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Application of Biosecurity Strategies for the Control of MAS (*Motile Aeromonas Septicemia*) in *Tubifex* Worms (*Tubifex* sp.) for Larval Catfish (*Pangasius* sp.) Culture

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ABSTRACT

Background: Catfish (*Pangasius* sp.) is one of the high-value freshwater aquaculture commodities in Indonesia. To ensure sustainable production, hatchery management plays a crucial role, as the larval and juvenile stages are the most vulnerable to environmental stress and disease outbreaks. During the larval phase (7–14 days post-hatch), *Tubifex* worms (*Tubifex* sp.) are commonly used as a natural feed due to their high nutritional content and digestibility.
Aims & Methods: This study aimed to evaluate the effectiveness of formalin in suppressing *Aeromonas hydrophila* in *Tubifex* worms (*Tubifex* sp.), which are used as natural feed for larval catfish (*Pangasius* sp.). The research was conducted in two phases: in vitro and in vivo assays. The in vitro assay was carried out to determine the minimum inhibitory concentration (MIC) and inhibition zone of formalin against *A. hydrophila*. The in vivo assay consisted of two parts: a toxicity test of formalin on *Tubifex* worms and an evaluation of the effect of treated worms as feed on the survival of catfish larvae. A completely randomized design (RAL) was employed, consisting of four treatments with three replicates each: K (control—untreated *Tubifex*), A (400 ppm formalin immersion without rinsing), B (400 ppm formalin immersion with one rinse), and C (400 ppm formalin immersion with two rinses).
Result: The results demonstrated that a 400 ppm formalin concentration effectively inhibited the growth of *A. hydrophila*. Treatment C (two rinses following immersion in 400 ppm formalin) significantly reduced the toxic effects of formalin on the *Tubifex* worms used as natural feed. Consequently, this treatment led to an improvement in the survival rate of catfish larvae, reaching 44.6 ± 11.5% over a 14-day rearing period.

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1. Introduction

Catfish (*Pangasius* sp.) is one of the high-value freshwater aquaculture commodities in Indonesia, with steadily increasing market demand. According to data from the Ministry of Marine Affairs and Fisheries (KKP 2020), catfish production grew at an average annual rate of 10.40% from the year 2015 to 2019. To ensure sustainable production, hatchery management plays a crucial role, as the larval and juvenile stages are the most vulnerable to environmental stress and disease outbreaks. During the larval phase (7–14 days post-hatch), *Tubifex* worms (*Tubifex* sp.) are commonly used as a natural feed due to their high nutritional content and digestibility.

However, the use of *Tubifex* worms in aquaculture systems poses certain risks. These worms inhabit organic-rich aquatic environments and can act as vectors for pathogenic bacteria such as *Aeromonas* sp., *Vibrio* sp., and *Streptococcus* sp. (Khairuman & Amri, 2002; Hatmanti *et al.,* 2009). One of the most common diseases affecting catfish is *Motile Aeromonas Septicemia* (MAS), caused by *Aeromonas hydrophila* infection. This opportunistic bacterium can lead to high mortality rates—up to 80%—especially under suboptimal environmental conditions (Sanoesi, 2008; Harikrishnan *et al.,* 2005).

A potential approach to mitigate the risk of pathogen transmission through natural feed is disinfection treatment, such as the application of formalin. Formalin exhibits broad-spectrum antimicrobial properties and is effective against various bacterial pathogens. However, its application must be carefully managed to avoid toxic effects on both the feed organisms and the cultured fish (Leal *et al.,* 2018).

This study was conducted to evaluate the effectiveness of formalin in reducing *A. hydrophila* populations in *Tubifex* worms through two approaches: in vitro testing to assess antibacterial efficacy, and in vivo experiments to evaluate toxicity to *Tubifex* and the effect of rinsing treatments prior to feeding them to catfish larvae. This study aimed to evaluate the effectiveness of formalin in suppressing *Aeromonas hydrophila* in *Tubifex* worms (*Tubifex* sp.), which are used as natural feed for larval catfish (*Pangasius* sp.).

