Research article

Physiological Characteristics of Viable-but-nonculturable *Vibrio parahaemolyticus* upon Prolonged Exposure to the Refrigerator Temperature

Running title: Cellular properties of VBNC *V. parahaemolyticus*

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ABSTRACT

Although it has been reported that **v**iable-but-nonculturable (VBNC) cells of *Vibrio parahaemolyticus* can be developed by a prolonged duration of cold-starvation there are restricted cellular characteristics available on understanding the exact mechanisms governing the entry of pathogens into the VBNC state. Therefore, this research was aimed at determining the cellular profile of VBNC cells of *V. parahaemolyticus* upon exposure to the refrigerator temperature. Strains of *V. parahaemolyticus* were incubated in artificial sea water (ASW) microcosms (pH 6) added with different amounts of NaCl at 4^{o}C until these pathogens entered into such a dormant state. At a regular time-interval, both culturability and viability of these bacteria were enumerated, and then cellular profiling were carried out in terms of cellular membrane permeability, enzymatic activity, hydrophobicity, fatty acid composition, and morphological changes after cells of *V. parahaemolyticus* became the VBNC state. Three strains of *V. parahaemolyticus* used in this study showed that VBNC cells retained the strong virulent properties to Vero and CACO-2 cell lines, re-gained the cytotoxicity even after resuscitation, became permeabilized in terms of the outer membrane, showed lower levels of enzymatic (catalase and glutathione-S-transferase) activities, exerted the increasing hydrophobicity, and then exhibited increasing amounts of saturated fatty acids.

IMPORTANCE

To the current best knowledge, there are restricted information available on understanding the physiological characterization of viable-but-nonculturable cells. Most

47 previous studies are still making a degree of efforts in discovering the causative effector
48 causing microorganisms to be induced into the VBNC state. Herein, the present study showed
49 that pathogenic *V. parahaemolyticus* can enter into the VBNC state when challenged by a
50 certain environmental stress where higher amounts of NaCl combined with acidic pHs was
51 artificially controlled. Importantly, it was indicated that VBNC *V. parahaemolyticus*
52 maintained peculiarly different physiological characteristics. Furthermore, this study
53 proposed a novel approach on the transient/stepwise conversion of the bacteria into the
54 VBNC state. Specific alternative tools for measuring and controlling the incidence of VBNC
55 pathogens on food are not established until now. In this aspect, results obtained from this
56 study will used to provide an effective insight in determining physiological properties of
57 viable-but-nonculturable *V. parahaemolyticus*.
58
59 **KEYWORDS** fatty acid composition, cytotoxicity, NaCl, pathogen, viable-but-nonculturable
60

61

62 **INTRODUCTION**

63

64 *V. parahaemolyticus* has been recognized as one of the major food-borne pathogens
65 commonly found in estuarine environments such as seawater, costal area, and marine
66 sediment (1-2). It has been also reported that human pathogens such as *V. parahaemolyticus*,
67 *Vibrio vulnificus*, and *Vibrio cholerae* can be isolated from a wide variety of raw aquatic
68 products, including clam, mussel, oyster, scallop, and shrimp (3-4). Consumption of marine
69 products contaminated with these pathogens could result in serious clinical symptoms,
70 ranging from acute abdominal pain, vomiting, and nausea to fatal septicemia (5). Especially,
71 it has been determined that cells of *V. parahaemolyticus* are able to enter into a viable-but-
72 nonculturable (VBNC) state in response to a physiological ecology such as higher amounts of
73 NaCl (6) and low temperatures of $\leq 10^{o}$C (7). Until now, more than 65 species of
74 microorganisms, including *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *Campylobacter*
75 *jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* serovar
76 Typhimurium, *Shigella dysenteriae*, and *Staphylococcus aureus* are proven to be induced into
77 the VBNC state upon exposure to environmental stresses (8-13). It should be noted that
78 VBNC bacteria cannot represent the colony-forming capability on agar plates, on which these
79 organisms can grow routinely, thereby indicating that viable-but-nonculturable *V.*
80 *parahaemolyticus* might not be detected by means of the standardized cultivation-based
81 methods. Although lots of studies have been conducted to find out the exact mechanisms
82 governing the entrance of bacteria into such a dormant state there are still restricted scientific
83 evidences available on understanding physiological characteristics of VBNC organisms. After
84 producing VBNC *V. parahaemolyticus*, these bacteria showed a significantly higher
85 resistance to ethanol shock, as compared with the actively growing cells cultured in tryptic

soy broth (14). Above all, it was demonstrated that co-culturing VBNC pathogens such as *V. parahaemolyticus* and *Shig. dysenteriae* with clinical cell lines resulted in reducing a magnitude of viable animal cells, disrupting the cellular substructure undiscriminatingly (13, 15). Thus, it appeared that VBNC pathogens would be associated with the food-borne disease outbreaks, thereby threatening public health concerns. Nevertheless, it might be difficult to construct an effective microbial risk assessment for controlling the pathogenic bacteria due to a significant lack of cellular properties of the VBNC cells. In the present study, cellular properties of viable-but-nonculturable *V. parahaemolyticus* were characterized by measuring cytotoxic effects to human cell lines, the membrane permeabilization with ß-galactosidase and ß-lactamase assays, intracellular leakages of nucleic acid and protein, enzymatic activities of catalase and glutathione-S-transferase (GST), and the cellular hydrophobicity. Meanwhile, cellular fatty acid composition assays were conducted between pure cultures and VBNC cells of *V. parahaemolyticus* for the purpose of revealing the kinetic of mechanisms mediating the conversion of bacterial cells into the VBNC state. Furthermore, not only the ability of *V. parahaemolyticus* strains to be recovered from the VBNC state, but morphological changes before and after the entry into the VBNC state were examined to develop a well-standarized mean for determining the physiological characterization of *V. parahaemolyticus* in the VBNC state.

RESULTS AND DISCUSSION

Loss of the culturability of *V. parahaemolyticus* strains

In order to induce cells of *V. parahaemolyticus* into the VBNC state, three strains of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus*

ATCC 27969 were incubated in ASW microcosms (pH 6) supplemented with different amounts of NaCl at 4°C, respectively. Bacterial populations were enumerated at regular time-intervals by means of the cultivation-based method on agar plates. As shown in Fig. 1, initial densities of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 ranged from 6.0 $\log_{10}$ CFU/ml to 8.0 $\log_{10}$ CFU/ml. Populations of *V. parahaemolyticus* ATCC 17082 exceeded approximately 4.0 $\log_{10}$ CFU/ml until incubated in ASW microcosms containing 0.75% NaCl or amended with 5% NaCl at 4°C for 40 days, whereas microbial loads of *V. parahaemolyticus* ATCC 17082 dropped to below the detectable levels (<1.0 $\log_{10}$ CFU/ml) at 4°C within 80 days. Especially, loss of the culturability of *V. parahaemolyticus* ATCC 17082 was accelerated more progressively at increasing NaCl concentrations in ASW solutions, showing that cells of *V. parahaemolyticus* ATCC 17082 in ASW microcosms added with 10% and 30% NaCl were induced into the nonculturable state at 4°C within 21 days. There were no differences in the bacterial reductions between three strains of *V. parahaemolyticus* incubated in ASW microcosms containing 0.75% NaCl or supplemented with 5% NaCl. During the cold-starvation challenge, the culturability of these pathogens declined below the detectable levels within 80 days. Within 21 days, cells of *V. parahaemolyticus* ATCC 33844 entered into the nonculturable state in ASW microcosms added with ≥10% NaCl at 4°C. Cells of *V. parahaemolyticus* ATCC 27969 were also converted into the nonculturable state when incubated in either formal ASW microcosms or those amended with 5%, 10%, and 30% NaCl at 4°C for 7, 21, 80, and 80 days, respectively. These results indicated that higher degrees of NaCl facilitated the loss of bacterial culturablity more rapidly at the refrigerator temperature. However, it seemed likely clear that changes of the culturability are not the determination of making a decision on the true bacterial viability. In order to determine if bacterial cells are not dead, but still alive, it is

inevitable to evaluate the degree by which organisms are sincerely damaged. Accordingly, a part of fluorescent probes can reflect the level of cellular integrities quantitatively. Recently, staining dyes of SYTO9 and propidium iodide (PI) are widely applied in a field of the microbiology because these dyes are well-known for the ability to penetrate inside the cellular membrane with increasing cell injuries. SYTO9 is capable of penetrating bacteria with the intact membrane, interacting with the cell nucleic acid, and then displaying green colors for the live cell with the fluorescence microscopy. Propidium iodide can only penetrate bacterial cells through damaged membranes, labeling the dead cell only as red-coloured fluorescence. Thus, staining bacterial cells with SYTO9 and propidium iodide can distinguish between live and dead cells. Loss of the permeability barrier of stained cells represents irreparable membrane damages and thus cell death (16-17). In the present study, three strains of *V. parahaemolyticus* showed the viable numbers in the levels of >5.5 $\log_{10}$ CFU per a slide uniformly until these pathogens were challenged by cold-starvation stress (nutrient-deprivation and cold temperature) for more than 100 days, regardless of the controlled environmental effector such as excessive concentrations of NaCl and acidic pHs, which means that although the culturable count of *V. parahaemolyticus* were below the detectable levels when incubated in ASW microcosms at 4^{o}C for 80 days bacterial cells were proven to be still alive, maintaining the intact cell membrane. Accordingly, it was demonstrated that a prolonged period of cold-starvation challenge enabled three strains of *V. parahaemolyticus* to enter into the VBNC state within 80 days.

Cytotoxic effects of VBNC pathogens

As shown in Fig. 2A, there was no difference in the cellular virulence (cytotoxicity)

159 between the cells of *V. parahaemolyticus* ATCC 17082 either actively grown overnight in
160 TSB at 37°C or incubated in ASW microcosms at 4°C for ≤80 days. In contrast, VBNC *V.*
161 *parahaemolyticus* ATCC 17082 exhibited decreased cellular damages in the levels of ≤45%
162 against Vero cells when incubated in ASW microcosms added with 5% NaCl at 4°C for 80
163 days previously (Fig. 2B). Cells of *V. parahaemolyticus* ATCC 33844 and *V.*
164 *parahaemolyticus* ATCC 27969 showed gradual decreases in the cytotoxicity during cold-
165 starvation. In particular, less than 40% of the cellular toxicity was achieved when these
166 bacteria were incubated in ASW microcosms at 4°C for 21 days. Clearly, it appeared that the
167 cellular virulence of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969
168 against Vero cells declined in the reverse proportion to a prolonged duration of cold-
169 starvation. Especially, VBNC *V. parahaemolyticus* ATCC 33844 showed no cytotoxic effects
170 on Vero cells as maintained in ASW microcosms supplemented with 30% NaCl at 4°C for 80
171 days (Fig. 2B). However, it was shown that once challenged by the cold-starvation for 80
172 days VBNC *V. parahaemolyticus* ATCC 27969 represented the strong cytotoxicity, disrupting
173 the cellular structure of animal cell lines apparently (*data not shown*). Of much importance,
174 strains of *V. parahaemolyticus* which were converted from the nonculturable state recovered
175 the whole cytotoxicity in the levels of ≥100%. It has been well-reported that VBNC *V.*
176 *parahaemolyticus* can be recovered back to the culturable state by eliminating the causative
177 environmental stress. Several studies showed that strains of *V. parahaemolyticus* and *V.*
178 *vulnificus* in such a dormant state were converted to the culturable state on solid agar plates,
179 followed by culturing these long-term-stressed cells in liquid nutrient-rich media at ambient
180 temperatures for several days (6, 35). Dwidjosiswojo et al. (18) reported that while VBNC-
181 induced cells of *Pseudomonas aeruginosa* did not show the cytotoxicity these cells re-
182 cultured in a nutrient-rich culture broth (which recovered back to the culturable state) killed

100% Chinese hamster ovary cells within 24 hrs, supporting these findings. According to Wong et al. (2), cells of *V. parahaemolyticus* were induced into the VBNC state when incubated in Morita minimal salt broth at 4^{o}C for 35 days. Consequently, either VBNC or resuscitated cells of *V. parahaemolyticus* maintained higher levels of the cellular virulence, causing 100% cell death of Hep-2 cells within 24 hrs. Findings of Cappelier et al. (12) were also in an agreement with these results. Thus, it should be noted that VBNC cells of *V. parahaemolyticus* were not dead, but rather maintained peculiar metabolic activities to survive in an unfavorable environment such as cold-starvation. In this study, it was revelated out that three strains of *V. parahaemolyticus* in the VBNC state still remained the cellular toxicity against human cell lines, including Vero and CACO-2. Notably, VBNC *V. parahaemolyticus* strains were able to be resuscitated after temperature upshift, and then resulted in lethal injuries to the human cell lines. Therefore, it could be postulated that VBNC cells of pathogenic bacteria, including *V. parahaemolyticus*, would retain the infectious virulence or re-gain the infectivity after resuscitation process. VBNC-induced pathogen on marine products may present a potential risk of causing public health hazards. Necessarily, it should be further required for determining the environmental stress which activates either the transition of the food-borne pathogens into the VBNC state or the resuscitation-availability of VBNC bacteria to the culturable and infectious state.

