Journal of Fisheries
Volume 14 Issue 3 December 2026
Peer Reviewed | Open Access

eISSN 2311-3111
pISSN 2311-729X

DOI: https://doi.org/10.17017/j.fish.1258

Original Article

Biosafety improvement of aquaculture-associated bacteriophage preparations via endotoxin reduction: implications for *Flavobacterium psychrophilum* control

Mustafa Ustundag[1] • **Berrin Ustundag**[2]

[1] *Department of Chemistry and Chemical Management Technologies, Muradiye Vocational School, Van Yuzuncu Yıl University, Van, Türkiye*
[2] *Department of Scientific Research Project Office, Van Yuzuncu Yıl University, Van, Türkiye*

Joint correspondence
Mustafa Ustundag; Department of Chemistry and Chemical Management Technologies, Muradiye Vocational School, Van Yuzuncu Yıl University, Van, Türkiye
mustafaustundag@yyu.edu.tr

Manuscript history
Received 22 March 2026 | Accepted 24 May 2026 | Published online 2 June 2026

Citation
Ustundag M, Ustundag B (2026) Biosafety improvement of aquaculture-associated bacteriophage preparations via endotoxin reduction: implications for *Flavobacterium psychrophilum* control. Journal of Fisheries 14(3): 143201. DOI: 10.17017/j.fish.1258

Abstract
The use of bacteriophages specific to *Flavobacterium psychrophilum* in aquaculture has shown promising results in controlling infections and reducing fish mortality. However, the biosafety and cytocompatibility of these phage preparations, especially under long-term exposure conditions, remain largely unexplored. In this study, bacteriophages specific to *F. psychrophilum* were isolated and characterized. To enhance biosafety, the phages were subjected to endotoxin reduction procedures. Endotoxin levels and phage titers were quantitatively determined before and after purification. Cytocompatibility was evaluated using the MTT assay on the mammalian cell line MCF-7 over a 120-hour exposure period. The purification process reduced endotoxin levels by 97.3%, while phage titers were preserved or increased, indicating that biological activity was maintained. Time-dependent analyses revealed that crude phage preparations caused a decrease in cell viability, with statistically significant reductions observed at 72, 96, and 120 hours ($p < 0.05$). In contrast, no difference in cell viability was observed in cells treated with purified phage preparations compared to the control group ($p > 0.05$). These findings demonstrate that the cytotoxic profile of phage preparations is determined by endotoxin content and prolonged exposure duration. This study highlights that endotoxin removal is critically important for ensuring cytocompatibility and demonstrates that endotoxin content in phage preparations used in aquaculture may lead to reduced cell viability in mammalian cells under long-term exposure conditions.

Keywords: bacteriophages; cell culture; cytocompatibility; endotoxin

1 | INTRODUCTION

Antimicrobial resistance has become an increasing concern in aquaculture, largely driven by intensive production practices and the widespread use of antibiotics. In particular, the routine use of antibiotics for preventive purposes has accelerated this problem, turning it into a global issue (Bhat and Altınok 2023). The accumulation of antibiotic residues in aquatic environments, along with the development of resistance mechanisms, poses risks not only to fish health but also to environmental and hu-

man health (Yang *et al*. 2024). These challenges highlight the need for alternative control strategies that are more sustainable, environmentally friendly, and economically feasible.

In recent years, bacteriophages (phages), applied for disease control in aquaculture, have attracted increasing research interest as alternative biotherapeutic agents due to their host specificity and rapid replication capacity (Ramesh *et al*. 2021). In particular, in vivo and in vitro studies conducted on pathogens such as Flavobacterium, Vibrio, and Aeromonas have demonstrated the effectiveness of lytic phages (Nithin *et al*. 2024). *Flavobacterium psychrophilum* has been identified as the causative agent of Bacterial Cold-Water Disease (BCWD), including its clinical manifestation known as Rainbow Trout Fry Syndrome, in salmonid fish, leading to high mortality rates and significant economic losses, particularly in hatchery conditions (Strepparava *et al*. 2014). Recent studies have highlighted that the epidemiology, host susceptibility, and disease progression of *F. psychrophilum* infections in aquaculture systems are highly complex and influenced by multiple interacting factors (Avila *et al*. 2022). Moreover, antibiotic-based approaches used to control this pathogen are progressively losing their effectiveness due to the development of resistance mechanisms (Pereira *et al*. 2022). Although the primary objective of phage applications in aquaculture is pathogen control, the interactions between phages and mammalian cells have largely been overlooked. Studies have demonstrated that phages can interact with mammalian cells and be internalized via macropinocytosis and endocytosis (Lehti *et al*. 2017; Bichet *et al*. 2021). Moreover, internalized phages may not only persist within cells but also modulate cellular functions (Van Belleghem *et al*. 2019; Bichet *et al*. 2021). Although contaminants such as endotoxins (lipopolysaccharides), cellular debris, and bacterial DNA present in some phage preparations have not been conclusively shown to cause direct cytotoxicity in mammalian cells, these findings suggest that phage–cell interactions may induce not only acute but also time-dependent cumulative effects (Dufour *et al*. 2017; Podlacha *et al*. 2021). Therefore, beyond antimicrobial efficacy, evaluating the in vitro cytotoxicity profiles and cellular biocompatibility of phage-based applications is essential for a comprehensive assessment of their applicability (Elgendy *et al.* 2024). In the context of aquaculture, such biosafety evaluations are particularly important, as phage preparations may be directly or indirectly exposed to fish tissues, aquatic microbiota, and the surrounding environment.

Current literature on bacteriophages developed against aquaculture pathogens at the cell line level remains limited. Existing studies have evaluated the cytotoxicity of *Yersinia ruckeri*, *Aeromonas hydrophila*, and *Lactococcus petauri* phages in the EPC cell line under exposure durations of 24–72 hours (Türe *et al*. 2022; Altınok *et al*. 2026a; Altınok *et al*. 2026b). Studies investigating the effects of aquaculture-derived phages on mammalian cell lines are extremely scarce, with only the study by Wu *et al*. (2026) providing notable insight. In that study, *Edwardsiella tarda* phages were shown to interact with and be internalized by Caco-2 cells; however, cytotoxicity was assessed using qualitative methods such as fluorescent live/dead staining rather than quantitative analyses. Consequently, cellular metabolic activity, dose–response relationships, and long-term toxicity effects were not comprehensively evaluated (Wu *et al*. 2026). Notably, most of these limited studies have focused on short-term exposures (24–72 hours) (Henein *et al*. 2016; Podlacha *et al*. 2021).

Although it is known that phages used in aquaculture can interact with human cells, no study has systematically investigated the effects of long-term phage exposure on mammalian cell lines under conditions resembling aquaculture environments. Due to the predominance of short-term experimental designs, the cellular effects and potential cumulative consequences of prolonged exposure (≥96 hours) remain insufficiently characterized. In this context, investigating the long-term interactions between phages specific to *F. psychrophilum*, an important aquaculture pathogen, and mammalian cells is of particular significance.