2. Methods

2.1 Study Site

The experiment was conducted from March 25 to May 18, 2024, at two locations: the Vocational School Laboratory of IPB University in Sukabumi and the IPB University Vocational School in Bogor. The laboratory in Sukabumi, located at Jalan Sarasa No. 45, Babakan, Cibeureum District, Sukabumi City, West Java, was used for MIC and inhibition zone assays. Meanwhile, the IPB Vocational School located at Jalan Kumbang No. 14, RT.02/RW.06, Babakan, Central Bogor District, Bogor City, West Java, served as the site for the in vivo experimental treatments.

2.2 Experimental Design

The experiment was designed using a Completely Randomized Design (RAL). The study consisted of two main phases. The first phase was an in vitro test to determine the optimal concentration of formalin to suppress the population of *A. hydrophila*. The second phase involved an in vivo toxicity test of formalin on *Tubifex* worms, followed by an evaluation to determine the best rinsing procedure before the treated worms were fed to catfish larvae.

2.3 In Vitro Assay

The in vitro assay was conducted to assess the effectiveness of formalin in inhibiting the growth of *A. hydrophila* in *Tubifex* worms. The assay comprised two stages: inhibition zone test and Minimum inhibitory concentration (MIC) test, aimed at determining the most effective concentration of formalin. The inhibition zone test was conducted using the disk diffusion method on TSA media inoculated with *A. hydrophila*. Treatments included a negative control (PBS), a positive control (oxytetracycline), and 4% formalin in serial dilutions (12.5-800 ppm). The experimental design for the inhibition zone and MIC

tests is presented in table 1. Sterile paper disks were immersed in each treatment solution, placed aseptically on the media, and incubated for 48 hours at 28°C. The clear zone diameter was measured horizontally and vertically using a caliper, subtracting the 5 mm disk diameter to determine the actual inhibition strength.

The MIC test was performed by mixing 2 mL of formalin solution with 2 mL of bacterial suspension in a test tube, then incubating for 24 hours. The lowest concentration that resulted in a clear medium, compared to the negative control, was recorded as the MIC. This concentration was then used as the reference for subsequent in vivo testing.

Table 1. Design of the inhibition zone and Minimum inhibitory concentration (MIC) test experiment on bacteria in vitro

No	Treatment	Description
1	K-	TSB and bacteria without formalin
2	K+	TSB+antibiotic+bacteria
3	12,5 ppm	TSB+12,5 ppm formalin+bacteria
4	25 ppm	TSB+25 ppm formalin+bacteria
5	50 ppm	TSB+50 ppm formalin+bacteria
6	100 ppm	TSB+100 ppm formalin+bacteria
7	200 ppm	TSB+200 ppm formalin+bacteria
8	400 ppm	TSB+400 ppm formalin+bacteria
9	800 ppm	TSB+800 ppm formalin+bacteria

2.4 Total Plate Count (TPC)

Total plate count (TPC) was performed by taking a 0.1 g sample of *Tubifex* worms (Abdi *et al.,* 2022), which was homogenized and diluted with 0.9 mL of phosphate buffer saline (PBS). The diluted sample was then serially diluted and plated on Rimler-Shotts (RS) agar at a 10^{-7} dilution level, followed by incubation for 18 hours at 37°C. Colony counts in the range of 30–300 CFU were recorded and analyzed.

2.5 In Vivo Assay

The in vivo assay was conducted following the in vitro phase to evaluate the direct effects of formalin on living organisms.

- **Formalin Toxicity Test on *Tubifex* Worms**

This test aimed to determine the potential toxicity of formalin to *Tubifex* worms. The experimental design included two treatments (see Table 2), each consisting of 20 worms. The worms were immersed in a formalin solution for 30 minutes and then transferred to petridishes for observation. Mortality was recorded every 2 hours for 24 hours.

