



**HAL**  
open science

## Zebrafish as an effective model for evaluating phage therapy in bacterial infections: a promising strategy against human pathogens

Lucile Plumet, Denis Costechareyre, Jean-Philippe Lavigne, Karima Kissa, Virginie Molle

### ► To cite this version:

Lucile Plumet, Denis Costechareyre, Jean-Philippe Lavigne, Karima Kissa, Virginie Molle. Zebrafish as an effective model for evaluating phage therapy in bacterial infections: a promising strategy against human pathogens. *Antimicrobial Agents and Chemotherapy*, 2024, 68 (10), 10.1128/aac.00829-24 . hal-04742103

**HAL Id: hal-04742103**

**<https://hal.science/hal-04742103v1>**

Submitted on 23 Oct 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# 1 Zebrafish as an Effective Model for Evaluating Phage Therapy in 2 Bacterial Infections: A Promising Strategy Against Human 3 Pathogens

4 Lucile Plumet <sup>1</sup>, Denis Costechareyre <sup>2</sup>, Jean-Philippe Lavigne <sup>3</sup>, Karima Kissa <sup>1</sup> and Virginie  
5 Molle <sup>1,3,\*</sup>

6 <sup>1</sup> VBIC, INSERM U1047, University of Montpellier, 34095 Montpellier, France

7 <sup>2</sup> Greenphage, Cap Alpha, 34830 Clapiers, France

8 <sup>3</sup> VBIC, INSERM U1047, University of Montpellier, Department of Microbiology and Hospital Hygiene, CHU Nîmes,  
9 30908 Nîmes, France

10 \*Correspondence: VBIC, INSERM U1047, University of Montpellier, Place E. Bataillon, Bat 15, Case 2401, 34095  
11 Montpellier cedex 05, France (virginie.molle@umontpellier.fr)

## 12 ABSTRACT

13 The escalating prevalence of antibiotic-resistant bacterial infections necessitates urgent alternative therapeutic  
14 strategies. Phage therapy, which employs bacteriophages to specifically target pathogenic bacteria, emerges  
15 as a promising solution. This review examines the efficacy of phage therapy in zebrafish models, both embryos  
16 and adults, which are proven and reliable for simulating human infectious diseases. We synthesize findings  
17 from recent studies that utilized these models to assess phage treatments against various bacterial pathogens,  
18 including *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Mycobacterium abscessus*, *Staphylococcus aureus*, *Klebsiella*  
19 *pneumoniae*, *Acinetobacter baumannii*, and *Escherichia coli*. Methods of phage administration, such as circulation  
20 injection and bath immersion, are detailed alongside evaluations of survival rates and bacterial load  
21 reductions. Notably, combination therapies of phages with antibiotics show enhanced efficacy, as evidenced  
22 by improved survival rates and synergistic effects in reducing bacterial loads. We also discuss the transition  
23 from zebrafish embryos to adult models, emphasizing the increased complexity of immune responses. This  
24 review highlights the valuable contribution of the zebrafish model to advancing phage therapy research,  
25 particularly in the face of rising antibiotic resistance and the urgent need for alternative treatments.

26 **KEYWORDS:** Zebrafish, animal model, bacteria, phage therapy, antibiotics

27 The escalating challenge of antibiotic resistance poses a significant threat to global health, necessitating  
28 innovative approaches to combat bacterial infections (1). The extensive use and misuse of antibiotics have  
29 accelerated the evolution of resistant strains, notably those classified among the ESKAPE pathogens  
30 (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas*  
31 *aeruginosa*, and *Enterobacter* species). These pathogens are responsible for the majority of hospital-acquired  
32 infections worldwide and are particularly alarming due to their frequent resistance to multiple antibiotics,  
33 rendering many conventional treatments ineffective (2, 3). This crisis has underscored a critical need for  
34 alternative therapeutic strategies. Phage therapy, which utilizes bacteriophages (or phages) to target and  
35 eliminate bacterial pathogens, stands out as one of the most promising options. It offers numerous advantages  
36 over traditional antibiotics, such as high specificity to target bacteria, minimal disruption to native microbiota,  
37 and the ability to evolve in response to bacterial resistance mechanisms (4, 5). However, advancing phage  
38 therapy from basic research to a viable clinical option necessitates comprehensive in vivo studies to explore  
39 its dynamics, efficacy, and safety (6–8). In this context, the zebrafish (*Danio rerio*) model has emerged as a  
40 valuable tool in pre-clinical research, offering several unique advantages that make them particularly suitable  
41 for these studies (9, 10). Their genetic, physiological, and immunological similarities to humans render them

42 an essential model for studying human diseases (11). Additionally, the high reproductive rate of zebrafish  
43 ensures a consistent supply of subjects, thereby facilitating large-scale studies. This is economically  
44 advantageous, enabling researchers to conduct extensive testing at a fraction of the cost associated with mouse  
45 models, which is particularly critical in the early stages of research where multiple trials are often necessary  
46 to optimize therapeutic strategies. Regulatory frameworks mandate data from indispensable mammalian  
47 models before progressing to human clinical trials. Therefore, incorporating zebrafish studies allows  
48 researchers to refine their hypotheses and treatment protocols, thereby shortening the duration and reducing  
49 the scope of subsequent, more costly and time-consuming, mouse studies (12, 13). By utilizing the versatility  
50 of zebrafish models, researchers can accelerate the development of phage therapy, gaining crucial insights into  
51 its efficacy, mechanisms, and potential limitations in treating bacterial infections. Finally, the zebrafish model  
52 complies with the 3Rs (Replacement, Reduction, Refinement) rule, an ethical principle aimed at improving the  
53 welfare of animals used in scientific experiments. This review highlights how the integration of the zebrafish  
54 model into phage therapy research underscores its valuable contribution to advancing this promising field,  
55 particularly in the face of rising antibiotic resistance and the urgent need for alternative treatments. To achieve  
56 this, we conducted a comprehensive search on PubMed and other relevant databases using a combination of  
57 keywords tailored to our study focus. Specifically, we used terms such as "zebrafish model," "embryo," "adult,"  
58 "bacterial pathogen," "human infection," and "phage therapy." We included studies that specifically employed  
59 either zebrafish embryos or adults as models for human bacterial infections treated with phage therapy and  
60 explicitly excluded any studies focusing on pathogens specific to fish. All articles that met these criteria were  
61 thoroughly reviewed and are cited in Table 1, ensuring a comprehensive representation of the current research  
62 landscape in this field.

## 63 ZEBRAFISH EMBRYO MODEL IN THE DEVELOPMENT OF PHAGE THERAPY TO TREAT 64 BACTERIAL INFECTIONS

65 The zebrafish embryo model offers distinct advantages compared to mammalian models for the study of  
66 infectious diseases and treatment responses, in accordance with the 3Rs. Embryo studies are generally cost-  
67 effective and not subject to the same ethical regulations as vertebrate animals, as when using embryos under  
68 5 days post fertilization (dpf), they are not classified as *in vivo* assays under European Directive 2010/63/EU,  
69 thus simplifying experimental approval process. This makes them an excellent replacement for early-stage  
70 studies, significantly reducing the number of mammals required for preliminary research (14, 15). One of their  
71 most notable features is their transparency, which allows for real-time observation and imaging of cellular  
72 behaviors, infection dynamics and treatment effects. This transparency, combined with the rapid development  
73 of embryos, during which major organ systems form within five days, provides valuable insights into the  
74 dynamics of bacterial infection and phage activity within a living organism (16, 17). The model versatility  
75 allows for testing various methods of infection and sites, enhancing its utility and interest for detailed  
76 experimental exploration (Figure 1). Importantly, the zebrafish embryo innate immune system shares  
77 significant similarities with the human immune system, making it an excellent model for understanding how  
78 treatments interact with immune responses, which is essential for optimizing treatment strategies (18).  
79 However, the zebrafish model also has distinct disadvantages when compared to mammalian models.  
80 Anatomical and physiological differences, such as the presence of gills instead of lungs and hematopoiesis  
81 occurring in the anterior kidney rather than the bone marrow, can limit the applicability of results to mammals.  
82 Additionally, the regulatory frameworks currently mandate data from mammalian models before progressing  
83 to human clinical trials, meaning zebrafish studies alone are not sufficient for regulatory approval. This  
84 situation could change in the future as regulatory perspectives evolve, but at present, it remains a significant  
85 limitation. While the zebrafish model excels in the principles of reduction and refinement by allowing detailed  
86 *in vivo* studies with fewer ethical concerns, it is not yet a complete replacement for mammalian models in the  
87 regulatory landscape.

