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RESEARCH ARTICLE

Coinfection of channel catfish (*Ictalurus punctatus*) with virulent *Aeromonas hydrophila* and *Flavobacterium covae* exacerbates mortality

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Abstract

Flavobacterium covae and virulent *Aeromonas hydrophila* are prevalent bacterial pathogens within the US catfish industry that can cause high mortality in production ponds. An assessment of in vivo bacterial coinfection with virulent *A. hydrophila* (ML09-119) and *F. covae* (ALG-00-530) was conducted in juvenile channel catfish (*Ictalurus punctatus*). Catfish were divided into seven treatments: (1) mock control; (2) and (3) high and low doses of virulent *A. hydrophila*; (4) and (5) high and low doses of *F. covae*; (6) and (7) simultaneous challenge with high and low doses of virulent *A. hydrophila* and *F. covae*. In addition to the mortality assessment, anterior kidney and spleen were collected to evaluate immune gene expression, as well as quantify bacterial load by qPCR. At 96 h post-challenge (hpc), the high dose of virulent *A. hydrophila* infection (immersed in 2.3×10^{7} CFU mL^{-1}) resulted in cumulative percent mortality (CPM) of $28.3\pm9.5\%$, while the high dose of *F. covae* (immersed in 5.2×10^{6} CFU mL^{-1}) yielded CPM of $23.3\pm12.9\%$. When these pathogens were delivered in combination, CPM significantly increased for both the high- ($98.3\pm1.36\%$) and low-dose combinations ($76.7\pm17.05\%$) ($p<.001$). Lysozyme activity was found to be different at 24 and 48 hpc, with the high-dose vAh group demonstrating greater levels than unexposed control fish at each time point. Three proinflammatory cytokines (*tnfα*, *il8*, *il1b*) demonstrated increased expression levels at 48 hpc. These results demonstrate the additive effects on mortality when these two pathogens are combined. The synthesis of these mortality and health metrics advances our understanding of coinfections of these two important catfish pathogens and will aid fish health diagnosticians and channel catfish producers in developing therapeutants and prevention methods to control bacterial coinfections.

KEYWORDS
catfish, challenge, immune responses, mixed infections, polymicrobial

1 | INTRODUCTION

Catfish aquaculture in the United States is a key contributor to the agricultural economies of multiple southern states (Osmundsen et al., 2020), yielding $447 million in sales in 2022 (NASS, 2023). Aquaculture practices have improved over the past several decades yielding more efficient land use and greater production (Kumar et al., 2016). Along with enhanced rearing techniques, increased stocking densities associated with more intensive production practices result in more fish produced per acre. However, these increased stocking densities and intensive systems also increase the risk of disease (Bosworth et al., 2015; Cole et al., 2009).

Nearly 45% of inventory losses in catfish aquaculture are attributed to infectious diseases, of which 60% are caused by single or mixed bacterial infections (Hawke & Khoo, 2004). Bacterial infections have significant economic consequences in catfish aquaculture. In addition to direct losses in the form of mortality, the diminished feeding activity of sick fish reduces productivity through lost feed days. Furthermore, reactive treatments with medicated feeds are expensive and can lead to antibiotic resistance, which hinders the efficacy of the limited approved antibiotics (Ananda Raja & Jithendran, 2015; Chuah et al., 2016; Tekedar et al., 2020; Wise et al., 2015). Though several factors contribute to losses within the catfish industry, the gram-negative pathogens *Flavobacterium covae* (formerly *F. columnare* genetic group 2; LaFrentz et al., 2022) and an atypical, virulent strain of *Aeromonas hydrophila* (vAh) are important disease agents and responsible for significant losses within this sector (Baumgartner et al., 2017; Wise et al., 2021; Zhou et al., 2018).

In catfish, *F. covae* is the primary cause of columnaris disease, with losses approaching $30 million annually (Abdelrahman et al., 2023). Columnaris typically manifests as an external infection but can also cause systemic bacteraemia (Hawke & Thune, 1992), with mortality approaching 90% in severe outbreaks. The pathogen often colonizes the gills, resulting in a destructive necrosis that impairs gill function leading to asphyxiation (Shoemaker et al., 2008). Clinical signs associated with columnaris disease include, but are not limited to, necrotic gills and/or skin, yellow-pigmented bacterial aggregates on the mouth and fins, as well as a distinct 'saddleback' lesion on the dorsal surface (Declercq et al., 2013). There are limited treatment and preventative strategies for *F. covae*. Medicated feeds are available but come at a significant cost to the producer. While there is a commercially available vaccine, there has been limited adoption due to an inability to meet producer expectations (Bebak & Wagner, 2012).

Aeromonas hydrophila, a gram-negative bacterium, is the causative agent of motile aeromonad septicaemia (MAS) and infects many fish hosts. Generally considered an opportunistic pathogen, *A. hydrophila* can have devasting effects when coupled with other stressors and disease agents (Plumb & Hanson, 2010). Fish afflicted with MAS can present multiple clinical signs, including a severe haemorrhagic septicaemia. In 2009, an atypical strain of *A. hydrophila* emerged as a significant disease agent in the catfish farming regions of west Alabama and east Mississippi (Wise et al., 2021; Zhou et al., 2018). Since these first outbreaks, this virulent strain continues to plague the industry with catastrophic fish kill events, particularly in large, market-size fish. Losses attributed to *A. hydrophila* approach $35 million annually. Though *A. hydrophila* is often opportunistic, this virulent strain causes substantial mortality over a short period. At present, it is unknown if this strain can act as a primary pathogen or requires an additional as-yet-identified stressor to trigger infection (Baumgartner et al., 2017; Hemstreet, 2010; Richardson et al., 2021).

Coinfections are a common occurrence in aquaculture (Kotob et al., 2016). The intensive nature of fish farming provides an ideal environment for the transmission of disease, with combinations of pathogens (bacterial, viral, fungal, parasitic) afflicting a variety of hosts (Erfanmanesh et al., 2019; Figueroa et al., 2017; Han et al., 2021). Coinfections have been well documented in the catfish industry, with diagnostic reports from the Louisiana Aquatic Diagnostic Laboratory at Louisiana State University indicating the majority (86%) of cases involving columnaris disease involved mixed infections with other bacterial species, such as *Edwardsiella ictaluri* and *A. hydrophila* (Hawke & Khoo, 2004; Hawke & Thune, 1992). Likewise, reports from the Alabama Fish Farming Center in Greensboro, AL indicate coinfections of columnaris-causing bacteria and *A. hydrophila* are a common occurrence, accounting for nearly 10% of diagnostic submissions in 2019–2020 (Wise et al., 2021). The likelihood of coinfections may also increase due to potential portals of entry created as a result of columnaris. Damaged gill tissue and lesions leave fish vulnerable to other opportunistic pathogens, increasing the severity of infection (Labrie et al., 2004; LaFrentz et al., 2022; Zhou et al., 2018). Coinfective interactions and their impact on mortality, particularly the potential to exacerbate losses in otherwise subclinical infections are unknown. Assessing mortality and immune responses through an experimental challenge model provides crucial information on the severity and a preliminary understanding of immunological effects. Given the severe mortality each pathogen can elicit, it is hypothesized that coinfection of vAh and *F. covae* would increase mortality in coinfected treatment groups above that of single infections of each pathogen alone. Herein, the coinfective effects of vAh and *F. covae* on mortality and the effects on select immune parameters are reported for channel catfish, advancing our understanding of the coinfection dynamics and bacterial interactions of these two economically important fish pathogens.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Experimental procedures involved in this study were approved by the Auburn University Animal Care and Use Committee (Protocol # 2021-3979). Additionally, all pathogens were handled under Biological Use Agreement #926 (Bruce), approved by Auburn University's Department of Risk Management and Safety.

