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Original Article

Biosafety and cellular cytocompatibility assessment of endotoxin-reduced *Flavobacterium psychrophilum* bacteriophages

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Abstract
Flavobacterium psychrophilum causes bacterial cold-water disease in salmonids, leading to substantial losses in juvenile rainbow trout aquaculture. Although bacteriophages represent promising antibiotic alternatives, biosafety concerns persist due to endotoxin contamination originating from Gram-negative hosts. In this study, five lytic phages (FpV30–FpV34) were isolated from trout hatchery effluent using *F. psychrophilum* ATCC 49418 and characterized by one-step growth, adsorption assays, host range analysis, and transmission electron microscopy (TEM). Phages exhibited adsorption constants between 1.21×10^{-9} and 1.58×10^{-6} mL·min^{-1}, latent periods of 3–6.5 h, and burst sizes of 11–34.5 particles per cell. Spot tests demonstrated high specificity toward *F. psychrophilum*, with negligible activity against non-target bacteria. TEM revealed icosahedral capsids (≈75–83 nm) and long tails (≈129–142 nm), consistent with Myoviridae-like morphology. Crude lysates were purified by PEG/NaCl precipitation followed by dialysis, resulting in a ~40-fold increase in phage titer (from 1×10^{8} to 4×10^{9} PFU mL^{-1}) and a 99.8% reduction in endotoxin levels (from 1.2×10^{3} to 2.3 EU mL^{-1}), confirmed by chromogenic LAL assay with interference control. Cellular cytocompatibility assessed in MCF-7 cells revealed that crude preparations reduced viability to 58% at 48 h, whereas endotoxin-reduced phages maintained >90% viability. These findings demonstrate that PEG/NaCl-based purification enables high-titer, low-endotoxin *F. psychrophilum* phage preparations with favorable cytocompatibility, supporting their biosafe application in aquaculture.

Keywords: bacteriophage therapy; cytocompatibility; endotoxin reduction; *Flavobacterium psychrophilum*; MTT

1 | INTRODUCTION

Bacterial diseases in aquaculture significantly affect fish health and directly impact the economic performance of farming operations (Donati 2021). In particular, pathogens such as *Flavobacterium psychrophilum* cause an important disease known as Bacterial Cold-Water Disease in juvenile trout, which is associated with high mortality rates (Hoare *et al*. 2017). Some studies have reported that *F. psychrophilum* infections can lead to extremely high losses under certain conditions (Hoare *et al*. 2017).

In combating this pathogen, the increasing antimicrobial resistance and drug residue issues associated with conventional antibiotic treatments have revealed the need for alternative therapeutic strategies (Helmy *et al*.

2023). In this context, bacteriophage (phage) therapy, which involves viruses that specifically target and eliminate bacteria, is considered a sustainable control method in aquaculture (Ustundag and Ustundag 2026). In recent years, the use of phages against fish pathogens has increased, and effective phage cocktails have been developed against various Vibrio, Aeromonas, Pseudomonas, and Flavobacterium species (Donati 2021).

On the other hand, while applied phages eliminate their specific host bacteria, their effects on aquatic ecosystems and, indirectly, on human health are not yet fully understood. This uncertainty renders the sustainability of phage applications a subject of debate.

The One Health approach in aquaculture evaluates fish, human, and ecosystem health as an inseparable whole and aims to develop holistic and sustainable solution strategies for controlling zoonotic diseases caused by aquatic pathogens such as *Vibrio* spp., *Aeromonas* spp., and *Mycobacterium* spp., preventing antibiotic resistance development, ensuring environmental sustainability, promoting alternative biological control methods, and safeguarding food safety (Ansari and Nagar 2024). The use of phages in aquaculture should also be considered within this perspective. In aquaculture facilities where phage applications are implemented, phage preparations may come into contact with farm workers or spread through the aquatic ecosystem. Therefore, phages intended for use in aquaculture must be evaluated not only in terms of pathogen control but also with respect to biosafety implications related to human health (Aly and Fathi 2024).

Accordingly, phage preparations intended for aquaculture should be assessed not only for antibacterial efficacy but also, in the context of potential human exposure, through biocompatibility screening in well-established and commonly used human cell lines. At this point, a critical issue is distinguishing between the biocompatibility of the phage particle itself and the potential effects of bacterial residues carried over during the production process. Phage preparations propagated in Gram-negative host bacteria may be contaminated with endotoxins such as lipopolysaccharide (LPS), which can induce strong biological responses in mammalian cells (Henry *et al*. 2015).

It is generally accepted that phages cannot infect cells other than their specific host bacteria (Clokie *et al*. 2011). Based on this, phages used to control fish pathogens are not expected to cause direct harm to human cells (Van Belleghem *et al*. 2019). Indeed, the literature reports that phage particles and capsid proteins used in aquaculture are generally non-toxic to mammalian cells (Romero *et al*. 2012). However, a key limitation often overlooked is that in cell culture experiments conducted with crude phage lysates or insufficiently purified phage preparations, the observed biological responses may be driven not by the phage particle itself but by contaminants such as lipopolysaccharide (LPS), bacterial membrane fragments, or residual proteins/DNA. This complex interaction may lead to incorrect conclusions suggesting that phages are toxic in mammalian cell safety tests, thereby obscuring the true biocompatibility profile of phages (Melo *et al*. 2019).

