

Original Research Articles

Mixed infection of *Pseudomonas aeruginosa* and Non-O1/O139 *Vibrio cholerae* in tilapia eggs: Pathogen confirmation, synergistic virulence, and effective control measures

Xianli Ke^{1,2†}, Ling Li^{1,2†}, Yan Cai^{1,2}, Jiawen Wang^{1,2}, Zhangding Zhao³, Shifeng Wang^{1,2} 

¹ Collaborative Innovation Center of Marine Science and Technology, Hainan University, Haikou, Hainan 570228, PR China, ² School of Marine Biology and Fisheries, Hainan University, Haikou, Hainan 570228, PR China, ³ Hainan Baolu Aquatic Products Technology Co., Ltd, Haikou, Hainan 571822, PR China

Keywords: egg, mixed infection, *Pseudomonas aeruginosa*, *Oreochromis niloticus*, non-O1/O139 *Vibrio cholerae*

<https://doi.org/10.46989/001c.172006>

Israeli Journal of Aquaculture - Bamidgeh

Vol. 78, Issue 4, 2026

Nile tilapia (*Oreochromis niloticus*) egg survival is critical for hatchery productivity. In March 2023, a commercial hatchery in Hainan, China, experienced severe egg mortality, with hatching rates below 10%. This study identified the causative pathogens, evaluated pathogenicity, and developed control strategies. Eight bacterial isolates from two species were recovered from diseased eggs; the most virulent isolate of each species was selected via challenge testing (three replicates, 50 eggs each), yielding two dominant strains identified as *Pseudomonas aeruginosa* TLV-01 and non-O1/O139 *Vibrio cholerae* LHV-07 based on physiological, biochemical, and 16S rRNA analyses. Both isolates showed β -haemolytic activity, and LHV-07 tested negative for major cholera toxin genes (ctxA, toxR). Artificial infection assays showed both strains were pathogenic, with 7-day LD₅₀ values of 2.0×10^8 CFU/mL (TLV-01) and 2.0×10^6 CFU/mL (LHV-07). Co-infection markedly enhanced lethality, lowering the LD₅₀ to 2.0×10^5 CFU/mL—1,000-fold lower than TLV-01 alone and 10-fold lower than LHV-07 alone. To assess deviation from expected independent effects, the Bliss independence model was applied as the primary analytical tool, with dose-response curves fitted using a four-parameter log-logistic model and forward interpolation used to estimate expected combined mortality. Results indicated weak synergism at low concentrations (1.0×10^4 – 1.0×10^5 CFU/mL), additive effects at 1.0×10^6 CFU/mL, and a lethality saturation effect at high concentrations (1.0×10^7 – 1.0×10^8 CFU/mL), likely due to microenvironmental resource limitation. The Loewe additive model, used for cross-validation, showed combination index (CI) values below 1 (0.021–0.662) across 1.0×10^4 – 1.0×10^7 CFU/mL, exceeding 1 at 1.0×10^8 CFU/mL—consistent with the Bliss model trend. These findings confirm a potentiating effect of co-infection on lethality, providing a quantitative basis for hatchery risk assessment. Antibiotic susceptibility testing revealed multidrug resistance in TLV-01, while LHV-07 remained broadly susceptible. Disinfection trials identified chloramine-T (4 mg/L) as most effective, raising hatchability to $65.2 \pm 2.5\%$ without larval deformities. This study provides the first evidence of concurrent co-infection with *P. aeruginosa* and non-O1/O139 *V. cholerae* in tilapia eggs, linked to increased mortality, and offers practical antimicrobial and disinfection strategies to improve hatchery biosecurity and the sustainability of seed production.

1. INTRODUCTION

Tilapia (*Oreochromis niloticus*) ranks as the world's second-most farmed freshwater fish, with global production exceeding 4.5 million tonnes annually.¹ Intensive aquaculture

systems (e.g., pond culture, recirculating aquaculture systems [RAS]) significantly increase microbial load and nutrient accumulation during critical developmental stages such as egg incubation.² The underdeveloped immune defenses of teleost eggs, including those of tilapia, render

† Joint first authors

a Corresponding author e-mail: 991055@hainanu.edu.cn (S. Wang)

them highly susceptible to bacterial pathogens — a vulnerability exacerbated in high-density farming.³

Among emergent pathogens, *Pseudomonas aeruginosa* (Gram-negative, ubiquitous in aquatic environments) causes severe infections in cyprinids like *Cyprinus carpio* and *Labeo rohita*.^{4,5} Its virulence arsenal—exotoxins, elastases, biofilm formation, and quorum-sensing systems—enables opportunistic pathogenesis.^{6,7} Similarly, non-O1/O139 *Vibrio cholerae* strains persist in freshwater ecosystems, and may harbor virulence-associated genes despite lacking *ctxAB*.^{8,9} These strains have triggered outbreaks in crustaceans and amphibians,^{10,11} yet their impact on teleost eggs remains uncharacterized. At the same time, it can also cause sporadic cases of gastroenteritis and extraintestinal infections in humans, and has become a public health issue.

Critically, mixed bacterial infections in intensive systems may amplify pathogenicity through synergistic mechanisms—quorum-sensing crosstalk, immunosuppression, and epithelial barrier disruption. Despite this threat, embryonic-stage co-infections are poorly documented, impeding disease management in hatcheries. We hypothesize that when *Pseudomonas aeruginosa* and non-O1/O139 *Vibrio cholerae* are present simultaneously in tilapia eggs, the lethal effect of the co-infection system differs from the lethal effects of monoinfections with either strain alone. In this study, the Bliss independent model and the four-parameter log-logistic model were employed to fit the dose-response curves for each of the two isolates separately.^{12,13} Building on this, a dual-model quantitative assessment framework was established through cross-validation by applying the Loewe additive model as an auxiliary tool (using the combination index CI as the criterion, where $CI < 1$ is defined as synergy).¹⁴

2. MATERIALS AND METHODS

2.1. STUDY SITE AND SAMPLE COLLECTION

This study was conducted at a commercial tilapia hatchery in Hainan Province, China. In 2023, mortality events occurred in all three batches of eggs at the facility. Many eggs were not scattered individually but were clustered; the dead eggs turned white and gradually dissolved, resulting in a hatch rate of less than 10%. The affected eggs were collected under sterile conditions for pathogen isolation and subsequent analysis. Healthy tilapia eggs were collected from a batch at the same hatchery that was unaffected by the mortality events. These healthy eggs were plump, uniform in color, and had intact, transparent egg membranes; no signs of whitening or dissolution were observed, and embryonic development was normal. They were used for subsequent artificial infection experiments. To prevent the spread of pathogens, the artificial infection experiments were conducted using these healthy eggs in a controlled, recirculating laboratory aquaculture system rather than directly in the hatchery environment. During the experiment, water quality parameters in the hatching water were maintained at a temperature of 30 ± 1 °C, a pH of 8.2 ± 0.5 , dis-

solved oxygen of 5 ± 1 mg/L, total ammonia (NH_3/NH_4) < 0.3 mmol/L, and salinity of 4‰.

2.2. BACTERIAL PATHOGEN ISOLATION AND PURIFICATION

Approximately 1.0 ± 0.1 g of infected tilapia eggs (equivalent to ~50 eggs) were rinsed three times with sterile phosphate-buffered saline (PBS, pH 7.4) to remove surface contaminants. The eggs were then homogenized in 1 mL of sterile PBS using a pre-chilled, sterile mortar and pestle on ice. A 100- μL aliquot of the resulting homogenate was subjected to ten-fold serial dilutions in sterile PBS, yielding dilutions from 10^{-1} to 10^{-6} . Aliquots (100 μL) of the 10^{-2} , 10^{-3} , and 10^{-4} dilutions were spread onto separate Luria-Bertani (LB) agar plates. All plates were incubated at 37°C for 24 hours. Two distinct colony morphotypes that were predominant—defined as morphologically uniform colonies constituting $\geq 40\%$ of the total colony count on the 10^{-3} dilution plate—were selected. To obtain pure cultures, each morphotype was subcultured by streaking onto fresh LB agar plates three times consecutively, and purity was confirmed by Gram staining and microscopic examination.

2.3. MOLECULAR IDENTIFICATION OF PATHOGENS

Genomic DNA was extracted from each isolate using a bacterial genomic DNA extraction kit (Tiangen Biotechnology (Beijing) Co., Ltd.). The full-length 16S rRNA gene was amplified using primers 27F and 1492R. PCR conditions were pre-denaturation at 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min; followed by a final extension at 72°C for 10 min and a 16°C hold. The PCR system (25 μL) consisted of 13 μL LA Taq, 1 μL of each primer, 1 μL DNA template, and 9 μL ddH₂O. Amplicons were sequenced by BGI Shenzhen. Sequence analysis was performed using BLAST, and phylogenetic trees were constructed using MEGA 7.0.

2.4. VIRULENCE TESTING

To assess the relative pathogenicity of the isolated strains against tilapia eggs and select the two most virulent isolates for subsequent LD₅₀ determination, the experiment comprised nine treatment groups (eight experimental groups and one negative control group), with three independent biological replicates per group; each replicate contained 50 healthy eyed-spot stage eggs. During the challenge phase, eggs from each experimental group were placed in sterile 250-mL glass beakers and immersed in 100 mL of the corresponding bacterial suspension at a concentration of 1.0×10^8 CFU/mL under gentle aeration for a 2-hour bath exposure. The control group underwent an identical 2-hour immersion in 100 mL of sterile phosphate-buffered saline (PBS). Following the challenge, all eggs were immediately transferred to a small-scale recirculating hatching system with strictly controlled water quality parameters for a 7-day observation period. Water quality in the hatching system was maintained at a temperature of 30 ± 1 °C, pH of 8.2 ± 0.5 , dissolved oxygen of 5 ± 1 mg/L, total

ammonia nitrogen ($\text{NH}_3/\text{NH}_4^+$) < 0.3 mmol/L, and salinity of 4‰. Egg mortality was recorded daily as complete opacification and whitening of the egg, accompanied by the absence of an embryonic heartbeat, observed under a dissecting microscope. Based on the 7-day cumulative mortality rate, the two most virulent strains were selected for dose-response experiments.

2.5. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISATION

The two most virulent strains (TLV-01 and LHV-07) were identified using the HuanKai Microbial Identification Kit (Guangdong Huan Kai Microbiology Technology Co., Ltd.). The testing parameters included carbohydrate fermentation (glucose, lactose, inositol), enzyme activity (lysine decarboxylase), gelatin hydrolysis, and DNA hydrolysis. The results were compared against Bergey's Manual of Systematic Bacteriology.