2.2 | Bacteria and culture conditions

An archived cryostock of vAh isolate ML09-119 (Hossain et al., 2013; Tekedar et al., 2013) was revived by isolation streaking on tryptic soy agar (TSA) and culture at 28°C for 24 h. An individual colony was expanded in 20 mL of tryptic soy broth (TSB) containing 0.4 mM xenosiderophore (iron chelator) deferoxamine mesylate (DFO; Sigma, St. Louis, MO, USA; Peatman et al., 2018). The culture was incubated with shaking (175 rpm) at 28°C for 12 h and subsequently used to inoculate 1 L of TSB with 0.4 mM of DFO (12 h at 28°C, 175 rpm). The final inoculation broth was adjusted to $OD_{600}=2.026$ using a Biophotometer Plus spectrophotometer (Eppendorf; Enfield, CT). *Flavobacterium covae* (ALG-00-530; LaFrentz et al., 2022) was similarly revived from archived cryostock by isolation streaking on modified Shieh agar (MSA; LaFrentz & Klesius, 2009) for 24 h at 28°C. A single colony was subsequently transferred to a 50 mL conical tube containing 12 mL of sterile, modified Shieh broth (MSB) and expanded for 12 h at 28°C with shaking (175 rpm). An aliquot (6 mL) was used to seed 200 mL of MSB and expanded for 12 h under the same conditions. The challenge culture was adjusted using sterile MSB to an $OD_{540}=0.707$. Colony forming units (CFU) mL^{-1} of the adjusted cultures were determined using standard spread plate count techniques and appropriate media for each pathogen (virulent *A. hydrophila*: TSA; *F. covae*: MSA).

2.3 | Experimental design

F. covae and vAh coinfections were characterized in vivo using ~22 g channel catfish (Marion strain; Table 1). Challenges consisted of seven treatment groups, with six tanks per treatment (20 fish $tank^{-1}$). Treatment groups 1 and 2 were challenged by immersion with two doses (high and low) of vAh, while groups 3 and 4 were challenged with two doses (high and low) of *F. covae*. Groups 5 and 6 (coinfection group) were similarly challenged but received combinations of either the high (Group 5) or low (Group 6) doses of each pathogen. Group 7 consisted of mock-challenged fish, and fish were exposed to sterile phosphate-buffered saline (PBS; pH 7.2) in lieu of bacterial inoculum (Figure 1). Three tanks were designated for sampling for each treatment to collect tissues at various timepoints without biasing mortality data. Similarly, three tanks were designated as non-sampling and used to establish the mortality of the challenge.

2.4 | Immersion challenge

Prior to the challenge, fish were pooled and distributed among 60 L tanks (20 fish $tank^{-1}$) containing 37.9 L of water. Catfish were acclimated for 2 d, in which they were monitored and fed twice daily a commercial floating catfish diet (Optimal Fish Food, Omaha, Nebraska). Tanks were supplied with 28°C ± 1°C dechlorinated municipal water at a rate of 0.4 L min^{-1}, and supplemental aeration was provided via airstones, which maintained dissolved oxygen (DO) at 8.0 ± 1.0 mg L^{-1}. All channel catfish, including controls, were fed to satiety 4 h before the challenge. Fish were anaesthetised in water containing 100 mg L^{-1} of tricaine methanesulfonate (MS-222; Syndel, Ferndale, Washington) and 100 mg L^{-1} of sodium bicarbonate and the adipose fin clipped according to Zhang, Xu and Shoemaker (2016). Fish were subsequently returned to their respective tanks, water flow suspended, and the volume lowered to 10 L prior to the addition of respective inoculums of each bacterial culture (PBS for the mock challenge). Fish were exposed for 1 h, after which water flow was restored to 0.5 L min^{-1} (Peatman et al., 2018). For the individual pathogen challenges, tanks were inoculated with 100 mL of culture for the high dose and 50 mL for the low dose. Coinfected groups received 200 mL (100 mL of vAh; 100 mL of *F. covae*) for the high-dose treatment and 100 mL (50 mL of vAh; 50 mL of *F. covae*) for the low-dose treatment, which were delivered simultaneously. Based on plate counts, groups exposed to vAh received 2.3×10^7 CFU mL^{-1} for the high dose and 1.1×10^7 CFU mL^{-1} for the low dose. Likewise, fish exposed to *F. covae* were exposed to 5.2×10^7 CFU mL^{-1} (high dose) and 2.6×10^7 CFU mL^{-1} (low dose) (Table 1).

2.5 | Collection and sampling

For each treatment group, fish were sampled from sampling tanks ($n=3$ tanks; 3 fish $tank^{-1}$) at 6, 12, 24, 48 and 96 h post-challenge. Anterior kidney, spleen and blood were collected aseptically and used to extract RNA, DNA and sera, respectively. Kidney and spleen

TABLE 1 Description of treatment groups, including which bacterium was administered, volume and final dose for challenged channel catfish.

Treatment	Inoculum	Volume administered	Final dose administered
High-dose *vAh*	vAh	100 mL	2.3×10^7 CFU mL^{-1}
Low-dose *vAh*	vAh	50 mL	1.1×10^7 CFU mL^{-1}
High-dose *F. covae*	*F. covae*	100 mL	5.2×10^7 CFU mL^{-1}
Low-dose *F. covae*	*F. covae*	50 mL	2.6×10^7 CFU mL^{-1}
High-dose coinfection	vAh; *F. covae*	100 mL; 100 mL	2.3×10^7 CFU mL^{-1}; 5.2×10^7 CFU mL^{-1}
Low-dose coinfection	vAh; *F. covae*	50 mL; 50 mL	1.1×10^7 CFU mL^{-1}; 2.6×10^7 CFU mL^{-1}
Controls	PBS	100 mL	NA

Abbreviation: PBS, phosphate-buffered saline.

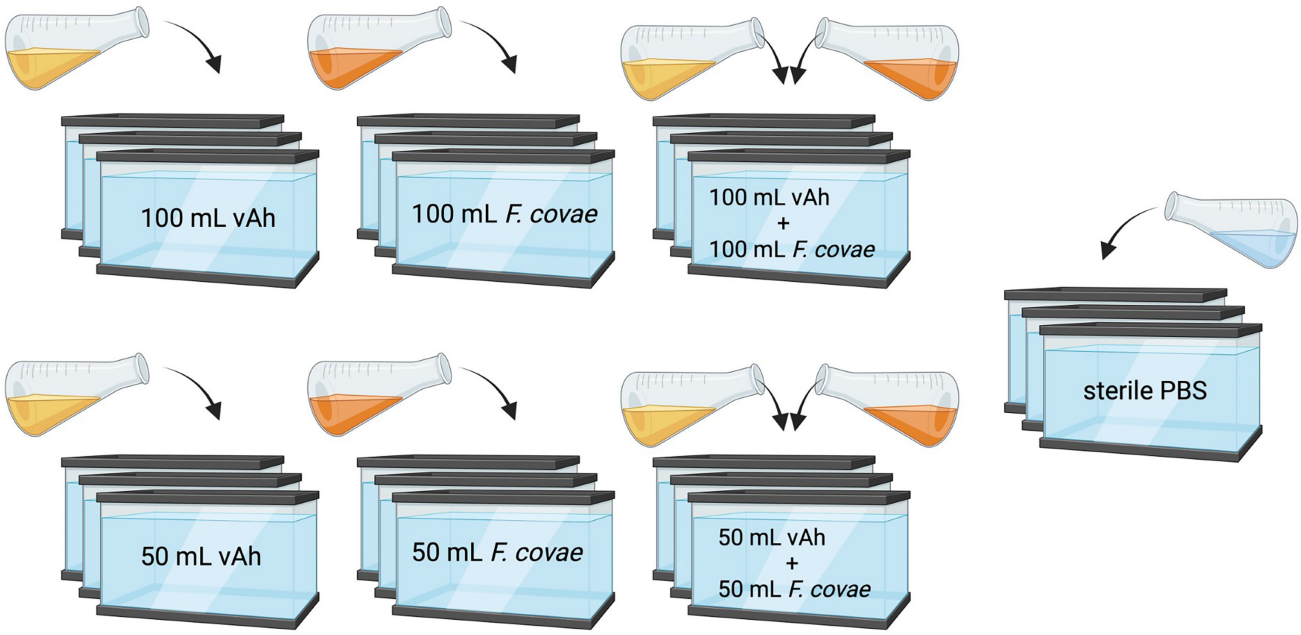

FIGURE 1 Graphic depicting experimental design to assess high and low doses of both single and coinfective treatment groups.