88 In this section, we provide a detailed comparison of the zebrafish embryo model, demonstrating its  
89 effectiveness in studying methodologies, outcomes, and implications for treating a variety of human bacterial  
90 pathogens with phage therapy. Initially, we present model of non-pathological embryos to establish a baseline  
91 for normal responses to bacterial infections and phage treatments. Subsequently, we focus on zebrafish  
92 embryos mimicking cystic fibrosis (CF) as a pathological context, providing insights into how this specific  
93 disease model can influence the efficacy and dynamics of phage therapy.

#### 94 **Phage Therapy Studies in Healthy Zebrafish Embryo Model**

95 *Enterococcus faecalis*. Al-Zubidi *et al.* (2019) focused on the isolation and characterization of phages against  
96 *E. faecalis* and highlighted the efficacy of the phage SHEF2 in clearing systemic lethal infection in zebrafish. *E.*  
97 *faecalis* is an opportunistic pathogen often implicated in nosocomial infections and exhibits intrinsic resistance  
98 to many standard antibiotics. In this study, zebrafish embryos were infected by intravenous injection of *E.*  
99 *faecalis* OS16 in the duct of Cuvier (30,000 colonies forming units (CFU)/embryo at 30 hours post-fertilization  
100 (hpf), and subsequently treated with either a live phage SHEF2 ( $6 \times 10^5$  plaque forming units PFU/embryo at 2  
101 hours post-infection (hpi)) or a heat-killed SHEF2, and the results were monitored for 72 hpi. The authors  
102 found that *E. faecalis* OS16 caused significant time-dependent lethality in embryos, which was markedly  
103 higher than in the control groups treated with phosphate-buffered saline (PBS) or phage alone. While the heat-  
104 killed SHEF2 did not improve survival rates, with a 73% mortality rate identical to that of the infection-only  
105 group, the live SHEF2 phage significantly reduced the mortality rate to 16%, demonstrating an 84% survival  
106 rate. The zebrafish infected with *E. faecalis* exhibited severe symptoms such as lack of blood circulation, yolk  
107 sac oedema and eye abnormalities, pericardial edema, and spinal curvature. Conversely, the majority of  
108 phage-treated zebrafish remained healthy, displaying a health status comparable to that of the PBS-injected  
109 controls and those injected with phage only. Overall, the study demonstrates that systemic phage treatment  
110 following infection significantly reduced mortality rates and enhanced the health of the zebrafish embryos,  
111 thus highlighting the phage effectiveness in treating systemic *E. faecalis* infections. Importantly, the study also  
112 confirmed that neither the phage nor the bacterial components released upon bacterial lysis exhibited toxicity  
113 to the embryos, suggesting that the phage could be safely used for systemic applications. The zebrafish model  
114 proved to be extremely valuable not only for assessing the efficacy of the phages against systemic bacterial  
115 infections but also as a dynamic tool for real-time monitoring of infection progression and phage-mediated  
116 clearance (19).

117 *Staphylococcus aureus*. The study by Plumet *et al.* (2024) offers significant insights into the use of the zebrafish  
118 embryo model for assessing phage therapy efficacy against *S. aureus*, a pathogenic bacterium responsible for  
119 a broad spectrum of infections. Zebrafish embryos (30 hpf) were infected with methicillin-resistant *S. aureus*  
120 (MRSA, USA300 and NSA0799 strains) and methicillin-susceptible *S. aureus* (MSSA, SH1000 strain) with  
121 15,000 CFU/embryo in the hindbrain ventricle and subsequently treated 2 hpi with varying doses of the phage  
122 SAVM02. Untreated embryos experienced 100% mortality, whereas those receiving phage treatment showed  
123 significantly improved survival rates in a concentration-dependent manner, with the highest concentrations  
124 achieving 100% survival over 72 hours post-treatment (hpt). Control groups treated with either PBS or phage  
125 alone also showed 100% survival, confirming the non-toxicity of the phage. Heat-inactivated SAVM02 phage  
126 did not improve survival, verifying that the survival benefit was due to the active phage therapeutic action.  
127 Throughout the study, phage-treated embryos remained healthy, underscoring the safety and effectiveness of  
128 phage therapy in treating *S. aureus* infections in this model. Furthermore, phage treatment ( $7.10^7$  PFU/embryo)  
129 significantly reduced the bacterial load in zebrafish embryos infected with MRSA USA300, decreasing it from  
130 15,000 to fewer than 100 CFU/embryo over 72 hpt. Microscopic analysis supported these results, showing a  
131 marked reduction in green fluorescence bacteria, which underscores the strong bacteriolytic capability of the  
132 SAVM02 phage in effectively diminishing the bacterial burden in the zebrafish infection model. The authors

133 also demonstrated that the phage load increased considerably over 24 hpt before gradually decreasing.  
134 Additionally, the study compared the efficacy of phage therapy with vancomycin for treating MRSA  
135 infections. It was found that the minimum inhibitory concentration (MIC) for vancomycin against *S. aureus*  
136 strains ranged from 0.5 to 1 µg/mL, confirming their susceptibility to the antibiotic. When administered at  
137 different concentrations, vancomycin achieved 100% survival rates in embryos infected with USA300 at the  
138 highest concentrations tested, while lower concentrations were progressively less effective. In this specific  
139 study, a comparison between phage therapy and a single dose of vancomycin (300 µg/mL or 600x MIC)  
140 showed that although both initially reduced bacterial loads, phage therapy demonstrated a more sustained  
141 antibacterial effect. However, it should be noted that this is not a head-to-head comparison, as vancomycin in  
142 clinical settings is administered in multiple doses due to its limited half-life, which might affect its long-term  
143 efficacy. While vancomycin effectively decreased bacterial counts within the first 24 hpt, its effect diminished  
144 over time. In contrast, phage therapy maintained a greater reduction in bacterial load throughout the 72 hpt.  
145 While vancomycin can be effective at high doses for acute infections, its long-term effectiveness diminishes as  
146 it is metabolized and cleared. In contrast, phage therapy not only ensures survival but also provides a longer-  
147 lasting reduction in bacterial counts, making it potentially more suitable for chronic infections. This study  
148 illustrates that the zebrafish embryo model provides crucial insights into the interactions among the host,  
149 pathogen, and therapeutic agents (20).

150 ***Klebsiella pneumoniae***. Assafari *et al.* (2021) focused on the isolation and characterization of the phage  
151 UMP2146, which targets *K. pneumoniae*, a pathogenic bacterium commonly associated with nosocomial  
152 infections. In their study, the impact of phage UPM2146 was evaluated using an immersion method with  
153 zebrafish embryos (72 hpf). The embryos were exposed to *K. pneumoniae* 2146 ( $3.3 \times 10^6$  CFU/mL) for either 30  
154 or 90 min before direct phage treatment ( $10^6$  PFU/mL). This study showed that embryos infected for 30 min  
155 had significantly lower mortality rates, surviving up to 24 hours. Conversely, those infected for 90 min reached  
156 100% mortality by 10 hpi. Subsequent treatment with UPM2146 led to a drastic reduction in bacterial load.  
157 Specifically, in the phage-treated group, bacterial counts dropped from initial levels to zero by 10 hpt and  
158 remained at zero until 20 h. This contrasts with untreated embryos, where bacterial counts increased sharply.  
159 These findings underscore UPM2146 potent bactericidal effect and its potential as a therapeutic agent.  
160 Additionally, the study confirmed the safety of UPM2146, positioning this phage as a promising candidate for  
161 the safe and effective treatment of *K. pneumoniae* infections (21).

162 ***Acinetobacter baumannii***. The study by Neto *et al.* (2023) validated the use of a bath infection model with tail-  
163 injured zebrafish embryos to evaluate the efficacy of phage treatments against *A. baumannii* strains, a major  
164 bacterial pathogen associated with antibiotic resistance-related deaths. Phage treatments were administered  
165 at a concentration of  $10^9$  PFU/mL at 12 hpi to target bacteria introduced at  $10^4$  CFU/mL and 48 hpf, focusing  
166 on highly virulent strains such as K2, K9, K32, and K45. Control groups within the study underscored the non-  
167 toxicity of the phage treatments and highlighted the need for phage specificity, as demonstrated when the  
168 non-specific K1 phage failed to improve survival in embryos infected with the K32 strain. Specific phage  
169 treatments markedly enhanced survival rates, boosting survival from an average of 35% to 74% for the K32  
170 strain. The effectiveness of these phages was illustrated in significant improvements in survival, particularly  
171 against the most virulent strains (22).

