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Induction of a viable but nonculturable state in *Vibrio parahaemolyticus* by a high concentration of salt and its impact on fatty acid composition profile and membrane potential

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ABSTRACT

The study aimed to investigate the effects of a high concentration of salt on the induction of a viable but nonculturable (VBNC) state in *V. parahaemolyticus* and its impact on fatty acid (FA) composition profile and membrane potential during the persistence of a VBNC state. When three *V. parahaemolyticus* strains were incubated in artificial sew water (ASW) microcosms containing maximally 30% salt at 4 °C, these bacteria became uncultivable within 50~70 d. On day 100, the viable numbers of *V. parahaemolyticus* that maintained its membrane integrity were ≥ 6.0 log CFU/slide in ASW microcosms stored at 4 °C, implying that a high concentration of salt can be an inducer causing the phase transition of a VBNC state in *V. parahaemolyticus*. Especially, there was a strong correlation between increased saturated FA proportion and decreased membrane potential (as determined by using *N*-phenyl-*1*-napthylamine and propidium iodide probes) during the persistence of a VBNC state in *V. parahaemolyticus*, indicating that VBNC cells had increasingly permeable membrane properties. Knowledge on the characteristics of VBNC cells may provide better understanding of the ecology of bacteria, as well as their survival mechanisms.

1. Introduction

Vibrio parahaemolyticus has been found in marine environments and readily isolated from a wide variety of raw aquatic products during warmer months, when the incidence of food-borne diseases and illnesses is the highest (Wong, Shen, Chang, Lee, & Oliver, 2004; Wong, Wang, Chen, & Chiu, 2004; Yu et al., 2013; Yue, Liu, Xiang, & Jia, 2010). Consumption of raw marine products contaminated with *V. parahaemolyticus* results in multiple clinical symptoms, ranging from acute abdominal pain, vomiting, and nausea to septicemia (Piñeyro et al., 2010). Especially, human-pathogenic bacteria, such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, are known to enter into a viable but nonculturable (VBNC) state upon exposure to citral, copper, CO_2, refrigeration, or starvation (Ayibieke, Nishiyama, Senoh, & Hamabata, 2023; Hung, Jane, & Wong, 2013; Luo et al., 2024; Oliver, 1995; Ramesh, Sathiyamurthy, Meganathan, & Athmanathan, 2024; Wagley, 2023; Zhang et al., 2015; Zhang et al., 2023). In this nonculturable but metabolically active and viable state, VBNC bacteria fail to grow on routine media on which they normally proliferate and represent a specific modification in cellular membrane ultrastructure (Brenzinger et al., 2019; Cai, Liu, Li, Wong, & An, 2021; Chaiyanan et al., 2007; Xu, Zhu, Sheng, Tang, & Zhang, 2024), membrane fatty acid (FA) composition profile (Jia et al., 2014; Pazos-Rojas et al., 2024), and RNA/DNA or protein synthesis (Asakura et al., 2007; Cheng et al., 2023; İzgördü, Gurbanov, & Darcan, 2024), while the cytoplasmic membrane remains integrated in response to adverse stressful conditions. Furthermore, recent studies revealed the involvement of increased salt amendment/supplementation in earlier induction of a VBNC state in gram-negative bacteria during nutrient deficiency incorporated with refrigerated temperature (NIR) (Song & Lee, 2021; Yoon, Bae, & Lee, 2017; Yoon et al., 2021; Yoon, Moon, Choi, Ryu, & Lee, 2019; Zhao et al., 2024). As food-borne pathogenic bacteria can be induced into a VBNC state, but undergo a resuscitation process from a VBNC state to an actively metabolizing state in a favorable condition where provides rich nutrients to encourage their biological metabolism functions (Alam et al., 2007; Fu et al., 2020; Hu et al., 2024; Liu, Yang, Kjellerup, & Xu, 2023; Oliveira, de Almeida, Baglinière, de Oliveira, & Vanetti, 2021; Oliver, 2000; Oliver & Bockian, 1995), the emergence of VBNC *V.*

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parahaemolyticus in salted foods would pose serious risks to public health and food safety.

To date, as well documented by Pazos-Rojas et al. (2024) and Zhang et al. (2023), a substantial portion of microbial species (more than 100 microorganisms) can exhibit the VBNC phenomenon when faced with harsh external environments. Previously, several studies (Song & Lee, 2021; Zhao et al., 2024) have been undertaken to investigate the dynamic change and the ability to food-borne pathogenic bacteria, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* serovar Enteritidis, to enter a VBNC state driven by exposing these bacteria to different concentrations of salt (10, 20 or 30%) incorporated with freezing (− 20 °C) or refrigeration (4 °C). However, little is known regarding a high concentration of salt, which is commonly used to inhibit the growth of spoilage and/or pathogenic microorganisms, on the entry of a VBNC state in *V. parahaemolyticus* and biochemical characterization of the resultant cells with regard to the concomitant modulation of FA composition and membrane potential in VBNC cells. Despite the fact that salt has been shown to have a broad spectrum of growth-inhibiting activities against microorganisms, there is no systematic study on whether it can induce *V. parahaemolyticus* persisted under a NIR-inducible condition to form a VBNC state.

In the present study, three strains of *V. parahaemolyticus* were incubated in artificial sea water (ASW) microcosms, which were amended with up to 30% salt and adjusted to pH 6.0 using lactic acid (LA), at 4 °C until induced into a VBNC state. Once upon entering a VBNC state, the resultant cells were analyzed in terms of total FA composition profile and membrane potential with N-phenyl-1-napthylamine (NPN) and propidium iodide (PI) probes. Importantly, this study highlights that i)*V. parahaemolyticus* became VBNC when persisted in ASW microcosms of high salt concentrations (≤ 30%) at 4 °C for 7–60 d, which was in parallel with the mostly green (SYTO9®)-fluoresced cells of this bacterium (otherwise, elevated salt concentrations can be an inducer not only causing the phase transition of a VBNC state in *V. parahaemolyticus*, but also making this bacterium more prone to enter a VBNC state under NIR; ii) there was a strong correlation between increased saturated FA proportion and decreased membrane potential during the persistence of a VBNC state in *V. parahaemolyticus*; iii)a transmission electron microscopy (TEM) assay revealed that the formation of an irregular cell morphology (from a common arc to ellipsoidal or spherical shapes) of VBNC cells would be due to cytoplasmic condensation and cell wall deformation, leading to limit their exchange of substances through the specific cell surface areas with the external environments which in turn would minimize the related energy-consuming metabolic activities enabling VBNC cells to maintain the basic need for survival and adaptation. Exploring cellular properties of *V. parahaemolyticus* upon entering a VBNC state by a high concentration of salt will offer a new insight for better understanding the ecology of this bacterium, as well as its survival mechanisms. Especially, the data on the appropriate concentration ranges of salt to which food-borne pathogenic bacteria can be induced into a VBNC state are very limited. Our findings may assist the food industry with the establishment of appropriate control measures that ensure the microbiological safety of foods with an intermediate or high amount of salt.

