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RESEARCH ARTICLE

Synergistic infection of *Edwardsiella ictaluri* and *Flavobacterium oreochromis* in cage cultured tilapia (*Oreochromis* sp.)

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Doan Thi Nhinh: Data curation, Formal analysis, Methodology, Roles/Writing - original draft, Writing - review & editing. Dang Thi Hoa: Data curation, Formal analysis; Nguyen Thi Huong Giang: Formal analysis; Validation; Visualization; Kim Van Van: Supervision, Writing - review & editing; Dang Thi Lua: Supervision, Writing - review & editing; Mags Crumlish: Supervision, Writing - review & editing; Ha Thanh Dong: Conceptualization; Data curation; Supervision, Writing - review & editing; Truong Dinh Hoai: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page. Ethical approval for the challenge experiments was obtained from the Faculty of Fisheries, Vietnam National University of Agriculture Animal Care and Use committee FFVNUA-ACUC, approval number: 110422-1-KHCN-FFVNUA.

Abstract

Widespread distribution of a highly pathogenic *Edwardsiella ictaluri* strain in farmed tilapia in northern Vietnam has recently been reported. The subsequent investigation noticed a disease outbreak occurred at five nearby tilapia farms with floating cages, in which the clinical signs of both edwardsiellosis and columnaris diseases were observed on the same infected fish and caused 65 to 85% fish mortality. Naturally diseased fish (n=109) were sampled from the five infected farms for bacterial identification and conducting challenge tests. The two bacteria *Edwardsiella ictaluri* and *Flavobacterium oreochromis* were identified by a combination of biochemical tests, PCR and 16SrRNA sequencing methods. Experimental challenge tests on Nile tilapia resulted in the median lethal dose (LD_{50}) of *E. ictaluri* and *F. oreochromis* at 70 CFU/fish by intraperitoneal (i.p.) injection and 3.6×10^6 CFU/ml by immersion, respectively. The experimentally co-infected challenged fish exposed to LD_{50} doses resulted in 83 ± 6 % mortality, with the infected fish exhibiting clinical signs of both edwardsiellosis and columnaris diseases, mimicking the naturally diseased fish. This finding suggests that the co-infection of *E. ictaluri* and *F. oreochromis* may interact in a synergistic manner, to enhance the overall severity of the infection and elevates the need for efficient methods to control both pathogens.

Keyword: *co-infection, Flavobacterium oreochromis, Edwardsiella ictaluri, tilapia, disease outbreak*

1. Introduction

Tilapia (*Oreochromis* spp.) is one of the most popular aquaculture species and is widely promoted for farming due to its unique characteristics in growth and ease of culture (Yue et al., 2016). This species is commercially cultured in more than 140 countries, with the global production in 2020 reaching over 6 million tons (Market, 2021). Vietnam is among the top ten tilapia producers with reported production in 2019 of 250,000 tons and aims to reach 400,000 tons by 2030 (MARD, 2019). Despite the rapid growth of tilapia production, high stocking densities within intensively cultured farms is likely to increase the risk of disease outbreaks. Diseases often occur following an initial stressor, and in the case of infectious disease, it is common that more than one pathogen can simultaneously infect the fish (Abdel-Latif et al., 2020a; Dong et al., 2015b; El-Sayed, 2019).

Concurrent infection is the simultaneous presence of more than one pathogen, which often drastically alters the host's susceptibility to the infection and makes the situation more severe

(Abdel-Latif et al., 2020a; Nicholson et al., 2020; Wanja et al., 2020). Several events of naturally occurring concurrent infections have been reported in tilapia, e.g. multiple bacteria (Assis et al., 2017; Delphino et al., 2019; Dong et al., 2017; Lee and Wendy, 2017), parasite-parasite (Echi et al., 2009; Pinto et al., 2014; Zhi et al., 2018), parasite-bacteria (Abdel-Latif and Khafaga, 2020b; Xu et al., 2009; Xu et al., 2007), bacteria-virus (Dong et al., 2015b; Nguyen et al., 2020; Nicholson et al., 2017), and fungi-bacteria (Cutuli et al., 2015; Eissa et al., 2013; Oda et al., 2016). In tilapia, natural co-infections act synergistically to worsen the health status of infected fish, resulting in heavy mortalities and economic loss to farms (Abdel-Latif et al., 2020a; Amal et al., 2018).

Flavobacterium columnare is one of the oldest known bacterial pathogens in aquaculture, having a global impact on freshwater fish farming, infecting most of the cultured species including tilapia, carp and catfish (Anderson and Conroy, 1969; Barony et al., 2015; Chockmangmeepisan et al., 2020; Dong et al., 2016; Tien et al., 2012). Recently, LaFrentz et al. (2022) reclassified *Flavobacterium columnare* into four distinct species: *F. columnare*, *F. covae*, *F. davisii*, and *F. oreochromis.* Of which, *F. oreochromis* are the species causing diseases in tilapia. Meanwhile, *E. ictaluri* has been considered one of the most pathogenic bacteria in catfish worldwide for a long time. This bacterial species was reported to infect tilapia in Western Hemisphere in 2012 (Soto et al., 2012), Vietnam and possibly other tilapia farming countries relying on imported tilapia fry or fingerlings (Dong et al., 2019; Nhinh et al., 2022).

The present study describes a natural disease outbreak occurring in several floating-cage fish farms resulting from co-infection with a highly pathogenic strain of *E. ictaluri* and *F. oreochromis*. Experimental infection studies demonstrated the synergistic effect of co-infection contributing to increased fish mortality and highlighted the need for improved disease management strategies targeted to tackle co-infections by these two bacteria.

