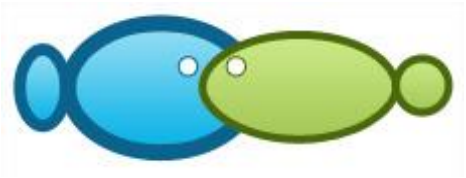

High salinity reduces *Vibrio* spp. while maintaining nauplii survival during *Artemia franciscana* (Kellog, 1906) culture: a sustainable approach for aquaculture

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Abstract. The use of contaminated brine shrimp, *Artemia franciscana* (Kellog, 1906), as live feed leads to vibriosis outbreaks and threats to global aquaculture productivity. This study highlights a potential cost-effective and environmentally friendly approach to suppress *Vibrio* spp. load in *Artemia* live feed. Axenic *A. franciscana* nauplii were reared with *Vibrio campbellii* and *Vibrio parahaemolyticus* under 30-150 PSU of salinity. A salinity of 80 PSU can eliminate more than 90% of both strains, while maintaining over 90% survival of *A.* franciscana nauplii. Another experiment demonstrated that the *Vibrio* spp. load in non-axenic cyst-hatching tanks at 30 PSU was high but reduced with increasing salinities. The addition of 1 g L^{-1} *Pandanus tectorius* leaf extract in the high salinity rearing water significantly reduced cyst-derived bacterial load. Findings showed that 80-110 PSU of salinity is effective in suppressing almost all the *Vibrio* spp. without adverse effects on *A. franciscana* growth, removing the need for cyst disinfection or nauplii tank transfer.
Keywords: brine shrimp, disease, infection, pathogen, sterilization.

Introduction. Aquaculture is among the fastest-growing industries, with annual global production reaching 130.9 million tonnes and a trade value of USD 313 billion in the year of 2022 (Food and Agriculture Organization of the United Nations, 2024). However, its sustainability is increasingly threatened by several challenges, notably disease outbreaks that can result in substantial economic losses (Leung et al., 2013). One of the most severe threats is vibriosis, a bacterial disease caused by *Vibrio* spp., particularly the highly pathogenic strains within the Harveyi clade, such as *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* (Sheikh et al., 2022). These strains carry virulent genes that contribute to high mortality rates in key aquaculture species, such as Penaeid shrimps (De Schryver et al., 2014), Eastern oyster (*Crassostrea virginica*) (Alfaro et al., 2019), and mud crab (*Scylla* spp.) (Kwok et al., 2024), especially during early life stages. Among them, *V. parahaemolyticus* is notably responsible for early mortality syndrome (EMS) in penaeid shrimps, a disease later termed acute hepatopancreatic necrosis disease (AHPND), which causes massive mortalities, particularly when shrimp larvae are compromised by infection (Choi et al., 2017; Kumar et al., 2020; Lozano-Olvera et al., 2024). Although the hepatopancreas is the main target of *V. parahaemolyticus* infection (Aguirre-Guzmán et al., 2010), this chitinase-producing strain may also penetrate the shrimp through the cuticular structure (Quiroz-Guzmán et al., 2013; Deng et al., 2025). Several virulence

factors were identified from pathogenic strains isolated from infected *P. vannamei*, including genes related to hemolysins and type three secretion system 1 and 2 (Paria et al., 2021). Besides that, other *Vibrio* spp., such as *V. coralliilyticus* and *V. tubiashii,* were also associated with high mortality in oyster culture (Richards et al., 2015).

Vibrio spp. can enter and proliferate in aquaculture systems through various methods. Key contributing factors include the use of untreated seawater, poor water quality, contaminated equipment, interactions with wild animals and vectors, and the introduction of live feed (Quiroz-Guzmán et al., 2013; Sanches-Fernandes et al., 2022). Among live feeds, the *Artemia* spp., is a well-documented vector for *Vibrio* spp. and other bacterial pathogens. During *Artemia* spp. hatching, high levels of organic material are released from the cysts, creating nutrient-rich conditions that promote rapid *Vibrio* spp. growth (Lavens & Sorgeloos 1996).

Antibiotics are commonly used to inhibit *Vibrio* spp. growth, but prolonged use is detrimental to the environment and leads to the emergence of antibiotic-resistant strains. Additionally, excessive antibiotic application may enter the food chain, posing potential risks to human health (Loo et al., 2020). One of the earliest methods developed to remove bacterial pathogens from *Artemia* spp. cysts is decapsulation. In this process, cysts are treated with a hypochlorite solution to remove the chorion (the hard outer shell) where *Vibrio* spp. contamination frequently occurs. Following sterilization, the *Vibrio*-free cysts can be used for hatching. While effective in eliminating harmful bacteria, this method involves the use of chemicals that are hazardous to both human health and the environment, making it unsuitable for large-scale application. As alternatives, essential oils, probiotics, and nano-silver particles have shown promise as less invasive and more efficient means of inhibiting *Vibrio* spp. contamination in *Artemia* spp. (Zheng et al., 2021; Fernandes et al., 2023). However, these approaches are often costly. Meanwhile, the use of aqueous ozone may be effective in controlling *V. parahaemolyticus,* but high concentrations must be applied to take effect (Feng et al., 2018). Therefore, more affordable and sustainable alternatives are needed for a long-term solution.

