

ANTIMICROBIAL POTENTIAL OF MINERAL ACIDIFIER AGAINST PATHOGENIC *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is a gram-negative, halophilic, facultative anaerobic bacterium that poses a serious threat to vaname shrimp (*Litopenaeus vannamei*) aquaculture and human food safety. The increasing prevalence of this pathogen in coastal and brackish water environments necessitates the development of effective and safe antibacterial alternatives to conventional antibiotics. This study aimed to evaluate the antibacterial potential of Intra AquaAcid Mineral, a mineral acidifier product, against *V. parahaemolyticus* through inhibition zone testing and Total Vibrio Count (TVC) assessment. The antibacterial activity was tested using the agar disc diffusion method on Mueller Hinton Agar (MHA), with seven treatment groups consisting of one negative control and six concentrations of mineral acidifier diluted in NaCl solution (50%, 60%, 70%, 80%, 90%, and 100%), each with three replications arranged in a Completely Randomized Design. Inhibition zones were measured using a digital caliper, and culture water testing was performed at 0, 6, 12, and 24 hours using TCBS agar. Results showed that all tested concentrations produced inhibition zones ranging from 34.50 mm to 44.50 mm, classified as very strong antibacterial activity according to Davis and Stout (1971) criteria (>20 mm). The largest inhibition zone was observed at 100% concentration (44.50 mm). TVC results demonstrated complete inhibition of *V. parahaemolyticus* in treated culture water, while control groups showed detectable Vibrio counts throughout the observation period. These findings indicate that Intra AquaAcid Mineral exhibits strong dose-dependent antibacterial activity and holds significant potential as an alternative antibacterial agent in shrimp aquaculture systems.

Keywords: Antibacterial, *Inhibitory effect*, *Litopenaeus vannamei*, Mineral acidifier, *Vibrio parahaemolyticus*.

INTRODUCTION

Litopenaeus vannamei (whiteleg shrimp) is one of the leading commodities in global fisheries (Fendjalang et al., 2016). The demand for vannamei shrimp is driven by its short life cycle and ease of cultivation (Sun et al., 2023). However, disease outbreaks remain a major challenge in shrimp aquaculture. One of the bacterial pathogens affecting vannamei shrimp is *Vibrio parahaemolyticus*. According to Scallan et al. (2011), this bacterium has been identified as a primary cause of seafood-borne illness and mortality in the United States.

Among the most threatening pathogens in vannamei shrimp farming is *Vibrio parahaemolyticus*. This bacterium is Gram-negative, halophilic, motile, comma-shaped, facultatively anaerobic, non-spore-forming, and zoonotic (Siddique et al., 2021). *V. parahaemolyticus* grows optimally in environments with NaCl concentrations of 2.5–3.5%, making it highly adaptive to pond and coastal ecosystems (Fan et al., 2025). It is recognized as the causative agent of Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS), which results in mass shrimp mortality within the first seven days of culture (Markus, 2015). The virulence of *V. parahaemolyticus* is determined by the production of Thermostable Direct Hemolysin (TDH) and Thermostable Related Hemolysin (TRH), which damage the host hepatopancreas (Broberg et al., 2011; Zhang and Orth, 2013; Letchumanan et al., 2014). Beyond aquaculture, this bacterium is also a leading cause of seafood-borne diseases in humans across multiple countries (Scallan et al., 2011).