Assessment of cell membrane permeabilization

In the present study, inner and outer cell membrane permeabilization assays were carried out by using ß-galactosidase and ß-lactamase, respectively (Fig. 3). Outer cell membranes of *V. parahaemolyticus* were damaged under cold-starvation challenge at 4^{o}C for 30 days as

being assessed with the ß-lactamase activity. Commonly, optical densities at 482 nm of *V. parahaemolyticus* ATCC 17082 grown overnight in TSB and in the VBNC state were increased with the prolonged duration. In contrast, ß-galactosidase activities of VBNC *V. parahaemolyticus* were not different from the pure cultures of *V. parahaemolyticus* ATCC 17082. Given that ß-galactosidase is localized in cytoplasm and is capable of hydrolyzing ONPG if the inner membrane of bacteria is compromised (19), it seemed likely that such a cold-starvation stress interacted with the outer cell membrane either mainly or preferentially. In addition, ß-lactamase, which is present in the periplasmic space, responds to pyridine-2-azo-4'-(N', N'-dimethylaniline) cephalosporin (PADAC) unless permeability of the outer membrane is decreasing (20). From these results, it could be accepted that the release of intracellular materials contributed to the loss of cell membrane permeabilization during a long-term of cold-starvation stress.

The cellular leakage of nucleic acid and protein into microcosm fluids was also measured to evaluate the degree to which cell membrane permeabilization of VBNC *V. parahaemolyticus* was truly damaged. In Table 1, initial densities of nucleic acid ranged from 0.417 to 1.567 when *V. parahaemolyticus* began to be exposed at 4^{o}C in ASW microcosms. After 150-days-incubuation at 4^{o}C, higher levels of nucleic acid were released from these cells; leakages of nucleic acid were 2.611, 2.550, 2.315, and 2.683 from VBNC *V. parahaemolyticus* ATCC 33844 in ASW microcosms at controlled NaCl concentrations of 0.75%, 5%, 10%, and 30%, respectively. Similarly, it has been shown that intracellular proteins were permeabilized through the membrane of VBNC cells into bacterial microcosms. After the entry of *V. parahaemolyticus* into the VBNC state, nucleic acid and protein outflowed more than twice over those of the actively growing cells. These results implied that cold-starvation stress could increase the membrane permeability of *V. parahaemolyticus*,

231 resulting in the cellular leakages of nucleic acid and protein from the bacterial cytoplasm to
232 external fluids. According to Zhang et al. (21), these authors stated that the release of nucleic
233 acid and protein would be strongly involved in persisting the integrity of cell membrane. At
234 this point of view, the temporary accumulation of these cellular components in ASW
235 microcosms was implicated with the loss of membrane integrity (22).
236
237 **Enzymatic activities of catalase and GST**
238
239 While culturing aerobically, reactive oxygen species (ROS) such as O_2^-, $^{\cdot}OH$, and H_2O_2 are
240 generally accumulated in the cell of bacteria. Especially, hydrogen peroxide (H_2O_2) has been
241 recognized as one of the leading toxic compounds due to its ability to penetrate the cellular
242 wall and membrane, thereby damaging nucleic acids directly (23). During exposure to
243 environmental stressors such as starvation, bacteria might encounter intracellularly
244 accumulated H_2O_2 (24). Hydrogen peroxidase is able to separate ROS into a molecule of H_2O
245 and O_2. Generally, detoxifying enzymes, including catalase, GST, and peroxidase, prevented
246 microbial cells from being damaged by ROS. It was determined that when *Ralstonia*
247 *solanacearum* was induced into the VBNC state upon exposure to 200 ㎛M copper,
248 significantly ($p < 0.05$) higher amounts of H_2O_2 were accumulated in the bacterial pellets (25).
249 Such a cellular damage caused by H_2O_2 could have a strong influence on the entry of
250 pathogens into the VBNC state. In particular, it has been reported that cells of *V. vulnificus*
251 incubated in ASW microcosms added with ≤500 ㎛M H_2O_2 at 4^{o}C were converted to the
252 nonculturable state more rapidly (26). Thus, effects of H_2O_2 to the cellular damage could be
253 examined by measuring the amounts of catalase produced intracellularly. In Fig. 4, *V.*
254 *parahaemolyticus* ATCC 17082 grown in TSB exerted <20 U/mg/protein catalase, whereas

VBNC-induced cells in ASW microcosms containing 0.75% NaCl and supplemented with 5%, 10%, and 30% NaCl showed <60, ≤100, <30, and <30 U/mg/protein catalase, respectively. When incubated in ASW microcosms amended with ≥10% NaCl at 4°C for 100 days *V. parahaemolyticus* ATCC 27969 exhibited a less decreased catalase activity (<50 U/mg/protein), as compared with that of the stationary-phase cells. While initial catalase activities of *V. parahaemolyticus* ATCC 33844 were <30 U/mg/protein, these organisms suspended in ASW microcosms added with ≥5% NaCl at 4°C for 100 days displayed enhanced catalase activities, ranging from 80 to <140 U/mg/protein. These results indicated that H_2O_2-degrading capability of *V. parahaemolyticus* would be dependent on strain-to-strain variables. GST was also recognized as one of the important agents associated with oxidative stress response of pathogens. Three strains of *V. parahaemolyticus* showed no differences among GST activities before and after a prolonged duration of cold-starvation (Fig. 5). Similarly, GST activities of these organisms ranged from >1.5 to <2.0 U/mg/protein. In the present study, there was no close correlation between the loss of culturability and ROS-detoxifying activities.

Assessment of the Cellular Hydrophobicity

In Table 3, hydrophobicity of *V. parahaemolyticus* in the VBNC state at 4°C for 100 days was measured for determining a hallmark of VBNC-induced bacteria. When subjected to the refrigerator temperature for 100 days, levels of hydrophobicity of *V. parahaemolyticus* ATCC 17082 in ASW microcosms added with 5%, 10%, and 30% NaCl were 37.5%, 46.8%, and 62.2%, respectively. Surface hydrophobicity of VBNC *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 increased dramatically more than those of the pure cultures.

279 Strains of *V. parahaemolyticus* used in this study exhibited the highest hydrophobicity of
280 ≤62.2%, followed by cold-starvation in ASW microcosms supplemented with 30% NaCl at
281 4^{o}C for 100 days. Probably, hydrophobicity of bacterial cells was closely related with a
282 change of fatty acid composition, thereafter implying that the increasing amounts of saturated
283 fatty acid (SFA) would result in a rise of the hydrophobicity of bacterial cells. Wong et al. (2)
284 reported that total amounts of saturated fatty acid were largely declined in cells of *V.*
285 *parahaemolyticus* ST550 during cold-starvation for 40 days, ranging from 60% to <40%. In
286 this point of view, our results shown in Table 4 would indicate that decreases of the content of
287 SFA, followed by a decline of the hydrophobicity, in VBNC cells of *V. parahaemolyticus*
288 would correspond to the increasing resistances to environmental challenges. Several studies
289 reported that nonculturable cells of bacteria, including *V. parahaemolyticus*, *V. vulnificus*, and
290 *Campylobacter jejuni*, exerted notably higher resistances to 20 ppm H_2O_2, antibiotics, and
291 heat (55^{o}C for 3 min), supporting our findings (14, 27-28).
292
293 **Morphological changes of VBNC cells**
294
295 In a stationary-phase grown cell of *V. parahaemolyticus* ATCC 17082 (Fig. 6A), the
296 cytoplasmic matrix was filled with granules and the cell membrane became intact without
297 minor damages. On contrast, VBNC cells of *V. parahaemolyticus* ATCC 17082 were shown
298 to have less densed cytoplasmic spaces than those of the stationary-phase cells. In particular,
299 cellular membranes of VBNC cells were largely loosed, resulting in generating empty spaces
300 between the outer and inner membranes (Fig. 6B-C). When it comes to a study conducted by
301 Zhao et al. (29), it was reported that the number of ribosomes was notably reduced in VBNC
302 cells of *Escherichia coli* O157:H7. These authors also pointed out that this change in the

303 interior structure of cells would be implicated with decreases of both deoxyribonucleic acid
304 (DNA) amplification and protein translation, thereby causing these VBNC cells to minimize
305 cell maintenance requirements (compactness)
306
307 **Fatty acid compositions of VBNC *V. parahaemolyticus***
308
309 Cells of *V. parahaemolyticus* ATCC 17082 grown overnight in TSB accounted for 41.34%
310 of the saturated fatty acid and 58.67% of unsaturated fatty acid out of the whole fatty acid
311 composition. Normal cells exerted the highest amounts of palmitic acid ($C_{16:0}$, 25.64%) and
312 palmitoleic acid ($C_{16:1\ w7c}$, 26.76%), respectively. When cells of *V. parahaemolyticus* ATCC
313 17082 were incubated in ASW microcosms added with higher concentrations of NaCl at 4^{o}C
314 for 90 days, contents of palmitic acid were increased slightly, ranged from 23.05% to 23.88%.
315 Among the saturated fatty acid, contents of lauric acid ($C_{12:0}$), 2-hydroxylauric acid ($C_{12:0\ 2OH}$),
316 and myristic acid ($C_{14:0}$) were increased as *V. parahaemolyticus* ATCC 17082 became the
317 viable-but-nonculturable state. In terms of the unsaturated fatty acid, VBNC cells exhibited
318 increasing concentrations of palmitoleic acid, ranging from 32.98% to 35.77%. In particular,
319 it was shown that unsaturated fatty acids were more synthesized with the increasing
320 concentrations of NaCl. On the other hand, levels of cis-vaccenic acid ($C_{18:1\ w7c}$) declined
321 largely upon exposure of *V. parahaemolyticus* to the low temperature for 90 days in ASW
322 microcosms with the addition of increasing amounts of NaCl, showing by 18.1%, 14.90%,
323 14.16%, and 13.18%, respectively. In addition, it seemed clear that several saturated fatty
324 acids, including lauric acid, 2-hydroxylauric acid, and myristic acid, were responsible for the
325 increasing amounts of total saturated fatty acid in VBNC cells of *V. parahaemolyticus* ATCC
326 17082. In a study conducted by Jia et al., (30), four strains of *V. parahaemolyticus* isolated

from seafood showed that palmitic acid ($C_{16:0}$) was decreased dramatically, ranging from 47.6% to 9.9% when these organisms were incubated in TSB at 4^{o}C for 30 days, whereas palmitoeleate ($C_{16:1}$) increased greatly after the entry into the VBNC state. This study was in an agreement with our findings. It was also reported that palmitic acid contents (%) of *V. vulnificus* C7184 were significantly ($p < 0.05$) lower once this organism was incubated in ASW microcosms at 5^{o}C for 24 hrs (31). In VBNC cells of *V. vulnificus*, palmitoleic acid ($C_{16:1}$) and stearic acid ($C_{18:0}$) increased significantly ($p < 0.05$), while margaric acid ($C_{17:0}$) declined remarkably at the refrigerator temperature within 24 hrs, supporting our findings. Furthermore, it was demonstrated that VBNC cells of *V. parahaemolyticus* ATCC 17082 were recovered to the culturable state reversibly as being resuscitated in a nutrient-rich medium such as TSB (pH 8) and brain hearth infusion broth (pH 8) at 25^{o}C for several days (*data not shown*). Thus, resuscitated cells were further used to perform the fatty acid composition analysis in the present study. As shown in Table 6, the total density of unsaturated fatty acids was increased more than normal and VBNC cells. Especially, lauric acid ($C_{12:0}$), 2-Hydroxylauric acid ($C_{12:0\ 2OH}$), and unknown ($C_{14:0\ 3OH}$) were increased comparatively, whereas palmitic acid ($C_{12:0}$) was decreased remarkably among the total saturated fatty acid of resuscitated cells. After resuscitation, cis-vaccenic acid ($C_{18:1\ w7c}$) increased largely, ranging from 20.65% to 32.17%. Of which on the resuscitated cells of *V. parahaemolyticus* ATCC 17082, the total amounts of saturated fatty acid declined, but unsaturated fatty acids increased to that extent. In our preliminary studies, VBNC *V. parahaemolyticus* exhibited higher resistances through a simulated human gastric digestion (*data not shown*). Along with our findings in this study, it would be suggested that increasing synthesis of saturated fatty acids in the VBNC cells correspond to the improved environmental resistance. As saturated fatty acids display a straight-line structure VBNC cells would acquire more compact cell