2. Materials and methods

2.1. Preparation of microcosm and inoculation

ASW was prepared by dissolving 30 g of sea salt powder (Sigma-Aldrich® Co., St Louis, MO, USA) in 1 L of distilled water according to the instruction provided by the manufacturer. The formal ASW fluids (pH 8.0) were amended with 5% (5P-ASW), 10% (10P-ASW) or 30% (30P-ASW) salt, and the ASW microcosms with or without salt were autoclaved at 120 °C for 20 min. After cooled down in a laminar biosafety hood at 25 °C for 6 h, all the microcosms, including ASW, 5P-ASW, 10P-ASW, and 30P-ASW were further adjusted to pH 6.0 using 0.2-μm membrane-filtered LA (Daejung Co. Ltd., Siheung-si, Republic of Korea).

V. parahaemolyticus ATCC 17802, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon-si, Republic of Korea). The stocks were maintained at − 75 °C and activated in tryptic soy broth (TSB; Difco® Laboratories Inc., Detroit, MI, USA) added with 3% salt (TSBS) at 37 °C for 24 h. The cells of *V. parahaemolyticus* in the stationary growth phase were harvested by centrifugation at 10,000 × *g* for 3 min at 4 °C, washed thrice in 0.1 M phosphate buffered saline (PBS; pH 7.0), and the final pellets were resuspended in 1 mL of ASW (pH 8.0) without LA, corresponding to approximately $10^{8\sim9}$ CFU/mL. The bacterial suspensions were inoculated in the formulated microcosms (pH 6.0), including ASW, 5P-ASW, 10P-ASW, and 30P-ASW. The ASW microcosms were kept at 4 °C until culturable counts of *V. parahaemolyticus* decreased to below the detection limits (⟨ 1.00 log CFU/mL).

2.2. Enumeration

V. parahaemolyticus was plating-counted on tryptic soy agar (TSA; Difco® Laboratories Inc.) supplemented with 3% salt (TSAS). Decimal dilutions were prepared in alkaline peptone water (APW; Difco® Laboratories Inc.) consisting of 10 g/L of peptone and 10 g/L of salt. Then, the 100-μL-aliquots were spread on TSAS, followed by 24 h of incubation at 37 °C. Colonies of *V. parahaemolyticus* developed on TSAS were quantified to determine the culturable counts of this bacterium.

Total and viable numbers of *V. parahaemolyticus* were measured via Live/Dead® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific™, Inc., Waltham, MA, USA) comprising two fluorescent probes, SYTO9® and PI. Briefly, an equal volume of SYTO9® and PI was combined in a sterile microtube, and 3 μL of this mixture was added to 1 mL of each of the bacterial solutions. After 15 min of incubation at 25 °C in the dark, 5–8 μL of the suspension was attached on a sterile glass slide. Microscopic images of *V. parahaemolyticus* before and after 100 d of incubation in ASW microcosms stored at 4 °C were demonstrated using a TE 2000-U electron-fluorescence microscope (Nikon, Inc., Tokyo, Japan).

2.3. FA composition profile

After 90 d of incubation in ASW microcosms stored at 4 °C, *V. parahaemolyticus* ATCC 17802 used in Fig. 1 was further collected by centrifugation at 15,000 × *g* for 3 min at 4 °C, washed thrice in 0.1 M PBS, and resuspended in 5 mL of TSBS, following 7 d of enrichment at 25 °C. Then, the turbid culture was plated on TSAS, and we confirmed the formation of *V. parahaemolyticus* colonies as identified by using the API 20E diagnostic kit (bioMérieux, Inc., Marcy l'Etoile, France) at 99.9% similarity rates.

FA analysis was conducted according to the standard protocol provided by the Microbial Identification System (MIDI®; Microbial ID Inc., Newark, Del, USA). The 90-d-old cells of *V. parahaemolyticus* were withdrawn from a low temperature incubator at a given temperature (4 °C), harvested by centrifugation at 15,000 × *g* for 3 min at 4 °C, and underwent saponification, methylation, and extraction of carboxylic acid derivatives from long chained aliphatic molecules. The extracted lipid content was analyzed sing gas chromatography and further identified at the TSBA6 database available at the MIDI® system.

2.4. Membrane potential measurement

The permeabilizing ability of the cell envelopes of *V. parahaemolyticus* was measured using NPN and PI probes as described previously by Hyun, Choi, & Lee (2020) with some modifications. Before and after 30 d of incubation in ASW microcosms stored at 4 °C, 1 mL of the bacterial solutions were centrifugated at 15,000 × *g* for 3 min at 4 °C, washed twice in *4*-(*2*-hydroxyethyl)-*1*-piperazineethanesulfonic acid