Therefore, in aquaculture phage studies, safety assessments should not be reduced solely to cell viability outcomes; instead, they must be conducted within an experimental design that verifies phage preparation purity and endotoxin load, which constitutes a mandatory standard for scientific reliability. Studies emphasize that safety concerns represent one of the major barriers to large-scale phage application and that the content and purity standardization of phage preparations constitute a critical obstacle even in non-clinical fields such as aquaculture (Cooper *et al*. 2016). In this context, comparing crude and purified phage preparations used in aquaculture and distinguishing whether the observed cellular effects are attributable to the phage particle itself or to endotoxin/residual load is crucial for the sustainable use and biosafety of phages.

In the present study, phages isolated from *F. psychrophilum* were evaluated for safety in a human cell line. The MCF-7 cell line, which is standardized, comparable, suitable for biocompatibility screening, sensitive to cellular stress/cytotoxic responses, and widely accepted in cytotoxicity and biological agent safety studies, was selected as the experimental model (Soule *et al*. 1973). Although fish-derived cell lines are commonly used in aquaculture research, MCF-7 cells were deliberately selected to address human-relevant biosafety under the One Health framework, as phage applications may lead to indirect human exposure via handling, environmental release, or consumption of treated fish. Moreover, MCF-7 cells are highly sensitive to endotoxin-mediated stress, enabling discrimination between intrinsic phage biocompatibility and lipopolysaccharide-related effects. The study investigated whether *F. psychrophilum* phages induce cytotoxicity in MCF-7 cells, thereby assessing their potential use as safe biological agents in aquaculture. Furthermore, although endotoxin/LPS contamination is a critical confounding factor in interpreting mammalian cell responses to aquaculture phage preparations, studies systematically disentangling this effect are limited. This study aims to provide a standardized evaluation framework for safe phage formulation by distinguishing the biocompatibility of the phage particle from endotoxin-related effects.

2 | METHODOLOGY

2.1 Bacterial culture

In this study, *F. psychrophilum* reference strain ATCC 49418 was used. The bacterial culture was incubated at 15°C for 5–7 days. At the end of the incubation period,

the culture was revived and prepared for phage isolation (Castillo *et al*. 2021).

2.2 Phage isolation and purification

Water used for bacteriophage isolation was collected from the effluent of trout hatcheries located in the region. Each water sample was transferred into 15 mL Falcon tubes and centrifuged at 10,000 × g for 10 min. The pellet containing solid debris was discarded and the supernatant was collected. The supernatant was then slowly filtered through a 0.22 µm membrane filter. For each sample, 30 mL of the 0.22 µm–filtered water was mixed with 5 mL of bacterial culture and 15 mL of the selective medium used for bacterial isolation, and incubated at 15°C for 24–48 h. After incubation, chloroform (50 µL mL^{-1}) was added to the supernatant. The supernatant was subsequently collected and passed through a 0.22 µm filter. The presence of phages in the supernatant was determined using the double-layer agar plaque method (Molina *et al*. 2020). Exponentially growing (18–24 h) bacterial cultures were mixed with bacteriophages and kept at 15°C for 30 min. After incubation, the mixture was combined with molten top agar (0.4%) at 48–50°C, immediately poured onto pre-prepared plates, and incubated at the optimum temperature for the bacterium for 24–48 h (Stenholm *et al.* 2008).

For purification of phages showing lytic activity, the single-plaque isolation method was used. Serial dilutions of phage suspensions were prepared in SM buffer (50 mM Tris-Cl, pH 7.5; 99 mM NaCl; 8 mM $MgSO_4$; 0.01% gelatin) up to 10^{-8} to dilute the phage titers in the stock preparations. The diluted phage suspensions were mixed with exponentially growing (18–24 h) pathogens and incubated for 30 min at the optimum temperature specific to the bacterium. After incubation, the mixture was combined with molten top agar (0.4%) at 48–50°C, immediately poured onto pre-prepared plates, and incubated at the optimum temperature for 24–48 h. A single, well-isolated plaque was picked using a sterile Pasteur pipette, transferred into SM buffer, and mixed for at least 2 h. Following chloroform extraction (32 µL mL^{-1}) and centrifugation (9,000 × g, 20 min), the filtrate was transferred into a sterile tube. This procedure was repeated at least three times (Stenholm *et al*. 2008). After lysis, the phage-containing supernatant was filtered through a 0.22 µm membrane filter to remove bacterial cells, yielding a crude phage lysate (Bruttin and Brüssow 2005).

2.3 Phage titration (PFU mL^{-1} determination)

Phage titers were determined by calculating the number of bacteriophages present per milliliter of sample, and results were expressed as plaque-forming units per milliliter (PFU mL^{-1}). Serial dilutions (10^{-1} to 10^{-8}) of the phage stock in liquid culture were prepared using SM buffer. From each dilution, 300 µL of phage suspension was mixed with 1,000 µL of bacterial culture, then combined with 4,000 µL of molten top agar (0.4%) at 48–50°C, poured onto plates, and incubated at 15°C for 3–5 days. After incubation, phage titers were determined by counting plaques on the plates (Donati *et al*. 2022).

2.4 Determination of multiplicity of infection

To determine the multiplicity of infection (MOI), a fresh 24 h culture in the exponential phase (approximately 10^8 CFU mL^{-1}) was transferred into Eppendorf tubes. Diluted phage lysates were added and mixed. After allowing phage adsorption for 15 min, the tubes were centrifuged at 5,000 × g for 10 min and the supernatants were removed. The pellet was resuspended in Anacker and Ordal medium and incubated at 15°C. At the end of this period, the bacteriophage count was determined (Hyman and Abedon 2009).