tissues were preserved in DNA/RNA Shield (Zymo Research Corp., Irvine, California) and stored at −20°C until nucleic acid extraction. Sera for lysozyme activity was collected by bleeding fish from the caudal vein using 22-gauge syringes and placing blood into microtubes. Blood was allowed to clot overnight at 4°C, centrifuged at 16,000×*g* (Eppendorf 5420; Enfield, CT) for 5 min, and then sera were harvested and stored at −80°C until needed. Aside from sampling, only dead fish were removed from tanks. Kidney and spleen tissue from 20% of the daily dead were cultured to confirm the presence of challenge bacteria. Tissues from coinfected groups were plated on TSA and MSA to target both bacteria.

2.6 | DNA and RNA extraction

Spleen tissues were homogenized with pestles within 1.5 mL microtubes. Genomic DNA was extracted using the Omega E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Inc., Norcross, Georgia). Kidney tissue samples were manually homogenized with pestles in DNA/RNA Shield (Zymo Research Corp., Irvine, California). RNA was extracted using a Zymo Research Quick-RNA™ MiniPrep Plus kit (Zymo Research Corp., Irvine, California). Similarly, genomic DNA was isolated from recovered single *F. covae* colonies, grown in broth and pelleted. Pellets collected from fresh mortalities were used for endpoint PCR to confirm pathogen identity. Genomic DNA from bacterial pellets was isolated using the Omega E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Inc., Norcross, Georgia, USA). Virulent *A. hydrophila* isolates were confirmed through colony pick PCR using a vAh-specific primer set (Griffin et al., 2013). All DNA and RNA samples were eluted with 100 µL of nuclease-free water. Extracted RNA and DNA samples were quantified spectrophotometrically with Nanodrop One^{c} (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at −20°C until needed.

2.7 | Gene expression analysis

Extracted RNA was diluted to 50 ng µL^{-1} using nuclease-free water and converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Each 20 µL reaction contained 2 µL of 10× R.T. buffer, 0.8 µL of 25× dNTP Mix, 2 µL of 10× R.T. random primers, 1 µL of Multiscribe™ reverse transcriptase, 500 ng of template RNA and nuclease-free water to volume and cDNA samples synthesized in a MiniAmp Plus thermal cycler (Applied Biosystems, Carlsbad, California) programmed for a single cycle of 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. Synthesized cDNA was then adjusted to 2.5 ng µL^{-1} with nuclease-free water for qPCR reactions. Four genes were evaluated for expression analysis, namely *il-1β*, *tnfα* (Wang et al., 2021), *il8* (Jiang et al., 2016) and *tgfβ1* (Moreira et al., 2017). The housekeeping genes *ef1α* (Jiang et al., 2017) and *actb* (Hao et al., 2021) were used for normalization. The PCR was performed in 10-µL volumes consisting of 5 µL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 0.25 µM of each forward and reverse primer (Table S1), 3 µL of sample cDNA (7.5 ng total) and nuclease-free water to volume. Each sample was run in duplicate along with no-template controls consisting of nuclease-free water in place of template cDNA. qPCR was run on a QuantStudio™ 5 Real-Time PCR system (Applied Biosystems) programmed for initial steps of 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s and 72°C for 30 s, with data collection occurring after the 72°C elongation. For each

gene target, reaction efficiencies were assessed using serial dilutions of cDNA covering five orders of magnitude, run in duplicate, and starting at 50 µg. For each gene, reaction efficiencies ranging from 90% to 110% were considered acceptable (Taylor et al., 2010). The $2^{-\Delta\Delta Ct}$ method was used to calculate all gene expression values (Schmittgen & Livak, 2008), combining both housekeeping genes (*ef1α* and *actβ*) and control groups for each sampling period, thus allowing each fold change of each gene to be expressed relative to control averages.

2.8 | Lysozyme activity assay

Lysozyme activity was quantified following previously published protocols (Welker et al., 2011). Lysozyme standards consisted of serial dilutions of chicken lysozyme egg white (Rockland Immunochemicals, Pottstown, Pennsylvania, USA) dissolved in sodium phosphate buffer (SPB; 0.04 M Na_2HPO_4; pH 6.0) and diluted to create a standard curve with a range of 0–16 µg mL^{-1}. Freeze-dried *Micrococcus lysodeikticus* (Worthington Biochemical, Lakewood, New Jersey, USA) was resuspended into 40 mL SPB at 0.25 mg µL^{-1}, and 250 µL of the bacterial suspension added to each well, along with 10 µL of sera. Each sample was run in duplicate. Absorbances at OD_{450} were collected after a 20 min incubation at 37°C with a Synergy HTX™ Multimode Reader (BioTek, Winooski, Vermont, USA) and compared with a standard curve assembled from prepared standards mentioned above run in tandem.

2.9 | Quantification of bacterial load

Quantitative polymerase chain reaction (qPCR) was performed with QuantStudio 5 Real-Time PCR instrument (Applied Biosystems, Carlsbad, California) to quantify the bacterial load within sampled spleens. Extracted DNA samples were diluted to 10 ng µL^{-1}. To quantify vAh present in splenic tissue, each qPCR reaction consisted of 9.5 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, California), 1 µL of forward and reverse primers (20 mM; S1), 1 µL of vAh probe (2 mM) (Table S1; Griffin et al., 2013), 1 µL of spud template DNA (500 copies), 0.5 µL of SPUD primers (Table S1), 0.5 µL of SPUD probe (Table S1; Nolan et al., 2006), 5 µL of template (50 ng reaction^{-1}) and 6.5 µL of nuclease-free water. Run conditions were programmed with a denaturation for 15 min at 95°C, 40 cycles of another 15-s denaturation at 95°C, and an annealing/extension step at 60°C for 1 min, with data collection following the annealing/extension step at the end of each cycle. Similarly, *F. covae* was quantified in spleen tissue using the same reaction conditions, only with *F. covae*-specific primers and probe (Table S1; Gibbs et al., 2020). Primer concentrations and run conditions were identical for quantifying both *vAh* and *F. covae*. SPUD DNA and probes served as internal positive controls to test for PCR inhibition. Each reaction plate contained samples run in duplicate along with negative controls (no template, only TE buffer) and 5, 10-fold serially diluted genomic DNA standards (1.4×10^3–1.4×10^7 genome equivalents) from each respective target bacteria (Richardson et al., 2021).