In this study, the effects of time-dependent phage exposure—extending up to 120 hours—on cytotoxicity and biocompatibility in mammalian cells were investigated. The MCF-7 cell line, a standardized and reproducible mammalian cell system, was selected as the model. Although widely used in cancer biology, targeted therapy, and drug delivery studies, it was employed here to evaluate biosafety conditions. The use of purified *F. psychrophilum* phage preparations minimized the effects of residual endotoxins and other bacterial contaminants, allowing for a more controlled assessment of cellular responses. Cellular responses were evaluated using the MTT assay. The findings are expected to contribute to the current understanding of the biocompatibility of endotoxin-reduced phage preparations under long-term exposure conditions in mammalian cells.

2 | METHODOLOGY

2.1 Bacteriophage isolation and purification

2.1.1 Bacterial culture: In this study, the *F. psychrophilum* reference strain ATCC 49418 was used. The bacterial culture was inoculated into Anacker-Ordal medium and incubated at 15°C for 5 days. End of the incubation period, the culture was reactivated. Cultures in the 24-hour growth phase were prepared for phage isolation (Chen *et al*. 2023).

2.1.2 Bacteriophage isolation: Water used for bacteriophage isolation was collected from the effluent of trout

hatcheries in the region and from five different sampling points. Each water sample was transferred into 15 mL Falcon tubes were subjected to centrifugation at 10,000 × g for 10 minutes, after which the supernatant was collected and filtered through a 0.22 µm membrane. Each sample consisted of 25 mL of 0.22 µm-filtered water combined with 5 mL of bacterial culture and 15 mL of Anacker-Ordal medium, followed by incubation at 15°C for 48 hours. Chloroform (50 µL mL^{-1}) was introduced to the supernatant, which was subsequently filtered again through a 0.22 µm membrane (Molina *et al*. 2020). The presence of phages was assessed using the double-layer agar plate method, with bacterial cultures incubated alongside phages at 15°C. (Ustundag and Ustundag 2026). For phage purification, the single plaque isolation method was employed, involving serial dilutions of phage suspensions and subsequent incubation with *F. psychrophilum* cultures, ultimately yielding a crude phage lysate after filtration (Kalatzis *et al*. 2016).

2.2 Characterization of bacteriophages

2.2.1 Phage titration (determination of PFU mL^{-1}): Phage titers were determined by calculating the number of phages present per milliliter in a sample and were expressed as PFU mL^{-1}. Serial dilutions (10^{-1} to 10^{-8}) of phage stocks in liquid culture were prepared using SM buffer. From each dilution, 300 µL of phage suspension was mixed with 900 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, plaques were counted and phage titers were determined (Anand *et al*. 2016).

2.2.2 Determination of kinetic parameters of bacteriophages: A total of 300 µL of phage suspension was mixed with 900 µL of exponentially growing *F. psychrophilum* culture and incubated at 15°C for 10 minutes. The mixture was centrifuged at 10,000 × g for 10 minutes. After centrifugation, the cells were resuspended in 2000 µL of cold Anacker and Ordal medium. Samples were then taken every 30 minutes for 8 hours and plated using the double-layer agar method, followed by incubation at 15°C. After incubation, plaques were counted and recorded. The latent period was determined based on the time interval during which the phage titer measured by the double-layer agar method remained constant. Burst size was calculated using the following equation (Lallo *et al*. 2014):

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

To determine the adsorption rate of bacteriophages to host cells, bacteriophages were mixed with 20 mL of *F. psychrophilum* culture in the 18–24-hour growth phase and incubated at 15°C. During incubation, samples were taken every 5 minutes for 30 minutes, diluted 1:10 with SM buffer containing chloroform (50 µL mL^{-1}), thoroughly mixed, and centrifuged at 5,000 × g for 3 minutes. The samples were then rapidly plated using the double-layer agar method and incubated at 15°C. After incubation, plaques were counted and recorded. The phage titer in the supernatant containing unabsorbed phages was determined, and the adsorption percentage was calculated using the following formula (Islam *et al*. 2023):

> % adsorption = [(initial phage titer (PFU/mL) − phage titer after adsorption (PFU/mL)) / (phage titer during the latent period (PFU/mL))] × 100

The phage adsorption constant (K) was calculated as follows: $K = 2.3/(B \times t) \times \log(P_o/P)$; where, K = phage adsorption constant; B = bacterial concentration (bacteria per mL; determined as CFU/mL by plate counting); t = time; P_o = initial PFU; P = PFU in the supernatant.

2.2.3 Determination of phage host range: In order to evaluate the specificity of phages toward *F. psychrophilum*, the following strains were used: *F. psychrophilum* (ATCC 49418), *F. psychrophilum* strains 1–6, *Enterobacter hormaechei*, *Chryseobacterium* spp., *Staphylococcus aureus*, *Salmonella typhimurium*, *Chryseobacterium chaponense*, *Pseudomonas aeruginosa*, *Enterococcus casseliflavus*, *Bacillus subtilis spizizenii*, and *Escherichia coli*. The lytic activities of the phages were investigated using the spot-test method. For each group, 500 µL of bacterial culture was mixed with 4000 µL of soft agar and spread onto a pre-poured top agar layer. After the agar dried, 15 µL of phage isolates was spotted, and lysis formation was examined (Alharbi and Alshaikh 2022).

2.2.4 Examination of bacteriophages by electron microscopy: Phage suspensions were mixed with 2.5% glutaraldehyde buffer at a ratio of 1:10 and incubated at room temperature for 5 minutes. The phage suspension was then inoculated onto Formvar–carbon-coated copper grids and allowed to stand at room temperature for 2 minutes. Phages were stained with 2% uranyl acetate and incubated at room temperature for 2 minutes. Subsequently, the phages were visualized using TEM (Cornuault *et al*. 2018).