## 172 173 **Phage Therapy Studies in Pathological Zebrafish Embryo Model of Cystic Fibrosis.**

174 The significant genetic homology shared between zebrafish embryos and humans, along with the ease of  
175 genetic manipulation, through techniques like CRISPR/Cas9, transgenesis, and morpholinos (chemically  
176 modified oligonucleotides designed to bind to specific mRNA sequences and inhibit protein translation),  
177 allows researchers to dissect gene functions and effectively model human diseases (23). Notably, the

178 morpholino of the cystic fibrosis transmembrane regulator (CFTR) gene in zebrafish embryos is a valuable  
179 model for CF research. Embryos share structural similarities with human CFTR channels and exhibit increased  
180 susceptibility to infections, mimicking CF patient responses. Despite lacking lungs, zebrafish embryos possess  
181 mucins similar to those overexpressed in the lungs of CF patients, enabling relevant studies of infection  
182 responses across various organs. Zebrafish embryos also exhibit CF-related symptoms such as pancreatic  
183 dysfunction and hematopoietic anomalies, providing insights into aspects of CF not observed in mouse model.  
184 Additionally, the absence of an adaptive immune system response during the first weeks of development  
185 makes them ideal for exploring exclusively innate immune defenses against infections, which are pertinent to  
186 human CF (24, 25). The distinction between wild-type (WT) and CF zebrafish embryos plays a pivotal role in  
187 understanding the interaction between genetic backgrounds and treatment outcomes in infectious disease  
188 research. WT embryos typically exhibit a robust immune response that can be indicative of general immune  
189 capabilities in a non-compromised biological system. In contrast, CF embryos, which genetically mimic the  
190 human condition of cystic fibrosis, often display altered immune responses and heightened sensitivity to  
191 infections. This sensitivity is primarily due to the mutation in the *cfr* gene, which affects various physiological  
192 responses, including those related to bacterial infections.

193

194 *Pseudomonas aeruginosa*. Cafora *et al.* conducted two studies, in 2019 and 2022, that provide valuable insights  
195 into the development and optimization of phage therapy for treating *P. aeruginosa* infections in zebrafish  
196 embryos in both WT and CF contexts. The studies utilized similar phage cocktails, including two *Podoviridae*  
197 vB\_PaeP\_PYO2, vB\_PaeP\_DEV, and two *Myoviridae* vB\_PaeM\_E215, and vB\_PaeM\_E217. These phages were  
198 chosen due to their proven efficacy in preliminary tests on *Galleria mellonella* larvae against *P. aeruginosa*, a  
199 pathogen that is notably difficult to treat due to its inherent and acquired resistance mechanisms.

200 In the study by Cafora *et al.* (2019), *P. aeruginosa* PAO1 strain (30 CFU/embryo) were injected into the duct  
201 of Cuvier of WT and CF embryos at 48 hpf to assess their susceptibility, revealing that CF embryos exhibited  
202 significantly higher lethality rates compared to WT counterparts (83% vs. 66% at 20 hpi). To test the efficacy  
203 of phage therapy in zebrafish, they first administered the phage cocktail into the yolk sac of PAO1-infected  
204 WT embryos (300-500 PFU/embryo) at either 0.5 or 7 hpi, leading to a notable reduction in lethality from 70%  
205 to 42% and 38% for early and late injection, respectively. Further experiments confirmed the efficacy of phage  
206 therapy in both WT and CF embryos with earlier infection time, showing a decrease in lethality from 66% to  
207 35% in WT and from 83% to 52% in CF embryos. Additionally, phage treatment significantly reduced the  
208 bacterial burden in both embryo types by about 80% at 8 hpi, indicating substantial bacterial clearance. Time-  
209 lapse analyses revealed that while untreated WT embryos showed increased fluorescence due to bacterial  
210 proliferation of the PAO1 strain labeled with GFP, those treated with phages showed no increase, suggesting  
211 effective antibacterial activity by the phages. Similar patterns were observed in CF embryos, though with  
212 inherently higher bacterial levels. Interestingly, Cafora *et al.* (2021) showed that CF zebrafish embryos exhibit  
213 significantly higher levels of pro-inflammatory cytokines TNF- $\alpha$  and IL- $\beta$  even without induced PAO1  
214 infection, indicating that the absence of *Cfr* function inherently triggers a chronic inflammatory state, similar  
215 to human CF patients. They also found that administering the phage cocktail to these CF embryos, in the  
216 absence of bacterial infection, reduced TNF- $\alpha$  and IL- $\beta$  levels closer to those observed in wild-type embryos.  
217 This implies that phages might have a secondary role as immunomodulators, potentially moderating  
218 inflammation rather than exacerbating it, especially in environments with pre-existing inflammation, such as  
219 in CF. Moreover, their study also examined the combined effects of phage and antibiotic therapy  
220 (ciprofloxacin, 100  $\mu$ g/mL in fish water). CF embryos treated with both phages and ciprofloxacin exhibited the  
221 lowest lethality rates compared to those treated with either treatment alone. Thus, the results demonstrate that  
222 while both the phage cocktail and ciprofloxacin effectively combat *P. aeruginosa* infection, their combination  
223 provides a superior benefit. This synergistic effect allows for the same therapeutic outcome with reduced  
224 dosages of each agent, potentially lowering the risk of developing bacterial resistance against either treatment

225 Taken together, this study shows that phage therapy was effectively applied in a CF zebrafish model, marking  
226 its first successful *in vivo* use in such a model. Phage therapy not only combatted bacterial infections but also  
227 modulated immune responses, reducing inflammation in CF model while mildly increasing cytokine  
228 expression in WT. This immunomodulatory potential of phages could make them valuable as anti-  
229 inflammatory agents in CF treatment (26).

230 Cafora *et al.* (2022) subsequently explored the efficacy of prophylactic and therapeutic treatments of  
231 phosphatidylserine/phosphatidic acid (PS/PA) liposomes and the phage cocktail mentioned above on PAO1-  
232 infected WT or CF embryos. The rationale behind this combination is that while phages target extracellular  
233 bacteria, PS/PA liposomes can potentially address intracellular bacteria and stimulate macrophage activity,  
234 which is crucial as *P. aeruginosa* can reside inside macrophages where phages cannot reach (27). The research  
235 initially focused on the prophylactic administration of PS/PA liposomes (28 hpf) followed by a PAO1 infection  
236 (100-300 CFU/embryo, 48 hpf) and subsequent phage cocktail treatment (500 PFU/embryo, 3 hpi) into the duct  
237 of Cuvier. In WT embryos, which exhibit a natural immune response, PS/PA liposomes alone were  
238 surprisingly more effective than phage treatment in reducing PAO1 infection, and the combination of PS/PA  
239 liposomes with the phage cocktail did not enhance the antimicrobial effect beyond that achieved by the  
240 liposomes alone. However, in CF embryos, both PS/PA liposomes and phages showed comparable  
241 effectiveness when used individually but acted synergistically when combined, significantly lowering the  
242 bacterial burden. The therapeutic potential of PS/PA liposomes and phage cocktail was then assessed under  
243 conditions that mimic a clinical scenario where embryos were first infected with PAO1 and then treated.  
244 Although both treatments reduced the bacterial load in infected embryos, the therapeutic application of PS/PA  
245 liposomes was notably less effective than the prophylactic approach, likely due to the shorter time available  
246 for full antimicrobial macrophage activation. Phage cocktail was effective in significantly reducing bacterial  
247 burdens in both WT and CF embryos to a similar extent. When used together, PS/PA liposomes and phage  
248 cocktail only further decreased the bacterial load in WT embryos, with no significant difference observed in  
249 CF embryos compared to phage treatment alone. The studies also examined the effect of these treatments on  
250 a phage-resistant PAO1 strain. Treatment with either phage cocktail or PS/PA liposomes reduced the overall  
251 bacterial burden by about 40-50% in both WT and CF embryos. However, the combination therapy was more  
252 effective than either treatment alone, suggesting a synergistic effect. Interestingly, the proportion of phage-  
253 resistant bacteria was significantly reduced by the combination treatment in both embryo types, indicating  
254 that PS/PA liposomes effectively helped to control the infection, including the one caused by the phage-  
255 resistant strain. These findings underscored the potential of using PS/PA liposomes in conjunction with phage  
256 therapy to treat PAO1 infections effectively but also to enhance outcomes in settings where phage resistance  
257 is a concern. The ability of PS/PA liposomes to augment the effects of phage therapy, particularly against  
258 resistant strains, provides a promising avenue for the development of comprehensive treatment strategies for  
259 bacterial infections in CF and other clinical contexts (28).