As *Artemia* spp. is a hypersaline organism, we hypothesize that high salinity (80-110 PSU) can inhibit *Vibrio* spp. proliferation while maintaining *A. franciscana* nauplii survival, providing a sustainable alternative to antibiotics in aquaculture hatcheries. Moreover, integrating the use of cheap antimicrobial alternatives, such as *Pandanus tectorius* leaf extract (PLE) (Andriani et al., 2019), serves as an efficient and suitable method of sterilization used to attenuate *Vibrio* spp. In this study, the effects of using high salinity exposure to suppress the *Vibrio* spp. load were examined in axenic and non-axenic *A. franciscana* culture systems. Decapsulated *A. franciscana* was hatched without bacterial interference, and the live *Vibrio* spp. were introduced into axenic *A. franciscana* reared under high salinity to assess the *Vibrio* spp. inhibition and *A. franciscana* survival. The outcomes generated from the axenic culture system were then tested in a non-axenic hatchery setup. The study outcomes provided insights into vibriosis control through a low-cost and environmental-friendly strategy.

Material and Method

Axenic and non-axenic Artemia franciscana cysts hatching. *A. franciscana* cysts originated from the Great Salt Lake, USA (Ocean Star International, INC). Axenic *A. franciscana* nauplii were hatched from *the* cysts following Sung et al., (2007); Baruah et al., (2009). The decapsulated cysts were then collected and rinsed thoroughly with filter-sterilized seawater. Subsequently, decapsulated cysts were transferred to a sterile 500 mL screw-cap bottle containing 300 mL of sterile seawater (~30 PSU). Then, cysts were allowed to hatch under filtered aeration and constant illumination (2000 lx), with the temperature controlled at 28°C for 28 h.

To simulate the hatchery operation, non-axenic *A. franciscana* nauplii were hatched from 0.5 g unprocessed cysts in 500 mL of non-sterile seawater (32-34 PSU) under constant illumination (2000 lx) and unfiltered aeration at 28°C for 28 h.

Vibrio spp. culture. Isolates of the bacterial strains, *V. campbellii* and *V. parahaemolyticus,* were revived from 20% glycerol stocks in respective Marine broth 2216 (Difco) by overnight incubation at 28 ℃ and agitation at 0.3 × g. Bacterial cells were harvested in 50 mL centrifugation tubes by spinning at 10,000 × g for 10 min. The supernatant was discarded, and the cell pellet was resuspended with sterile seawater. The bacterial cell density was measured by BioSpectrometer (Eppendorf) at 600 nm, and cell count was determined (Sung et al., 2007).

Experimental design. The axenic and non-axenic *A. franciscana* nauplii were used in the following experiments to test for the efficiency of high salinity in suppressing *Vibrio* spp. (Figure 1). All experiments were conducted in triplicate. The survival of *A. franciscana* nauplii was calculated following Tiong et al., (2024).

Experiment 1: Axenic Artemia franciscana* nauplii *exposed to Vibrio spp. A total of 30 axenic *A. franciscana* nauplii were transferred into a 50-mL centrifuge tube containing 30 mL of seawater at different salinities (30, 60, 70, 80, 90, 100, 110, 120, 130, 140, and 150 PSU, respectively). Each group of the nauplii was then exposed to a single *Vibrio* sp. (either *V. campbelli* or *V. parahaemolyticus*) with varying concentrations (10^7 and 10^8 cells mL^{-1}, separately for 48 h at 28°C with continuous illumination. Two control groups were included: one group was 10^7 cells mL^{-1} of *Vibrio* sp. in seawater without *A. franciscana* nauplii; the other one was axenic *A. franciscana* nauplii cultured in seawater without *Vibrio*. The conditions were maintained at 28°C with constant illumination (2000 lx) and aeration.

Experiment 2: Non-axenic Artemia franciscana* nauplii *cultured in seawater. Right after cysts hatched, the salinity of the hatching seawater, which contained nauplii and a high bacterial load (> 10^7 cells mL^{-1}) derived from the hatching process, was adjusted to 30, 50, and 60 to 200 PSU, respectively, without removing the cyst shells and unhatched cysts. In the separate run, *A. franciscana* nauplii were also cultured in the presence of 1 g L^{-1} *Pandanus tectorius* leaf extract, a condition known to effectively remove *Vibrios* in Penaeid shrimp culture system (Anirudhan et al., 2023). PLE was prepared by dissolving methanolic extract *of P. tectorius* leaves in dimethyl sulfoxide (DMSO). in a ratio of 2:3 (v/v), following Anirudhan et al., (2023). DMSO was tested in prior and is not lethal to either *A. franciscana* nauplii or *Vibrio* spp. The parameters for light, temperature and aeration remained the same as the axenic *A. franciscana* culture.