2.6. TYPING OF *VIBRIO CHOLERA* LHV-07

Hemolytic Activity: The strain was streaked onto 5% sheep blood agar plates (Solarbio) and incubated at 30°C for 24 hours. Haemolysis was classified as α , β , or γ , and the diameter of β -haemolysis zones was measured with a Vernier calliper.

Serum Agglutination Test: The strain in the logarithmic growth phase was resuspended in sterile PBS to 1.0×10^8 CFU/mL. Ten microlitres of this suspension were mixed with 10 μL of O1/O139 *Vibrio cholerae* antiserum (Guangzhou Siyue Biotechnology Co., Ltd Catalog No.HN1001, Lot No. 20250109) on a microscope slide. Agglutination was assessed within 60 seconds under bright-field microscopy; a positive result was defined as the formation of distinct, macroscopic clumps against a clear background, while weak or peripheral granular aggregation without central clearing was considered negative. PBS served as the negative control, and agglutination was observed.

Virulence Gene PCR: Six virulence genes (*ctxA*, *toxR*, *wbeO1*, **wbfO139*, *VcO1*, and *VcO139*) (Table 1) were detected by PCR. The reaction system (20 μL) contained 10 μL 2 $\times$ Taq Master Mix, 0.8 μL of each primer (10 μM), 2 μL DNA template, and 6.4 μL ddH₂O. PCR conditions: 95°C pre-denaturation for 5 min; 30 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. Products were visualised on 1.5% agarose gels.

Primers VcO1 and VcO139 were designed in this study based on conserved sequences within the O1- and O139-specific O-antigen gene clusters, respectively, retrieved from GenBank reference genomes (NC_002505 for O1, NC_012668 for O139). Specificity was verified in silico using BLASTn against the NCBI nr database to ensure no cross-reactivity with non-target *Vibrio* species or other common aquaculture pathogens. Due to institutional biosafety restrictions prohibiting the handling of pandemic O1/O139 strains, experimental validation employed negative controls and confirmed the absence of amplification.

2.7. ARTIFICIAL INFECTION EXPERIMENTS

Single infection: The two most virulent isolates identified in the preliminary screening (TLV-01 and LHV-07) were evaluated separately to establish dose-response relationships. For each strain, five final bacterial concentrations were tested: 2.0×10^4 , 2.0×10^5 , 2.0×10^6 , 2.0×10^7 , and 2.0×10^8 CFU/mL. Three independent biological replicates were performed for each concentration, with each replicate containing 50 healthy eyed-spot-stage eggs. The challenge was performed by immersing the eggs in 100 mL of the corresponding bacterial suspension in sterile glass beakers under gentle aeration for 2 hours; the PBS control group was processed in parallel. After the 2-hour challenge, the eggs were transferred to the aforementioned recirculating hatching system for a continuous 7-day observation period. Water quality in the small-scale hatching system was maintained as in the virulence testing. Daily mortality was recorded and 7-day dose-response curves were constructed to estimate the LD₅₀ values with their 95% confidence intervals. Dead eggs were randomly selected, homogenized, and subjected to pathogen re-isolation and molecular confirmation to fulfill Koch's postulates.

Mixed infection: To evaluate the combined pathogenicity of TLV-01 and LHV-07 in mixed infection, suspensions of the two strains were combined at a 1:1 volume ratio. The total bacterial concentration in the mixed suspensions was set at 2.0×10^4 , 2.0×10^5 , 2.0×10^6 , 2.0×10^7 , and 2.0×10^8 CFU/mL, corresponding to a final concentration of 1.0×10^4 , 1.0×10^5 , 1.0×10^6 , 1.0×10^7 , and 1.0×10^8 CFU/mL for each individual strain, respectively. For each total concentration, three independent biological replicates were prepared, each containing 50 healthy eyed-spot-stage eggs. The challenge procedure was identical: eggs were immersed in the mixed suspension for 2 hours, then transferred to the controlled recirculating hatching system for a 7-day observation period. Water quality in the small-scale hatching system was maintained as in the virulence testing. Daily mortality was recorded, and the 7-day LD₅₀ for the mixed challenge was estimated from the data. Pathogen re-isolation and molecular confirmation were performed on dead eggs as described for the single-infection experiments to verify the co-involvement of both pathogens.

To quantitatively evaluate the combined lethal effects of co-infection with TLV-01 and LHV-07, and to circumvent the dose mismatch arising from the fact that the actual inoculation concentration of each strain in the co-infection groups (1.0×10^4 – 1.0×10^8 CFU/mL) was only half of that in the corresponding single-infection groups (2.0×10^4 – 2.0×10^8 CFU/mL), direct point-to-point comparisons were avoided. Instead, a dual-model quantitative framework based on dose-response curve fitting was established, with the Bliss independent model serving as the primary quantitative assessment method and the Loewe additive model providing supplementary cross-validation. Firstly, using the log₁₀-transformed inoculation concentration as the x-axis and the 7-day mortality rate (%) as the y-axis, four-parameter log-logistic (LL.4) dose-response

Table 1. List of genes and primers used in this study¹⁵

Gene	Primers	Sequence (5'-3')	Reference
ctxA	ctxA-F	TCCGGAGCATAGAGCTTGGA	
	ctxA-R	TCGATGATCTTGAGCATTCC	
toxR	toxR-F	CCGAATAACCACCCTGATCTTT	
	toxR-R	ACCTGTGGCAATGACTTCTATC	
wbeO1	wbeO1-F	GTTGAGAAGGGCGGTCTAATAA	
	wbeO1-R	TGTCTGGTACTTGAGTTGGTAAG	
wbfO139	wbfO139-F	AGAAGCCAGTCGCAGTAAAG	
	wbfO139-R	TCGCCATCTTCCAGCATAAA	
Vc-01	Vc-01-F	GCGAGTTAATCAACCCATTTA	This study
	Vc-01-R	CAGGCCATTCATGTTCAATAT	
Vc-0139	Vc-0139-F	AGTGGTCAACCTTCGGTCTGG	This study
	Vc-0139-R	AACGTACCCAACGCTCGTGAG	

curves were fitted to the monogonal infection data for TLV-01 and LHV-07. The model equations are as follows¹²:

$$E = c + \frac{d - c}{1 + \exp [b (\log_{10}(C) - \log_{10}(EC_{50}))]}$$

Where E is the mortality rate (%), C is the inoculation concentration (CFU/mL), b is the slope factor, EC₅₀ is the median effective concentration, and c and d represent the lower and upper asymptotic limits, respectively. For TLV-01, the fitted parameters were: c≈5.2% (lower asymptote), d≈94.8% (upper asymptote), b≈-0.6 (slope factor), and EC₅₀ ≈ 2.0×10⁷ CFU/mL. For LHV-07, the fitted parameters were: c≈3.8%, d≈96.2%, b≈-1.2, and EC₅₀ ≈ 5.0×10⁶ CFU/mL. Fitting was performed using the drc function of the drc package (v3.0-1) in R software (v4.0.0) via the least-squares method.

Based on the fitted curves, forward interpolation was applied to derive the expected single-strain mortality rates E_A (TLV-01) and E_B (LHV-07) at each co-infection concentration gradient (1.0×10⁴, 1.0×10⁵, 1.0 × 10⁶, 1.0 × 10⁷, 1.0 × 10⁸ CFU/mL). The interpolated results for E_A were 13.80%, 17.80%, 30.18%, 38.49%, and 46.29%, respectively; for E_B, they were 17.09%, 30.09%, 36.10%, 54.47%, and 71.79%, respectively. The expected combined mortality rate was then calculated according to the general Bliss mortality formula¹⁶:

$$E_{\text{expected}} = E_A + E_B - E_A \times E_B$$

The calculated E_{expected} values at the five concentration points were 28.53%, 42.53%, 55.38%, 72.00%, and 84.85%, respectively. If the observed mortality rate E_{obs} is higher than E_{expected}, a synergistic trend is indicated; if close to E_{expected}, an additive effect is indicated; if significantly lower than E_{expected}, the deviation is interpreted as a high-density saturation effect due to microenvironmental resource limitations. For statistical evaluation, based on three independent replicates (N=150 per concentration point), the observed mortality frequencies were compared with the expected mortality frequencies based on E_{expected} using the Chi-square (χ²) test, with p<0.05 considered statistically significant.

As a complementary validation, the Loewe additivity model was also employed. Using the observed mortality rate E_{obs} as the target effect level, the equivalent single-agent concentrations D_A (TLV-01) and D_B (LHV-07) required for each strain alone to achieve this mortality level were derived by log-linear interpolation on the fitted curves¹⁷:

$$\log_{10}(D) = \log_{10}(C_1) + \frac{E_1 - E_{\text{obs}}}{E_1 - E_2} \times [\log_{10}(C_2) - \log_{10}(C_1)]$$

When E_{obs} fell outside the known data range, linear extrapolation using the terminal slope was applied. The Combination Index (CI) was then calculated as: CI = $\frac{d_A}{D_A} + \frac{d_B}{D_B}$. Where d_A and d_B are the actual final inoculation concentrations of TLV-01 and LHV-07 in the co-infection groups (both at 1.0 × 10⁴, 1.0 × 10⁵, 1.0 × 10⁶, 1.0 × 10⁷, and 1.0 × 10⁸ CFU/mL, respectively). CI < 1 indicates a synergistic trend, CI ≈ 1 indicates additivity, and CI > 1 indicates a deviation trend. The 95% confidence intervals of the CI values were estimated by bootstrap resampling (1000 resamples, with experimental replicates as sampling units). The Loewe model results were used solely as cross-validation references for the primary Bliss model conclusions.¹⁴

2.8. ANTIBIOTIC SUSCEPTIBILITY TESTING

In accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2024), antimicrobial susceptibility testing was performed using the disc diffusion (Kirby-Bauer) method. A bacterial suspension at a concentration of 1.0 × 10⁸ CFU/mL was prepared and evenly spread onto Mueller-Hinton (M-H) agar plates (Beijing BioDee Biotechnology Co., Ltd.). Antibiotic discs (Hangzhou Microbial Reagents Co., Ltd.) were placed on the plates, which were then incubated at 37°C for 18–24 hours. The diameter of the inhibition zone was measured (to the nearest 0.1 mm) and classified into three categories—sensitive (S), moderately sensitive (I) or resistant (R)—in accordance with CLSI standards. Each antibiotic was tested in triplicate. The use of antibiotic discs containing substances prohibited or

suspended for use in aquaculture in China was strictly for laboratory research and not for any commercial applications. It should be noted that the incubation temperature of 37°C was selected to align with standard clinical protocols for human pathogens as defined by CLSI; however, this differs from the typical growth temperature (25–30°C) of these vibrios in aquaculture environments. Consequently, the interpretive criteria (breakpoints) applied herein are those established for clinical isolates and may not fully reflect the in situ antimicrobial response under fish farming conditions.