Control of *V. parahaemolyticus* has traditionally relied on antibiotics. However, uncontrolled antibiotic use has led to antimicrobial resistance and harmful residues in aquaculture products (Siddique et al., 2021). Therefore, the development of safe and effective alternative antibacterial agents is urgently needed. One promising approach is the application of acidifiers or organic acids. Recent studies have demonstrated that hexanal, an organic acid, can inhibit *V. parahaemolyticus* growth at a minimum inhibitory concentration (MIC) of 0.4 mg/mL through mechanisms involving cell membrane disruption, reduction of intracellular ATP levels, and inhibition of biofilm formation (Fan et al., 2025). In addition, phenyllactic acid (PLA) has been reported to effectively suppress *V. parahaemolyticus* by interfering with metabolism and gene expression (Li et al., 2024). However, these previous studies have primarily focused on single organic acid compounds characterized through in vitro molecular or transcriptomic approaches, whereas empirical evidence on multi mineral based acidifier formulations which are increasingly marketed and applied in commercial shrimp farming remains scarce. Intra AquaAcid Mineral is a commercial mineral acidifier product claimed to possess broad-spectrum antibacterial activity; however, its scientific efficacy against *V. parahaemolyticus* has not yet been systematically reported.

This study aims to evaluate the antibacterial potential of Intra AquaAcid Mineral against *Vibrio parahaemolyticus* through inhibitory assays (disc diffusion) at various concentrations and Total Vibrio Count (TVC) analysis in culture water. The findings are expected to provide a scientific basis for the application of mineral acidifiers as safe alternative antibacterial agents in vannamei shrimp aquaculture systems.

MATERIALS AND METHODS

Time and Place

The study was conducted at the MAXMAR Group Laboratory on October 04, 2025. The study consisted of two experimental stages, namely laboratory-scale antibacterial testing and culture water testing. All microbiological analyses were performed under standard in vitro antimicrobial testing procedures.

Research Material

The primary material used in this study was the commercial product Intra Aqua Acid Mineral. The exact composition and proportion of organic acids in Intra Aqua Acid Mineral are proprietary information not disclosed by the manufacturer; the product is characterized as a blend of organic acids combined with chelated zinc (and copper in some formulations). The bacterial isolate employed for antibacterial testing was *Vibrio parahaemolyticus*, obtained from laboratory stock cultures. The microbiological media included TCBS (Thiosulfate Citrate Bile Salts Sucrose agar) for Total Vibrio Count (TVC) enumeration, and Chrom Agar Vibrio (CHROMagar, France) for species identification. Additional reagents comprised sterile distilled water, 70% ethanol, sterile physiological NaCl 0.9%, and McFarland standard 0.5 (bioMérieux, France). Laboratory equipment consisted of Petri dishes (90 mm), sterile paper discs (6 mm), an autoclave (Tomy SX-700, Japan), an incubator (Mettler IN55, Germany), a laminar airflow cabinet (ESCO, Singapore), a vortex mixer (Thermo Scientific, USA), a digital caliper (Mitutoyo, Japan), micropipettes (Eppendorf, Germany), a colony counter, an analytical balance (Ohaus, USA), and other standard microbiological laboratory apparatus.

Research design

This study employed a Completely Randomized Design (CRD) with seven treatments and three replicates, resulting in a total of 21 experimental units. The concentration range (50–100%, with 10% increments) was selected based on the general principle that the antimicrobial efficacy of liquid-based antibacterial agents declines markedly below 50% dilution, with optimal bactericidal activity typically observed between 60–90% concentration (CDC, 2008). The treatments consisted of: A = Negative control (without acidifier); B = Mineral acidifier + NaCl diluent at 50% concentration; C = Mineral acidifier + NaCl diluent at 60% concentration; D = Mineral acidifier + NaCl diluent at 70% concentration; E = Mineral acidifier + NaCl diluent at 80% concentration; F = Mineral acidifier + NaCl diluent at 90% concentration; and G = Mineral acidifier + NaCl diluent at 100% concentration.

Work Procedure

Preparation of Bacterial Isolates

All laboratory equipment was first thoroughly washed and dried, then sterilized using an autoclave for 15 minutes at 121 °C, followed by oven-drying for 30 minutes. *Vibrio parahaemolyticus* was re-cultured on selective Chrom Agar medium using the streak plate method and incubated at 35 °C for 18–24 hours to obtain fresh and active colonies. Subsequently, a culture of *V. parahaemolyticus* was prepared by suspending the cells into a test tube containing 5 mL of sterile 0.9% NaCl solution, homogenized, and adjusted to the turbidity of McFarland standard 0.5 (1.5×10^8 CFU/mL).