Vibrio spp. viability assay. The rearing water of each group was diluted (10×, 100×, and 1000×) with sterile seawater following Herigstad et al., (2001). A total of 50 uL of each diluted rearing water was then spread onto *Vibrio*-selective Thiosulfate Citrate Bile Sucrose (TCBS, GranuCult® prime) agar plates (López-Torres and Lizárraga-Partida 2001) and incubated overnight at 28°C. Colony-forming units (CFU) on the TCBS plates were calculated.

Vibrio spp. detection and identification. PCR was used to detect *Vibrio* spp. in non-axenic *A. franciscana* rearing water and nauplii. DNA extraction was conducted from *A. franciscana* rearing water, mixed TCBS-grown colonies, and 50 individuals of nauplii, by using the Wizard® Genomic DNA Purification Kit (Promega, USA), according to the manufacturer's instruction for gram-negative bacteria. *Vibrio*-specific primers used were VG C2694352 F46 (5'-GTC ARA TTG AAA ARC ART TYG GTA AAG G -3') and VG C2694352 R734 (5'-ACY TTR ATR CGN GTT TCR TTR CC -3') (Kim et al., 2015). The reaction mixture contained 12.5 µL of 2× MyTaq Mix (Bioline, UK), 1 µL of each 10 µM forward and reverse primer, and 18 ng of DNA template. and sterile double-distilled water to a final volume of 25 µL. PCR conditions were as follows: an initial denaturation at 95℃ for 5 min, followed by 25 cycles of 95℃ for 30 s, 52℃ for 30 s, 72℃ for 30 s, and a final extension at 72℃ for 10 min.

For further verification, 50 uL of axenic and non-axenic *A. franciscana* rearing water (salinity of 30 PSU) was spread on a TCBS agar plate after 48 h of culture. The TCBS plate was then incubated at 28°C overnight. Three and 14 single colonies were randomly selected

from axenic and non-axenic *A. franciscana* cultures for DNA extraction, as aforementioned. The V5-V6 region of the 16S rRNA gene sequence was amplified by using primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). The amplicon was then sent for Sanger sequencing by Apical Scientific (Selangor, Malaysia) on the BigDye® Terminator v3.1 Cycle Sequencing platform (Thermo Fisher Scientific). The 16S gene sequences were then queried against the NCBI nucleotide database through Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