2.10 | PCR confirmation

The presence of each bacterial pathogen in both single and co-infected treatment groups was evaluated from fresh dead fish suitable for bacterial culture. All PCR reactions were conducted on a MiniAmp thermal cycler (Applied Biosystems, Carlsbad, CA). Colony PCR was performed on presumptive vAh by harvesting a single colony from isolation plates and resuspending it in reaction tubes containing 12.5 µL hot-start master mix (Trilink BioTechnologies, San Diego, CA), 0.5 µL (10 µM stock) each of vAh-specific primers 2968F and 2968R (Griffin et al., 2013) and 11.5 µL nuclease-free water (Griffin et al., 2013). Positive controls consisting of genomic DNA isolated from vAh strain ML09-119 and negative (nuclease-free water) controls were run in tandem with samples. Reactions were run with the following conditions: 95°C for 5 min, followed by 30 cycles at 95°C for 15 s, 58°C for 15 s and 72°C for 15 s, with a final extension step of 72°C for 5 min. Aliquots of PCR products (5 µL) were separated by electrophoresis through 2.0% agarose gels in Tris-acetate-EDTA (TAE) buffer, stained with GelRed (Biotium Inc., Fremont, California, USA) and visualized by ultraviolet transillumination in a Gel Doc Go imaging system (Bio-Rad, Inc., Hercules, California, USA). Samples were run alongside concurrently run molecular weight standards to confirm the presence of appropriately sized bands (167 bp).

Genomic DNA extracted from presumptive *F. covae* colonies from dead fish was confirmed by multiplex PCR (LaFrentz et al., 2019, 2022). Each 25-µL reaction contained 12.5 µL hot-start master mix (Trilink BioTechnologies, San Diego, CA), 2 µL of a primer cocktail (0.5 µM GG-forward, 0.1 µM GG1-reverse, 0.45 µM GG2-reverse, 0.45 µM GG3-reverse, 0.3 µM GG4-reverse), 5 ng of DNA template and nuclease-free water to volume. Reactions were performed with the following program: 95°C for 5 min, 40 cycles of 94°C for 30 s, 56°C for 20 s, and 72°C for 1 min, followed by 10 min at 72°C. Genomic DNA from *F. covae* AL-02-36^T type strain was run as a positive control, with nuclease-free water as no-template, negative controls. As described above, PCR products (5 µL) were resolved on a 2.0% agarose gel via electrophoresis, visualized by ultraviolet light, and identity confirmed by the presence of a 320 bp amplicon, revealed by direct comparisons to concurrently positive controls and molecular weight standards.

2.11 | Statistical analyses

The cumulative percent mortality (CPM) for the immersion pathogen trial was analysed using a one-way ANOVA, following an arcsine square root transformation. Comparisons between treatment groups and time periods for lysozyme activity, and gene expression analyses were performed using a repeated measures two-way ANOVA ($\alpha=0.05$) evaluating treatment, time, treatment × time and

incorporating tanks as a random factor. Gene expression data were log-transformed for the analyses. When a significant interaction was detected, one-way ANOVAs were conducted at each separate time point. Data from 48 and 96 hpc were not included for the high-dose coinfection treatment due to a lack of surviving fish (tanks had reached 100% mortality in sampling tanks). Tukey's HSD post hoc testing was incorporated when significant differences were observed ($p < .05$). For the bacterial load analyses, nonparametric Kruskal–Wallis tests were conducted to discern treatment effects at each time point, and multiple comparisons were made using Dunn's test. Statistical analysis was conducted using R statistical software (ver 4.3.0; R Core Team, 2021) and GraphPad Prism (ver 10.0.3). All errors reported represent the standard error of the mean between treatments.

3 | RESULTS

3.1 | Mortality due to in vivo pathogen challenge

Mortality was recorded daily throughout the trial, and endpoint CPM was calculated (96 h; Figure 2). The first mortality was observed 6 h post-challenge in both high and low vAh treatments and both coinfected groups. Mortality in the high-dose coinfection group stopped after 24 h, while mortality in both vAh treatments abated after approximately 48 h. Mortality for high and low *F. covae* treatment groups started 24–48 h post-challenge and ceased after 84 h. The results from the ANOVA indicated treatment-related differences in mortality $F\ (5,12) = 13.52$, $p < .05$. Mortality for all single infection groups (both high and low doses; *F. covae* and vAh) had significantly lower CPM than either of the high- or low-dose co-infected treatments ($p < .05$). Mortality in the high-dose coinfected group was 98.3 ± 0.3% CPM, while mortality in the low-dose coinfection treatment was 76.7 ± 4.2%. High dose, single infections for *F. covae* (23.3 ± 2.6%) and vAh (28.33 ± 1.5%) were significantly lower than coinfected groups ($p < .05$) but did not significantly differ from the low-dose treatments (*F. covae*: 10.0 ± 1.0%; vAh: 23.3 ± 1.9%; $p > .05$). No mortality was observed in any control tanks throughout the experiment.

3.2 | Presence of clinical signs

Consistent with diagnostic reports (Baumgartner et al., 2017), fish exposed to vAh exhibited severe ocular haemorrhaging along with external haemorrhaging of the anal and caudal fins (Figure 3). Internally, fish demonstrated haemorrhaging within the gastrointestinal tract. Clinical signs due to *F. covae* were also consistent with diagnostic reports (Hawke & Khoo, 2004), with skin discoloration, gill necrosis and dorsal lesions (saddleback). Fish coinfected with both pathogens demonstrated an amalgam of clinical signs. Clinical signs also differed by time and stage of infection. In the coinfected treatments, deceased fish collected soon after the initial infection (12 h) either had discoloration consistent with *F. covae* infections or mild external and internal haemorrhaging consistent with vAh infections (Figure 4). However, dead and moribund fish at 36 h and