2. Materials and methods

2.1. Disease outbreaks and sample collection

In February 2022, a disease outbreak occurred in Nile tilapia approximately 1-2 months post stocking at five nearby farms (approximate distance between 0.5-2 km) in Hoa Binh reservoir, Vietnam (Figure S1). Fish were transferred from hatchery farms in North Vietnam and cultured in 144 m^3-floating cages (6m × 6m × 4m-width × length × depth) at stocking densities from 2,500 to 3,500 fish at the initial size of 10-15 gram/fish. Approximately 65-85% of the fish died within 3-7 days, presenting clinically with pale discolouration of the skin, fins and gills, along with gross lesions

of white sports in the internal organs including kidney, spleen and liver (Table 1). The water temperature at the time of the disease outbreak was 22-24°C. Onsite examination of the affected fish showed the clinical signs indicative of both edwarsiellosis and columnaris diseases. At the time of the peak mortality, a total of 109 moribund fish were sampled for disease diagnostics. The sampled fish were kept in closed plastic bags partially filled with oxygenated water during transportation (2 - 3 hours) to the laboratory. The screening of sampled fish showed that all fish were negative with Tilapia Lake Virus (TiLV) and the gills occasionally infected with *Dactylogyrus* sp. (5 out of 109 examined fish) at low infestation (4-6 parasites/fish). Thus, we presumptively diagnosed that the disease outbreaks were likely caused by bacteria.

2.2. Disease diagnosis and bacterial isolation

Clinical examination was immediately conducted when the collected samples arrived the laboratory. The fish were weighed, examined gross lesions recorded. Tissue smears were made at the time of sampling from each fish including gill, head kidney, spleen, and liver and Gram stained to identify the presence of bacteria in the organs.

Samples of infected gill, skin, kidney, spleen, and liver of each fish were aseptically streaked onto Tryptic soy agar (TSA; Merck, Darmstadt, Germany) and tryptone yeast extract salts agar (TYES) (Holt, 1987), incubated for 36-48h at 28°C. The predominant colony types were subsequently sub-cultured as required, and pure isolates were preserved in the respective Tryptic Soy Broth (TSB) containing 15% glycerol, stored at -80°C.

2.3. Morphological and biochemical characterizations of bacteria

Five representative isolates presumptively identified as *Edwardsiella* sp. and *Flavobacterium* sp. from each farm were randomly selected for full species identification. They were selected based on colony morphology in the respective agars. The *Flavobacteria* sp. were identified based on conventional tests described by Bernardet et al. (1996) and Bernardet et al. (2002). The morphology of colonies on TYES plates, bacterial cells and their motility were observed under a light microscope. The presence of flexirubin pigment was detected using a 20% (w/v) potassium hydroxide (KOH) solution, while congo red adsorption was tested using a 0.01% dye solution. The production of cytochrome oxidase was differentiated using tetramethyl-p-phenylene diamine dihydrochloride reagents (bioMerieux, Marcy-l'Étoile, France).

For putative *Edwardsiella* sp. isolates, the morphology of Gram-staining bacterial cells and colonies on TSA plates was examined under a microscope. Oxidase and catalase tests were

performed as described by Crumlish et al. (2002). Other phenotypic tests were conducted using the API 20E kit (BioMeriux) following the instruction of the manufacturer.

2.4. DNA extraction

Genomic DNA of all bacterial isolates (n = 5 for each pathogen) was extracted using the InstaGene Matrix kit (Bio-Rad, California, USA) following the manufacturer's protocol. The extracted DNA was preserved at -20°C for sequencing and PCR assays.

2.5. Polymerase chain reaction (PCR) assays

PCR assays were conducted using universal primers targeting the 16S rRNA gene and species-specific primers targeting the fimbrial gene of *E. ictaluri* (470 bp; Sakai et al., 2009; Table 2). Since specific PCR was not available to identify *F. oreochromis*, this study employed a previously reported PCR assay targeting the ISR gene of *F. columnare,* which later split into four species, for the initial confirmation of the *Flavobacterium* genus (450-550 bp; Welker et al., 2005; Table 2). Nuclease-free water was used as a negative control. The DNA of *E. ictaluri* LMG 7860 and *F. columnare* LMG 13035 purchased from BCCM/LMG Bacteria Collection, Gent, Belgium was used as the respective positive controls. Each PCR reaction mixture (25 µL) included 12 µL Gotaq Green Master Mix (Promega, Wisconsin, USA), 1.5 µL (10 µM) of each respective primer (forward and reverse), 5 µL DNA template, and 5 µL DNA-free distilled water. The mixtures were then placed in a thermocycler for the amplification process under the following conditions: initial denaturation for 4 min at 94 °C; 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s; and a final extension for 7 min at 72 °C. The amplified products were then analyzed by electrophoresis on a 1.3% agarose gel containing a RedSafe nucleic acid staining solution (Intron, Gyeonggi-do, Korea). The images were digitally captured using a gel image system (Bio-Rad, California, USA).

2.6. Sequence and phylogenetic analysis of the 16S rRNA gene

The PCR products of the 16S rRNA gene were purified using the QIAquick PCR extraction kit (Qiagen) and sequenced (Macrogen, Seoul, Korea). The obtained sequences were assembled using Bio Edit version 7.0 (Hall, 1999) and deposited in the GenBank database to issue the accession numbers. The sequences were aligned with related sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) nucleotide search.

The phylogenetic analysis was conducted to match the 16S rRNA sequences of the five representative isolates with the closely related sequences retrieved from GenBank using the ClustalW program (Thompson et al., 1994). Phylogenetic trees were then performed by MEGA 10 software (Kumar et al., 2018) using the neighbour-joining method (Saitou and Nei, 1987). A boot-strap value of 1000 replicates was applied for the robustness of phylogeny.

2.7. Challenge experiments

Single-pathogen challenges

Nile tilapia juveniles (*Oreochromis* sp.) at approximately 35 g with a health certificate were obtained from a commercial tilapia hatchery in northern Vietnam for bacterial challenge experiments. The fish were acclimatized to the experimental conditions for one week before conducting experiments. Bacterial isolates recovered from the naturally infected fish, which were representative of the identification profiles per species, were selected for the challenge studies. The *E. ictaluri strain* Fo-VN0522 and *F. oreochromis strain* EdTil-VN0522 were randomly selected for experimental infection, cultured aseptically in 100ml-flasks of TSB and TYES broth, respectively and incubated at 28°C for 36h with gentle shaking. The viable bacterial density of the stock suspensions was adjusted to approximately 1 x 10^8 colony forming units (CFUs)/mL, and confirmed using Miles and Misra method (Miles et al., 1938).

