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Escherichia coli phages isolated from broiler chickens showed ideal characteristics in gut modulation

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Abstract. Phage has gained interest as an alternative antibiotic growth promoter (AGPs) in poultry production. Most phage studies only focus on phages that target pathogens. In this study, we isolated and characterised phages that target non-pathogens from chicken intestines. This study aimed to isolate and characterise phages that target non-pathogenic *Escherichia coli* for gut modulation study in broiler chickens. Based on a morphological study, the C1 phage belonged to the *Podoviridae* family, whereas C2, C3, and C4 phages belonged to the *Siphoviridae* family. The C1, C2, C3, and C4 phages appeared to be unique based on restriction fragment length polymorphisms (RFLPs), amplification of phage signature genes, and protein profiling (SDS-PAGE). The C1 phage had an ideal multiplicity of infection (MOI) of 0.001, followed by 0.1 for the C2, C3, and C4 phages. C1 had the highest adsorption rate of 99.7% in 1 min, followed by C2 (98%), C3 (98.7%), and C4 (98.2%), all of which were within 2 min. C1 also exhibited the largest burst size (72 PFU/infected cell) and the shortest latent period (5 min). The latent period of the C2, C3, and C4 phages was longer, lasting 10 minutes, and their burst sizes were 70, 77, and 46 PFU/infected cells, respectively. All phages had optimum lytic activity at pH 7 and 37°C. Each phage was unique and possessed favourable lytic characteristics, making all of them suitable for gut modulation study in chickens.

Keywords: *E. coli*, bacteriophage, antibiotic growth performance, alternative, poultry production

INTRODUCTION

Antibiotic-resistant bacteria have emerged due to the widespread use of antibiotic growth promoters (AGPs) in poultry production (Brüssow, 2009; Gill & Hyman 2010; Heilmann *et al.,* 2010). This has motivated researchers to study a practical substitute for enhancing host growth performance and health. The use of phage as a feed supplement has since attracted research attention. Phage has the advantage of a narrow killing spectrum, where it will only kill specific bacterial strains or species, in which other gut microbes will not be affected (Skurnik & Strauch 2006; Gill & Hyman, 2010; Loc-Carrillo & Abedon, 2011). Consequently, the disruption to other gut microorganisms will be lessened (Hyman & Abedon, 2009; Loc-Carrillo & Abedon, 2011). Phages are also capable of self-sustaining in the hosts, owing to their self-replication mechanisms (Abedon & Thomas-Abedon, 2010; Loc-Carrillo & Abedon, 2011). Although phages are commonly used in therapeutic studies, they have also been used to modulate the gut microbiota to improve growth performance and health in chickens (Yan *et al.,*

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2012; Zhao *et al.,* 2012; Wang *et al.,* 2013; Kim *et al.,* 2014).

Phages are ubiquitous bacteria-infecting viruses found in various environments (Pelzek *et al.,* 2008; Brüssow, 2009; Gill & Hyman, 2010; Heilmann *et al.,* 2010). Two types of phages are classified based on their life cycle, which is lytic and temperate. For lytic phages, they follow a direct lytic life cycle, where phages will obligately lyse their hosts at the end of the cycle. Temperate phages can choose between two cycles, which are lytic cycles like the lytic phage or the lysogenic cycle (Clokie *et al.,* 2011; Gandon, 2016). In the lysogenic cycle, the temperate phage can incorporate its genetic materials into the bacterial host genome (Clokie *et al.,* 2011; Gandon, 2016). Temperate phages must be avoided in therapeutic or gut modulation studies due to the risk of virulence genes that can be transferred to the host (Skurnik & Strauch, 2006, Gill & Hyman, 2010, Loc-Carrillo & Abedon, 2011).

Escherichia coli is a bacterium mainly found as gut residents in chicken intestines (Lu *et al.,* 2003; Amit-Romach *et al.,* 2004; Chambers & Gong 2011; Mohd Shaufi *et al.,* 2015). They can be found as commensals or pathogens, influencing gut microbial dynamics and chickens' health (Lu *et al.,* 2003; Amit-Romach *et al.,* 2004; Kaper *et al.,* 2004). The reduction of the *E. coli* population in the chicken gut might reduce the competition with other gut microbes, especially the good ones. Prior research has mainly focused on isolating pathogen-specific phages (Yan *et al.,* 2012; Zhao *et al.,* 2012; Wang *et al.,* 2013; Kim *et al.,* 2017). This is the first study to discuss the isolation and characterisation of phage that targets non-pathogens from chicken intestines. Specifically, non-pathogenic *E. coli* was targeted to avoid the contamination of phage lysate with pathogenic bacteria components (e.g., bacterial endotoxin) during large-scale phage production. The presence of pathogenic bacteria components in phage lysate might lead to activation of the host immune response or cause disease and, even worse, can result in death (Skurnik & Strauch, 2006; Loc-Carrillo & Abedon, 2011). Although phage can be thoroughly purified using Caesium Chloride (CsCl) centrifugation techniques, the yield from the procedure is very low and impractical for large-scale production.

The characterisation of newly isolated phage based on their molecular, biological and physiological properties are necessary for ensuring practical phage application *in vivo* (Jończyk *et al.,* 2011; Bardina *et al.,* 2012). Previous studies showed that poor phage selection, ineffective preparation and phage viability during and after supplementation are the main reasons that cause failure in phage application (Merril *et al.,* 2003; Przerwa *et al.,* 2006; Gill & Hyman, 2010). For instance, the effectiveness of phage supplementation *in vivo* can be affected by several environmental factors, such as pH and temperature (Jończyk *et al.,* 2011). Therefore, phages need to be thoroughly characterised so that specific strategies can be implemented to ensure that phages can survive in an extreme environment. This study aimed to characterise the morphological, biological and physiological properties of isolated *E. coli* phages and investigate the interaction between phage and host at specific pH and temperature.

MATERIALS AND METHODS

Sample preparation

The intestinal samples from previous studies were used to isolate *E. coli* and phage (Mohd Shaufi *et al.,* 2015; Shaufi *et al.,* 2017). Briefly, one hundred and twenty-eight samples of ileal and caecal contents from 7, 14, 21, and 42 d birds were scraped and individually stored in a sterile tube. The intestinal contents were always kept in ice before storing at -80°C.

E. coli isolation, characterisation, and confirmation

One gram of the sample was serially diluted in 9 ml of Maximum Recovery Dilution (MRD) water (Difco, BD, USA) at appropriate dilutions and spread plated on ethylene-methylene blue (EMB) agar (Difco, BD, USA). After 24 h of incubation at 37°C, presumptive blue-black and green metallic sheen colonies were isolated and purified. The distinctive isolates were further purified by streaking on new EMB plates. This step was repeated three times until consistent morphology was observed. The isolates were stored in 15% glycerol at -80°C freezer for further analysis. The isolates were further confirmed based on their

biochemical patterns (71 carbon source utilisation assays and 23 chemical sensitivity assays) using Biolog GEN III MicroPlate™ (Biolog, USA) by following the manufacturer's instructions. In order to ascertain the pathogenicity of the isolates, the samples were sent for serotyping to the Veterinary Research Institute (VRI) in Ipoh, Malaysia. The serotyping is based on *E. coli* polyclonal antiserum against pathogenic serogroups of O1, O2, O157, K1 and K80 (Orskov *et al.,* 1977). Any of the isolates serotyped to these serogroups were then removed. The final bacterial hosts chosen were subjected to full-length amplification of the 16S rRNA gene for their species confirmations. They were then sent to First BASE Laboratories Sdn Bhd (Seri Kembangan, Malaysia) for Sanger sequencing. Sequences obtained were blasted with the GenBank database (http://www.ncbi.nlm.nih.gov/) to confirm the bacterial host species by referring to their highest identity (Zhang *et al.,* 2000).

Phage isolation and purification

Phage was isolated based on direct and enrichment methods. For direct screening, intestinal contents were mixed with sterile SM buffer (50 mM Tris-HCl [pH 7.5], 0.10 M NaCl, 8 mM $MgSO_4.7H_2O$) at a 1:10 ratio, homogenised by vigorous vortexing, and centrifuged at 4,000 x *g* in 4°C for 10 min to remove bacteria and intestinal debris. Supernatants were subjected to filter sterilisation using a 0.22 µm filter (Sartorius, Germany) to remove any bacterial carry-over. For enrichment screening, an equal volume of a mid-log phase of *E. coli* host was mixed with the intestinal sample and incubated at 37°C with continuous shaking (180 rpm) for 24 h. Overnight cultures were then centrifuged and filter sterilised as described earlier. Phage was screened from the filtrates based on double-layer agar assay methods (Adams, 1959). Plates were checked for plaque formation with clear halo zones. Plaques with different morphology were observed and extracted from the plate by picking using truncated pipette tips. Phages were then inoculated into 10 ml LB broth with 500 µl of mid-log phase *E. coli* hosts. The mixture (phage + *E. coli*) was incubated at 37°C with continuous shaking (180 rpm) for 2 – 3 h until the cells were lysed. Following the lysis step, the sample was centrifuged at 4,000 x *g* for 10 min at 4°C and filter sterilised by using a 0.22 µm filter (Sartorius, Germany). This procedure was repeated three times until consistent shape and size of plaques were obtained.

Large-scale phage propagation and purification

A highly concentrated phage was produced from PEG 8000 precipitation, and the purification was conducted using caesium chloride (CsCl) (Amresco, USA) density gradient centrifugation. Both procedures were performed as described in the Novagen T7 Select System Manual (Novagen 2002). Phage was then purified by layering on top of four different density gradients of CsCl solutions in clear SW 41 ultracentrifuge tubes (Beckman, USA) at ratios CsCl:TE of 1:2, 1:1, 2:1, and 1:0. They were centrifuged in Avanti J-25I (Beckman, USA) at 209,700 x *g* and for 1 h at 4°C. Purified phage lysate was collected from the turbid, clear, and opalescent blue band. The purified phage lysate was dialysed at the ratio of 1:100 of phage lysate and dialysis buffer with SnakeSkin Dialysis Tubing (10K MWCO, 22mm) (Termoscientific, USA). The phage was stored at 4°C and -80°C for short- and long-term storage, respectively.

Phage morphological study based on transmission electron microscopy (TEM)

The concentrated and purified phage lysates were dropped onto a carbon-coated copper grid and incubated at room temperature for 6 min. The lysates were then negatively stained with 2% (w/v) uranyl acetate and left for another 10 min before being observed under Philips HMG 400 transmission electron microscope (TEM) (Philips, The Netherlands) at a magnification of 300,000 x. The morphology of each phage was analysed, compared, and classified based on the International Committee of Taxonomy of Viruses (ICTV) described in detail by Ackermann (2009).

Phage host range

The host range of isolated phages was determined based on various bacterial hosts (Table 1). Five hundred microliters of mid-log phase cultures (OD_{600nm} = 0.6 – 0.8) were mixed with 4.5 ml LB top agar in test tubes, where they were then poured evenly onto LB agar. Plates were left dry for a few minutes in the biosafety cabinet. Then,

10 µl of phage lysate was spotted on the respective plates and let dry for 20 minutes. All cultures were then incubated at 37°C for 24 h, while *Lactobacillus* and *Clostridium perfringens* cultures were incubated under anaerobic conditions. The presence and absence of plaques were then recorded as (+) and (-), respectively.

Table 1. List of bacterial hosts and their sources used for phage host range study.