Table 2. Experimental design for the toxicity test of formalin on *Tubifex* worms

No	Experiment	Description
1	Control	Immersed in plain water without formalin treatment
2	Treatment	Immersed in formalin

- **Feeding Trial of Treated *Tubifex* Worms to Catfish Larvae**

Three-day-old *Pangasius* larvae were used as test organisms at a stocking density of 1,000 individuals per aquarium (20 fish/L). Twelve aquaria, each measuring 60 × 40 × 20 cm with 20 cm water depth (48 L), were used. The larvae were fed with *Tubifex* worms treated with the following four protocols: K (Control) – no formalin treatment; A – 400 ppm formalin immersion without rinsing; B –

400 ppm formalin immersion followed by one rinse; C – 400 ppm formalin immersion followed by two rinses. The 400 ppm concentration (0.4 mL of formalin in 1 L water for 30 minutes) was determined based on the in vitro MIC results. For each treatment, 1 g of worms was used as feed. Feeding was carried out 8 times per day, starting at 02:00 WIB. The experiment followed a Completely Randomized Design (RAL) with three replicates. Siphoning was conducted before feeding, and water volume was replenished as needed. The feeding trial design is presented in table 3.

Table 3. Experimental design for feeding tubifex worms to catfish larvae

No	Treatment	Description
1	K (control)	*Tubifex* worm
2	A (400 ppm)	*Tubifex* worm immersed in formalin without rinsing
3	B (400 ppm)	*Tubifex* worm immersed in formalin rinsed once
4	C (400ppm)	*Tubifex* worm immersed in formalin rinsed twice

2.6 Water Quality Monitoring

Water quality parameters including temperature, dissolved oxygen (DO), and pH were measured daily at 08:00 and 16:00 WIB.

2.7 Observed Parameters

The observed parameters in this study included the Minimum inhibitory concentration (MIC), inhibition zone diameter, toxicity, total bacterial count, survival rate (SR), and clinical symptoms. The MIC test was conducted to determine the lowest concentration of formalin that could inhibit bacterial growth (Sacher *et al.,* 2000). The inhibition zone was measured as the diameter of the clear area surrounding the disk (Toy *et al.,* 2015). The toxicity test was carried out over a 24-hour period to evaluate the effect of formalin on the survival of *Tubifex* worms. Total pathogenic bacterial count was determined using the total plate count method (Lestari *et al.,* 2016). The survival rate (SR) of *Pangasius* larvae was calculated at the end of the maintenance period. Feeding behavior was observed descriptively through direct visual observation of the larvae during the rearing period. The observations included larval responses to feed, feeding speed, and general feeding activity in each treatment group.

2.8 Data Analysis

The collected data included MIC values, inhibition zone diameters, tubifex worm toxicity levels, total pathogenic bacterial counts, survival rate (SR) of catfish larvae, and clinical symptoms. Quantitative data (MIC, inhibition zone, toxicity, total bacteria, and SR) were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine significant differences among treatments. The analyses were performed using Microsoft Excel 2016 and IBM SPSS Statistics 22. Qualitative data, such as larval feeding behavior, were analyzed descriptively.

3. Results

3.1 In Vitro Results

The results of the Minimum inhibitory concentration (MIC) and inhibition zone assays of formalin on *A. hydrophila* are presented in table 4. The result showed that a formalin concentration of 400 ppm was the most effective treatment for inhibiting the growth of *A. hydrophila*.

Table 4. Minimum Inhibitory Concentration (MIC) and Inhibition Zone Diameter of Formalin against *A. hydrophila*.

No	Treatment	MIC	Inhibition Zone (mm)
1	K-	Turbid	3,7
2	K+	Turbid	5,7
3	12,5	Turbid	4,2
4	25	Turbid	3,3
5	50	Turbid	3,9
6	100	Turbid	3,3
7	200	Turbid	4,3
8	400	Clear	4,2
9	800	Clear	4,2

3.2 Total Plate Count (TPC)

The total plate count (TPC) values are presented in table 5. It was observed that the bacterial count prior to formalin treatment was 5×10^8 CFU/mL, which decreased to 0 CFU/mL after the application of formalin.

Table 5. Total plate count (TPC) values before and after formalin treatment

No	Treatment	Total bacteria (CFU/mL)
1	*Tubifex* worm without formalin	5×10^8
2	*Tubifex* worm added formalin	0

3.3 In Vivo Test Results

3.3.1 Formalin Toxicity on Tubifex Worms

The results of the toxicity test of formalin on *Tubifex* worms are shown in Figure 1. The graph indicates that the highest mortality occurred between the 4th and 10th hour post-immersion. The total mortality of worms over the 24-hour observation period for each replicate was 18 individuals (Replicate 1), 19 individuals (Replicate 2), and 19 individuals (Replicate 3). Conversely, no mortality was recorded in the control group across all replicates, with all worms surviving until the end of the observation period.