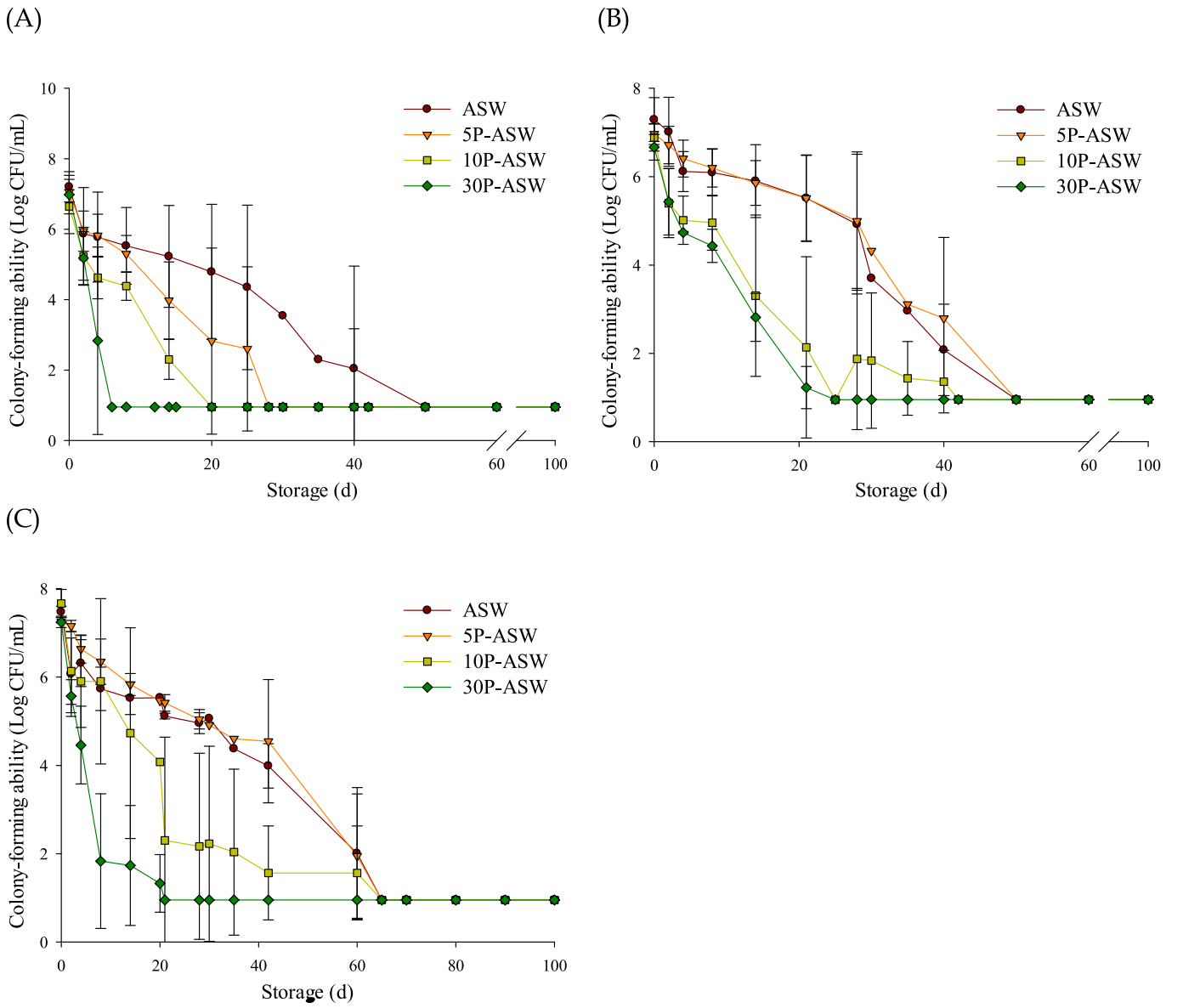

Fig. 1. Change in the colony-forming ability of (A) *V. parahaemolyticus* ATCC 17802, (B) *V. parahaemolyticus* ATCC 33844, and (C) *V. parahaemolyticus* ATCC 27969 in ASW (●), 5P-ASW (▼), 10P-ASW (■), and 30P-ASW (◆) stored at 4 °C.

(HEPES; Thermo Fisher Scientific™, Inc.), and resuspended in 2 mL of HEPES containing 10 μM NPN. The background fluorescence was recorded using a Gemini XPS spectrophotometer (Molecular Devices, Inc., CA, USA). Excitation or emission wavelength was adjusted to 350 or 420 nm. The cultures of *V. parahaemolyticus* grown in TSBS at 37 °C overnight were harvested by centrifugation at 15,000 × *g* for 3 min at 4 °C, washed thrice in HEPES, and resuspended in 2 mL HEPES containing 50 μg/ml polymyxin B (Pol-B; Sigma-Aldrich® Co.), following 15 min of incubation at 25 °C in the dark. The Pol-B-treated cells were used as negative control groups to compare the outer membrane (OM)-permeabilizing properties with those cells of *V. parahaemolyticus* persisting in ASW, 5P-ASW, 10P-ASW or 30P-ASW stored at 4 °C for 30 d.

Briefly, a 1.5 mM PI stock (Sigma-Aldrich® Co.) was dissolved in sterile deionized water, corresponding to 30 μM/mL, and stored at 4 °C for 15 min in the dark prior to its use. One mL of 30 μM/mL PI was added to the collected pellet in a microtube at its final concentration of 15 μM. Untreated cells were used as a negative control. After 15 min in the dark, each of the samples was washed twice in 0.1 M PBS to remove any residual dye. Finally, fluorescence was measured using a Gemini XPS spectrophotometer (excitation: 485 nm; emission: 635 nm) to determine the inner membrane (IM) properties of *V. parahaemolyticus* after the evolution of a VBNC state as follows:

PI uptake rates (%) = [(*Fluorescence of PI − stained cells suspended in a buffer*) − (*Fluorescence of cells in a buffer*)]/[(*Fluorescence of a buffer containing PI*) − (*Fluorescence of a buffer without PI*)] × 100

2.5. A TEM assay

V. parahaemolyticus cells either grown in TSBS at 37 °C for 24 h or persisted in ASW and 5P-ASW at 4 °C for 100 d were centrifugated at 12,000×*g* for 3 min, rinsed in 0.1 M PBS (pH 7.0) three times, and were resuspended in 0.1 M PBS. The cell fluids then were prefixed in 2% paraformaldehyde overnight at 4 °C. Each of the bacterial solutions was washed in 0.1 M PBS, postfixed in 1% osmium tetroxide, and were serially dehydrated by 30%, 50%, 70%, 95%, and 100% ethanol solutions, following the infiltration with 2 mL of epoxy resin. Polymerization of the resins was performed at 60 °C for 24 h. The resins were cut (section: approximately 120 nm thickness) and were photographed with a JEOL JEM 1200 EX transmission electron microscope (JEOL USA Inc.,

Peabody, MA, USA).