No	Bacterial host	Source
1	*Escherichia coli* (C1)	This study
2	*Escherichia coli* (C2)	This study
3	*Escherichia coli* (C3)	This study
4	*Escherichia coli* (C4)	This study
5	*Escherichia coli* (7I 1E)	This study
6	*Escherichia coli* (7I 2E)	This study
7	*Escherichia coli* (7I 2X)	This study
8	*Escherichia coli* (7I 3X)	This study
9	*Escherichia coli* (14I 1E)	This study
10	*Escherichia coli* (14I 2E)	This study
11	*Escherichia coli* (14I 3E)	This study
12	*Escherichia coli* (14C 2E)	This study
13	*Escherichia coli* (21I 2E)	This study
14	*Escherichia coli* (42I 2E)	This study
15	*Escherichia coli* (42I 6E)	This study
16	*Escherichia coli* O78:K80 5886/05	This study
17	*Escherichia coli* O157:H7	VRI
18	*Salmonella enterica* serovar Typhimurium 8720/06	VRI
19	*Salmonella enterica* serovar Pullorum 8214/06	VRI
20	*Salmonella enterica* serovar Enteritidis 692/06	VRI
21	*Shigella sonnei* (ATCC® 25931™)	VRI
22	*Enterobacter aerogenes* (ATCC® 13048™)	ATCC®
23	*Lactobacillus casei* strain Shirota	ATCC®
24	*Enterococcus faecalis* (ATCC® 2912™)	ATCC®
25	*Clostridium perfringens* (7I)	From lab
26	*Clostridium perfringens* (21I)	From lab
27	*Klebsiella pneumonia* (K36)	From lab

Phage genomic analysis

The DNA of four phages was extracted based on phenol/chloroform/isoamyl alcohol DNA extraction methods described by Thurber *et al.* (2009). The phage genomic DNA (gDNA) was mixed with 6X loading dye (Vivantis, Malaysia), and resolved on 0.7% agarose gel electrophoresis in 1X Tris-acetate-EDTA (TAE [40 mM Tris-acetate; 1 mM EDTA] buffer at 90 V for 1 h. The VC Lambda/*Hind*III marker (Vivantis, Malaysia) was loaded at both gel ends. The gel was photographed under UV light in Gel Doc™ XR+ System (Biorad, USA).

Phage restriction fragment length polymorphisms (RFLPs) analysis

Phage genomic DNA was digested with restriction enzymes of *Eco*RI, *Hind*III and *Bam*III (Promega, USA) following the manufacturer's protocol. The genomic DNA of four phages was standardised to 0.2 µg and incubated with restriction enzyme 10X buffer, acetylated BSA and respective restriction enzymes for 1 h at 37°C. The RFLPs analysis was performed in triplicate. The digested DNA was then subjected to electrophoresis alongside DNA ladder of VC 1kb-Ex (Vivantis, Malaysia).

Phage signature genes amplification analysis

PCR amplifications were performed in a reaction mixture (25 µl) containing 50 ng template DNA, NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs, UK) and 0.5 µM primer each (Table 2), using SureCycler 8800 (Agilent, USA). PCR amplifications were carried out in triplicate. The amplicons were then resolved on 1.5% agarose gel. The GeneRuler 100 bp ladder (Thermofisher Scientific, UK) was then loaded into three wells at both ends and the middle of the gel.

Phage component protein profiling based on SDS-PAGE

Phage structural proteins were analysed based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Laemmli (Laemmli 1970) buffer system in Mini-PROTEAN® 3 Cell (Bio-Rad, USA). Concentrated and purified phages were diluted in 2X sample buffer (SDS reducing buffer) [0.06 mM Tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 25% glycerol, 0.01% (w/v) bromophenol blue]. The proteins were then heated at 95°C for 4 min and loaded into the well of the prepared gel (12% stacking and 6% resolving gel at 37.5:1 ratio of 30% Acrylamide/Bis solutions (Sigma-Aldrich, USA)). The samples were resolved alongside Prestained Precision Plus Protein Standard (Bio-rad, USA) at

180 V for 1 h. Subsequently, the gel was washed three times for 5 min with Mili-Q water to remove excess SDS. The gel was then stained with Bio-Safe Coomassie Blue G-250 (Bio-Rad, USA) and was shaken gently for 1 h. The gel was then destained with water for 30 min and photographed. The SDS-PAGE study was performed in triplicate.

Table 2. List of target genes and the primers designed for phage signature genes amplification analysis.

Target gene	Gene product	Target phage Family	Primer (5'-3')	Product length (bp)
LD32_gp10	Major capsid protein	*Siphoviridae* of *Escherichia phage bV_EcoS_AHP42*	F: TACGGCGAAATGGATGGTGT R: GTTGCAGGAACTCCACCTGT	520
ABF05_gp07	Major capsid protein	*Siphoviridae* of *Enterobacteria phage phi80*	F: AGCGGTGTGGTCAACATCAT R: ATTCCCCAGCACCATTGTCA	255
F856_gp22	Major capsid protein	*Siphoviridae* of *Enterobacteria phage vB_EcoS_Rogue1*	F: GGTATGGTCAGGCTCTACTGC R: CACGGAAGTGCAAATCCTTCGG	422
AGC_0135	DNA polymerase	*Siphoviridae* of *Enterobacteria phage EPS7*	F: ACGTTCTGATGCCTGGTTGT R: ACGGAGCTGCTTAACAGAGT	206
pol	DNA polymerase	*Siphoviridae* of *Enterobacteria phage T5*	F: AGGCTGCTAAAGCAATCACCT R: GCTGCTTGTTCTAGTAATGCTTCGT	94
orf124	DNA polymerase	*Siphoviridae* of *Escherichia phage bV_EcoS_AKFV33*	F: GTTGCGGATGCGAAGGACTA R: CCACGGTCTTCGGAATGGAT	161
T7p44	Major capsid protein	*Podoviridae* of T7 phage	F: CAGAACAAGGCCGCACTTAC R: TTACCCTCACCTTTATTGGCAGG	335
T7p29	DNA polymerase	*Podoviridae* of T7 phage	F: TTGCTGGTGCTCCTTACACC R: AGCTCAAGACCGGATGCGT	481

Phage multiplicity of infection (MOI)

The optimum multiplicity of infection (MOI) ratio of phage was investigated in the range of 0.001 to 10. Phages and mid-log phase bacteria (OD_{600nm}= 0.6 – 0.8) were mixed at the respective ratio and incubated at 37°C with continuous shaking (180 rpm). The sample was collected every 20 min for 120 min, where both phage and bacterial host were quantified by double layer agar assay and spread plated on EMB agar, respectively. The experiment was repeated in triplicate.

Phage adsorption rate

Phages were mixed with mid-log phase bacterial cultures at the multiplicity of infection (MOI) ratio of 0.001 and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). A hundred microlitres of the sample were taken out at 1, 2, 3, 4, 6, 8, 10, 15 and 20 min and centrifuged at 5,000 x *g* for 3 min at 4°C. The supernatant was collected for titration of unadsorbed phages (phages that are not adsorbed to the host) based on the double-layer agar assay method. The experiment was repeated in triplicate. The calculation for adsorption rate was based on the formula:

$$\text{Adsorption rate} = \frac{\text{Initial phage titre - unadsorbed phage titre}}{\text{initial phage titre}} \times 100$$

Phage single-step growth curve

At MOI ratio of 0.001, phages and mid-log phase bacterial culture were mixed and incubated based on the pre-determined adsorption rate. They were then centrifuged at 5,000 x *g* for 3 min at 4°C. Pellets were collected and re-suspended in 10 ml LB broth in a glass tube and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). The mixtures were titred at various

intervals for each phage based on pilot experiments. The experiment was repeated in triplicate. Phage latent periods and burst sizes were determined from plotted curves of phage titre against time intervals. The latent period is identified between the phage adsorption period (0 min) and until the lysis of phage (the start of rising phage titre). The calculation for burst size is as per described by Swamy (2008):

$$\text{Burst size} = \frac{\text{Final PFU-Initial PFU}}{\text{Number of infected bacterial cells}}$$

Effect of pH on phage-host interaction

At MOI ratio of 0.1, phages and mid-log phase bacteria were mixed in 10 ml LB broth. Negative control of both phage and bacteria was prepared. Samples and control were incubated at pH 3, 5, 7 and 9 in an orbital shaker at 37°C with continuous shaking (180 rpm) for 1 h for C1 phage, while 2 h for C2, C3 and C4 phages. The samples were collected at the end of the incubation period. Double-layer agar and spread plate techniques measured the phage and bacteria, respectively. The experiment was repeated in triplicate. Phage increment rate was calculated based on:

$$\text{Phage increment rate} = \frac{\text{Final phage titre - initial phage titre}}{\text{Final phage titre}} \times 100$$

For host reduction rate:

$$\text{Host reduction rate} = \frac{\text{Initial host concentration - final host concentration}}{\text{Initial host concentration}} \times 100$$

Effect of temperature on phage host interaction

The procedure for the temperature sensitivity experiment was as described earlier, except those samples and control were incubated at a temperature of 25°C, 37°C, 42°C, and 60°C with continuous shaking (180 rpm).

Statistical analysis

Physiological characterisations of phages were repeated in triplicate for both double-layer agar assays and spread plates. All readings were analysed based on a one-way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) Statistics version 22 (IBM, USA). Results were recorded as means or means ± standard error (SE). Statistical significance was defined as $P < 0.05$ based on Duncan's multiple range test (Duncan 1955).

RESULTS

E. coli and phage isolation

Sixteen non-pathogenic *E. coli* were isolated and identified based on BIOLOG, serotyping and 16S rRNA gene Sanger sequencing (Table 1). These isolates were then used for the isolation of phages. The best four phages (most effective in killing *E. coli* host) based on broth lysis (results not included) were selected for further characterisations.

Phage host range

The host range of C1, C2, C3 and C4 phages was investigated on various bacterial hosts (Table 3). C1 phage could only infect their originally isolated host, *E. coli* (C1). On the other hand, C2, C3, and C4 phages can cross-infect *E. coli* (C2), (C3) and (C4).

Phage morphology

All four phages were observed under transmission electron microscopy (TEM), and their morphologies were analysed. All phages had a hexagon nucleocapsid head structure with a short tail for C1 and a long tail for C2, C3 and C4 phages (Figure 1). Table 4 shows that tail length was significantly ($P < 0.05$) different between all phages.

Restriction fragment length polymorphisms (RFLPs) of phage DNA

All four phages' DNA was subjected to restriction enzymes (REs) digestion of *Eco*RI, *Hind*III and *Bam*III to discriminate between phages. Figure 2 shows that C1 phage gDNA was digested into 2 different fragments (15,000 bp and 8,000 bp), whilst C2 phage gDNA to 5 different fragments (15,000 bp, 10,000bp, 3,500 bp, 1,300 bp and 1,200 bp) based on *Eco*RI REs. No digestions were observed for C3 and C4 phage gDNA from all three REs.

Amplification of phage signature genes

Out of eight PCR amplifications performed on C1, C2, C3 and C4 phages, only genes encoding the structural proteins of T7p44 major capsid protein and T7p29 DNA polymerase were amplified in C1 phage, with the band size of 335 bp and 481 bp, respectively (Figure 3(a)).

SDS-PAGE analysis of phage protein

Molecular masses of C1, C2, C3 and C4 phage proteins were determined based on SDS-PAGE analysis. C1 phage had a very distinctive protein profile compared to C2, C3 and C4 phages (Figure 4). C2, C3, and C4 phages had slightly similar protein profiles, but C4 phages had fewer visible proteins detected. One major protein of 40 kDa size and seven minor proteins ranging from 53 kDa to 160 kDa were observed for the C1 phage. For C2, C3 and C4 phages, three major proteins of 35 kDa, 28 kDa and 15 kDa were observed. The rest of the bands represented minor proteins which were barely visible, and they could be seen in the size ranging from 10 and 37 to 110 kDa, in which C2 and C3 phages had ten bands compared to four bands in C4 phage.

Table 3. Host range results of C1, C2, C3 and C4 phages based on spot tests on various bacterial hosts.