2.3 Purified phage preparation and endotoxin control

To minimize bacterial debris, endotoxin/LPS, and medium-derived contaminants present in the crude phage lysate, the PEG/NaCl precipitation method was applied. NaCl was added to the crude phage lysate to reach a final concentration of 1.0 M and mixed until completely dissolved. Subsequently, PEG 8000 was added to a final concentration of 10% (w/v). The mixture was incubated at 4°C at 50 rpm for at least 12 hours. After incubation, the suspension was centrifuged at 4°C at 10,000 × g for 20–30

minutes, 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 completely dissolved to obtain a concentrated phage preparation (Pirnay *et al*. 2024). To remove residual PEG/NaCl and low molecular weight contaminants, the concentrated phage suspension was dialyzed against PBS at 4°C using a sterile dialysis membrane. The dialysis buffer was changed three times. The final preparation was passed through a 0.22 µm sterile filter prior to cell culture applications. Phage titers were determined using the double-layer agar method and adjusted to the desired concentration before experimental use. The sterility of the preparations was confirmed by plating aliquots on non-selective agar media and observing no bacterial growth after 48 hours of incubation at 15°C. Endotoxin levels in crude and purified phage preparations were quantitatively determined using a chromogenic Limulus Amebocyte Lysate (LAL) assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. Measurements were performed using a standard curve prepared in the range of 0.01–10 EU mL^{-1}. Samples were serially diluted with pyrogen-free water (Ketchum and Novitsky 2000). Spike recovery tests were performed to evaluate the potential of phage preparations to inhibit or enhance the LAL reaction, and recovery rates were confirmed to be within the acceptable range (50–200%). All measurements were conducted in triplicate and expressed as endotoxin units per milliliter (EU mL^{-1}). Endotoxin levels were also normalized to phage load and reported as EU per 10^9 PFU.

Endotoxin removal efficiency was calculated using the formula:

$[(C_{initial} - C_{final}) / C_{initial} \times 100]$ based on the initial ($C_{initial}$) and post-purification (C_{final}) endotoxin concentrations (Magalhães *et al*. 2020).

Total phage recovery was calculated based on PFU mL^{-1} × total volume for both crude and purified preparations (Szermer-Olearnik and Boratyński 2015; Dufour *et al*. 2019; Van Belleghem *et al*. 2019).

2.4 MCF-7 cell line and cell culture conditions

The MCF-7 human breast cancer cell line (ATCC, HTB-22) was cultivated in a medium RPMI-1640, supplemented by 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 2.5 mM L-glutamine under 37°C and 5% CO_2. Cell counts were conducted using the Cedex XS cell counter (Roche, Mannheim, Germany) (Eker *et al*. 2019). During the digestion, cells were washed with 37°C sterile phosphate salts (dPBS) and incubated for 5 minutes with 37°C trypsin-EDTA. After incubation, cells were transferred to a 15mL tube, added a culture medium and centrifuged for 5 minutes at 130 g. After removal of the supernate, the cell pellet is returned to the medium with 1 mL of medium and cultured in a 5% CO_2 incubator. The cells used in the experiment were found to be P5–P20 in the passages and to be negative to mycoplasma (Huang and Nitin 2019).

2.5 Determination of phage doses and application protocol

When expressing the unit of phage doses, plaque-forming units (PFU mL^{-1}) were taken as the basis. The phage concentration to be used in the experiments was determined as 1×10^8 PFU mL^{-1}, considering preliminary experiments and commonly used dose ranges in the literature. The double-layer agar method was used to determine phage titer. Phage preparations were filtered through a 0.22 µm filter prior to cell culture applications and applied to the cell culture medium in equal volumes (Van Belleghem *et al*. 2019).

2.6 Experimental groups and controls

The cell groups to be used in the experiments were designed as follows:

I. Control group: cells containing only cell culture medium
II. PBS control group: cells treated with an equivalent volume of sterile PBS without phage
III. Crude phage group: cells treated with crude phage preparation at a concentration of 1×10^8 PFU mL^{-1}
IV. Purified phage group: cells treated with purified phage preparation at a concentration of 1×10^8 PFU mL^{-1}

Applications were carried out for all experimental groups at time points of 24, 48, 72, 96, and 120 hours.

2.7 Evaluation of cell viability by the MTT method

In this research, FpV37 was chosen due to its exceptional lytic activity, characterized by the shortest latent period and the largest burst size among the six isolated phages. Cytotoxicity and biocompatibility assessments were conducted utilizing this phage. The impact of both crude and purified phage preparations on MCF-7 cells was evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell counting was executed using a Cedex XS device (Roche, Mannheim, Germany). Cells were plated into 96-well plates at a density of 1×10^4 cells per well in 200 µL of complete medium and incubated for 24 hours at 37°C with 5% CO_2 to facilitate attachment (Abedon 2011).

After the attachment phase, cells were treated according to the experimental groups defined earlier. Crude and purified phage preparations were titrated using the double-layer agar method and standardized to a final concentration of 1×10^8 PFU mL^{-1} (Kropinski *et al*. 2009). Before application to cell cultures, all phage preparations were filtered through a 0.22 µm membrane filter (Merabishvili *et al*. 2009; Bonilla *et al*. 2016). Specifically, after

the 24-hour attachment period, 100 µL of culture medium was extracted from each well and replaced with 100 µL of the relevant treatment solution. Thus, the treatment volume applied to each well was maintained at 100 µL, and the total final volume was kept constant at 200 µL (Riss *et al*. 2016). The endotoxin exposure per well (EU per well) was calculated using the endotoxin values determined in Section 2.3 according to the following equation: EU/well = endotoxin concentration (EU mL^{-1}) × volume added to the well (mL). This equation aids in determining the effective endotoxin dose administered to the cells (Petsch and Anspach 2000; Magalhães *et al*. 2020).

Furthermore, the endotoxin load was adjusted according to the phage quantity and represented as EU per 10^9 PFU, which facilitated the comparison of different preparations (Bonilla *et al*. 2016; Dufour *et al*. 2019). Cells were subjected to incubation with phage preparations for durations of 24, 48, 72, 96, and 120 hours following a time-dependent extended exposure protocol. Each experimental condition included 8 technical replicates, and a minimum of three independent biological replicates were conducted for all experiments (Riss *et al*. 2016). Upon completion of the incubation period, 20 µL of MTT stock solution (prepared in PBS at a concentration of 5 mg mL^{-1}) was introduced to each well. Consequently, the final MTT concentration per well was approximately 0.45 mg mL^{-1}, with the total volume adjusted to 220 µL. The plates were incubated at 37°C for 4 hours to facilitate the formation of formazan crystals by viable cells (Mosmann 1983; Riss *et al*. 2016). After incubation, the supernatant was meticulously removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the generated formazan crystals. The plates were gently agitated at room temperature for a brief period to ensure the complete dissolution of the crystals. Absorbance readings were taken at 570 nm using a microplate reader. Wells devoid of cells (medium + MTT + DMSO) served as blanks for background correction (Mosmann 1983).

Cell viability (%) was calculated according to the following formula: % Cell viability = (Abssample − Absblank) / (Abscontrol − Absblank) × 100

2.8 Data analysis

All experimental data were evaluated by calculating the mean values from a minimum of three independent biological replicates, with results presented as mean ± standard deviation (SD). To minimize intra-plate variation, technical replicates (n = 8) within each biological replicate were utilized and averaged before analysis. The normality of the data was examined through the Shapiro–Wilk test. Levene's test was employed to assess the homogeneity of variances. Group comparisons were conducted using a two-way analysis of variance (two-way ANOVA), which incorporated the factors of time (24, 48, 72, 96, and 120 hours) and treatment (control, PBS, crude phage, and purified phage). To identify significant differences, Tukey's multiple comparison test was utilized. All statistical analyses were carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The threshold for statistical significance was set at $p < 0.05$ (Motulsky 2014).