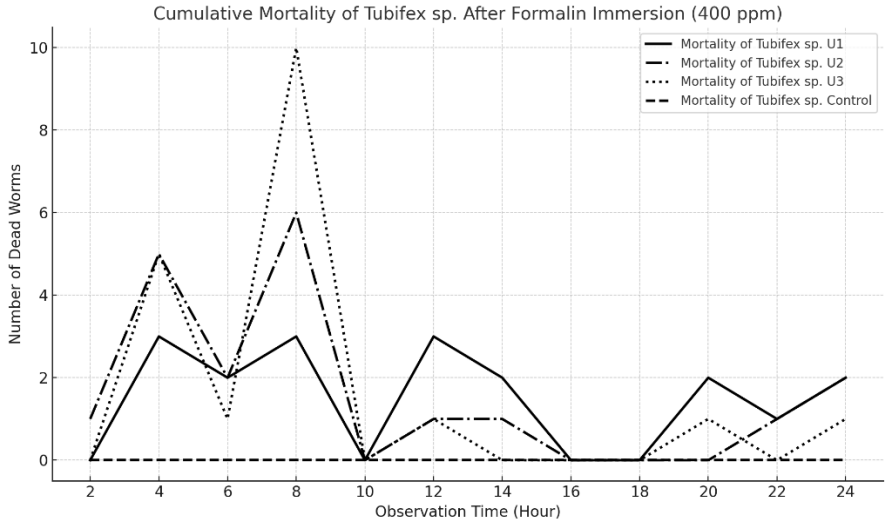

Figure 1. Cumulative mortality of *Tubifex* worms following immersion in 400 ppm formalin solution for 30 minutes, observed over 24 hours. U1 (replicate 1), U2 (replicate 2), U3 (replicate 3).

3.3.2 Survival Rate (SR) of Pangasius Larvae

The survival rate (SR) of *Pangasius* larvae is presented in Figure 2. The highest survival rate was observed in the treatment group with double rinsing, while the lowest was found in the control group. The control group showed a significantly lower SR of $16.3 \pm 1.0\%$. The single formalin immersion treatment and the single-rinse group resulted in survival rates of $33.6 \pm 2.6\%$ and $29.7 \pm 10.7\%$, respectively, which were not significantly different from each other. However, the double-rinse treatment significantly improved the survival rate to $44.6 \pm 11.5\%$, showing a significant difference compared to all other treatments.

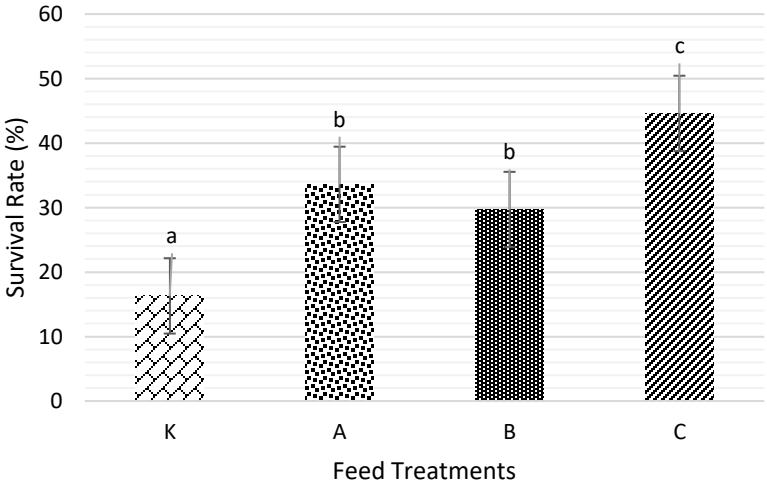

Figure 2. Survival rate (%) of catfish larvae (*Pangasius* sp.) fed with natural feed (*Tubifex* worms) under different treatment conditions: (K) untreated control, (A) immersed in 400 ppm formalin without rinsing, (B) immersed in 400 ppm formalin and rinsed once, and (C) immersed in 400 ppm formalin and rinsed twice. The result of ANOVA test indicate statistically significant differences ($P<0.05$).