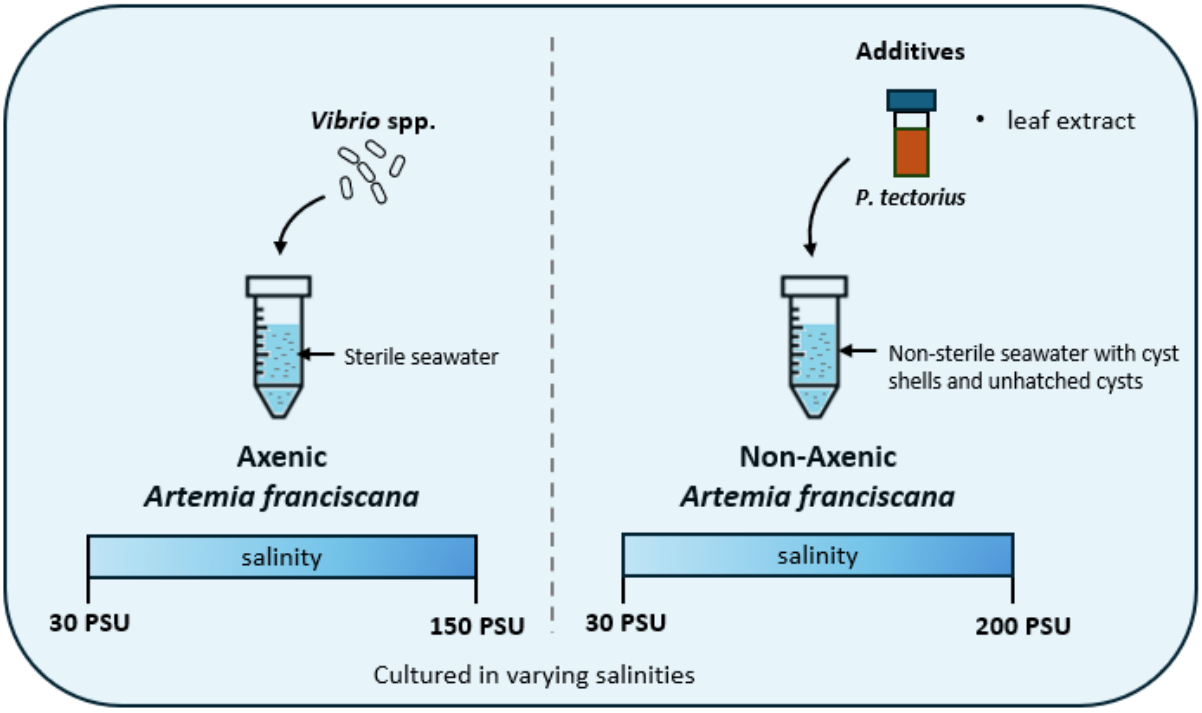

Figure 1. The experimental design. (Left) Decapsulated cysts were hatched and cultured in the presence of a single *Vibrio* sp. (*V. campbelli or V. parahaemolyticus*) with varying salinities. (Right) Non-treated *Artemia franciscana* cysts were hatched and cultured in the same flask under varying salinities with high bacterial load, which derived from the non-treated cysts' hatching process. In the separate run, *Pandanus tectorius* leaf extract was added into the non-axenic *Artemia franciscana* rearing water to examine the *Vibrio* spp. suppression effect.

Statistical analysis. All data were statistically analyzed in GraphPad Prism v9.5. Homogeneity of variances was assessed, then one-way ANOVA was used to compare differences among experimental groups. Significant difference was flagged by post-hoc Dunnett's test at $p < 0.05$.

Results

Artemia franciscana* nauplii *survival trend upon Vibrio sp. challenge in high salinity conditions. Axenic *A. franciscana* cultured in salinity ranging from 30 to 80 PSU were recorded with increasing survival (Figure 2a). The survival reduced when salinity reached 90 PSU, and the reducing trend continues with increasing salinity. By 120 PSU, *A. franciscana* survival dropped under 30% (Figure 2a).

The presence of *Vibrio* sp. affected the survival trend of axenic *A. franciscana* cultured in varying salinity. When cultured with 10^7 and 10^8 cells mL^{-1} *V. campbelli*, *A. franciscana* survival increased gradually when salinity increased from 30 PSU to 80 PSU (Figure 2a). Then, survival declined when salinity went above 90 PSU. At a salinity of 150 PSU, less than 30% of *A. franciscana* nauplii co-cultured with *V. campbelli* survived (Figure 2a).

Unlike axenic *A. franciscana* challenged with *V. campbelli*, axenic *A. franciscana* recorded higher survival upon incubation with *V. parahaemolyticus* at a salinity of 30 PSU (Figure 2a). The survival dropped more than 30% when salinity increased to 60 PSU (Figure

2a). Meanwhile, *V. parahaemolyticus* was more lethal to *A. franciscana* at moderate salinities. When challenged with 10^7 and 10^8 cells mL^{-1} of *V. parahaemolyticus*, *A. franciscana* survival elevated at salinities of 90 PSU and 80 PSU (Figure 2a). The survival of *A. franciscana* challenged with *V. parahaemolyticus* declined significantly when salinity exceeded 100 PSU, and survival was low at a salinity of 150 PSU (Figure 2a). Meanwhile, under non-axenic culture conditions, *A. franciscana* survived better under salinity varying from 30 PSU to 200 PSU (Figure 2b), with the highest and lowest survival recorded at salinities of 80 PSU and 200 PSU (Figure 2b).