Ten-fold serial dilutions of *E. ictaluri* Fo-VN0522 were prepared to achieve seven bacterial concentrations from ~1 × 10^2 to ~1 × 10^8 CFU/ml. 240 fish were divided into 21 experimental tanks of seven bacterial concentrations (10 fish in each tank with three replicates) and 3 control tanks. For each bacterial concentration, fish were intraperitoneally injected with 0.1 ml of bacterial suspension, while in the control group, fish were injected with 0.1 ml PBS.

For the *F. oreochromis* challenge experiment, fish were exposed to the pathogen by immersion challenge at a bacterial concentration from 1 × 10^1 to 1 × 10^7 CFU/ml. The other 240 fish were divided into eight groups of 30 fish. Of which seven groups were immersed in the seven corresponding bacterial suspensions and one in freshwater with PBS addition for one hour. The 30 fish in each immersion group were then divided into three 120L-tanks (three replicates) for monitoring for 14 days. The medium lethal doses (LD_{50}) of *E. ictaluri* and *F. oreochromis* on tilapia were calculated as described by Reed and Muench (1938).

Combined-pathogen challenge

195 Fish were divided into four groups of ten fish per tank: (1) received 0.1 ml of the LD_{50} dose
196 of *E. ictaluri* by i.p. injection; (2) received LD_{50} dose of *F. oreochromis* for one hour by immersion; (3)
197 received both 0.1 ml of the LD_{50} dose of *E. ictaluri* by i.p. injection then immersed in LD_{50} dose of *F.*
198 *columnare* for one hour; and (4) treated the same way as group 3 but not exposed to bacteria and
199 instead injected with PBS and immersed in clean water for one hour (control group). Three replicate
200 tanks were included for each treatment group. Mortality/morbidity was observed daily for two
201 weeks. All affected fish were collected for gross inspection of external and internal clinical signs.
202 Moribund fish from each challenge group and healthy fish from the control groups (at the end of
203 the experiment) were subjected to bacterial re-isolation and histopathological analysis.

204

205 **2.7. Histopathological examination**

206 Affected tissues (gills, kidney, liver, spleen) from natural co-infected fish, moribund and
207 healthy tilapia from each challenge experiment group described (3-5 fish/group) were observed for
208 histopathological examination. The tissues were collected and preserved in 10% buffered formalin
209 for 24 hours. The sampled tissues were then dehydrated in an ethanol series, embedded in paraffin,
210 and sectioned at 5 µm thickness before being stained with hematoxylin and eosin following the
211 standard histological protocol. Histopathological changes in the infected tissues were examined
212 under a light microscope equipped with a digital camera (Olympus, Tokyo, Japan).

213 **2.8. Statistical analysis**

214 The mortality rates of fish between treatment groups exposed to the bacteria in the challenge
215 tests were compared by one-way ANOVA which was performed using the SPSS program 20.0, and
216 the significance was identified as $P \leq 0.05$.

217 **3. RESULTS**

218 **3.1. Clinical signs and gross lesions of diseased tilapia**

219 In the present study, naturally diseased tilapia from the five affected farms ranged from 25-
220 215g in weight, with the estimated mortality rate between 65-85% (Table 1). Macroscopic
221 examination showed that the diseased fish (n=109) presented grossly with signs of both freshwater
222 columnaris disease which included whitish or yellow areas on the gill, pale discolouration areas on
223 the skin and the fin base with more clearly shown in the caudal fin and edwardsiellosis as shown by
224 the presence of white spots on the spleen, kidney, and occasionally on the liver (Figure 1).

225

226 **3.2. Bacterial isolation and identification**

227 Microscopically, the damaged tissues of the gill and skin revealed the presence of bundles of
228 Gram-negative, filamentous bacteria, which were suspected to be *F. oreochromis*. (Figure 2A). On
229 the same fish, there were Gram-negative, intra-cellular, rod-shaped bacteria, which were similar to
230 *E. ictaluri,* on the spleen, kidney, and liver (Figure 2B). Consistently, there were two dominant types
231 of bacterial colonies retrieved from the same fish representing the co-infection of columnaris and
232 edwarsiellosis diseases after isolation. The first one was yellow rhizoid colonies on TYES agar (Figure
233 2C), isolated from the whitish gills and pale areas on the skin of all diseased fish and occasionally from
234 the kidney and spleen. The bacteria were Gram-negative, slender, and variable length but
235 predominantly long rod-shaped cells (2-10µm) and showed gliding motility (Video footage-S1). These
236 yellow-pigmented bacteria produced positive results in flexirubin pigment, congo red, and oxidase
237 tests (Table 3), identical to the reference strains *F. columnare*. In addition, LaFrentz et al. (2018, 2022)
238 demonstrated that *F. columnare* represents four distinct species, in which *F. oreochromis* is the species
239 causing disease in tilapia. Thus, the rhizoid-colony bacteria were identified as belonging to the putative
240 *F. oreochromis*. The second bacteria presented with whitish, pinpoint size colonies on TSA, isolated
241 from the spleen, kidney, and liver of the infected fish with white spots lesion (Figure 2B and 2D) and
242 were identified as Gram-negative, rod-shaped (1.5-2.5µm), oxidase-negative and catalase-positive
243 organisms. Other biochemical characteristics were identical to the *E. ictaluri* strains from red tilapia
244 (Dong et al., 2019) and striped catfish (Crumlish et al., 2002) (Table 4).