3.3.3 Feeding Behavior of Catfish Larvae

Observations of feeding behavior in catfish (*Pangasius* sp.) larvae for each treatment group (K, A, B, and C) during the 14-day rearing period are presented in table 6.

Table 6. Observations of feeding behavior in *Pangasius* sp. larvae fed with *Tubifex* worms under different treatments

No	Treatment	Observation
1	Control (*Tubifex* worm)	Clear water; fish swam normally without stress
2	A (*Tubifex* worm immersed in formalin)	Cloudy water; fish consumed *Tubifex* worms at the bottom of the aquarium
3	B (*Tubifex* worms immersed in formalin for 30 minutes and rinsed once)	Slightly cloudy water; fish showed responsive feeding behavior
4	C (*Tubifex* worms immersed in formalin for 30 minutes and rinsed twice)	Clearer water; fish exhibited more responsive feeding behavior

3.3.4 Water Quality

Measurements of water temperature, pH, and dissolved oxygen (DO) were conducted daily at 08:00 and 16:00 WIB. The recorded water quality parameters throughout the experimental period are presented in table 7. Water quality measurements showed that the temperature remained at 25°C, pH at 7.0, and dissolved oxygen (DO) at 5 mg/L for all treatment.

Table 7. Water quality parameters during the rearing period of *Pangasius* sp. larvae

No	Parameters	Treatment				Water quality standard	Source
		K+	A	B	C		
1	Temperature (°C)	25	25	25	25	28-30	SNI 7256–2006
2	pH	7	7	7	7	6.5-7.5	SNI 7256–2006
3	DO (mg/L)	5	5	5	5	>5	SNI 7256–2006

4. Discussion

4.1 In Vitro

The in vitro analysis demonstrated that a formalin concentration of 400 ppm was the most effective dose for inhibiting the growth of *A. hydrophila*. This was evidenced by the change in the medium from turbid to clear following incubation, and the formation of a 4.2 mm inhibition zone at that concentration. According to the Minimum inhibitory concentration (MIC) test, media treated with formalin concentrations below 400 ppm remained turbid, indicating active bacterial growth. In contrast, media treated with 400 ppm and 800 ppm formalin appeared clear, suggesting complete bacterial inhibition. The largest inhibition zone was recorded in the positive control (oxytetracycline), measuring 5.7 mm, while the 400 ppm formalin treatment produced a smaller inhibition zone classified as weak (Surjowardojo *et al.,* 2015). Nonetheless, the presence of a clear zone confirmed that formalin exhibited notable antibacterial activity against *A. hydrophila*.

Bacteria *A hydrophila* is a common opportunistic pathogen in freshwater aquaculture environments and is a known causative agent of *Motile Aeromonas Septicemia* (MAS), which has significant implications for fish health (Austin & Austin, 2016). Formalin is a potent disinfectant containing 35–40% formaldehyde, which effectively disrupts microbial defense systems, leading to cell death (Saptarini *et al.,* 2011; Santi, 2017). The efficacy of formalin was also confirmed by the Total plate count (TPC), which showed a reduction in *A. hydrophila* from 5×10^8 CFU/mL (pre-treatment) to 0 CFU/mL (post-treatment) with 400 ppm formalin. This aligns with findings by Floyd & Pouder (2023), who reported that formalin effectively reduces bacterial populations in aquaculture systems. Additionally, post-incubation observations using RS media revealed a yellow coloration, indicating the presence of *Aeromonas* spp. (Setiadi & Wadjdy, 2021).

Formalin use as an antibacterial agent presents a promising alternative to antibiotics, especially given the growing concern over antimicrobial resistance (Cabello, 2006). Although the inhibition zone at 400 ppm was smaller than that of the positive control, this dose was selected as the minimum effective concentration that inhibits bacterial growth without causing harm to catfish larvae. This is consistent with the principle of optimal dosing, whereby excessively high concentrations do not necessarily offer superior protection and may instead induce toxicity (Nikoskelainen *et al.,* 2001; Widanarni *et al.,* 2010).