2.9. EFFICACY ASSESSMENT OF ANTIMICROBIAL AGENTS ON TILAPIA EGG HATCHING

During this outbreak of fish egg disease, we collected fish eggs specifically for this purpose and incubated them alongside various common commercial antimicrobial agents, including environmental disinfectants and therapeutic antibiotics, to identify emergency interventions. Six treatment groups were established, each with three replicates: control (CT, PBS), enrofloxacin (EN, 4 mg/L), iodine (PO, 20 mg/L), bromochlorohydrin (DI, 10 mg/L), chloramine-T (LT, 4 mg/L), and tetracycline (TE, 4 mg/L). Each replicate consisted of ~300 eggs in 30 mL of water in the hatching chamber of a recirculating system connected to a 1.25 L reservoir. Disinfectants were continuously applied for 2 days, with 50% water changes daily. Hatching rates were calculated after 7 days.

$$\text{Hatching rate (\%)} = \frac{\text{Number of surviving fry}}{\text{Total number of eggs}} \times 100$$

2.10. DATA PROCESSING AND ANALYSIS

Experimental data in this study were analyzed and plotted using SPSS 27.0 (IBM Corp., Armonk, NY, NY, USA) and GraphPad Prism 10.0.1 (GraphPad Software, San Diego, CA, USA). Probit regression analysis was performed to estimate the median lethal dose (LD₅₀) and its 95% confidence interval for the *Pseudomonas aeruginosa* TLV-01 single-infection group, the *Vibrio cholerae* LHV-07 single-infection group, and the co-infection group, respectively. The differences in survival curves between groups were compared using the Log-rank (Mantel-Cox) test. For the Bliss model, synergy was assessed by comparing observed and expected mortality frequencies at each concentration using the Chi-square (χ²) test. For the Loewe model, the statistical reliability of CI values was evaluated by estimating 95% confidence intervals via bootstrap resampling (1000 resamples, with experimental replicates as sampling units). *P* < 0.05 was considered statistically significant.

3. RESULTS

3.1. ISOLATION OF PATHOGENS

After 24 hours of incubation on LB agar, multiple morphologically distinct colonies were observed. Two colony types predominated:



Figure 1. Colony Morphology of *Pseudomonas aeruginosa*



Figure 2. Colony Morphology of *Vibrio cholerae*

1. colonies with a slightly raised centre, circular shape, moist surface, relatively small size, and a noticeable odour (Fig. 1);
2. colonies that were smooth, moist, flat, translucent, larger, and with well-defined margins (Fig. 2).

Four colonies were selected from each colony type, yielding eight isolates in total. These isolates were designated TLV-01, TLV-02, TLV-03, TLV-04, LHV-05, LHV-06, LHV-07, and LHV-08.

3.2. PATHOGEN IDENTIFICATION

PCR amplification using universal 16S rRNA primers produced ~1500 bp fragments from all eight isolates. BLAST analysis confirmed taxonomic identity as follows:

- i. TLV group (4 strains): >97.50% similarity with *Pseudomonas aeruginosa*
- ii. LHV group (4 strains): >98.95% similarity with *Vibrio cholerae*

Phylogenetic analysis further supported these results. TLV isolates grouped with *P. aeruginosa* strain RDLN43 (Fig. 3), while LHV isolates clustered with *V. cholerae* strain RC782 (Fig. 4).

3.3. PATHOGENICITY TESTING

All eight isolates (TLV-01, TLV-02, TLV-03, TLV-04, LHV-05, LHV-06, LHV-07, and LHV-08) caused mortality in tilapia eggs. The most virulent strains, TLV-01 and LHV-07, were selected as representative strains for the LD₅₀ artificial infection assay. These correspond to the dominant isolates of *Pseudomonas aeruginosa* and *Vibrio cholerae*, respectively. The morphological and biochemical characteristics of the re-isolated strains were fully consistent with those of the original inoculum. Furthermore, all re-isolated strains were also confirmed by 16S rRNA gene sequencing, thus fulfilling the Koch's postulates. (Fig. 5).

Fig.5 The 7-day mortality rate in tilapia eggs infected with different bacterial isolates.

3.4. BIOCHEMICAL PROFILING

Biochemical assays showed that both bacterial groups fermented glucose, lactose, maltose, sucrose, sorbitol, and silicon and were positive for gelatine and cellobiose hydrolysis. Neither group utilized xylose, maltose, or β -galactosidase, nor showed activity for Simon's citrate, lysine decarboxylase, or ornithine decarboxylase. A distinguishing feature was inositol utilization: TLV-01 did not utilize inositol; LHV-07 did, which aligns with the biochemical characteristics of *Pseudomonas aeruginosa* and *Vibrio cholerae*, respectively.

3.5. TYPING OF *VIBRIO CHOLERA*

Both pathogens produced β -haemolysis on blood agar. Haemolytic zones measured 2.05 ± 0.08 cm for TLV-01 (Fig. 6A) and 1.60 ± 0.05 cm for LHV-07 (Fig. 6B). Serological agglutination tests showed that LHV-07 did not agglutinate with antisera from the O1/O139 serogroup, confirming that it belongs to a non-O1/O139 serogroup. PCR screening confirmed that it does not carry virulence genes from the O1 or O139 *Vibrio cholerae* serogroups tested.

3.6. PATHOGENICITY AND INTERACTION ANALYSIS

TLV-01 Infection: Following infection, the mortality rate of fish eggs gradually increased as bacterial concentration rose; mortality rates in all groups stabilized between 5 and 7 days. Its 7-day half-lethal concentration was 2.0×10^8 CFU/mL (Fig. 7).

LHV-07 infection: The mortality rate continued to rise following infection, and the survival rate of fish eggs was

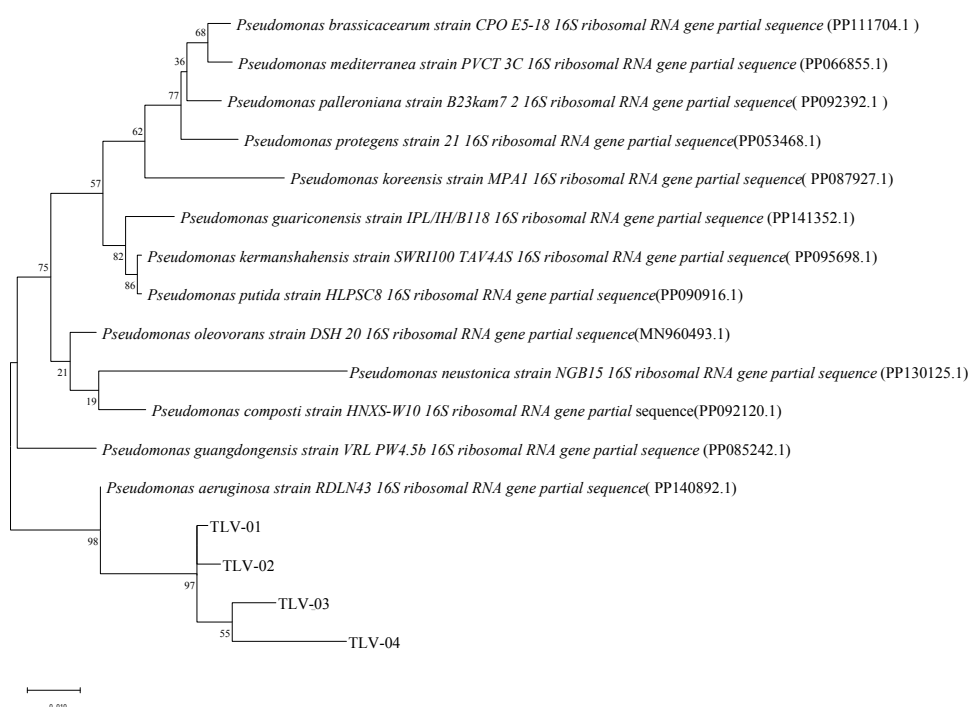


Figure 3. Phylogenetic tree of *Pseudomonas aeruginosa* based on 16S rRNA gene sequences.

Phylogenetic tree analysis of t based on 16S rRNA nucleotide sequences using the MEGA software and the nearest-neighbor method. The numbers next to the branches indicate the percentage values from 1,000 bootstrap replicates.

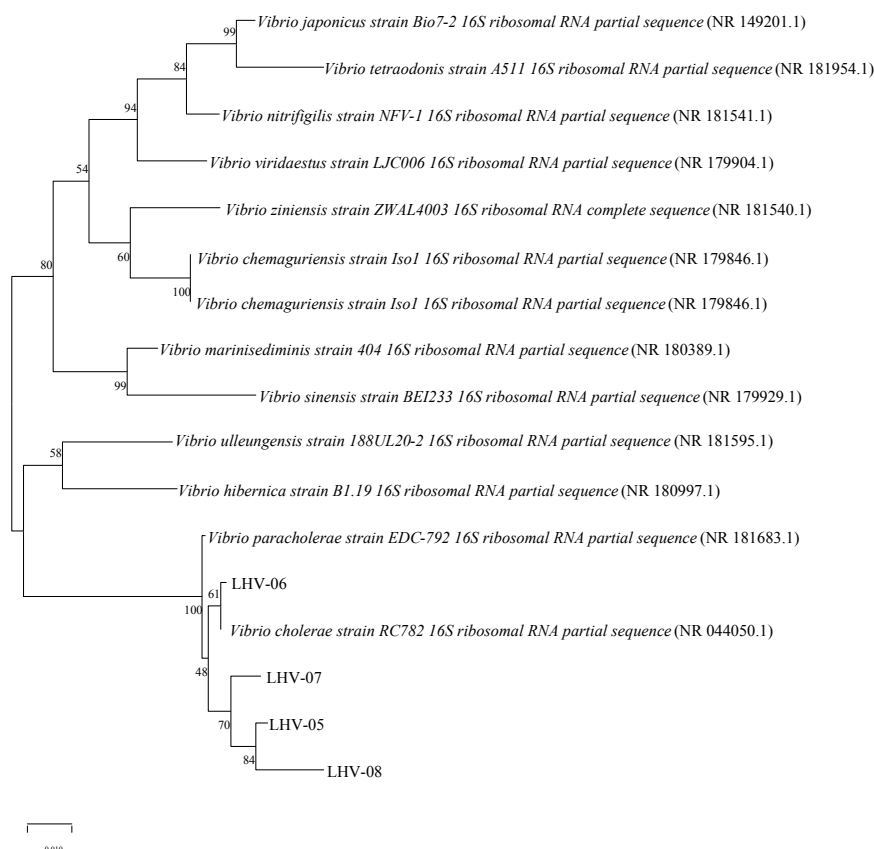


Figure 4. Phylogenetic tree of *Vibrio cholerae* based on 16S rRNA gene sequences.