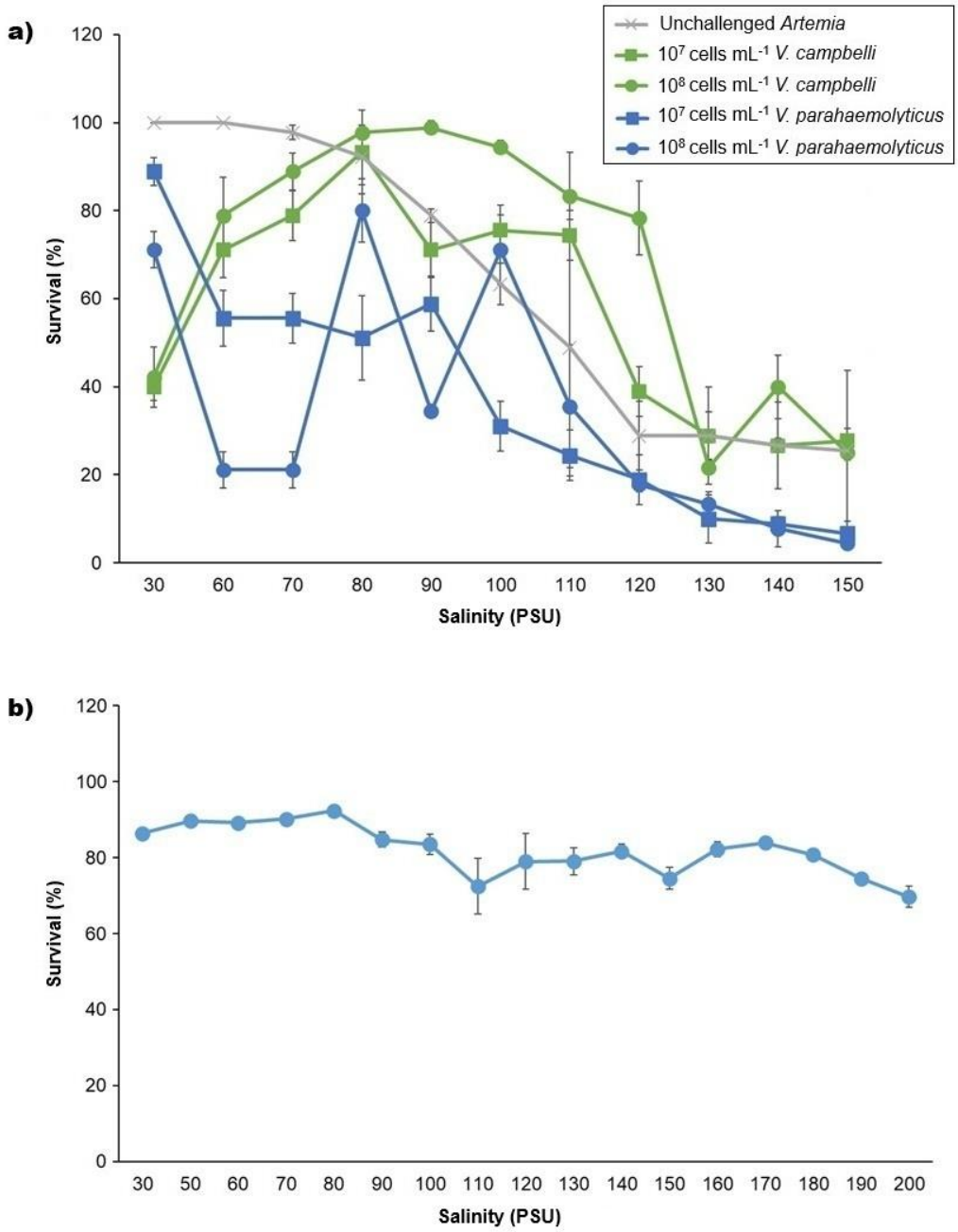

Figure 2. The *Artemia franciscana* nauplii survival when cultured under a) axenic and b) non-axenic conditions with varying salinities. Two concentrations (10^7 and 10^8 cells mL^{-1}) of *V. campbelli* and *V. parahaemolyticus* were inoculated into axenic rearing water. Non-axenic rearing water consisted of > 10^7 cells mL^{-1} of bacterial load, which derived from the non-treated cysts' hatching process. Experiment was conducted in triplicate. Data is presented in mean±SD.

High salinity attenuated Vibrio spp. proliferation in axenic Artemia franciscana culture. The abundance of live *V. campbellii* and *V. parahaemolyticus* in axenic *A. franciscana* rearing water with varying salinities was monitored on selective TCBS agar. In *A. franciscana* rearing water with an initial inoculation of 10^7 cells mL^{-1} of *V. campbelli*, high abundance of live *V. campbelli* was recorded after 48 h of culturing at 30 PSU of salinity (Table 1). The number reduced to an undetectable level when salinity went beyond 80 PSU (Table 1). When inoculated with 10^8 cells mL^{-1} of *V. campbelli*, the abundance was uncountable from 30 PSU to 70 PSU of salinity, and the number dropped at 80 PSU to an undetectable level when salinity reached 100 PSU and above (Table 1).

A similar trend was observed in axenic *A. franciscana* rearing water consisting of *V. parahaemolyticus* (Table 2). In rearing water with an initial number of 10^7 cells mL^{-1}, the abundance of live *V. parahaemolyticus* decreased to undetectable level at salinity above 60 PSU (Table 2). Rearing water inoculated with 10^8 *V. parahaemolyticus* still consisted of numerous live *V. parahaemolyticus* at salinity of 60 PSU, and by 90 PSU salinity, live *V. parahaemolyticus* dropped and was no longer viable at 110 PSU salinity (Table 2).

Table 1

Vibrio campbelli abundance on TCBS agar after cultured in varying salinities for 48 h. Treatments started with two initial *Vibrio campbelli* concentration, 1×10^7 and 1×10^8 cells mL^{-1}. Experiment was conducted in triplicate. Data is presented in mean±SD

Salinity (PSU)	*TCBS colony count (total CFU)*	
Initial inoculation (cells mL^{-1})	1×10^7	1×10^8
30	297±6	TNTC
60	186±14	TNTC
70	66±3	TNTC
80	TFTC	107±8
90	0	46±14
100	0	TFTC
110	0	0
120	0	0
130	0	0
140	0	0
150	0	0

Note: TNTC - too numerous to count; TFTC - too few to count; TCBS - thiosulfate-citrate-bile salts-sucrose.