4.2 In Vivo

Toxicity test results revealed that formalin at a concentration of 400 ppm exerted toxic effects on *Tubifex* worms, resulting in a mortality rate of 90% (18 out of 20 individuals) within 24 hours. This indicates that while formalin at this concentration is effective as an antibacterial agent against *A. hydrophila*, it exceeds the physiological tolerance threshold of *Tubifex* worms, leading to rapid mortality. Nevertheless,

the 400 ppm concentration was retained for subsequent testing on catfish (*Pangasius* sp.) larvae, with a modified protocol involving rinsing of the *Tubifex* worms after formalin immersion. This step was intended to reduce residual formalin on the worms' surface and minimize secondary toxicity risks to catfish larvae consuming the worms as live feed. The strategy was based on the principle of partial detoxification through repeated washing using clean water or an appropriate culture medium. Fitriadi & Putri (2016) reported that effective rinsing procedures can significantly reduce residues of harmful chemical compounds.

The study found that immersion of *Tubifex* worms in 400 ppm formalin solution for 30 minutes, followed by two rinsing steps, significantly improved the survival rate (SR) of catfish larvae (*Pangasius* sp.). The highest SR was recorded in this treatment group at 44.6 ± 11.5%, which was statistically different from the other treatments. In contrast, the untreated control group (no formalin immersion) had the lowest SR at 16.3 ± 1.0%, suggesting that the use of untreated live feed increases the risk of infection by pathogenic bacteria such as *A. hydrophila*.

The effectiveness of formalin in reducing *A. hydrophila* populations was demonstrated by Chinabut *et al.,* (1988), who reported that a concentration of 25 ppm was capable of killing 99.9% of bacteria within 24 hours, while concentrations of 50 and 75 ppm eliminated 100% of bacteria in a shorter time. However, the use of formalin at high concentrations may exert toxic effects on non-target organisms. In the present study, immersion of *Tubifex* worms in 400 ppm formalin without rinsing resulted in 90% mortality within 24 hours, indicating a high level of toxicity. Nonetheless, a modified treatment involving two rinsing steps following immersion proved effective in reducing residual formalin and thus lowering toxicity to catfish larvae. The two-step rinsing treatment is considered to significantly reduce residual formalin carried over by the live feed, thereby achieving a balance between antibacterial efficacy and safety for the test organisms. Consequently, this method shows potential as an alternative to antibiotics in freshwater aquaculture for the control of pathogenic bacterial infections such as *A. hydrophila*.

Water quality measurements during the experiment showed that the temperature remained at 25°C, pH at 7.0, and dissolved oxygen (DO) at 5 mg/L. According to the Indonesian National Standard (SNI 7256-2006), the pH and DO levels were within the optimal range for the maintenance of catfish larvae, which are pH 6.5-7.5 and DO >5 mg/L. However, the recorded temperature of 25°C was considered suboptimal, as it falls below the ideal range for catfish larvae rearing (28-30°C). Research by Sherif *et al.,* (2024) indicated that low temperatures can suppress the immune response in Nile tilapia and increase susceptibility to *A. hydrophila* infection. Additionally, low temperatures may reduce fish appetite (Jaya, 2011). The survival rate (SR) of catfish larvae observed in this study was below the national standard threshold of 50% after 15 days of rearing (BSN, 2000), with the highest SR recorded at only 44.6 ± 11.5% in the treatment involving formalin immersion followed by two rinses. This finding suggests that although formalin treatment effectively reduced pathogenic bacterial density and improved SR compared to the control, the suboptimal temperature may have been a limiting factor that prevented the achievement of optimal survival rates.

5. Conclusions

The application of formalin at a concentration of 400 ppm effectively inhibited the growth of *Aeromonas hydrophila*, and treatment C (immersion followed by two rinsing steps) successfully reduced the toxic effects of formalin on live feed (*Tubifex* worms), thereby increasing the survival rate of catfish larvae (*Pangasius* sp.) over a 14-day rearing period.

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