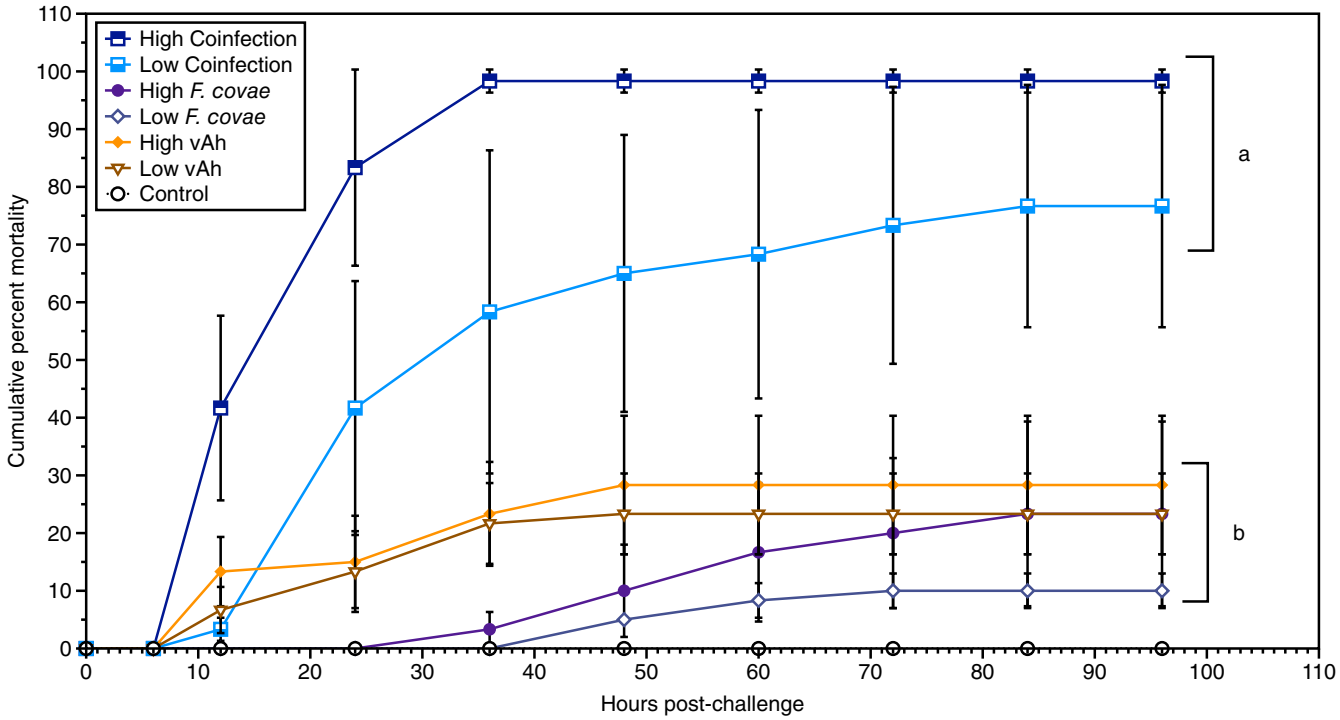

FIGURE 2 Cumulative percent mortality due to single infections of virulent *Aeromonas hydrophila* and *Flavobacterium covae* and coinfections from both pathogens throughout the trial (96 h). Each treatment group had three tanks ($n = 3$). Bars represent the standard error of the mean for each day.

FIGURE 3 Images document catfish with clinical signs due to singly infected vAh: ocular haemorrhaging and haemorrhaging of all fins (a) and *Flavobacterium covae*; skin discoloration, frayed fins, and damaged operculum (b).

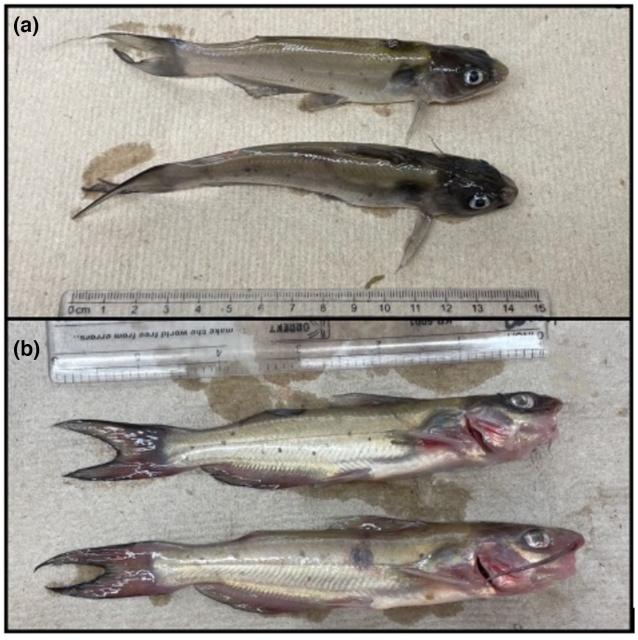

FIGURE 4 Images documenting catfish with clinical signs due to coinfection with *Flavobacterium covae* and vAh during early-stage infection (12 hpc). Image (a) depicts discoloration of deceased fish (saddleback lesion), and (b) depicts mild and external haemorrhaging in fins and operculum.

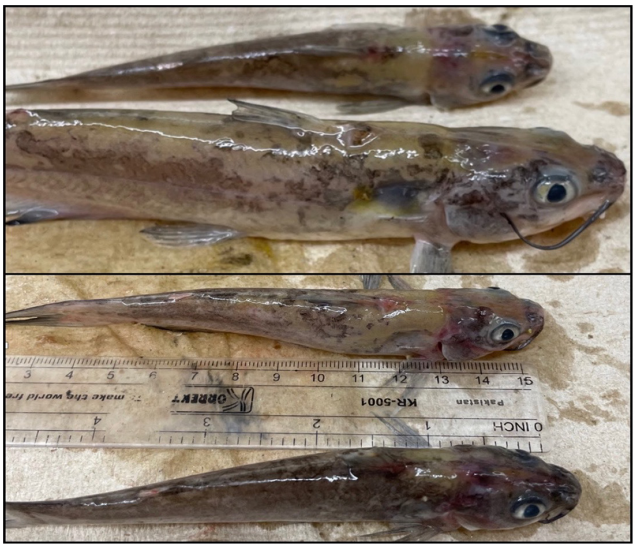

FIGURE 5 Images documenting catfish with clinical signs due to coinfection with *Flavobacterium covae* and vAh during later-stage infection (36 hpc).

beyond exhibited extreme external and internal haemorrhaging and epithelial sloughing consistent with clinical symptoms from both pathogens (Figure 5).

3.3 | PCR confirmations

Yellow-pigmented rhizoid colonies were reisolated from 38 out of 47 necropsied trial mortalities sampled for treatments containing *F. covae*. A subsample ($n = 21$) of these colonies we subjected to DNA extraction and 95.2% were confirmed as *F. covae* via multiplex PCR. On culture plates, colonies displaying morphology of vAh were reisolated from spleen all of the sampled challenge mortalities examined, and a subsample of these were confirmed via PCR, with 97.5% of reactions found positive as vAh.

3.4 | Sera lysozyme activity

Sera lysozyme activity was compared among all treatment groups over time at 6, 12, 24, 48 and 96 hpc and revealed a significant interaction effect ($F_{22,65} = 2.86$; $p < .001$) between treatment and time using the two-way repeated measures ANOVA (Figure 6). Resulting one-way ANOVAs were conducted at individual time points and discerned treatment differences. There was a significant difference between treatment groups at 24 hpc ($F_{6,14} = 6.71$; $p < .001$), where the high-dose vAh challenged catfish displayed increased lysozyme when compared to the controls and the *F. covae* groups. At 48 hpc,

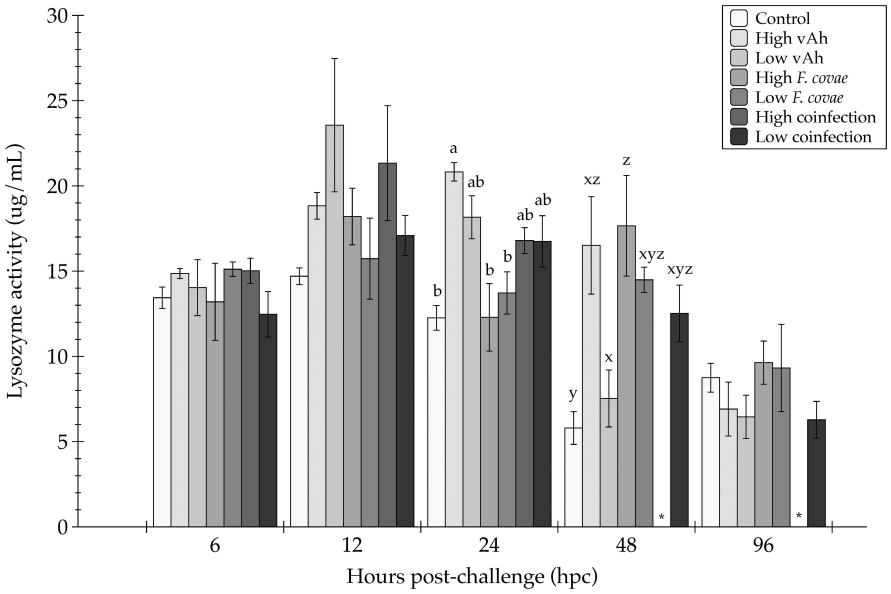

FIGURE 6 Lysozyme activity ($\mu g\,mL^{-1}$) in sera from sampled fish at 6, 12, 24, 48, and 96 h post-challenge. Each treatment group was analysed in triplicate ($n = 3$) using a one-way ANOVA at each time point. Capital letters indicate significant differences in activity between treatment time periods (6, 12, 24, 48, 96 hpc), and lowercase letters represent significance within treatment groups. Coinfected groups at 48 and 96 hpc are not included due to no surviving fish. Error bars represent the standard error of the mean for each treatment group.

treatment differences were also observed ($F_{5,12} = 5.84$; $p < .05$), with the high-level *F. covae* fish having a higher lysozyme activity than the control and low-dose vAh treatments. The high-dose vAh treatment group was also found to have higher lysozyme activity than the control group at 48 hpc.