245 The PCR assays conducted on the specific-species genes confirmed that the rod-shaped
246 bacteria were *E. ictaluri* which produced a specific band at 470 bp (Figure S2). Further identification
247 of the bacterial species was done by sequence analysis of their 16S rRNA genes. For the five tentative
248 *E. ictaluri* isolates, the sequences of 16S rRNA (~1.5kb) displayed 99.93%-100% nucleotide identity
249 to the reference strain *E. ictaluri* ATCC 33202 (NR024769). In addition, phylogenetic trees performed
250 on the 16S rRNA gene showed that the selected isolates were placed in the same cluster with other
251 *E. ictaluri* strains, including the isolates infected in tilapia in other Northern provinces of Vietnam;
252 and were phylogenetically distinct from other *Edwardsiella* species (Figure S3). The 16S rRNA
253 sequences of these isolates have been deposited in GenBank under accession numbers OP604351-
254 OP604355.

The PCR results revealed that the filamentous bacteria were positive with the *Flavobacterium* genus (Figure S2). The 16S rRNA sequences of five putative *F. oreochromis* isolates yielded fragments of 1437 bp, which have been deposited in the GenBank database under the following accession numbers: OP604326-OP604330. The BLAST analysis of the 16S rRNA gene sequences revealed that they shared 100% similarity with the species *F. oreochromis* on GenBank, strain TI2056 (KX711900.1) and TI982 (MG516961.1). Also, the sequences of the five isolates matched 100% with the *F. columnare* strains isolated from tilapia, such as CUVET1343 (KF274048.1) and CUVET1350 (KF774291.1), which were identified in 2013, prior to the re-classification of *F. columnare* into four genetic groups, and later four species conducted by LaFrentz et al. (2018; 2022). Meanwhile, these sequences showed 98.04% nucleotide identity with the reference strains, *F. columnare* ATCC 23463 from chinook salmon and *F. columnare* ATCC 49513 from catfish. The phylogenetic analysis showed that the five isolates in this study clustered with the strains of *F. oreochromis* isolated from tilapia (Figure S3). In conclusion, the five filamentous bacterial isolates were identified as *F. oreochromis*.

3.3. Bacterial challenge experiments

The challenge experiments revealed that the LD_{50} doses for *E. ictaluri* and *F. oreochromis* were 70 CFU/fish and 3.6×10^6 CFU/ml, respectively. Fish exposed to high concentrations of *E. ictaluri* (10^4-10^7 CFU/fish) became sick and died quicker than the fish in lower concentrations, with the mean percent mortality from 63-93% on 4 dpi and reached 100% after 6-10 dpi (Figure 3A). The low concentrations from 10^1 – 10^3 CFU/fish of *E. ictaluri* caused 27 - 77% mortality at 14 dpi. No mortalities or morbidities were observed in any of the fish exposed to *F. oreochromis* by immersion at 10^1-10^4 CFU/ml. The mean percent mortality was from 23 - 63% when challenged with the higher concentrations of 10^5-10^7 CFU/ml (Figure 3B). The moribund/dead fish in the *E. ictaluri* treatment showed a typical gross presentation of white spots in the viscera, particularly spleen and kidney, which were not observed in the fish exposed to *F. oreochromis* only. On the other hand, pale to white discoloration on the gill lamellae of the diseased fish were observed in the *F. oreochromis* challenge group. The fish experimental challenged with single pathogen were successfully reisolated and confirmed for the respective pathogens of *E. ictaluri* and *F. oreochromis*.

The tilapia challenged with the LD_{50} dose of single *F. oreochromis* (3.6×10^6 CFU/ml) or *E. ictaluri* (70 CFU/fish) resulted in a mean percent mortality of 47 ± 6 % and 53 ± 6 %, respectively, on the 14 dpi (Figure 4). Meanwhile, the simultaneous challenge with the LD_{50} dose of *F. oreochromis* and *E. ictaluri* resulted in 83 ± 6%, which is significantly higher than individual infection ($P<0.01$).

The infected fish in the simultaneous exposure group presented clinical signs of both columnaris and edwardsiellosis, mimicking the naturally diseased fish (Figure 5). No mortality was observed in the control group during the challenge period. Isolates of *F. oreochromis* and *E. ictaluri* were successfully recovered and species were confirmed from the same infected fish exposed to both pathogens (data not shown).

3.4. Histopathology

Histopathological changes observed in fish exposed to *F. oreochromis* only, were primarily observed in the gills, with degeneration and extensive necrosis of the gill lamellae, as well as colonization of filamentous bacteria. No significant lesions were found in the kidney, spleen, and liver of the affected fish. In contrast, fish challenged with *E. ictaluri* only, showed no gill changes but exhibited severe lesions of focally-extensive to confluent areas of necrosis in the spleen, kidney, and liver. These findings demonstrate the specific pathogenic effects of each pathogen, with *F. oreochromis* targeting the gills and *E. ictaluri* causing multi-organ necrosis.

In the fish receiving a combined pathogen challenge, the mixed histopathological features were observed in both external and internal organs including the gills, kidney, spleen and liver. The lesions were distinguishable by the severe degeneration of the fish gill, such as the extensive necrosis of primary and secondary gill filaments, as well as lamellae (Figure 6). In severe cases, areas of the gill filaments were partially or completely degenerated and the colonization of massive basophilic filamentous bacteria was found on the gill filaments. The spleen of the affected fish in the groups subjected to combined pathogen challenge exhibited notable focal areas of necrosis within the parenchyma, accompanied by the development of pyogranulomas. (Figure 7A and 7B). The affected livers showed vascular congestion, lipidosis and multifocal necrotic areas with the infiltration of inflammatory cells resembling macrophages and lymphocytes. Hepatocytes also showed loss of storage lipid, cell degradation, and pyknosis (Figure. 7C). Similarly, the kidneys of infected fish showed disruption of typical normal structure of kidney glomerulus and tubules, accompanied by several histopathological lesions including pyogranulomas, focal necrosis and signs of haemorrhages (Fig. 7D).