Preparation of Mineral Acidifier

The mineral acidifier was prepared in liquid form prior to testing. The acidifier was diluted with NaCl at different concentrations, namely 50%, 60%, 70%, 80%, 90%, and 100% (v/v). The test solutions were prepared immediately before use to prevent any reduction in biological activity.

Antibacterial Assay of Mineral Acidifier Against *Vibrio parahaemolyticus*

The antibacterial activity of the mineral acidifier was tested using the agar diffusion method with Mueller Hinton Agar (MHA) as the test medium. Sterile paper discs previously immersed in Intra Aqua Acid Mineral solutions of varying concentrations were placed evenly on the surface of the medium that had been inoculated with *Vibrio parahaemolyticus*. The plates were then incubated at 37 °C for 18–24 hours (Rahmadeni et al., 2019). The presence of clear inhibition zones indicated bacterial susceptibility to the tested antibacterial agent, expressed as the diameter of the inhibition zone. The inhibition zones formed were measured using a digital caliper to determine the level of effectiveness (Harlina et al., 2023). The determination of inhibition zones was conducted following the method described by Ramadhani and Febria (2024).

$$R = (p + q) / 2$$

The inhibition zone diameter (R) was determined by measuring the longest diameter (p) and the shortest diameter (q) of the clear zone, with R expressed in millimeters. The strength categories of antibacterial activity were classified according to Davis and Stout (1971), namely: weak (<5 mm), moderate (5–10 mm), strong (11–20 mm), and very strong (>20 mm).

Total Vibrio Count (TVC)

Total Vibrio Count (TVC) testing was conducted using the spread plate method on TCBS agar medium. Water samples from the hatchery pond were collected at time intervals of 0, 6, 12, and 24 hours. Serial dilutions were performed using sterile 0.85% NaCl solution by transferring 100 µL of the sample into 900 µL of 0.85% NaCl, followed by homogenization. A 100 µL aliquot of the diluted sample was then spread evenly on the surface of TCBS agar using a sterile triangular spreader. The plates were incubated at 35 °C for 24 hours. Colonies were manually counted based on color differentiation: yellow colonies (*Vibrio cholerae*) and green colonies (*V. parahaemolyticus*). Colony enumeration followed the Standard Plate Count (SPC) method with an acceptable range of 25–250 colonies per plate. The threshold for TVC was determined according to the Indonesian National Standard (SNI 7311:2009), namely $\leq 1 \times 10^3$ CFU/mL.

Data Analysis

Data analysis was performed both descriptively and quantitatively using SPSS version 26 (IBM Corp., Armonk, NY, USA). The inhibition zone diameter data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Data meeting these assumptions were analyzed using one-way Analysis of Variance (ANOVA) at a 5% significance level ($p < 0.05$). Where a significant treatment effect was detected, Tukey's Honestly Significant Difference (HSD) post hoc test was performed for pairwise comparisons among treatments, with results expressed using superscript letter notation, where different letters indicate statistically significant differences ($p < 0.05$). The strength and direction of the relationship between acidifier concentration and inhibition zone diameter were assessed using Pearson's correlation coefficient (r). TVC data were presented descriptively in the form of tables and graphs.

RESULTS AND DISCUSSION

Antibacterial Activity of Mineral Acidifier Against *Vibrio parahaemolyticus*

The results of the antibacterial activity assay of Intra AquaAcid Mineral against *Vibrio parahaemolyticus* using the agar diffusion method at various concentrations are presented in Table 1. All tested concentrations produced inhibition zones classified as very strong (>20 mm) according to the criteria established by Davis and Stout (1971).