3.5 | Gene expression analysis

No significant interactions were found between time and treatment for all genes evaluated ($p > .05$), and proinflammatory cytokines expression was increased at 48 hpc. Expression of *il1ß* was evaluated at 6, 12, 24, 48, and 96 hpc (Figure 7). Time was found to be significant in the model ($F_{4,65} = 7.14$; $p < .001$), and an increase in expression was observed at 48 hpc compared to all other timepoints; however, although there was a significant treatment effect ($F_{6,65} = 2.30$; $p = .045$), no differences were discerned with Tukey's HSD for treatment groups. Similar expression patterns were observed with the *il8* gene with a significant effect of time ($F_{4,65} = 5.02$; $p < .05$), but differences were not observed between 96 and 48 hpc (Figure 7). With respect to treatment effect ($F_{6,65} = 4.71$; $p < .001$), *il8* expression was higher than the *F. covae*-challenged catfish and the high coinfection group. The expression of *tnfα* and *tgfβ* genes were evaluated, and differences were detected over time for both genes (Figure 8). A treatment effect ($F_{6,65} = 2.26$; $p < .05$) was discerned for *tnfα* expression increase in was noted across treatments, with lower expression in the low-dose *F. covae* catfish compared with the unchallenged controls.

3.6 | Quantification of bacterial load

Bacterial loads of each pathogen were quantified at 6, 12, 24, 48 and 96 hpc (Figure 9a). Unfortunately, high dose, coinfected catfish at 48 and 96 h could not be measured due to a lack of surviving fish. The estimated bacterial loads for *F. covae* were highly variable (Figure 9). Treatment effects were detected at 6 hpc ($H_5 = 9.93$; $p < .05$) and 12 hpc ($H_4 = 8.19$; $p < .05$), although post hoc analyses did not discern any differences from the multiple comparisons at 6 hpc. However, at 48 h the high dose of only *F. covae* had significantly higher bacterial loads than the low-dose *F. covae* group ($p_{adj} = .048$).

For vAh, there were no treatment differences discerned at each sampling time point (Figure 9b). Bacterial loads were present in all groups exposed to vAh at 6 h and at 12 hpc, vAh was only detected in splenic tissue in the high-dose vAh treatment and the high-dose coinfected groups. Furthermore, the high-dose coinfected treatment was the only treatment with detectable vAh bacterial loads at 24 hpc. At 48 and 96 hpc, no bacterial loads were detected in any treatments.

4 | DISCUSSION

Coinfections occur in multiple aquaculture industries, causing significant mortality and economic loss (Jansen et al., 2019; Ma et al., 2019; Nicholson et al., 2020). Furthermore, coinfections among opportunistic pathogens can augment the severity of mortality above what would be caused by opportunistic pathogens alone, thus increasing the potential economic loss (Crumlish et al., 2010). Within the catfish industry, coinfections of vAh and *F. covae* have increased in recent years. While coinfections are a common problem in production ponds, little is known concerning infection mechanics, severity and mitigation strategies (Machimbirike et al., 2022), while the majority of research focuses on the effects of single infections (Declercq et al., 2013). As such, there is a need for more research into coinfective bacterial pathogens to fill a knowledge gap within the catfish industry (Mohammed & Peatman, 2018). This study evaluated the effects of two critically important bacterial pathogens (vAh and *F. covae*) on mortality in channel catfish in single and concurrent infections. Previous studies have documented the role

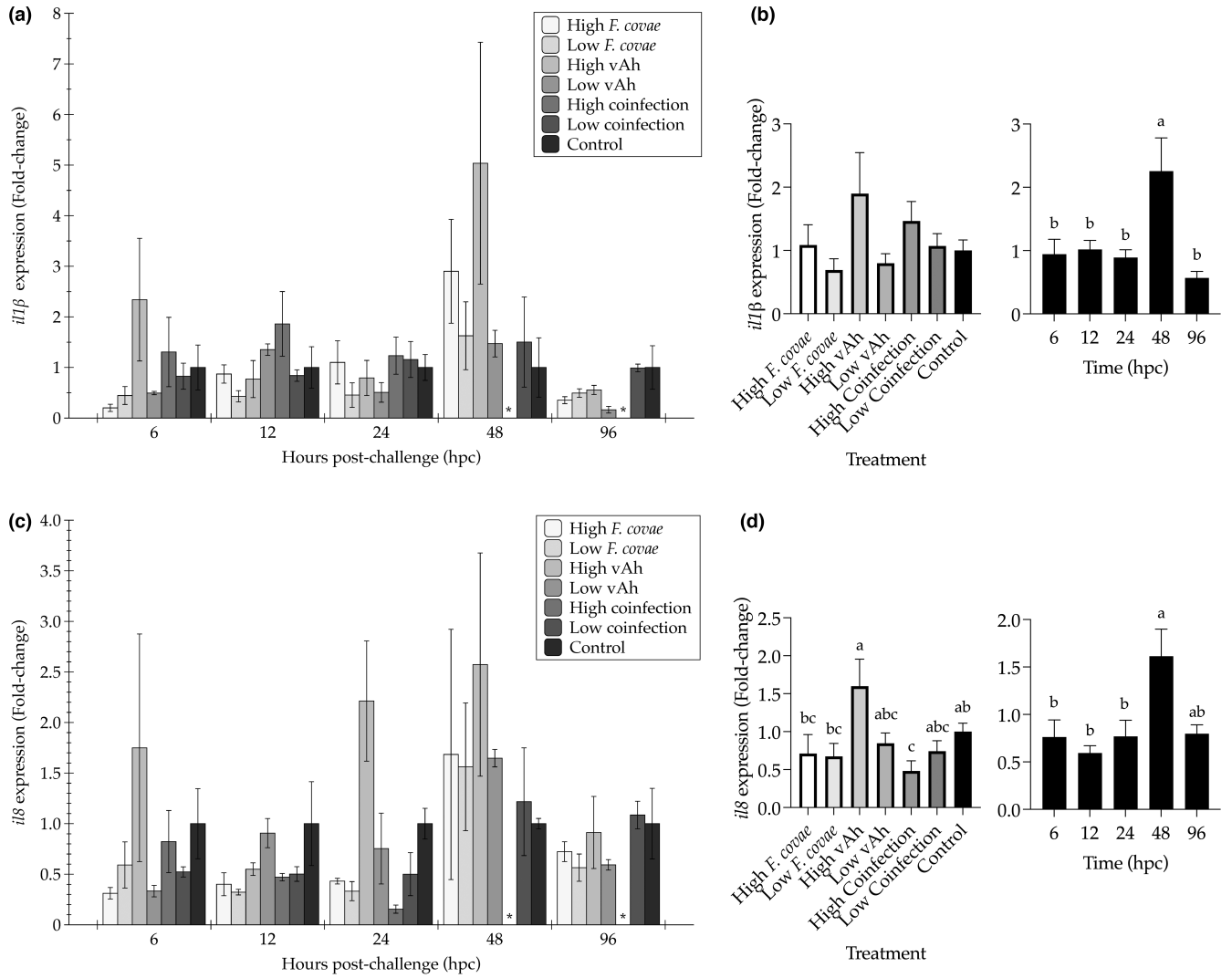

FIGURE 7 Summary of main effects (Treatment and Time) and interaction (Treatment × Time) from the two-way repeated measures analysis. Relative expression of *il1b* (a and b; fold change) and *il8* expression (c and d; fold change) in channel catfish anterior kidney. Letters indicate significant differences for the respective main effects and the analysis was performed on log-transformed data. Error bars represent the standard error of the mean for each treatment.