2.6. Data analysis

The obtained results were expressed as the mean ± standard deviation. Significant ($p < 0.05$) differences among the groups were determined using analysis of variance (ANOVA) with Duncan's multiple range test (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Measurement of culturable count and membrane integrity

Fig. 1 shows the culturable cell count (log CFU/mL) of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 incubated in ASW microcosms amended with different concentrations of salt (pH 6.0) at 4 °C. Initial loads of *V. parahaemolyticus* were between 6.1–7.8 log CFU/mL. *V. parahaemolyticus* ATCC 17802 declined by approximately 6.0 log CFU/mL when incubated in ASW and 5P-ASW at 4 °C for up to 40 d, and this bacterium further dropped below the detection limits within 50 d. *V. parahaemolyticus* ATCC 17802 was undetectable in ASW, 5P-ASW, 10P-ASW, and 30P-ASW, following 50, 28, 20, and 12 d of incubation at 4 °C, respectively. During the first 20 d of incubation at 4 °C, the culturable cell number of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 ranged from 4.4 log CFU/mL to 5.9 log CFU/mL in ASW microcosms containing ≤ 10% salt and remained constantly culturable at levels of 2.2–4.6 log CFU/mL until day 40. By contrast, *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 became uncultivable in 30P-ASW after 21 d of incubation at 4 °C and then decreased at the undetectable levels in all microcosms within 50 or 65 d. A cell membrane integrity can be measured using an epifluorescence microscopy with membrane-permeabilizing probes SYTO9® and PI (Liao, Jiang, & Zhang, 2018). Herein, the membrane integrity of three *V. parahaemolyticus* strains persisting in ASW microcosms stored at 4 °C for 100 d was measured in the Supplementary Fig. S1. The cell numbers with intact membranes were stable in ASW microcosms stored at 4 °C for over three months, yielding more than 5.9–6.5 log CFU/slide on day 100, particularly irrespective of the amendment of the microcosms with salt.

3.2. FA composition profile

Fig. 2 represents the total membrane FA composition profiles (%) of *V. parahaemolyticus* ATCC 17802 before and after 90 d of incubation in ASW microcosms (pH 6.0) stored at 4 °C. Palmitic acid and palmitoleic acid were the most abundant in *V. parahaemolyticus* cells grown overnight in TSBS at 37 °C. The FA composition ratios of palmitic acid were 28.7%, 23.9%, 23.4%, and 23.1% in *V. parahaemolyticus* exposed to 90 d of NIR in ASW, 5P-ASW, 10P-ASW, and 30P-ASW, respectively, at 4 °C. Among the SFAs identified, an increase in the levels of lauric acid, 2-hydroxylauric acid, and myristic acid was observed in VBNC *V. parahaemolyticus*, accounting for 14.4–16.1% of the total FA composition contents higher than those (10.9%) of its actively grown counterpart. Particularly, the pure culture possessed 26.8% of palmitoleic acid out of the total FA composition proportion, whereas VBNC cells exhibited increased contents of palmitoleic acid, ranging from 33.0% to 35.8%, in ASW microcosms after 90 d. By contrast, there were some decreases in the *cis*-vaccenic acid content of VBNC cells exposed to ASW, 5P-ASW, 10P-ASW, and 30P-ASW by 2.6%, 5.7%, 6.5%, and 7.4%, respectively. Some FAs, such as *3*-hydroxy-*9*-methyldecanoic acid, cetyl alcohol, and *cis-11*-palmitoleic acid, were newly detected from the 90-d-old cells of *V. parahaemolyticus* in ASW microcosms amended with more than 10% salt at 4 °C. This study revealed that the amount of palmitic acid, (*7Z*)-*13*-methyl-*7*-hexadecenoic acid, and *cis*-vaccenic acid had a positive correlation with the decreasing amendment of microcosms with salt. A PCA analysis revealed that the evolution of a VBNC state in *V. parahaemolyticus* might be strongly involved in a considerable increase in the proportions of palmitoleic acid, followed by *2*-hydroxylauric acid, myristic acid, lauric acid, and *cis-10*-palmitoleic acid, and the FA profile obtained from ASW clearly differed from that of 5P-ASW, 10P-ASW, and 30P-ASW (Fig. 3).

3.3. Membrane potential

Membrane potential, such as NPN and PI, of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 before and after 100 d of incubation in ASW microcosms stored at 4 °C is measured in Fig. 4. After 30 d, *V. parahaemolyticus* ATCC 17802 exerted increased fluorescence intensities between 1769 and 1875 when incubated in microcosms amended with more than 5% salt as compared with those of the actively grown counterpart. All *V. parahaemolyticus* strains yielded the highest NPN uptake capacity of 1875∼2643 RFU in 10P-ASW after 30 d. PI uptake values of the 30-d-old cells of *V. parahaemolyticus* were significantly ($p < 0.05$) lower than that of the negative controls. *V. parahaemolyticus* ATCC 17802 and *V. parahaemolyticus* ATCC 33844 had decreased PI uptake values with increasing salt concentrations. By contrast, the PI uptake intensity significantly ($p < 0.05$) increased with the increasing salt concentration of ASW microcosms in *V. parahaemolyticus* ATCC 27969, with the exception of 30P-ASW.

3.4. Morphological changes of VBNC cells

The pure cultures of *V. parahaemolyticus* ATCC 17802 were filled with lots of granules in cytoplasm and their cell membranes were shown to become intact without minor damages (Fig. 5A). By contrast, VBNC *V. parahaemolyticus* ATCC 17802 cells had the less organized cytoplasmic layers. Particularly, cell membrane of VBNC *V. parahaemolyticus* was largely loosened, with the generation of empty gaps between the inner and the outer membranes (Fig. 5B and C). Importantly, *V. parahaemolyticus* cells acquired the aberrantly-shaped coccal morphologies after the entry into the VBNC state.

4. Discussion

In the present study, while 100 d of NIR resulted in the inability of *V. parahaemolyticus* to grow, the viable cell numbers with intact membranes were consistently stable over several months. Particularly, *V. parahaemolyticus* was induced into a VBNC state in ASW microcosms (pH 6.0) amended with a high concentration of salt at 4 °C within 21 d and persisted for 150 d in a NIR condition. Generally, the addition of salt in acidic and/or acidified foods is known to inhibit the growth of undesirable microorganisms that have a negative impact on the safety and quality of foods, the results obtained from this study indicate that salt can be an important determinant that induces and/or accelerates the evolution of a VBNC state in *V. parahaemolyticus* cells. However, there may be a matter of debate whether *V. parahaemolyticus* cells would be still and truly alive in ASW microcosms containing 5, 10 or 30% salt for more than 100 d at 4 °C. *V. parahaemolyticus* is a moderate halophilic, with its optimal growth at 3% salt (Kalburge, Whitaker, & Boyd, 2014), and some strains can grow at 9.6% salt (Miles, Ross, Olley, & McMeekin, 1997). Alam et al. (2007) determined the effects of NIR on the viability of VBNC *Vibrio cholerae* O1. Consequently, *V. cholerae* persisted in a VBNC state for 495 d during NIR, and the VBNC cells of *V. cholerae* within biofilms were recoverable through an animal passage challenge even after subjected to a NIR stress for more than one year. The ability to resuscitate from a VBNC state would be one possible explanation for measuring the viability of VBNC cells. In this sense, we demonstrated that the 150-d-old cells of *V. parahaemolyticus* were reverted to a culturable state, followed by a temperature upshift method using a formulated resuscitation-promoting buffer (pH 8.0) composed of 3%

