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Activity of a Bacteriophage Cocktail to Control *Salmonella* Growth *Ex Vivo* in Avian, Porcine, and Human Epithelial Cell Cultures

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Abstract

We examined the activity of phages to control the growth of chicken and swine *Salmonella* strains in avian (CHIC-8E11), porcine (IPEC-1), and human (HT-29) cell cultures. We optimized a six-phage cocktail by selecting the five most effective myoviruses and a siphovirus that have optimal lysis on prevalent serovars. We observed ∼20% of 7 $\log_{10}$ PFU/well phage and 3–6 $\log_{10}$ CFU bacterial adhesions, and 3–5 $\log_{10}$ CFU bacterial invasion per 2 cm² of the cultured cells at 2 h post-treatment. The invasive bacteria when plated had a variable reduced susceptibility to the phages. After phage application at an MOI of 10, the prophylaxis regimen had better efficacy at controlling bacterial growth with an up to 6 $\log_{10}$ CFU/well reduction as compared with the 1–2 $\log_{10}$ CFU/well bacterial reduction observed in the remedial and coinfection regimens. Our data support the development of these phages to control salmonellosis in chickens, pigs, and humans.

Keywords: *Salmonella enterica*, gastroenteritis, bacteriophage therapy, HT-29, IPEC-1, CHIC-8E11

Introduction

GASTROENTERITIS DUE TO NONTYPHOIDAL *Salmonella* spp. is a major health concern with >93.8 million cases of salmonellosis and 155,000 deaths in humans reported every year globally.[1] Approximately 85% of the infection cases are foodborne and markedly linked to poultry and pork products. The economic burden due to the infection from poultry and pork sources was estimated to be $2.8 and $1.9 billion, respectively.[1–3] There is a growing demand for these products in Europe and Southeast Asia (including the United Kingdom and Thailand, respectively) that comes with an associated risk of salmonellosis.[4–6]

Although *Salmonella* control and surveillance strategies are in place in these countries, the number of *Salmonella* cases is still high, and this highlights the urgent need for a robust and more effective intervention strategy in poultry, pigs, and humans. To address how treatment may change with respect to the target, this study focuses on applying a holistic approach using bacteriophages (or phages). Phages

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are viruses that specifically target and kill bacteria and can be used to control prevalent serovars from the United Kingdom and Thailand potentially in animals, during food processing and in humans.[7–10]

Conventional treatment of salmonellosis has mainly relied on the use of antibiotics; however, antimicrobial resistance (AMR) to many frontline antibiotics such as fluoroquinolones and cephalosporins is common among the U.K. and Thai *Salmonella* strains.[6,11] The situation is further compounded by few or dwindling antibiotic innovations to combat the infection.[9,12] To control *Salmonella* effectively and prevent the general impending health crisis by AMR, novel solutions are needed and one such approach is to use phages.[13,14]

Phages have many advantages over antibiotics as they have been shown to specifically target and kill bacteria, including antibiotic resistant strains, and have gained high recognition and acceptance lately.[15] In the United States, phages are generally regarded as safe and have a huge potential to control bacteria in various model systems, hence, many preparations are commercially available or currently under investigation in multiple clinical trials.[7,8]

Among the ~2500 *Salmonella* serovars identified, only a subset of the *Salmonella enterica* subspecies *enterica* is linked to human salmonellosis outbreaks.[16] Some of the most common zoonotic serovars implicated in the United Kingdom and Thailand are *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Agona, *S.* Newport, *S.* Stanley, *S.* Kentucky, *S.* Virchow, *S.* Java, *S.* Bareilly, *S.* Hadar, *S.* Arizonae, *S.* Saint Paul, *S.* Group B†, *S.* Group C1‡, and *S.* Mikawasima.[6,17,18]

However, high salmonellosis prevalence in humans is more closely linked to *S.* Enteritidis (~27%) and *S.* Typhimurium (~21%) (including the monophasic strains) from poultry and swine, thus, specific reduction targets have been set for these two serovars.[6] In addition, *S.* Hadar, *S.* Infantis, and *S.* Virchow are included as reduction targets for breeding chicken flocks by the *Salmonella* National Control Program.[6] The prevalent serovars encode multiple AMR genes in their genomes that play a significant role in the evolution and spread of the pathogen, and the emergence of clones that are responsible for multiple independent local and global outbreaks.[9,12,19–21]

Salmonella can be waterborne or contracted through consumption of contaminated and undercooked food products by humans or contaminated feeds by animals.[9,22] When ingested, *Salmonella* develops acid tolerance to evade the low pH of the gizzard or stomach gastric juice, thus promoting its survival and colonization in the gut.[23,24] As *Salmonella* migrates through the digestive tract, it interacts with the gut lining by adhering to and invading epithelial cells. The secretion of effector proteins, intestinal secretory and inflammatory responses, and translocation proteins trigger invasion and bacterial survival and replication within the cells resulting in systemic infection.[25–27]

The invasion largely occurs through the specialized type III protein secretion system encoded on *Salmonella* pathogenicity islands (SPI), SPI-1 and SPI-2.[28–30] However, despite the deletion of the invasion factors in an *S.* Typhimurium strain, it was still able to invade HT-29, Hep G2, HeLa, IPI-2I, LMH, Ma-104, Caco-2, and IPEC-1 Cells.[25] This suggests that other factors such as iron levels and mucus could affect the invasion of *Salmonella* to eukaryotic cells.[31,32]

Studies using several intestinal epithelial cell lines from chickens, swine, and humans showed that different *Salmonella* serovars have variable degrees of adherence and invasion to the cell lines.[20,23–25] For example, ~5 $\log_{10}$ colony-forming unit (CFU) of *S.* Typhimurium was shown to invade $2\,cm^2$ of chicken kidney cell chick cells within 2 h and this invasion rate is higher than the ~3 $\log_{10}$ CFU invasion of *S.* Gallinarum, *S.* Dublin, and *S.* Enteritidis to the same cell lines.[33] Similarly, phage interactions with epithelial cells have been shown to occur in nature.[34] Work conducted on T84 and CaCo2 showed that phages penetrate the cell membrane and spread within the cytoplasm reaching to the golgi apparatus.[34]

The interaction of two phages, T4 and HAP1 with immortalized cells HS294T and A549, was shown to be mediated by the GP24 protein with integrin β3 found on the cells.[35] Phage adherence can also increase therapeutic effectiveness as shown by *Clostridioides difficile* phage, phiCDHS1 with human colon tumorigenic cell, HT-29.[36] However, there is a paucity of information regarding phages and *Salmonella* interaction with eukaryotic cells in the gut environment. To the best of our knowledge, only two reports have shown the impact of phage treatment (phages P22, ST-W77, and SE-W109) on the adhesion and invasion of *Salmonella* (*S.* Typhimurium) to intestinal epithelial cell lines (INT-407, HD11, and T84).[37,38] Information in this case can guide the future development and usage of phages to treat salmonellosis.

Individual and multiple combinations of phages have been shown to reduce *Salmonella* burden *in vitro* using culture and biofilm-based assays and *ex vivo* using relevant cell cultures.[37–43] In addition to reduced bacterial burden, phage therapy was reported to improve symptoms of disease *in vivo* using established and refined animal infection models and animal trials.[38,41,44–47]

Our previous study on the optimization of a two- and three-phage cocktails from the phages studied here showed their therapeutic potential in a *Salmonella* mouse and *Galleria mellonella* infection model, respectively.[38,46] Clearly phages have to target relevant strains and our previously optimized three-phage cocktail was developed based upon a host range analysis of 23 chicken- and 10 swine-related isolates, which provides limited information for their efficacy on globally dominant strains.[46]

To increase host range to encompass more diverse strains, here, we expanded our host range studies to include 83 poultry- and 86 swine-related isolates and carried out extensive virulence assays. From these studies, a broader spectrum cocktail of six phages was optimized. We determined the ability of this six-phage cocktail to reduce bacterial burden of representative poultry and swine *Salmonella* strains in infections of avian (CHIC-8E11), porcine (IPEC-1), and human (HT-29) intestinal epithelial cell lines during prophylaxis, remedial, and coinfection therapy regimens.