351 membranes, thereby resulting in more decreased cellular permeabilization than that of the
352 normal cells. Consequently, it would administer higher resistances to various environmental
353 challenges for these VBNC cells. Previously, several studies disclosed that there were
354 significantly different fatty acid compositions on the bacterial membranes between actively
355 growing and VBNC cells (2, 31). Interestingly, it has been shown that ratios of saturated fatty
356 acids/unsaturated fatty acids increased significantly after forming VBNC cells. Probably, this
357 change would be closely associated with a distinct physiology of VBNC cells. Given that
358 VBNC cells might be capable of re-gaining the platable-capability on culture media, followed
359 by further resuscitation efforts, there are still restricted information available on
360 understanding cellular properties of VBNC *V. parahaemolyticus*. By comparing the fatty acid
361 composition between VBNC and resuscitated cells, it would provide a meaningful insight in
362 revealing out mechanisms governing the entry of *V. parahaemolyticus* into the VBNC state.
363 Based on all the findings obtained from the present study, a novel hypothesis explaining the
364 entrance of *V. parahaemolyticus* into the viable-but-nonculturable state would be established
365 (Fig. 7). It would be plausible that the morphological transformation of bacterial cells either
366 from rods to coccus or the dwarfing in the cellular size has to be preceded before the actively
367 growing cells could turn into the VBNC state. Previously, it was determined that 100-days-
368 stressed cells of VBNC. *V. parahaemolyticus* displayed the increasing cellular leakages such
369 as nucleic acid and protein as much twice as the pure cultures. Besides, it was also shown that
370 VBNC *V. parahaemolyticus* exhibited the increasing permeabilization through the outer
371 membrane, indicating that the loss of cell membrane integrity caused by the increasing
372 membrane permeabilization could result in the cellular leakage before VBNC cells could be
373 steadily developed. Hence, such a transient conversion of bacterial cells could aim to alter the
374 surface area as compact as possible, minimizing the cellular damage and the consumption of

375 energy sources under continuous environmental stresses (nutrient-deprivation and cold
376 temperature). One of our findings about the morphological change of VBNC *V.*
377 *parahaemolyticus* can be in an agreement with this hypothesis strongly. Subsequently, fatty
378 acid composition of these cells will begin to be altered, showing the increasing levels of
379 saturated fatty acid. This procedure might be significantly associated with the complete
380 compactness of the cellular membrane structure, and then could diminish the increased
381 permeabilization of outer membranes into normal ranges, thereby stabilizing VBNC cells at
382 the end. Seriously, as most VBNC cells of bacteria exerted higher resistances to various
383 environmental conditions more than the actively grown cells enhanced survivals of VBNC
384 cells could be elucidated by the alternation of fatty acid compositions after forming VBNC
385 cells. Overall, it can be defined that once challenged by environmental stresses nonculturable
386 bacteria, which show cellular modifications such as the increasing synthesis of saturated fatty
387 acid, causing the cellular dwarfing in sizes to minimize the exposure of bacterial surfaces and
388 to save the energy consumption, and then prevent intracellular periplasms from being
389 seriously damaged by ROS, maintaining the integrity of bacterial membranes, could be called
390 as viable-but-nonculturable cells.
391
392 In conclusion, this research was conducted to determine the cellular characteristics of
393 VBNC *V. parahaemolyticus*. As shown in Table 4, three strains of *V. parahaemolyticus* used
394 in this study showed that VBNC cells (I) ratained the cellular virulence to Vero and $CaCO_2$
395 cell lines, (II) re-gained the 100% cytotoxicity even after resuscitation process, (III) became
396 permeabilized mainly at the binding sites of the outer membrane, (IV) showed lower levels of
397 enzymatic (catalase and GST) activities, (V) exerted the increasing hydrophobicity, (VI)
398 showed increasing amounts of SFA, and then (VII) displayed flappy outer membranes out of

399 the cytoplasm in the cellular morphology. As pathogens can encounter a number of
400 environmental stresses through a wide variety of channels contaminated food materials are
401 subjected to heat, desiccation, cold temperature, freeze, and others in the middle of the
402 preparation and the processing. As one of the surviving strategies, pathogens on food respond
403 to these challenges, could turn into the VBNC state, and then can pose a risk of causing the
404 food-borne outbreaks potentially. Clearly, it was demonstrated that VBNC cells could be the
405 source of food-borne outbreaks upon eliminating the initiative environmental stress.
406 Therefore, understanding the cellular characterization of nonculturable pathogens is of much
407 importance in an attempt to designing adequate control measures for assuring the food safety.
408 Although these results cannot reveal out all of the properties of VBNC bacteria, it will
409 provide useful basic natures of a bacterial surviving strategy, understanding a mechanism
410 governing the transition into the VBNC state.
411

412 **MATERIALS AND METHODS**
413

414 **Preparation of microcosm solutions** ASW solutions (Sigma-Aldrich, St. Louis, MO, USA)
415 were prepared according to the manufacturer's instruction. Formal ASW microcosms (pH
416 7.2-7.8) contained 19,290 mg of Cl, 10,780 mg of Na, 2,660 mg of SO_4, 420 mg of K, 400
417 mg of Ca, 200 mg of CO_3, 8.8 mg of Sr, 5.6 mg of B, 56 mg of Br, 0.24 mg of I, 0.3 mg of Li,
418 1.0 mg of F, and 1,320 mg of Mg per 1 liter of sterile distilled water. These microcosms were
419 autoclaved at 125^{o}C for 20 min before use. Then, NaCl concentrations of these microcosms
420 were adjusted to 0.75%, 5%, 10%, and 30% (m/v), respectively. Each of these microcosms
421 was adjusted to pH 6.0–6.2, using a membrane-filtered 1N NaOH solution (Kanto chemical,
422 Tokyo, Japan), facilitating the induction of *V. parahaemolyticus* into the VBNC state.

Preparation of bacterial inoculums Cells of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 27969, and *V. parahaemolyticus* ATCC 33844 were purchased from the Korean Collection for Type Cultures (KCTC, Daejon, Korea). Bacterial stocks were maintained at -75^{o}C and further activated by a series of transfers in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 3% NaCl (TSB) at 37^{o}C for 24 hrs before use. Each strain of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 grown overnight in 35 ml of TSB at 37^{o}C for 24 hrs was harvested by the centrifugation at 10,000 X g for 3 min, washed twice, and then bacterial pellets were re-suspended in 1 ml of ASW solutions (pH 6), corresponding to the bacterial density of approximately $10^{8\text{-}9}$ CFU/ml. Finally, cells of these pathogens were inoculated in 100 ml of ASW microcosms (pH 6) added with 0.75%, 5%, 10% and 30% NaCl, respectively. Bacterial fluids were kept at 4^{o}C and ASW microcosms were withdrawn from the incubator at regular time-intervals to conduct the following process.

Enumeration of the bacterial population In order to count the bacterial number, cells of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 were plating-counted on typtic soy agar (Difco) supplemented with 3% NaCl (TSA). Decimal dilutions (10^{-1}) of these cells were prepared in alkaline peptone water (APW, pH 8) consisting 10 g of peptone and 10 g of NaCl per 1 liter of distilled water and 100 µl of these aliquots was spread on TSA. These media were further incubated at 37^{o}C for 24 hrs. Colonies developed on media were further enumerated. At the same time, the numbers of living and dead cells of *V. parahaemolyticus* were measured using the Live/Dead$^{(R)}$ BacLight Bacterial Viability kit (Life technologies, Eugene, Oregon, USA). Briefly, equal volumes (1:1)

447 of SYTO9 and propidium iodide (PI) were combined and 3 µl of this mixture was added to
448 each 1 ml of the bacterial cell. After a short time of incubation (approximately 15 min) at an
449 ambient temperature in the dark, 5-8 µl of this aliquot was attached on a glass slide and a
450 coverslip was placed on it. Then, bacterial images were demonstrated using an electron
451 microscope (TE 2000-U, Nikon, Japan).
452
453 **Preparation of human cell lines** CACO-2 and Vero cells were cultured in 5-10 ml of
454 Dulbecco's modified eagle's medium (DMEM, Corning, NY, USA) supplemented with 5%
455 and 20% fetal bovine serum (FBS, Corning) at 37^{o}C for 1-2 days in 5% CO_2, respectively.
456 The medium was removed in a petri-dish and washed 5 ml of PBS three times, and then 5 ml
457 of trypsin (Corning) was added to facilitate the cell lysis. Each of these cells was incubated
458 at 37^{o}C for 5 min in 5% CO_2. To alleviate the enzymatic activity caused by trypsin, 2-3 ml
459 of the medium was added to all the cell cultures. Cell fluids were further transferred into 15
460 ml of a sterile cap tube and centrifugated at 15,000 X g for 3 min. The supernatant was
461 eliminated, re-suspended in 5 ml of the medium, corresponding to the cellular density of
462 10^4/ml, and then 100 µl of the eukaryotic cell solution was loaded into a 96-well plate
463 containing 100 µl of the DMEM medium. CACO-2 and Vero cells were incubated at 37^{o}C
464 for 24 hrs in 5% CO_2 before use.
465
466 **Measurement of the cytotoxicity of VBNC *V. parahaemolyticus* against the cell line**
467 Cellular virulence of *V. parahaemolyticus* in the VBNC state against the human cell line was
468 measured according to several studies conducted by Beattie and Williams (32), Jeßberger et
469 al. (33), and Dektas et al. (34). As mentioned above, prepared cell lines, corresponding to
470 10^4 CFU/ml, were added into a 96-well plate, respectively. Serial dilutions (0.1 ml/well) of

471 the filtered cell-free bacterial supernatants were placed into 96-well plates. Or bacterial
472 suspensions, corresponding to 10^8 CFU/ml, was added in each of wells. Animal cell
473 suspensions (0.1 ml/well), corresponding to 10^4 cells per a well for Vero cells and 2 X 10^4
474 cells per a well for CACO-2 cells, were supplemented into 96-well plates, respectively. Cell
475 fluids were incubated at 37°C for 24 hrs under the atmospheric environment containing 5%
476 CO_2, replaced with either 100 μl/well of EMEM containing 5 mg/ml 3-(4, 5-
477 dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT, Corning), and then
478 incubated at 37°C for 1 hr.
479 These mixtures were further removed in each of the wells, 100 μl of dimethyl-sulphoxide
480 (DMSO, Corning) was added into a 96-well plate, and then read on a microtiter plate reader
481 at 570 and 620 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa,
482 Finland).
483
484 **Analysis of the cell membrane permeabilization** The release of cytoplasmic ß-
485 galactosidase from cells of VBNC *V. parahaemolyticus* was measured to determine the inner
486 membrane permeabilization. Briefly, 10 μl of VBNC *V. parahaemolyticus* cells in ASW
487 microcosms (pH 6) were transferred onto each well of a 96-well plate containing 50 μl of *O*-
488 Nitrophenyl-$_{L}$-$_{D}$-galactoside (Sigma) and 40 μl of ASW solutions (pH 6). Once bacterial
489 suspension was incubated at 30°C for 30 min in a dark room the absorbance was read on a
490 microtiter plate reader at 420 nm (Multiskan GO Microplate Spectrophotometer). When it
491 comes to determine the outer membrane permeabilization of VBNC cells, 10 μl of VBNC *V.*
492 *parahaemolyticus* cells in ASW microcosms were added to each well of a 96-well plate
493 containing 80 μl of ASW solutions and 10 μl of nitrocefin (Sigma), incubated at 30°C for 30
494 min in a dark room, and then the absorbance was read on a microtiter plate reader at 482 nm

(Thermo Scientific).