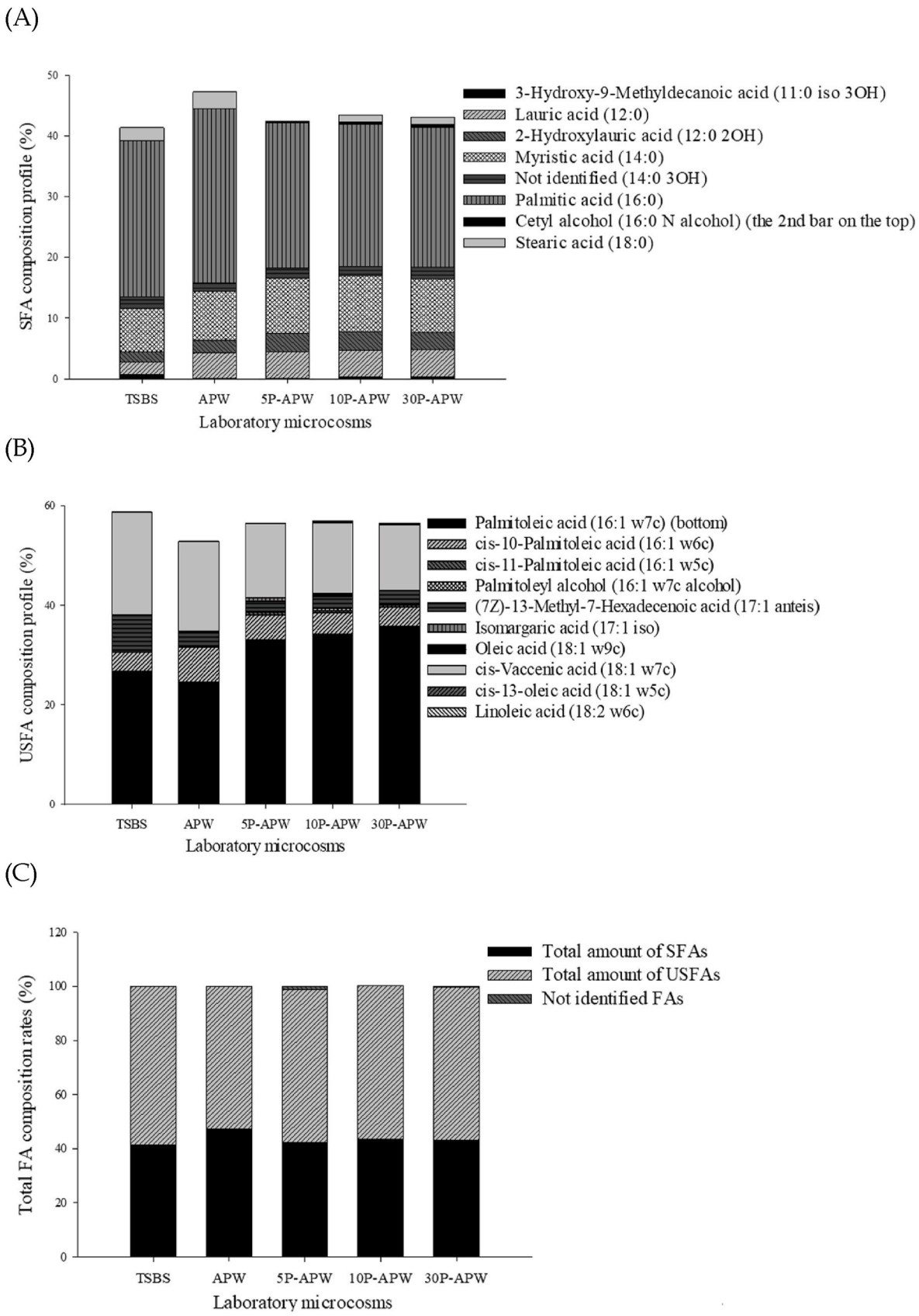

Fig. 2. Comparison of membrane FA composition profiles (%) of *V. parahaemolyticus* ATCC 17802 before and after 90 d of incubation in ASW microcosms stored at 4 °C {(A), SFA composition profile; (B), USFA composition profile; (C), total FA composition rates}. The overnight cultures of *V. parahaemolyticus* grown in TSBS at 37 °C were used as control groups in this study.