Materials and Methods

Bacterial isolates examined, sources, and growing conditions

A total of 169 *Salmonella* strains comprising 26 of the major serovars of *S. enterica* subsp. *enterica* (including monophasic strains) responsible for serious zoonotic outbreaks globally were examined as targets for phage host

range analysis in this study (Supplementary Tables S1 and S2). Among the isolates, 60 (36%) were obtained from Dr. Sunee Korbsrisate's laboratory in Thailand and 109 (64%) were U.K. strains sourced from Animal and Plant Health Agency (APHA) (Supplementary Tables S1 and S2).

The proportions of strains from swine and poultry (chickens and turkeys) were 51% ($n = 86$) and 49% ($n = 83$), respectively. An additional chicken isolate, SL1344 (serovar *S.* Typhimurium), (kindly donated by Dr Primrose Freestone from the University of Leicester, United Kingdom), was used as the propagation host for all the phages, and to optimize and test our new phage cocktail in various conditions. The APHA swine isolate, MSG44-S01 (serovar *S.* 4:i:-), and the phage propagating host, SL1344, were used as test strains in the tissue culture experiments.

All strains were routinely cultured by streaking out from 25% glycerol $-80°C$ stocks on Luria–Bertani (LB) (Oxoid, United Kingdom) 1.5% agar and subsequently in broth by inoculating 2–3 of the 18–24 h colonies into 5 mL of LB broth and incubating at 37°C aerobically with shaking at 100 rpm for 2–3 h.

Phages examined in this study and purification method

The 22 phages (21 myoviruses and a siphovirus) examined here were propagated as described before and used for phage host range analysis, cocktail optimization, and therapeutic *ex vivo* testing (Supplementary Table S1).[42,43,46] The phages used in the cell tissue work were buffer exchanged to PBS using Amicon Ultra-15 centrifugal filter columns (Merck Millipore Ltd., Cork, Ireland) to remove the nutritive LB. In brief, 14 mL (5 mL at a time) of 10^9 plaque-forming unit (PFU)/mL phage samples was added to the columns and centrifuged at 5000 g for 15 min. Afterward, 10 mL of PBS was added and incubated at room temperature for 5 min before being aspirated into a sterile tube and enumerated on confluent cultures of SL1344 in LB 0.75% soft agar as described previously.[46]

Host range analysis of the phages and optimization of new cocktail

Phage host range analysis was conducted on the 169 *Salmonella* strains using spot test analysis by applying 10 μL volume of 10^8 PFU/mL individual phages onto confluent agar cultures of the bacteria as previously described.[46] Phage activity on the agar plates and lysis in aerobic broth cultures of SL1344 at multiplicity of infection (MOI) of 10 were used to optimize the new phage cocktail.[46] The phage combination with the lowest resultant bacterial growth and regrowth, and with the widest combined host range, was selected for further analysis. Once an optimal cocktail was developed on SL1344 at 37°C and 40°C, its activity was also determined on MSG44-S01 using the same temperatures and MOI.

Procurement of cells lines, growth conditions, and seeding of culture plates

The efficacy of the cocktail to clear the two test strains was further determined in avian CHIC-8E11 (passage 32; Micromol, Germany), porcine IPEC-1 (passage 135; DMSZ, Germany), and human HT-29 (passage 3; ECACC, Salisbury, United Kingdom) epithelial cell lines.[36,48,49] The two former cell lines were kindly donated by Prof. Paul Wigley, Uni-

versity of Liverpool, United Kingdom. All cell lines were used within 10 subsequent passages.

IPEC-1 was cultured in DMEM/F-12 (1:1) (1×) Ham's medium, whereas CHIC-8E11 and HT-29 cell lines were cultured in DMEM high-glucose medium. Both media were supplemented with final concentrations of 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics; penicillin (100 U/mL) and streptomycin (1 μg/mL). All media and reagents were sourced from ThermoFisher Scientific (Paisley, United Kingdom).

Thawed cryostocks of the cell lines were added to 10 mL of growth medium in 25 cm^2 culture flask, incubated at 37°C and 5% CO_2 for 24 h at 95% humidity and examined under an inverted phase contrast microscope (Nikon TMS-F, Japan).[36,48,49] Media were refreshed every 24–48 h until 80–90% confluence was attained. Cells were harvested using Trypsin–EDTA (ThermoFisher Scientific) before being quickly mixed with 8 mL growth medium and transferred to a 75 cm^2 flask containing 10 mL growth medium and incubated. When 80–90% confluence was achieved, cells were harvested and resuspended in 20 mL medium and tested for viability using trypan blue (Merck Life Science Limited, Dorset, United Kingdom, according to manufacturer's instructions) and cells adjusted to 10^5 cells/mL.

Approximately, 1 mL each of the cell cultures was added to a Nunc™ Cell-Culture-treated 24-well plates (Thermo-Fisher Scientific), and incubated until 80–90% confluence. Afterward, cells were washed twice with 1 mL PBS, and 900 or 800 μL (according to the experiment, given hereunder) of fresh medium excluding antibiotics was added to each well.

Phage interaction with cell lines

Before examining the phages/cell lines, phage stability was determined in the tissue culture cell growth medium alone to ascertain whether the growth medium was detrimental to the phages. To do this 10% of the individual phages were added to the volume of the growth medium. Phages were titered at 1 and 2 h postincubation. Data were compared with phages incubated in equal volume of LB broth medium at 37°C aerobically (data not shown).

The interaction of the phages with the three cell lines was tested.[50] To do this, 100 μL of $\sim 10^8$ PFU/mL of individual phages was added to the culture wells of each cell line (to give final concentration of $\sim 10^7$ PFU/well), and control wells containing just the medium without the cells. Phages from the wells were enumerated at 0, 30, 60, 90, and 120 min time points using plaque assay on confluent culture of SL1344.[46] Phage adherence to the cells was calculated as the percentage of the difference between the number of phages recovered from the control wells and those recovered from wells containing the cell lines against the phages added.

Bacterial adherence to the cell lines

The activity of the optimized phage cocktail was tested on the two test isolates grown with relevant cell lines. Before this, we tested whether the bacteria would interact with the appropriate cell lines by adherence assay.[30,31,37] Therefore, we exposed SL1344 to CHIC-8E11, MSG44-S01 to IPEC-1, and both SL1344 and MSG44-S01 to HT-29.

Bacterial isolates were prepared by inoculating colonies into LB broth and culturing as already mentioned. Afterward,

a 10% subculture was made in fresh LB and grown to OD_{600} $\sim 0.7 \pm 0.02$ at 37°C with shaking at 100 rpm aerobically. The bacterial cells were washed by centrifuging twice at $15,000 \times g$ for 5 min, resuspending in an equal volume of PBS, and adjusted to 10^7 CFU/mL in PBS. A 100 μL volume of the PBS culture was added to 24-well plates (10^6 CFU/well) containing the appropriate cell lines in 900 μL medium and incubated for 0, 30, 90, and 120 min.

Control plates were set up but without the cell lines and incubated for the same time points. At each time point, a set of plates (treated with bacteria and control) were harvested. Each well was washed twice with 1 mL PBS and then treated with 250 μL of Trypsin–EDTA and incubated for 10 min to dislodge the cells from the wells. This was followed by the addition of 750 μL/well PBS and the bacteria enumerated on Xylose Lysine Deoxycholate.[46]

Bacterial invasion assay and phage sensitivity on recovered invaded bacterial cells

After the bacterial–cell lines interaction assay, we determined how many bacterial cells invaded the cell lines in each animal/human cell type.[24,51] To do this, we set up the bacterial cultures for the invasion assay and control containing cell lines for the time points as mentioned. At each invasion assay time point, the culture wells were washed with PBS and treated with gentamycin to 150 μg/mL final concentration and incubated for 1 h to kill surface bacteria. Residual culture was tested using a streak assay. Wells were washed twice, treated with 250 μL of Trypsin–EDTA for 10 min, and with 250 μL of 0.2% Triton X-100 (0.1% final concentration) for 10 min before 500 μL of PBS was added.

The control plates (containing cell lines and bacteria not treated with gentamycin) were treated with 100 μL of 1% Triton X-100.[33,37] Total bacteria (both invaded and free bacteria) were enumerated as already mentioned. Recovered invaded bacteria were cultured in LB broth and used to conduct plaque assays with the original individual phage stocks.[46] Efficiency of plating (EoP) was calculated by dividing the phage titer determined on the recovered invaded cells against the titer of phages conducted on the propagation host, SL1344.