Phylogenetic analysis of *Vibrio cholerae* based on 16S rRNA nucleotide sequences using the nearest-neighbor-joining method in MEGA software. The numbers next to the branches indicate the percentage values based on 1,000 bootstrap replicates.

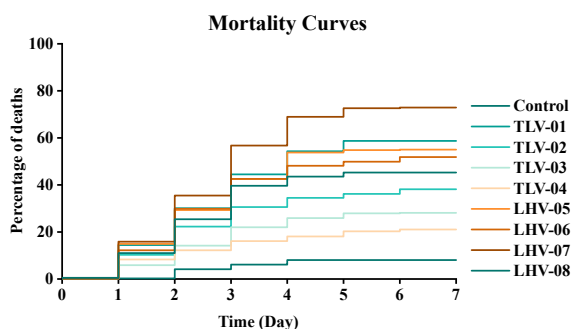


Figure 5. The 7-day mortality rate in tilapia eggs infected with different bacterial isolates.

significantly reduced in all infected groups (Fig. 8). The higher the concentration of the pathogenic strain, the more rapidly the mortality curve for tilapia eggs in the corresponding experimental group rose. The 7-day half-lethal concentration was 2.0×10^6 CFU/mL.

The mixed challenge with TLV-01 + LHV-07 yielded an estimated 7-day LD₅₀ of 2.0×10^5 CFU/mL, which was lower than the corresponding single-strain LD₅₀ estimates for

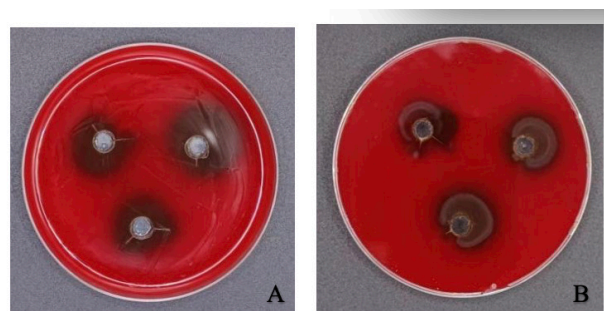


Figure 6. Hemolysis identification test: A. *Pseudomonas aeruginosa* TLV-01; B. non-O1/O139 *Vibrio cholerae* LHV-07.

LHV-07 (2.0×10^6 CFU/mL) and TLV-01 (2.0×10^8 CFU/mL) (Fig. 9). This comparison indicates enhanced lethality of the mixed challenge at the dose-response level, but the reduction in LD₅₀ alone was not used as evidence of statistical synergism. Aggregation and attachment of eggs were also observed during the infection experiments.

Based on the four-parameter Log-logistic fitted curves and forward interpolation, the expected single-strain mortality rates (E_A , E_B) of TLV-01 and LHV-07 at each co-infection concentration were derived, and the expected combined mortality rate was calculated according to the general Bliss mortality formula. The results are summarized in

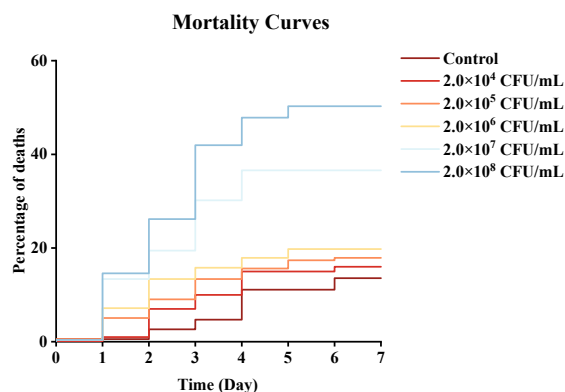


Figure 7. The 7-day mortality rate of tilapia eggs infected with *Pseudomonas aeruginosa* TLV-01 at different concentration gradients.

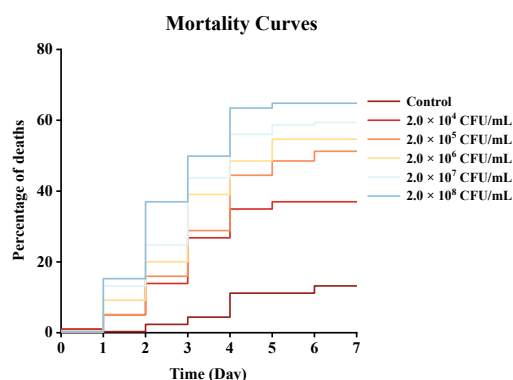


Figure 9. The 7-day mortality curves for tilapia eggs infected with a 1:1 mixture of *Pseudomonas aeruginosa* TLV-01 and non-O1/O139 *Vibrio cholerae* LHV-07 at varying concentration gradients.

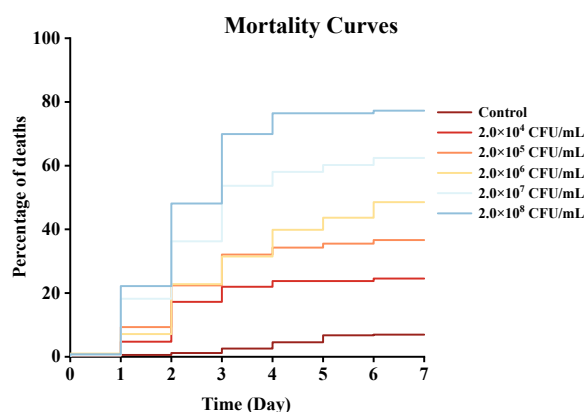


Figure 8. The 7-day mortality rate of tilapia eggs infected with non-O1/O139 *Vibrio cholerae* LHV-07 at serial concentrations.

Table 2. In the low concentration range (1.0×10^4 – 1.0×10^5 CFU/mL), the observed mortality rates (35 %, 47 %) were slightly higher than the expected mortality rates (28.53 %, 42.53 %), suggesting a synergistic trend. At a concentration of 1×10^6 CFU/mL, the observed mortality rate (54 %) was almost exactly in line with the expected mortality rate (%), strictly conforming to the additive model.

To cross-validate the results obtained from the Bliss model, the Loewe additivity model (Combination Index, CI) was additionally employed as a complementary analysis. The results are summarized in [Table 3](#). Within the concentration range of 1.0×10^4 – 1.0×10^7 CFU/mL, all CI values were below 1 (ranging from 0.021 to 0.662), and the upper limits of the 95% guided confidence intervals were all below 1, indicating a trend towards synergism. The lowest CI value (CI = 0.021) was observed at 1.0×10^5 CFU/mL, indicating the strongest potential for synergism at this con-

centration. At 1.0×10^7 , CI = 0.662 (95% CI: 0.581–0.728), which remains well below 1.

3.7. ANTIBIOTIC SENSITIVITY

TLV-01 was sensitive to 12 antibiotics, moderately responsive to 10, and resistant to 8. The most effective agents were: Chloramphenicol (7.14 ± 0.57 mm) (Prohibited Drugs), Trimethoprim (7.35 ± 0.36 mm), and Streptomycin (7.83 ± 0.31 mm). Resistant antibiotics included Enrofloxacin (29.70 ± 0.16 mm), Ciprofloxacin (21.52 ± 0.10 mm) (Prohibited Drugs), Ofloxacin (19.73 ± 0.63 mm) (Prohibited Drugs).

LHV-07 showed high sensitivity to 6 antibiotics, intermediate sensitivity to 9, and resistance to 15. The greatest inhibition was observed with: Trimethoprim (0.00 ± 0.00 mm), Polymyxin B (6.21 ± 1.16 mm), and Furazolidone (8.35 ± 0.59 mm) (Prohibited Drugs). Resistance was recorded against Ofloxacin (33.13 ± 0.39 mm) (Prohibited Drugs), Ciprofloxacin (29.97 ± 0.99 mm) (Prohibited Drugs), Chloramphenicol (28.67 ± 0.70 mm) (Prohibited Drugs) ([Table 4](#)).

3.8. DISINFECTION EFFICACY

The highest egg survival rate occurred with 4 mg/L chloramine-T ($65.2 \pm 2.5\%$), followed by 4 mg/L enrofloxacin ($60.1 \pm 1.8\%$), 10 mg/L bromochlorohydrin ($58.5 \pm 2.1\%$), 20 mg/L iodine ($54.7 \pm 2.2\%$), and 4 mg/L tetracycline ($52.3 \pm 1.9\%$).

The control group showed the lowest survival rate ($48.9 \pm 2.3\%$). No deformities were observed in LT, EN, or DI treatments, indicating that these concentrations are safe and effective for tilapia egg disinfection ([Fig.10](#)).

4. DISCUSSION

Through a comprehensive analysis, including examination of colony morphology, physiological and biochemical char-

Table 2. Comparison of Bliss-expected combined mortality and observed co-infection mortality

Co-infection (CFU/mL)	TLV-01 expected mortality E_A (%)	LHV-07 expected mortality E_B (%)	Bliss expected mortality E_{expected} (%)	Observed co-infection mortality E_{obs} (%)	Observed - expected	χ^2 value	p-value	Interaction trend interpretation
1.0×10^4	13.80	17.09	28.53	35.00	6.47	3.08	>0.05	Weak synergy
1.0×10^5	17.80	30.39	42.53	47.00	4.17	1.22	>0.05	Weak synergy
1.0×10^6	30.18	36.10	55.38	54.00	-1.38	0.12	>0.05	Strictly additive
1.0×10^7	38.49	54.47	72.00	59.00	-13.00	12.57	<0.001	High density saturation effect
1.0×10^8	46.29	71.79	84.85	62.00	-22.85	61.07	<0.001	High density saturation effect

Note: $E_{\text{expected}} = E_A + E_B - E_A \times E_B$;

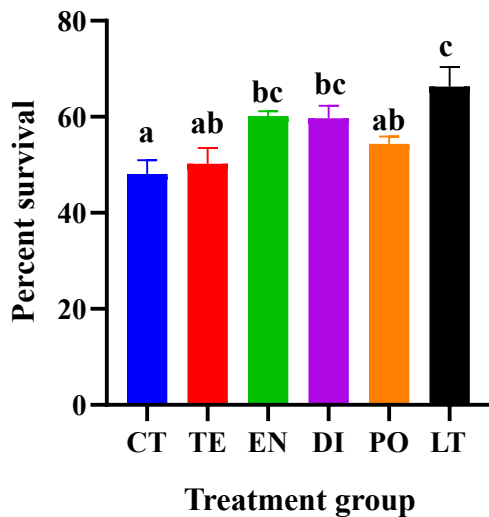


Figure 10. Effects of different disinfectant treatments on the hatching rate of tilapia eggs after bacterial challenge.