3 | RESULTS

3.1 Isolation and plaque morphology of bacteriophages

The phages to be used in the study were identified on *F. psychrophilum* using the double-layer agar plate method. Following inoculation, they were incubated at 15°C for 24–48 hours. After incubation, inhibition zones (plaques) were observed on the petri dishes. The formed plaques were determined to be circular in shape and to have well-defined boundaries. Plaque-forming phages were purified using the single plaque isolation method, and six phages (FpV35–40) were isolated.

3.2 Kinetic parameters of the phages

The adsorption rate constants (K) of the isolated bacteriophages are presented in Table 1. The highest adsorption rate constant was determined in phage FpV37 ($121.01 \times 10^{-8} \pm 1.02 \times 10^{-7}$ mL min^{-1}). The adsorption rate constants of the other phages were found to be on the order of 10^{-9} mL min^{-1} and therefore exhibited lower adsorption rates.

The latent periods were determined to range between 4.0 and 6.5 hours. The shortest latent period was observed in FpV37 (4.0 hours), while the longest latent period was observed in FpV36 (6.5 hours). In terms of burst size, the highest value was recorded for FpV37 (31±5 phage $cell^{-1}$), followed by FpV38 (30.5±11 phage $cell^{-1}$) and FpV36 (21±1 phage $cell^{-1}$).

3.3 One-step growth curve and replication kinetics

FpV37 displayed the shortest latent period and the largest burst size among the six isolates, it was selected for detailed one-step growth curve analysis. Following the adsorption phase, samples were taken from infected *F. psychrophilum* cultures at 30-minute intervals between 0 and 480 minutes. Free phage titers were determined in PFU mL^{-1} using the double-layer agar method. In the growth curve, the phage titer remained largely constant until approximately 120 minutes. Subsequently, a distinct increase phase occurred between 120 and 240 minutes, and the titers reached a plateau level after approximately 240 minutes.

3.4 Phage host range

Spot-test analyses showed that the isolated phages produced clear lysis on all tested *F. psychrophilum* strains, and no inhibition zones were observed on any of the other bacteria (Table 1).

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

Phage	Capsid diameter (nm)	Tail length (nm)	Adsorption constant (min^{-1} mL)	Latent period (h)	Burst size (phage/cell)
FpV35	78 ± 3	133 ± 7	$(1.98 \pm 0.11) \times 10^{-9}$	6.0	12.5 ± 10
FpV36	80 ± 2	139 ± 4	$(1.09 \pm 0.11) \times 10^{-9}$	6.5	21 ± 1
FpV37	82 ± 3	140 ± 8	$(1.21 \pm 0.10) \times 10^{-6}$	4.0	31 ± 5
FpV38	75 ± 4	131 ± 9	$(9.98 \pm 1.76) \times 10^{-8}$	4.5	30.5 ± 11
FpV39	79 ± 3	135 ± 6	$(2.03 \pm 0.10) \times 10^{-9}$	6.0	10 ± 8
FpV40	77 ± 2	127 ± 5	$(1.01 \pm 0.10) \times 10^{-9}$	5.5	15 ± 10

Values are presented as mean ± standard deviation (n = 3). Capsid diameter and tail length were determined by transmission electron microscopy. Adsorption constants and one-step growth parameters were calculated based on independent triplica experiments.

3.5 Morphological characterization of bacteriophages by transmission electron microscopy (TEM)

Prepared bacteriophage suspensions were negatively stained with uranyl acetate and prepared for imaging by TEM. TEM analyses revealed the presence of phage particles (Figure 1). Capsid diameters were determined to be approximately 75–82 nm, while tail lengths were approximately in the range of 127–140 nm (Table 1). The examined images showed that the phages possessed structural integrity. Representative TEM images showing the morphology of the isolated bacteriophage are presented. FpV37 exhibited an icosahedral capsid and a long non-contractile tail, consistent with siphovirus-like morphology.

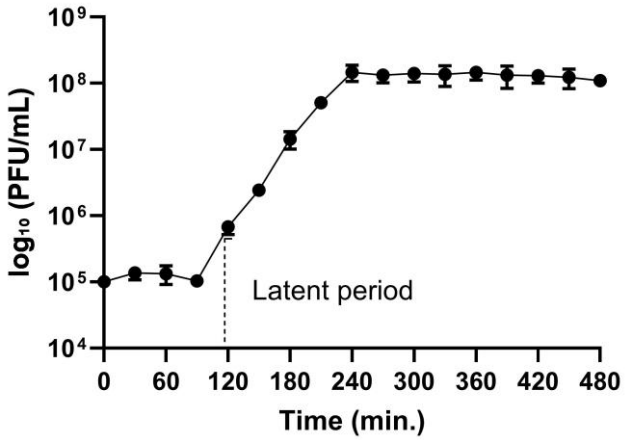

FIGURE 1 One-step growth curve of bacteriophage FpV37. Phage titers (log_{10} PFU/mL) were determined at 30-minute intervals over 480 minutes. The curve demonstrates a distinct latent phase, followed by a rapid rise phase and a stable plateau. Data represent mean ± SD (n = 3).

3.6 Purification yield and phage recovery

Following the purification process, the concentration changes of bacteriophage preparations and the total phage recovery efficiency were evaluated. Before purification, the average titer of the crude phage suspension was determined as $2.78 \times 10^7 \pm 1.04 \times 10^7$ PFU mL^{-1}, and the initial volume was 30 mL. After purification, the final preparation volume was reduced to 0.5 mL, and the average phage titer was measured as $1.00 \times 10^9 \pm 5.0 \times 10^8$ PFU mL^{-1}. According to these data, an approximately 36-fold (35.97-fold) increase in phage titer was achieved after purification. Considering the total number of phage particles, the total phage load before purification was calculated as 8.34×10^8 PFU, while the total phage load after purification was calculated as 5.00×10^8 PFU. Accordingly, the total phage recovery rate was determined to be approximately 60% (59.95%).

3.7 Endotoxin reduction after purification

Following the purification process, endotoxin levels of the phage preparations were evaluated using the LAL (Limulus Amebocyte Lysate) assay. Before purification, the endotoxin level was measured as $1.0 \times 10^{-5} \pm 3.0 \times 10^{-6}$ EU mL^{-1}, while after purification, this value decreased to $2.7 \times 10^{-7} \pm 1.2 \times 10^{-7}$ EU mL^{-1} (n = 3). This result indicates that approximately a 97.3% reduction in endotoxin levels was achieved with the applied purification protocol. Spike recovery analyses yielded values within the acceptable range of 50–200%, indicating that the phage preparations did not cause significant inhibition or enhancement of the LAL assay. In addition to volumetric endotoxin quantification, endotoxin burden was normalized to phage titer and expressed as EU/10^9 PFU. The crude preparation exhibited an endotoxin load of approximately 3.60×10^{-4} EU/10^9 PFU, whereas the purified preparation showed a substantially lower value of 2.7×10^{-7} EU/10^9 PFU (Table 2).