Phage therapy assays

To determine the ability of the phages to clear the test strains in the presence of the cell lines, we explored three therapeutic regimens: prophylaxis, remedial, and phage–bacteria coinfection regimens as described hereunder.

Prophylaxis and remedial phage therapy assays. The prophylaxis and remedial assays were conducted by adding the phage cocktail or bacteria, respectively, to the wells and incubating for 0, 30, 60, 90, and 120 min before the subsequent treatments. This was done by adding 100 μL of 10^7 PFU/mL of phage cocktail or 10^6 CFU/mL of bacteria to the 800 μL growth medium in the wells containing the different cell lines. The control wells were treated with 100 μL of PBS instead.

At each time point, 100 μL of either 10^7 PFU/mL phage cocktail for the remedial assay, of 10^6 CFU/mL of bacterial cells for the prophylaxis assays, or PBS for the control plates was added to the appropriate wells. The treated plates were

then incubated for 1 h before the supernatants were removed and immediately centrifuged at 15,000 g for 5 min. Phages were enumerated from the supernatants using plaque assays, and the cell pellets resuspended in 1 mL of PBS and enumerated.

Coinfection phage therapy assay. For the coinfection assay, 100 μL each of 10^7 PFU/mL of the phage and 10^6 CFU/mL of bacteria were simultaneously added to the 800 μL volume of the culture medium containing the growing cell lines. A control containing 100 μL each of PBS and bacteria added to culture wells was also prepared. Experiments were conducted for 0, 1, 2, 4, and 24 h. At each time point, the supernatants were mixed by pipetting three times before aspirating the ~ 1 mL into a sterile 1.5 mL microtube and centrifuging at 15,000 g for 5 min and enumerating for phage and bacteria.

Data analysis

All experiments were conducted in duplicate and repeated twice. Data generated were analyzed using GraphPad Prism 9.

Results

Host range analysis of the 22 phages examined in this study

To determine the initial clinical importance of the phages studied here, analysis of their host range was conducted on the 169 swine and poultry isolates. Our data showed the variable lysis capabilities of the phages (Supplementary Tables S1, S2 and Fig. 1A, B). The phages lysed between 105 (SPFM11) and 126 (SEW-109) of the isolates. Four of the phages SPFM19, SPFM14, SPFM10, and SPFM12 have the lowest host range, individually lysing only 45–60 isolates (Fig. 1A, B). Put together, 83% of the total strains investigated (73% and 97% of the Thai and U.K. isolates, respectively) were susceptible to phage lysis.

We also observed that the specificity of the phages was independent of their geographical source or that of the bacterial strains. For example, all the phages, including those from Thailand (ST-W77 and SE-W109), could efficiently lyse all *S.* Enteritidis and *S.* Typhimurium serovars from U.K. chicken isolates; however, the phages had variable individual lysis effects on the Thai bacterial strains with SPFM19, SPFM10, and SPFM12 unable to lyse any strains used in our assays (Supplementary Table S1). None of the phages could lyse the serovars *S.* Schwarzengrund, *S.* Hadar, *S.* Duesseldorf, *S.* Anantum, *S.* Corvallis, *S.* Kentucky, *S.* Mbandaka, or *S.* Singapore, and work is currently ongoing in our laboratories to isolate phages for these serovars.

An optimized six-phage cocktail showed broad combinatorial host range and lysis efficacy at 37°C and 40°C

We aimed to design a phage cocktail with the minimal number of phages needed to have the broadest clinical coverage and optimal combinatorial lysis effect.[39,46] Our previously optimized three-phage cocktail comprised SPFM17, ST-W77, and SE-W109, which also demonstrated a wide coverage in this study, so were included as candidate phages for our new cocktail.[46] Having expanded the number of
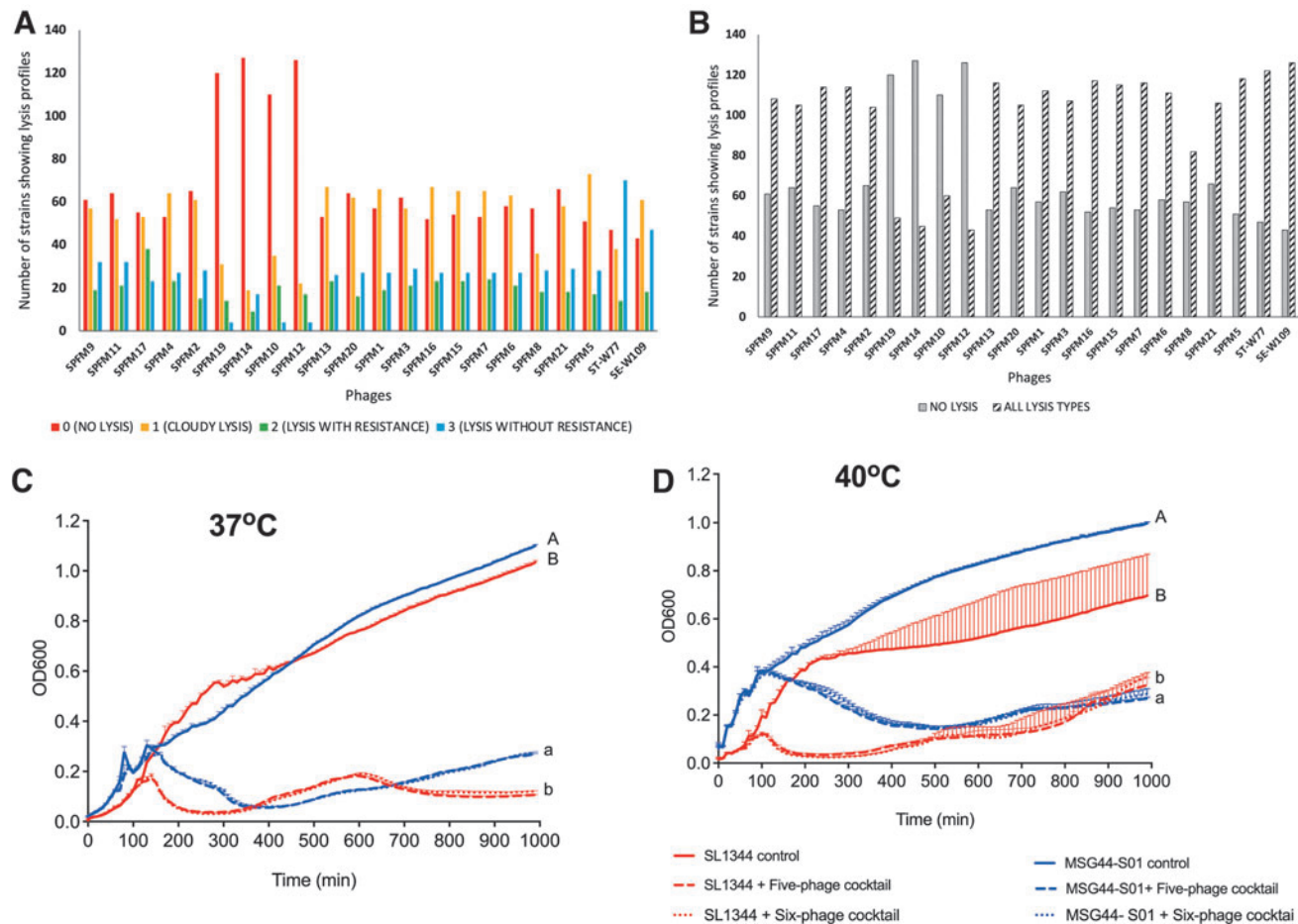
FIG. 1. Lytic activity of the 22 phages examined in this study showing **(A)** host range coverage showing all lysis types and **(B)** cummulative lysis types (1–3) put together versus no lytic activity (0) of the phages on all 169 *Salmonella* strains tested here. Broth lytic activity of the optimized six-phage cocktail on the two test strains, SL1344 (chicken)- and MSG44-S01 (swine)-related *Salmonella* strains at **(C)** 37°C and **(D)** 40°C. Host range activity was conducted using spot test technique by applying 10 μL aliquots of the 10^8 PFU/mL of the phages on confluent cultures of the host bacteria in soft agar. Lysis was scored as displayed. Lytic activity of the five- and six-phage cocktail on cultures of SL1344 and MSG44-S01 in broth was conducted at an MOI of 10 in 200 μL volume in 96-well plates for 16 h aerobically at temperatures displayed. Host range agar assay was conducted in duplicates and repeated twice, whereas the broth killing assay was conducted in triplicates and repeated twice. Data were analyzed in GraphPad Prism 9 with standard error of mean of all replicates shown. MOI, multiplicity of infection; PFU, plaque-forming unit.

strains tested from 33 from our previous study to 169 here, the three-phage cocktail could now only lyse 71% of the total updated strains. To improve this coverage, we carefully investigated the host range of each phage and selected additional candidates that would add value to the clinical coverage and lysis potential of the three preselected phages.