4. Discussion

Infectious disease outbreaks in farmed tilapia remain a global challenge to this aquatic food production sector. Traditionally the emphasis has been on single diseases or single pathogens,

however coinfection by two or more pathogens simultaneously has been reported previously (Dong et al., 2015b; Kotob et al., 2017). The present study described the first natural coinfection in tilapia of *E. ictaluri*, an emerging disease in tilapia (Nhinh et al., 2022) and *F. oreochromis*, one of four distinct species in fish pathogen *F. columnare* (LaFrentz et al., 2022). Dong et al. (2015c) demonstrated co-infection in striped catfish (*Pangasianodon hypophthalmus*) species with isolates from these two bacteria under experimental conditions. It is postulated from this study that co-infections from *E. ictaluri* and *F. oreochromis* occurs more frequently in tilapia farms due to their similar optimal temperature range (20 - 28 °C) for invasion and disease manifestation, but may not be widely recognised. In Northern Vietnam, during the winter and early spring, the air temperature often falls below 20°C, but due to the water depth, the large reservoirs still keep the water cool and make opportunities for the prevalence of both columnaris and edwardsiellosis leading to the coinfection of these two diseases at this time. Moreover, due to the widespread infection of *E. ictaluri* in tilapia farms in Northern Vietnam, as reported by Nhinh et al., (2022), and the ubiquitous presence of *Flavobacterium* spp. in freshwater (Declercq et al., 2013), the coinfection of these two diseases is likely to appear frequently in tilapia culture systems exacerbated by lower temperature, including floating-cage and pond cultures.

In aquatic pathology, fish are often experimentally challenged by either i.p injection, cohabitation, or immersion. In the present study, fish were challenged with *E. ictaluri* by i.p injection, which is an efficient method that shortens the time to develop signs of disease and is preferred due to the reduction in time and cost (Meza et al., 2019). More importantly, this method produces more reproducible results than other exposure methods (Avila et al., 2022) as every fish is exposed to the pathogen at the same concentrations. However, a different exposure method was adopted for the fish in the *Flavobacterium* group as it does not cause a systemic disease. The isolation process in our study also revealed that *F. oreochromis* existed in affected fish at low concentrations and can be difficult to isolate from internal organs, similar to the previous report by Dong et al. (2015a). In addition, Staroscik et al. (2008) suggested that *Flavobacterium* spp. could utilize fish skin mucus as a substrate for growth, hence lack of systemic invasion by these bacteria. Subsequently, several studies have demonstrated that the immersion exposure route was most suitable experimental model for challenging fish with *Flavobacterium* spp. (Declercq et al., 2015; Dong et al., 2015c). Therefore, tilapia exposed to *E. ictaluri* via intraperitoneal injection and *F. oreochromis* through immersion could serve as a suitable model for effectively reproducing clinical signs and pathological features observed in naturally coinfected fish with these two bacteria.

In both macroscopic and microscopic levels, fish experimentally challenged with only *F. oreochromis* did not exhibit systemic lesions. However, the gills were predominantly affected, potentially impairing normal respiratory function. This was attributed to the formation of *F. oreochromis* biofilm on the gill surface, which hindered the uptake of oxygen into the fish's body, ultimately leading to mortality as suggested by previous studies (Dong et al., 2015a; Declercq et al., 2015). On the other hand, fish experimentally infected with *E. ictaluri* displayed severe damage to internal organs, particularly the head kidney (a central lymphoid and hematopoietic organ) and the spleen (a peripheral lymphoid organ). These lesions were consistent with previous reports (Dong et al., 2019; Nhinh et al., 2022). Interestingly, when the two bacteria were simultaneously introduced, synergistic effects of two respective diseases were observed in both gross signs and histopathological lesions. This implies that co-infection with both pathogens results in concurrent lesions and dysfunction in multiple vital organs. As a result, the ability of the fish to recover is diminished due to the disruptive synergistic effects on normal physiological functions of the gills and crucial internal organs. Consequently, the mortality rate in co-infected fish is significantly higher compared with single infections.

Overall, this study reports the first case of naturally concurrent infection of *F. oreochromis* and *E. ictaluri* and experimentally proves their synergistic effects of the coinfection on tilapia. The results raise awareness and the requirement for management strategies with a simultaneous approach, such as bivalent vaccines or immunological enhancement, to control the infection and mitigate economic losses for farmers.

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558

559 **TABLES**

560 **Table 1.** Information on the disease outbreak in this study

Farm code	Fish size (g)	Mortality rate (%)(*)	Number of affected cages	No of collected fish	Number of fish co-infected	Representative isolates	Water temperature (°C)
F1	25-96	80-85	6	21	21	Fo-VN0122 EdTil-VN0122	22
F2	75-118	65-70	8	26	26	Fo-VN0222 EdTil-VN0222	23
F3	48-73	70-75	5	18	18	Fo-VN0322 EdTil-VN0322	22.5
F4	85-127	65-75	4	16	16	Fo-VN0422 EdTil-VN0422	24
F5	131-215	65-70	8	28	28	Fo-VN0522 EdTil-VN0522	23

561 (*) estimated by the farm holder; the isolates with bold names were used for the challenge
562 experiments in Section 2.7.
563 Fo, *Flavobacterium oreochromis*
564 EdTil, *Edwardsiella ictaluri* from tilapia
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572 **Table 2.** Primers used for 16r-RNA sequencing and PCR assays in this study

Primer sequence (5'→3')	Target Gene (size)	References
Sequencing		
Uni-Bact-F/AGAGTTTGATCMTGGCTCAG	*16S rRNA*	Weisburg et al. (1991)
Uni-Bact-R/ACGGHTACCTTGTTACGACTT	(~1500 bp)	
Specific PCR assay for F. columnare determination		
F.columnare-F/TGCGGCTGGATCACCTCCTTTCTAGAGACA	ITS	Welker et al. (2005)
F.columnare-R/TAATYRCTAAAGATGTTCTTTCTACTTGTTTG	(450-550 bp)	
Specific PCR assay for E. ictaluri determination		
Ed-ictaluri-F/GTAGCAGGGAGAAAGCTTGC	Fimbrial gene	Sakai et al. (2009)
Ed-ictaluri-R/GAACGCTATTAACGCTCACACC	(470 bp)	