No	Bacterial host	C1 phage	C2 phage	C3 phage	C4 phage
1	*Escherichia coli* (C1)	+	-	-	-
2	*Escherichia coli* (C2)	-	+	+	+
3	*Escherichia coli* (C3)	-	+	+	+
4	*Escherichia coli* (C4)	-	+	+	+
5	*Escherichia coli* (7I 1E)	-	-	-	-
6	*Escherichia coli* (7I 2E)	-	-	-	-
7	*Escherichia coli* (7I 2X)	-	-	-	-
8	*Escherichia coli* (7I 3X)	-	-	-	-
9	*Escherichia coli* (14I 1E)	-	-	-	-
10	*Escherichia coli* (14I 2E)	-	-	-	-
11	*Escherichia coli* (14I 3E)	-	-	-	-
12	*Escherichia coli* (14C 2E)	-	-	-	-
13	*Escherichia coli* (21I 2E)	-	-	-	-
14	*Escherichia coli* (42I 2E)	-	-	-	-
15	*Escherichia coli* (42I 6E)	-	-	-	-
16	*Escherichia coli* O78:K80 5886/05	-	-	-	-
17	*Escherichia coli* O157:H7	-	-	-	-
18	*Salmonella enterica* serovar Typhimurium 8720/06	-	-	-	-
19	*Salmonella enterica* serovar Pullorum 8214/06	-	-	-	-
20	*Salmonella enterica* serovar Enteritidis 692/06	-	-	-	-
21	*Shigella sonnei* (ATCC® 25931™)	-	-	-	-
22	*Enterobacter aerogenes* (ATCC® 13048™)	-	-	-	-
23	*Lactobacillus casei* strain Shirota	-	-	-	-
24	*Enterococcus faecalis* (ATCC® 2912™)	-	-	-	-
25	*Clostridium perfringens* (7I)	-	-	-	-
26	*Clostridium perfringens* (21I)	-	-	-	-
27	*Klebsiella pneumonia* (K36)	-	-	-	-

(+) = presence of plaque. (-) = absence of plaque.

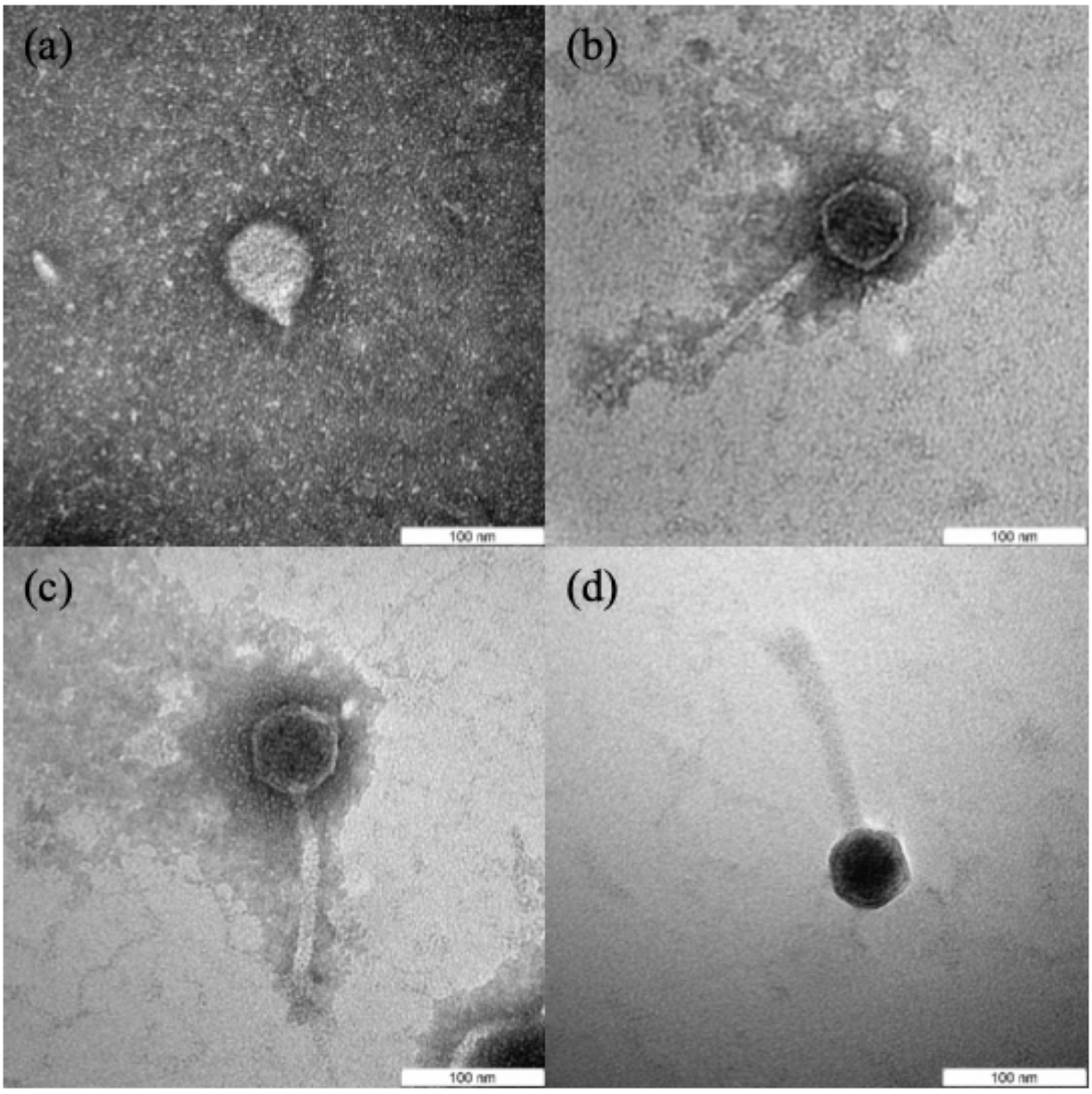

Figure 1. Transmission electron micrograph (TEM) of (a) C1, (b) C2, (c) C3, and (d) C4 phages that were negatively stained with 2% uranyl acetate under 300 k magnification. Bar = 100 nm.

Table 4. Summary of phage morphological properties based on transmission electron microscopy.

Phage	Family	Head Diameter (nm)	Tail Length (nm)
C1	*Podoviridae*	62.05 ± 1.26	17.84 ± 2.48[a]
C2	*Siphoviridae*	56.52 ± 3.19	119.25 ± 4.30[b]
C3	*Siphoviridae*	63.58 ± 1.42	136.99 ± 5.43[c]
C4	*Siphoviridae*	59.34 ± 2.41	150.38 ± 4.22[d]

Mean ± standard error (SE) of n = 5. Different alphabet from the same column showed significant of $P < 0.05$.

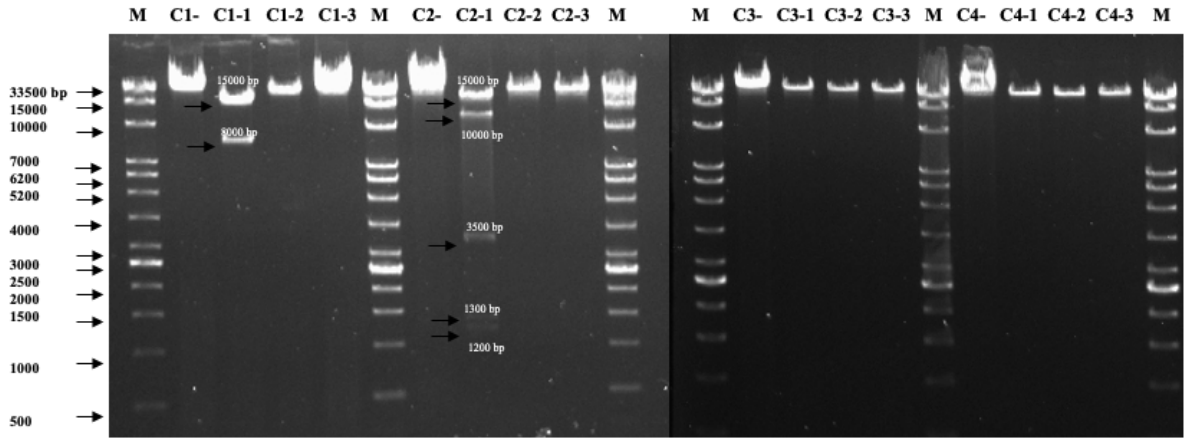

Figure 2. Restriction fragment length polymorphisms (RFLPs) analysis of C1, C2, C3 and C4 phages genomic DNA based on *Eco*RI, *Hind*III and *Bam*III visualised on 1% agarose gel electrophoresis. Only C1 and C2 phage genomic DNA were able to be digested by *Eco*RI. M = VC 1kb-Ex DNA Ladder; Cx = C1, C2, C3, and C4 phage genomic DNA; Cx-1 = *Eco*RI; Cx-2 = *Hind*III; Cx-3 = *Bam*III.

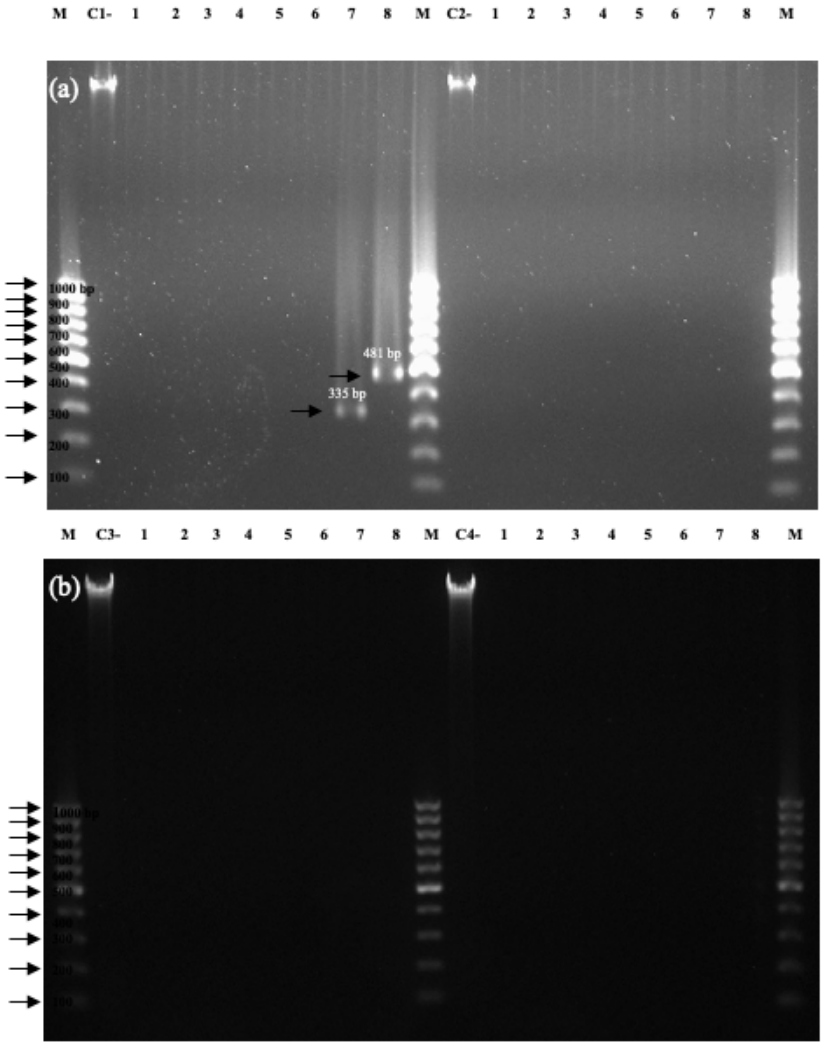

Figure 3. Amplification of phage signature genes of major capsid protein (1 = LD32_gp10; 2 = ABF05_gp07; 3 = F856_gp22; 7 = T7p44), DNA polymerase (4 = AGC_0135; 5 = polA; 6 = orf124; 8 = T7p29) for (a) C1, C2, (b) C3 and C4 phages. M = GeneRuler 100 bp DNA Ladder; (-) = negative control; C1 = C1 phage; C2 = C2 phage; C3 = C3 phage; C4 = C4 phage.