Initially, we selected SPFM4, SPFM9, SPFM11, SPFM13, and SPFM14 based on their wide and complementary host ranges. We conducted virulence assays on SL1344 at MOI of 10 using various permutations containing 1, 2, 3, 4, or 5 combinations of the selected phages added to the already optimized three-phage cocktail, SPFM17, ST-W77, and SE-W109 (data not shown). Analysis of our lysis data showed that the best cocktail comprised five phages: SPFM17, ST-W77, SE-W109, SPFM9, and SPFM14. This combination did not, however, target the single Thai isolate SK-Thai-54, of the serovar *S.* Singapore.

To increase host range coverage to 100% of the susceptible strains (83% of the total bacterial strains studied, Supple-

mentary Table S1), we added SPFM5 to the five-phage cocktail to make it a six-phage cocktail. Our virulence assay showed that both the five- and six-phage cocktails had comparable killing efficiencies on the two test bacterial hosts at MOI of 10 and at both 37°C and 40°C (Fig. 1C, D). The temperatures used represent the gut temperature conditions for propagating the bacterial strains and of human and pig (37°C), and chicken (40°C) guts.

The optimized six-phage cocktail has therapeutic potential to reduce Salmonella *growth in human (HT-29), porcine (IPEC-1), and avian (CHIC-8E11) epithelial cell cultures*

Having determined the host range properties of the phages from which we optimized a broad host range six-phage cocktail, we then determined the therapeutic potential of the cocktail to remove *Salmonella* in human, porcine, and avian cell lines.
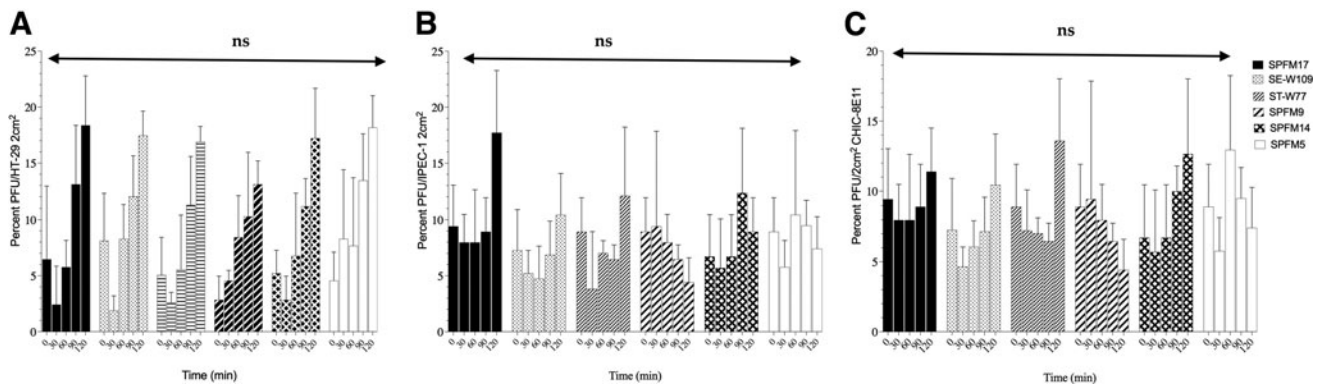
FIG. 2. Percentage phage adhesion to **(A)** human HT-29, **(B)** porcine IPEC-1, and **(C)** avian CHIC-8E11 epithelial cell lines. Phage adhesion assay was conducted in duplicate wells in 24-well plates and repeated twice. Approximately 10^7 PFU/mL of individual phages were exposed to 80–90% confluent epithelial cells and incubated at the times specified. Phages recovered were enumerated using plaque assay on confluent cultures of the propagation host, SL1344. Data were analyzed using analysis of variance in GraphPad Prism 9 and standard error of mean of all replicates and significance at $p < 0.05$ are presented.

Phages examined interacted with human, porcine, and avian cell lines. After establishing phages were viable in cell culture medium, phage interactions with the cell lines were determined using an adherence assay and enumerating recoverable viable phages as shown previously.[27,50] The number of phages was determined with respect to our negative control (culture wells containing just growth medium with no cell lines) and could be attributed to phage loss due to adherence to the cell lines. After exposing cell cultures to $\sim 10^7$ PFU of the individual phages, we recorded no significant change ($p < 0.05$) in the adherence of these phages to any cell line over the time measured (Fig. 2A–C).

Approximately, 18% of the phages adhered to HT-29 and IPEC-1, whereas 15% adhered to CHIC-8E11 (Fig. 2). The phages started to adhere immediately (just after 0 min) after encountering the cell lines. Afterward (from 30 min postinfection), the phages detached from the cell lines at various time points, but eventually accumulated on the cells over time. At the end of the experiment, relatively higher number of phages adhered to the cell lines than the start except SPFM9, SPFM14, and SPFM5 in the IPEC-1 and SPFM9 and SPFM5 in the CHIC-8E11 cell cultures (Fig. 2).

Salmonella showed adherence to human, porcine, and avian cells. After demonstrating that the individual phages could adhere to the cell lines, we determined whether bacteria could also bind to the eukaryotic cells.[30,31,37] After exposing the bacterial cultures to the cells in each well, we established that a significant ($p < 0.05$) number of the bacteria interacted with the relevant cell lines by adhering to them initially and remaining bound for the entire study time (Fig. 3). We observed that 10^2 CFU/well of both SL1344 and 44S01 adhered to HT-29 at the start, then this increased in a logarithmic manner and plateaued at $\sim 10^5$ CFU/well at 30 min.

The attachment remained relatively constant until 90 min, then slightly increased to 10^6 CFU/well at 120 min. When
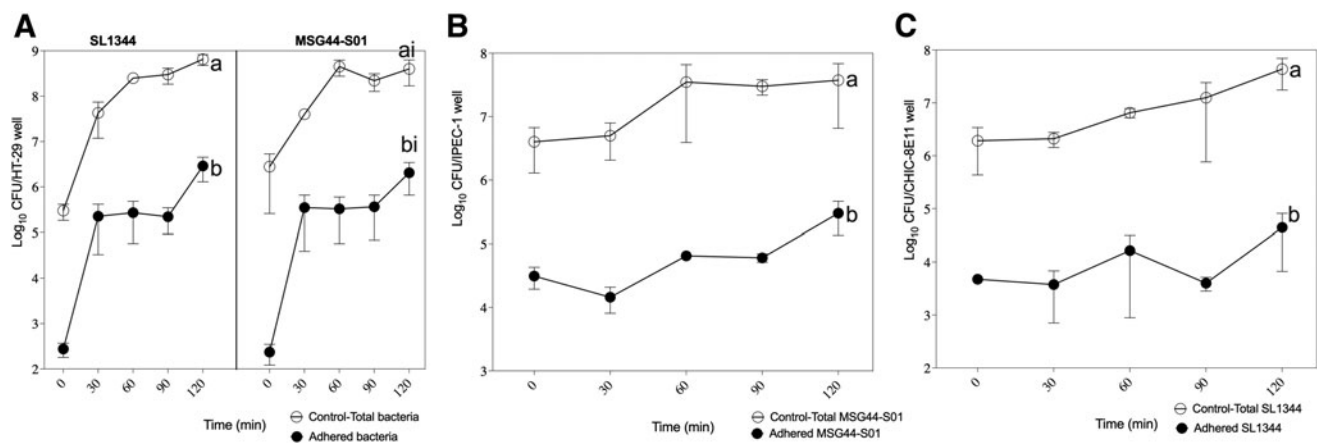

FIG. 3. Bacterial adhesion to **(A)** HT-29, **(B)** IPEC-1, and **(C)** CHIC-8E11 per well. Representative 10^6 CFU/mL each of *Salmonella* swine (MSG44–S01) and chicken (SL1344) cultures was added to 80–90% confluent epithelial cells and incubated at the times specified. Bacteria were recovered on XLD after washing with PBS and treatment with trypsin. Assay was conducted in duplicates and repeated twice. Standard error of means of replicates is shown. Data were analyzed using analysis of variance in GraphPad Prism 9. Codes (a/b and ai/bi) shown on each data set on graphs represent significance at $p < 0.05$. CFU, colony-forming unit; XLD, Xylose Lysine Deoxycholate.

compared with the estimated number of HT-29 cells present in each well, we deduced that only ∼1 bacterial cell adhered to 10^4 of HT-29 cells at 0 min and then increased to 1:1 ratio of cell line to bacteria at 120 min. It may be possible that multiple bacterial cells could adhere to one epithelial cell, thus some cells may have no bacteria. The bacterial strains adhered better to IPEC-1 and CHIC-8E11 (∼10^4 CFU/well) than HT-29 (10^2 CFU/well) at the 0 min start (Fig. 3A–C).

