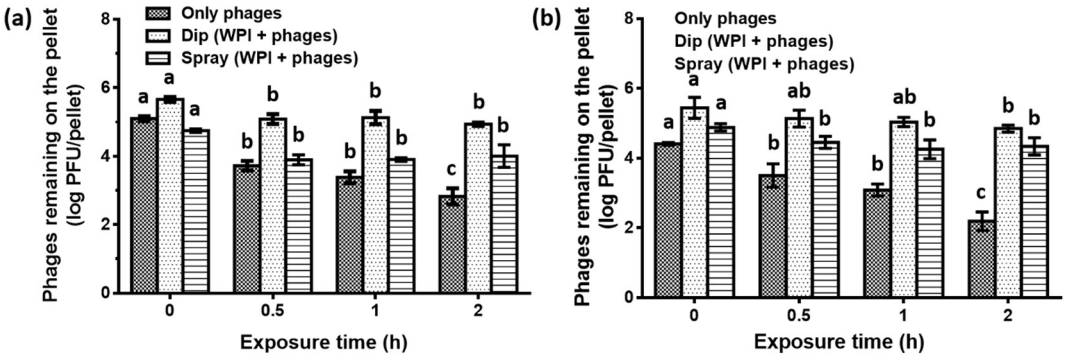

Fig. 5. Release of phages in simulated seawater: (a) T7 Phages, and (b) Vibrio phages.

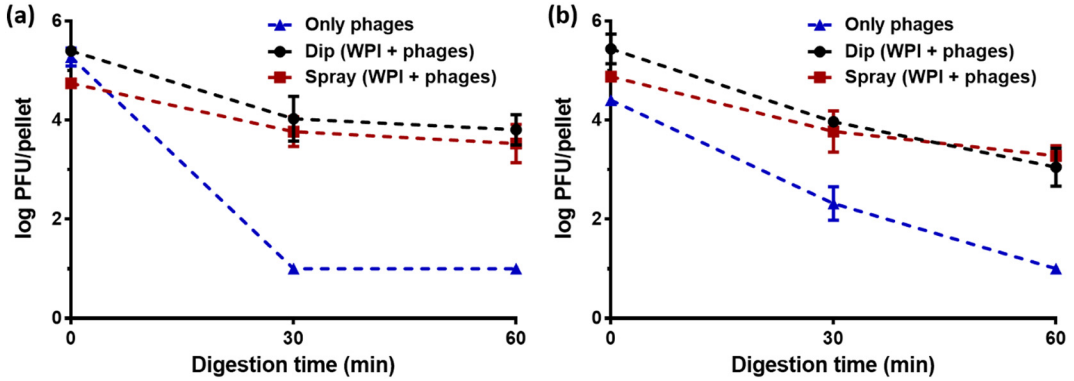

Fig. 6. Stability of phage activity in simulated gastric digestion: (a) T7 phages, and (b) *Vibrio* phages.