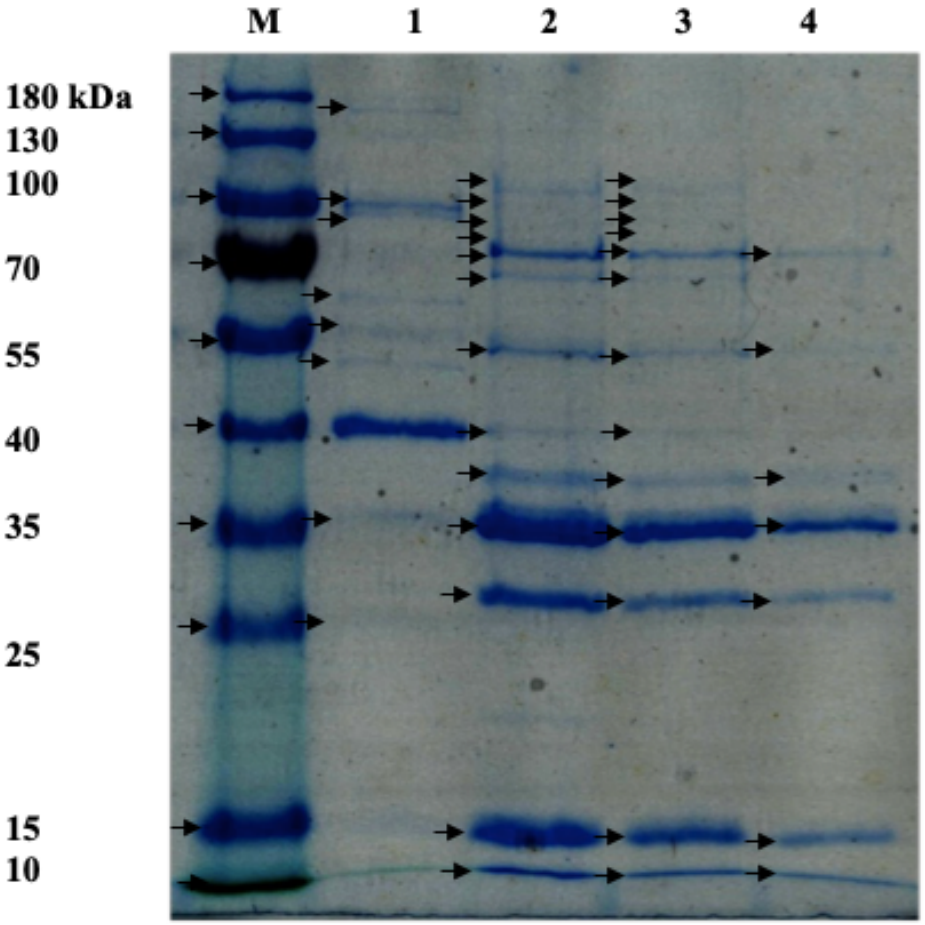

Figure 4. Protein profiling by SDS-PAGE analysis of C1, C2, C3, and C4 phages. Phage proteins were resolved on Laemmli buffer system in Mini-PROTEAN® 3 Cell (Bio-Rad, USA) at 180V for 1 h, where the gel was then washed 3 times for 5 min with Mili-Q water, stained with Bio-Safe Coomassie Blue G-250 (Bio-Rad, USA) for 1 h and destained with Mili-Q water for 30 min. Various major and minor protein bands were observed (as highlighted by the arrows). C1 phage had unique protein profiles compared to C2, C3 and C4 phages. M = PageRuler Prestained Protein Ladder (Thermofisher Scientific); 1 = C1 Phage;2 = C2 Phage; 3 = C3 Phage; 4 = C4 Phage.

Phage multiplicity of infection (MOI)

The multiplicity of infection (MOI) is the *in vitro* study of an optimal phage dosage. The host concentration was fixed at the mid-log phase period (OD_{600nm} = 0.6 – 0.8), while phage titre was varied at different phages to host ratios of 0.001, 0.01, 0.1, 1 and 10. The optimal MOI for C1 phage was observed at 0.001, where the highest phage titre increment of 5.97 log and the lowest reduction of the host at 2.85 log were observed (Figure 5). For C2 phage, the optimal MOI of 0.1 yielded the highest phage titre at 3.68 log increment, while the lowest host reduction was observed at 2.45 log (Figure 6). At MOI of 0.1, C3 phage had the highest phage increment at 3.87 log and the lowest host reduction at 2.37 log (Figure 7). The optimal MOI for C4 phage was observed at MOI of 0.1, where 4.76 log increments of phage titre and 2.35 log reduction of the host were recorded (Figure 8).

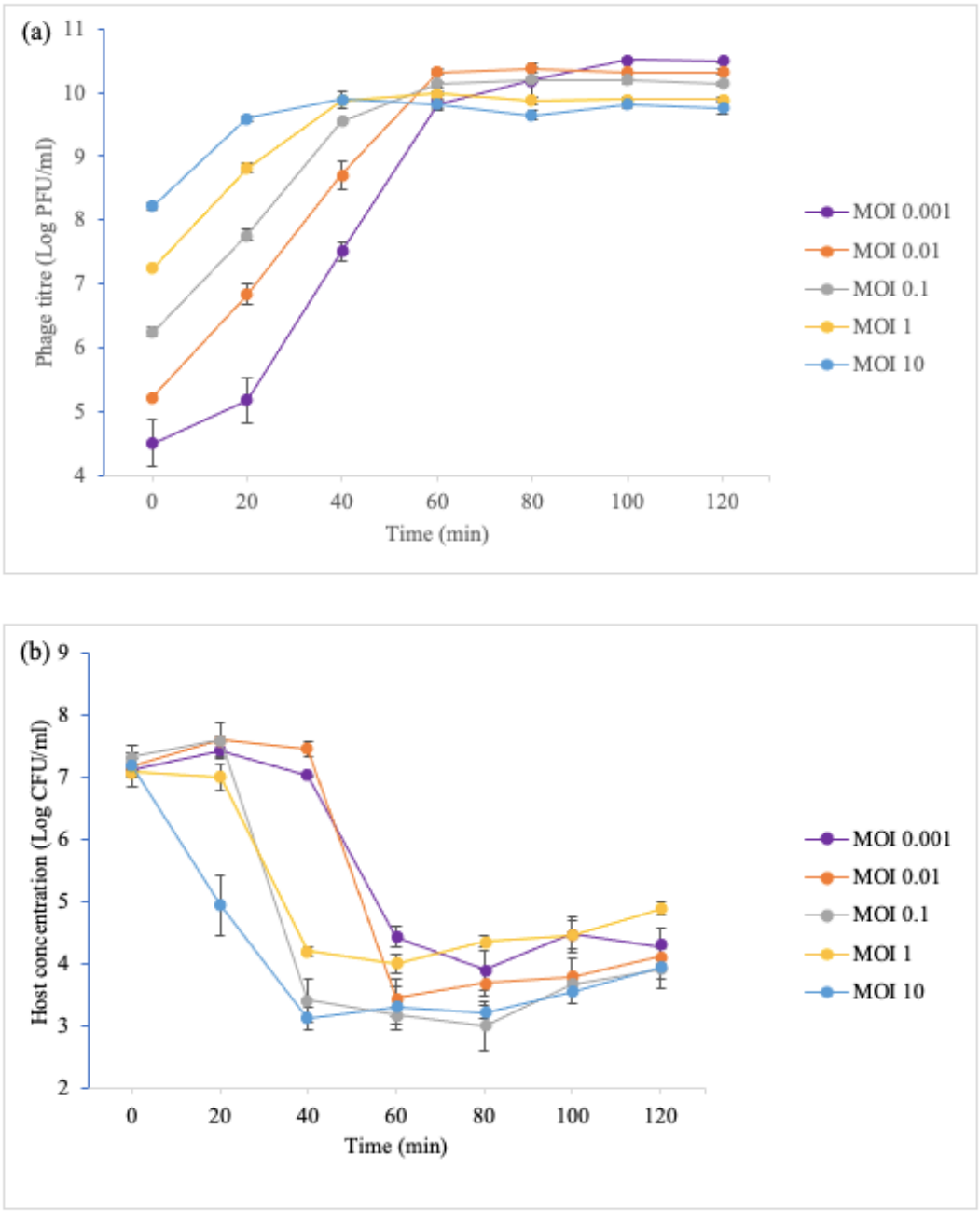

Figure 5. Phage MOI study at ratios of 0.001, 0.01, 0.1, 1 and 10 on (a) C1 phage titre increment, and (b) C1 host concentration reduction measured every 20 min for 120 min. C1 phage optimal MOI was observed at MOI 0.001. Error bar represents standard error (SE) of the mean of 3 biological replicates.

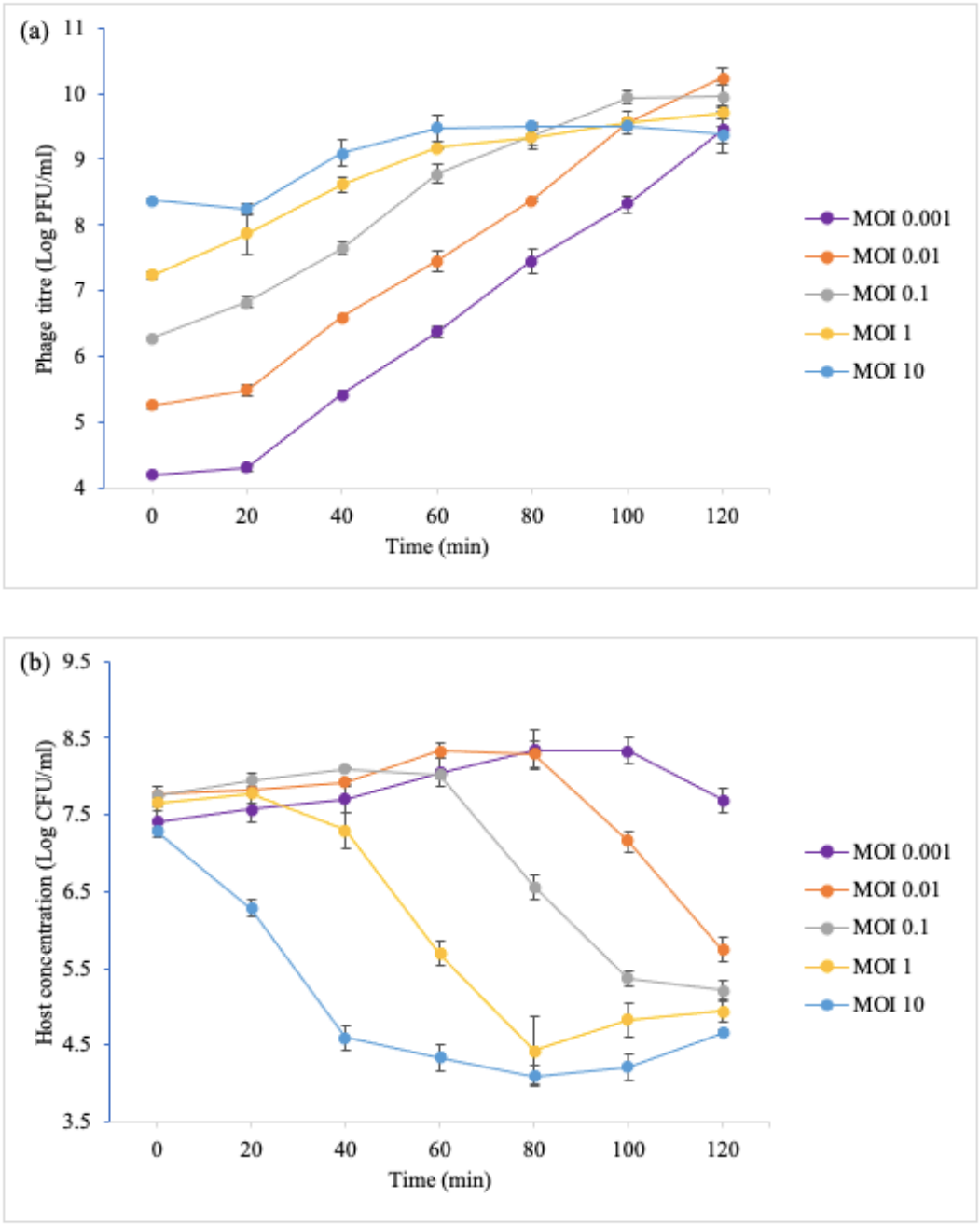

Figure 6. Phage MOI study at ratios of 0.001, 0.01, 0.1, 1 and 10 on (a) C2 phage titre increment, and (b) C2 host concentration reduction measured every 20 min for 120 min. C2 phage optimal MOI was observed at MOI 0.1. Error bar represents standard error (SE) of the mean of 3 biological replicates.