The number of bacteria increased to 10^6 CFU/well that is equivalent to 1:1 ratio of each of the bacterial strain to the corresponding cell line and is comparable with the number of bacteria that adhered to HT-29. In addition, the bacterial cells tended to adhere then detach on IPEC-1 and CHIC-8E11 during alternate time points as shown in Figure 3B and C.

Salmonella can invade human, porcine, and avian cell lines. During interaction with epithelial cell lines, *Salmonella* can become invasive.[52] We, therefore, determined the rate at which our test bacterial strains would invade the relevant cell lines after adhering to them. A significant ($p < 0.05$) number of the two test bacterial strains invaded HT-29 most effectively, with up to 10^5 CFU/well (estimated 3–5 CFU/HT-29 cell) at the 60-min time point, and this remained consistent until the end of the experiment (Fig. 4A). For the two other cell lines, only 10^3 CFU/well of 44S01 invaded the IPEC-1 cell line with only an estimated 1 out of 10^{3-4} of the cells were invaded by *Salmonella* (Fig. 4B).

We observed the lowest bacterial invasion rate in CHIC-8E11, where internalized number of bacteria were undetectable during the first 60 min (Fig. 4C). At 90 min, however, 10^2 CFU/well of SL1344 cells were recovered from the culture wells and the number of intracellular bacteria grew to 10^5 CFU/well at 120 min. Similarly, considering the individual CHIC-8E11 cells, we could deduce that only 1 out of 10^3 of the cells were invaded by a single SL1344 bacterium at

90 min. The invasion later increased to ∼1:1 ratio of the CHIC-8E11 cells to SL1344 bacterium at 120 min (Fig. 4C).

Recovered invaded *Salmonella* have variable efficiency of plating to the phages. After recovering the invasive bacteria (Supplementary Tables T3–T5), their sensitivity to the phages was tested as shown by a previous report.[37] Plaque assay data demonstrated that the phage EoPs were variable among the recovered invasive bacteria (Fig. 5A–F). Interestingly, the phages showed much reduced EoPs on the majority of the recovered invasive bacterial strains isolated from HT-29 at the later time points, 90–120 min (Fig. 5A, B, and Supplementary Table S3), and strains from IPEC-1 and CHIC-8E11 from 0 min time points (Fig. 5C–F, Supplementary Tables S4 and S5).

The optimized phage cocktail showed variable efficacy under different therapeutic regimens in the presence of human, porcine, and avian cell lines. Having established the interactions of the phage cocktail and test bacteria individually with the three cell lines, we went further to determine the interaction of the three biological entities (phage, bacteria, and cell lines) together using three different therapeutic regimens (Figs. 6A–F, and 7A–F).[37] In general, we observed that the prophylaxis was significantly ($p < 0.05$) more effective at removing the bacteria faster and more efficiently in all the cell lines investigated than the remedial regimen as shown in other *Salmonella* infection studies (Fig. 6A, C, F).[46,53,54]

Concomitantly, lower number of bacteria were recovered in the prophylaxis than the remedial regimen (Fig. 6A, C, E). Interestingly, at the end of the experiment, SL1344 was undetectable in the HT-29 prophylaxis regimen, and this is significantly ($p < 0.05$) better than the same therapy on MSG44-S01/HT-29 with 10^3 CFU/well bacteria recovered (Fig. 6A).
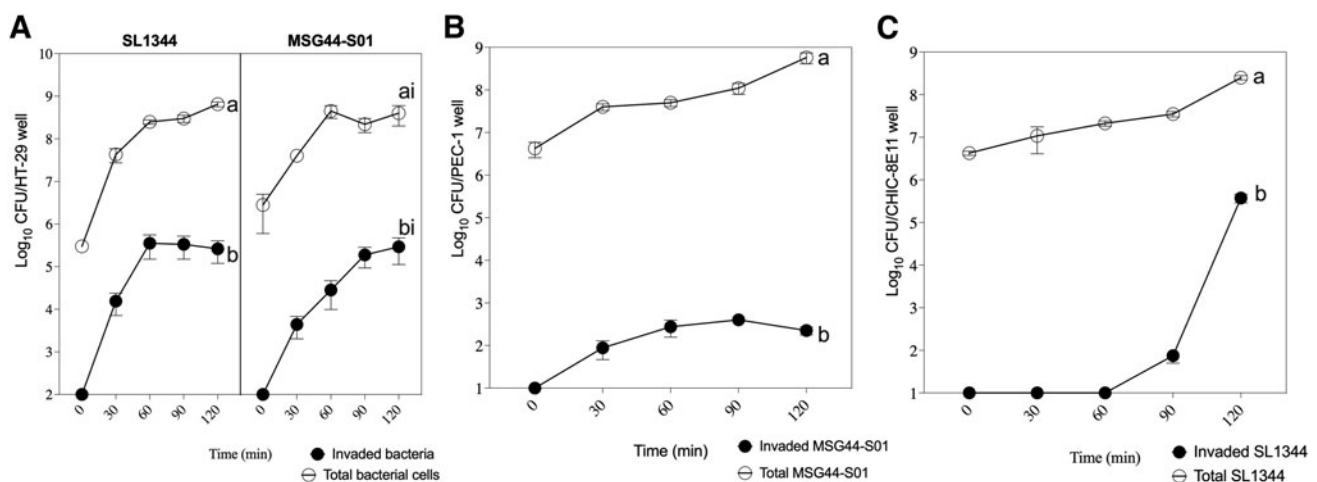