Table 2

Vibrio parahaemolyticus abundance on TCBS agar after culturing in varying salinities for 48 h. Treatments started with two initial *Vibrio campbelli* concentrations, 1×10^7 and 1×10^8 cells mL^{-1}. Experiment was conducted in triplicate. Data is presented in mean±SD.

Salinity (PSU)	*TCBS colony count (CFU)*	
Initial inoculation (cells mL^{-1})	1×10^7	1×10^8
30	294±6	TNTC
60	167±10	283±10
70	TFTC	250±22
80	0	208±15
90	0	94±6
100	0	64±3
110	0	TFTC
120	0	0
130	0	0
140	0	0
150	0	0

Note: TNTC - too numerous to count; TFTC - too few to count; TCBS - thiosulfate-citrate-bile salts-sucrose.

Hatching of Artemia franciscana cysts promoted Vibrio spp. proliferation in the rearing water. Prior to the experiment, sterile and non-sterile seawater was plated on TCBS agar. Colony was absent, indicating the non-existence or undetectable level of *Vibrio* spp. Axenic *A. franciscana* rearing water inoculated with *V. parahaemolyticus* at 30 PSU salinity was spread on TCBS agar, and all colonies were sequenced. The colonies were 99.40% identical to *V. parahaemolyticus* (Table 3), indicating that no other *Vibrio* spp. contamination was present in the axenic *A. franciscana* rearing water.

Upon cyst hatching at 30 PSU salinity under non-axenic conditions, random colonies grown from cyst-hatching water on TCBS agar were collected and sequenced. The bacteria were identified as *V. mediterranei*, *V. paucivorans* and *V. tubiashii*, implying that these *Vibrio* spp. were likely derived from the *A. franciscana* cyst-hatching process (Table 3).

Table 3

Bacterial identification of colonies derived from axenic and non-axenic *Artemia franciscana* cultures. Random TCBS-selective colonies were sent for 16S sequencing and the sequences matched *Vibrio* spp. in GenBank database

Culture condition	*Sample no.*	*Salinity (PSU)*	*Bacterial identity*	*Similarity (%)*
Axenic	1	30	*Vibrio parahaemolyticus*	99.40
	2	30	*Vibrio parahaemolyticus*	99.40
	3	30	*Vibrio parahaemolyticus*	99.44
Non-axenic	1	30	*Vibrio mediterranei*	97.00
	2	30	*Vibrio tubiashii*	98.07
	3	30	*Vibrio mediterranei*	97.87
	4	30	*Vibrio mediterranei*	97.82
	5	30	*Vibrio mediterranei*	97.57
	6	30	*Vibrio mediterranei*	97.15
	7	30	*Vibrio mediterranei*	97.37
	8	30	*Vibrio mediterranei*	97.79
	9	30	*Vibrio mediterranei*	97.29
	10	30	*Vibrio aestivus*	98.31
	11	30	*Vibrio aestivus*	97.08
	12	30	*Vibrio mediterranei*	98.66
	13	30	*Vibrio mediterranei*	98.99
	14	30	*Vibrio mediterranei*	98.53

Pandanus tectorius leaf extract suppresses Vibrio spp. proliferation in non-axenic conditions. *A. franciscana* cysts were hatched, and the nauplii were cultured under non-axenic conditions with varying salinity. PCR results showed that salinity as high as 200 PSU failed to eliminate the presence of *Vibrio* spp. in *A. franciscana* rearing water, TCBS-grown colonies, and *A. franciscana* nauplii.

To examine if the *Vibrio* spp. growth in non-axenic conditions was affected by increasing levels of salinity, the abundance of *Vibrio* spp. in rearing water and *A. franciscana* nauplii was measured. At 50 PSU of salinity, the viable *Vibrio* spp. load in *A. franciscana* nauplii and rearing water were high in number. The numbers fluctuated along with increasing salinity, and by 100 PSU, the *Vibrio* spp. abundance reduced in *A. franciscana* nauplii and rearing water samples. When salinity increased beyond 110 PSU, *Vibrio* spp. were significantly suppressed in both rearing water and nauplii samples with < 20 CFU.

Since maintenance of rearing water with salinity above 110 PSU was commercially infeasible, PLE addition into rearing water was tested alongside high salinity to examine the *Vibrio* spp. inhibition strength. Findings showed that the addition of 1 g L^{-1} PLE into rearing water did not enhance the *Vibrio* spp. suppression effect when salinity was below 60 PSU. The *Vibrio* spp. inhibition effect was greater in PLE-added rearing water when salinity was 70 PSU and above, where the *Vibrio* spp. count was significantly lower than in rearing water without PLE. At 70 PSU of salinity, *Vibrio* spp. load in PLE-added and not PLE-added rearing water were inhibited. By increasing salinity to 100 PSU, only minimal *Vibrio*

spp. load was observed in PLE-added rearing water compared to that of rearing water without PLE.