Analysis of the cellular leakage in the bacterial suspension Cell membrane integrity was assessed by measuring cellular leakages such as nucleic acid and protein from VBNC cells of *V. parahaemolyticus*. Cell suspensions (1.5 mL) of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 which had been incubated in ASW microcosms at 4°C for 100 days were transferred onto a sterile microtube, respectively. Pure cultures of these pathogens grown overnight in TSB at 37°C were centrifugated at 13,000 X g for 3 min, washed twice, and then re-suspended in ASW solutions (pH 8) added with 0.75%, 5%, 10%, and 30% NaCl, respectively. These pathogens were centrifuged at 10,000 X g at 4°C for 15 min to collect bacterial pellets. Each of these supernatants was collected in a sterile microtube and further used to assess the cellular leakage such as nucleic acid and protein by measuring the absorbance at 260 and 280 nm via multi-scan Go spectrophotometer (Thermo Scientific Inc.).

Measurement of enzymatic activities Catalase activity was measured in terms of H_2O_2-degradation with a spectrophotometric assay (CAT100, sigma). This assay was undertaken following by a method introduced in several studies (Santander et al., 2017; Molina-Cruz et al., 2008). Overnight grown cultures of *V. parahaemolyticus* and VBNC cells incubated in ASW (pH 6) microcosms at 4°C for 100 days were re-suspended in 50 mM potassium phosphate buffer (pH 7) containing 1 g of 3-mm-sized glass bead (Sigma), vortexed for 25 min, and then centrifugated at 13,000 X g for 3 min. According to a manual provided by a manufacture, supernatant was separately transferred into a sterile microtube. In a total of 100 µl of volume, 15 µl of supernatant was combined with 5 mM H_2O_2. Such a reaction was

519 carried out at 25^{o}C for 15 min and ceased with 900 µl of 15 mM sodium azide. Then,
520 absorbance was colorimetrically read at 520 nm via multi-scan Go spectrophotometer
521 (Thermo Scientific Inc.). Glutathione-S-Transferase (GST) activity was measured, using GST
522 assay kit (CS0410, Sigma). In this assay, 1-Chloro-2, 4-DiNitroBenzene (CDNB) was used as
523 a substrate. Twenty µl of bacterial supernatants were added to 180 µl of a substrate solution in
524 each well of a 96-well plate, respectively. Then, absorbance was read at 340 nm via multi-
525 scan Go spectrophotometer.
526
527 **Microbial adhesion to hydrocarbons (MATHs) assay** Cells of *V. parahaemolyticus* grown
528 overnight in TSB and incubated in ASW microcosms at 4^{o}C for 50 days were centrifugated at
529 8,000 X g for 1 min, washed twice, and then re-suspended in 0.1 M PBS to fit an optical
530 density of 1.0 (A_o) at 600 nm via a UV-Visible Spectrometer (Multiskan GO, Thermo
531 Scientific). One hundred µl of hexadecane was added to 1 ml of these bacterial suspensions
532 and incubated at an ambient temperature for 10 min. Optical density of these bacteria in the
533 aqueous phase was measured at 600 nm (A_1). The degree of hydrophobicity was calculated,
534 following as (Choi, et al., 2013);
535
536 $[1-A_1/A_o]$ X 100 (%).
537
538 **Transmission electron microscopic (TEM) Assay** Bacterial cells incubated in ASW
539 microcosms (pH 6) containing 0.75% NaCl or supplemented with 5%, 10%, and 30% NaCl at
540 4^{o}C for 7 days were centrifugated at 10,000 X g for 3 min, rinsed three times with 0.1M
541 Phosphate Buffered Saline (PBS, pH 7.2), and then re-suspended in 0.1M PBS to collect the
542 pellets. These cells were fixed in 2% paraformaldehyde overnight at 4^{o}C. After washing with

PBS, cells of *V. parahaemolyticus* were post-fixed in 1% osmium tetroxide in 0.1M PBS and dehydrated in a series (30%, 50%, 70%, 95%, and 100%) of ethanol solutions. Thereafter, each of bacterial cells were infiltrated with 2 ml of epoxy resin. Polymerization of the resin was performed at 60^{o}C for 24 hrs. Then, sections, approximately 120 nm thickness, were cut randomly and photographed with a JEOL JEM 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA, USA).

Fatty acid composition analysis Whole cell fatty acid analysis was conducted according to a standardized method by Microbial Identification System (MIDI, Microbial ID Inc., Newark, Del., USA). Cells from 5 ml of a stationary-phase culture and 5 ml of VBNC *V. parahaemolyticus* were harvested by centrifugation at 10,000 X g for 5 min. Bacterial cells were subjected to a series of procedures, including saponification, methylation, and washing to extract carboxylic acid derivatives of long-chain aliphatic molecules. Then, extracted lipids were used to analyze the fatty acid composition of *V. parahaemolyticus* in the VBNC state, following several procedures provided by the supplier (Microbial Identification System, Newark, Del.)

Assessment of VBNC bacterial resistances to an acidic environment Ten ml of an exponential-phase culture and 10 ml of VBNC *V. parahaemolyticus* ATCC 17082were harvested by centrifugation at 10,000 X g for 5 min, washed twice, and then re-suspended in 10 ml of PBS, respectively. A simulated human gastric juice was prepared according a study conducted by Lee et al. (2016). This human digestion model (pH 1.5) consisted of 6.5 ml of HCl (37 g per 1 L of DW), 18 ml of $CaCl_2 \cdot 2H_2O$ (37 g per 1 L of DW), 1 g of bovine serum albumin (Corning), 2.5 g of pepsin (Sigma), and 3 g of mucin (Sigma). These chemicals and

567 enzymes used in the present study were either autoclaved or filtered through a 0.2-um-size a
568 polycarbonate membrane (ADVANTEC) before use. Thus, 10 ml of the bacterial cell was
569 mixed with 10 ml of the gastric juice. Each of *V. parahaemolyticus* ATCC 17082 cells in a
570 simulated stomach digestion model was incubated in a shaking water bath (120 rpm) at 37^{o}C
571 for 60 min. At a regular time-interval, the bacterial population was plating-counted on TSA
572 and the viability of nonculturable *V. parahaemolyticus* ATCC 17082 was also enumerated
573 with the fluorescence microscopic assay.
574

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579

580 **REFERENCES**
581

582 1. Yue X, Liu B, Xiang J, Jia J. 2010. Identification and characterization of the
583 pathogenic effect of a *Vibrio parahaemolyticus*-related bacterium isolated from clam
584 *Meretrix meretrix* with mass mortality. J Invertebr Pathol 103:109-115.
585 2. Wong HC, Shen CT, Chang CN, Lee YS, Oliver JD. 2004. Biochemical and virulence
586 characterization of viable but nonculturable cells of *Vibrio parahaemolyticus*. J Food
587 Prot 67:2,430-2,435.
588 3. Yu WT, Jong KJ, Lin YR, Tsai S, Tey YH, Wong H. 2013. Prevalence of *Vibrio*
589 *parahaemolyticus* in oyster and clam culturing environments in Taiwan. Int J Food
590 Microbiol 160:185-192.

4. Hackney CR, Bay B, Speck ML. 1980. Incidence of *Vibrio parahaemolyticus* in and the microbiological quality of seafood in North Carolina. J Food Prot 43:769-773.
5. Piñeyro P, Zhou X, Orfe LH, Friel PJ, Lahmers K, Call DR. 2010. Development of two animal models to study the function of Vibrio parahaemolyticus Type III secretion systems. Infect Immun 78:4,551-4,559.
6. Yoon JH, Bae YM, Lee SY. 2017. Effects of varying concentrations of sodium chloride and acidic conditions on the behavior of *Vibrio parahaemolyticus* and *Vibrio vulnificus* cold-starved in artificial sea water microcosms. Food Sci Biotechnol 26:829-839.
7. Gribbon LT, Barer MR. 1995. Oxidative metabolism in nonculturable *Helicobacter pylori* and *Vibrio vulnificus* cells studies by substrate-enhanced tetrazolium reduction and digital image processing. Appl Environ Microbiol 61:3,379-3,384.
8. Mishra A, Taneja N, Sharma M. 2011. Demonstration of viable but nonculturable *Vibrio cholerae* O1 in fresh water environment of India using ciprofloxacin DFA-DVC method. Lett Appl Microbiol 53:124-126.
9. Liu Y, Wang C, Tyrrell G, Hrudey SE, Li XF. 2009. Induction of *Escherichia coli* O157:H7 into the viable but nonculturable state by chloraminated water and river water, and subsequent resuscitation. Environ Microbiol Rep 1:155-161.
10. Muela A, Seco C, Camafeita E, Arana I, Orruño M, López JA, Barcina I. 2008. Changes in *Escherichia coli* outer membrane subproteome under environmental conditions inducing the viable but nonculturable state. FEMS Microbiol Ecol 64:28-36.
11. Besnard V, Federighi M, Declerq E, Jugiau F, Cappelier J. 2002. Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*. Vet Res

615 33:359-370.

616 12. Cappelier JM, Minet J, Magras C, Colwell RR, Federighi M. 1999. Recovery in
617 embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and
618 maintenance of ability to adhere to HeLa cells after resuscitation. Appl Environ
619 Microbiol 65:5,154-5,157.

620 13. Rahman I, Shahamat M, Chowdhury MA, Colwell RR. 1996. Potential virulence of
621 viable but nonculturable *Shigella dysenteriae* type 1. Appl Environ Microbiol 62:115-
622 120.

623 14. Chiang ML, Ho WL, Chou CC. 2008. Ethanol shock changes the fatty acid profile
624 and survival behavior of *Vibrio parahaemolyticus* in various stress conditions. Food
625 Microbiol 25:359-3765.

626 15. Rahman I, Shahamat M, Kirchman PA, Russek-Cohen E, Colwell RR. 1994.
627 Methionine uptake and cytopathogenicity of viable but nonculturable *Shigella*
628 *dysenteriae* type 1. Appl Environ Microbiol 60:3,573-3,578.

629 16. Antolinos V, Esteban MD, Ros-Chumillas M, Huertas JP, Periago PM, Palop A,
630 Fernández PS. 2014. Assessment of the of acid shock effect on viability of *Bacillus*
631 *cereus* and *Bacillus weihenstephanensis* using flow cytometry. Food Res Int 66:306-
632 312.

633 17. Mizunoe Y, Wai SN, Ishikawa T, Takade A, Yoshida SI. 2000. Resuscitation of viable
634 but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under
635 starvation. FEMS Microbiol Lett 115-120.

636 18. Dwidjosiswojo Z, Richard J, Moritz MM, Dopp E, Flemming HC, Wingender J. 2011.
637 Influence of copper ions on the viability and cytotoxicity of *Pseudomonas aeruginosa*
638 under conditions relevant to drinking water environments. Int J Hyg Environ Health

214:485-492.