MOI = (phage titer (PFU)) / (bacterial count (CFU))

2.5 Determination of kinetic parameters of bacteriophages

2.5.1 Determination of latent period and burst size

A total of 500 µL of phage suspension was mixed with 2,000 µL of exponentially growing *F. psychrophilum* and incubated at 15°C for 10 min. The mixture was centrifuged at 10,000 × g for 10 min. After centrifugation, the cells were resuspended in 2,000 µL of cold Anacker and Ordal medium. Thereafter, samples were taken every 30 min for 8 h and plated using the double-layer agar method, followed by incubation at 15°C. After incubation, plaques were counted and recorded (Stenholm *et al*. 2008). The latent period was determined based on the time interval during which the phage titer remained constant as measured by the double-layer agar method. Burst size was calculated using the following equation (Hyman and Abedon 2009):

Burst size = (highest phage titer (PFU mL^{-1})) / (phage titer during latent period (PFU mL^{-1}))

2.5.2 Phage adsorption rate and adsorption constant

To determine the adsorption rate of bacteriophages to host cells, bacteriophages were mixed with 20 mL of exponentially growing *F. psychrophilum* and incubated at 15°C. During incubation, samples were collected every 5 min for 30 min, diluted 1:10 in SM buffer containing chloroform (50 µL mL^{-1}), thoroughly mixed, and centrifuged (5,000 × g, 3 min). The samples were then rapidly plated using the double-layer agar method and incubated at 15°C. After incubation, plaques were counted and recorded (Lallo *et al*. 2014).

The phage titer in the supernatant containing non-adsorbed phages was determined and percent adsorption was calculated using the following formula (Moldovan *et al*. 2007):

% adsorption = [(initial phage titer (PFU mL^{-1}) − post-

adsorption phage titer (PFU mL^{-1})) / (phage titer during latent period (PFU mL^{-1}))] × 100

The phage adsorption constant (K) was calculated as:

$K = 2.3/(B \cdot t) \times \log(P_o/P)$;

K = phage adsorption constant; B = bacterial concentration (bacteria per mL; determined as CFU mL^{-1} by plate count); t = time; P_o = PFU at time zero; P = PFU in the supernatant

2.6 Determination of phage host range

To assess whether the phages were specific to *F. psychrophilum*, the following strains were used: *F. psychrophilum* (ATCC 49418), *F. psychrophilum* strains 1–5, *Chryseobacterium chaponense*, *Chryseobacterium* spp., *Enterococcus casseliflavus* (ATCC 700327), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633), *Enterobacter hormaechei* (ATCC 700323), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis*, and *Pseudomonas putida*. Lytic activity of the phages was investigated using the spot-test method. For each group, 500 µL of bacterial culture was added to 4,000 µL of soft agar and spread over the prepoured top agar layer. After the agar dried, 15 µL of each phage was spotted onto the marked areas, and lysis formation was examined (Mutai *et al*. 2022).

2.7 Electron microscopy of bacteriophages

Phage suspensions were mixed at a 1:10 ratio with 2.5% glutaraldehyde buffer and kept at room temperature for 5 min. The phage suspension was then inoculated onto Formvar–carbon-coated copper grids and allowed to stand for 2 min at room temperature. Excess sample was removed using filter paper. The phages were stained with 2% uranyl acetate and kept at room temperature for 2 min. Excess stain was removed with filter paper, and the grids were washed with distilled water. The phages were then visualized by TEM (Stenholm *et al*. 2008).

2.8 Purified phage preparation (PEG/NaCl + dialysis) and endotoxin/LPS control

To minimize bacterial residues, endotoxin/LPS, and medium components that may be present in the crude phage preparation, PEG/NaCl precipitation was applied to the crude lysate. NaCl was added to the crude phage lysate to a final concentration of 1.0 M and mixed until fully dissolved. Subsequently, PEG 8000 was added to obtain a final PEG concentration of 10% (w/v). The mixture was incubated at 4°C for at least 12 h at 50 rpm. It was then centrifuged at 4°C and 10,000 × g for 20–30 min, and the phage-containing pellet was carefully collected. The pellet was gently resuspended in 0.5 mL sterile PBS (pH 7.2–7.4) until fully dissolved to obtain a concentrated phage preparation (Bonilla *et al*. 2016).

To remove residual PEG/NaCl and low-molecular-weight contaminants, the concentrated phage suspension underwent buffer exchange/desalting. Buffer exchange was performed by dialysis against PBS. For this purpose, the concentrated phage suspension was dialyzed against PBS at 4°C using a sterile dialysis membrane, and PBS was replaced at least three times to ensure removal of PEG/NaCl residues. The final product was passed through a 0.22 µm filter prior to cell culture applications. The phage titer was standardized, and the absence of bacterial growth after inoculation onto appropriate medium and incubation for 24–48 h was used to confirm sterility. In addition, endotoxin levels in crude and purified preparations were measured using an LAL-based endotoxin assay and reported as EU mL^{-1}.

Endotoxin levels were determined according to the manufacturer's protocol using a Limulus Amebocyte Lysate (LAL) chromogenic endotoxin kit (e.g. Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, USA). Measurements were performed based on a standard curve generated over the range of 0.01–10 EU mL^{-1}. Samples were serially diluted in pyrogen-free water to prevent potential matrix interference.

To evaluate potential inhibitory or enhancing effects of phage preparations on the LAL reaction, a spike recovery (interference control) test was performed, and recovery rates were confirmed to be within the 50–200% range. All measurements were carried out in three technical replicates and reported as EU mL^{-1}.