260 These studies collectively highlight the versatility and potential of the zebrafish embryo model for  
261 evaluating phage therapy in treating *P. aeruginosa* infections, particularly in a CF context. Although zebrafish  
262 embryos do not replicate the chronic *P. aeruginosa* infections typical in CF patients, they offer a rapid, cost-  
263 effective system for *in vivo* testing. The conservation of the innate immune response between fish and humans  
264 allows for insights into how macrophages, which are often defective in CF, can be activated by liposomes.  
265 Despite lacking lungs, zebrafish offers a valuable genetic model for studying CF due to their optical  
266 transparency and the ease of genetic manipulation. This model helps in understanding CF pathophysiology  
267 and developing new treatments. Additionally, these studies emphasize the need for innovative approaches  
268 that combine phages with other therapeutic modalities to optimize treatment efficacy and enhance patient  
269 outcomes.

270 *Mycobacterium abscessus*. The study by Johansen *et al.* (2021) focused on the application of phage therapy to  
271 treat *M. abscessus* infections in zebrafish embryos in WT and CF contexts (29). Two phages were used in this  
272 research, Muddy, which efficiently kills the *M. abscessus* GD01 strain, and Gabriela, which infects and kills  
273 *M. abscessus* very inefficiently compared to Muddy. Following initial confirmation that *M. abscessus* infection  
274 could be established via caudal vein injection (300 CFU/embryo, 30 hpf), the application of Muddy ( $1.5 \times 10^4$   
275 PFU/embryo, 1 to 5 days post-infection, dpi) showed no significant difference in survival rates of infected WT  
276 embryos. However, a notable reduction in bacterial burden was achieved with Muddy, as measured by  
277 fluorescent pixel count (FPC), contrasting with the lesser effect observed with Gabriela. This reduction in  
278 bacterial load by Muddy did not translate into increased survival but did significantly decrease pathological  
279 signs associated with infection, such as cord formation and abscess development. Specifically, Muddy  
280 treatment resulted in about a two-fold decrease in cord formation, a hallmark of acute infection in zebrafish,  
281 at 2, 4, and 6 dpi. In contrast, Gabriela alone did not produce a significant change in cord formation, and  
282 combining Muddy with Gabriela did not enhance the effect over Muddy alone. Regarding abscess formation,  
283 which indicates severe infection, both Muddy alone and in combination with Gabriela significantly reduced  
284 the occurrence of abscesses at 4 and 6 dpi, while Gabriela alone did not affect abscess formation. These findings  
285 underscore Muddy ability to effectively lyse *M. abscessus* and reduce severe infection markers in zebrafish  
286 embryos, though it did not extend larval survival within the study's 12-days observation period. This suggests  
287 that while phage therapy with Muddy can reduce the bacterial load and pathology, extending treatment  
288 duration might be necessary to observe potential improvements in survival.

289 The authors then assessed the role of functional innate immunity, particularly the involvement of  
290 macrophages, in supporting the efficacy of the phage Muddy in treating *M. abscessus* infections in zebrafish  
291 embryos. Macrophages are crucial for controlling this infection, as they are integral to granuloma formation  
292 and the containment of mycobacterial growth. Depletion of macrophages using liposomal clodronate led to  
293 rapid larval death within days, emphasizing the vital role these cells play in managing mycobacterial  
294 infections. When macrophages were depleted, infection with *M. abscessus* resulted in 100% mortality by 8 dpi,  
295 a significantly more severe outcome compared to infections in WT embryos or those treated with liposomal  
296 PBS. Despite the administration of phages (Muddy, Gabriela, or both), no improvement in survival rates was  
297 observed in macrophage-depleted embryos, with median times to 50% mortality remaining at 6 and 7 days,  
298 respectively. Furthermore, phage therapy did not alter the bacterial burden in these embryos, nor did it reduce  
299 the incidence of cords and abscesses, which were drastically increased in macrophage-depleted larvae  
300 compared to controls. This was further evidenced by the presence of large infection foci in phage-treated  
301 embryos compared to untreated ones, as demonstrated by FPC analysis and whole-embryo imaging. These  
302 findings underscore the indispensable role of macrophages in the effectiveness of phage therapy against  
303 *M. abscessus*, highlighting the necessity of a functional innate immune system to achieve successful outcomes  
304 in phage therapy.

305 Moreover, the study validated phage treatment against *M. abscessus* in CF zebrafish embryos. The results  
306 showed that *M. abscessus*-infected CF embryos were highly susceptible to infection, displaying rapid and  
307 severe symptoms including increased bacterial burden, pronounced cording, and abscess formation. Phage  
308 treatment, using either Muddy alone or in combination with Gabriela, significantly improved outcomes in CF  
309 embryos. Specifically, treatment extended survival (median time to 50% mortality increased from 8 to 10 days)  
310 and enhanced overall survival rates (20% in untreated *vs.* approximately 40% in Muddy-treated embryos at  
311 12 dpi). Moreover, phage therapy markedly reduced the physical manifestations of infection such as cords  
312 and abscesses at various dpi, as well as the bacterial load, compared to untreated controls. These findings  
313 suggest that CF zebrafish, like young CF patients, are hypersensitive to *M. abscessus* infection, but respond  
314 favorably to phage therapy. The effective reduction in infection severity and improvement in survival rates  
315 highlight the potential of phage therapy as a treatment option for CF-related infections, underscoring the  
316 utility of the zebrafish CF model in studying the effectiveness of therapeutic interventions in a context



317 mimicking human disease. In addition, this study also investigated the efficacy of combining phage therapy  
318 (Muddy) with the antibiotic rifabutin in CF embryos with *M. abscessus* infection. The results demonstrated that  
319 while treatment with rifabutin alone or Muddy alone significantly improves survival rates compared to  
320 untreated CF embryos, the combination of both treatments substantially enhances the therapeutic outcomes.  
321 Specifically, the combination of Muddy and rifabutin not only increases the survival rate of infected CF  
322 zebrafish to 70% at 12 dpi, comparable to survival rates in WT zebrafish, but also extends the median time to  
323 50% mortality beyond the calculation threshold, indicating a significant reduction in mortality. The results  
324 underscore the complex interactions between phages, the host immune system, and bacteria, highlighting the  
325 need to consider a patient's immunological status before implementing phage therapy. Overall, the zebrafish  
326 embryo model supports the development of phage therapies and could serve as a pre-clinical platform for  
327 assessing effective phage and antibiotic combinations, providing crucial insights before clinical application in  
328 *M. abscessus* patients.

### 329 THE ZEBRAFISH ADULT MODEL FOR ADVANCED PHAGE THERAPY

330 The adult zebrafish model provides complementary advantages to the embryo model, making it a valuable  
331 tool for preclinical research. Its fully developed immune system, which includes both innate and adaptive  
332 immunity, allows for studies that closely mimic human immune responses, offering relevant insights into  
333 disease mechanisms and treatment outcomes. Additionally, adult zebrafish facilitate long-term studies  
334 essential for chronic diseases research and sustained treatment evaluations. With fully formed and matured  
335 organs, they are pivotal for investigating organ-specific diseases and functions. Moreover, the increased size  
336 of adult zebrafish allows for the administration of larger quantities of bacteria, phages, or active substances,  
337 which is advantageous when studying the effects of these agents at realistic physiological levels. However,  
338 this can sometimes pose a problem when compound is precious or limited in quantity. Unlike their embryonic  
339 counterparts, adult zebrafish lose the transparency that facilitates direct observation, necessitating the fixation  
340 of tissues and the production of histological sections for detailed study. This requirement can complicate the  
341 research process and may limit the immediate visual assessment of internal processes (9).

342 In this section, we highlight the adult zebrafish model's effectiveness in validating the phages efficacy  
343 against human bacterial pathogens.

344 *Klebsiella pneumoniae*. In their 2021 study, Sundaramoorthy *et al.* explored the safety and therapeutic  
345 potential of phage KpG in treating *K. pneumoniae* infections within adult zebrafish model. They monitored  
346 liver and brain enzyme profiles for potential toxicity and assessed the therapeutic impact through phages  
347 administration for treating *K. pneumoniae* infections. Zebrafish injected with phage KpG showed no significant  
348 variations in liver enzyme profiles, and a slight but statistically insignificant increase in brain acetylcholine  
349 esterase levels. Histological analysis of muscle and liver tissues, stained with hematoxylin and eosin,  
350 confirmed the absence of morphological or biochemical aberrations, suggesting that phage treatment does not  
351 induce adverse effects in zebrafish. To assess the *in vivo* efficacy of the phages, they infected zebrafish  
352 intramuscularly with the MTCC 432 strain of *K. pneumoniae* ( $10^5$  CFU/mL). Treatments included streptomycin,  
353 phage KpG ( $10^8$  PFU/mL), or a combination of both administered intramuscularly 2 hpi. Results indicated that  
354 phage KpG alone reduced bacterial counts in muscle tissue by 77%, while streptomycin alone achieved a 62.8%  
355 reduction. The combination of both treatments significantly enhanced efficacy, leading to a 98% decrease in  
356 bacterial counts. These findings underscore the utility of the adult zebrafish model in advancing our  
357 understanding of infectious diseases and treatment strategies (30).