3.8 Endotoxin exposure per well (EU per well)

In order to evaluate biologically relevant endotoxin exposure in cell culture experiments, the measured endotoxin levels were calculated as endotoxin amount per well (EU/well). When 100 µL of sample was applied to each well, endotoxin exposure was determined as 1.0×10^{-6} EU/well for the crude preparation and 2.7×10^{-8} EU/well for the purified preparation.

TABLE 2 Quality characteristics of crude and purified bacteriophage preparations.

Parameters	Crude phage preparation	Purified phage preparation	Fold change / % reduction
Phage titer (PFU/mL)	$2.78 \times 10^7 \pm 1.04 \times 10^7$	$1.00 \times 10^9 \pm 5.0 \times 10^8$	35.97-fold increase
Endotoxin (EU/mL)	$1.0 \times 10^{-5} \pm 3.0 \times 10^{-6}$	$2.7 \times 10^{-7} \pm 1.2 \times 10^{-7}$	97.30% reduction (37.04-fold decrease)
Endotoxin normalized to phage load (EU/10^9 PFU)	3.60×10^{-4}	2.70×10^{-7}	1333.33-fold reduction
Endotoxin exposure per well (EU/well)	1.0×10^{-6}	2.7×10^{-8}	37.04-fold reduction

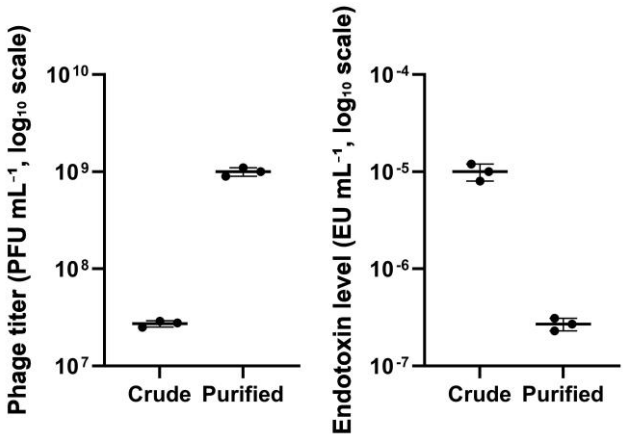

FIGURE 2 Endotoxin reduction and phage titer following purification.

3.9 Time-dependent cell viability (MTT assay)

MTT assay results revealed that the effects of crude and purified phage preparations on MCF-7 cells differed in a time-dependent manner. In the control and PBS groups, cell viability was maintained at a high level at all time points and exhibited a similar time-dependent pattern. In the control group, cell viability was determined as 97.83%, 95.03%, 90.97%, 88.83%, and 85.30% at 24, 48, 72, 96, and 120 hours, respectively, while in the PBS group, these values were measured as 96.77%, 95.57%, 90.33%, 88.67%, and 85.57%, respectively, and no statistically significant difference was observed between the control and PBS groups ($p > 0.05$). In the group treated with the crude phage preparation, cell viability was found to decrease gradually over time. While cell viability was measured as 97.23% at 24 hours, this value decreased to 93.07% at 48 hours, 75.47% at 72 hours, 70.07% at 96 hours, and 68.10% at 120 hours. The decrease in cell viability became particularly more pronounced after 72 hours. Statistical analyses showed that, particularly at 72, 96, and 120 hours, there was a significant loss of viability in the crude phage group compared to the control group ($p < 0.05$) (Table 3).

TABLE 3 Time-dependent cell viability of MCF-7 cells following exposure to crude and purified bacteriophage preparations. Data are presented as mean ± SD ($n = 3$). Cell viability values were calculated based on MTT absorbance measurements and expressed as percentage viability relative to relative to the control group. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns: not significant).

Time (h)	Control (% viability)	PBS (% viability)	Crude (% viability)	Purified (% viability)
24	97.83 ± 0.75	96.77 ± 0.75	97.23 ± 0.50	96.67 ± 0.40
48	95.03 ± 0.47	95.57 ± 0.35	93.07 ± 0.51	94.50 ± 0.50
72	90.97 ± 0.40	90.33 ± 0.55	75.47 ± 1.10***	94.53 ± 0.55
96	88.83 ± 0.91	88.67 ± 0.40	70.07 ± 0.90***	90.73 ± 0.75
120	85.30 ± 0.76	85.57 ± 0.55	68.10 ± 1.05***	89.07 ± 1.05

In contrast, in the group treated with the purified phage preparation, cell viability was maintained at a high level at all time points. Cell viability was measured as 96.67%, 94.50%, 94.53%, 90.73%, and 89.07% at 24, 48, 72, 96, and 120 hours, respectively. No statistically significant difference was found between the phage group and the control group ($p > 0.05$). In addition, when crude and purified phage preparations were directly compared, statistically significant differences in cell viability were observed, particularly at 72 hours and beyond ($p < 0.05$). Statistical analyses were performed using GraphPad Prism software (version 10.1), and all data were expressed as mean ± standard deviation (mean ± SD). In the present study, cellular responses were evaluated based on metabolic activity measurements using the MTT assay. Although morphological assessment was not performed, the

consistent preservation of viability in the purified phage group and the absence of statistically significant differences compared to the control group suggest that no pronounced cytotoxic effects are likely to occur under the tested conditions.

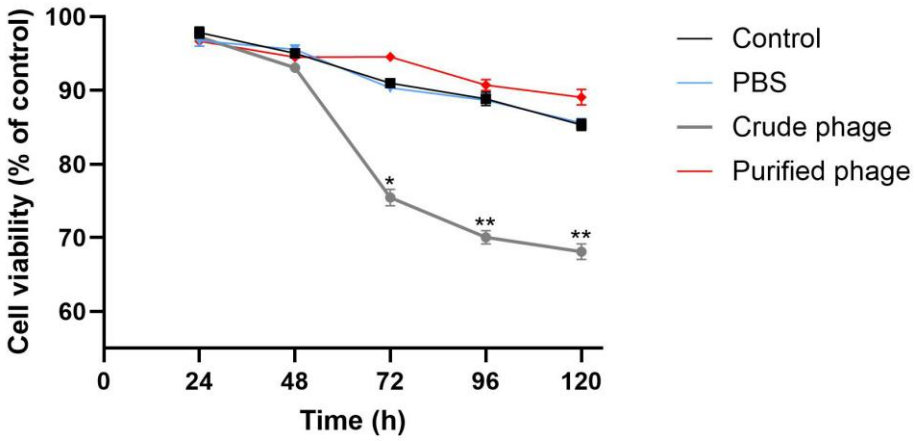

FIGURE 3 Time-dependent effects of crude and purified bacteriophage preparations on MCF-7 cell viability.