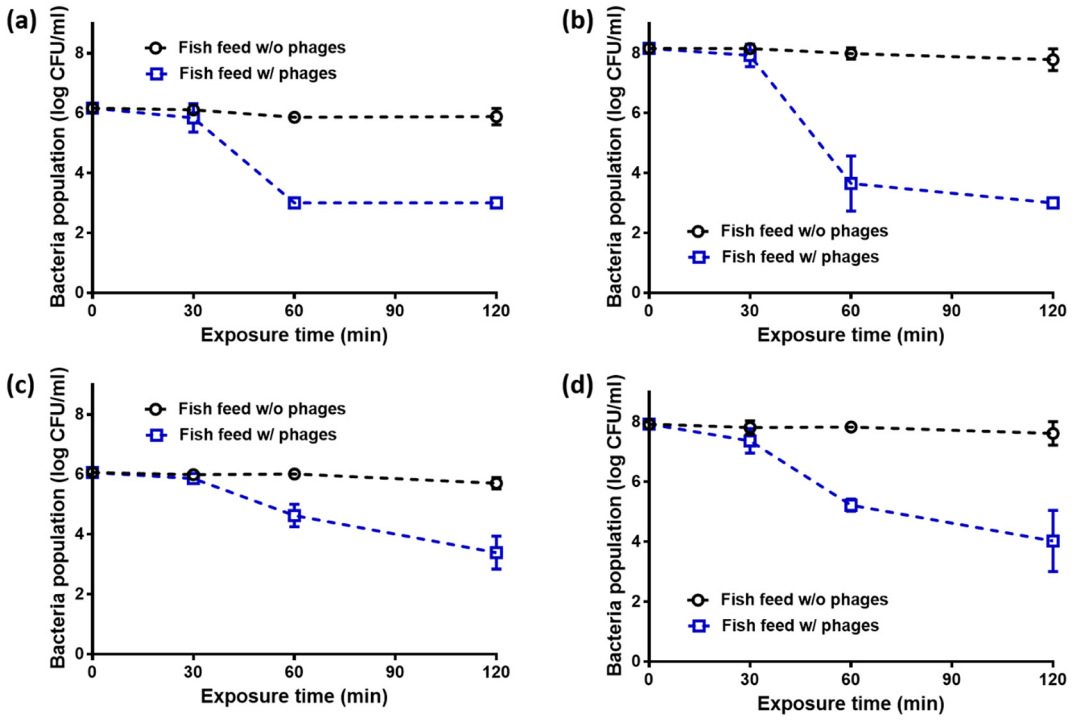

Fig. 7. Antimicrobial activity of WPI dip-coating with phages on fish feed: (a) using T7 phage coated fish feed to treat the digestion mixture inoculated with 6 log CFU/mL *E. coli*, (b) using T7 phage coated fish feed to treat the digestion mixture inoculated with 8 log CFU/mL *E. coli*, (c) using *Vibrio* phage coated fish feed to treat the digestion mixture inoculated with 6 log *Vibrio* spp., and (d) using *Vibrio* phage coated fish feed to treat the digestion mixture inoculated with 8 log *Vibrio* spp.

3.5. In-vitro challenge assay in simulated intestinal digestion

The results in Fig. 7 demonstrate that the edible WPI coatings incorporated with phages are effective for inactivating 3 to 5 log reduction of target bacteria in a simulated intestinal digestion. In this in-vitro challenge assay, the phage coated feed pellets were added to a simulated intestinal fluid containing 6 log and 8 log CFU/mL of target bacteria, respectively. Addition of regular fish feed without phage coatings did not cause significant reduction in *E. coli* and *Vibrio* spp. populations after exposure to simulated intestinal fluid for 2 h ($P > .05$). The fish feed dip-coated with T7-WPI coatings resulted in approximately 3.1 log and 5.2 log reduction in *E. coli* BL21 with an initial bacterial concentration of 6 log CFU/mL and 8 log CFU/mL, respectively. A slightly lower reduction of *Vibrio* spp. was achieved from the samples treated by Vibrio phage coated fish feed. Incubation at 37 °C for 2 h resulted in approximately 2.6 log and 3.8 log reduction with an initial bacterial concentration of 6 log and 8 log CFU/mL, respectively. In summary, these results show that edible phage coatings on fish feed has a potential application for controlling fish disease. These observations agree with other reported data regarding phage based biopolymer formulations can achieve a similar inactivation of target bacteria ranging from 1 to 4 log reduction (Colom et al., 2015, pp. 4841–4849; Lone et al., 2016, pp. 49–58; Vonasek et al., 2018, pp. 1871–1879; Vonasek et al., 2014, pp. 7–13), although the treatment time in this study was shorter when compared to those studies. Furthermore, these results are comparable to previous studies that have achieved a similar reduction in microbial count using antibiotics to treat bacterial infections in fish. For example, a 2–3 log reduction in fish pathogens (*Francisella asiatica* infection in Tilapia) was observed upon an in-vitro treatment with Florfenicol for 24–48 h (Soto et al., 2010, pp. 4664–4670). Similarly, another study reported a 1–3 log reduction of Aeromonas populations in Atlantic salmon by treatment with oxytetracycline for 1–10 days (Navarrete et al., 2008, pp. 177–183). However, the change of composition of the intestinal microbiota in fish with conventional antibiotics and the emergence of antibiotic resistance is of significant concern for the aquaculture industry (Assefa and Abunna, 2018, pp. 10; Kent et al., 2008, pp. 240–248; Navarrete et al., 2008, pp. 177–183). The edible antimicrobial WPI coatings on fish feed can be