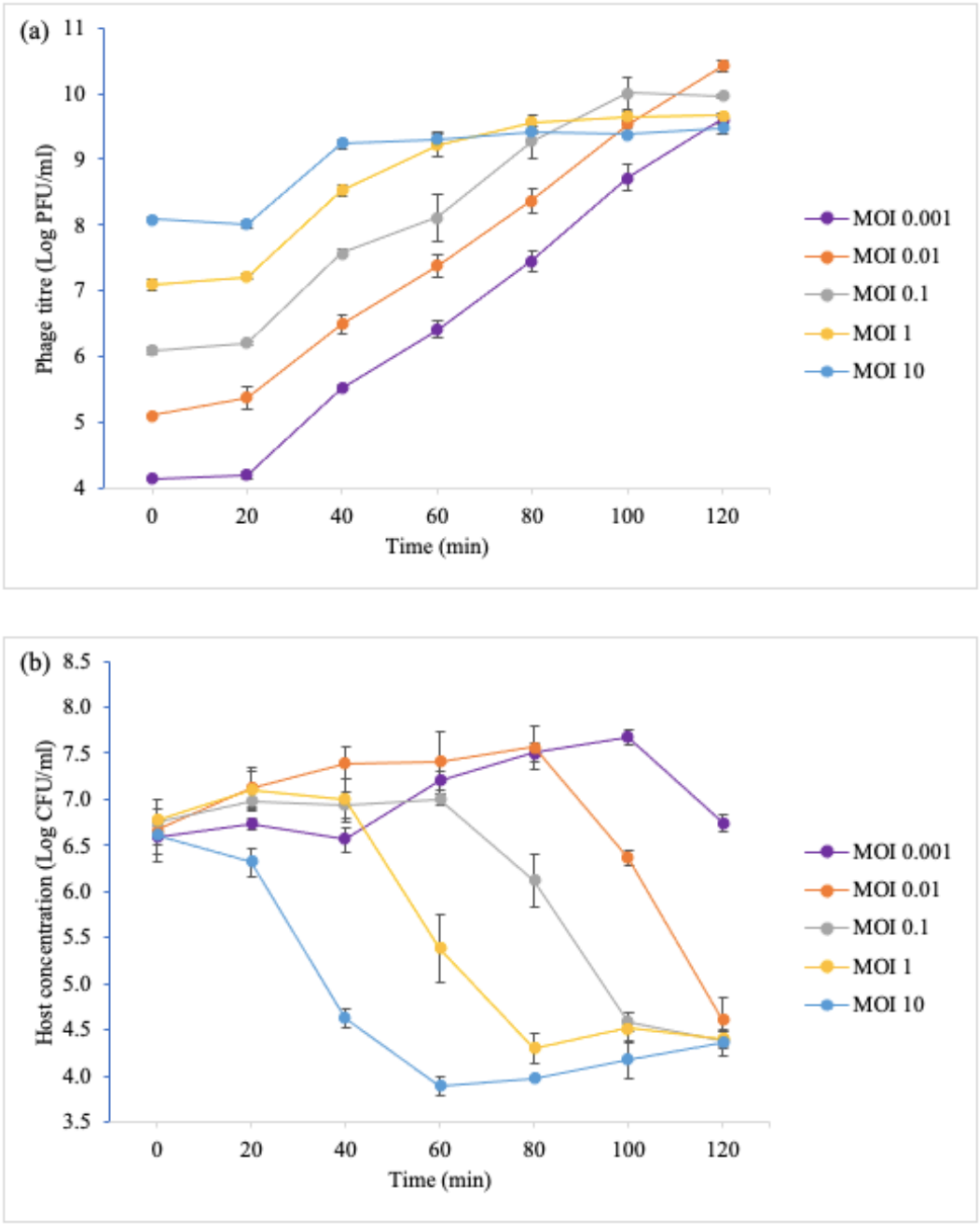

Figure 7. Phage MOI study at ratios of 0.001, 0.01, 0.1, 1 and 10 on (a) C3 phage titre increment, and (b) C3 host concentration reduction measured every 20 min for 120 min. C3 phage optimal MOI was observed at MOI 0.1. Error bar represents standard error (SE) of the mean of 3 biological replicates.

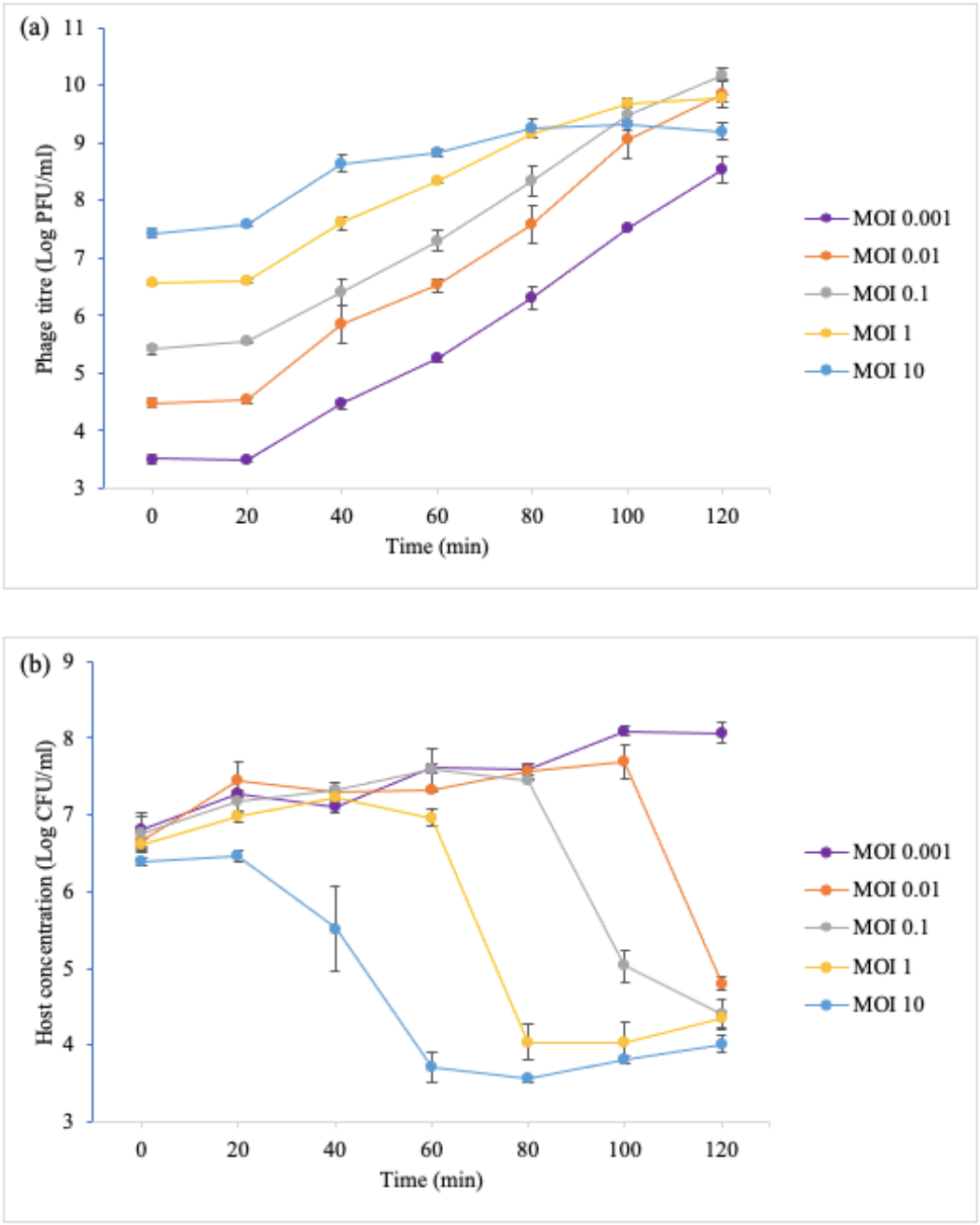

Figure 8. Phage MOI study at ratios of 0.001, 0.01, 0.1, 1, and 10 on (a) C4 phage titre increment, and (b) C4 host concentration reduction measured every 20 min for 120 min. C4 phage optimal MOI was observed at MOI 0.1. Error bar represents standard error (SE) of mean of 3 biological replicate.

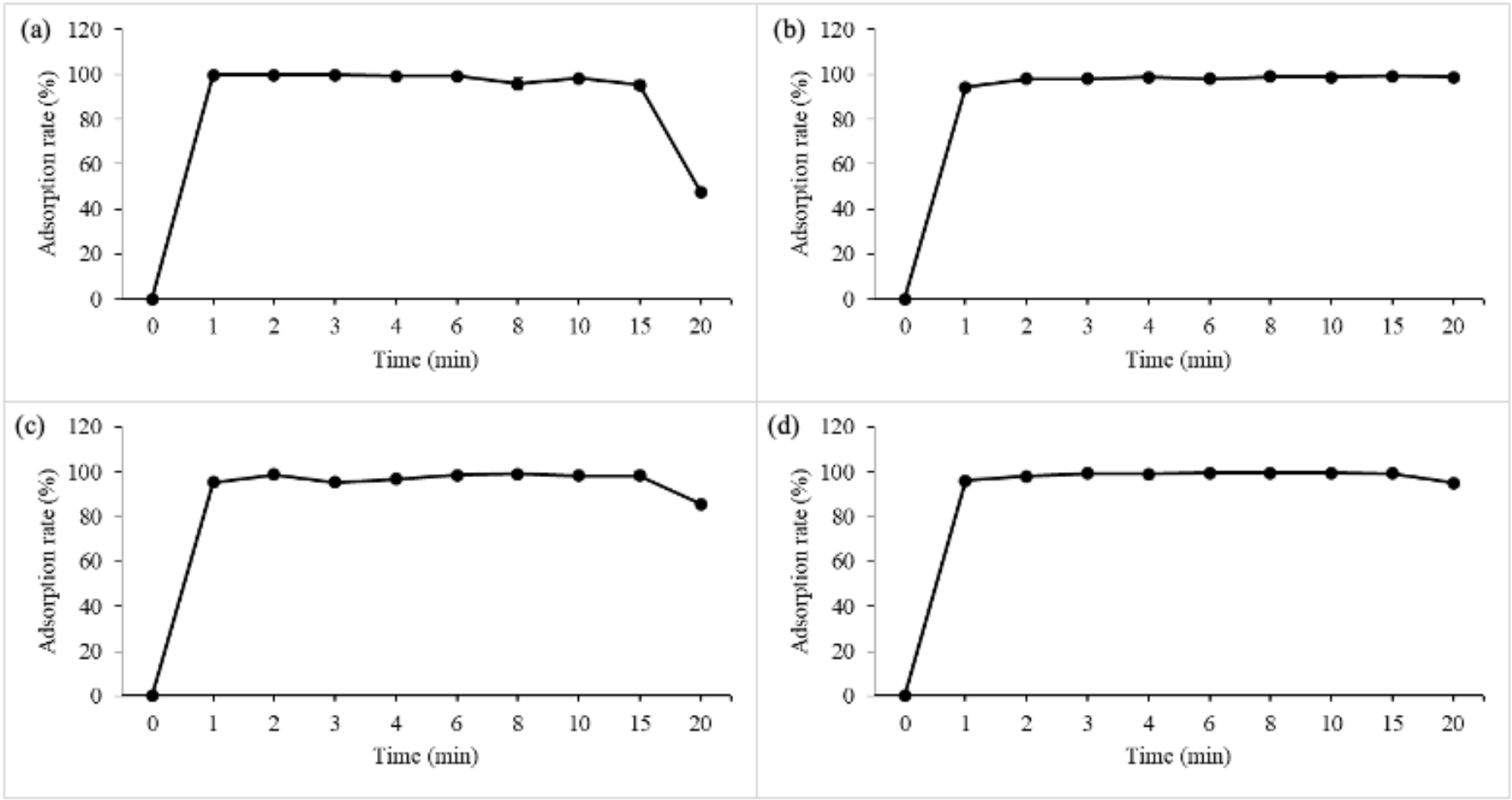

Figure 9. Phage adsorption curves of (a) C1, (b) C2, (c) C3, and (d) C4 phages. Phages were mixed with mid-log phase hosts at MOI 0.001 and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). Phage lysates were centrifuged, and supernatants were titred for 1, 2, 3, 4, 6, 8, 10, 15 and 20 min sampling. C1 phage had the fastest adsorption rate at 1 min (99.65%), compared to 2 min for C2 (98.02%), C3 (98.72%) and C4 (98.17%) phages. Error bar represents standard error (SE) of the mean of 3 biological replicates.