Endotoxin removal efficiency was calculated using the initial (Cinitial) and post-purification (Cfinal) endotoxin concentrations according to [(Cinitial − Cfinal)/Cinitial × 100] (Salah *et al*. 2022). Total phage recovery was calculated for crude and purified preparations based on (PFU mL^{-1} × total volume). For calculation of purification efficiency, both concentration factor and total phage recovery were determined. Concentration factor was defined as the ratio of purified titer to crude titer (PFU mL^{-1}), whereas total phage recovery was calculated from the total infectious phage content before and after purification using the formula PFU mL^{-1} × volume.

2.9 Cell culture (MCF-7) and experimental conditions

The MCF-7 human breast cancer cell line (ATCC, HTB-22) was cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 2.5 mM L-glutamine under conditions of 37°C and 5% CO_2. Cell counting was performed using the Cedex XS cell counting system (Roche, Mannheim, Germany).

During passaging, cells were washed with sterile phosphate-buffered saline (dPBS) pre-warmed to 37°C and then incubated with 0.25 mL Trypsin-EDTA at 37°C for 5 min. Cells were transferred into 15 mL tubes, culture medium was added, and the suspension was centrifuged at 130 × g for 5 min. After removing the supernatant, the cell pellet was resuspended in 1 mL supplemented medi-

um and incubated in a 5% CO_2 incubator. For experiments, mycoplasma-negative MCF-7 cells within passage numbers P5–P20 were used (Taghavifar *et al*. 2025).

2.10 Cell viability analysis (MTT)

In this study, among the five isolated phages, FpV33 was selected because it exhibited the shortest latent period and the highest burst size, indicating that it was the most biologically active isolate. Thus, cytotoxicity was evaluated using the phage with the highest lytic capacity.

The biocompatibility/cytotoxicity effects of crude and purified phage preparations on MCF-7 cells were analyzed using the MTT assay according to a previously described method. Cell counting was performed using a Cedex XS cell analyzer (Roche). For the MTT assay, cells were seeded into 96-well plates at 1×10^4 cells/well in 200 µL. After seeding, plates were incubated for 24 h to allow cell attachment.

After the attachment period, experimental groups were arranged as follows: (i) Control: cells + culture medium only, (ii) PBS control: sterile PBS in an equivalent volume without phage, (iii) Crude phage preparation: 1×10^8 PFU/mL, (iv) Purified phage preparation: 1×10^8 PFU/mL, standardized to match the crude preparation.

Doses of crude and purified phage preparations were standardized based on PFU mL^{-1}. Prior to cell applications, phage preparations were sterilized by 0.22 µm filtration, and endotoxin levels of crude and purified preparations were measured using an LAL-based endotoxin assay and reported as EU mL^{-1}. Cells were incubated for 24 and 48 h; n = 8 technical replicates were used for each condition, and experiments were performed in at least three independent repeats (Smulders *et al*. 2018). For the MTT experiments performed in 96-well plates, the final application volume was 200 µL per well at a phage concentration of 1×10^8 PFU mL^{-1}. Based on the measured endotoxin concentrations, the estimated endotoxin exposure corresponded to 240 EU per well for the crude phage preparation and 0.46 EU per well for the purified preparation. Prior to cell exposure, both crude and purified phage preparations were diluted in sterile PBS and standardized to the same final concentration of 1×10^8 PFU mL^{-1} to ensure that observed cellular responses reflected differences in preparation purity rather than differences in phage dose.

At the end of incubation, 20 µL of MTT solution was added to each well on top of the existing medium, standardizing the final volume to 220 µL per well. Plates were incubated at 37°C for 4 h. After MTT incubation, 100 µL of DMSO was added to each well. Absorbance was measured at 570 nm using an ELISA microplate reader. Wells without cells (culture medium + MTT + DMSO) were used as blanks for background correction. Cell viability percentages were calculated (Zeybek *et al*. 2023).

%Cell viability = (Abssample − Absblank) / (Abscontrol − Absblank) × 100.

The resulting % cell viability values obtained from experiments and calculations were interpreted as the biocompatibility of crude and purified phage preparations on MCF-7 cells.

2.11 Data analysis

Data from experiments performed in triplicate are presented as mean ± standard deviation (SD). Group comparisons were conducted using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using GraphPad Prism sSoftware. Data are presented as mean ± standard deviation (SD). Experiments were performed in at least three independent biological replicates. Where appropriate, experiments involving multiple groups and time points were analyzed using two-way ANOVA (group × time) followed by Sidak's multiple comparisons test. For comparisons between two groups, Student's t-test was applied. Prior to parametric analyses, the assumptions of normality and homogeneity of variances were evaluated. A p value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 Plaque formation and lytic activity of the isolated phages

Phages isolated from the effluent waters of trout hatcheries were detected on *F. psychrophilum* using the double-layer agar plaque method. Following inoculation, plaques were observed on the bacterial lawn after 24–48 h of incubation at 15°C. The plaques were circular in shape with well-defined margins. Plaque-forming phages were purified using the single-plaque isolation method, and five phages (FpV30–34) were isolated.

3.2 Kinetic parameters of the phages

The isolated phages were evaluated by one-step growth analysis. Adsorption constant values showed marked differences among the phages. The highest adsorption constant was observed in phage FpV30 ($1.583\times10^{-6}\pm3.41\times10^{-8}$ min^{-1} mL), whereas the adsorption constants of the other phages were in the order of 10^{-9} (Table 1).