358 *Escherichia coli*. In 2020, Easwaran *et al.* explored the therapeutic potential of phage  $\Phi$ EcSw in treating *E. coli*  
359 bacterial infections in adult zebrafish that were infected intraperitoneally with the *E. coli* Sw1 strain ( $4.10^6$   
360 CFU/fish) and simultaneously treated with phage  $\Phi$ EcSw ( $4.10^7$  CFU/fish). Zebrafish treated with  $\Phi$ EcSw  
361 exhibited a 100% survival rate up to 84 hpi, markedly higher than the 70% survival rate observed in the *E. coli*

362 Sw1-infected group without phage treatment. The findings from the Easwaran *et al.* (2020) study demonstrate  
363  $\Phi$ EcSw potential as an effective therapeutic agent against *E. coli* Sw1 infections in adult zebrafish, reinforcing  
364 the relevance of using adult zebrafish to study specific phage therapy (31).

## 365 DISCUSSION

366 The zebrafish embryo model has become a useful tool for studying the dynamics of infectious diseases and  
367 evaluating treatment responses in a controlled environment (Figure 2 and Table 2). Some of the research  
368 mentioned above have focused on comparing WT and CF zebrafish embryos to understand how genetic  
369 variations influence their responses to bacterial infections and phage therapy treatments. Studies have shown  
370 that CF embryos exhibit higher susceptibility to severe infections and increased mortality rates compared to  
371 WT embryos when exposed to the same pathogenic challenges. For instance, CF embryos infected with the  
372 same concentration of *P. aeruginosa* show significantly higher mortality rates due to the impact of the *cftf*  
373 mutation on their ability to manage bacterial threats (26, 28). Similar vulnerability is observed in CF embryos  
374 infected with *M. abscessus*, resulting in bacterial burdens and more severe clinical manifestations (such as  
375 cording and abscess formation) compared to WT embryos (29). Phage therapy efficacy also varies between the  
376 two genetic types, with WT embryos responding better to phage treatments due to their more robust immune  
377 system synergizing with phage bactericidal actions. These findings from the described studies illustrate that  
378 genetic differences between WT and CF zebrafish significantly influence their response to infections and  
379 treatments. Additionally, in CF embryos, the mutation contributes to other complications, such as pancreatic  
380 dysfunction, which can affect the overall health and treatment outcomes in these models. Therefore,  
381 understanding these differences is crucial for tailoring phage therapy strategies to enhance efficacy and reduce  
382 mortality, particularly in vulnerable populations, and CF zebrafish embryos represent a valuable tool in  
383 achieving these goals. Currently, only the CF model has been tested for phage therapy treatment; however,  
384 other pathological models could also prove highly useful for testing various infections.

385 In addition, it is also important to discuss various parameters that are essential for comparing the models,  
386 including experimental variables such as the timing, methods of administration, dosages used, and how these  
387 correlate with mortality rates. The age of zebrafish embryos during experimentation impacts study results on  
388 infectious diseases and treatments. The physiological characteristics and immune system capabilities of  
389 embryos undergo rapid developmental changes within the first few days post-fertilization, which can  
390 influence their response to infections and treatments. Innate immune response begins emerging around 24  
391 hpf, but complete functional maturity occurs around 72 hpf with the presence of functional macrophages and  
392 neutrophils (32–34). Consequently, embryos at different developmental stages may exhibit varied  
393 susceptibility to pathogens and differing efficacies of therapeutic interventions. For example, younger  
394 embryos (less than 24 hpf) may be more susceptible to bacterial infections due to their less mature immune  
395 system and the absence of neutrophil population, potentially leading to higher mortality rates. Conversely,  
396 older zebrafish embryos and larvae demonstrate more robust immune responses and, therefore, higher  
397 survival rates when exposed to the same pathogenic load.

398 The method of administering treatments in zebrafish embryos is crucial for optimizing therapeutic efficacy  
399 and understanding the dynamics of infection control. Various administration routes can impact the  
400 distribution, absorption, and effectiveness of treatments, particularly in the context of phage therapy.  
401 Intravenous injection, typically into the duct of Cuvier or the caudal vein, is a common method for introducing  
402 phages directly into the bloodstream of zebrafish embryos. This route ensures rapid dissemination of the  
403 treatment throughout the organism, providing immediate therapeutic effects. For instance, intravenous  
404 administration of phage cocktails in CF embryos significantly reduced bacterial loads and improved survival  
405 rates, demonstrating the efficacy of this approach for systemic infections (19, 26, 28, 29). Intraperitoneal  
406 injections which potentially exposes multiple organs to phages, also demonstrated no adverse effects

407 suggesting that phages may be safely used without triggering harmful systemic immune responses in adult  
408 zebrafish (31). Administering treatments intramuscularly or into the hindbrain ventricle target specific regions  
409 for localized infections. These methods were used to treat *E. coli* in adult zebrafish and *S. aureus* infections in  
410 zebrafish embryos, resulting in significant reductions in mortality rates. The localized administration helps  
411 concentrate the phage at the site of infection, enhancing the localized immune response and therapeutic  
412 effectiveness (20, 30). The immersion method involves exposing zebrafish embryos to a solution containing  
413 phages or antibiotics, allowing for a more generalized treatment application. This is particularly useful for  
414 treating external infections or when a less invasive method is preferred (21, 22). Studies using the immersion  
415 approach with embryos exposed to *K. pneumoniae* demonstrated its potential, with treated embryos showing  
416 drastically lower mortality rates and reduced bacterial loads compared to controls. While each administration  
417 route has its advantages, the choice of method depends on the type of infection, the desired speed of  
418 therapeutic action, and the specific physiological targets. Therefore, intravenous and hindbrain ventricular  
419 injections provide controlled and immediate effects, ideal for acute or localized infections. In contrast,  
420 immersion method is more suitable for external infections or when a broader treatment distribution is needed.  
421 The efficacy of these methods depends on the pathogen involved and the health status of the embryos. For  
422 example, the effectiveness of immersion can be influenced by the water solubility and stability of the phage or  
423 drug ability to penetrate biological tissues. Carefully selecting the method of treatment administration can  
424 significantly influence the outcome of therapeutic interventions. Understanding the nuances of each method  
425 allows for the development of more tailored and effective treatment strategies, thereby improving overall  
426 success rates in combating infectious diseases.

427 The relationship between phage dosages and mortality rates in zebrafish embryos infected with various  
428 bacterial species is pivotal in optimizing phage therapy. The studies reviewed demonstrate how dosage levels  
429 can significantly affect the survival outcomes, emphasizing the importance of precision in phage application.  
430 The research on *S. aureus* indicated a concentration-dependent survival benefit, where higher doses of phages  
431 correlated with better survival rates. For instance, untreated embryos experienced 100% mortality, while those  
432 receiving the highest phage concentration had a 100% survival rate over 72 hpt, highlighting the need for  
433 adequate phage dosing against aggressive strains like MRSA (20). Moreover, consistently between the *S.*  
434 *aureus* and *E. faecalis* studies, the authors showed that live phage significantly reduced mortality rates  
435 compared to heat-killed phages, emphasizing the importance of phage viability alongside dosing (19) (Plumet  
436 *et al.*, 2024). Finally, study on treatments against *A. baumannii* requiring high doses of specific phages to  
437 improve survival rates, underscored the need for phage specificity in addition to dose optimization (22). These  
438 examples illustrate the critical role of phage dosage in determining the success of infection control. Optimal  
439 dosages not only ensure the reduction of bacterial loads but also contribute to the health and survival of the  
440 host.

441 Moreover, combining phage therapy with antibiotics has shown promise in enhancing therapeutic efficacy  
442 and addressing complex bacterial infections in adults and zebrafish embryos. However, it is essential to  
443 evaluate the effectiveness of each combination independently due to varying synergistic effects. In the study  
444 involving *S. aureus* by Plumet *et al.* (2024), phage SAVM02 was tested against MRSA infections in zebrafish  
445 embryos. The study found that high doses of SAVM02 alone were as effective as the combination of the phage  
446 with vancomycin, indicating that in some cases, phage therapy alone may be sufficient to effectively manage  
447 infections (20). In addition, a study by Cafora *et al.* (2022) demonstrated that liposomes combined with phages  
448 effectively helped to control the infection, including those caused by resistant strains (28). Furthermore, when  
449 phage therapy was combined with the rifabutin in CF embryos, the therapeutic outcomes were substantially  
450 enhanced, showing a greater reduction in mortality and pathology than when either treatment was used alone.  
451 This synergistic effect underscores the potential for combined treatments in managing severe infections,  
452 especially in genetically predisposed populations (29).