4 | DISCUSSION

Phage therapy has gained increasing attention in aquaculture as a potential alternative to antibiotics, particularly in response to the growing problem of antimicrobial resistance. However, evaluating the applicability of these approaches requires more than assessing their antimicrobial activity alone. It is also important to consider their cytocompatibility and overall biosafety profiles. Current literature shows that studies investigating the cytotoxic effects of bacteriophages against aquaculture pathogens are still limited, and most of them focus primarily on short-term exposure conditions (Türe *et al*. 2022; Altınok *et al*. 2026a; Altınok *et al*. 2026b; Wu *et al*. 2026).

In recent years, in vitro cell culture techniques have been widely used in research. The most commonly used cell lines include RTgill-W1 (gill epithelium), RTG-2 (gonadal), RTL-W1 (liver), RTS11 (macrophage), EPC (epithelial origin), ZFL (zebrafish liver), and RF (fin cell line) (Fent 2001; Segner 2004; Tan *et al*. 2007; Kolarova *et al*. 2021). However, despite their widespread use, the majority of studies focus on acute exposure windows (20–72 hours) (Ahmed *et al*. 2020; Demir *et al*. 2020; Kolarova *et al*. 2021). For example, studies conducted with the RTG-2 cell line typically rely on 20-hour EC50 measurements (Kolarova *et al*. 2021), while heavy metal studies often calculate IC50 values based on 24-hour exposure periods (Goswami *et al*. 2014). Similarly, nanoparticle-based toxicity studies generally prefer 48-hour exposure durations, during which significant cytotoxic effects may not be observed (Demir *et al*. 2020). However, a study by Hernandez-Moreno *et al*. (2022) clearly demonstrated that while no toxicity was observed at 24-hour exposure in the RTgill-W1 cell line, prolonged exposure of up to 28 days resulted in pronounced cytotoxic and metabolic effects. This finding indicates that agents considered "non-toxic" under short-term exposure may produce entirely different biological outcomes under long-term conditions.

When evaluating the effects of aquaculture-derived phage preparations at the cell line level, it is evident that existing studies are largely limited to the EPC cell line and exposure durations of 24–72 hours. In a study where YP3 and YP10S2 phages specific to *Yersinia ruckeri* were evaluated in EPC cells under 24, 48, and 72-hour exposure conditions, no significant decrease in cell viability or morphological changes were observed (Altınok *et al*. 2026a). Similarly, *Aeromonas hydrophila* phages have been reported to exhibit no cytotoxic effects in EPC and other fish cell lines under 24–72-hour exposure conditions (Türe *et al*. 2022). In a study conducted with the Lp ACM616_1 phage targeting *Lactococcus petauri*, no cytotoxicity was observed in the EPC cell line (Altınok *et al*. 2026b). Taken together, these findings suggest that aquaculture-derived phages are generally biocompatible under short-term in vitro conditions. However, the fact that nearly all these studies are limited to exposure durations of 72 hours or less suggests that the results primarily reflect acute effects.

From the perspective of mammalian cell–phage interactions, the study conducted by Wu *et al*. (2026) provides an important reference point. Our results show that *Edwardsiella tarda* phages can interact with Caco-2 cells and undergo internalization. However, cytotoxicity assessment was largely based on qualitative methods such as fluorescent live/dead staining, and quantitative metabolic analyses were not employed. In addition, the short exposure duration and the lack of systematic investigation of dose–time relationships limit the evaluation of long-term effects. Similarly, studies in the literature have shown that phages can be internalized by mammalian cells and may modulate cellular processes (Lehti *et al*.

2017; Bichet *et al*. 2021; Bichet *et al*. 2023). However, it is noteworthy that most of these studies also involve short exposure durations.

Unlike previous studies, this work quantitatively demonstrates the determining effect of long-term exposure in mammalian cells. In the control group, cell viability at 24, 48, 72, 96, and 120 hours was measured as 97.83%, 95.03%, 90.97%, 88.83%, and 85.30%, respectively, whereas in the group treated with crude phage preparations, these values were 97.23%, 93.07%, 75.47%, 70.07%, and 68.10%. A marked decrease in cell viability was observed particularly after 72 hours, and statistically significant reductions were detected at 72, 96, and 120 hours compared to the control group ($p < 0.05$). In contrast, in the group treated with purified phage preparations, cell viability was measured as 96.67%, 94.50%, 94.53%, 90.73%, and 89.07% at the same time points, with no statistically significant difference compared to the control group ($p > 0.05$). These findings indicate that cytotoxic effects are not only time-dependent but also directly related to the purity of the phage preparation.

In this context, the present study was deliberately designed based on a "worst-case biosafety model" approach. Although fish-derived cell lines (e.g., EPC, RTgill-W1) are commonly preferred in aquaculture studies, mammalian cell lines provide a more sensitive and protective model, enabling the detection of low-level cytotoxic and metabolic effects. The MCF-7 cell line is widely used and is considered a reliable model for cytotoxicity assessment due to its high reproducibility and extensive use in the literature. Furthermore, considering that aquaculture products are ultimately intended for human consumption, evaluating the potential effects of phage preparations on mammalian cells provides an additional layer of translational safety. Therefore, in this study, the use of a mammalian cell model was not intended to replace fish cell lines but rather to complement them by representing a more stringent, upper-bound risk scenario. Although a mammalian cell line was used in this study, the findings have important implications for aquaculture applications. Endotoxin contamination in bacteriophage preparations represents a general biosafety concern that is not limited to mammalian systems but is also highly relevant for fish and other aquatic organisms. In this respect, mammalian cell-based cytotoxicity assays can serve as a sensitive preliminary screening approach to evaluate the safety of phage preparations prior to their application in aquaculture settings. Furthermore, given the conserved nature of eukaryotic cellular responses to endotoxins, the observed effects may provide indirect but meaningful insights into potential responses in aquatic vertebrates, including aquatic mammals. Given that *F. psychrophilum* is a major pathogen in aquaculture, improving the biosafety of phage preparations directly supports the development of safer phage-based control strategies in fish farming systems.

Additionally, a single phage concentration (1×10^8 PFU mL^{-1}), representing a commonly used therapeutic dose in aquaculture, was selected. The primary aim of the study was to determine the time-dependent effects of long-term exposure on cytocompatibility, independent of concentration effects. Therefore, the concentration was kept constant, and exposure duration was treated as the variable parameter.