Examination of the one-step growth curves showed that latent periods ranged between 3 and 6.5 h. The shortest latent period was observed in FpV33 (3 h), while FpV31 and FpV34 exhibited latent periods of 6.5 h. In terms of burst size, the highest value was recorded for FpV33 (34.5±19 phage $cell^{-1}$), followed by FpV32 (29±5 phage $cell^{-1}$) and FpV30 (22.5±12 phage $cell^{-1}$).

Considering the one-step growth curves, FpV33 was determined to have the shortest latent period (3 h) and the highest burst size (34.5±19 phage $cell^{-1}$). Therefore, FpV33, representing the highest lytic activity, was selected as the representative isolate for subsequent purifica-

tion and cell culture experiments.

TABLE 1 Infection kinetics of the isolated *Flavobacterium psychrophilum* bacteriophages, including adsorption constant (*K*), latent period, and burst size determined from one-step growth experiments.

Phage	Adsorption constant (min^{-1} mL)	Latent period (h)	Burst size (phage $cell^{-1}$)
FpV30	$1.583\times10^{-6} \pm 3.41\times10^{-8}$	4	22.5±12
FpV31	$3.69\times10^{-9} \pm 1.27\times10^{-8}$	6.5	11±1
FpV32	$3.01\times10^{-9} \pm 1.01\times10^{-8}$	4	29±5
FpV33	$4.98\times10^{-9} \pm 1.76\times10^{-8}$	3	34.5±19
FpV34	$1.21\times10^{-9} \pm 1.31\times10^{-8}$	6.5	12±8

3.3 Phage host range

Spot-test analyses demonstrated that the isolated phages produced clear lysis on all tested *F. psychrophilum* strains. In contrast, no lytic activity was observed against *Chryseobacterium chaponense*, *Chryseobacterium* spp., *Enterococcus casseliflavus*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus subtilis spizizenii*, *Enterobacter hormaechei*, *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas putida*.

Weak lysis zones (±) were detected in Pseudomonas aeruginosa ATCC 27853 (Table 2). However, this observation likely reflects spot inhibition rather than productive infection and replication, as spot assays may occasionally produce localized clearing due to bacteriostatic effects or other non-replicative mechanisms.

TABLE 2 Evaluation of the host range of isolated phages by spot-test method.

Bacterial strain	Reference	Lysis*
Flavobacterium psychrophilum	ATCC 49418	+
Flavobacterium psychrophilum	İsolate 1	+
Flavobacterium psychrophilum	İsolate 2	+
Flavobacterium psychrophilum	İsolate 3	+
Flavobacterium psychrophilum	İsolate 4	+
Flavobacterium psychrophilum	İsolate 5	+
Chryseobacterium chaponense	–	–
Chryseobacterium spp.	–	–
Enterococcus casseliflavus	ATCC 700327	–
Salmonella typhimurium	ATCC 14028	–
Staphylococcus aureus	ATCC 29213	–
Pseudomonas aeruginosa	ATCC 27853	±
Bacillus subtilis subsp. *spizizenii*	ATCC 6633	–
Enterobacter hormaechei	ATCC 700323	–
Escherichia coli	ATCC 25922	–
Enterococcus faecalis	–	–
Pseudomonas putida	–	–

*+ lysis, – no lysis, ± partial or variable lysis

3.4 Transmission electron microscopy (TEM)–based morphological characterization of phages

Prepared bacteriophage suspensions were negatively stained with uranyl acetate and processed for visualization by TEM. TEM analysis revealed intact phage particles possessing icosahedral capsid structures and long tails (Figure 1).

The capsid diameters were approximately 75–83 nm, and tail lengths ranged between approximately 129–142 nm. No structural abnormalities or empty capsids were observed in the examined images, indicating a high structural integrity of the phage preparations.

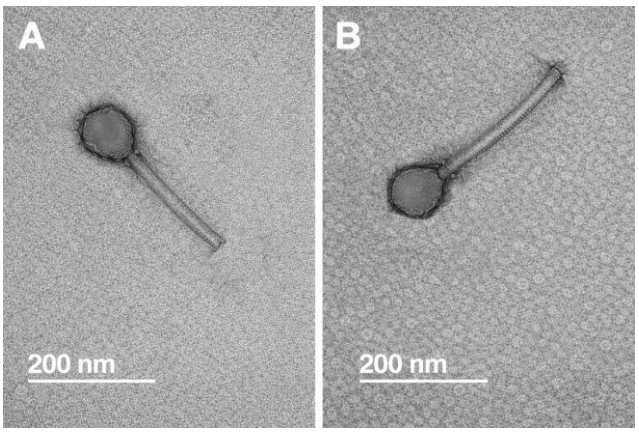

FIGURE 1 Transmission electron microscopy (TEM) images of isolated *Flavobacterium psychrophilum* bacteriophages (A:FpV31, B: FpV33). Representative TEM micrographs showing the morphology of the isolated bacteriophages. Panel (A) and (B) illustrate intact phage particles exhibiting icosahedral capsids and long tails, consistent with members of the Myoviridae family. Variations in tail orientation reflect natural structural flexibility during sample preparation.

3.5 Phage Titration and Concentration After PEG/NaCl Purification

As a result of double-layer agar plaque assays performed using serial dilutions (10^{-1}–10^{-8}), the mean titer of the crude phage lysate was calculated as $1\times10^{8}\pm0.2\times10^{8}$ PFU mL^{-1}. Following PEG/NaCl precipitation and dialysis against PBS, a marked concentration of phage particles was observed. The mean phage titer of the purified preparation was determined to be $4\times10^{9}\pm0.3\times10^{9}$ PFU mL^{-1}. This corresponds to a 40-fold increase (≈1.60 $\log_{10}$ increase) compared to the crude lysate ($p < 0.05$), demonstrating the high efficiency of the PEG/NaCl purification protocol in concentrating phage particles (Figure 2a).