453 Zebrafish models could also be used to gain valuable insights into several other critical aspects of phage  
454 therapy research, beyond just assessing therapy efficacy. For example, zebrafish models could be used to study  
455 the pharmacodynamics and pharmacokinetics (PD/PK) of phage therapy. It would be very interesting to test  
456 if the zebrafish model could allow real-time tracking of phages within a living organism, potentially providing  
457 critical data on absorption, distribution, metabolism, and excretion (ADME) of phages, though this remains to  
458 be demonstrated. This could be particularly useful in determining optimal dosing and understanding the  
459 interaction between phages and the host biological systems. The timing of phage administration is another  
460 crucial aspect for maximizing therapeutic efficacy. While our review primarily focuses on efficacy  
461 assessments, it is important to note that zebrafish models can be used to investigate the critical aspect of  
462 therapy timing. For instance, Cafora *et al.* (2019) tested different times of phage administration post-infection,  
463 finding similar results for both early (about 30 minutes) and late (7 hours) injections. This suggests that the  
464 timing of phage administration may not significantly impact efficacy, though this remains to be confirmed by  
465 further studies as it is the only known study addressing this aspect. Additionally, the innate immune response  
466 to phages is a critical area of study, as it can influence the efficacy and safety of phage therapy. Zebrafish, with  
467 their well-characterized innate immune system, provide an excellent model for studying these interactions as  
468 studies have shown that phages can impact or require the host immune response (29, 35).

## 469 CONCLUSIONS

470 In conclusion, zebrafish models have significantly advanced our understanding of phage therapy in the  
471 context of bacterial infections, making a rapidly evolving field bolstered by recent publications. These models  
472 are essential for elucidating bacterial pathogenesis and gauging the efficacy of treatments, particularly in the  
473 exploration of diverse therapeutic combinations. This research complements broader studies that demonstrate  
474 the efficacy of phage-antibiotic synergies against multidrug-resistant organisms, thereby establishing phage  
475 therapy as a vital element of comprehensive antimicrobial strategies. Despite anatomical and physiological  
476 distinctions from mammals, such as possessing gills rather than lungs and conducting hematopoiesis within  
477 the anterior kidney instead of bone marrow, zebrafish models provide critical preclinical insights. They  
478 expedite the drug discovery and development trajectory by swiftly identifying compounds that are either  
479 ineffective or unsafe, thereby conserving both time and resources. This initial screening is pivotal, ensuring  
480 that only the most promising therapeutic candidates progress to more intricate animal models and, ultimately,  
481 to human clinical trials. Zebrafish models offer a rapid, cost-effective, and ethically responsible approach for  
482 assessing novel therapeutic agents, thereby serving an intermediary role in the translation of new treatments  
483 from laboratory research to clinical practice. However, it is important to recognize several limitations of the  
484 zebrafish model. There is significant variability in experimental protocols across different laboratories, such  
485 as variations in inoculums and administration times, leading to inconsistent results. Anatomically, zebrafish  
486 have gills instead of lungs and undergo hematopoiesis in the anterior kidney rather than the bone marrow,  
487 which can affect the generalization of results to mammals. The smaller size of zebrafish limits complex surgical  
488 interventions, and the reduced complexity of some organs compared to mammals may impact the study of  
489 certain diseases. Additionally, while the zebrafish immune system is similar to that of mammals, there are key  
490 differences that can influence the outcomes of phage therapy studies, particularly in embryos that lack a fully  
491 developed adaptive immune system. Finally, some human pathologies, such as chronic or long-term  
492 conditions, may not be perfectly mimicked in zebrafish. Despite these limitations, the zebrafish model remains  
493 a valuable tool in preclinical research, providing critical insights towards the development of new therapeutic  
494 strategies.

495

## 496 REFERENCES

- 497 1. Kulshrestha M, Tiwari M, Tiwari V. 2024. Bacteriophage therapy against ESKAPE bacterial pathogens:  
498 Current status, strategies, challenges, and future scope. *Microb Pathog* 186:106467.

- 499 2. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, Paterson DL, Walker  
500 MJ. 2020. Antimicrobial Resistance in ESKAPE Pathogens. *Clin Microbiol Rev* 33:e00181-19.
- 501 3. Mancuso G, Midiri A, Gerace E, Biondo C. 2021. Bacterial Antibiotic Resistance: The Most Critical  
502 Pathogens. *Pathogens* 10:1310.
- 503 4. Hampton HG, Watson BNJ, Fineran PC. 2020. The arms race between bacteria and their phage foes.  
504 *Nature* 577:327–336.
- 505 5. Wang Y, Fan H, Tong Y. 2023. Unveil the Secret of the Bacteria and Phage Arms Race. *Int J Mol Sci*  
506 24:4363.
- 507 6. Lin DM, Koskella B, Lin HC. 2017. Phage therapy: An alternative to antibiotics in the age of multi-  
508 drug resistance. *World J Gastrointest Pharmacol Ther* 8:162–173.
- 509 7. Luong T, Salabarria A-C, Roach DR. 2020. Phage Therapy in the Resistance Era: Where Do We Stand  
510 and Where Are We Going? *Clinical Therapeutics* 42:1659–1680.
- 511 8. Plumet L, Ahmad-Mansour N, Dunyach-Remy C, Kissa K, Sotto A, Lavigne J-P, Costechareyre D,  
512 Molle V. 2022. Bacteriophage Therapy for *Staphylococcus aureus* Infections: A Review of Animal Models,  
513 Treatments, and Clinical Trials. *Front Cell Infect Microbiol* 12:907314.
- 514 9. Lawrence C. 2007. The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture* 269:1–20.
- 515 10. Rasheed S, Fries F, Müller R, Herrmann J. 2021. Zebrafish: An Attractive Model to Study *Staphylococcus*  
516 *aureus* Infection and Its Use as a Drug Discovery Tool. *Pharmaceuticals* 14:594.
- 517 11. Gomes MC, Mostowy S. 2020. The Case for Modeling Human Infection in Zebrafish. *Trends in*  
518 *Microbiology* 28:10–18.
- 519 12. Lieschke GJ, Currie PD. 2007. Animal models of human disease: zebrafish swim into view. *Nat Rev*  
520 *Genet* 8:353–367.
- 521 13. Renshaw SA, Trede NS. 2012. A model 450 million years in the making: zebrafish and vertebrate  
522 immunity. *Dis Model Mech* 5:38–47.
- 523 14. Strähle U, Scholz S, Geisler R, Greiner P, Hollert H, Rastegar S, Schumacher A, Selderslaghs I, Weiss  
524 C, Witters H, Braunbeck T. 2012. Zebrafish embryos as an alternative to animal experiments—A commentary  
525 on the definition of the onset of protected life stages in animal welfare regulations. *Reprod Toxicol* 33:128–132.
- 526 15. Aleström P, D'Angelo L, Midtlyng PJ, Schorderet DF, Schulte-Merker S, Sohm F, Warner S. 2020.  
527 Zebrafish: Housing and husbandry recommendations. *Lab Anim* 54:213–224.
- 528 16. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic  
529 development of the zebrafish. *Dev Dyn* 203:253–310.
- 530 17. Torraca V, Mostowy S. 2018. Zebrafish Infection: From Pathogenesis to Cell Biology. *Trends Cell Biol*  
531 28:143–156.
- 532 18. Meijer AH, Spaink HP. 2011. Host-Pathogen Interactions Made Transparent with the Zebrafish Model.  
533 *CDT* 12:1000–1017.
- 534 19. Al-Zubidi M, Widziolek M, Court EK, Gains AF, Smith RE, Ansbro K, Alrafaie A, Evans C, Murdoch  
535 C, Mesnage S, Douglas CWI, Rawlinson A, Stafford GP. 2019. Identification of Novel Bacteriophages with  
536 Therapeutic Potential That Target *Enterococcus faecalis*. *Infect Immun* 87:e00512-19.
- 537 20. Plumet L, Magnan C, Ahmad-Mansour N, Sotto A, Lavigne J-P, Costechareyre D, Kissa K, Molle V.  
538 2024. The Zebrafish Embryo Model: Unveiling its Potential for Investigating Phage Therapy Against  
539 Methicillin-Resistant *Staphylococcus aureus* Infection. *Antimicrob Agents Chemother in press*.
- 540 21. Assafiri O, Song AA-L, Tan GH, Hanish I, Hashim AM, Yusoff K. 2021. *Klebsiella* virus UPM2146 lyses  
541 multiple drug-resistant *Klebsiella pneumoniae* *in vitro* and *in vivo*. *PLoS One* 16:e0245354.
- 542 22. Neto S, Vieira A, Oliveira H, Espiña B. 2023. Assessing *Acinetobacter baumannii* virulence and treatment  
543 with a bacteriophage using zebrafish embryos. *FASEB J* 37:e23013.
- 544 23. Varshney GK, Burgess SM. 2014. Mutagenesis and phenotyping resources in zebrafish for studying  
545 development and human disease. *Brief Funct Genomics* 13:82–94.
- 546 24. Navis A, Bagnat M. 2015. Loss of *cfr* function leads to pancreatic destruction in larval zebrafish. *Dev*  
547 *Biol* 399:237–248.
- 548 25. Semaniakou A, Croll RP, Chappe V. 2019. Animal Models in the Pathophysiology of Cystic Fibrosis.  
549 *Front Pharmacol* 9:1475.
- 550 26. Cafora M, Deflorian G, Forti F, Ferrari L, Binelli G, Briani F, Ghisotti D, Pistocchi A. 2019. Phage  
551 therapy against *Pseudomonas aeruginosa* infections in a cystic fibrosis zebrafish model. *Sci Rep* 9:1527.