CT: control group treated with sterile PBS; EN: enrofloxacin (4 mg/L); PO: iodine (20 mg/L); DI: bromochlorohydrin (10 mg/L); LT: chloramine-T (4 mg/L); TE: tetracycline (4 mg/L). Eggs were experimentally exposed to a mixed bacterial infection of *Pseudomonas aeruginosa* TLV-01 and *Vibrio cholerae* LHV-07. Disinfectants were applied continuously for 48 h under recirculating aquaculture conditions. Hatching rates were recorded after 7 days.

acteristics, and 16S rRNA sequencing, this study confirmed that the primary pathogens isolated from the eggs of diseased tilapia were *Pseudomonas aeruginosa* and non-O1/O139 *Vibrio cholerae*. To our knowledge, this study is the first to report a mixed pathogen infection involving both *Pseudomonas aeruginosa* and non-O1/O139 *Vibrio cholerae* in the eggs of farmed fish, thereby extending the known host range of these two pathogens from fry and adult fish to the embryonic stage.

Previous studies have shown that *Pseudomonas aeruginosa* causes erythema, abdominal swelling, corneal opacity, scale loss, and gill inflammation in tilapia¹⁸; gill ischemia

and hepatopancreatic congestion in crucian carp (*Carassius auratus gibelio*)¹⁹; and hemorrhagic lesions in the abdomen and pallor of the liver in grass carp (*Ctenopharyngodon idella*).²⁰ Furthermore, non-O1/O139 *Vibrio cholerae* have been reported in tilapia, loach (*Misgurnus anguillicaudatus*),²¹ giant freshwater prawn (*Macrobrachium rosenbergii*),²² and the American bullfrog (*Rana catesbeiana*)²³; however, to date, there have been no reports of separate or mixed infections of tilapia eggs with *Pseudomonas aeruginosa* and non-O1/O139 *Vibrio cholerae*. Our findings indicate that fertilized eggs are equally susceptible, likely due to weakened innate immune defenses during early developmental stages. The ability of *Vibrio cholerae* to colonize tilapia during early development poses new challenges for disease control in hatcheries.

The estimated LD₅₀ values obtained from the mixed-infection trials were significantly lower than those from any of the single-strain infection trials, suggesting that the lethality of mixed infections is enhanced at the dose-response level. This finding is consistent with previous reports that mixed bacterial infections can alter the severity of disease in farmed fish.^{24,25} However, a reduction in LD₅₀ values alone does not prove the presence of synergism. Furthermore, when the concentrations of each strain in the mixed-infection group were only half those in the single-strain group, the Bliss independence model based on the 'equal-dose assumption' could not perform direct point-to-point statistical comparisons. To address this methodological limitation, this study abandoned the traditional strategy of direct point-to-point comparisons and instead obtained the expected combined mortality rates at various concentration points of the mixed infection through four-parameter log-logistic fitting and forward interpolation.²⁶

The Bliss model indicates that, within the low-concentration range (1.0×10^4 – 1.0×10^5 CFU/mL), mortality rates tend to be higher than expected, suggesting that mixed infections at low concentrations primarily exhibit weak synergistic effects. At medium concentrations (1.0×10^6), the observed results are almost entirely consistent with expectations, strictly adhering to the additive model, which fur-

Table 3. Auxiliary Combination Index (CI) analysis based on the Loewe additivity model

Co-infection (CFU/mL)	$d_A = d_B$ (CFU/mL)	E_{obs} (%)	TLV-01 equivalent (D_A) (CFU/ mL)	LHV-07 equivalent (D_B) (CFU/mL)	D_A/d_A	D_B/d_B	CI	95%CI	Interaction trend interpretation
1.0×10^4	1.0×10^4	35	2.0×10^6	4.3×10^5	200	43	0.028	(0.025,0.031)	Synergy
1.0×10^5	1.0×10^5	47	1.2×10^8	5.0×10^6	1200	50	0.021	(0.018,0.025)	Synergy
1.0×10^6	1.0×10^6	54	7.1×10^8	9.6×10^6	718	9.6	0.106	(0.095,0.118)	Synergy
1.0×10^7	1.0×10^7	59	2.6×10^9	1.5×10^7	258	1.5	0.662	(0.581,0.728)	Synergy
1.0×10^8	1.0×10^8	62	5.6×10^9	2.0×10^7	56	0.2	5.018	(4.523,5.612)	High density deviation trend

Note: D_A : Derived from TLV-01 curve interpolation/extrapolation targeting E_{obs} ; D_B : Derived from LHV-07 curve interpolation/extrapolation targeting E_{obs} ; $CI = d_A/D_A + d_B/D_B$; 95%CI: Bootstrap resampling (1000 resamples, with experimental replicates as sampling units); When $CI > 1$, this indicates that the equivalent dose increment is significantly lower than the actual dose.

Table 4. Antibiotic Susceptibility Profile of *Pseudomonas aeruginosa* TLV-01 and *Vibrio cholerae* LHV-07

Antibiotics	Dose of disk Potency (µg / slip tablet)	Criteria for determining sensitivity to antibiotic zones of inhibition (mm)			<i>Pseudomonas aeruginosa</i> TLV-01		Non-O1, non-O139 <i>Vibrio cholerae</i> LHV-07	
		Sensitivity	Intermediately sensitive	Resistant	Inhibition diameter (mm)	Results	Inhibition diameter (mm)	Results
Meloxicillin	75	≤17	--	≥18	18.73±0.09	R	22.04±0.68	R
Amoxicillin	20	≤13	14-17	≥18	19.12±0.24	R	25.14±0.92	R
Cefotiam	30	≤14	15-17	≥18	15.75±1.08	I	21.91±0.75	R
Cefalexin	30	≤14	15-17	≥18	12.11±1.00	S	16.49±0.42	I
Cefazolin	30	≤14	15-17	≥18	17.80±0.50	I	19.88±1.59	R
Cefaladin	30	≤14	15-17	≥18	15.58±0.45	I	16.99±1.11	I
Cefoperazone	75	≤15	16-20	≥21	18.70±0.48	I	21.89±1.19	R
Cefotaxime	30	≤14	15-22	≥23	15.45±0.64	I	26.03±0.10	R
Ceftazidime	30	≤14	15-17	≥18	16.87±0.39	I	18.85±0.57	R
Gentamicin	10	≤12	13-14	≥15	15.67±0.63	R	13.99±0.41	I
Kanamycin	30	≤13	14-17	≥18	8.45±0.28	S	15.45±1.55	I
Tobramycin	10	≤12	13-14	≥15	14.24±0.25	I	15.45±1.55	S
Neomycin	30	≤12	13-16	≥17	9.23±0.48	S	15.06±1.35	I
Streptomycin	10	≤11	12-14	≥15	7.83±0.31	S	12.45±1.15	I
Erythromycin	15	≤13	14-22	≥23	12.63±1.44	S	20.34±0.75	I
Medimycin	30	≤13	14-17	≥18	11.40±0.97	S	18.29±1.72	R
Tetracycline	30	≤14	15-18	≥19	12.48±0.45	S	17.19±1.39	I
Ciprofloxacin	5	≤15	16-20	≥21	21.52±0.10	R	29.97±0.99	R
Enoxacin	10	≤14	15-17	≥18	15.19±0.42	I	25.80±0.90	R
Ofloxacin	5	≤12	13-15	≥16	19.73±0.63	R	33.13±0.39	R
Chloramphenicol	30	≤12	13-17	≥18	7.14±0.57	S	28.67±0.70	R
Enrofloxacin	10	≤21	22-28	≥29	29.70±0.16	R	28.22±0.44	R
Clindamycin	2	≤14	15-20	≥21	12.94±0.32	S	10.47±0.17	S
Furantoin	300	≤14	15-16	≥17	15.81±0.41	I	11.99±0.70	S
Furazolidone	300	≤14	15-16	≥17	9.67±0.37	S	8.35±0.59	S
Norfloxacin	10	≤12	13-16	≥17	14.97±0.23	R	27.51±1.10	R
Polymyxin B	300	≤8	8-11	≥12	12.47±0.23	R	6.21±1.16	S

Antibiotics	Dose of disk Potency (μg / slip tablet)	Criteria for determining sensitivity to antibiotic zones of inhibition (mm)			<i>Pseudomonas aeruginosa</i> TLV-01		Non-O1, non-O139 <i>Vibrio cholerae</i> LHV-07	
		Sensitivity	Intermediately sensitive	Resistant	Inhibition diameter (mm)	Results	Inhibition diameter (mm)	Results
Trimethoprim	5	≤ 10	11-15	≥ 16	7.35 $\pm$ 0.36	S	0.00 $\pm$ 0.00	S
Florfenicol	30	≤ 12	13-17	≥ 18	11.94 $\pm$ 0.65	S	22.43 $\pm$ 1.35	R
novobiocin	30	≤ 12	13-16	≥ 17	15.30 $\pm$ 0.12	I	14.14 $\pm$ 1.27	I