The crude phage lysate showed an average titer of $1\times10^{8}\pm0.2\times10^{8}$ PFU mL^{-1}, whereas PEG/NaCl precipitation followed by dialysis increased the phage concentration to $4\times10^{9}\pm0.3\times10^{9}$ PFU mL^{-1}, corresponding to an approximately 40-fold increase in titer. This increase reflects the concentration of phage particles per unit volume after purification and should not be interpreted as total particle recovery.

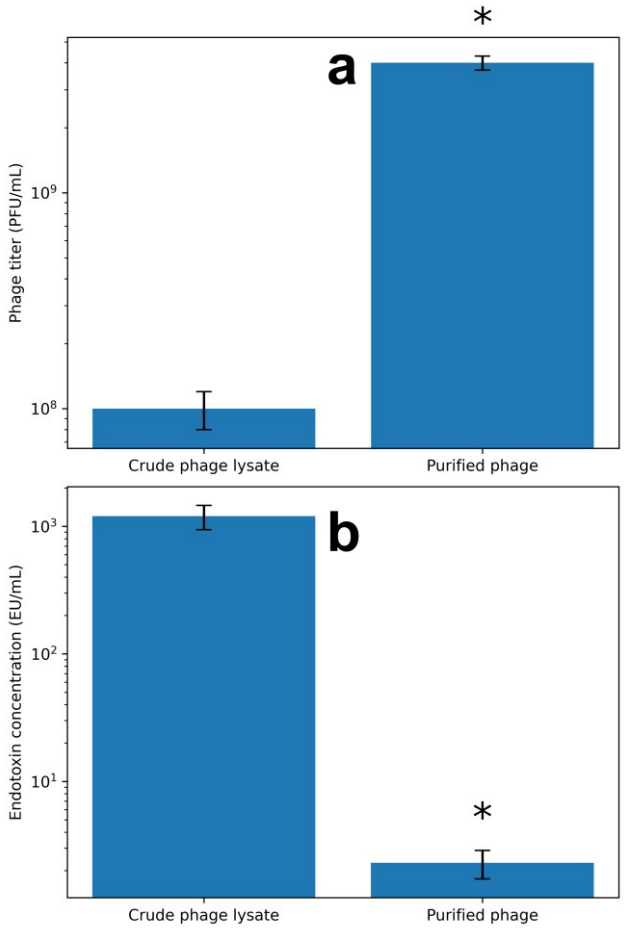

FIGURE 2 a) Phage titers were determined by double-layer agar plaque assay and expressed as plaque-forming units per milliliter (PFU/mL); b) Reduction of endotoxin levels following PEG/NaCl precipitation and buffer exchange. *p < 0.05 vs. crude phage lysate (Student's t-test). Results are presented as mean ± standard deviation (SD) from at least three independent experiments ($n \geq 3$). The y-axis is displayed on a logarithmic scale.

Total phage recovery was calculated from the total number of infectious particles before and after purification using the formula PFU mL^{-1} × volume. The initial volume of crude lysate used for purification was 33 mL, and the final purified preparation was resuspended in 0.5 mL PBS. Accordingly, the total phage content before purification was 3.3×10^9 PFU, whereas the total phage content after purification was 2×10^9 PFU, corresponding to approximately 60% total phage recovery. Preservation of lytic activity was confirmed based on plaque morphology and incubation outcomes, indicating that the purification process did not adversely affect phage infectivity. The crude lysate used for purification had an initial volume of 33 mL, whereas the final purified preparation was resuspended in 0.5 mL PBS. Based on the total infectious phage content (PFU mL^{-1} × volume), the crude preparation contained approximately 3.3×10^9 PFU prior to purification, while the purified preparation contained approximately 2×10^9 PFU after purification, corresponding to an overall phage recovery of approximately 60%.

3.6 Reduction of endotoxin (LPS) levels and evaluation of purification efficiency

Endotoxin levels in crude phage preparations were determined using an LAL-based assay. The endotoxin concentration measured in the crude phage lysate was $1.2\times10^3\pm2.6\times10^2$ EU mL^{-1} (Figure 2b). After PEG/NaCl precipitation and multiple buffer exchanges against PBS, the endotoxin level in the purified phage preparation decreased to 2.3±0.58 EU mL^{-1}, and this reduction was statistically significant ($p < 0.05$, Student's t-test). Endotoxin removal efficiency was calculated to be approximately 99.8%. Endotoxin concentrations in crude phage lysates and purified phage preparations were quantified using a Limulus Amebocyte Lysate (LAL)–based assay. A significant decrease in endotoxin levels was observed after purification (Student's t-test, $p < 0.05$).

Considering the experimental conditions used in the cell culture assays (200 µL per well at 1×10^8 PFU mL^{-1}), the measured endotoxin concentrations correspond to an estimated endotoxin exposure of 240 EU per well for the crude preparation and 0.46 EU per well for the purified preparation.

3.7 Biocompatibility and cytotoxicity profile of phage preparations in MCF-7 cells

3.7.1 General cell viability results (24 and 48 h)

MTT assays were performed in at least three independent biological replicates, with n = 8 technical replicates per experimental condition. Within each independent experiment, eight technical replicate wells per condition were used to calculate the mean value for that experiment. Statistical analyses were performed using the independent biological replicate values rather than pooled technical replicates.