From a physiological perspective, a significant portion of these effects is thought to be associated with the endotoxin load present in the preparation. In this study, endotoxin levels were reduced from 1.0×10^{-5} EU mL^{-1} to 2.7×10^{-7} EU mL^{-1}, corresponding to a 97.3% reduction. This decrease directly correlates with the observed differences in cellular biocompatibility. Lipopolysaccharides (LPS) present in phage preparations derived from Gram-negative bacteria can activate NF-κB and MAPK signaling pathways via TLR4, thereby triggering inflammatory and metabolic responses (Zhang *et al*. 2018; Van Belleghem *et al*. 2019). Although these processes may not cause immediate cell death, under long-term exposure conditions they may result in cumulative metabolic stress, mitochondrial dysfunction, and suppression of cell proliferation.

From an aquaculture application perspective, these findings provide important translational implications. A limitation of this study is the absence of fish-derived cell lines or in vivo aquaculture models. Therefore, future studies should validate these findings using fish cell cultures and aquatic organism-based systems to further strengthen their applicability. Under real production conditions, phages are often applied repeatedly, leading to prolonged exposure of organisms. Therefore, it is considered that short-term in vitro studies alone offer limited predictive value in terms of biosafety (Duarte *et al*. 2025).

5 | CONCLUSIONS

This study shows that the cytotoxic effects of aquaculture-derived bacteriophage preparations in mammalian cells cannot be fully understood based only on short-term exposure data. While no clear toxicity was observed under short-term conditions, prolonged exposure resulted in a noticeable decrease in cell viability. These findings suggest that the biosafety of phage preparations is influenced by exposure duration and should be evaluated accordingly. Considering that repeated and long-term exposure is likely in aquaculture applications, incorporating long-term in vitro models into safety assessments is essential. In addition, the differences observed between crude and purified preparations underline the importance of production and purification processes for biosafety. Overall, these results support the need to expand current evaluation approaches by including long-term exposure analyses to ensure the safe and sustainable use of phage-

based applications in aquaculture.

CONFLICT OF INTEREST

The authors declare that they have 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..

REFERENCES

Abedon S (2011) Phage therapy pharmacology: calculating phage dosing. Advances in Applied Microbiology 77: 1–40.

Ahmed F, Soliman FM, Adly MA, Soliman HAM, El-Matbouli M, Saleh M (2020) In vitro assessment of the antimicrobial efficacy of chitosan nanoparticles against major fish pathogens and their cytotoxicity to fish cell lines. Journal of Fish Diseases 43(9): 1049–1063.

Alharbi N, Alshaikh SK (2022) Isolation and characterization of lytic phage against *Salmonella typhimurium*. Biosciences Biotechnology Research Asia 19(4): 927.

Altınok I, Türe M, Ustaoğlu D, Cebeci A, Öztürk RÇ (2026b) Genomic characterisation and in vivo therapeutic evaluation of the novel phage Lp ACM616_1 targeting *Lactococcus petauri*. Journal of Fish Diseases: e70124.

Altınok I, Türe M, Ustaoğlu D, Cebeci A, Öztürk RÇ, … Kaygusuz Ö (2026a) Isolation and characterization of novel *Yersinia ruckeri* bacteriophages for potential use in aquaculture. Aquaculture 612: 743219.

Anand T, Bera BCh, Vaid RK, Barua S, Riyesh T, … Tripathi BN (2016) Abundance of antibiotic resistance genes in environmental bacteriophages. Journal of General Virology 97(12): 3458–3466.

Avila BW, Huyvaert KP, Winkelman DL, Fetherman ER (2022) Factors affecting post-challenge survival of *Flavobacterium psychrophilum* in susceptible rainbow trout from the literature. Pathogens 11(11): 1318.

Bhat RAH, Altınok İ (2023) Antimicrobial resistance (AMR) and alternative strategies for combating AMR in aquaculture. Turkish Journal of Fisheries and Aquatic Sciences 23(11).

Bichet MC, Adderley J, Avellaneda-Franco L, Magnin-Bougma I, Torriero-Smith N, … Barr JJ (2023) Mammalian cells internalize bacteriophages and use them as a resource to enhance cellular growth and survival. PLoS Biology 21(10): e3002341.

Bichet MC, Chin WH, Richards W, Lin YW, Avellaneda-Franco L, … Barr JJ (2021) Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. iScience 24(4): 102287.

Bonilla N, Rojas MI, Netto Flores Cruz G, Hung SH, Rohwer F, Barr JJ (2016) Removal of endotoxins from bacteriophage preparations by extraction with organic solvents. PLoS ONE 11(4): e0152672.

Chen F, Wang D, Lu T, Li S (2023) Identification of a novel type II-C Cas9 from the fish pathogen *Flavobacterium psychrophilum*. Frontiers in Microbiology 14.

Cornuault JK, Petit M, Mariadassou M, Benevides L, Moncaut E, … Paepe MD (2018) Phages infecting *Faecalibacterium prausnitzii* belong to novel viral genera that help to decipher intestinal viromes. Microbiome 6(1): 65.

Demir V, Bucher J, Kropf C, Arenz M, Segner H (2020) Comparative study of cytotoxicity by platinum nanoparticles and ions in vitro systems based on fish cell lines. Toxicology in Vitro 66: 104859.

Duarte J, Pereira C, Calado R, Almeida A (2025) Phage stability research: a systematic review bridging methodological gaps and paving the way for industrial and clinical applications. PHAGE 6(3): 189–200.

Dufour N, Delattre R, Ricard JD, Debarbieux L (2017) The lysis of pathogenic *Escherichia coli* by bacteriophages releases less endotoxin than by β-lactams. Clinical Infectious Diseases 64(11): 1582–1588.

Dufour N, Delattre R, Ricard JD, Debarbieux L (2019) The lysis of pathogenic Escherichia coli by bacteriophages releases less endotoxin than by β-lactams. Clinical Infectious Diseases 68(9): 1582–1588.

Eker ED, Çetin B, Şahin NÖ (2019) In vitro cytotoxic effects and quality control studies on black cumin seed capsules and oils in Turkey market. Journal of Medical Case Reports and Reviews 2(7).

Elgendy MY, Ali SE, Dayem AA, Khalil RH, Moustafa MM, Abdelsalam M (2024) Alternative therapies recently applied in controlling farmed fish diseases: mechanisms, challenges, and prospects. Aquaculture International 32: 9017–9078.

Fent K (2001) Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicology in Vitro 15(4–5): 477–488.

Goswami M, Yadav K, Dubey A, Sharma BS, Konwar R, … Lakra WS (2014) In vitro cytotoxicity assessment of two heavy metal salts in a fish cell line (RF). Drug and Chemical Toxicology 37(1): 48–54.