of these two pathogens in coinfections but focused on different co-infective agents and hosts (Crumlish et al., 2010; Ma et al., 2019). Channel catfish exposed to both pathogens experienced severely augmented mortality at all administered doses, suggesting a synergistic coinfection and lack of antagonism between the two bacteria, which are more lethal in combination than alone. In pathogen coinfections, synergistic effects may be attributed to an increased level of disease severity, and the potential for coinciding, elevated mortality rates (Okon et al., 2023). There have been several previous reports of this phenomenon with columnaris-causing bacteria. For instance, Dong et al. (2015) conducted coinfection testing with striped catfish (*Pangasianodon hypopthalmus*) immersion-challenged with *F. columnare* and *E. ictaluri*. A low dose of columnaris administered in combination with *E. ictaluri* resulted in an approximate 16% CPM increase compared to the *E. ictaluri* single dose, while the single dose of *F. columnare* only reached approximately 3% CPM. In a Nile tilapia (*Oreochromis niloticus*) coinfection trial reported by Nhinh et al. (2023), *F. oreochromis* (immersion) and *E. ictaluri* (intraperitoneal injection) also demonstrated a synergistic increase in CPM when the pathogens were co-administered.

Fish rely on the innate immune system as the first line of defence against an array of pathogens, of which lysozyme plays a key role (Saurabh & Sahoo, 2008). Lysozyme acts as a primary immunological barrier, and the antibacterial activity is well documented. Given its ubiquitous presence in fish mucus, lymphoid tissue, sera and other bodily fluids, measurements of lysozyme activity are an important indicator of the fish's innate immunity (Bladen et al., 1973; Magnadottir et al., 2005; Saurabh & Sahoo, 2008). Herein, lysozyme activity was investigated as a measure of the host's innate immune response to coinfections with *F. covae* and vAh. The present study detected few differences in lysozyme activity among treatment groups, which contrasts with previous work by Wise et al. (2023), which showed fish coinfected with *E. ictaluri* and *F. covae* had significantly greater lysozyme activity than single-infected groups,

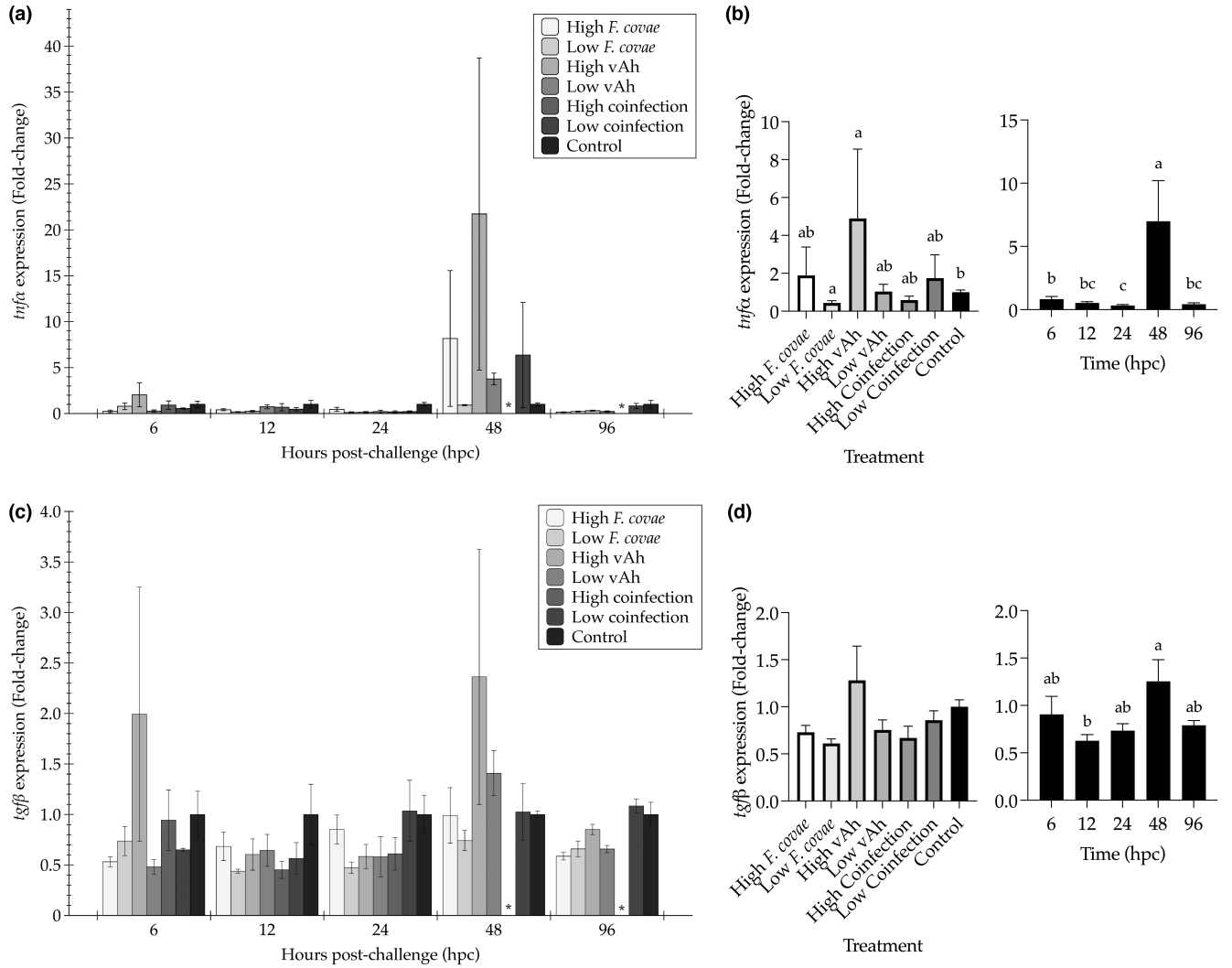

FIGURE 8 Summary of main effects (Treatment and Time) and interaction (Treatment × Time) from the two-way repeated measures analysis. Relative expression of *tnfα* expression (a and b; fold change), and *tgfβ* expression (c and d; fold change) in channel catfish anterior kidney. Letters indicate significant differences for the respective main effects and the analysis was performed on log-transformed data. Error bars represent the standard error of the mean for each treatment.

however, *E. ictaluri* acted as a primary pathogen and was the driver of mortality and lysozyme activity. Comparably, coinfection with two opportunistic pathogens (*F. covae* and vAh) resulted in highly varied lysozyme activity, and single high-dose infections were the only treatments resulting in increased lysozyme activity compared to controls. For *Aeromonas* spp. infections, it has been previously documented that *A. veronii* infection increased lysozyme expression in grass carp (*Ctenopharyngodon idellus*; Chen et al., 2022), and infections of *A. hydrophila* increased lysozyme activity for up to 21d in blunt nose sea bream (*Megalobrama amblycephala*; Xia et al., 2017). Unfortunately, samples from coinfected treatment groups were not obtainable at 48 or 96h due to a lack of surviving fish at the high doses. At the lower doses, coinfected groups did not present any difference in activity to other treatments. While there were limited differences between treatments, there were differences in lysozyme activity over time. This provides previously unknown information pertaining to the onset and abatement of infection, evident by increased lysozyme activity observed at 12 hpc compared to 6, 24 and 48h. Furthermore, it appears that the infection was initiated between 6 and 12 hpc, which coincided with peak observed mortality. Similarly, the decline in lysozyme activity at 96 hpc and diminishing mortality suggest the infection had run its course. While previous investigations have investigated *A. hydrophila*'s effects on lysozyme activity in other hosts (Xia et al., 2017), there is limited data regarding sera lysozyme activity in channel catfish exposed to vAh. While *F. covae* and vAh coinfections do not elicit a significant increase in lysozyme activity compared to single infections, these results provide insight into the infection dynamics (beginning, peak, and end of infection) of both single and coinfections of *F. covae* and vAh.