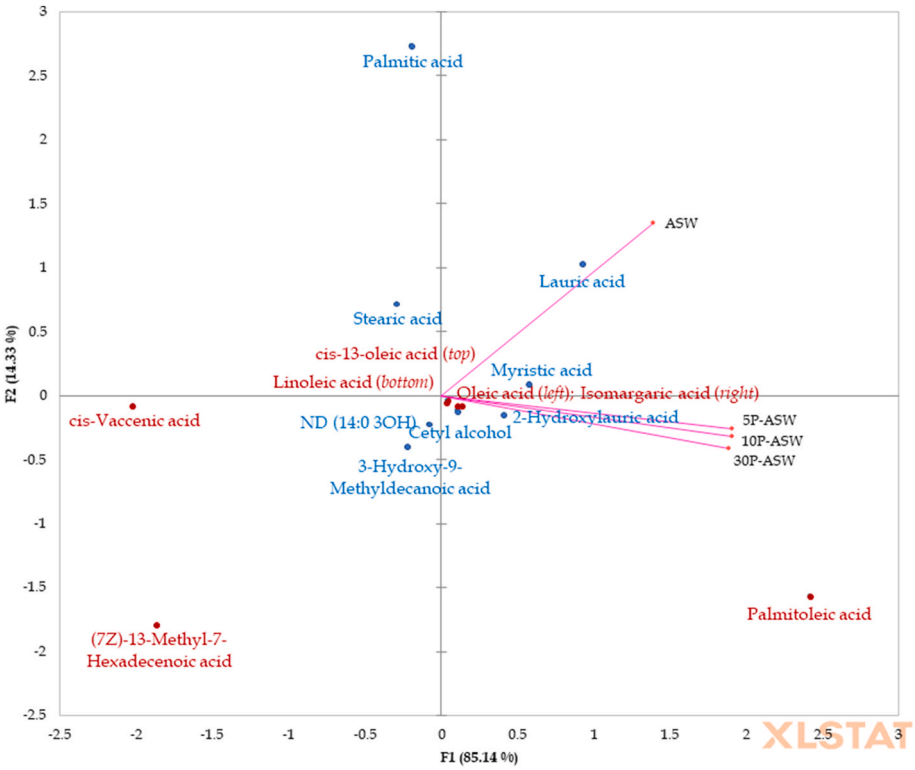

Fig. 3. PCA analysis of the factors affecting the modification of the membrane FA composition profiles in *V. parahaemolyticus* ATCC 17802 incubated in ASW microcosms at 4 °C for 90 d. The multivariable data (n = 72) differentiated all FAs on the relatively increased ratios $\{(A_{after} - A_{before}) \times 100\}$ obtained from each ASW microcosm (A_{before}, the ratio of a FA obtained from *V. parahaemolyticus* grown in TSBS; A_{after}, the ratio of a FA obtained from this bacterium persisting in ASW, 5P-ASW, 10P-ASW or 30P-ASW at 4 °C for 90 d), and their plot-scores were analyzed by means of PCA using XLSTAT 2021 program (Addinsoft Corporation, NY, USA).

salt, 10,000 U/mg catalase, 2% sodium pyruvate, 20 mM $MgSO_4$, 5 mM EDTA, and a cell-free supernatant derived from the stationary phase-grown cells of *V. parahaemolyticus* ATCC 17802 (Yoon et al., 2021). Accordingly, it was found that *V. parahaemolyticus* was able to enter a VBNC state under a NIR condition, allowing VBNC cells to retain their membrane structure and integrity consistently. Until now, although a number of previous studies were undertaken to investigate the phase transition of microorganisms into a VBNC state as significantly affected by a variety of stressful conditions, there is still insufficient information to determine whether the combination of two or more stressful factors would facilitate the formation of a VBNC state in food-borne pathogenic bacteria. So far, further studies should be necessary to ensure the accurate and effective identification of VBNC bacteria, as well as their pathogenic potentials.

As shown in Fig. 1, the considerable variability was noted in the time periods that it took three *V. parahaemolyticus* strains to reach a VBNC state under the same conditions. In the cases of *V. parahaemolyticus* ATCC 17802 lasting in ASW, the time required for this bacterium to enter a VBNC state largely varied, ranging from 40 d to 60 d even under the same NIR-inducible condition; after repeating the experiment three times, *V. parahaemolyticus* ATCC 27969 was capable of entering a VBNC state in ASW stored at 4 °C for 40, 50 or 60 d (Fig. 1A). Similarly, there was a significant variation (approximately at least one or more months) in the time periods needed to induce a VBNC state in *V. cholerae* O139 between the duplicate experiments repeatedly conducted under the same NIR condition (Bates & Oliver, 2004; Oliver, 2000; Sung, Chen, Shih, & Hsu, 2006; Yoon & Lee, 2019), which is in agreement with our findings. Although the resultant phenomenon remains unclarified, the colony-forming capability of *V. parahaemolyticus* would be highly sensitive to various indigenous factors (such as those found in solid agar plates), including the amount/level of salt or pH, the presence of oxygen, the formation of oxidative agents, and others, during the cultivation process. Particularly, Oliver (2000) observed a nonconsistent decrease in the platable counts of the same *Vibrio* sp. exposed to the same NIR stress, suggesting that such a variation regarding the time periods required for pathogenic bacteria to enter a VBNC state would be markedly affected by the physiological age of inoculums and the salt content of culture media.

The 90-d-old *V. parahaemolyticus* had increased total SFA contents in the levels of 42.4–47.2. Similarly, lauric acid, myristic acid, pentadecanoic acid, and palmitic acid were found to be largely increased in *V. parahaemolyticus* ST550 induced into a VBNC state by 35 d of starvation in minimum mineral salt (MMS) stored at 4 °C (Wong, Wang, et al., 2004). Among the total SFA composition profile, Jia et al. (2014) also observed a clear increase of decanoic acid, tridecanoic acid, and myristic acid in several *V. parahaemolyticus* strains of food origin persisting at 4 °C for 30 d. As supported by Chiang, Wu, & Chen (2014), adaptation of *V. parahaemolyticus* to pH 5.5 for 1.5 h resulted in a significant increase in the ratio of SFA/unsaturated fatty acid (USFA), implying that the microcosms acidified to pH 6.0 by using LA could be partially linked to the increased proportion of SFAs in VBNC *V. parahaemolyticus*. In addition, an increase in the amount of palmitic acid and stearic acid was shown to be involved in an increasing membrane rigidity in *V. parahaemolyticus* cells (Danevčič, Rilfors, Štrancar, Lindblom, & Stopar, 2005). Gram-negative bacteria typically alter their membrane fluidity with significant changes in the ratio of SFA to USFA, the levels of cyclopropane fatty acid, and *cis/trans* isomerization in response to external environmental conditions (Yoon et al., 2021).

In this study, the increases in the fluorescence unit due to the partitioning of the NPN probe into OM were observed in the 100-d-old *V. parahaemolyticus*; particularly, *V. parahaemolyticus* ATCC 17802 persisted in a VBNC state in ASW and 5P-ASW for 100 d had the increased NPN uptake levels higher than its pure culture. Moreover, the induction of a VBNC state in *V. parahaemolyticus* by a high concentration of salt