- 552 27. Fajardo-Lubian A, Venturini C. 2023. Use of Bacteriophages to Target Intracellular Pathogens. *Clinical*  
553 *Infectious Diseases* 77:S423–S432.
- 554 28. Cafora M, Poerio N, Forti F, Loberto N, Pin D, Bassi R, Aureli M, Briani F, Pistocchi A, Fraziano M.  
555 2022. Evaluation of phages and liposomes as combination therapy to counteract *Pseudomonas aeruginosa*  
556 infection in wild-type and CFTR-null models. *Front Microbiol* 13:979610.
- 557 29. Johansen MD, Alcaraz M, Dedrick RM, Roquet-Banères F, Hamela C, Hatfull GF, Kremer L. 2021.  
558 Mycobacteriophage–antibiotic therapy promotes enhanced clearance of drug-resistant *Mycobacterium*  
559 *abscessus*. *Dis Model Mech* 14:dmm049159.
- 560 30. Sundaramoorthy NS, Thothathri S, Bhaskaran M, GaneshPrasad A, Nagarajan S. 2021. Phages from  
561 Ganges River curtail *in vitro* biofilms and planktonic growth of drug resistant *Klebsiella pneumoniae* in a  
562 zebrafish infection model. *AMB Express* 11:27.
- 563 31. Easwaran M, De Zoysa M, Shin H-J. 2020. Application of phage therapy: Synergistic effect of phage  
564 EcSw ( $\Phi$ EcSw) and antibiotic combination towards antibiotic-resistant *Escherichia coli*. *Transbound Emerg Dis*  
565 67:2809–2817.
- 566 32. Herbomel Philippe, Thisse B, Thisse C. 1999. Ontogeny and behaviour of early macrophages in the  
567 zebrafish embryo. *Development* 126:3735–3745.
- 568 33. Herbomel P, Thisse B, Thisse C. 2001. Zebrafish Early Macrophages Colonize Cephalic Mesenchyme  
569 and Developing Brain, Retina, and Epidermis through a M-CSF Receptor-Dependent Invasive Process.  
570 *Developmental Biology* 238:274–288.
- 571 34. Le Guyader D, Redd MJ, Colucci-Guyon E, Murayama E, Kissa K, Briolat V, Mordelet E, Zapata A,  
572 Shinomiya H, Herbomel P. 2008. Origins and unconventional behavior of neutrophils in developing zebrafish.  
573 *Blood* 111:132–141.
- 574 35. Cafora M, Brix A, Forti F, Loberto N, Aureli M, Briani F, Pistocchi A. 2021. Phages as  
575 immunomodulators and their promising use as anti-inflammatory agents in a *cftr* loss-of-function zebrafish  
576 model. *Journal of Cystic Fibrosis* 20:1046–1052.

577

**TABLE 1. Main features of studies using zebrafish models (embryo and adult) to assess the efficacy of phage therapy against human pathogenic bacteria.**

Bacteria	Route of infection and treatment	Bacterial dose and zebrafish stage	Phage treatment	Combination therapy	Outcomes	Ref.
<b>Zebrafish healthy embryo model</b>						
<i>Enterococcus faecalis</i>	Circulation (duct of Cuvier)	30,000 CFU/embryo (30 hpf)	Phage SHEF2, 6×10 <sup>5</sup> PFU/embryo (2 hpi)	NA	↗ Survival rate (~57%)	(19)
<i>Staphylococcus aureus</i>	Local (hindbrain ventricle)	15,000 CFU/embryo (30 hpf)	Phage SAVM02, 70 PFU/embryo (2 hpi)	Vancomycin (300 µg/mL)	↗ Survival rate with phage, vancomycin or combination of both (~100%) ↘ Bacterial load with phage (~99%), vancomycin (~97%) or combination of both (~97%)	(20)
<i>Klebsiella pneumoniae</i>	Bath immersion	3.3×10 <sup>6</sup> CFU/mL (72 hpf)	Phage UPM2146, 1×10 <sup>6</sup> PFU/mL (0.5 hpi)	NA	↘ Bacterial load (~100%)	(21)
<i>Acinetobacter baumannii</i>	Bath immersion	10,000 CFU/mL (48 hpf)	Phages K2, K9, K32 and K45 alone, 10 <sup>9</sup> PFU/mL (12 hpi)	NA	↗ Survival rate (~40%)	(22)
<i>Pseudomonas aeruginosa</i>	Circulation (duct of Cuvier/yolk sac)	30 CFU/embryo (48 hpf)	Phages vB_PaeP_PYO2, vB_PaeP_DEV, vB_PaeM_E215 and vB_PaeM_E217 in cocktail, 300-500 PFU/embryo (0.5 hpi)	NA	↗ Survival rate with phages with treatment at 0.5 hpi (~28%) and 7 hpi (~30%) ↘ Bacterial load with phages (~80%) ↘ Inflammatory response with phages	(26)
<i>Pseudomonas aeruginosa</i>	Circulation (duct of Cuvier)	100-300 CFU/embryo (48 hpf)	Phages vB_PaeP_PYO2, vB_PaeP_DEV, vB_PaeM_E215 and vB_PaeM_E217 in cocktail, 500 PFU/embryo (3 hpi)	Phosphatidylserine/ phosphatidic acid liposomes (prophylactic treatment 24 hpf or therapeutic treatment 3pi)	↘ Bacterial load with phages (~80%) ↘ Bacterial load with liposomes in prophylaxis (~95%) and in therapy (~60%) ↘ Bacterial load with phages and liposomes (~95%)	(28)

<i>Mycobacterium abscessus</i>	Circulation (caudal vein)	300 CFU/embryo (30 hpf)	Phage Muddy, 1.5×10 <sup>4</sup> PFU/embryo (1 to 5 dpi)	NA	No significant difference in survival rates with phage ↘ Bacterial burden with phage ↘ Pathological signs with phage	(29)
<b>Zebrafish cystic fibrosis embryo model</b>						
<i>Pseudomonas aeruginosa</i>	Circulation (duct of Cuvier/yolk)	30 CFU/embryo (48 hpf)	Phages vB_PaeP_PYO2, vB_PaeP_DEV, vB_PaeM_E215 and vB_PaeM_E217 in cocktail, 300-500 PFU/embryo (0.5 hpi)	Ciprofloxacin immersion (100 µg/mL)	↘ Bacterial load with phages (~80%) ↘ Inflammatory response with phages ↗ Survival rate with phages (~30%), ciprofloxacin (~40%) and combination of both (~45%)	(26)
<i>Pseudomonas aeruginosa</i>	Circulation (duct of Cuvier)	100-300 CFU/embryo (48 hpf)	Phages vB_PaeP_PYO2, vB_PaeP_DEV, vB_PaeM_E215 and vB_PaeM_E217 in cocktail, 500 PFU/embryo (3 hpi)	Phosphatidylserine/ phosphatidic acid liposomes (prophylactic treatment 24 hpf or therapeutic treatment 3pi)	↘ Bacterial load with phages (~70%) ↘ Bacterial load with liposomes in prophylaxis (~70%) and in therapy (~40%) ↘ Bacterial load with phages and liposomes in prophylaxis (~90%) and in therapy (~80%)	(28)
<i>Mycobacterium abscessus</i>	Circulation (caudal vein)	300 CFU/embryo (30 hpf)	Phage Muddy, 1.5×10 <sup>4</sup> PFU/embryo (1 to 5 dpi)	Rifabutin immersion (50 µg/mL)	↗ Survival rate with phage (~20%), rifabutin (~20%), or combination of both (~40%) ↘ Bacterial burden and pathological signs with phage, rifabutin or combination of both	(29)
<b>Zebrafish adult model</b>						
<i>Klebsiella pneumoniae</i>	Local (intramuscular)	10 <sup>5</sup> CFU/mL	Phage KpG, 10 <sup>8</sup> PFU/mL (2 hpi)	Streptomycin	↘ Bacterial load with phage (~77%), streptomycin (~63%), or combination of both (~98%)	(30)
<i>Escherichia coli</i>	Local (intraperitoneal)	4.10 <sup>6</sup> CFU/adult	Phage ΦEcSw, 4.10 <sup>7</sup> PFU/adult (simultaneously)	NA	↗ Survival rate (~30%)	(31)

580 CF, cystic fibrosis; hpf, hours post-fertilization; dpf, days post-fertilization; hpi, hours post-infection; dpi, days post-infection; ↗, increase; ↘, decrease; NA, non-available.