FIG. 4. Bacterial invasion to **(A)** HT-29, **(B)** IPEC-1, and **(C)** CHIC-8E11 epithelial cells per well. Representative 10^6 CFU/24-well each of *Salmonella* swine (MSG44-S01) and chicken (SL1344) cultures was added to 80–90% confluent epithelial cells and incubated for the specified times. Extracellular bacteria were killed by treating with gentamycin. After treating with trypsin to dislodge the epithelial cells and with Triton X-100 to lyse the cells, bacteria were recovered and enumerated on XLD. Assays were conducted in duplicates and repeated twice. Standard error of means of replicates is shown. Data were analyzed using analysis of variance in GraphPad Prism 9. Codes (a/b and ai/bi) shown on each data set represent significance at $p < 0.05$.
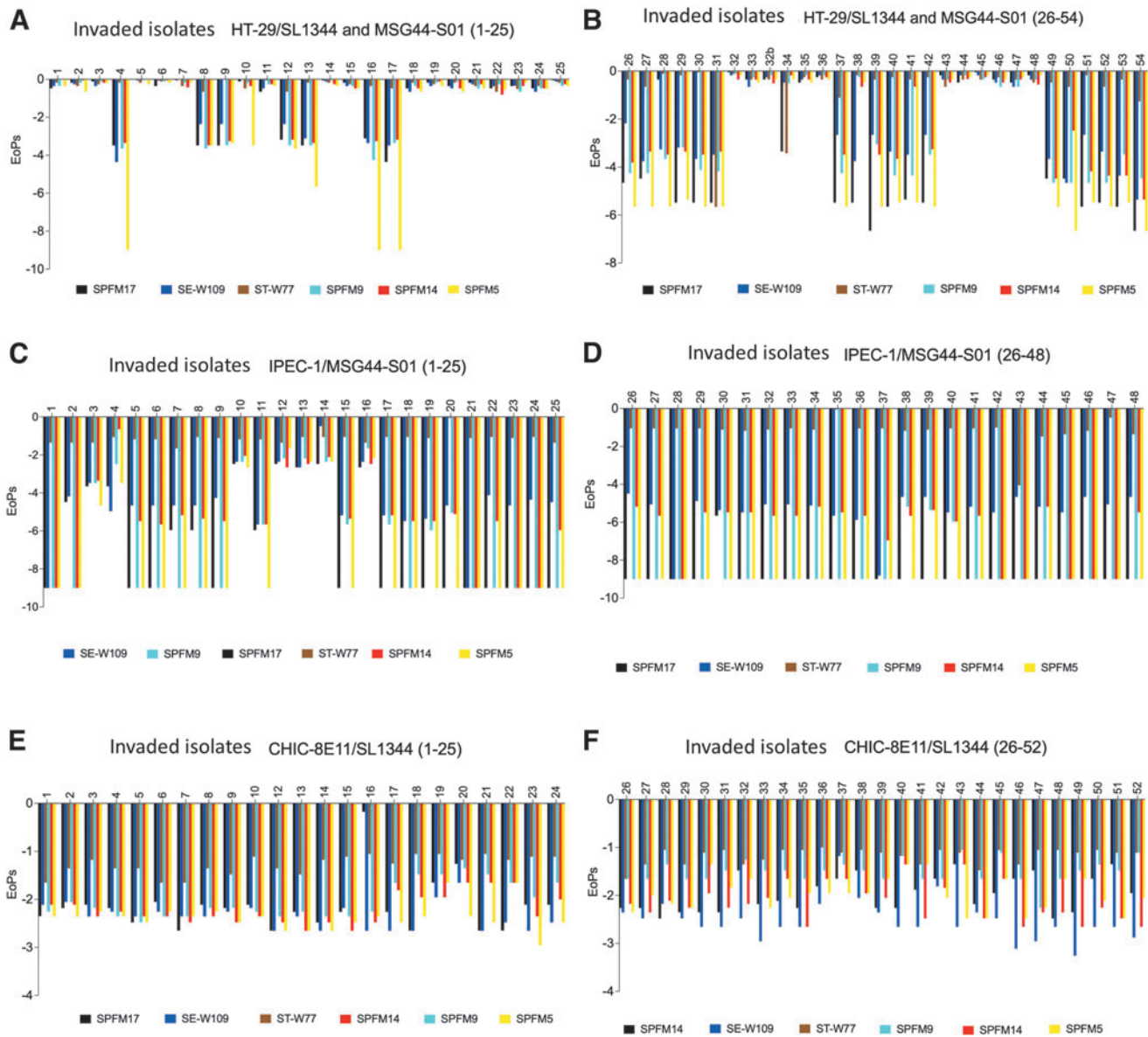
FIG. 5. Efficiency of plating of invaded test *Salmonella* strains to HT-29 **(A, B)**, IPEC-1 **(C, D)**, and CHIC-SE11 **(E, F)** epithelial cells. Invaded bacteria were recovered and tested for their susceptibility to the original individual phage stocks using efficiency of plating technique. This was done by conducting a plaque assay using the individual phages with soft agar confluent cultures of recovered invaded isolates. Phage titer values obtained from the invaded bacteria were divided by values obtained from the host, SL1344. Data were analyzed using GraphPad Prism 9. Means of two biological replicates are presented.

Although the therapeutic efficacy of the remedial regimen was inferior to the prophylaxis regimen, the remedial regimen also significantly ($p < 0.05$) reduced the number of bacteria compared with the bacterial control except in the IPEC-1/MSG44-S01 assays, where relatively equal number of bacteria were recovered (Fig. 6A, C, E). Comparing both therapies, we did not observe bacterial regrowth in prophylaxis and remedial except in HT-29/SL1344 remedial assay (Fig. 6A, C, E).

In the coinfection regimen, we observed a significant ($p < 0.05$) reduction of 4 $\log_{10}$ CFU/well in number of bacteria at 1 and 2 h postphage treatment of HT-29 with the cultures of MSG44-S01 and SL1344, respectively, compared

with the bacterial control. However, no significant difference ($p < 0.05$) in reduction of number of bacteria between the two phage-treated strains was observed (Fig. 6B). Although a slight increase, followed by a decrease in number of MSG44-S01 was observed at the 1 and 2 h time points afterward, respectively, the culture continued to grow exponentially until the end of the experimental time, 24 h for the HT-29 wells (Fig. 6B).

For SL1344, however, a regrowth started at 3 h and bacterial growth remained relatively constant until the end of the experiment (Fig. 6B). Decreased MSG44-S01 growth was observed 2–4 h post-treatment with phage in the presence of IPEC-1 cell lines, however, a regrowth was observed at 24 h
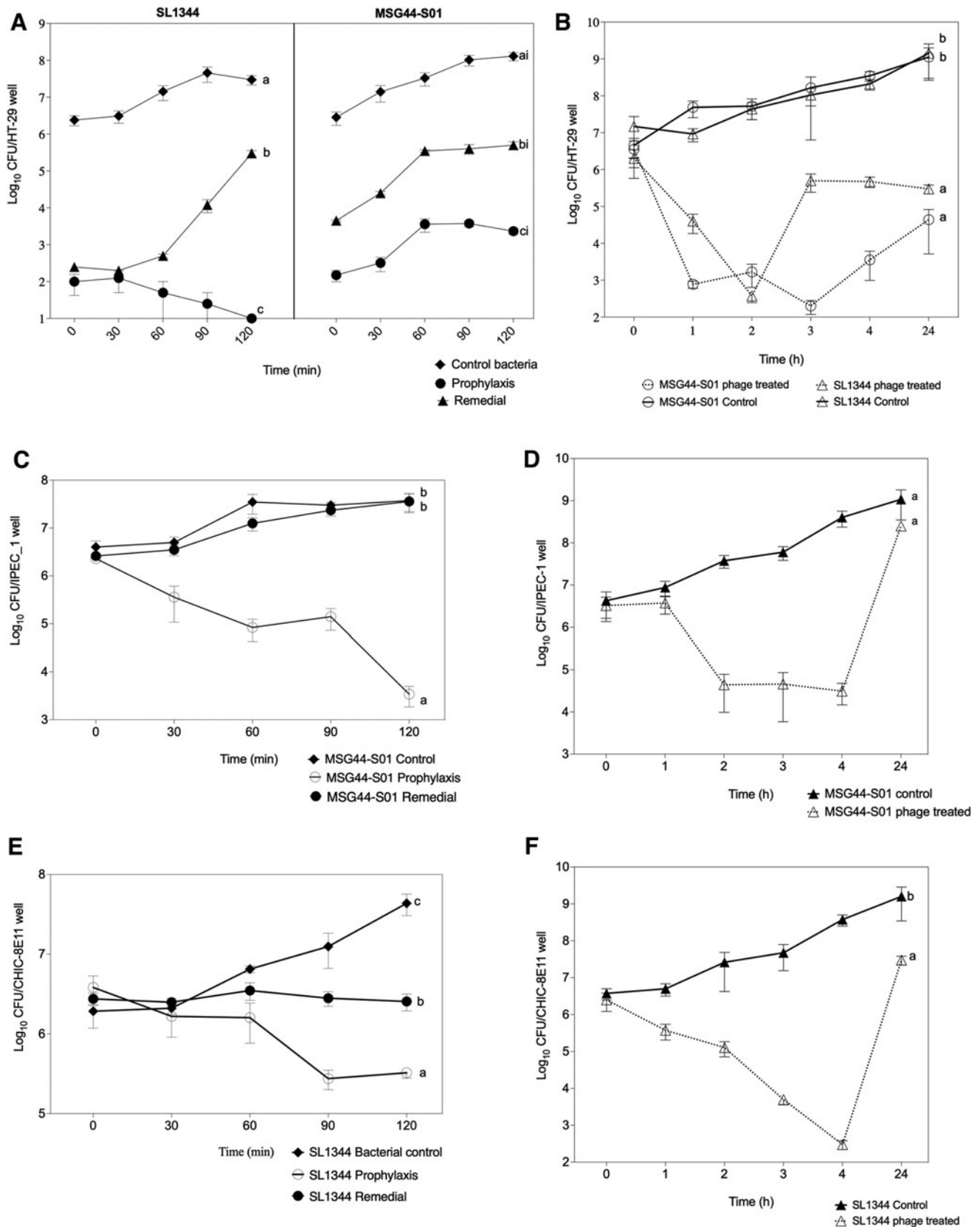
FIG. 6. Activity of the six-phage cocktail on bacterial recovery during prophylaxis, remedial, and coinfection therapy assays on HT-29 (**A, B**), IPEC-1 (**C, D**), and CHIC-8E11 (**E, F**) epithelial cells, respectively. For the prophylaxis and remedial assays, the phage cocktails or bacteria were added to the 80–90% confluent epithelial cells, respectively, and incubated for 0, 30, 60, 90, and 120 min. Afterward, bacterial cultures were added to the phage-treated wells or phages to the bacterial-treated wells, and both treated plates were further incubated for 1 h before bacterial recovery on XLD. For the coinfection assay, phages and bacteria were added to the wells simultaneously and incubated for 0, 1, 2, 3, 4, and 24 h before bacterial recovery as already mentioned. Assays were conducted in duplicate wells and repeated twice. Standard error of means of replicates is shown. Data were analyzed using analysis of variance using GraphPad Prism 9.