19. Li N, Luo M, Fu YJ, Zu YG, Wang W, Zhang L, Yao LP, Zhao CJ, Sun Y. 2013. Effect of corilagin on membrane permeability of *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Phytother Res 27:1,517-1,523.
20. Thennarasu S, Nagaraj R. 1996. Specific antimicrobial and hemolytic activities of 18-residue peptides derived from the amino terminal region of the toxin pardaxin. Protein Eng 9:1,219-1,224.
21. Zhang Z, Liu X, Wang Y, Jiang P, Quek SY. 2016. Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. Food Cont 59:282-289.
22. Balakumaran MD, Ramachandran R, Balashanmugam P, Mukeshkumar DJ, Kalaichelvan PT. 2016. Mycosynthesis of silver and gold nanoparticles: Optimization, characterization, and antimicrobial activity against human pathogens. Microbiol Res 182:8-20.
23. Linley E, Denyer SP, McDonnell G, Simons C, Maillard JY. 2012. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. J Antimicrob Chemother 67:1,589-1,596.
24. McDougald D, Gong L, Srinivasan S, Hild E, Thompson L, Takayama K, Rice SA, Kjelleberg S. 2002. Defences against oxidative stress during starvation in bacteria. Antonie Van Leeuwenhoek. 81:3-13.
25. Um HY, Kong HG, Lee HJ, Choi HK, Park EJ, Kim ST, Murugiyan S, Chung E, Kang KY, Lee SW. 2013. Altered gene expression and intracellular changes of the viable but nonculturable state in *Ralstonia solanacearum* by copper treatment. Plant Pathol J 29:374-385.

663 26. Abe A, Ohashi E, Ren H, Hayashi T, Endo H. 2007. Isolation and characterization of
664 a cold-induced nonculturable suppression mutant of *Vibrio vulnificus*. Microbiol Res
665 162:130-138.
666 27. Nowakowska J, Oliver JD. 2013. Resistance to environmental stresses by *Vibrio*
667 *vulnificus* in the viable but nonculturable state. FEMS Microbiol Ecol 84:213-222.
668 28. Klančnik A, Guzej B, Jamnik P, Vučković D, Abram M, Možina SS. 2009. Stress
669 response and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation.
670 Res Microbiol 160:345-352.
671 29. Zhao F, Bi X, Hao Y, Liao X. 2013. Induction of viable but nonculturable *Escherichia*
672 *coli* O157:H7 by high pressure CO_2 and its characteristics. PLOS One 8:e6238.
673 30. Jia J, Chen J, Jiang Y, Tang J, Yang L, Liang C, Jia Z, Zhao L. 2014. Visualized
674 analysis of cellular fatty acid profiles of *Vibrio parahaemolyticus* strains under cold
675 stress. FEMS Microbiol Lett. 357:92-98.
676 31. Day AP, Oliver JD. 2004. Changes in membrane fatty acid composition during entry
677 of *Vibrio vulnificus* into the viable but nonculturable state. J Microbiol. 42:69-73.
678 32. Beattie SH, Williams AG. 1999. Detection of toxigenic strains of *Bacillus cereus* and
679 other *Bacillus* spp. with an improved cytotoxicity assay. Lett Appl Microbiol 28:221-
680 225.
681 33. Jeßberger N, Dietrich R, Bock S, Didier A, Märtlbauer E. 2014. *Bacillus cereus*
682 enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to
683 different human cell lines. Toxicon 77:49-57.
684 34. Dektas E, Daferera D, Sökmen M, Serdar G, Ertürk M, Polissiou MG, Sökmen A.
685 2016. In vitro antimicrobial, antioxidant, and antiviral activities of the essential oil
686 and various extracts from *Thymus nummularis* M. Bieb. Indian J Tradit Knowl

687 15:403-410.

688 35. Wong HC, Wang P, Chen SY, Chiu SW. 2004. Resuscitation of viable but non-
689 culturable *Vibrio parahaemolyticus* in a minimum salt medium. FEMS Microbiol Lett
690 233:269-275.

691

692

693 **Figure legends**

694

695 **FIG 1** Loss of the culturability (A, C, and E) and the viability (B, D, and F) of *V.*
696 *parahaemolyticus* ATCC 17082 (A-B), *V. parahaemolyticus* ATCC 33844 (C-D), and *V.*
697 *parahaemolyticus* ATCC 27969 (E-F) incubated in ASW (pH 6) microcosms supplemented
698 with varying concentrations of NaCl at 4^{o}C for 100 days.

699

700 **FIG 2** Changes of the cytotoxicity of *V. parahaemolyticus* ATCC (A and D) 17082, *V.*
701 *parahaemolyticus* ATCC (B and E) 33844, and *V. parahaemolyticus* ATCC (C and F) 27969
702 incubated in ASW (pH 6) microcosms at 4^{o}C for 80 days against (A-C) Vero and (D-F)
703 CACO-2 cells, respectively**.** (A-C); a, 0 day; b, 7 days; c, 21 days; d, resuscitated cells in
704 TSB after 21 days of cold-starvation; f, 80 days; g, resuscitated cells in TSB after 21 days of
705 cold-starvation; *, not determined. (D-F); a, 0 day; b, 7 days, c, resuscitated cells in TSB after
706 21 days of cold-starvation; d, 80 days; e, resuscitated cells in TSB after 80 days of cold-
707 starvation; *, not determined. Cells of VBNC *V. parahaemolyticus* suspended at 4^{o}C for 21
708 and 80 days were transferred onto TSB, and then incubated at 25^{o}C for consecutive 3 days,
709 respectively.

710

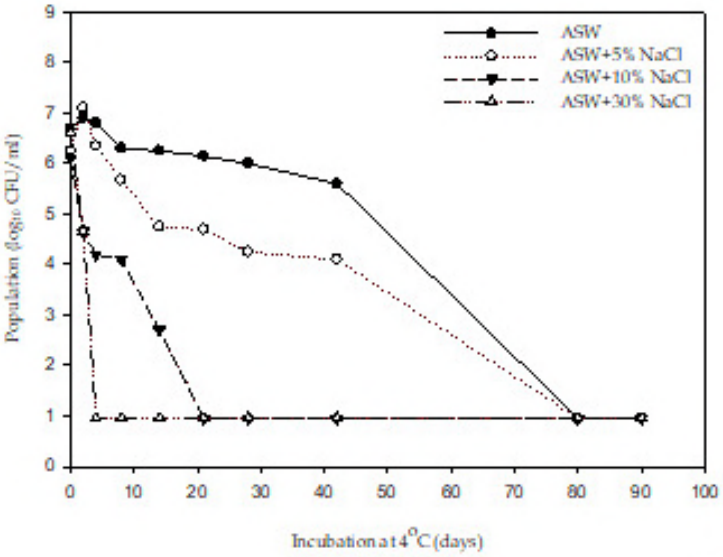
711 **FIG 3** Assessment of the cellular membrane permeability (A, ß-lactamase activity; B, ß-
712 galactosidase activity) of *Vibrio parahaemolyticus* ATCC 17082 and *V. parahaemolyticus*
713 ATCC 33844 incubated in ASW microcosms at 4^{o}C for 30 days.

714

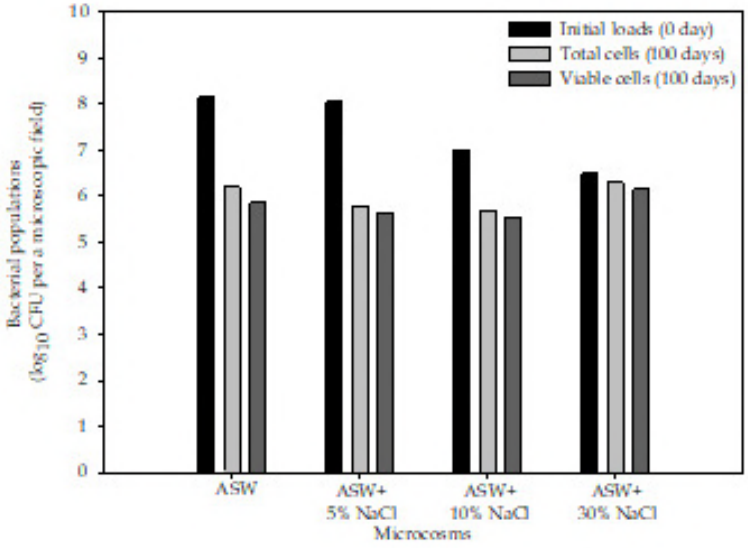
715 **FIG 4** Assessment of catalase activity (U/mg protein) of *V. parahaemolyticus* ATCC (A)

716 17082, *V. parahaemolyticus* ATCC (B) 33844, and *V. parahaemolyticus* ATCC (C) 27969 in
717 the VBNC state at 4^{o}C for 100 days.
718
719 **FIG 5** Investigation on the morphology of *V. parahaemolyticus* ATCC 33844 (A) before and
720 (B-C) after the entry into the VBNC state. Bacterial cells of *V. parahaemolyticus* ATCC
721 33844 were incubated in ASW (pH 6.1) microcosms containing (B) 0.75% NaCl or (C)
722 supplemented with 5% NaCl at 4^{o}C for 100 days.
723
724 **FIG 6** Comparison of fatty acid compositions between *V. parahaemolyticus* ATCC 17082
725 incubated in ASW (pH 6) microcosms at 4^{o}C for 90 days and resuscitated cells.
726
727 **FIG 7** A schematic diagram for elucidating the transition process of *V. parahaemolyticus* cells
728 into the viable-but-nonculturable state.
729
730

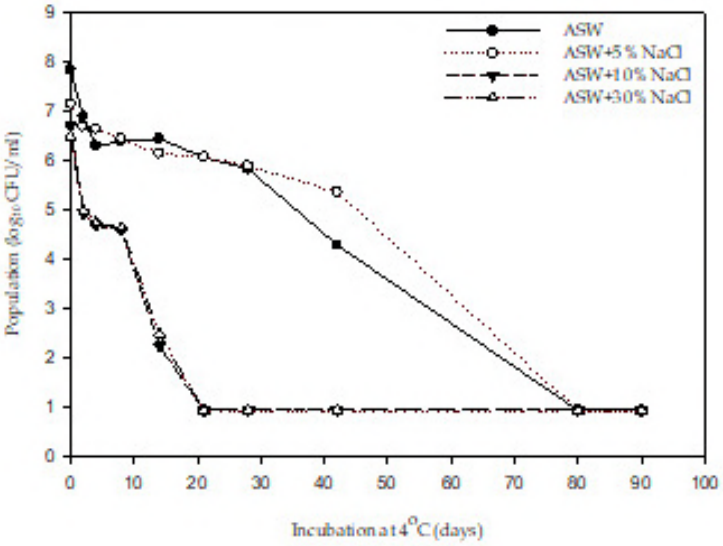
(A)


ASW
ASW+5% NaCl
ASW+10% NaCl
ASW+30% NaCl
Population (log10 CFU/ml)
Incubation at 4°C (days)

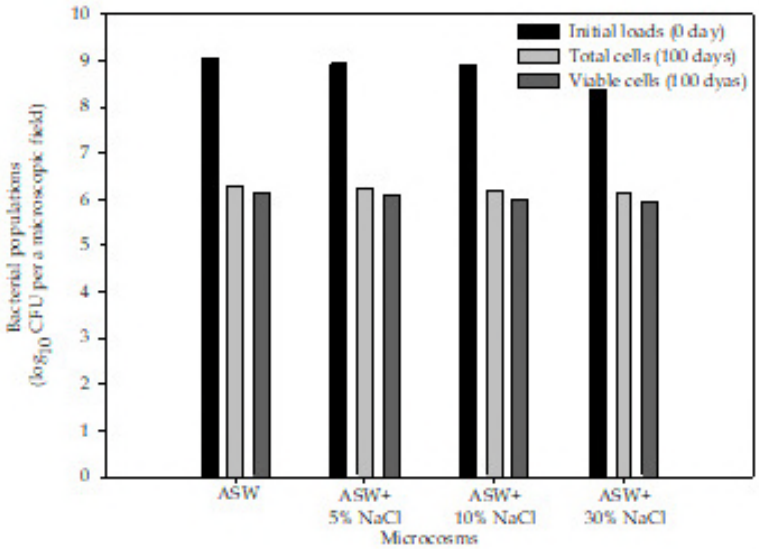
(B)