Table 1
Estimated cost of phage incorporated biopolymer coatings on fish feed.

Ingredient	Estimated cost	References
Phages	5×10^{-13} dollars/particle	(Krysiak-Baltyn et al., 2018, pp. 31–44)
WPI	10 dollars/kg	(USDA, 2018, pp. 1A)
Glycerol	1 dollar/kg	(Graff, 2009, pp. 1)
Coating solution	5.3×10^{-4} dollars/mL	
Coated fish feed	1×10^{-5} dollars/pellet	

widely used by incorporating cocktail of phages that target a single pathogen for enhanced inactivation and overcoming the potential of gaining resistance against individual phages (Fischer et al., 2013, pp. e78543; Pires et al., 2016, pp. 523–543).

3.6. Cost assessment

Table 1 summarizes the estimated cost of phage incorporating biopolymer coatings on fish feed. The cost of large-scale phage production was estimated as $\$5 \times 10^{-13}$ per phage particle (Krysiak-Baltyn et al., 2018, pp. 31–44). The estimated costs of WPI and glycerol were \$10/kg and \$1/kg, respectively. In this study, the compositions of phage incorporating biopolymer coating solution include 10^7 PFU/mL phages, 5% WPI, and 2.5% glycerol. Thus, the estimated cost of the formulation is $\$5.3 \times 10^{-4}$ /mL. As 1 mL of coating solution can effectively coat 50 fish feed pellets, the additional cost of adding phages coatings to fish feed is only 0.001 cents. This price is significantly lower when compared to antibiotic treatment, the cost of which is around \$ 0.05–0.1 per dose (Durborow and Francis-Floyd, 1996, pp. 1–4; Kirwan, 2017, pp. 1). In addition, bacterial infections are becoming increasingly resistant to antibiotics, resulting in more costly and longer treatments, as well as higher mortality in farm animals. Emergence of bacterial resistant to antibiotics significantly reduces or eliminates the effectiveness of the antibiotics resulting in a significant economic loss due to increase mortality and morbidity (Watts et al., 2017, pp. 158). There is a strong drive from consumers and governments to reduce the use of antibiotics in aquaculture production. This cost-effective phage incorporating WPI coatings on fish feed can be used as an alternative approach to effectively prevent and treat bacterial infections in aquaculture industry.

4. Conclusion

Overall, the results of this study demonstrate the feasibility of using phage incorporated biopolymer coating on fish feed to improve loading efficiency, enhance storage stability, control release of phage in salt water, and maintain antimicrobial activity of phages in a simulated gastric-intestinal digestion. The edible WPI coatings were effective in enhancing loading of phages on fish feed pellets and reducing the loss of phage activity during storage of feed. The edible coatings were also able to limit the release of phages in salt water within two hours of incubation compared to the control coatings on feed without the biopolymer coating. The simulated gastric digestion assay demonstrated that edible WPI coating significantly improved the stability of phages in a simulated gastric environment compared to the control coatings. The in-vitro antimicrobial challenge assay suggests that the edible coatings reduced 3 to 5 logs of target bacteria in a simulated intestinal digestion. These antimicrobial activity results were demonstrated using a model bacterium (*E. coli*) and a fish pathogen (*Vibrio spp.*). In summary, this study illustrates that phage incorporated WPI coatings on fish feed can be an cost-effective approach enhancing the treatment of fish infections, reducing the risk of antibiotic resistance in aquaculture environment, and improving the safety and sustainability of aquaculture industry.

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