After 24 h of incubation, cell viability in the PBS control group was 96.4±0.2%, showing no significant difference compared to the untreated control group (100%) ($p > 0.05$). At the same time point, treatment with crude phage preparation (1×10^8 PFU mL^{-1}) reduced cell viability to 86.2±0.3%, whereas the purified phage preparation resulted in 90.03±0.7% viability. Although a slight decrease was observed in both phage-treated groups compared to the control, the purified preparation did not produce a pronounced cytotoxic effect.

After 48 h of incubation, cell viability in the PBS group was maintained at 100±0.1%. In contrast, cells treated with crude phage preparation exhibited a marked decrease in viability to 58±0.5%. In the purified phage group, viability was 91±0.9%, remaining close to control levels (Figure 3).

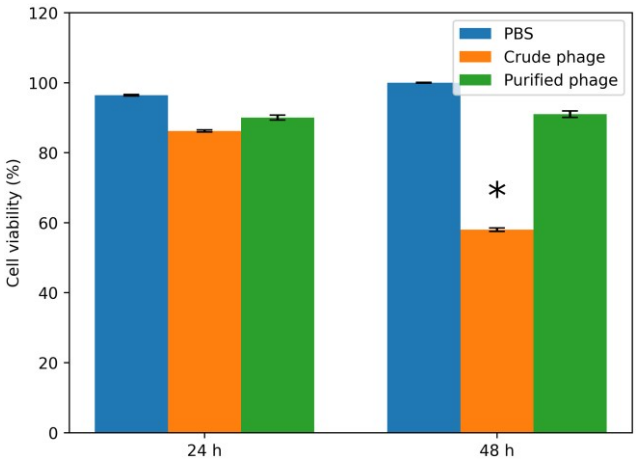

FIGURE 3 Cytocompatibility and cytotoxicity profile of crude and purified phage preparations in MCF-7 cells. Cell viability was assessed by MTT assay after 24 and 48 h of incubation. Data are presented as mean ± SD ($n \geq 3$ independent experiments with $n = 8$ technical replicates per condition).

Two-way ANOVA (group × time) analysis revealed that both group and time factors had statistically significant effects on cell viability ($p < 0.05$). As shown in Table 3, multiple comparison analysis demonstrated that the crude phage group exhibited significantly lower cell viability compared to the PBS control, particularly at 48 h ($p < 0.05$). In contrast, no significant difference was detected between the purified phage group and the control at either 24 or 48 h ($p > 0.05$).

TABLE 3 Cell viability (%) of MCF-7 cells treated with crude and purified phage preparations determined by MTT assay.

Group	Time (% viability ± SD)	
	24 h	48 h
Control	100% (values normalized to control)	100% (values normalized to control)
PBS	96.4 ± 0.2	100.0 ± 0.1
Crude phage (1×10^8 PFU/mL)	86.2 ± 0.3*	58.0 ± 0.5*
Purified phage (1×10^8 PFU/mL)	90.03 ± 0.7	91.0 ± 0.9

3.7.2 Comparative biocompatibility profile of crude and purified phages

MTT analyses showed that after 24 h, cell viability decreased to 86.2 ± 0.3% in the crude phage group, whereas it remained at 90.03 ± 0.7% in the purified phage group. After 48 h of incubation, viability in crude phage-treated cells decreased to 58 ± 0.5%, while the purified phage group maintained 91±0.9% viability. Two-way ANOVA analysis confirmed statistically significant effects of both group and time factors on cell viability ($p < 0.05$). Post-hoc comparisons indicated that the crude phage group exhibited significantly lower viability compared to the control group, particularly at 48 h, whereas no statistically significant difference was observed between the purified phage group and the control at either time point ($p > 0.05$). These findings demonstrate that crude and purified phage preparations applied at equivalent PFU levels exert time-dependent differential effects on MCF-7 cells and that the purified preparation maintains cell viability above 90% up to 48 h.

4. DISCUSSION

4.1 Biological characterization of isolated *Flavobacterium psychrophilum* bacteriophages

In this study, the biocompatibility of lytic bacteriophages specific to *F. psychrophilum*, isolated from environmental samples and purified using a PEG/NaCl precipitation and dialysis-based protocol for endotoxin reduction, was evaluated in the MCF-7 cell line.

The stable plaque morphology and preserved lytic activity of the purified phage isolates demonstrate that the phages were reliably purified (Jurczak-Kurek *et al*. 2016).

4.2 Infection kinetics and lytic capacity of the selected phage isolate

Following PEG/NaCl precipitation and dialysis against PBS, an approximately 40-fold (≈1.60 $\log_{10}$) increase in phage titers was achieved. This finding is consistent with previous studies reporting that PEG-based precipitation effectively concentrates phage particles (Bonilla *et al*. 2016). In this context, selecting the phage with the highest lytic capacity for cell culture experiments ensured that safety evaluation was conducted under worst-case scenario biological exposure conditions, allowing for a more conservative interpretation of biocompatibility outcomes.

4.3 Host specificity and implications for aquaculture biosafety

The weak lysis observed against Pseudomonas aeruginosa in the spot-test assay should be interpreted cautiously. Considering the substantial phylogenetic distance between *Flavobacterium psychrophilum* and *Pseudomonas aeruginosa*, this observation most likely reflects localized spot inhibition rather than productive infection and replication. Such weak inhibition zones in spot assays may arise from transient bacteriostatic effects or localized components of the phage lysate rather than true host susceptibility. In addition, localized inhibition in spot assays may also arise from residual non-phage components carried over from lysate preparation, including traces of chloroform or other processing-related substances, rather than true host susceptibility.