Note: S: susceptible; I : intermediate; R: resistant

ther confirms the predictability of the effects within this concentration range. Within the high-concentration range (1.0×10^7 – 1.0×10^8), the observed mortality rate was significantly lower than the extreme values predicted based on independent action. This phenomenon should be regarded as a ‘mortality saturation effect’ resulting from limited resources—such as space, nutrients and oxygen—in the infection microenvironment under high-density conditions, rather than a biologically significant negative interaction between the two strains. When pathogen density exceeds the carrying capacity of the microenvironment, bacterial proliferation is constrained by physical space and nutrient depletion; due to excessive density, quorum sensing signals may trigger a feedback-mediated downregulation of virulence factors, thereby preventing the actual mortality rate from reaching the extreme levels predicted by the independent action theory.²⁷ As supplementary validation, the CI values derived from the Loewe additive model were consistently below 1 within the low-to-medium concentration range (1.0×10^4 – 1.0×10^7), indicating a trend towards synergy. It should be noted, however, that this model is based on the ‘common target’ hypothesis, whereas the Bliss model is based on the ‘independent target’ hypothesis; consequently, the two models employ different evaluation criteria.²⁸ The Bliss cross-validation results support the conclusion of synergism in the low-concentration range (1.0×10^4 – 1.0×10^5 CFU/mL), but show discrepancies with the Loewe model’s output in the high-concentration range (1.0×10^6 – 1.0×10^8 CFU/mL). This discrepancy may also be attributable to the limitations of the two models in this study. The limitations of the Bliss independent model are primarily reflected in its core assumption: that the lethal effects of the two bacterial strains on the host act on mutually independent targets or pathways.²⁸ However, both TLV-01 and LHV-07 exert their pathogenic effects by infecting tilapia eggs; they share similar host tissue targets (embryonic cells in fish eggs) and a common pathogenic process (adhesion, invasion, toxin secretion, tissue destruction). This suggests that the ‘independent action’ assumption of the Bliss model may be too restrictive in the context of this study. When two bacterial strains share the same limited host resources, the Bliss model tends to overestimate the expected combined mortality rate under high-density conditions. This is precisely one possible reason why, in this study, the mortality rate observed in the concentration range of 1.0×10^7 – 1.0×10^8 CFU/mL was significantly lower than the extreme predicted values: the Bliss model ‘allows’ both strains to independently reach their theoretical maximum effect, but the actual microenvironment cannot simultaneously support both strains in reaching this extreme level. Furthermore, the Bliss model is, by its nature, a static probabilistic model that cannot capture the temporal dynamics of the infection process (such as differences in the proliferation rates of the two bacterial strains, the sequence of colonization, and the time window of the host immune response), which may lead to an oversimplification of the actual interaction patterns described by the model. Secondly, due to the lack of measured single-strain mortality data for TLV-01 and LHV-07 at mixed con-

centrations in this study, the calculation of Bliss predicted values relied on the fitting of a four-parameter log-logistic model and forward interpolation. Although the LL.4 model is widely accepted in toxicological dose–response analysis, the interpolation process in the high-concentration range of 1.0×10^7 – 1.0×10^8 —which lies at the ends of the two single-strain curves—inevitably introduces estimation errors. The accuracy of the interpolation is limited by the experimental design, which involved only five discrete concentration gradients for single-strain infection; minor fluctuations at the ends of the curves may be amplified by the interpolation process, thereby affecting the precision of the Bliss expected values in the high-concentration range.²⁹ The Loewe model is based on the principle of dose additivity, assuming that the mechanisms of action of two formulations are identical or extremely similar, and that the superposition of effects follows the logic of ‘equivalent dose substitution’. However, TLV-01 and LHV-07 exhibit fundamental differences in terms of taxonomy, pathogenicity profiles, host colonization strategies and quorum sensing systems; they are not ‘homologous’ or ‘same-target’ formulations. Theoretically, the applicability of this model is limited. Consequently, this study positions the Loewe model as a supplementary validation tool rather than a primary model; this is a proactive measure taken precisely to circumvent this theoretical limitation.³⁰ Secondly, this study tested only a 1:1 mixing ratio of TLV-01 and LHV-07; however, the accurate application of the Loewe model typically requires complete dose-response data for multiple fixed ratios (e.g. 1:1, 1:3, 3:1, etc.).²⁸ Although confidence interval (CI) values for a single ratio (e.g. CI = 5.018 at a concentration of 1.0×10^8 in this study) may indicate a trend towards interaction at that ratio, they cannot fully reflect the synergistic interaction patterns between the two bacterial strains across all possible ratio combinations.

Although there are discrepancies between the experimental results of the two models in this study, these do not indicate contradictory findings; rather, they strongly support the view that these two pathogens do not act on independent targets, but rather act in concert on the same set of susceptible pathways in tilapia eggs. One possible mechanism is cross-communication between the quorum-sensing systems of the two pathogens.³¹ Signaling molecules such as 3-oxo-C12-HSL from *Pseudomonas aeruginosa* and AI-2 from *Vibrio cholerae* could potentially alter virulence-factor expression within the shared infection microenvironment.³² This proposed mechanism remains hypothetical and requires direct experimental validation. Direct experimental evidence is still needed to determine whether quorum-sensing cross-communication occurs between *Pseudomonas aeruginosa* and *Vibrio cholerae* in the tilapia egg infection model and whether it contributes to concentration-dependent interaction. Furthermore, aggregation of infected eggs may increase local bacterial exposure and facilitate pathogen transmission within the population. Whether this physical aggregation is driven by bacterial products, host responses, or both, and how it contributes to mixed-infection pathogenesis, warrants further investigation.

This study represents the first systematic investigation of the antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* TLV-01 and non-O1/O139 *Vibrio cholerae* LHV-07 isolated from tilapia eggs. The results indicate that TLV-01 exhibits a typical pattern of multidrug resistance, being resistant to eight antibiotics, including fluoroquinolones and polymyxin B. In contrast, LHV-07 was resistant to 15 antibiotics, including florfenicol, but was susceptible to polymyxin B. It should be emphasised that the susceptibility/resistance data for chloramphenicol and furazolidone are of purely academic significance, as the use of both compounds is strictly prohibited in aquaculture production in China (Ministry of Agriculture and Rural Affairs Announcement No. 250).

The resistance of TLV-01 to most aminoglycosides except gentamicin likely reflects enhanced efflux pump activity, consistent with classical resistance mechanisms in *P. aeruginosa*. The inherent resistance of *Pseudomonas aeruginosa* is closely associated with the synergistic effects of its outer membrane's low permeability and various efflux pump systems.^{33,34} The ability to form biofilms may further exacerbate phenotypic resistance by limiting antibiotic penetration into the deeper layers of the biofilm, but the specific contribution of efflux pumps still needs to be verified through experiments with efflux pump inhibitors.³⁵ The polymyxin B resistance observed in LHV-07 is particularly concerning as it may limit therapeutic options. However, the high susceptibility of both strains to fluoroquinolones suggests this antibiotic class could serve as an effective treatment option for bacterial infections in tilapia eggs, provided that dosage and withdrawal periods are strictly controlled to prevent resistance development. Long-term use of fluoroquinolones alone can easily induce target mutations and overexpression of efflux pumps.³⁶ These findings underscore the critical importance of implementing precision antimicrobial use and stewardship programs in aquaculture, while also highlighting the ecological risk of fish eggs as potential vectors for pathogen transmission.

The highest hatching success was recorded in the 4 mg/L chloramine-T treatment group, achieving $65.2 \pm 2.5\%$. This effectiveness is likely associated with its sustained chlorine release, long antimicrobial persistence, and relatively low toxicity to developing eggs. Unlike iodine or oxytetracycline, chloramine-T retains bactericidal activity for more than 48 hours, preventing secondary pathogen proliferation.³⁷ Comparable findings were reported by, where 15 mg/L chloramine-T significantly improved goldfish survival, reinforcing its potential as a preferred disinfectant for tilapia eggs under the current experimental conditions.³⁸ The mechanism of action of chloramine-T in aquatic environments does indeed rely on its release of reactive chlorine species (such as hypochlorous acid, HOCl) in water, thereby achieving a broad-spectrum bactericidal effect. Studies on zebrafish (*Danio rerio*) embryos have shown that high concentrations of chloramine-T can induce cardiotoxicity and neurotoxicity³⁹; however, fertilized tilapia eggs are not typically consumed directly as food; besides, under standard application conditions (e.g. ≤ 10 mg/L, short-term

immersion), chloramine-T's ability to penetrate the egg membrane is limited, making it difficult for significant residues to accumulate in embryonic tissues.⁴⁰

Enrofloxacin is metabolized into antibacterial ciprofloxacin in farmed fish such as tilapia, yellow catfish, and carp; ciprofloxacin exhibits a slower elimination rate than the parent compound, and both compounds are subject to aquaculture residue monitoring.⁴¹⁻⁴³ Accordingly, strict global regulatory controls govern enrofloxacin use in food fish production, with China specifying a maximum residue limit of 100 $\mu\text{g/kg}$ for the combined residues of enrofloxacin and ciprofloxacin in fish muscle.⁴⁰ However, to date, no globally recognized statutory withdrawal periods based on systematic residue depletion data are available for fish egg treatment, and non-standard approaches including egg immersion cannot support the establishment of scientifically valid and enforceable withdrawal intervals.

Even with optimal chloramine T treatment, the final hatching rate (65.2%) was relatively low. The reasons might be: First, while this disinfection suppresses major pathogens, it cannot fully remove the whole pathogenic and opportunistic microbial community within egg microenvironments.⁴⁴ Second, chloramine T at safe working concentrations may induce subtle sublethal stress to embryos.³⁹ These results reveal a key obstacle in aquatic disease control: single-drug treatments hardly fully block multi-factor syndromic disease outbreaks driven by environmental stress and complex microbiota.⁴⁵ High hatch rates (>80%) documented in literature are obtained using pathogen-free broodstock under strict laboratory settings and seldom reproduced in commercial intensive hatcheries. Production records of the farms in this study showed baseline hatching rates stayed at 62.3%–71.5% across 12 production cycles under pathogen-free routine operations, far below the 80% theoretical maximum. Therefore, the observed 65.2% hatching rate represented restoration to regular commercial baseline. Though 65% hatching rate is suboptimal, this treatment saved the hatchery from catastrophic failure in emergencies. Breaking this 65% bottleneck requires integrated management including improved broodstock nutrition, UV treatment of inlet water, and probiotic regulation of hatching water.

It is noteworthy that one of the predominant pathogens identified in this study, LHV-07, was characterized as a haemolytic, environmental non-O1/non-O139 *Vibrio cholerae* strain. Although non-O1/non-O139 *V. cholerae* strains lack cholera toxin and thus do not pose zoonotic hazards originating from fish, they remain of considerable clinical and public-health concern.⁴⁶ These environmental vibrios are increasingly documented as causative agents of human extraintestinal infections such as wound infections, septicemia, and meningitis, which typically occur upon exposure to contaminated water or ingestion of raw or undercooked seafood.⁴⁷ The detection of these potentially pathogenic strains in aquaculture hatcheries highlights the necessity of integrated biosecurity strategies to protect fish health and reduce human-health hazards for farm personnel and seafood consumers.