Table 1. Inhibition Zone Diameters of Intra AquaAcid Mineral Against *Vibrio parahaemolyticus* at Different Concentrations

Treatment (Dose)	Replicate 1 (mm)	Replicate 2 (mm)	Replicate 3 (mm)	Mean $\pm$ SD (mm)	Category*
Negative control (0%)	0.00	0.00	0.00	0.00 ± 0.00	–
50% dose	34.00	35.00	34.50	34.50 ± 0.50	Very strong
60% dose	35.00	36.00	36.50	35.83 ± 0.76	Very strong
70% dose	38.00	39.00	38.50	38.50 ± 0.50	Very strong
80% dose	43.00	42.00	41.50	42.17 ± 0.76	Very strong
90% dose	42.00	43.00	41.00	42.00 ± 1.00	Very strong
100% dose	44.00	45.00	44.50	44.50 ± 0.50	Very strong

Notes: Categories are based on Davis and Stout (1971): weak (<5 mm), moderate (5–10 mm), strong (11–20 mm), and very strong (>20 mm); SD = Standard Deviation.

The data presented in Table 1 demonstrate a consistent trend of increasing inhibition zone diameters with higher concentrations of mineral acidifier. The minimum inhibition zone was observed at the 50% concentration (mean 34.50 ± 0.50 mm), while the maximum was recorded at the 100% concentration (mean

44.50 $\pm$ 0.50 mm). One-way ANOVA revealed a significant effect of mineral acidifier dosage on the inhibition zone diameter of *V. parahaemolyticus* ($F(5,12) = 95.98$, $p < 0.001$). Tukey's HSD post hoc test indicated that the 80%, 90%, and 100% concentrations produced significantly larger inhibition zones compared to the 50% and 60% concentrations ($p < 0.05$), whereas no significant difference was found between the 80% and 90% concentrations. Pearson's correlation analysis showed a very strong positive correlation between acidifier concentration and inhibition zone diameter ($r = 0.97$; $p < 0.01$), indicating that increasing concentrations consistently enhanced antibacterial effectiveness.

The strong inhibitory activity of mineral acidifier against *V. parahaemolyticus* across all tested concentrations can be explained through several complementary mechanisms. First, organic acid components in the acidifier lower the extracellular pH, forcing bacterial cells to expend energy maintaining intracellular pH homeostasis, which depletes ATP and ultimately inhibits growth. In their undissociated form, organic acids can penetrate the lipopolysaccharide membrane of Gram-negative bacteria such as *V. parahaemolyticus* and disrupt the transmembrane proton gradient (Fan et al., 2025). This mechanism aligns with transcriptomic evidence reported by Lin et al. (2024), who demonstrated that phenyllactic acid (PLA), another organic acid, alters the expression of genes involved in *V. parahaemolyticus* metabolism, membrane integrity, and stress response, indicating that organic acid stress triggers coordinated physiological disruption beyond simple pH reduction. Second, beyond the organic acid component, the chelated mineral ions (zinc and, in some formulations, copper) present in Intra AquaAcid Mineral may contribute an additional antibacterial mode of action, as divalent metal ions are known to interfere with bacterial membrane transport proteins and generate oxidative stress within the bacterial cytoplasm. The combined presence of organic acids and chelated minerals therefore suggests a potentially multi-target mechanism, which may explain the consistently strong inhibition observed even at the lowest tested concentration (50%).