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FIG. 7. Phage recovery from the phage-treated bacterial cultures during prophylaxis, remedial, and coinfection therapy assays on HT-29 **(A, B)**, IPEC-1 **(C, D),** and CHIC-8E11 **(E, F)** epithelial cells. At each time point, samples from the phage-treated bacterial/epithelial cell cultures were taken, centrifuged, and titered using plaque assay on confluent soft agar cultures of SL1344. Assays were conducted in duplicate wells and repeated twice. Standard error of means of replicates are shown. Data were analyzed using analysis of variance using GraphPad Prism 9.
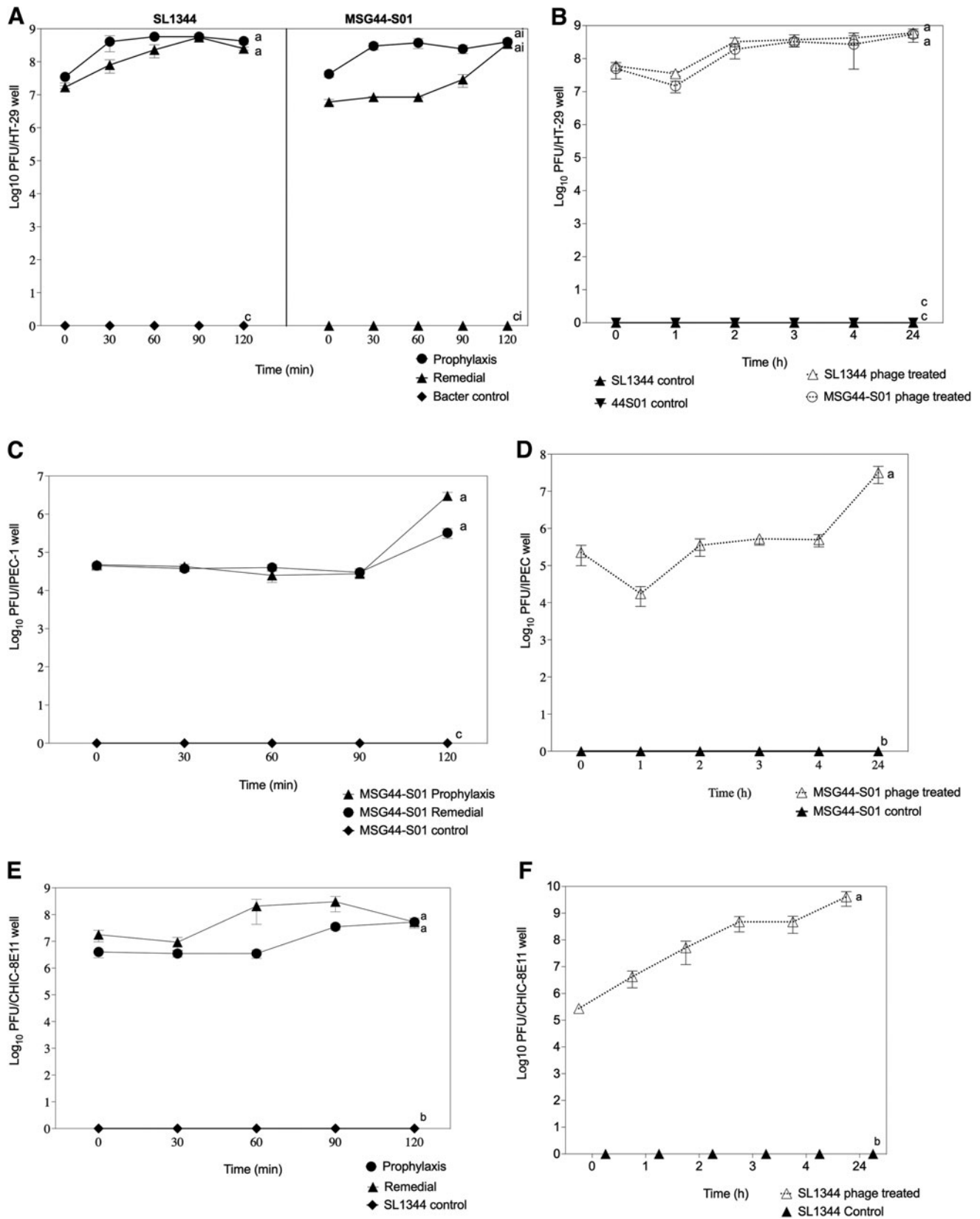
(Fig. 6D). For SL1344, phage treatment resulted in a decrease in bacterial growth in the first 4 h, but regrowth was observed at 24 h as seen in MSG44-S01, but SL1344 growth was not significantly different ($p < 0.05$) to the control (Fig. 6D, F). In all phage-treated cultures, high titers of phages were recovered at all time points in all regimens (Fig. 7A–F).

Discussion

The AMR threat in salmonellosis motivates a search for more effective measures to control the pathogen either in animals preharvest, during food and water processing, or in the final consumers, human.[7,9,39] Phage treatments have been shown to result in a significant reduction of *Salmonella* load on surfaces of poultry and pork meat products, colonization *in vivo*, *ex vivo* in relevant cell lines, and in chicken and pig guts, resulting in improved clinical and histopathological manifestations.[10,38,44,46] In this study, we built on existing knowledge in phage therapy research on zoonotic salmonellosis and present data to further strengthen the case for application of phages to control the infection in poultry, pigs, and humans.

We did this by developing an improved broad host range phage cocktail and showing its effectiveness to reduce *Salmonella* in relevant and important cell lines.[39,45,46,53] Knowledge of phage interaction with bacteria and epithelial cells is vital in determining their therapeutic potential or informing where improvements can be made to enhance stability and delivery, to achieve optimal downstream therapeutic effectiveness.[34,55–58]

In this study, we present analytical and viability assays to ascertain the therapeutic potential of phages to target chicken- and swine-associated *Salmonella* in the presence of relevant animal and human epithelial cell lines. The knowledge gained and concepts presented here are applicable to these model systems and may stimulate thoughts to investigate phage therapy in all zoonotic and plant-related bacterial infections.

We first identified a set of broad host range phages and determined their lytic potential on representative prevalent clinical *Salmonella* serovars.[43,59,60] To potentially construct a ''global multispecies product,'' strains from serovars found in poultry, pigs, and humans from across the United Kingdom and Thailand, and in global outbreaks and AMR, were assayed.[6,11,12,16,19,22] We previously studied the phages investigated here and showed that through synergistic effects, an optimized three-phage cocktail effectively lysed a subset of the U.K. isolates.[38,46] The cocktail also showed therapeutic efficacy in *G. mellonella* larvae infected with swine, chicken, and laboratory strains when tested in prophylaxis, remedial, and coinfection regimens.[46]

Numerous studies have confirmed that phage cocktails have superior therapeutic efficacy than single phage treatment.[39,41,43,45,46,53] Our data further support this and highlight the need to update phage cocktails to improve their clinical coverage and lysis efficacy especially when the host range is expanded. We showed that our previously reported three-phage cocktail that had 99% coverage on the subset of the strains studied in our earlier study only targeted ∼72% of the strains tested here.[46]

Our improved six-phage cocktail clearly had better clinical effect, targeting more of the U.K. and Thai serovars such that 83% of the strains investigated and 100% of the phage-susceptible strains were covered. Furthermore, the cocktail has comparable lysis efficacy to the three-phage cocktail, mitigating phage resistance *in vitro* at both of the experimental temperatures tested.[46]

After optimizing the six-phage cocktail *in vitro*, we determined its efficacy to reduce *Salmonella* in the presence of relevant avian, porcine, and human intestinal epithelial cell lines. Although the cell lines have different animal origins and characteristics, they were all derived from intestinal epithelium and have been used previously for bacterial infection and invasion studies and, thus, were a perfect fit to compare the behavior of phages and *Salmonella* serovars.