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574

575 **Table 3.** Bacterial identification of tentative *Flavobacterium* isolates

Characteristic	**Isolates in this study (n=5)**	***F. columnare* (Bernardet et al., 2002)**
Gram staining	-	-
Bacterial morphology	long, slender rod	long, slender rod
Colony morphology	Rhizoid	rhizoid
Gliding motility	+	+
Growth on TSA	-	-
Flexirubin pigments	+	+
Congo red	+	+
Cytochrome Oxidase	+	+

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577

578 **Table 4.** Biochemical characteristics of presumptive *E. ictaluri* isolates from diseased tilapia in this
579 study

Characteristics	*E. ictaluri* in this study (n=5)	*E. ictaluri* (Dong et al., 2019)
Gram	-	-
Morphology	rod	rod
Oxidase	-	-
Catalase	+	+
ONPG	-	-
Arginine dihydrolase	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	-	-
Citrate utilisation	+	V
H2S production	-	-
Urease	-	-
TDA	-	-
Indole production	-	-
Voges-proskauer	-	-
Gelatin	-	-
Acid production		
D-glucose	+	+
D-mannitol	-	-
Inositol	-	-
D-sorbitol	-	-
L-rhamnose	-	-
D-sucrose	-	-
D-melibiose	-	-
Amygdalin	-	-
L-arabinose	-	-

580 V, variable; (-), negative; (+), positive

581

582

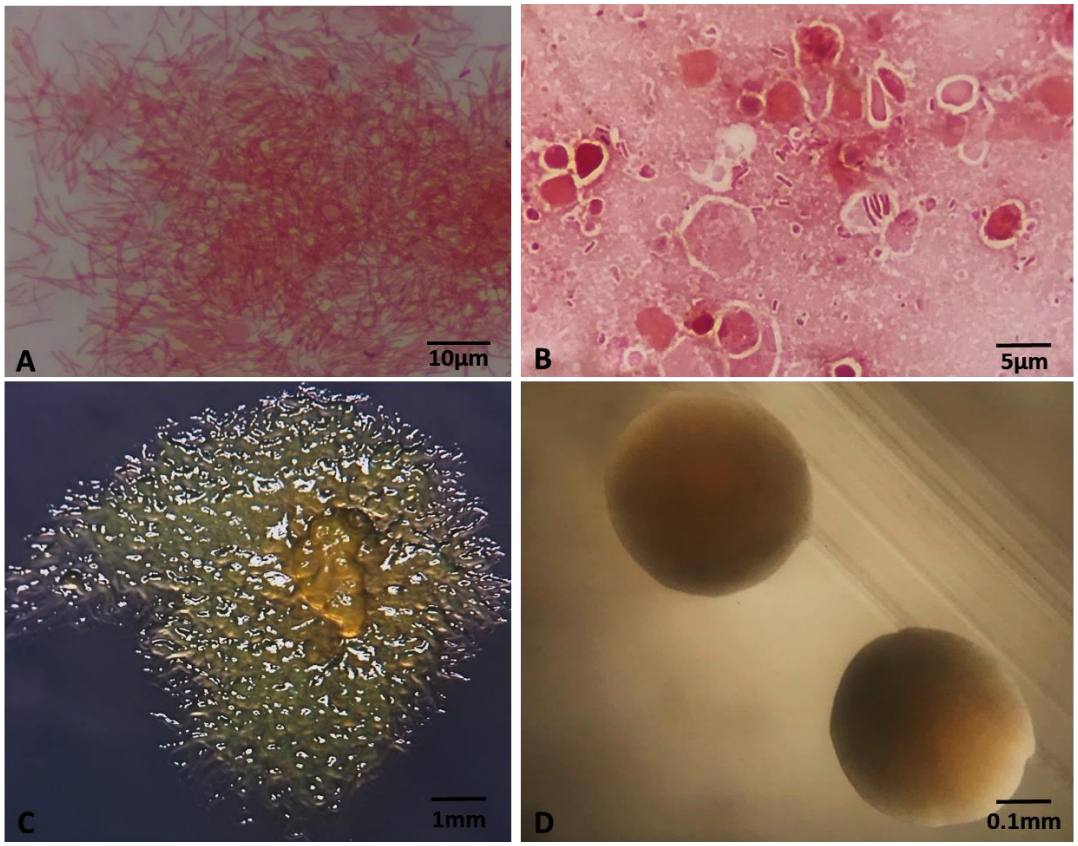
583 **FIGURES**

584

585 **Figure 1.** Clinical signs and gross finding of naturally infected fish collected in the outbreak. A-The
586 infected fish showed pale areas on skin and fins (arrow); B-C: discoloration of gills (arrows) and white
587 spots on the spleen and head kidney (arrow heads)

588

589



590

591 **Figure 2**. A- Gram-negative and filamentous bacteria observed on Gram-staining smear samples of
592 fish gill; B- Gram-negative and rod-shaped bacteria observed on Gram-staining smear samples of
593 head kidney of the same diseased fish; C, D- the respective bacterial colonies with presumptive
594 features of *F. columnare* complex and *E. ictaluri*