Differences in inhibition zone diameter among concentrations are attributed to variations in the density of active molecules diffusing from the discs into the agar medium, whereby higher concentrations result in a steeper diffusion gradient and larger measurable zones (Harlina et al., 2023). Nevertheless, it should be emphasized that the present study characterized inhibition at the phenotypic level only; molecular confirmation of these proposed mechanisms such as quorum-sensing gene suppression or membrane permeability assays was beyond the scope of this study and is addressed further in the Limitations section below. The inhibition zones obtained in this study (34.50–44.50 mm) consistently fell within the "very strong" category (>20 mm) according to Davis and Stout (1971), and were notably larger than inhibition zones reported for plant-based antibacterial agents against *V. parahaemolyticus* in comparable Indonesian

aquaculture studies, such as basil leaf extract (Harlina et al., 2023) and sponge-symbiont bacterial isolates (Ramadhani & Febria, 2024), which typically fall within the strong (11–20 mm) rather than very strong category. This comparison should nonetheless be interpreted cautiously, as inhibition zone diameter is influenced by compound diffusivity, molecular weight, and solvent polarity, which differ substantially between organic acid-based and plant-extract-based antibacterial agents; a direct quantitative comparison would require standardizing testing conditions (disc concentration, medium, inoculum density) across studies.

Direct comparison with organic acid-specific literature is similarly constrained by differing units of measurement Fan et al. (2025) and Lin et al. (2024) characterized hexanal and phenyllactic acid activity against *V. parahaemolyticus* using minimum inhibitory concentration (MIC) values (mg/mL) rather than inhibition zone diameter, precluding a direct numerical comparison with the present findings. This distinction itself underscores a methodological gap in the literature: while single organic acid compounds have been characterized primarily through molecular and MIC-based approaches, empirical, phenotype-level antibacterial data for multi-component mineral acidifier formulations of the type increasingly marketed for commercial aquaculture use remain scarce, reinforcing the exploratory contribution of the present study. Future work applying standardized MIC/MBC determination for Intra AquaAcid Mineral (as recommended in the Recommendations section) would allow a more rigorous, unit-consistent comparison with previously reported organic acid compounds.