Initial loads (0 day)
Total cells (100 days)
Viable cells (100 days)
Bacterial populations (log10 CFU per a microscopic field)
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosms

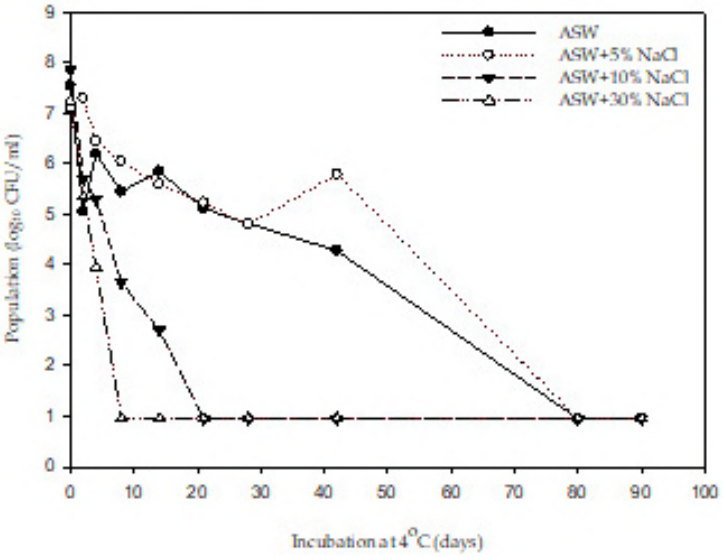
(C)


ASW
ASW+5 % NaCl
ASW+10% NaCl
ASW+30% NaCl
Population (log10 CFU/ml)
Incubation at 4°C (days)

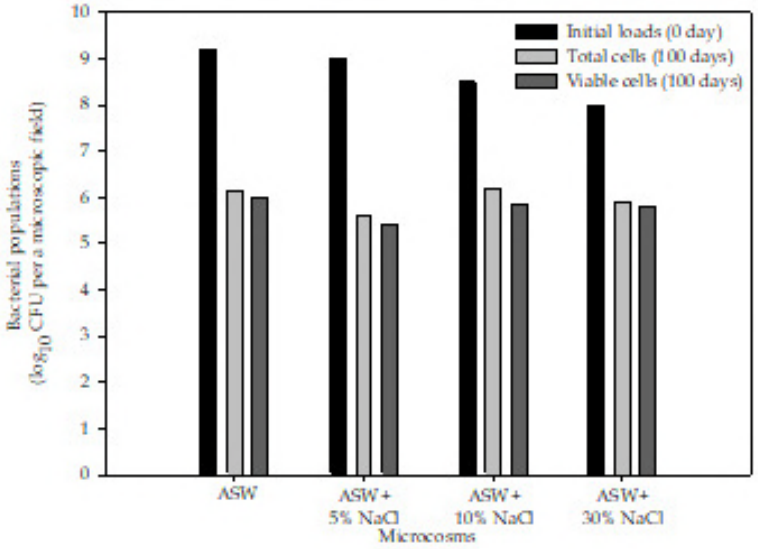
(D)

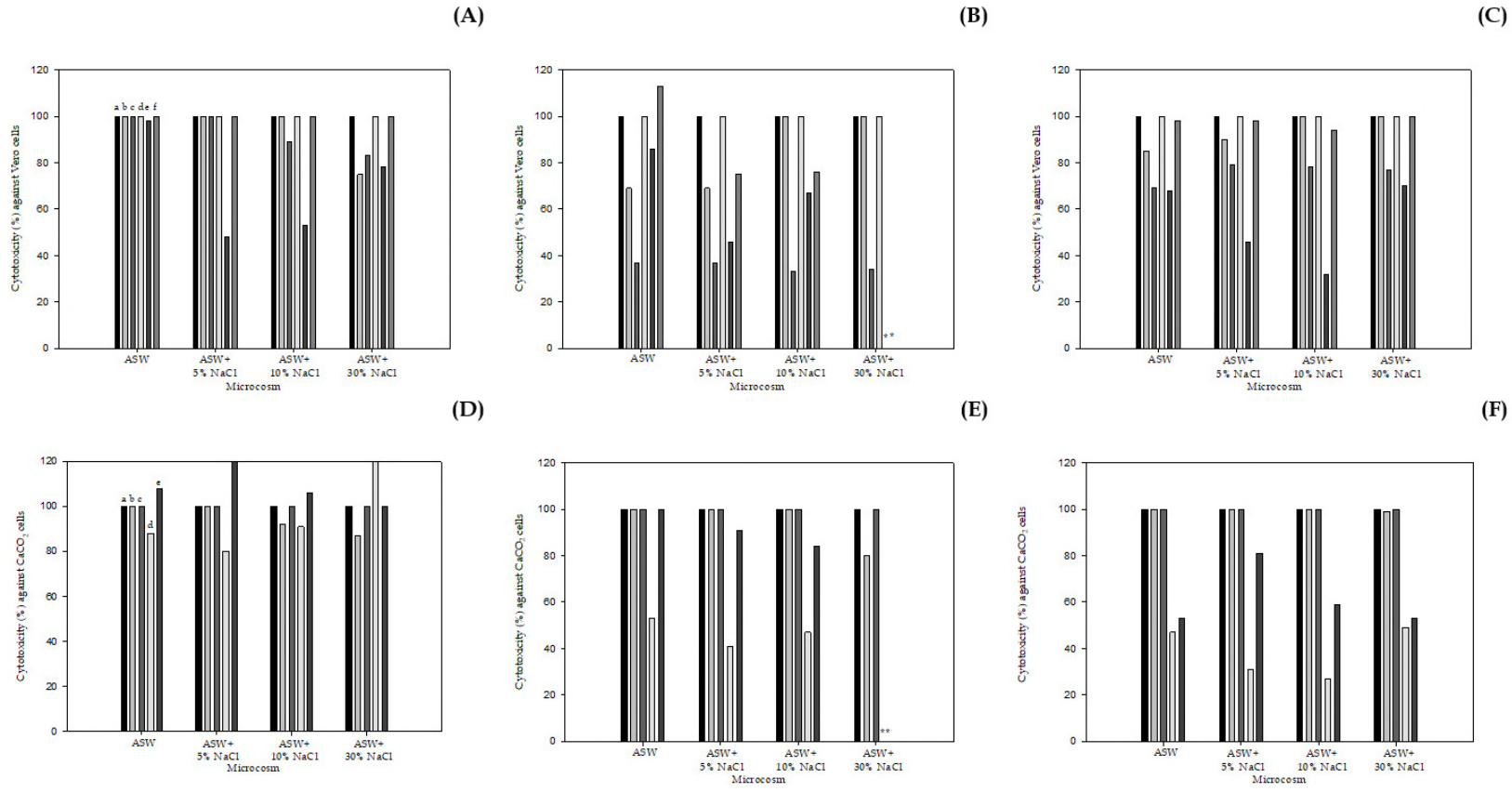

Initial loads (0 day)
Total cells (100 days)
Viable cells (100 dyas)
Bacterial populations (log10 CFU per a microscopic field)
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosms

(E)

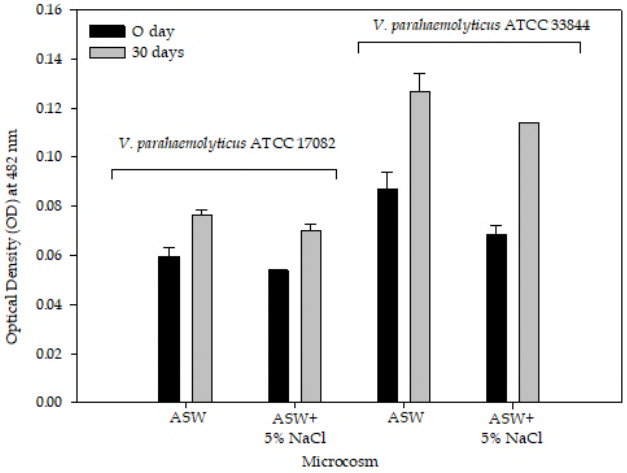

ASW
ASW+5% NaCl
ASW+10% NaCl
ASW+30% NaCl
Population (log10 CFU/ml)
Incubation at 4°C (days)

(F)

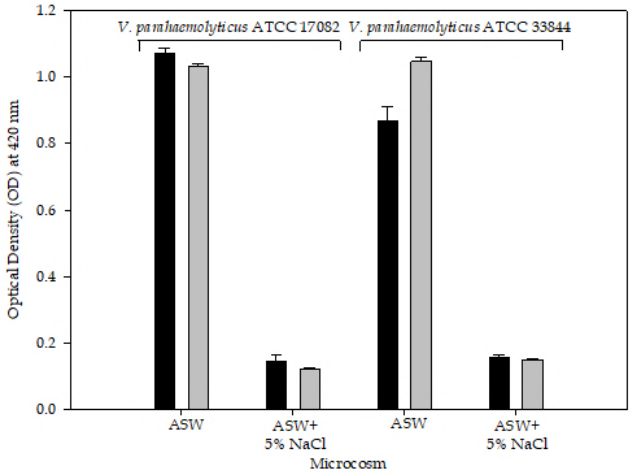

Initial loads (0 day)
Total cells (100 days)
Viable cells (100 days)
Bacterial populations (log10 CFU per a microscopic field)
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosms


(A)
(B)
(C)
(D)
(E)
(F)
Cytotoxicity (%) against Vero cells
Cytotoxicity (%) against CaCO3 cells
a b c de f
a b c
d
e
**
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosm
0
20
40
60
80
100
120

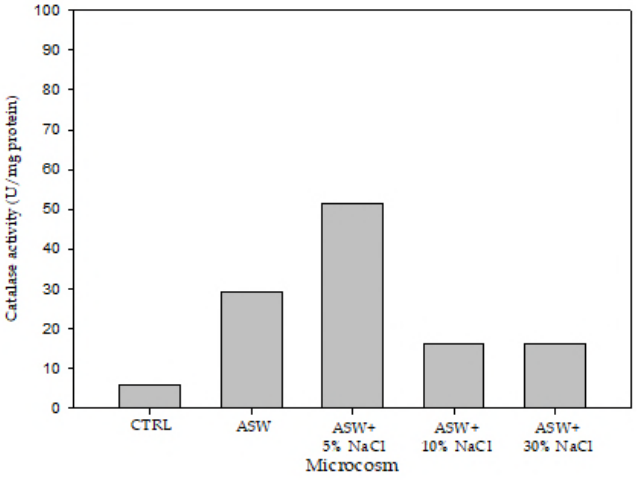
(A)


0 day
30 days
V. parahaemolyticus ATCC 33844
V. parahaemolyticus ATCC 17082
Optical Density (OD) at 482 nm
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ASW
ASW+ 5% NaCl
ASW
ASW+ 5% NaCl
Microcosm

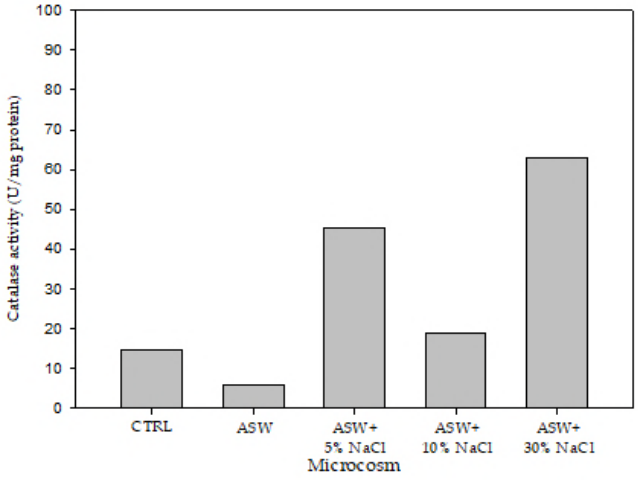
(B)


V. parahaemolyticus ATCC 17082
V. parahaemolyticus ATCC 33844
Optical Density (OD) at 420 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ASW
ASW+ 5% NaCl
ASW
ASW+ 5% NaCl
Microcosm