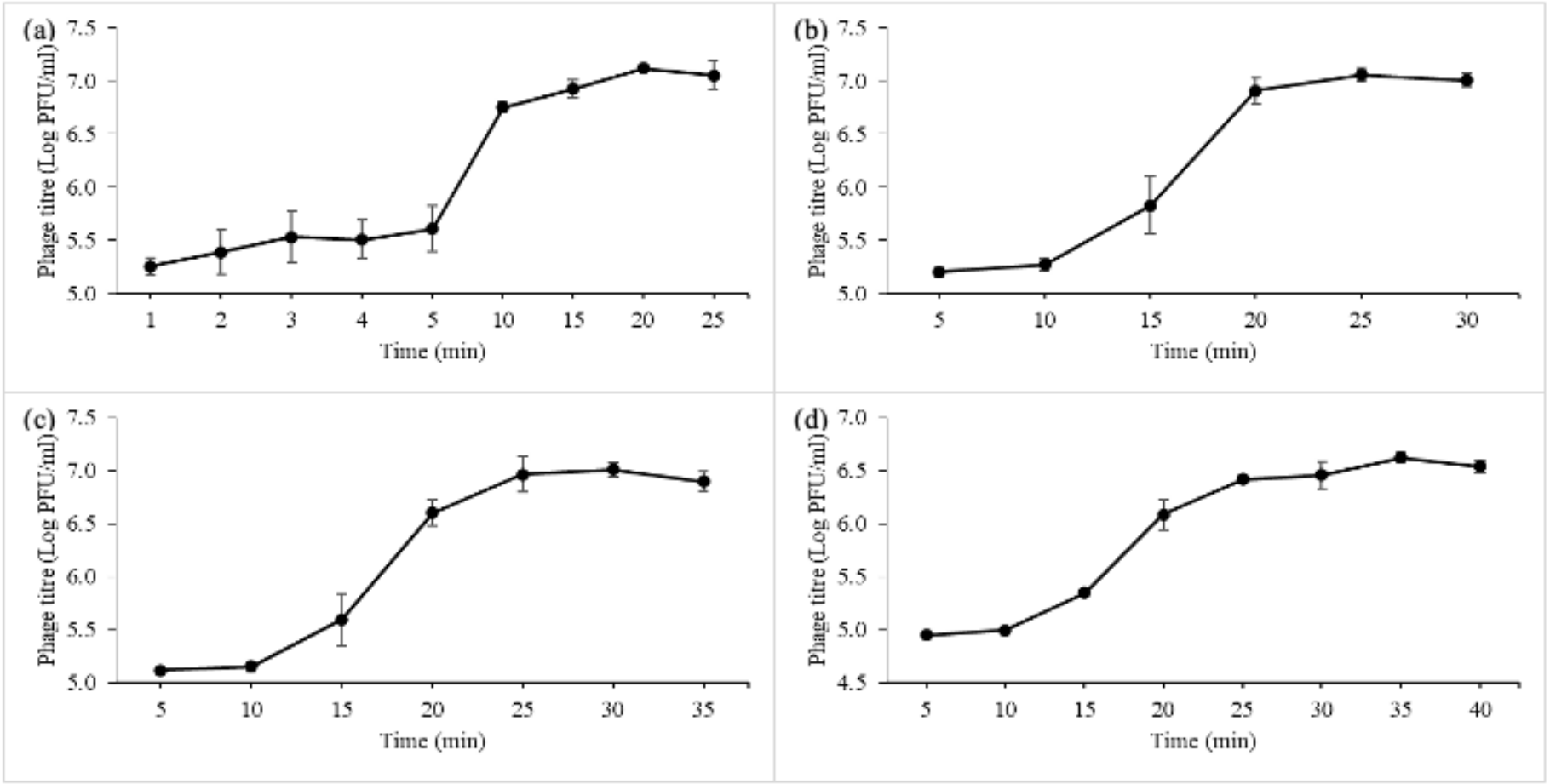

Figure 10. Phage single-step growth curves of (a) C1, (b) C2, (c) C3, and (d) C4 phages. Phages that adsorbed to the mid-log phase hosts at MOI 0.001 were incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). Phages were titred at various intervals depending from pilot experiments. Latent period-burst sizes determined were C1 (5 min-72 PFU/infected cell), C2 (10 min-70), C3 (10 min-73) and C4 (10 min-43). Error bar represents standard error (SE) of the mean of 3 biological replicates.

Phage adsorption rate

C1 phage was optimally adsorbed to its host within 1 min (99.65%), while C2 (98.02%), C3 (98.72%), and C4 (98.17%) phages were within 2 min (Figure 9). The adsorption of all phages started to drop after 15 min.

Phage single-step growth curve

C1 phage had the shortest latent period at 5 min compared to the C2, C3 and C4 phages at 10 min (Figure 10). Interestingly, C1 phage also had one of the highest burst sizes at 72 PFU/infected cell. On the other hand, the C2, C3 and C4 phages had the burst size of 70, 73 and 43 PFU/infected cell, respectively. The time taken for C1, C2, C3 and C4 phages to complete one lytic cycle was 20, 25, 30 and 35 min, respectively.

Effect of pH on phage-host interaction

The effect of pH on phage-host interactions was analysed at different pHs of 3, 5, 7 and 9 within 120 min incubation. Based on Figure 11-14, optimum phage activity was recorded at pH 7 for all phages, where the highest phage increment and the lowest host reduction were observed. At pH 9, C1 phage lytic activity could still be observed, but it was reduced significantly at pH 5 (Figure 11). However, C2, C3 and C4 phage lytic activities were reduced at pH 5 and 9, where the hosts increased compared to their initial concentration (Figure 12-14). All phages were nullified at pH 3.

Effect of temperature on phage-host interaction

The effect of temperature on phage-host interactions was studied at 25°C, 37°C, 42°C, and 60°C and incubated for 120 min. Based on Figure 15-18, the optimal lytic activity of all phages was observed at 37°C. For the C1 phage, lytic activity was slightly decreased at 42°C, followed by at 25°C (Figure 15). A different trend can be seen for C2, C3 and C4 phages, where phage lytic activity was slightly decreased at 25°C, followed by the lowest recorded at 42°C (Figure 16-18). There was no lytic activity observed for all phages at 60°C.

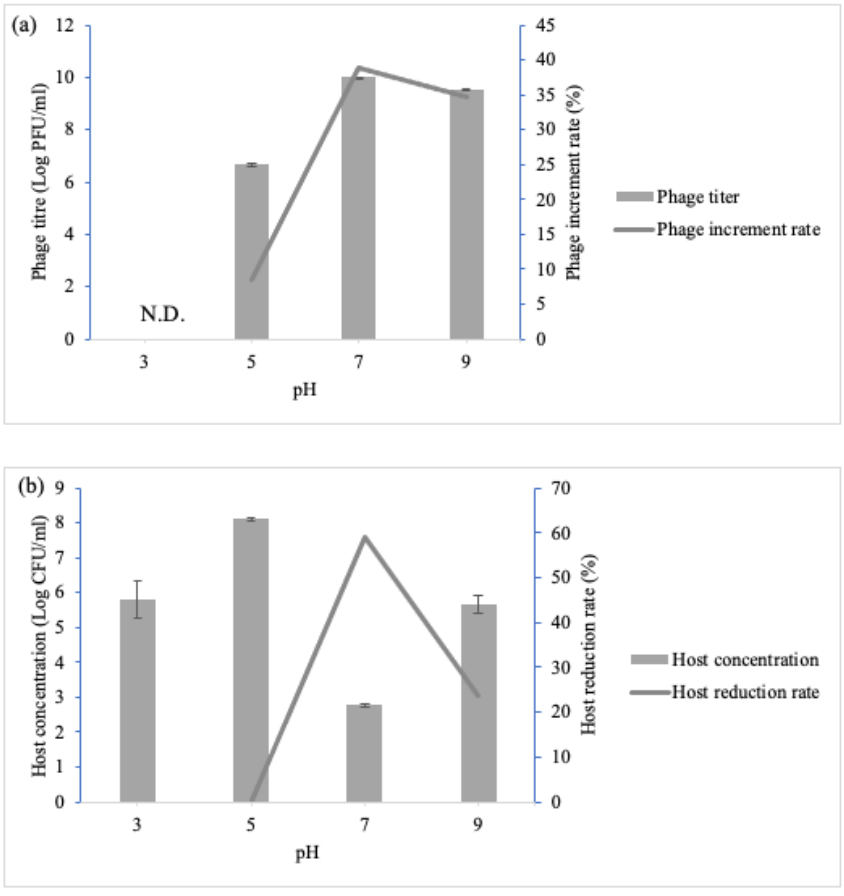

Figure 11. Effect of pH on C1 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth of pH 3, 5, 7 and 9, and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). Phages and hosts were quantified after 1 h. C1 phage optimal pH was observed at pH 7. Error bar represents standard error (SE) of the mean of 3 biological replicates.
N.D. = none detected.

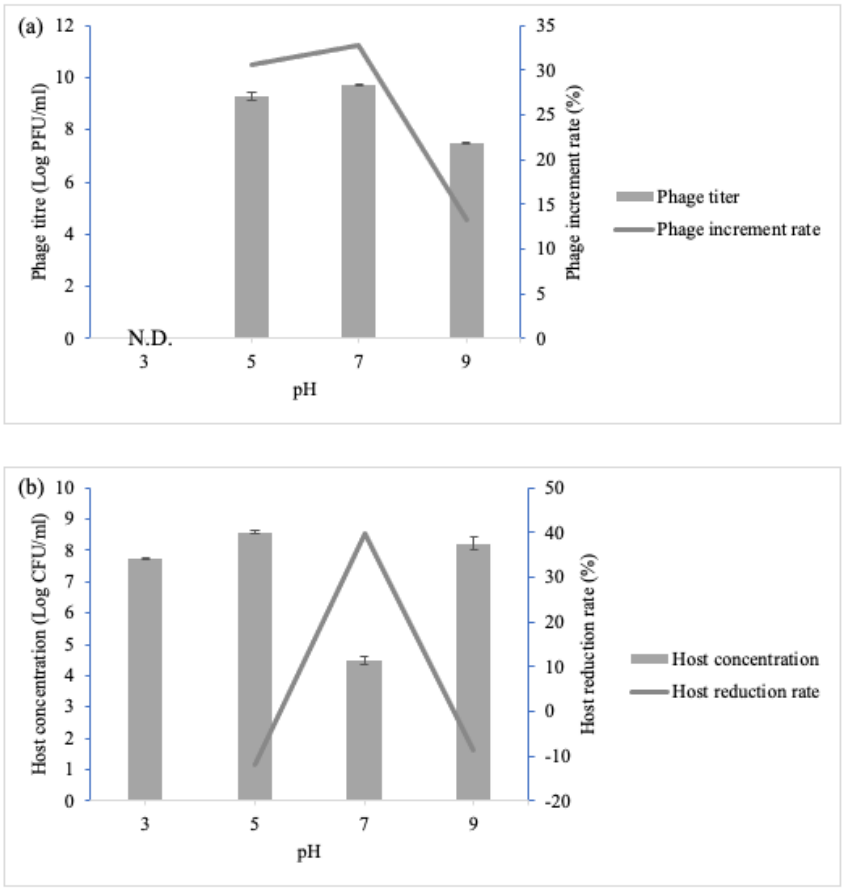

Figure 12. Effect of pH on C2 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth of pH 3, 5, 7 and 9, and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). Phages and hosts were quantified after 2 h. C2 phage optimal pH was observed at pH 7. Error bar represents standard error (SE) of the mean of 3 biological replicates.
N.D. = none detected.