Inhibition Assay Using NaCl Dilution

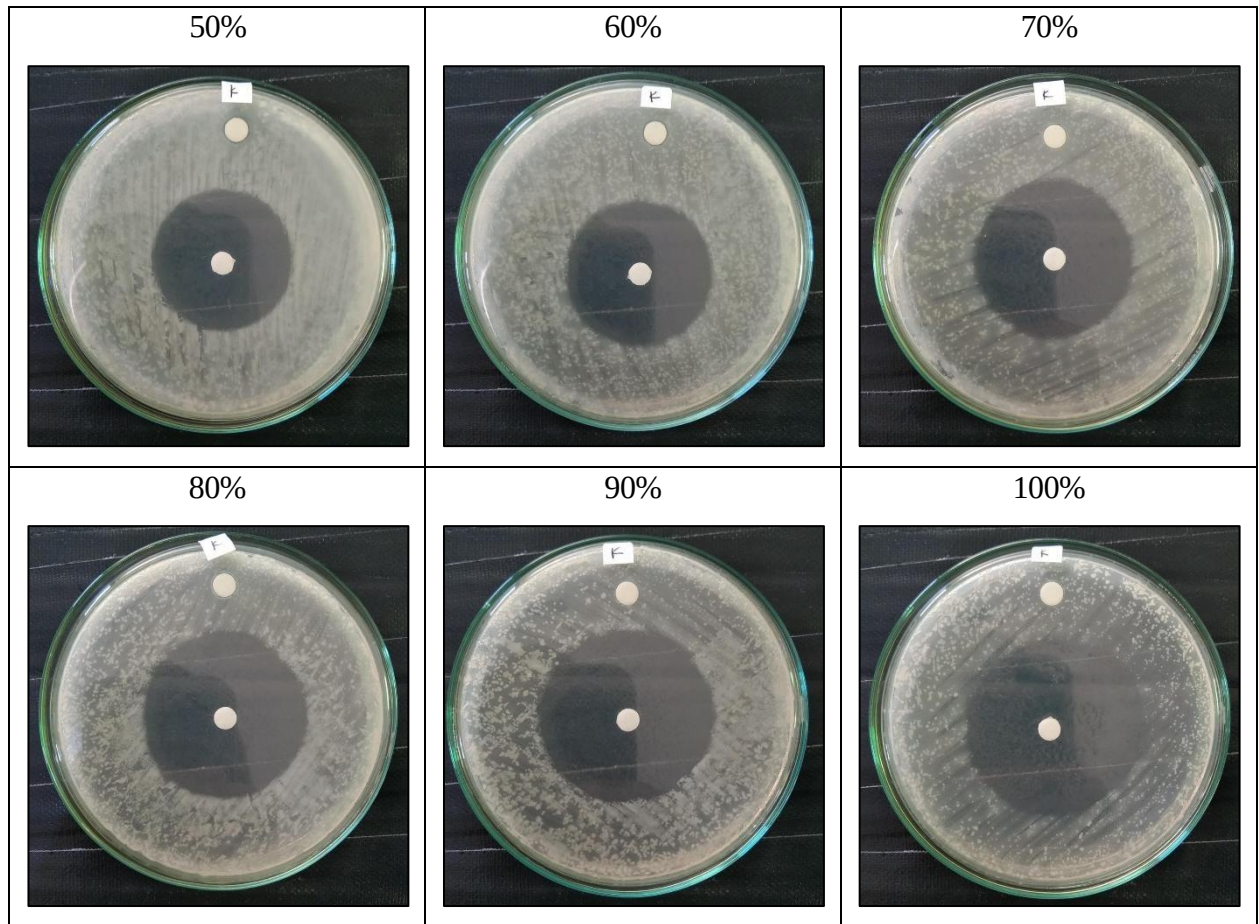


Figure 1. Inhibition Assay Using NaCl Dilution

Total Vibrio Count

The results of the Total Vibrio Count (TVC) assay in culture water revealed significant differences between the control group and the treatment group with Intra AquaAcid Mineral. Table 2 presents the TVC data for each group based on the observation time intervals.

Table 2. Total Vibrio Count (TVC) in Culture Water of Control and Intra AquaAcid Mineral Treatment Groups

Group	Replicate	TVC 0 h (CFU/mL)	TVC 6 h (CFU/mL)	TVC 12 h (CFU/mL)	TVC 24 h (CFU/mL)
Control	1	1.6×10^3	9.0×10^1	8.0×10^1	5.0×10^1
Control	2	1.4×10^3	8.0×10^1	8.0×10^1	7.0×10^1
Control	3	1.2×10^3	6.0×10^1	7.0×10^1	3.0×10^1
Intra AquaAcid Mineral	1	0 (negative)	0 (negative)	0 (negative)	0 (negative)
Intra	2	0 (negative)	0 (negative)	0 (negative)	0 (negative)

Group	Replicate	TVC 0 h (CFU/mL)	TVC 6 h (CFU/mL)	TVC 12 h (CFU/mL)	TVC 24 h (CFU/mL)
AquaAcid Mineral					
Intra AquaAcid Mineral	3	0 (negative)	0 (negative)	0 (negative)	0 (negative)

Notes: 0 (negative) = no *Vibrio* colonies detected in all replicates; TVC = Total *Vibrio* Count; maximum threshold according to SNI 7311:2009 = $\leq 1 \times 10^3$ CFU/mL.