581



582 **TABLE 2. Comparative summary of advantages and limitations of zebrafish embryo vs. adult models in**  
 583 **phage therapy research.**

Aspect	Zebrafish embryos	Adult zebrafish
Immune system	Simplified immune system; ideal for studying innate immunity	Fully developed immune system; includes both innate and adaptive responses
Real-time imaging	Transparency allows for real-time observation of infection dynamics and phage activity	Less transparency, making real-time imaging more challenging
Ethical considerations	Less regulated for embryos up to 5 days post-fertilization	More stringent ethical regulations due to vertebrate status
High-throughput screening	Suitable for large-scale studies due to high reproductive rate and lower cost	Lower throughput due to size and cost constraints
Genetic manipulation	Easier and quicker genetic manipulations (e.g., CRISPR/Cas9, morpholinos)	Genetic manipulations possible but more complex and time-consuming
Number of individuals	Large number of individuals can be tested in a single experiment	Fewer individuals can be tested; more labor-intensive to raise in large numbers
Complexity of organs	Less complex organ systems; may not fully mimic human physiology	Fully developed and more complex organ systems, better mimicking human physiology
Disease modeling	Limited to early developmental stages; not suitable for chronic or long-term disease studies	Suitable for chronic and long-term disease studies due to longer lifespan
Anatomical differences	Differences in hematopoiesis (anterior kidney vs. bone marrow) and respiratory system (gills vs. lungs)	Closer to human anatomy in terms of organ systems and physiological functions
Phage therapy specifics	Allows rapid screening of phage efficacy and dynamics in a controlled environment	Provides insights into long-term phage therapy effects and interactions with a fully developed immune system

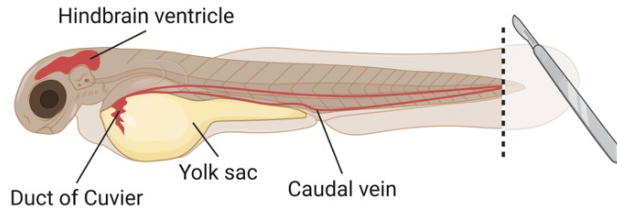
584

585

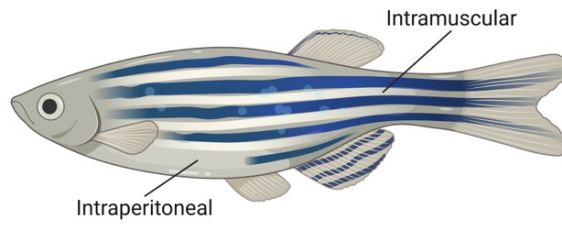
586

587 **FIGURE 1. Administration sites in zebrafish embryos and adults.** This illustration details the various  
588 administration sites used in both zebrafish embryos and adults as discussed in this review. Injections are  
589 categorized as systemic (duct of Cuvier, caudal vein, yolk), localized (hindbrain ventricle, intramuscular,  
590 intraperitoneal), or by bath immersion on healthy or tail-injured embryo. Created with BioRender.com.

**Embryo**



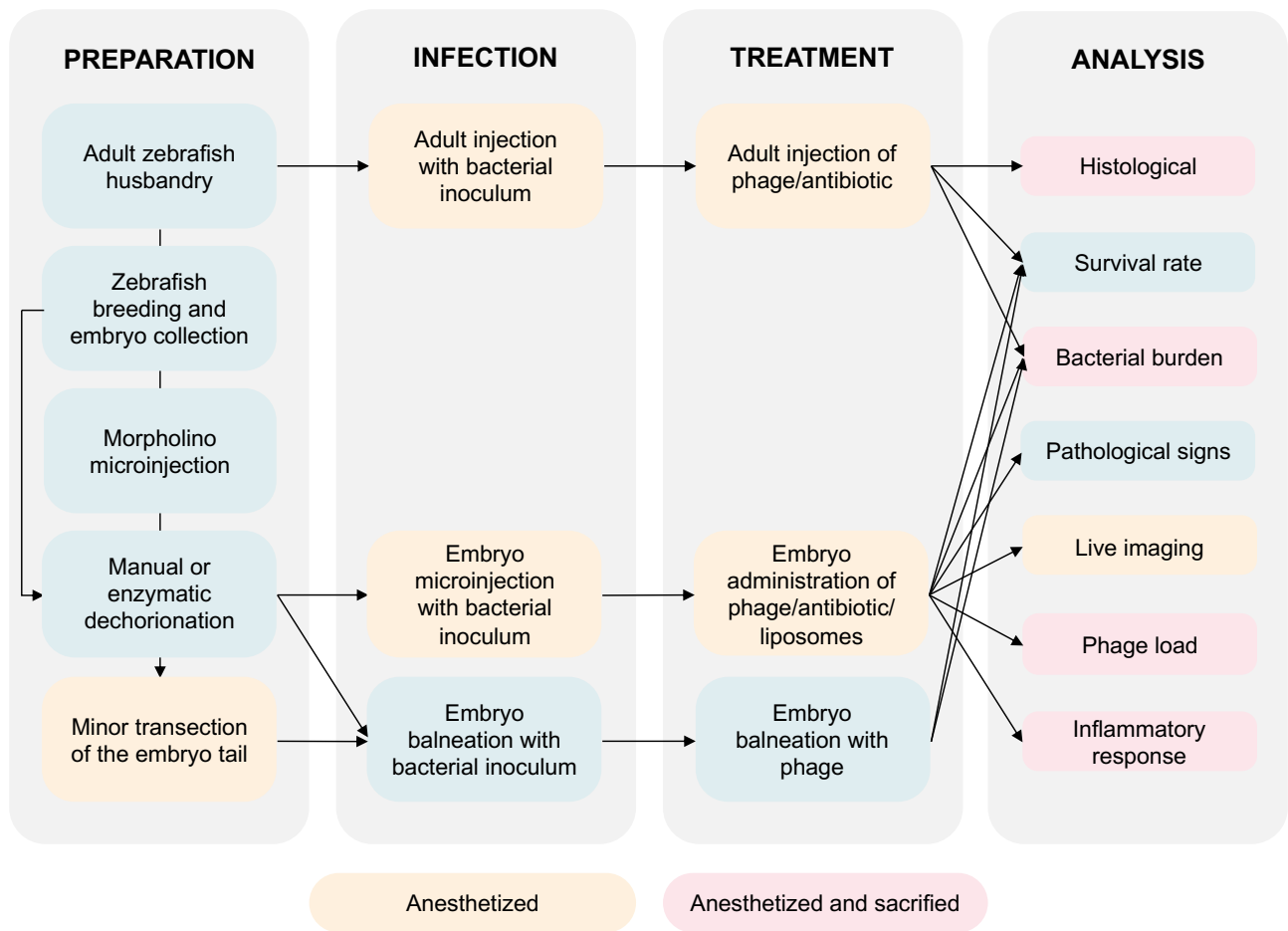
**Adult**



591

592

593 **FIGURE 2. Workflow for zebrafish model in phage therapy.** Adult zebrafish are maintained, crossed, and  
 594 raised according to general guidelines provided by the Zebrafish Information Network (ZFIN). Embryos are  
 595 collected, and when necessary morpholino microinjection is performed for gene knockdown studies. The  
 596 embryos are then manually or enzymatically dechorionated to remove the chorion. In the infection stage, adult  
 597 zebrafish are injected with a bacterial inoculum, and embryos can be either microinjected or bathed with  
 598 bacterial inoculum. During the treatment phase, adults receive an injection of phage or antibiotic, while  
 599 embryos undergo administration of phage, antibiotic, or liposomes through microinjection or balneation. The  
 600 analysis phase includes various methods to evaluate the outcomes, such as histological examination, survival  
 601 rate assessment, bacterial burden measurement, observation of pathological signs, live imaging, phage load  
 602 determination, and inflammatory response analysis. The color-coded legend indicates the different statuses of  
 603 the zebrafish during these procedures: yellow for anesthetized, and red for anesthetized and sacrificed.  
 604



605