Henein A, Hanlon GW, Cooper CJ, Denyer SP (2016) A partially purified *Acinetobacter baumannii* phage preparation exhibits no cytotoxicity in 3T3 mouse fibroblast cells. Frontiers in Microbiology 7: 1198.

Hernandez-Moreno D, Navas JM, Fernández-Cruz ML (2022) Short- and long-term effects of nanobiomaterials in fish cell lines: Applicability of RTgill-W1. Chemosphere 309(1): 136636.

Huang K, Nitin N (2019) Edible bacteriophage-based antimicrobial coating on fish feed for enhanced treatment of bacterial infections in aquaculture industry. Aquaculture 502: 18–25.

Islam MS, Nime I, Pan F, Wang X (2023) Isolation and characterization of phage ISTP3 for bio-control application against drug-resistant *Salmonella*. Frontiers in Microbiology 14.

Kalatzis PG, Bastías R, Kokkari C, Katharios P (2016) Isolation and characterization of two lytic bacteriophages, ϕSt2 and ϕGrn1; phage therapy application for biological control of *Vibrio alginolyticus* in aquaculture live feeds. PLoS One 11(3): e0151101.

Ketchum PA, Novitsky TJ (2000) Assay of endotoxin by *Limulus* amebocyte lysate. In: Septic Shock Methods and Protocols, pp. 3–12. Humana Press.

Kolarova J, Velisek J, Svobodova Z (2021) Comparison of in vitro (fish cell line) and in vivo (fish and crustacean) acute toxicity tests in aquatic toxicology. Veterinarni Medicina 66(8): 350–355.

Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP (2009) Enumeration of bacteriophages by double agar overlay plaque assay. In: Methods in Molecular Biology 501: 69–76.

Lallo GD, Evangelisti M, Mancuso FM, Ferrante P, Marcelletti S, … Thaller MC (2014) Isolation and partial characterization of bacteriophages infecting *Pseudomonas syringae* pv. *actinidiae*, causal agent of kiwifruit bacterial canker. Journal of Basic Microbiology 54(11): 1210–1221.

Lehti TA, Pajunen MI, Skog MS, Finne J (2017) Internalization of a polysialic acid-binding *Escherichia coli* bacteriophage into eukaryotic neuroblastoma cells. Nature Communications 8: 1915.

Magalhães PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TCV, Pessoa A (2020) Methods of endotoxin removal from biological preparations: a review. Journal of Pharmaceutical Sciences 96(6): 1471–1484.

Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Tediashvili M, … Vaneechoutte M (2009) Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS ONE 4(3): e4944.

Molina F, Simancas A, Tabla R, Gómez A, Roa I, Rebollo JE (2020) Diversity and local coadaptation of *Escherichia coli* and coliphages from small ruminants. Frontiers in Microbiology 11: 564522.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65(1–2): 55–63.

Motulsky H (2014) Intuitive biostatistics: a nonmathematical guide to statistical thinking. Oxford University Press, Oxford.

Nithin MS, Girisha SK, Boregowda KK, Ramakrishna NM, Suresh PK, … Abernathy JW (2024) Novel lytic bacteriophage AhFM11 as an effective therapy against hypervirulent *Aeromonas hydrophila*. Scientific Reports 14(1): 16882.

Pereira C, Duarte J, Costa P, Braz M, Almeida A (2022) Bacteriophages in the control of *Aeromonas* sp. in aquaculture systems: an integrative view. Antibiotics 11(2): 163.

Petsch D, Anspach FB (2000) Endotoxin removal from protein solutions. Journal of Biotechnology 76(2–3): 97–119.

Pirnay J, Djebara S, Steurs G, Griselain J, Cochez C, … Merabishvili M (2024) Personalized bacteriophage therapy outcomes for 100 consecutive cases: a multicentre, multinational, retrospective observational study. Nature Microbiology 9(6): 1434–1453.

Podlacha M, Grabowski Ł, Kosznik-Kwaśnicka K, Zdrojewska K, Stasiłojć M, … Węgrzyn A (2021) Interactions of bacteriophages with animal and human organisms– safety issues in the light of phage therapy. International Journal of Molecular Sciences 22(16): 8937.

Ramesh N, Royam MM, Manohar P, Leptihn S (2021) Application of bacteriophages and endolysins in aquaculture. Biological Control 160: 104678.

Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L (2016) Cell viability assays. In: Assay Guidance Manual.

Segner H (2004) Cytotoxicity assays with fish cells as an alternative to the acute lethality test with fish. Alternatives to Laboratory Animals 21(2): 117–124.

Strepparava N, Wahli T, Segner H, Petrini O (2014) Detection and quantification of *Flavobacterium psychrophilum* in water and fish tissue samples by quantitative real-time PCR. BMC Microbiology 14: 105.

Szermer-Olearnik B, Boratyński J (2015) Removal of endotoxins from bacteriophage preparations by extraction with organic solvents. PLoS ONE 10(3): e0122672.

Tan F, Wang M, Wang W, Lu Y (2007) Comparative evaluation of the cytotoxicity sensitivity of six fish cell lines to four heavy metals in vitro. Toxicology in Vitro 22(1):164–70.

Türe M, Cebeci A, Altınok I, Aygür E, Çalışkan N (2022) Isolation and characterization of *Aeromonas hy-*

drophila-specific lytic bacteriophages. Aquaculture 558(4): 738371.

Ustundag M, Ustundag B (2026) Antimicrobial resistance and phage sensitivity of *Chryseobacterium* sp. isolated from diseased rainbow trout (*Oncorhynchus mykiss*). Journal of Fisheries 14(1): 141207.

Van Belleghem JD, Dąbrowska K, Vaneechoutte M, Barr JJ, Bollyky PL (2019) Interactions between bacteriophage, bacteria, and the mammalian immune system. Viruses 11(1): 10.

Van Belleghem JD, Dąbrowska K, Vaneechoutte M, Barr JJ, Bollyky PL (2019) Interactions between bacteriophage, bacteria, and the mammalian immune system. Viruses 11(1): 10.

M Ustundag http://orcid.org/0000-0002-2990-4981
B Ustundag http://orcid.org/0000-0001-5211-7874

Wu Y, Ye S, Liu S, Yu J, Gao H, … Zheng J (2026) Characterization and biocontrol potential of two novel lytic phages vB_EtaS_2 and vB_EtaS_6 against *Edwardsiella tarda* in aquaculture. Aquaculture 612: 743245.

Yang L, Yang Q, Hu RG, Cong W, Li S, Kang Y (2024) The evaluation of bacteriophage therapy in aquaculture: a systematic review and meta-analysis. Aquaculture 588: 740925.

Zhang L, Hou X, Sun L, He T, Wei R, … Wang R (2018) *Staphylococcus aureus* bacteriophage suppresses LPS-induced inflammation in MAC-T bovine mammary epithelial cells. Frontiers in Microbiology 9: 1614.