Discussion Aquaculture systems are consistently threatened by pathogen infection, and antibiotic use has been the most common solution against aquaculture disease outbreaks (Bondad-Reantaso et al., 2023). The intensive use of antibiotics is disastrous to aquaculture systems, with serious environmental issues, such as pollution and the emergence of antimicrobial resistance strains (Wang et al., 2021). For example, the antibiotic use in Chinese freshwater aquaculture ponds has led to the widespread occurrence of numerous antibiotic resistance genes (Wang et al., 2021). Herein, we demonstrated an alternative (Figure 3) by using high salinity in rearing water to effectively eliminate common aquaculture pathogens, *Vibrio* spp.

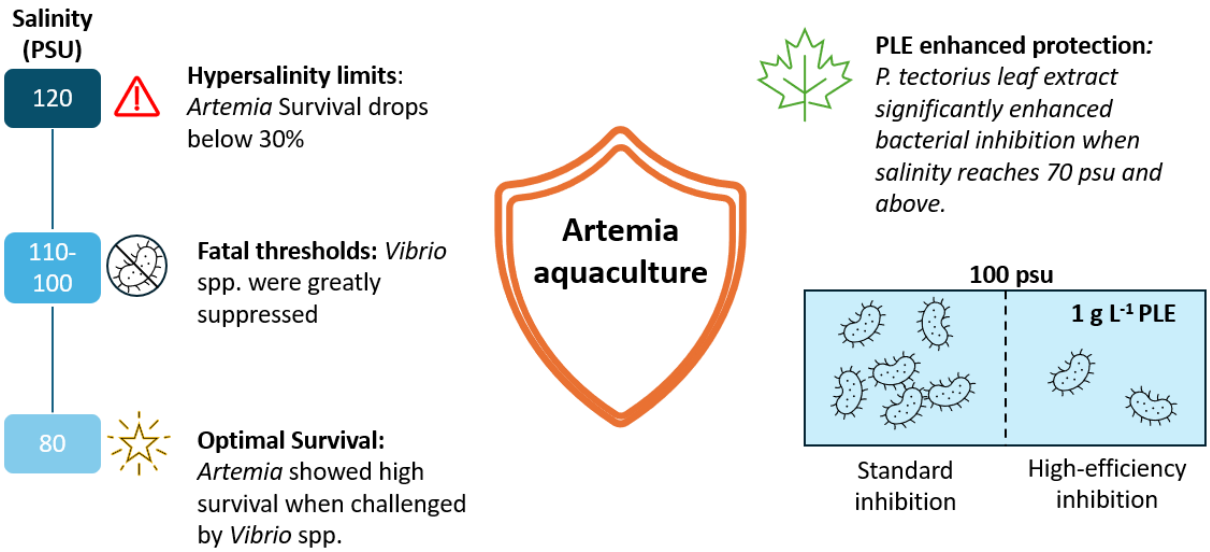

Figure 3. High salinity application in *Artemia* spp. aquaculture disease management.

Salinity efficiency. The hatching of *A. franciscana* nauplii from cysts usually occurs around 20 h, and they develop into the instar II stage by 8 h post-hatching (Støttrup & McEvoy 2003). Non-axenic cyst hatching is known to increase bacterial load in the production tank and release *Vibrio* spp., such as *V. mediterranei*, *V. paucivorans,* and *V. tubiashii,* as demonstrated in this study. Thus, FAO guidelines advise using disinfected or decaPSUlated *Artemia* spp. cysts before hatching or harvest and transfer hatched nauplii into new culture tanks to reduce the bacterial load (Van Stappen et al., 2024). Without these cyst pretreatment steps, use of the contaminated nauplii will severely affect hygiene and biosecurity in aquaculture. Moreover, as shown in this study, *A. franciscana* at the instar II stage that were reared at normal hatchery conditions (30 PSU of salinity) are susceptible to the *Vibrio* spp. infection, where both *Vibrio* sp. (10^8 cells mL^{-1}) led to at least 29% mortality. Thus, we propose the use of an optimum salinity level, which ensures high *A. franciscana* survival and concurrently inhibits the growth of *Vibrio* spp., is important in commercial *A. franciscana* production.