4.4 Efficiency of PEG/NaCl purification and endotoxin reduction

In cell culture applications, one of the most critical limiting factors affecting phage preparation biocompatibility, particularly in preparations derived from Gram-negative hosts, is lipopolysaccharide (LPS)-associated endotoxin contamination (Szermer-Olearnik and Boratyński 2015). In the present study, endotoxin levels measured in crude phage lysates were reduced by approximately 99.8% following PEG/NaCl precipitation and multiple buffer exchange steps, with residual endotoxin levels decreasing to 2.3 ± 0.58 EU mL^{-1} in the purified preparation. Importantly, when compared with values reported in the literature, under the experimental conditions used in this study, the marked reduction in endotoxin levels after purification coincided with improved cell viability in MCF-7 cells. This observation was biologically confirmed by the preservation of over 90% cell viability in MCF-7 cells up to 48 h following treatment with the purified phage preparation (Taghavifar *et al*. 2025).

MTT analyses clearly demonstrated that the effects of crude and purified phage preparations applied at equivalent PFU levels on cell viability were time-dependent. While the crude phage preparation led to a sharp decline in cell viability at 48 h, the purified phage group-maintained baseline viability levels at both 24 and 48 h. These findings strongly suggest that the cytotoxic effects observed in crude preparations were largely attributable to endotoxin and other bacterial residues. Importantly, these results support the relevance of human cell–based screening for evaluating the biosafety of aquaculture phage preparations under the One Health framework. By demonstrating that endotoxin-reduced phages preserve mammalian cell viability. These findings support the value of human cell-based screening for evaluating the in vitro cytocompatibility of aquaculture phage preparations under the tested experimental conditions. The parallel relationship between the sharp reduction in endotoxin levels and the improvement in cell viability further indicates that the PEG/NaCl plus dialysis protocol plays a decisive role in minimizing endotoxin-associated cellular stress (Mamat *et al*. 2015).

4.5 Cytocompatibility of endotoxin-reduced phage preparations

A clear inverse relationship was observed between residual endotoxin levels and MCF-7 cell viability, indicating that the cytotoxic effect was associated with LPS contamination rather than the phage particles themselves. Residual endotoxins are known to activate inflammatory and apoptotic signaling pathways in epithelial cells, and the reduced viability observed in crude phage preparations can be explained by this biological mechanism. Although inflammatory cytokines were not directly measured in this study, the strong parallel between the dramatic reduction in endotoxin levels and the significant improvement in cell viability strongly supports the conclusion that the observed cytotoxicity was primarily LPS-driven.

4.6 Study limitations and future perspectives

It should be noted that the cytocompatibility evaluation performed in this study was limited to a single human cell line (MCF-7) and relied on the MTT assay as an initial screening method. Therefore, the present findings should be interpreted as evidence of in vitro cytocompatibility under the tested experimental conditions rather than as direct evidence of broader biomedical or therapeutic applicability. Further studies involving additional cell types and mechanistic analyses, including inflammatory responses and immune activation, would be required to fully evaluate translational potential. A further limitation of the present study is the absence of an LPS-only control matched to the endotoxin levels measured in the crude and purified phage preparations; therefore, the relative contribution of endotoxin alone versus other residual bacterial components could not be experimentally separated in this study.

In recent years, the increasing application of phages in aquaculture has made cell culture compatibility of phage preparations a critical requirement (Henein *et al*. 2016). In this regard, the purification strategy applied in the present study offers a practical and scalable solution to overcome the endotoxin barrier, which is often considered a major limitation in phage-based applications. The current data clearly demonstrate a strong correlation between endotoxin removal and improved cell viability (Trend *et al*. 2018). However, genomic characterization of the isolated phages was not performed in the present study. Comprehensive phage safety evaluation typically includes whole-genome sequencing and bioinformatic screening for lysogeny-related genes, virulence factors, and antibiotic resistance determinants. Therefore, future studies should include genomic analyses to confirm the strictly lytic nature and genetic safety of the phage candidates prior to large-scale or translational applications.

5 | CONCLUSIONS

This study demonstrates that the combination of PEG/NaCl precipitation and dialysis enables the production of high-titer, low-endotoxin phage preparations that can be applied to MCF-7 cells without inducing significant cytotoxic effects. Endotoxin removal from phage preparations resulted in an approximately 40-fold increase in phage concentration while reducing endotoxin levels by 99.8%, thereby meeting critical biocompatibility requirements for cell culture applications.

Overall, these results demonstrate that practical and scalable production of high-titer, low-endotoxin phage preparations for aquaculture is achievable. These findings

demonstrate that PEG/NaCl purification can generate phage preparations with favorable in vitro cytocompatibility under the tested experimental conditions. Further studies involving multiple cell types and additional biological endpoints will be required to evaluate broader biomedical or translational applications. Future work exploring additional cell lines, dose–response effects, and phage-mediated cargo delivery will further advance phages as translational biomedical platforms.

ETHICAL APPROVAL

This study did not involve human participants or animal experiments. All experiments were performed using the commercially available MCF-7 human breast cancer cell line (ATCC HTB-22). Therefore, according to institutional and international guidelines, formal ethical approval was not required.

CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

Ustundag M: Conceptualization, Methodology, Phage isolation and characterization, Endotoxin reduction, Data analysis, Interpretation of results, Writing — original draft. Ustundag B: MTT cytocompatibility assays, Data analysis, Interpretation of results, Writing — review & editing.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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