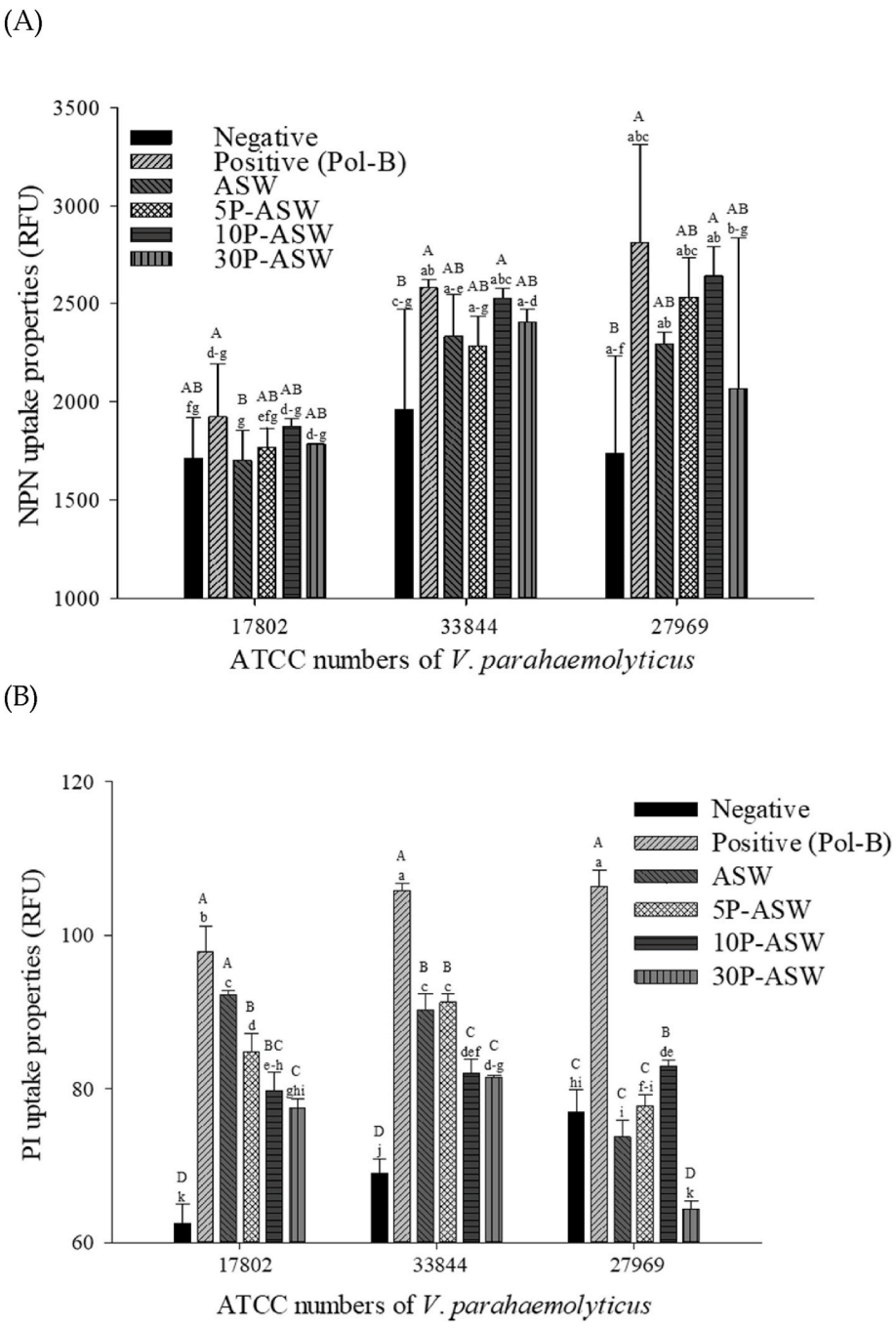

Fig. 4. Membrane potential measurement {(A), NPN; (B), PI} of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 before and after 100 d of incubation in ASW microcosms stored at 4 °C. Pol-B, polymyxin B. $^{A-D}$Mean values with different uppercase letters within the same *V. parahaemolyticus* strain group denote significant ($p < 0.05$) differences. $^{a-k}$Mean values with different lowercase letters among the three different strains of *V. parahaemolyticus* denote significant ($p < 0.05$) differences.

was further complemented by the increased PI uptake, indicating that the persistence of a VBNC state may be involved in the modification of the membrane potentials, particularly OM-permeabilizing activity (Tholozan, Cappelier, Tissier, Delattre, & Federighi, 1999). As well-documented in a study of Trevors, van Elsas, & Bej (2012), if bacteria entered into a VBNC state, the membrane became less fluid with intracellular K^+ leakage from the cytoplasm. Upon entering a VBNC state, there was a significant upregulation of some gene ontology groups involved in the transport and ATPase activity of molecular components in *E. coli*, concomitantly with an increased VBNC cell membrane permeability, which could excrete harmful substrates (Ye, Lin, Zhang, Chen, & Yu, 2020). When *Micrococcus luteus* was incubated in a lactate (0.01%) minimal medium at 4 °C, this organism became VBNC within 30 d and exerted a significant reduction in the membrane potential, as evidenced by a quantitative flowcytometry with the Rhodamine 123 probe that is indicative of viable or nonviable cells (Kaprelyants & Kell, 1992). Using a radioactive probe (tetra[^{3}H]phenylphosphonium bromide), VBNC *C. jejuni* strains persisted in natural water at 4 °C for 30 d showed dramatically reduced membrane potentials at levels of 2~14 mV (those for the stationary phase cells ranged from 54 to 79 mV) (Tholozan, Cappelier, Tissier, Delattre, & Federighi, 1999). The authors also reported that 30 d of NIR caused at least 10^2-fold decreases in internal K+ concentrations of VBNC *C. jejuni* cells. The alteration in membrane permeability may correspond to a decrease in cell membrane