Although complete elimination of 10^8 cells mL^{-1} *V. campbelli* and *V. parahaemolyticus* was possible in rearing water with salinity exceeding 110 PSU, such a high level of salinity was also imposing negative impacts on *A. franciscana* nauplii, where our findings revealed that axenic *A. franciscana* survival began to decline from 100 PSU of salinity. It is commonly perceived that 30-40 PSU of salinity is optimal for *A. franciscana* hatching, and most hatcheries maintain this salinity level for the *A. franciscana* nauplii culture. Interestingly, our findings suggested that immediate transfer of newly hatched *A. franciscana* nauplii from 30-40 PSU to 80-100 PSU of salinity would greatly reduce the risk of *Vibrio* spp. infection, and the huge change in salinity level is not lethal for *A. franciscana* nauplii (Vos, 1979). Besides that, a salinity range between 80-100 PSU did not significantly

impact the reproductive traits of *Artemia* spp. (Browne & Wanigasekera, 2000); thus, it is applicable for cyst production facilities.

Differences between Vibrio spp. Compared to *V. campbelli*, the pathology of *V. parahaemolyticus* seems to be more threatening to *Artemia* hatcheries, as the present study showed great elimination of *V. parahaemolyticus* at 60-70 PSU of salinity was still able to cause 80% *A. franciscana* mortality. It is, in fact, one of the most lethal strains to shrimp culture, where total mortality was recorded within 24 h in *L. vannamei* that was cultured at 25 PSU of salinity (Choi et al., 2017). A previous study also showed that *V. parahaemolyticus* is capable of growing rapidly in *Artemia* spp. hatching tanks, penetrating the cyst cuticular shell, negatively impacting embryo hatching, and killing hatched nauplii (Quiroz-Guzmán et al., 2013). Thus, maintaining a high level of salinity (between 80 and 100 PSU) is mandatory in post-hatching *A. franciscana* nauplii culture to suppress the proliferation of *V. parahaemolyticus* and prevent the cross-contamination from feeding *Artemia* spp. to marine larvae.

Applicability of Pandanus tectorius leaf extract. Unlike the exogenous introduction of *V. campbelli* and *V. parahaemolyticus* in an axenic experiment, the *V. mediterranei*, *V. paucivorans* and *V. tubiashii* that derived from untreated *A. franciscana* cyst hatching are likely not harmful to *A. franciscana* nauplii; even a high bacterial load was observed in both rearing water and *A. franciscana* nauplii. However, both *V. mediterranei* and *V. tubiashii* are emerging aquaculture pathogens. On the Mediterranean coastline, *V. mediterranei* led to total mortality in bivalves (*Pinna nobilis*) (Prado et al., 2020; Andree et al., 2021), whereas *V. tubiashii* was long known for causing mortality in larval shellfish facilities (Richards et al., 2015, 2021). Therefore, to reduce the risk of *Vibrio* spp. cross-contamination in facilities using *Artemia* spp. as live feed, our study proposed a better option without cyst pre-treatment and nauplii post-hatching tank transfer that was suggested by FAO (Van Stappen et al., 2024). The findings showed that high salinity can effectively control the bacterial load without the need to transfer hatched nauplii into a new culture tank. Although previous studies tested controlled temperature and florfenicol to contain the *V. mediterranei* outbreak (Prado et al., 2020), the addition of PLE alongside high salinity in *Artemia* spp. production in this study demonstrated significant suppression of bacterial growth in rearing water with possibly lower operational costs and minimal chemical use, as PLE was proven to exhibit antibacterial and immunoenhancing properties (Andriani et al., 2019; Patabandi et al., 2025).

Implications for hatcheries. Taking into consideration the results of the conducted study, salinity between 70 and 80 PSU can be considered a successful basis for *A. franciscana* production. Even though the high level of salinity (200 PSU) was not sufficient to kill all bacteria coming from the cyst-hatching process, the combination of high salinity and natural antimicrobial agents, which were used in this research, resulted in highly successful bactericide activity. In addition, the bacteria remained at a relatively low level without any pretreatment of cysts or movement of nauplii to the new water after hatching, hence reducing considerably both the amount of work involved and expenses. It should also be emphasized that compared to antibiotics, this method is more eco-friendly since there will be no chemical contamination.

Limitations and future directions. The proposed strategy is effective for the application during small-scale *A. franciscana culture*. Efforts to develop large-scale application protocols are required in the future.

Conclusions. The present study demonstrated that elevated salinity is an effective approach for suppressing *Vibrio* spp. proliferation while maintaining high survival of *A. franciscana* nauplii. A salinity range of 70-80 PSU provided a practical balance between nauplii performance and bacterial control, whereas the addition of *Pandanus tectorius* leaf extract further enhanced bacterial suppression under non-axenic culture conditions. This combined strategy may reduce the need for cyst pretreatment, post-hatch transfer, and

antibiotic use, thereby improving biosecurity and sustainability in Artemia production. Although the experiments were conducted under laboratory conditions, the findings provide a useful basis for developing practical disease management protocols for commercial hatcheries. Further studies are required to validate the effectiveness and economic feasibility of this approach under large-scale production conditions.

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Conflict of Interest. The authors declare that there is no conflict of interest.

Data Availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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