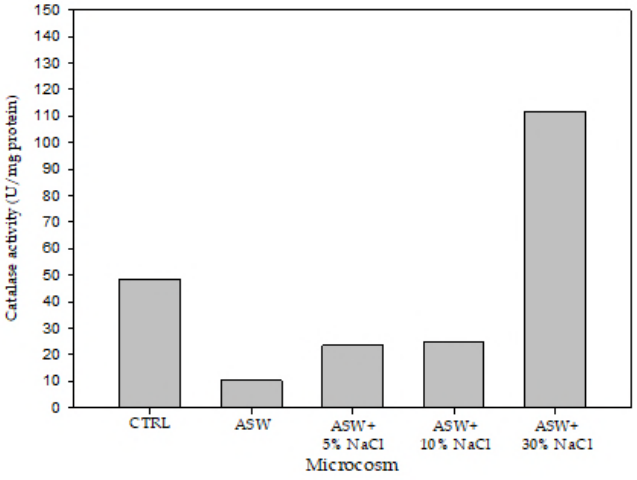
(A)


Catalase activity (U/mg protein)
100
90
80
70
60
50
40
30
20
10
0
CTRL
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosm

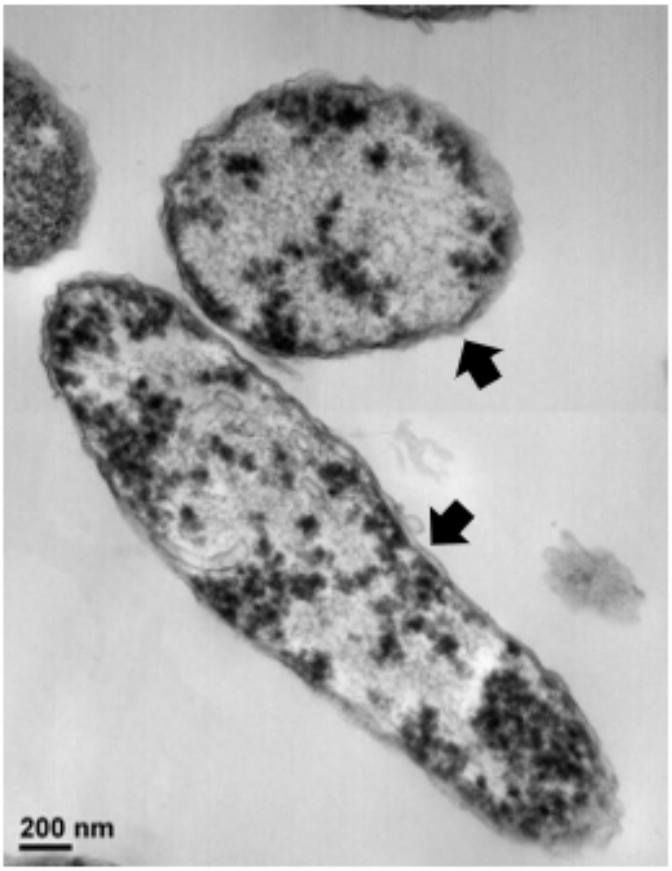
(B)


Catalase activity (U/mg protein)
100
90
80
70
60
50
40
30
20
10
0
CTRL
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosm

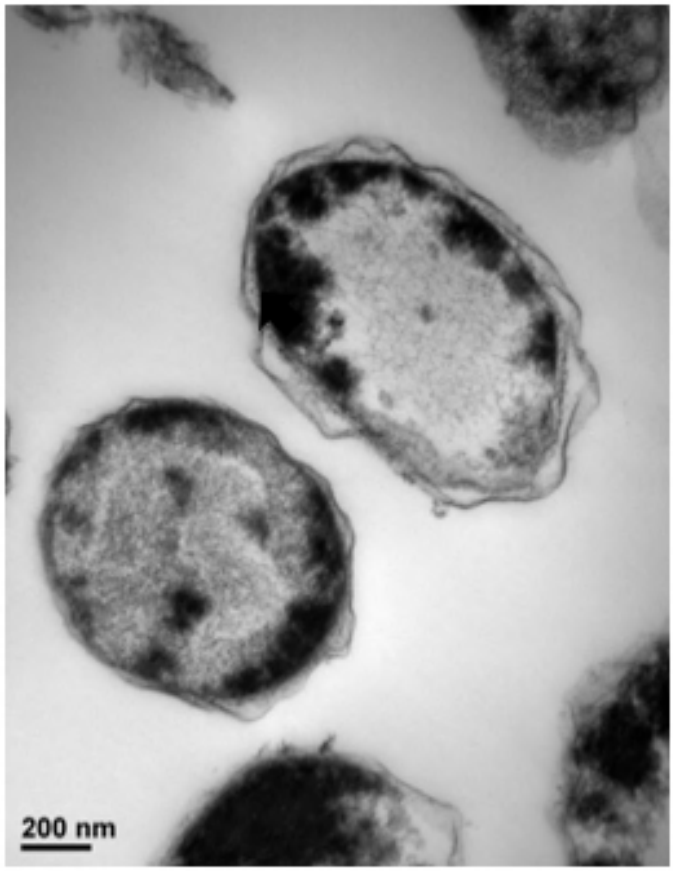
(C)


Catalase activity (U/mg protein)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
CTRL
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
Microcosm

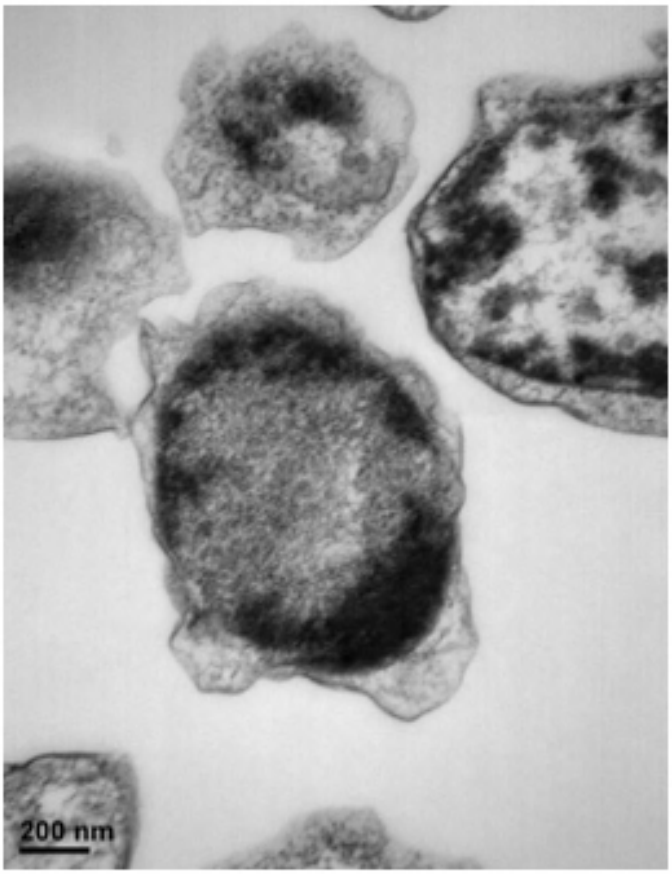
(A)