5. CONCLUSION

In this study, two major bacterial pathogens were isolated and identified from the eggs of diseased tilapia: *Pseudomonas aeruginosa* TLV-01 and non-O1/O139 *Vibrio cholerae* LHV-07. Both isolates exhibited β -haemolytic activity. Infection assays confirmed their pathogenicity, with the 7-day LD₅₀ estimated at 2.0×10^8 CFU/mL for TLV-01 and 2.0×10^6 CFU/mL for LHV-07, whilst the LD₅₀ value derived from mixed infection assays was lower, at 2.0×10^5 CFU/mL. The Loewe model showed that, within the concentration range of 1×10^4 – 1×10^7 CFU/mL, the CI value remained consistently below 1 (ranging from 0.021 to 0.662), which clearly confirmed the presence of synergy, exhibiting a trend of diminishing with increasing concentration; at the highest concentration (1×10^8 CFU/mL), the CI value rose sharply to 5.018, indicating a shift towards an antagonistic effect. The Bliss model indicated that, at low to medium concentrations, mixed infection primarily exhibited an additive effect. The Loewe additive model, used to validate the results, suggested a trend towards synergy in the low to medium concentration range. Antimicrobial susceptibility testing revealed marked differences in resistance among the isolates; of the intervention measures tested, 4 mg/L chloramine-T yielded the highest hatching rate ($65.2 \pm 2.5\%$), with no deformities observed in the fry. Taken together, these findings identify two relevant bacterial hazards present during the hatching process of tilapia eggs and provide a basis for improving biosecurity in hatcheries and implementing targeted disease prevention and control measures.

ACKNOWLEDGEMENTS

This work was supported by Innovative Fund for Scientific and Technological Personnel of Hainan Province (KJRC2023B22), and Hainan University Collaborative Innovation Center Fund (XTCX2022HYB03); Aquaculture Im-

provement Program (AIP)-Tilapia in Hainan, grant number HD-KYH-2020155; and Hainan Baolu Aquatic Products Technology Co., Ltd. Seed Industry Breeding and Promotion Technology Services, grant number HD-KYH-2022002.

CREDIT AUTHORS' CONTRIBUTION

Methodology: Xianli Ke (Equal), Ling Li (Equal), Jiawen Wang (Equal). Formal Analysis: Xianli Ke (Equal), Ling Li (Equal), Jiawen Wang (Equal). Investigation: Xianli Ke (Equal), Ling Li (Equal), Jiawen Wang (Equal). Visualization: Xianli Ke (Equal), Ling Li (Equal). Writing – original draft: Xianli Ke (Equal), Ling Li (Equal), Yan Cai (Equal). Conceptualization: Yan Cai (Equal), Shifeng Wang (Equal). Writing – review & editing: Yan Cai (Equal), Shifeng Wang (Equal). Supervision: Yan Cai (Equal), Shifeng Wang (Equal). Resources: Zhangding Zhao (Lead). Project administration: Zhangding Zhao (Equal), Shifeng Wang (Equal). Funding acquisition: Shifeng Wang (Lead).

ETHICAL CONDUCT APPROVAL – IACUC

In this study, all experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Hainan University. All operations were carried out in accordance with the relevant guidelines and regulations for the breeding and use of laboratory animals in China to minimize animal pain.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

Submitted: August 19, 2026 CDT. Accepted: September 19, 2026 CDT. Published: October 23, 2026 CDT.



This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (CCBY-4.0). View this license's legal deed at <http://creativecommons.org/licenses/by/4.0> and legal code at <http://creativecommons.org/licenses/by/4.0/legalcode> for more information.

REFERENCES

1. Wang B, Thompson KD, Wangkahart E, Yamkasem J, Bondad-Reantaso MG, Tattiyapong P, et al. Strategies to enhance tilapia immunity to improve their health in aquaculture. *Reviews in Aquaculture*. 2023;15:41-56. doi:[10.1111/raq.12731](https://doi.org/10.1111/raq.12731)
2. Zimmermann S, Kiessling A, Zhang J. The future of intensive tilapia production and the circular bioeconomy without effluents: biofloc technology, recirculation aquaculture systems, bio-RAS, partitioned aquaculture systems and integrated multitrophic aquaculture. *Reviews in Aquaculture*. 2023;15:22-31. doi:[10.1111/raq.12744](https://doi.org/10.1111/raq.12744)
3. Li M, Cai Y, Tian Y, Ke X, Zhao Z, Ni X, et al. First isolation of *Chryseobacterium* sp. as a pathogen of fish eggs. *Aquaculture Reports*. 2025;43(000). doi:[10.1016/j.aqrep.2025.102992](https://doi.org/10.1016/j.aqrep.2025.102992)
4. Bai J, Huo Y, Hu X, Lü A, Sun J. Characterization of pathogenic *Pseudomonas alcaligenes* isolated from Koi carp (*Cyprinus carpio*) in China. *Journal of Aquatic Animal Health*. doi:[10.1002/aa.10141](https://doi.org/10.1002/aa.10141)
5. Thanigaivel S, thanigaivel, Thomas J, Vickram AS, Gulothungan G, et al. Antioxidant and antibacterial efficacy of *Chaetomorpha linum* and its toxicological evaluation for the prophylactic treatment against *Pseudomonas aeruginosa* infection in *Labeo rohita*. doi:[10.1080/10454438.2021.1961966](https://doi.org/10.1080/10454438.2021.1961966)
6. Osman KM, Pires LDS, Franco OL, Orabi A, Ali AHM, Hamada M, et al. Nile Tilapia (*Oreochromis niloticus*) as an Aquatic Vector for *Pseudomonas* Species: Quorum Sensing Association with Antibiotic Resistance, Biofilm Formation and Virulence. *Preprints*. Published online 2019. doi:[10.20944/PREPRINTS201911.0282.V1](https://doi.org/10.20944/PREPRINTS201911.0282.V1)
7. Krell T, Matilla MA. *Pseudomonas aeruginosa*. *Trends in microbiology*. 2024;32(2):216-218. doi:[10.1016/j.tim.2023.11.005](https://doi.org/10.1016/j.tim.2023.11.005)
8. Zhao N, Weng X, Yu Y, Ge T, Tang C, He M, et al. Virulence gene profiles and antimicrobial resistance in clinical and environmental isolates of Non-O1/Non-O139 *Vibrio cholerae*. *BMC Microbiology*. 2026;26(1):267. doi:[10.1186/s12866-026-04836-9](https://doi.org/10.1186/s12866-026-04836-9)
9. Chen Z, Chen A, Cai X, Yin J, Liu Y, Dong Q, et al. Functional role of rpoN in regulating the virulence of non-O1/O139 *Vibrio cholerae*. *International Journal of Biological Macromolecules*. 2025;308:142439. doi:[10.1016/j.ijbiomac.2025.142439](https://doi.org/10.1016/j.ijbiomac.2025.142439)
10. Dehkordi SMH, Anvar SA, Rahimi E, Ahari H, Ataee M. Molecular investigation of prevalence, phenotypic and genotypic diversity, antibiotic resistance, frequency of virulence genes and genome sequencing in *Pseudomonas aeruginosa* strains isolated from lobster. *International Journal of Food Microbiology*. 2022;382:109901. doi:[10.1016/j.ijfoodmicro.2022.109901](https://doi.org/10.1016/j.ijfoodmicro.2022.109901)
11. Zhang Q, Alter T, Strauch E, Hammerl JA, Schwartz K, Borowiak M, et al. Genetic and Phenotypic Virulence Potential of Non-O1/Non-O139 *Vibrio cholerae* Isolated from German Retail Seafood. *Microorganisms*. 2023;11(11):2751. doi:[10.3390/microorganisms11112751](https://doi.org/10.3390/microorganisms11112751)
12. Benjamin D, Udo H, Monika R. Four-parameter nonlinear regression and maximum achievable effect in ecotoxicology: just visually appealing or relevant for risk assessment? *Integrated Environmental Assessment and Management*. 3:3. doi:[10.1093/inteam/vjaf085](https://doi.org/10.1093/inteam/vjaf085)
13. Ritz C, Baty F, Streibig JC, Gerhard D. Dose-Response Analysis Using R. *PLOS ONE*. 2015;10(12). doi:[10.1371/journal.pone.0146021](https://doi.org/10.1371/journal.pone.0146021)
14. Lee SI. Drug interaction: focusing on response surface models. *Korean Journal of Anesthesiology*. 2010;58(5):421-434. doi:[10.4097/kjae.2010.58.5.421](https://doi.org/10.4097/kjae.2010.58.5.421)
15. Greig DR, Hickey TJ, Boxall MD, Begum H, Gentle A, Jenkins C, et al. A real-time multiplex PCR for the identification and typing of *Vibrio cholerae*. *Diagnostic Microbiology and Infectious Disease*. 2018;90(3):171-176. doi:[10.1016/j.diagmicrobio.2017.11.017](https://doi.org/10.1016/j.diagmicrobio.2017.11.017)
16. Demidenko E, Miller TW. Statistical determination of synergy based on Bliss definition of drugs independence. *PLOS ONE*. 2019;14. doi:[10.1371/journal.pone.0224137](https://doi.org/10.1371/journal.pone.0224137)
17. Sridhar K, Charles AL. In vitro antioxidant activity of Kyoho grape extracts in DPPH and ABTS assays: Estimation methods for EC50 using advanced statistical programs. *Food Chemistry*. 2019;275:41-49. doi:[10.1016/j.foodchem.2018.09.040](https://doi.org/10.1016/j.foodchem.2018.09.040)
18. Aboyadak I, Ali NG. Enrofloxacin, Effective Treatment of *Pseudomonas aeruginosa* and *Enterococcus faecalis* Infection in *Oreochromis niloticus*. *Microorganisms*. 2024;12(5):901. doi:[10.3390/microorganisms12050901](https://doi.org/10.3390/microorganisms12050901)