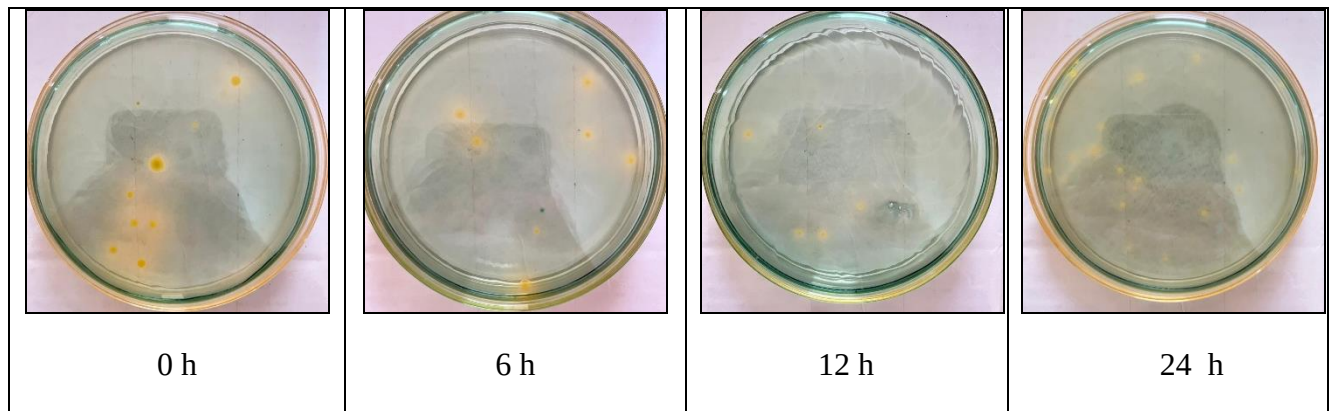
The data in Table 2 show that the control group had an initial mean TVC of 1.4×10^3 CFU/mL at 0 h, which was precisely at the maximum threshold set by SNI 7311:2009. At 6 h, a sharp decline was observed to $6.0\text{--}9.0 \times 10^1$ CFU/mL, with colonies still detected at 12 h and 24 h, indicating that *V. parahaemolyticus* remained viable despite fluctuations. In contrast, the Intra AquaAcid Mineral treatment group consistently showed negative results (no *Vibrio* colonies detected) across all observation intervals, including at 0 h. This finding indicates that the mineral acidifier was able to completely eliminate *V. parahaemolyticus* and exerted an immediate inhibitory effect upon initial contact. The total inhibitory effect observed in the TVC assay is consistent with the agar diffusion results, which also demonstrated very strong antibacterial activity. The mechanism of inhibition in water media is likely related to direct acidification, creating an environment unfavorable for bacterial survival. *V. parahaemolyticus* is known to be highly sensitive to pH changes and unable to persist under prolonged acidic conditions (Siddique et al., 2021). The use of selective TCBS medium in this study enabled specific differentiation between *V. parahaemolyticus* (green colonies) and *Vibrio cholerae* (yellow colonies), ensuring that the reported TVC values accurately reflected the presence of *V. parahaemolyticus* (Agung et al., 2020).

These findings confirm that Intra AquaAcid Mineral is not only effective under in vitro laboratory conditions but also capable of eliminating pathogens in aquatic environments representative of aquaculture systems. Considering that the maximum bacterial concentration in aquaculture waters is 10^4 CFU/mL, exceeding this threshold can result in up to 50% shrimp mortality within seven days (Markus, 2015). Therefore, the immediate and total inhibitory effect of mineral acidifier highlights its potential as a practical biosecurity intervention in shrimp hatchery management.

Several limitations of this study should be acknowledged. First, all antibacterial assays were conducted under controlled in vitro laboratory conditions using standardized culture media (Mueller Hinton Agar and TCBS agar) and a single bacterial isolate maintained under optimal growth conditions. Actual aquaculture pond or hatchery water contains substantially higher organic matter loads, fluctuating temperature and salinity, and a diverse resident microbiota, all of which may buffer or otherwise alter the effective antibacterial activity of the mineral acidifier compared to the clean water matrix used in the present TVC assay. Second, this study evaluated bactericidal efficacy only; the safety of Intra AquaAcid Mineral toward non-target organisms — including

vannamei shrimp larvae, beneficial nitrifying bacteria, and other components of pond microbiota — was not assessed, and complete elimination of *Vibrio* populations observed in vitro may not be desirable or achievable without unintended ecological side effects in a functioning biofloc or pond ecosystem. Third, the exact concentration and mode of application demonstrated to be effective under controlled conditions (50–100% v/v) may require substantial recalibration for practical field application, considering dilution by pond volume, water exchange rates, and continuous microbial recolonization from sediment and inflow water. Therefore, while the present findings provide strong preliminary evidence of the antibacterial potential of Intra AquaAcid Mineral against *V. parahaemolyticus*, translation of these results into practical hatchery or grow-out applications requires validation through in vivo challenge trials and pond-scale or mesocosm testing, as outlined in the Recommendations section.