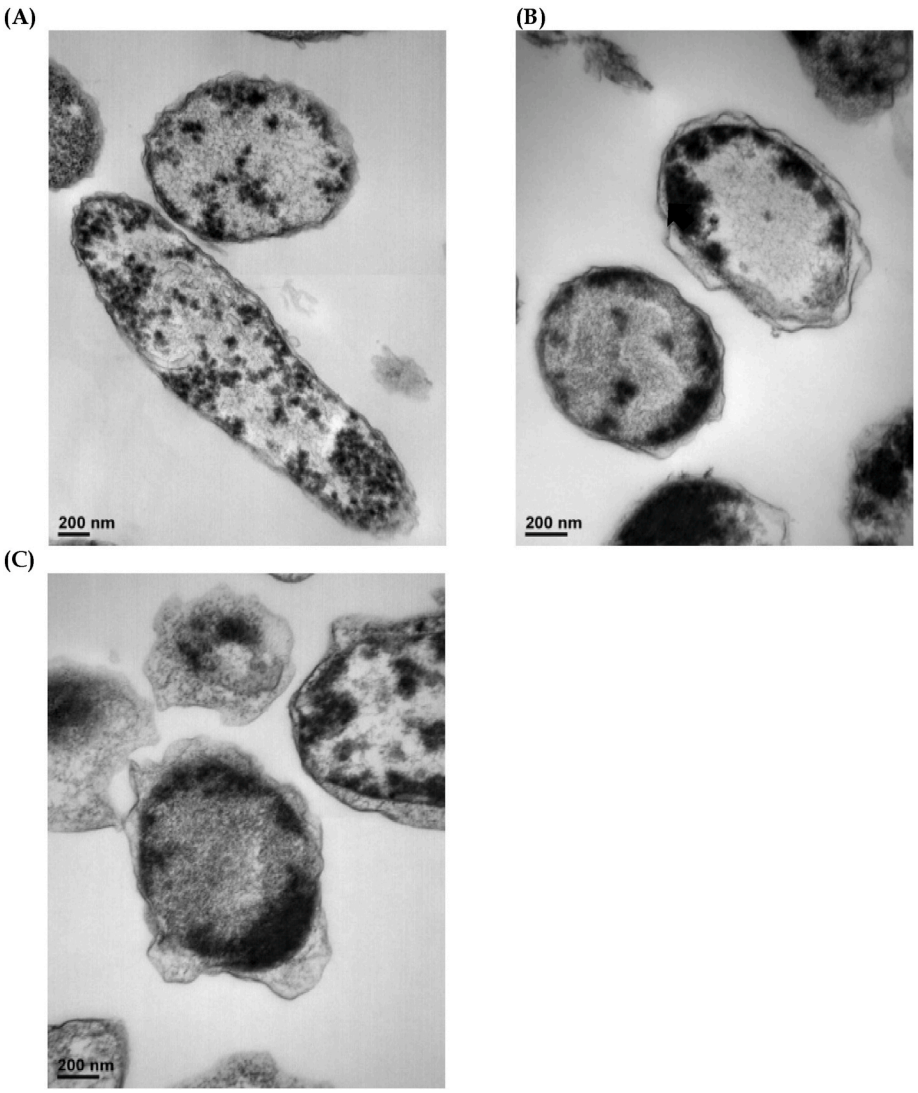

Fig. 5. TEM images of *Vibrio parahaemolyticus* ATCC 33844 either (A) grown in TSBS at 37 °C for 24 h or persisted in (B) ASW (pH 6.0) and (C) 5P-ASW (pH 6.0) at 4 °C for 100 d.

fluidity, as evidenced by an imbalance within the bacterial cells, which had the low membrane potential due to the penetration of NPN probes and the leakage of cellular contents, such as protein and DNA.

The modification in both FA composition profile and membrane potential would correspond to increasingly permeable membranes of *V. parahaemolyticus* during the persistence of a VBNC state. In a spite of that irreversible damage or loss of membrane permeability barriers indicates cell death in bacteria (Lowder et al., 2000; Zhang, Ye, Lin, Lv, & Yu, 2015), the NPN and PI uptake intensities of *V. parahaemolyticus* exposed to a high concentration of salt in ASW microcosms were significantly ($p < 0.05$) lower than the pol-B-treated controls. However, whether the membrane potential-associated values observed in this study truly represent the loss of the cell's vitality (integrity) due to compromised membranes, remains unclear. In a study of Brenzinger et al. (2019), a VBNC state was complemented by a comparison of structural proteome and peptidoglycan architecture in *V. cholerae*, determining that VBNC cells overexpressed iron acquisition and storage, peptide import, and arginine biosynthesis expected to be essential for maintaining its vitality. Otherwise, there may be a certain threshold between VBNC cells and dying/dead cells in terms of retaining the resuscitation capability. Once VBNC cells passed beyond this endpoint thereof, those cells became nonviable (dead). Hence, *V. parahaemolyticus* with maintenance of minimum vitality requirements resuscitates from a VBNC state under favorable environments, encouraging its regrowth and infection recurrence, which may pose a threat to public health and food safety. Overall, the VBNC state appears as one of the energy-saving survival strategies adopted by *V. parahaemolyticus* to preserve its viability and adaptability in response to constantly changeable environments (Fleischmann, Robben, Alter, Rossmanith, & Mester, 2021), rather than the cell death event.

In bacteria, the cell membrane fluidity plays an important role in a variety of cell physiological functions, including nutrient transport, protection from external adverse environments, and cell morphology. After shifting the VBNC state, the resultant changes of the FA profile may result in an physiological alteration in the cell structure of *V. parahaemolyticus* cells, concomitantly with the numbers of ribosome and organelle being notably decreased in VBNC cells, and these changes in the interior structure of VBNC cells may be involved in reduced cell volume that stems from the biological modulations, including FA composition and membrane potential. So far, the reduced cell fluidity would be implicated with a decrease of both DNA amplification and protein translation, causing VBNC *V. parahaemolyticus* to minimize the cell maintenance requirements under a NIR condition. *V. parahaemolyticus* would evolve a resistance to NIR by a responsive process of its cell size dwarfing and minimal maintenance requirement, which will be minimal to ATP, DNA, RNA or protein synthesis,

cytoplasmic volume, diffusion of macromolecular components, and gene expression (Balagurusamy et al., 2024; Oliver, 1995; Trevors et al., 2012; Wagley, 2023). Microorganisms have developed the survival mechanisms to withstand adverse environmental conditions, modifying the cell morphology and physiology.

5. Conclusion

At the onset of NIR, the higher the salt concentrations, the faster is the phase transition into a VBNC state. Particularly, a high concentration of salt inhibited the growth of *V. parahaemolyticus*, and this bacterium underwent a selected biochemical alteration, particularly in structural membrane and cytoplasmic layer, increased SFA proportion, decreased membrane potential, and retention of its viability, which may present potential health risks, during its persistence of a VBNC state. Thereby, an excessive amount of salt would cause the induction and persistence of a VBNC state in *V. parahaemolyticus* with increasingly permeable membrane properties. Theoretically, the physiological modulations may lead to the dwarfing of *V. parahaemolyticus* cells with the flappy outer membrane out of the cytoplasm, thereby minimizing their cell maintenance requirements. In conclusion, *V. parahaemolyticus* responds to a certain environmental stress, such as NIR, by inducing its phase transition into a VBNC state.

CRediT authorship contribution statement

Jae-Hyun Yoon: Writing – original draft, Visualization, Validation, Investigation. **Yeon-Jin Woo:** Investigation. **Sun-Young Lee:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Author declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117329.

Data availability

The authors do not have permission to share data.

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