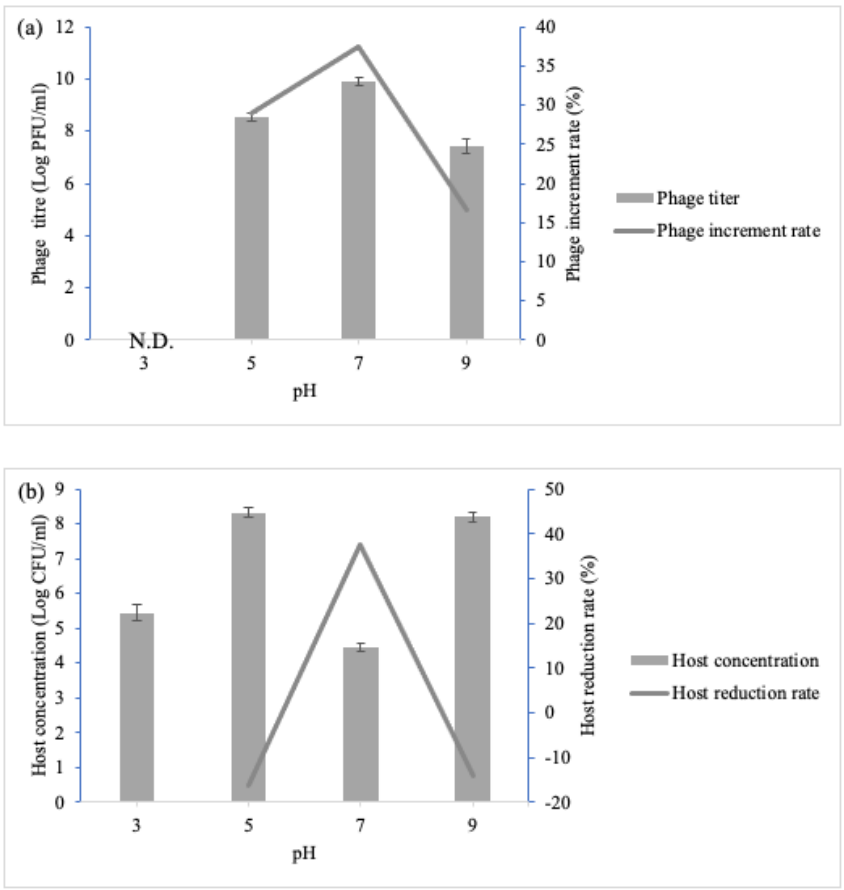

Figure 13. Effect of pH on C3 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth of pH 3, 5, 7 and 9, and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). Phages and hosts were quantified after 2 h. C3 phage optimal pH was observed at pH 7. Error bar represents standard error (SE) of the mean of 3 biological replicates.
N.D. = none detected.

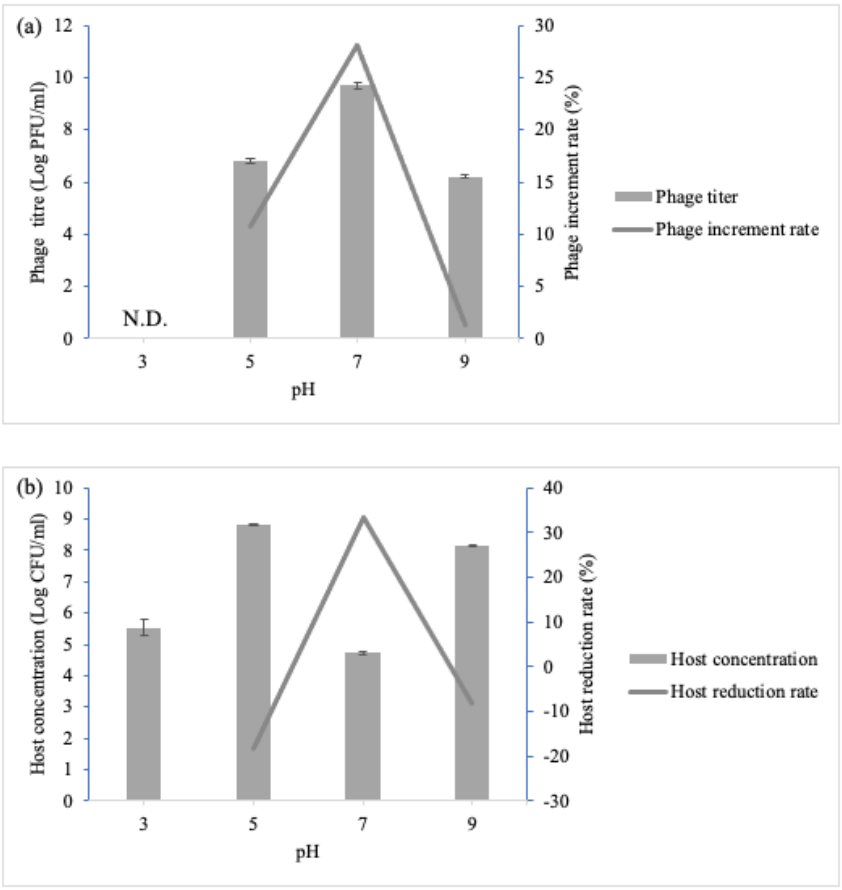

Figure 14. Effect of pH on C4 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth of pH 3, 5, 7 and 9, and incubated in an orbital shaker at 37°C with continuous shaking (180 rpm). Phages and hosts were quantified after 2 h. C4 phage optimal pH was observed at pH 7. Error bar represents standard error (SE) of the mean of 3 biological replicates.
N.D. = none detected.

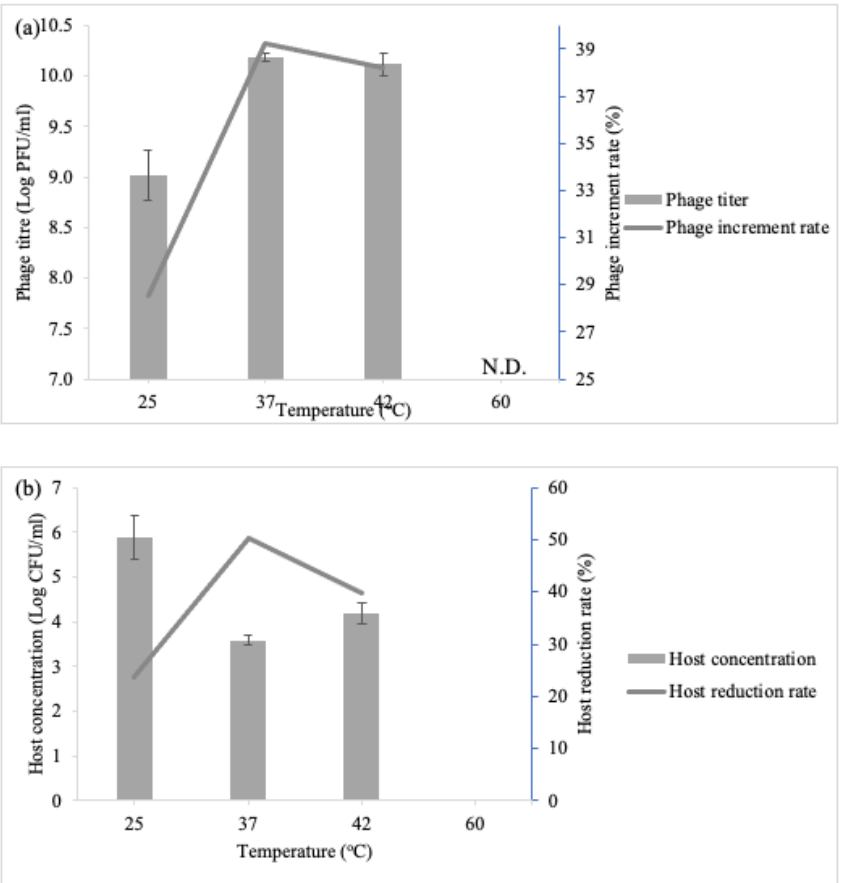

Figure 15. Effect of temperature on C1 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth and incubated in orbital shaker at temperatures of 25°C, 37°C, 42°C, and 60°C with continuous shaking (180 rpm). Phages and hosts were quantified after 1 h. C1 phage optimal temperature was observed at 37°C. Error bar represents standard error (SE) of mean of 3 biological replicates.
N.D. = none detected.

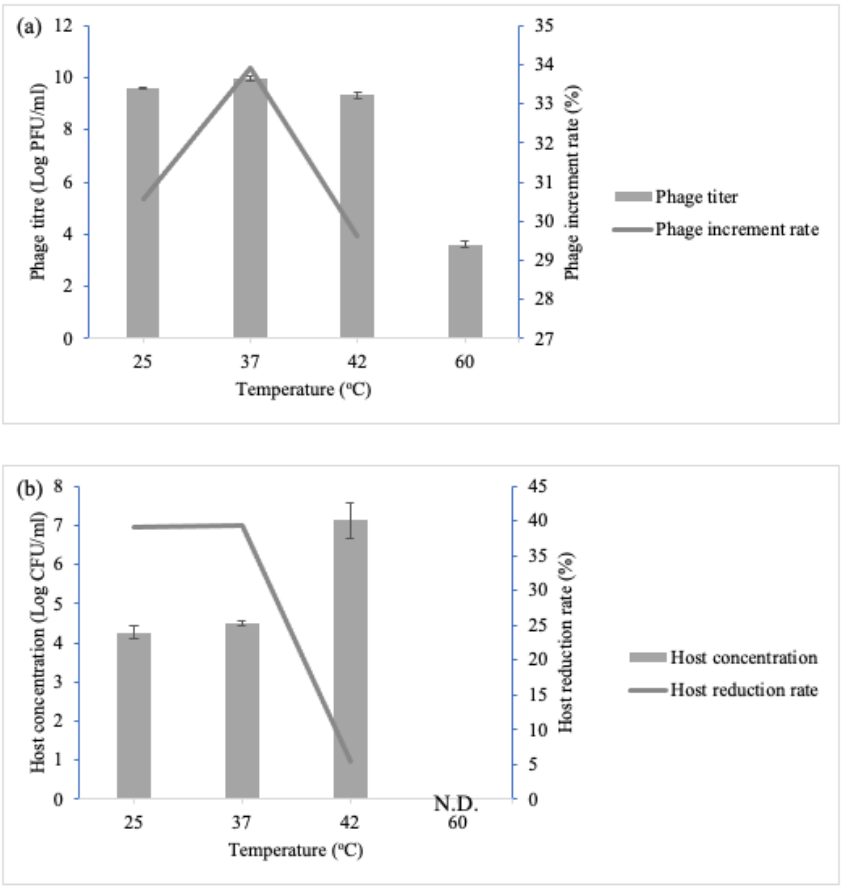

Figure 16. Effect of temperature on C2 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth and incubated in orbital shaker at temperatures of 25°C, 37°C, 42°C, and 60°C with continuous shaking (180 rpm). Phages and hosts were quantified after 2 h. C2 phage optimal temperature was observed at 37°C. Error bar represents standard error (SE) of mean of 3 biological replicate.
N.D. = none detected.