Proinflammatory cytokines (IL-8, TNF-α, and IL-1β) and an immunosuppressive cytokine (TGF-β) were measured to evaluate the dynamics of the innate immune system during coinfections. Cytokines are glycoproteins secreted by both innate and adaptive immune cells and aid in regulating the immune system. IL-1β

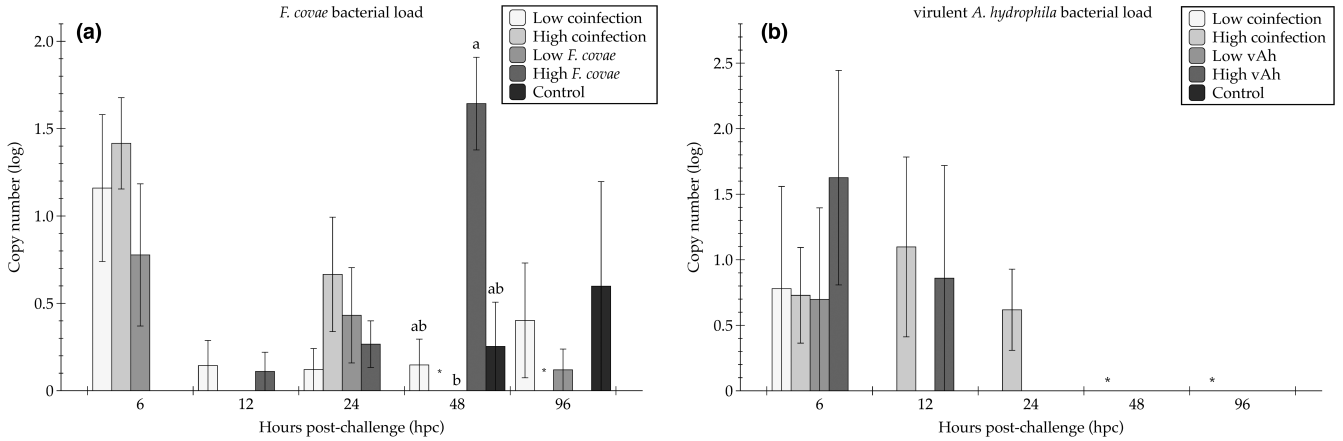

FIGURE 9 The bacterial load (log copy number) of channel catfish challenged with *Flavobacterium covae* (ALG-00-530) and vAh (ML09-119). Each analysis was performed to assess treatment effects at each time point using nonparametric analysis. The log of the copy number corresponds to 5 ng of input DNA for each sample. Each treatment group was analysed in triplicate ($n = 3$). Bars represent the standard error of the mean for each treatment.

serves as a chemoattractant for leukocytes and can influence the expression of additional cytokines throughout the fish (Zou & Secombes, 2016). In a previous study by Kumar et al. (2022), striped catfish exposed to a coinfection of *A. hydrophila* and the parasite *Icthyophthirius multifilis* exhibited a decrease in *il-1β* expression in liver tissue when compared to a non-pathogen exposed control group. IL-8 and TNF-α act as mediators for inflammation in the innate immune system, thus acting as important parameters to measure in order to monitor the innate immune response (Gao et al., 2012). In a zebrafish (*Danio rerio*) coinfection challenge study with *A. hydrophila* and *A. veronii*, *tnf-α* expression in kidney tissue of coinfected fish was highly induced when compared to non-infected controls (Chandrarathna et al., 2018). Liu et al. (2020) also reported a modulation of both *tnf-α* and *il-1β* expression in the head kidney tissue of Chinese perch (*Siniperca chuatsi*) following exposure to an experimental coinfection with *A. hydrophila* and infectious spleen and kidney necrosis virus (ISKNV). TGF-β functions to promote tissue repair and can inhibit the expression of proinflammatory cytokines to modulate the immune response (Baloch et al., 2022). Overall, there are limited reports of bacterial–bacterial coinfections and the fish immune response, and the current study sheds insight into the importance of better characterizing the host response to these complex infections. Interestingly in the current study, all genes demonstrated significantly higher expression at 48 hpc, primarily induced by vAh, but there were no differences between treatments for any evaluated cytokines. These results present integral information pertaining to coinfection immune responses. These cytokines levels were not affected during coinfection despite severely exponentiated mortality. However, differences in expression between single and coinfected treatment groups could not be made due to extreme sample variation and limited sample sizes.

Bacterial loads in the spleen were estimated by qPCR for each bacterium. Results for vAh coincide with observed mortality, indicating vAh infection is severe, but short-lived. This aligns with field observations, wherein outbreaks consist of rapid onset, catastrophic mortality events occurring over 1–2 days (Baumgartner et al., 2017; Richardson et al., 2021). There was no vAh DNA detected after 24 hpc. Furthermore, bacterial loads persisted longer in catfish exposed to higher vAh doses. This coincided with mortality data, where fish started dying between 6 and 12 hpc. This is consistent with a previous study by Zhang, Moreira, et al. (2016), who conducted a waterborne challenge and measured bacterial distribution and tissue targets of vAh in channel catfish. Those study results reported that the highest vAh bacterial loads in the spleen were observed between 1 and 4 hpc, and no vAh was detected after 48 hpc.

Results for *F. covae* were more variable, with the greatest bacterial loads observed early in the challenge and diminishing as the trial progressed. These analyses were complicated by the presence of qPCR-positive fish lacking signs of disease in the unexposed controls at 96 hpc. This could be due to the design of primers by Gibbs et al. (2020). These primers target what were previously considered *F. columnare* genomovar I and II, and thus would be expected to amplify DNA from *F. columnare* and *F. covae*. Since columnaris-causing bacteria are ubiquitous, the observed signal could represent underlying infection with less virulent *F. columnare*, or perhaps other closely related, yet non-pathogenic *Flavobacterium* spp.

In summary, coinfection between *F. covae* and virulent *A. hydrophila* under these laboratory conditions significantly increased mortality compared to single infections of both pathogens. Innate immunity parameters correlated with mortality trends, with the highest values occurring around 48 hpc, with minimal differences between treatments. Future studies should investigate the effects of currently approved antibiotics on coinfections and other mitigation strategies.

AUTHOR CONTRIBUTIONS

Timothy J. Bruce: Conceptualization; investigation; funding acquisition; writing – review and editing; visualization; validation; methodology; formal analysis; project administration; resources; supervision;

data curation. **Allison L. Wise:** Investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; software; data curation; conceptualization. **Benjamin R. LaFrentz:** Conceptualization; investigation; funding acquisition; writing – review and editing; methodology; project administration; supervision; resources. **Anita Kelly:** Methodology; writing – review and editing; supervision. **Mark Liles:** Methodology; writing – review and editing; supervision. **Matt Griffin:** Investigation; methodology; validation; writing – review and editing. **Benjamin Beck:** Funding acquisition; project administration; resources; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

All participating authors declare no conflicts of interest for these experiments.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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