595

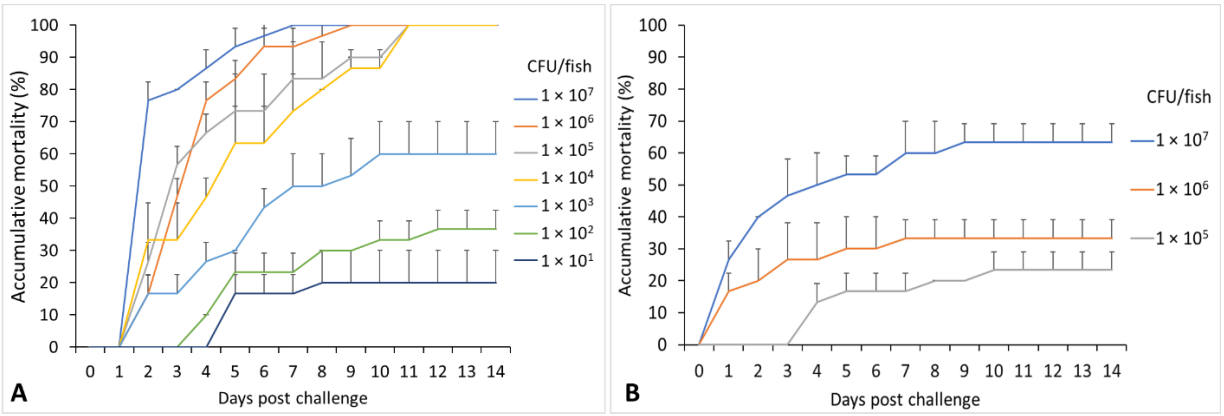


Figure 3. Cumulative percentage mortality of Nile tilapia challenged with a series of doses of *E. ictaluri* (A) and *F. oreochromis* (B), n=10, three replicates per treatment.

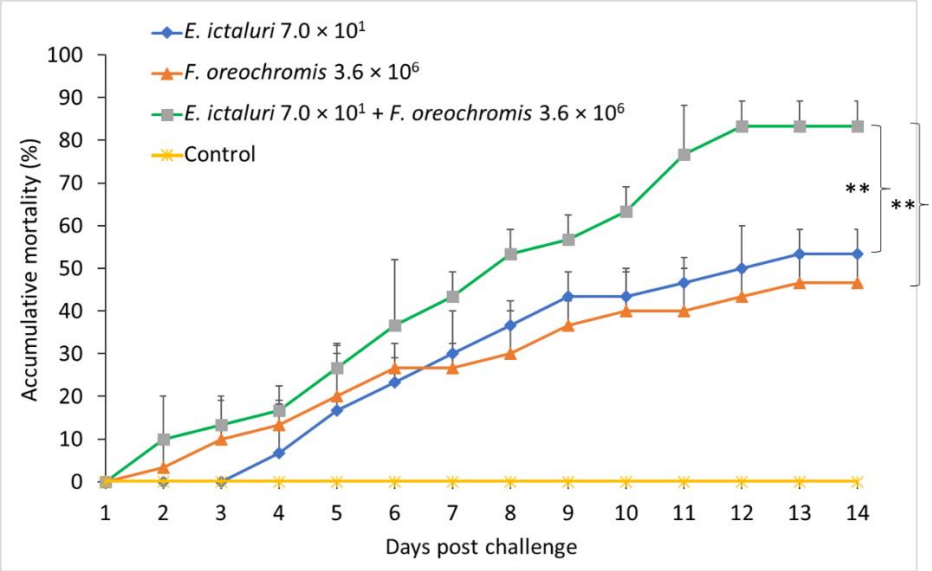


Figure 4. Cumulative percentage mortality of Nile tilapia challenged with LD_{50} doses of single *E. ictaluri*, single *F. oreochromis,* and both pathogens simultaneously. (**) indicates the significant difference in the mortality rate values at $p<0.01$, n=10, three replicates per treatment.

607

608 **Figure 5**. A-Fish challenged with single *F. oreochromis* showed only necrotic gill filaments (red
609 arrow); B-Fish challenged with single *E. ictaluri* showed only white-spots in the head kidney, spleen
610 and liver (black arrows); C-Fish challenged with two pathogens showed clinical signs of both
611 columnaris (red arrow) and edwarsiellosis (black arrows).