19. Ji T, Cao Y, Cao Q, Zhang Y, Yang H. The antagonistic effect and protective efficacy of gram-positive probiotics *Bacillus coagulans* to newly identified pathogens *Pseudomonas aeruginosa* in crucian carp *Carassius auratus gibelio*. *Aquaculture Reports*. Published online 2022. doi:[10.1016/j.aqrep.2022.101126](https://doi.org/10.1016/j.aqrep.2022.101126)
20. Li N, Xu Y, Bai X, Feng S, Luo Z, Hao S, et al. Recovery of *Pseudomonas aeruginosa* from diseased Grass Carp (*Ctenopharyngodon idella*) in China. *Israeli Journal of Aquaculture-Bamidgeh*. 2015;67. doi:[10.46989/001c.20687](https://doi.org/10.46989/001c.20687)
21. Zhang XJ, Yao DR, Yan BL, Bi KR, Liang LG, Qin GM. Identification of *Vibrio cholerae* as a causative bacterium for an ulcer disease of cultured loach *Misgurnus anguillicaudatus* in China. *African Journal of Microbiology Research*. 2012;6(9):2060-2070. doi:[10.5897/AJMR11.1344](https://doi.org/10.5897/AJMR11.1344)
22. Gao X, Miao Z, Li X, Chen N, Gu W, Liu X, et al. Pathogenicity of non-O1/O139 *Vibrio cholerae* and its induced immune response in *Macrobrachium rosenbergii*. *Fish & shellfish immunology*. 2019;92:300-307. doi:[10.1016/j.fsi.2019.06.032](https://doi.org/10.1016/j.fsi.2019.06.032)
23. Liao W, Wei D, Liu M, Ke K, Shi D, Li B, et al. Non-O1/O139 *Vibrio cholerae* causes severe intestinal disease in bullfrogs (*Rana catesbeiana*). *Animal Diseases*. 2023;3(1):27. doi:[10.1186/s44149-023-00092-w](https://doi.org/10.1186/s44149-023-00092-w)
24. Nair RR, John KR, Rajan P, Krishnan R, Safeena MP. Co-infection of *Lactococcus garvieae* and *Aeromonas hydrophila* in cultured Nile Tilapia in Kerala, India. *Brazilian Journal of Microbiology*. 2024;55(3):2071-2083. doi:[10.1007/s42770-024-01415-w](https://doi.org/10.1007/s42770-024-01415-w)
25. Xu H, Xu R, Wang X, Liang Q, Zhang L, Liu J, et al. Co-infections of *Aeromonas veronii* and *Nocardia seriolae* in largemouth bass (*Micropterus salmoides*). *Microbial Pathogenesis*. 2022;173:105815. doi:[10.1016/j.micpath.2022.105815](https://doi.org/10.1016/j.micpath.2022.105815)
26. Peng X, Lei C, Sun X. Comparison of Lethal Doses Calculated Using Logit/Probit-Log(Dose) Regressions With Arbitrary Slopes Using R. *Journal of Economic Entomology*. Published online 2021. doi:[10.1093/jee/toab044](https://doi.org/10.1093/jee/toab044)
27. Borer ET, Kendig AE, Holt RD. Feeding the fever: Complex host-pathogen dynamics along continuous resource gradients. *Ecology and Evolution*. 2023;13(7). doi:[10.1002/ece3.10315](https://doi.org/10.1002/ece3.10315)
28. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. *Pharmacology Research & Perspectives*. 2015;3(3). doi:[10.1002/prp2.149](https://doi.org/10.1002/prp2.149)
29. Fuentealba-Manosalva O, Mansilla M, Buelvas N, Martin-Martin A, Torres CG, López-Muñoz RA. Mind the Curve: Dose-Response Fitting Biases the Synergy Scores across Software Used for Chemotherapy Combination Studies. *International Journal of Molecular Sciences*. 2023;24(11):9705. doi:[10.3390/ijms24119705](https://doi.org/10.3390/ijms24119705)
30. Kifer D, Jaki D, Klari ME. Assessing the Effect of Mycotoxin Combinations: Which Mathematical Model Is (the Most) Appropriate? *Toxins*. 2020;12(3). doi:[10.3390/toxins12030153](https://doi.org/10.3390/toxins12030153)
31. Chu W, McLean RJC. Quorum Signal Inhibitors and Their Potential Use against Fish Diseases. *Journal of Aquatic Animal Health*. 2016;28(2):91-96. doi:[10.1080/08997659.2016.1150907](https://doi.org/10.1080/08997659.2016.1150907)
32. Siriken B, Öz V, Erol İ. Quorum sensing systems, related virulence factors, and biofilm formation in *Pseudomonas aeruginosa* isolated from fish. *Archives of Microbiology*. 2021;203(4):1519-1528. doi:[10.1007/s00203-020-02159-5](https://doi.org/10.1007/s00203-020-02159-5)
33. Ahmadian L, Haghshenas MR, Mirzaei B, Khalili Y, Goli HR. Role of MexAB-OprM efflux pump in the emergence of multidrug-resistant clinical isolates of *Pseudomonas aeruginosa* in Mazandaran province of Iran. *Molecular Biology Reports*. 2023;50(3):2603-2609. doi:[10.1007/s11033-022-08230-2](https://doi.org/10.1007/s11033-022-08230-2)
34. Sun J, Deng Z, Fung DKC, Yan A. Study of the expression of bacterial multidrug efflux pumps in anaerobic conditions. In: *Bacterial Multidrug Exporters: Methods and Protocols*. Springer; 2017:253-268. doi:[10.1007/978-1-4939-7454-2_14](https://doi.org/10.1007/978-1-4939-7454-2_14)
35. Ugwuanyi FC, Ajayi A, Ojo DA, Adeleye AI, Smith SI. Evaluation of efflux pump activity and biofilm formation in multidrug resistant clinical isolates of *Pseudomonas aeruginosa* isolated from a Federal Medical Center in Nigeria. *Annals of Clinical Microbiology and Antimicrobials*. 2021;20(1). doi:[10.1186/s12941-021-00417-y](https://doi.org/10.1186/s12941-021-00417-y)
36. Devi MS, Paria P, Kumar V, Parida PK, Maurye P, Behera BK, et al. Molecular identification and pathogenicity study of virulent *Vibrio cholerae* non O1/O139 serotype associated with mortality of farmed *Labeo rohita* (Hamilton, 1822), in India. *Aquaculture*. 2022;547:737529. doi:[10.1016/j.aquaculture.2021.737437](https://doi.org/10.1016/j.aquaculture.2021.737437)
37. Meinertz JR, Stehly GR, Greseth SL, Gaikowski MP, Gingerich WH. Depletion of the chloramine-T marker residue, para-toluenesulfonamide, from skin-on fillet tissue of hybrid striped bass, rainbow trout, and yellow perch. *Aquaculture*. 2004;235:65-75. doi:[10.1016/s0044-8486\(03\)00448-4](https://doi.org/10.1016/s0044-8486(03)00448-4)

38. Altinok I. Toxicity and therapeutic effects of chloramine-T for treating *Flavobacterium columnare* infection of goldfish. *Aquaculture*. 2004;239(1-4):47-56. doi:[10.1016/j.aquaculture.2004.05.035](https://doi.org/10.1016/j.aquaculture.2004.05.035)
39. Rivero-Wendt CLG, Fernandes LG, dos Santos AN, Brito IL, dos Santos Jaques JA, dos Santos dos Anjos E, et al. Effects of Chloramine T on zebrafish embryos malformations associated with cardiotoxicity and neurotoxicity. *Journal of Toxicology and Environmental Health, Part A*. 2023;86(11):372-381. doi:[10.1080/15287394.2023.2205271](https://doi.org/10.1080/15287394.2023.2205271)
40. Soleimani TA, Sattari A, Kheirandish R, Sharifpour I. Safety evaluation of chloramine-T on ornamental zebra fish (*Danio rerio*) using LC50 calculation and organ pathology. *IRANIAN JOURNAL OF FISHERIES SCIENCES*. 2017;(1). doi:[10.22092/IJFS.2018.114637](https://doi.org/10.22092/IJFS.2018.114637)
41. Rairat T, Chen SM, Lu YP, Hsu JCN, Liu YK, Chou CC. Determination of temperature-dependent optimal oral doses of florfenicol and corresponding withdrawal times in Nile tilapia (*Oreochromis niloticus*) reared at 25 and 30°C. *Aquaculture*. 2022;561:738719. doi:[10.1016/j.aquaculture.2022.738719](https://doi.org/10.1016/j.aquaculture.2022.738719)
42. Yang F, Zhang CS, Duan MH, Wang H, Song ZW, Shao HT, et al. Pharmacokinetics and Tissue Distribution of Enrofloxacin Following Single Oral Administration in Yellow River Carp (*Cyprinus carpio haematoperus*). *Frontiers in Veterinary Science*. 2022;9. doi:[10.3389/fvets.2022.822032](https://doi.org/10.3389/fvets.2022.822032)
43. Xu N, Sun W, Zhang YA, Xiaohui. The Assessment of Withdrawal Interval for Enrofloxacin in Yellow Catfish (*Pelteobagrus fulvidraco*) after Multiple Oral Administrations at Disparate Temperatures. *animals*. 2023;13(16). doi:[10.3390/ani13162568](https://doi.org/10.3390/ani13162568)
44. Najafpour B, Pinto PIS, Moutou KA, Canario AVM, Power DM. Factors Driving Bacterial Microbiota of Eggs from Commercial Hatcheries of European Seabass and Gilthead Seabream. *Microorganisms*. 2021;9(11):2275. doi:[10.3390/microorganisms9112275](https://doi.org/10.3390/microorganisms9112275)
45. Natrah I, Muthukrishnan S, Mohd-Padil H, Mohamad N, Alipiah NM, Shariff M, et al. Antimicrobial Resistance in Malaysian Shrimp Aquaculture and Strategies to Reduce Its Occurrence. *Reviews in Aquaculture*. 2025;17(3). doi:[10.1111/raq.70020](https://doi.org/10.1111/raq.70020)
46. Tan Q, Xu M, Li XX, Chen Y j., Wang R h., Tang L, et al. Research Progress on Non-O1 and non-O139 *Vibrio cholerae* Aquatic Animals and Its Public Health Significance. *Israeli Journal of Aquaculture - Bamidgeh*. 2025;77(3). doi:[10.46989/001c.142368](https://doi.org/10.46989/001c.142368)
47. Sánchez-Yebra Romera W, Caparrós Andújar A, Sánchez Gómez JA, Martínez Campos L, Lucerna Méndez MA, De Oña Baquero CM. Meningoencephalitis caused by Non-O1, non-O139 *Vibrio cholerae* in an infant from southern Spain. *New Microbes and New Infections*. 2024;56:101200. doi:[10.1016/j.nmni.2023.101200](https://doi.org/10.1016/j.nmni.2023.101200)