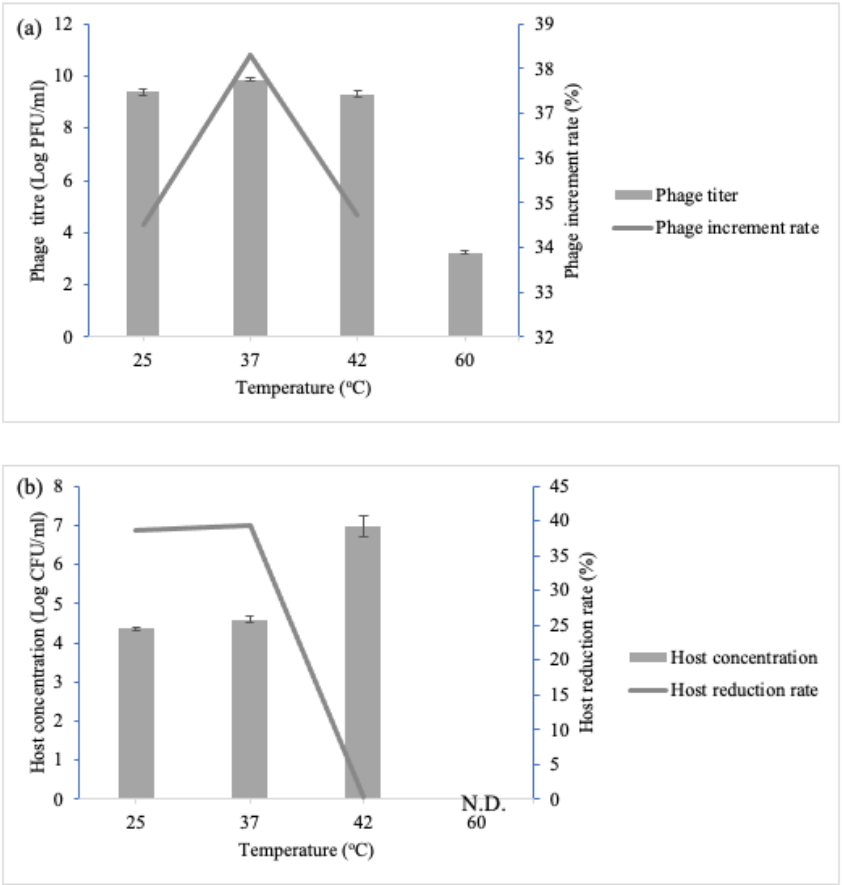

Figure 17. Effect of temperature on C3 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth and incubated in orbital shaker at temperatures of 25°C, 37°C, 42°C, and 60°C with continuous shaking (180 rpm). Phages and hosts were quantified after 2 h. C3 phage optimal temperature was observed at 37°C. Error bar represents standard error (SE) of mean of 3 biological replicate.
N.D. = none detected.

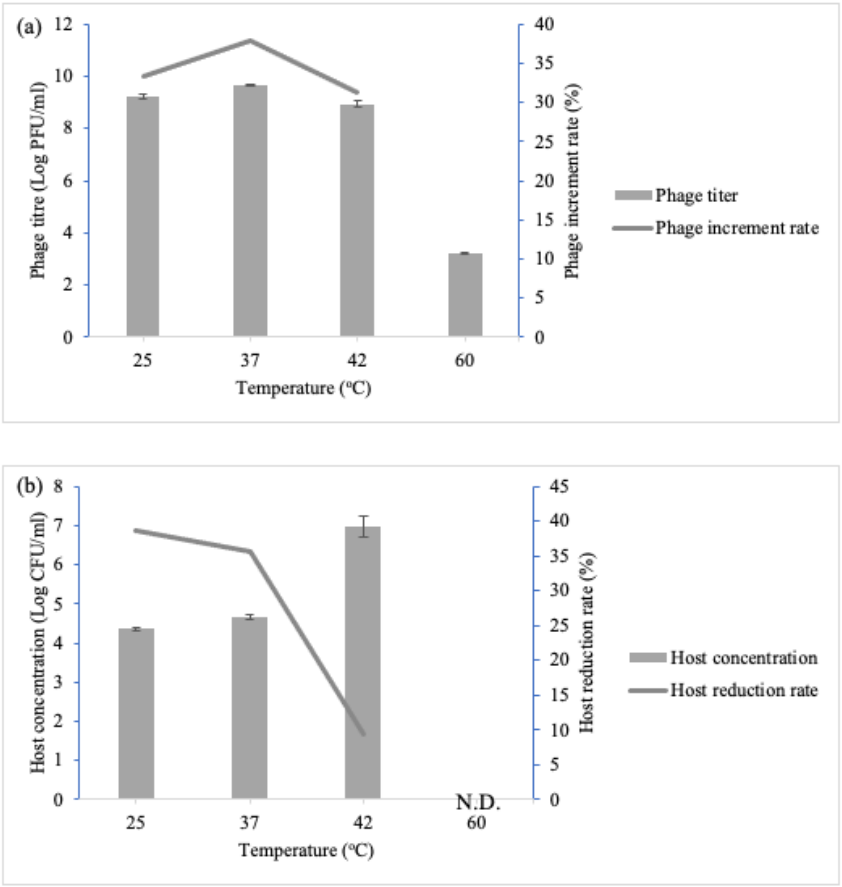

Figure 18. Effect of temperature on C4 (a) phages titre and (b) hosts concentration. Phages and mid-log phase hosts were mixed at MOI 0.1 in LB broth and incubated in orbital shaker at temperatures of 25°C, 37°C, 42°C, and 60°C with continuous shaking (180 rpm). Phages and hosts were quantified after 2 h. C4 phage optimal temperature was observed at 37°C. Error bar represents standard error (SE) of mean of 3 biological replicate.
N.D. = none detected.

DISCUSSION

Phage characterisation is crucial in determining phage lytic activity and properties to achieve optimal phage infection in the *in vivo* study (Skurnik & Strauch, 2006). This study showed that C1, C2, C3, and C4 *Escherichia coli* phages have good properties to be implemented in gut modulation studies. Desirable phage properties for therapeutic studies, such as tailed phages, fast adsorption rate, short latent period and large burst size, were demonstrated by all phages. However, a strategy must be implemented to ensure phage application is effective in harsh gut environments (e.g., low pH; high temperature). Although C2, C3, and C4 phages can co-infect each other, they only showed the highest lytic activity in their originally isolated host. Nevertheless, all phages were considered distinctive based on their morphology, molecular characterisations and physiological properties.

Phage morphology study based on a transmission electron microscope (TEM) is one of the most basic but essential techniques routinely used to characterise newly isolated phage (Ackermann, 2009). The phage morphological properties (e.g., type of capsid; tail length) can be used to classify phage to its family level (Ackermann, 2004). In the current study, the phage morphology study revealed that the C1 phage (short-tail) was distinctively different from those of the C2, C3 and C4 phages (long-tail). C1 phage was classified under the *Podoviridae* family, while C2, C3, and C4 phages were under the *Siphoviridae* family (Ackermann, 2004). *Podoviridae* and *Siphoviridae* are from a group of tailed phages of the *Caudovirales* order. The *Caudovirales* are among the most commonly classified lytic phages that account for more than 90% of all phages isolated (Ackermann, 2004; Ackermann, 2007; Ackermann, 2009). These two families are closely related, and the only difference is in the tail length, which could be due to the presence or absence of a molecular ruler controlling the tail length (Katsura & Hendrix, 1984; Katsura, 1987; Ackermann, 2004). *Podoviridae* (C1 phage) and *Siphoviridae* (C2, C3, and C4 phages) phages'

nucleic acids are classified as linear double-stranded deoxyribonucleic acid (dsDNA) (Ackermann, 2004; Ackermann, 2009). It is also noted that each phage had a significantly ($P < 0.05$) different tail length, suggesting their uniqueness.

The study of phage multiplicity of infection (MOI) is vital to determine an adequate ratio of phage-host replication, as each phage multiplies effectively at a different ratio from the host (Ly-Chatain 2014). C1 phage had an optimal MOI of 0.001, followed by 0.1 for C2, C3 and C4 phages. From the trend of the MOI of all phages, the lowest MOI (e.g., 0.001) tended to generate a higher number of phages compared to the highest MOI (e.g., 10) studied. The increment of phage was the lowest in MOI 10. At high MOI (e.g., MOI 10), the phage replication was low and eventually reached the plateau stage, indicating no new phages were replicated. This event occurred when phages were present at a high concentration while hosts were at a low concentration. As a result, multiple phages may infect the same host, and the lysis of the host will not increase the number of phage progeny, thus limiting phage replication (Kocharunchitt *et al.,* 2009; Shen *et al.,* 2012; Vieira *et al.,* 2012). This event is called "lysis from without", where no more new phage progeny is released (Delbrück, 1940; Delbrück, 1942; Abedon, 2011). Using too low MOI is also not recommended due to various factors that might reduce phage titre in the *in vivo* environment. Therefore, it is recommended that *in vivo* dosage study be performed.

Adsorption rate and single-step growth curve experiments highlighted the physiological information of infectivity processes of the phage lytic cycle. In this study, the MOI ratio was set at the lowest level (MOI = 0.001), where a shorter lytic cycle period can be expected due to the high concentration of bacterial host used (Wang, 2006; Hyman & Abedon, 2009). Adsorption is the first step of the phage life cycle, where phages attach to the receptor of their target host. A study by Shao and Wang (2008) suggested that a faster adsorption rate would lead to a shorter lytic cycle. The current study showed that all phages had a very fast adsorption rate, where C1 had the fastest adsorption rate of 1 min (99.7%), compared to C2 (98%), C3 (98.7%) and C4 (98.2%) phages which were within 2 min. This was reflected by their shorter lytic cycles of 20, 25, 30 and 35 min for C1, C2, C3 and C4 phages, respectively.

The single-step growth curve study highlighted the physiological information of one complete phage lytic cycle, where both latent period (period of phage progeny assembly before lysis) and burst size (number of phage progeny release for each lysis) can be determined (Wang *et al.,* 2000). Although large burst sizes are commonly associated with a long latent period (Abedon *et al.,* 2001; Abedon *et al.,* 2003), C1 phage had the best lytic characteristic of both. It had the fastest latent period with one of the highest burst sizes. This, however, was in contrast with the general characteristics of the *Podoviridae* family, which reported a short latent period and low burst size, and vice versa for *Siphoviridae* (Chibani-Chennoufi *et al.,* 2004).

Phage-host interactions are strongly influenced by pH, and the chicken gut environment has a pH range between 2.5 to 7 (Gauthier, 2002). Our study showed that all phages were very sensitive to acidic (pH 3 and 5) and alkaline (pH 9) environments. These suggested that phages will be nullified in an acidic gut environment of chicken (Gauthier, 2002), especially when they are supplemented through an oral administration route. Previous studies highlighted that phage lytic activity generally decreases as the pH decreases, and their replication is halted, especially when the pH is lower than 4.5 (Garcia *et al.,* 2009; Ly-Chatain, 2014). Using an antacid to ensure the phage's survival in the low pH of the gut is one technique that must be implemented to preserve phage lytic activity (Ly-Chatain, 2014).

The body temperatures of chickens range from 40.5°C to 42°C (Fonseca *et al.,* 2016). The survivability of phage within this temperature is required to ensure the efficacy of phage application. In the current study, all phages had optimal lytic activity at 37°C. Although the phage titre for all phages remained high at a high temperature, phage lytic activity (host reduction) was reduced at 42°C compared to 37°C. This observation suggests that phage activity will persist at 42°C but may take longer to reduce targeted hosts. Using an additive such as skim milk might effectively preserve the phage activity at a high temperature, as demonstrated in *Lactococcus* phage (Atamer *et al.,* 2009).

Tailed phage can be discriminated up to a certain level based on host range (species/strain), shape (family), RFLPs (species/strain), number of proteins (species), adsorption rate (species/strain), latent period (species/strain), and burst size (species/strain) (Ackermann, 2009). It was suggested that the significant difference between C1, C2, C3, and C4 phages was due to their family, which is *Podoviridae* and *Siphoviridae*, respectively. The C2, C3, and C4 phages could be closely related as they have the same host range, adsorption rate and latent period. Nevertheless, they were suggested to be distinct due to their difference in RFLPs and SDS-PAGE profiling. Physiological characterisations also supported the difference between each phage based on variations in burst size and period of the lytic cycle. Therefore, it was suggested that C2, C3, and C4 phages could be different. The discrimination of closely related phages might be difficult based on the current method. In this case, whole genome sequencing is a better approach but costly. A previous study has also encountered the same challenge on differentiating closely related phages (similar in terms of host range, morphology, SDS-PAGE protein profiles and genome sequences), in which whole genome sequencing even showed about 99% similarities between the phages (Fouts *et al.,* 2013). This showed that phages can still be considered different based on factors that need to be considered.

CONCLUSION

The findings of this study suggested that C1, C2, C3, and C4 phages had desirable lytic properties, where all phages were suitable for gut modulation study. These phages were also distinctively different, but C2, C3, and C4 phages could be closely related. On the other hand, a strategy such as preserving phage activity from harsh acidic and high-temperature environments must be implemented to maintain phage viability and lytic activity in the chicken gut.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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