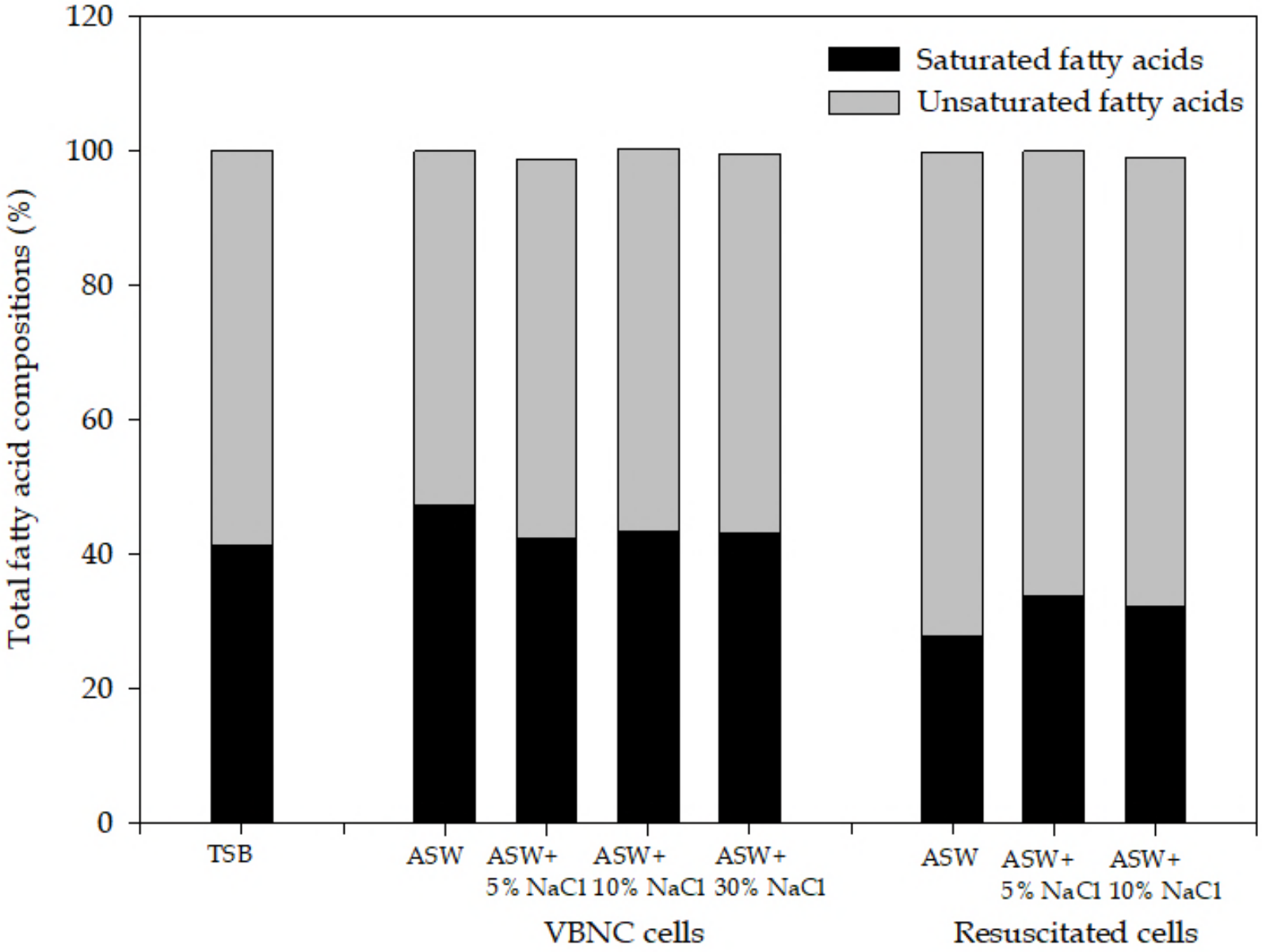


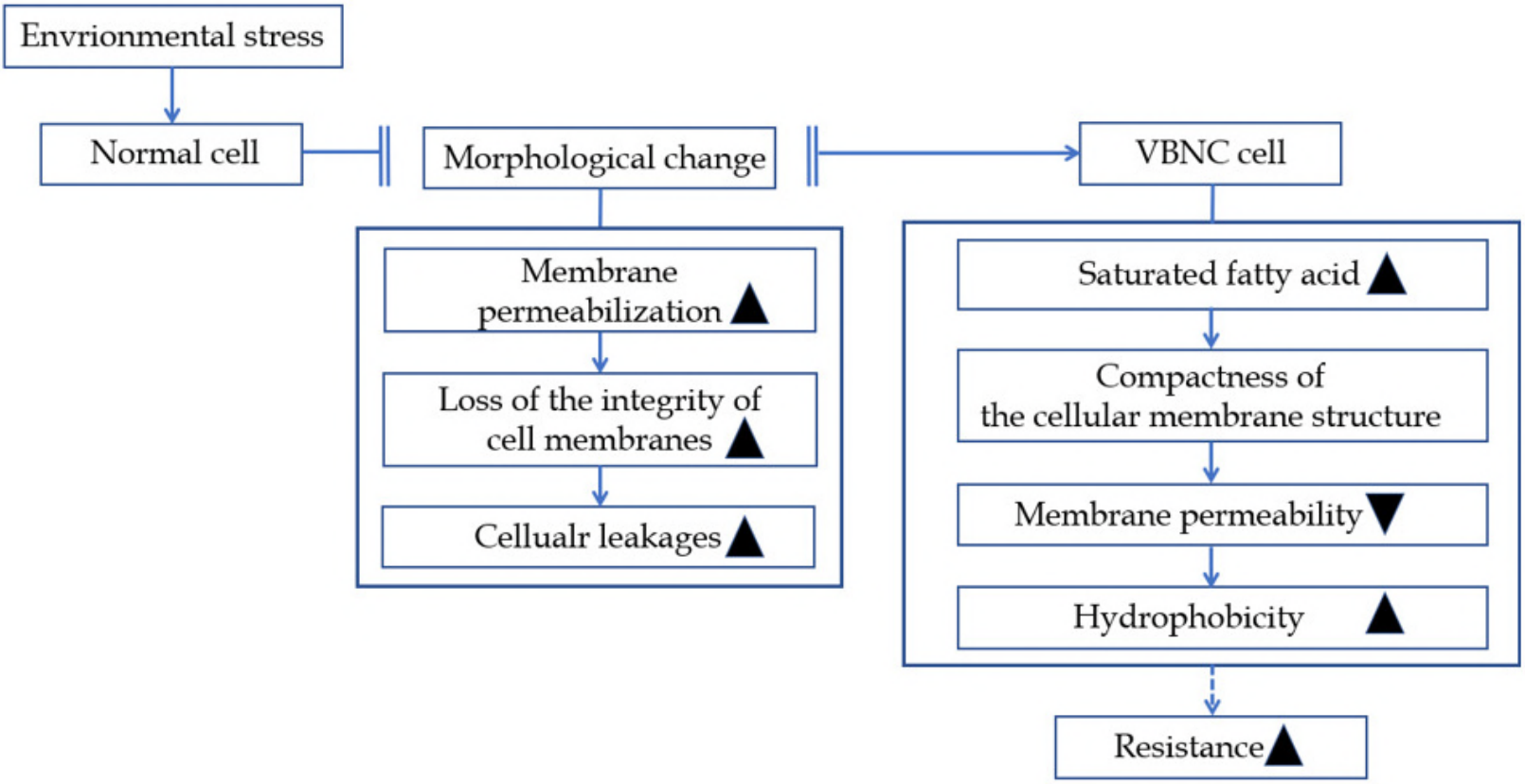
(B)



(C)




120
100
80
60
40
20
0
Total fatty acid compositions (%)
Saturated fatty acids
Unsaturated fatty acids
TSB
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
ASW+ 30% NaCl
ASW
ASW+ 5% NaCl
ASW+ 10% NaCl
VBNC cells
Resuscitated cells


Envrionmental stress
Normal cell
Morphological change
VBNC cell
Membrane permeabilization ▲
Loss of the integrity of cell membranes ▲
Cellualr leakages ▲
Saturated fatty acid ▲
Compactness of the cellular membrane structure
Membrane permeability ▼
Hydrophobicity ▲
Resistance ▲

1

TABLE 1 Measurement of the cellular leakage (nucleic acid, at OD 260 nm) of *V. parahaemolyticus* cells incubated in ASW (pH 6) microcosms at 4°C for 150 days

Pathogen	ATCC	Before (0 hrs):				After (150 days):			
		ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl	ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl
V. parahaemolyticus	17082	0.433	0.502	0.598	0.974	0.868	1.200	0.999	1.053
V. parahaemolyticus	33844	0.576	0.685	0.783	1.562	2.611	2.550	2.315	2.683
V. parahaemolyticus	27969	0.751	0.778	0.659	1.107	3.255	3.417	3.385	3.755

2

3

TABLE 2 Measurement of the cellular leakage (protein, at OD 280 nm) of *V. parahaemolyticus* cells incubated in ASW (pH 6) microcosms at 4°C for 150 days

Pathogen	ATCC	Before (0 hrs):				After (150 days):			
		ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl	ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl
V. parahaemolyticus	17082	0.390	0.435	0.465	0.797	0.426	0.602	0.600	0.638
V. parahaemolyticus	33844	0.466	0.537	0.630	1.316	1.270	1.218	1.226	1.433
V. parahaemolyticus	27969	0.573	0.597	0.521	0.910	1.588	1.747	1.872	2.351

4

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TABLE 3 Measurement of hydrophobicity (%) of viable-but-nonculturable *V. parahaemolyticus* at 4°C for 100 days

Pathogen	Microcosm	Incubation period (days)	
		0	**100**
V. parahaemolyticus ATCC 17082	ASW	37.1±2.7	ND[2]
	ASW+5% NaCl	26.3±3.2	37.5±0.2
	ASW+10% NaCl	45.8±3.6	46.8±5.0
	ASW+30% NaCl	27.2±2.5	62.2±2.8
V. parahaemolyticus ATCC 33844	ASW	22.0±1.9	ND
	ASW+5% NaCl	22.8±.04	20.9±2.5
	ASW+10% NaCl	22.2±2.4	37.1±3.7
	ASW+30% NaCl	29.8±1.8	41.1±0.9
V. parahaemolyticus ATCC 27969	ASW	22.0±0.8	25.2±16.6
	ASW+5% NaCl	23.0±2.0	17.5±1.2
	ASW+10% NaCl	23.1±1.9	23.1±6.4
	ASW+30% NaCl	20.8±15.8	43.7±5.8

[1] Surface hydrophobicity (%) of *V. parahaemolyticus* strains was calculated, following an equation as; $(A_O-A_1)/A_O$ X 100. Ao, the absorbance of the untreated cell suspension at 600 nm; A_1, the absorbance of the bacterial assay tube at 600 nm.

[2] ND, not determined.

6

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TABLE 4 Comparison on the fatty acid composition (%) of viable-but-nonculturable *V. parahaemolyticus* ATCC 17082 at 4°C for 90 days

Fatty acid	Name	Category	Microcosm				
			Ctrl	ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl
11:0 iso 3OH	3-Hydroxy-9-Methyldecanoic acid	Branched-chain	0.75%	ND[1]	ND	0.30%	0.32%
12:0	Lauric acid	Saturated	2.02%	4.37%	4.38%	4.37%	4.44%
12:0 2OH	2-Hydroxylauric acid	Hydroxy	1.63%	1.94%	3.12%	3.05%	2.92%
14:0	Myristic acid	Saturated	7.25%	8.05%	9.11%	9.19%	8.81%
14:0 3OH	ND	Hydroxy	1.86%	1.50%	1.68%	1.58%	1.84%
16:0	Palmitic acid	Saturated	25.64%	28.65%	23.88%	23.36%	23.05%
16:0 N alcohol	Cetyl alcohol	Saturated	-[2]	ND	0.20%	0.40%	0.50%
18:0	Stearic acid	Saturated	2.19%	2.71%	ND	1.23%	1.22%
	Total (saturated fatty acid)		41.34%	47.22%	42.37%	43.48%	43.10%
16:1 w7c	Palmitoleic acid	Unsaturated	26.76%	24.52%	32.98%	34.11%	35.77%
16:1 w6c	cis-10-Palmitoleic acid	Unsaturated	3.78%	7.13%	5.11%	4.44%	3.82%
16:1 w5c	cis-11-Palmitoleic acid	Unsaturated	-	-	0.50%	0.42%	0.53%
16:1 w7c alcohol	Palmitoleyl alcohol	Unsaturated	-	-	-	0.37%	-
17:1 anteiso w9c	(7Z)-13-Methyl-7-Hexadecenoic acid	Unsaturated	7.52%	3.12%	2.13%	2.42%	2.84%
17:1 iso	Isomargaric acid	Branched-chain	-	-	0.85%	0.24%	-
18:1 w9c	Oleic acid	Unsaturated	-	-	-	0.38%	-
18:1 w7c	cis-Vaccenic acid	Unsaturated	20.61%	18.01%	14.90%	14.16%	13.18%
18:1 w5c	cis-13-oleic acid	Unsaturated	-	-	-	0.35%	-
18:2 w6c	Linoleic acid	Unsaturated	-	-	-	-	0.37%
	Total (unsaturated fatty acid)		58.67%	52.78%	56.47%	56.89%	56.51%

[1] ND, not determined.
[2] -, not found.

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TABLE 5 Comparison of fatty acid composition (%) of *V. parahaemolyticus* ATCC 17082 to be recovered from the VBNC state

Fatty acid	Name	Category	Microcosm			
			Ctrl	ASW	ASW+5% NaCl	ASW+10% NaCl
11:0 iso 3OH	3-Hydroxy-9-Methyldecanoic acid	Branched-chain	0.75%	0.39%	-[1]	-
12:0	Lauric acid	Saturated	2.02%	2.71%	2.75%	2.95%
12:0 2OH	2-Hydroxylauric acid	Hydroxy	1.63%	2.48%	2.55%	2.78%
13:0 iso	Isotridecanoic acid	Branched-chain	-	0.65%	0.63%	0.65%
13:0 3OH	ND	Branched-chain	-	-	0.29%	0.28%
14:0	Myristic acid	Saturated	7.25%	4.50%	4.18%	4.70%
14:0 3OH	ND	Hydroxy	1.86%	2.79%	3.09%	2.40%
15:0 anteiso	12-Methylmyristic acid	Saturated	-	-	-	0.38%
16:0	Palmitic acid	Saturated	25.64%	10.40%	15.52%	14.65
16:0 iso	Isopalmitic acid	Branched-chain	-	-	0.33%	0.52%
16:0 N alcohol	Cetyl alcohol	Saturated	-	-	-	-
17:0	Margaric acid	Saturated	-	1.45%	2.11%	1.75%
17:0 anteiso	Anteisomargaric acid	Branched-chain	-	0.82%	0.47%	0.63%
17:0 iso	Isomargaric aid	Branched-chain	-	-	0.34%	0.44%
18:0	Stearic acid	Saturated	2.19%	1.68%	1.57%	0.55%
	Total (saturated fatty acid)		41.34%	27.87%	33.83%	32.30%
16:1 w7c	Palmitoleic acid	Unsaturated	26.76%	24.55%	23.14%	26.80%
16:1 w7c alcohol	Palmitoleyl alcohol	Unsaturated	-	-	0.22%	-
16:1 w6c	cis-10-Palmitoleic acid	Unsaturated	3.78%	7.03%	8.22%	10.16%
17:1 anteiso w9c	(7Z)-13-Methyl-7-Hexadecenoic acid	Unsaturated	7.52%	3.83%	1.09%	2.38%
17:1 w8c	cis-margaroleic acid	Unsaturated	-	2.00%	1.90%	1.84%
17:1 w6c	(11Z)-11-Heptadecenoic acid	Unsaturated	-	1.39%	1.22%	1.37%
18:1 w7c	cis-Vaccenic acid	Unsaturated	20.61%	32.17%	30.38%	23.98%
18:2 w6c	Linoleic acid	Unsaturated	-	0.93%	-	0.26%
	Total (unsaturated fatty acid)		58.67%	71.90%	66.17%	66.79%

[1] -, not found.

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TABLE 6. Determination of cellular properties of *V. parahaemolyticus* in the VBNC state

Property[1]		Category		
		Normal cells	**VBNC cells**	**Resuscitated cells**
Cell size		-	▼	-
Morphology		Rod	Coccus	Rod
Membrane damage		-	Blebbing	-
Cytotoxicity		▲	▼	▲
Hydrophobicity		-	▲	ND[2]
Fatty acid composition	SFA	-	▲	▼
	USFA	-	▼	▲
Enzyme activity	Catalase	-	-	ND
	GST	-	-	-
Cellular leakages		-	▲	ND

[1] SFA, saturated fatty acid; USFA, unsaturated fatty acid; GST, glutathione-S-transferase.
[2] ND, not determined.

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