612

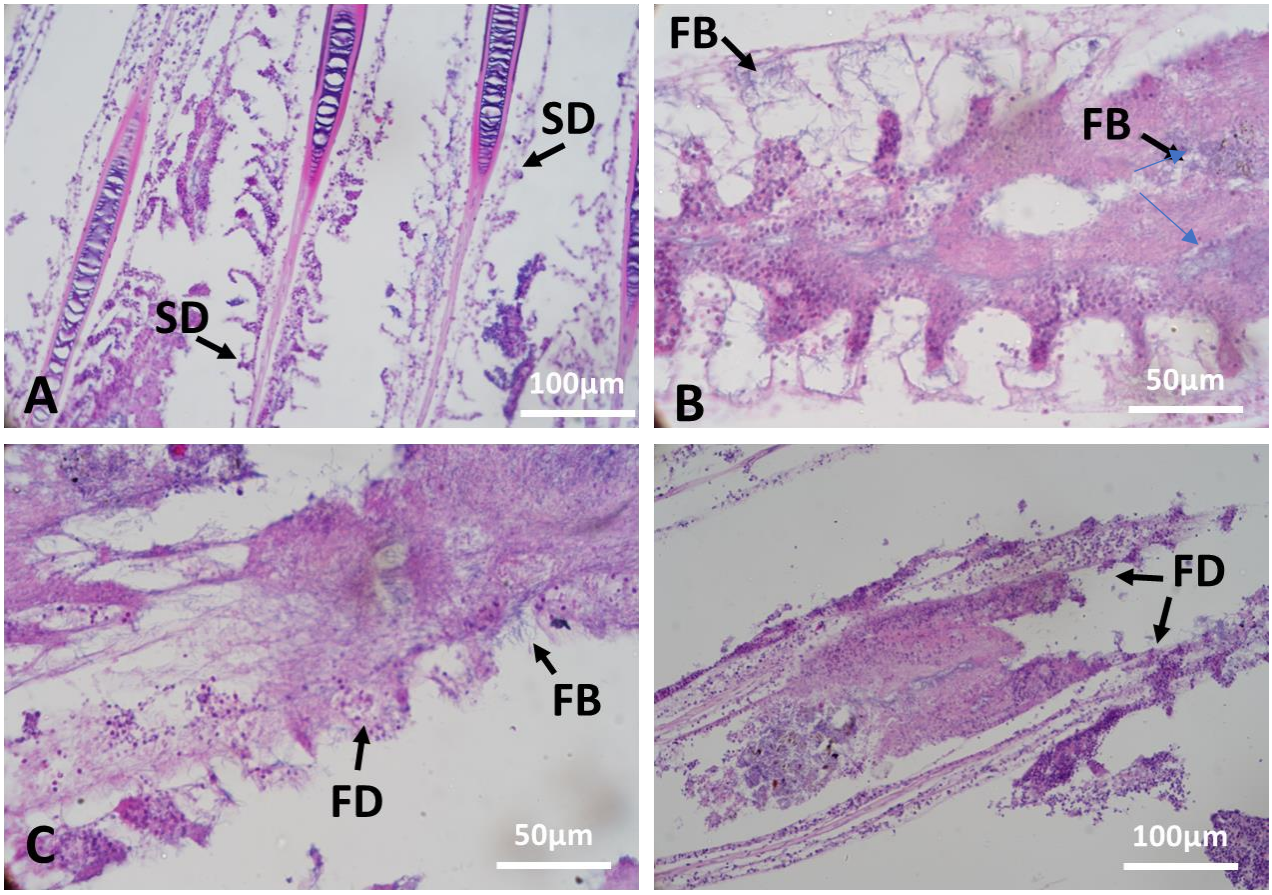


Figure 6. Representative photomicrographs showing histopathological changes on the gills of diseased fish in the dual challenged group of *F. oreochromis*. A- Severe distortion (SD) of the gill filaments and lamellae resulting in the loss of their normal architectural integrity; B-colonization of filamentous bacteria (FB) on gill filaments and lamellae; C- Filament degradation (FD) and clusters of filamentous bacteria (FB); D-Large areas of the filament degradation (FD)

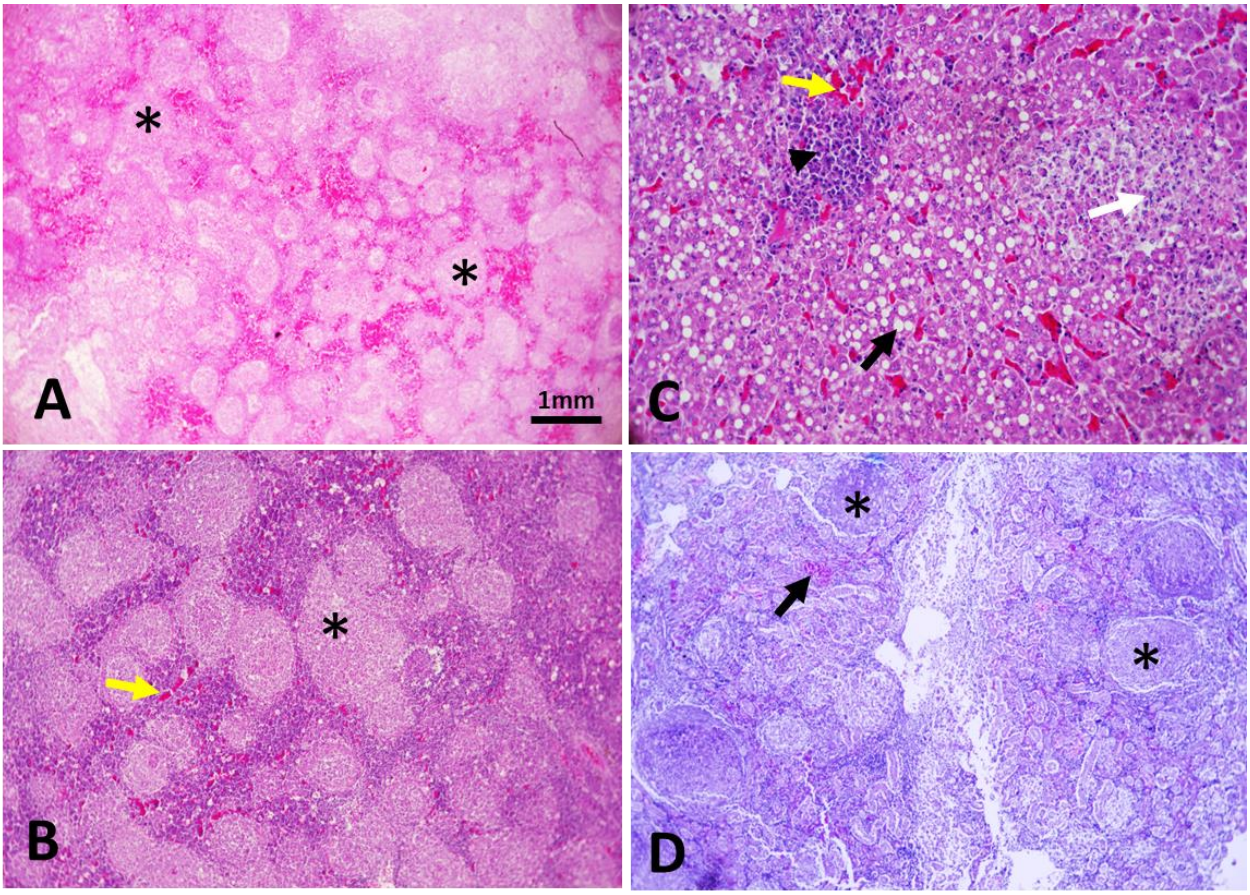


Figure 7. Representative photomicrographs showing histopathological changes of the infected fish in dual-pathogen challenges. A- The parenchyma of the spleen displays focally-extensive to confluent areas of necrosis (*), with some areas progressing to pyogranulomatous lesions; B-higher magnification of spleen showed multiple focal necrotic areas (*), and occasional congestion (yellow arrow); C-Liver exhibited blood vessel congestion (yellow arrow), lipidosis (black arrow), infiltration of inflammatory cells (black arrowhead), pyknosis (white arrow). D-kidney with multiple areas of pyogranulomas (*) and haemorrhages (black arrow).