CHIC-8E11 cell lines were derived from small and large intestines of leghorn chickens and are positive for the enterocyte markers, villin, E-cadherin, and cytokeratin.[48] The IPEC-1 was established from intestinal epithelium cells that were isolated from jejunum and ileum of neonatal pig. Finally, HT-29 is a colon tumorigenic cell established from small intestine of a Caucasian female.[61] Clearly, both bacterial strains grew well in the presence of the cell lines, but we achieved reduced infection of the cell that may lessen *Salmonella* infection with phage.

Before the phage–bacteria interaction assays, we determined the interaction of the individual phages with the relevant cell lines and showed that the phages did adhere to all the cell lines, although more adhered to HT-29 than to CHIC-8E11 or IPEC-1. Our observation with HT-29 concurs with previous study that *C. difficile* phages interacted with HT-29, but the previous study showed the phages did not accumulate on the cell lines.[36] We, however, saw slight cumulative adherence of the phages by the HT-29 cells after 30 min, the phages tend to detach and reattach at various times on the other two cell lines.

Although little is known on the mechanisms of phage interactions with eukaryotic cells, previous study has shown that this could depend on interaction between the Lys-Gly-Asp motif as shown in T4 phage protein and the beta3 integrin receptors on melanoma cells in mice.[35,50] Although it is not clear how phage interaction occurred in our setting, and work is ongoing to determine this, the adherence of our phages to cell lines at the onset of the experiment could greatly enhance phage therapeutic activity as shown previously.[36] Also, the subsequent phage detachment could also lead to the availability of more free phages to kill bacteria in the medium and reduce their adhesion and invasiveness as shown in other studies.[36,37,62]

Bacterial adhesion is an initial and important pathogenicity step leading to subsequent invasion into eukaryotic cells, and several factors have been shown to impact on the rate of adhesion and invasion of *Salmonella* to cell lines.[31,33,63,64] Adherence of *Salmonella* strains to eukaryotic cells was previously shown with epithelial cells and in blood using a murine hepatocyte and colonocytes T84 cell lines.[38,65] Data derived in this study showed that the *Salmonella* strains showed variable adhesion and invasion rates with the different cell lines, with higher adhesion rates to the human HT-29 compared with the porcine IPEC-1 or chicken CHIC-8E11 (comparable with HT-29 only at the final time point) cells lines.

The adherence of our strains to HT-29 is slightly higher than adherence seen with other human epithelial cells such as Caco-2.[66] Although previous study did not identify significant

differences between *Salmonella* adherence to the Caco-2 and HT-29 cell lines, the known ability of HT-29 to produce more mucus than Caco-2 could lead to the higher adhesion we saw in our studies.[32]

Our data on the adherence of strains to the chicken and porcine cell lines concur with another report of *S.* Typhimurium LT2 strain adherence to avian INT-407 cells.[37] This may be attributed to the similarity of the serovars used in both studies (both being *S.* Typhimurium).[37] The slightly higher *Salmonella* growth in our study may be linked to the medium used, with ours containing serum, whereas in the previous studies, the serum was excluded alongside the antibiotics.[37]

Our observed comparable invasion rates between our *S.* Typhimurium strain on CHIC-8E11 and the same serovars strains to HD11 and INT-407, which are both avian cell lines, could suggest that serovar strains have similar invasion rates to related (avian) cell lines.[33,37,67] The swine-associated *Salmonella* are more efficient at adhering to the human epithelial cells relative to the other serovars and the invasion level is less in the IPEC cells. This could be attributed to the number of passages our IPEC-1 cells have gone through and this might have affected its activity as shown in other cell lines.[68–70]

In addition to establishing the adherence and invasion of the bacteria to the three cell lines, we showed that >50% of the recovered invaded *Salmonella* have reduced susceptibility to phage infection and, therefore, hypothesize this may have contributed to the regrowth of phage-treated bacterial cultures observed at the 24 h time. Previous study showed that postinvasive *S.* Typhimurium were still susceptible to phage infection, however, only a $0.5 \log_{10}$ CFU/cm^2 reduction from the culture wells was observed after 24 h postinfection in that study.[37]

Also, the invasive bacteria generated from the previous report were combined and treated with phage.[37] It is difficult to ascertain how the individual isolated invaded strains responded to the phage infection since they were not characterized individually as we did here.[37] Work is ongoing to determine the possible role of changes in the genomes, surface layer proteins, lipopolysaccharides, toll-like receptors, antibacterial factors, and nitric oxide, in the observed reduced phage EoPs seen in invasive strains here. In addition to these factors, *Salmonella* has been shown to survive and replicate in *Salmonella*-containing vacuoles, its primary niche, and in cytosol.[71]

Thus, further investigations are needed here to ascertain how these changes and features affect the susceptibility of invasive bacteria to phage infection. An alternative explanation to this is that pretreatment with phages would eradicate/reduce the bacterium and prevent invasion as shown in our treatment regimens and discussed hereunder.

Finally, we compared three different therapeutic regimens to test the ability of our phage cocktail to reduce the bacterial load within the cell culture wells. Our observation that the prophylaxis treatment is more effective than remedial treatment concurs with our previous study and other published data on established *Salmonella* infection models.[41,46,53,54] For cell culture assays, prophylaxis treatment could reduce number of bacteria, limiting invasion of cell tissue and preventing systematic infection.[53] Also, we showed that the longer the phages were incubated with the cell culture medium, the better their infectivity in the prophylaxis regimen.

This also corresponded to the reduction in the attachment of the phages to the cell lines that means the phage are freely found in the medium in the phage adherence assay. Other published study also showed that phage remedial treatment is effective at controlling number of *Salmonella* in cell culture assays using a chicken cell line and, thus, reducing intracellular invasion of the bacterium.[37] The efficacy of *Salmonella* phages in both regimens as shown in our study and elsewhere further strengthens their possible application for the control of salmonellosis in the animals.[37,53]

Conclusion and Future Work

Our study seeks to provide fundamental insights into the therapeutic potential of phages to clear representative chicken and swine *Salmonella* strains in the presence of avian, porcine, and human cell lines. Our optimized cocktail of six phages (five myoviruses and one siphovirus) had a wide strain coverage and optimal lysis efficacy at both 37°C and 40°C. Our data showed the individual phages and bacterial strains have variable adherence rates to the different cell lines. After establishing the invasiveness of strains, we showed that recovered intracellular bacteria had varying susceptibility levels to phage infection.

Our phage cocktail had better therapeutic efficacy with prophylaxis rather than remedial and coinfection regimens. We are confident that our data strongly support the therapeutic development of these phages for clinical use to control *Salmonella* in chickens and pigs as an effective preharvest step to provide safer meat products, and as an excellent control strategy for human salmonellosis.

Future study will focus on whether our phages can invade the cell lines, or whether modification through formulation (encapsulating phages within a stabilizing substance) could improve their entry and delivery into the cell lines and kill *Salmonella* to prevent systemic infection. Further characterization of the postinvasive bacterial isolates will reveal potential genetic, structural, or physiological changes to disentangle the observed reduced phage susceptibility.

Authors' Contributions

J.Y.N., M.R.J.C., and E.E.G. conceived and designed the experiments. J.Y.N. optimized the six-phage cocktail and conducted the host range and virulence assays. J.Y.N. and B.A. conducted the cell culture assays. R.H. and J.S. provided useful technical support and consultations on the cell culture study. J.Y.N. analyzed the data and drafted the article. A.G., M.A.O., M.F.A., P.P., P.T., and S.K. isolated the *Salmonella* strains. All authors edited and agreed to be accountable for all aspects of the article and approved the final version to be published.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4
Supplementary Table S5

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