a. Control



b. Intra AquaAcid Mineral

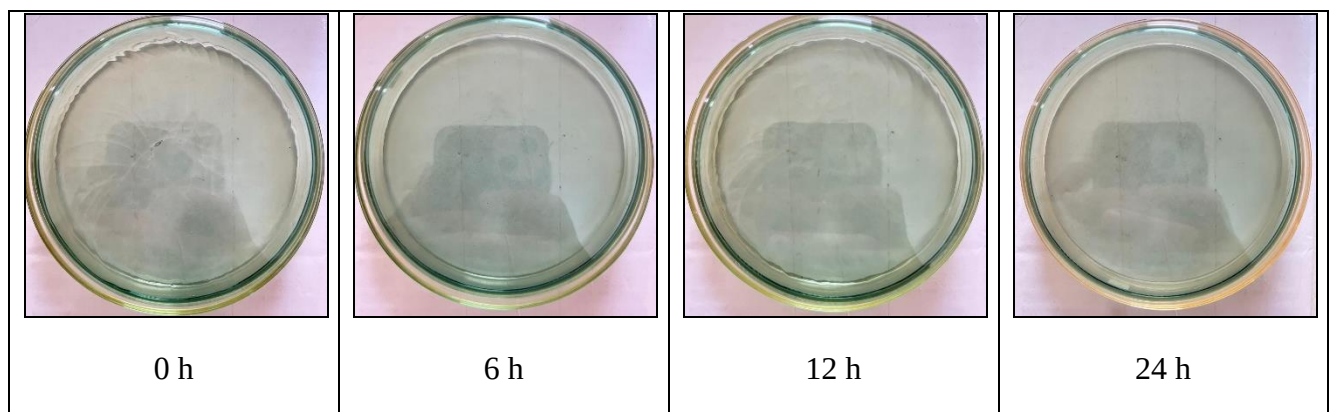


Figure 2. Total Vibrio Count (TVC) in Culture Water of Control Group and Intra Aqua Acid Mineral Treatment Group

Conclusion

Intra AquaAcid Mineral demonstrated very strong antibacterial potential against *Vibrio parahaemolyticus* at all tested concentrations (50%–100%). The inhibition zone diameters ranged from 34.50–44.50 mm, all of which were

classified as “very strong” (>20 mm) according to Davis and Stout (1971). A very strong positive correlation was observed between mineral acidifier concentration and inhibition zone diameter ($r = 0.97$; $p < 0.01$), and ANOVA confirmed significant differences among treatments ($p < 0.05$). The Total Vibrio Count assay further revealed complete inhibition of *V. parahaemolyticus* growth in the treatment group across all observation intervals (0, 6, 12, and 24 h), while the control group consistently showed the presence of pathogenic bacteria. These findings indicate that Intra AquaAcid Mineral has strong potential to be developed as an effective and safe antibacterial agent in vannamei shrimp aquaculture systems to control *V. parahaemolyticus* infections.

Recommendations

Further studies are recommended to: (1) Determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Intra AquaAcid Mineral against *V. parahaemolyticus* using precise dilution methods. (2) Evaluate its effectiveness under real aquaculture conditions through in vivo challenge tests in vannamei shrimp. (3) Investigate the molecular mechanisms of inhibition, including effects on virulence gene expression and biofilm formation. (4) Assess product safety for non-target organisms and comprehensively analyze its impact on aquaculture water quality.

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