

INFLUENCE OF LOW TEMPERATURE STORAGE AND SALINITY ON FATTY ACID PROFILE AND INACTIVATION BY SIMULATED GASTRIC JUICE OF HUMAN PATHOGENIC BACTERIUM *VIBRIO VULNIFICUS*

Under starvation conditions in artificial seawater, cells of *V. vulnificus* did not adjust their membrane fluidity to storage temperature within the investigated time frame. However, a significant switch ($p < 0.05$) from C18:1 ω 7c into C18:1 ω 6c by double bond relocation was observed. The relocation occurred faster at ambient temperatures compared to refrigerated temperatures. It is generally known that majority of *V. vulnificus* associated infections occur during summer hot months. *Vibrio vulnificus* ATCC 27562 was significantly less resistant to simulated gastric fluid (pH 4.0) after 7-day storage at 4 °C compared to the control, with D-values of 3.7 and 7.8 minutes, respectively. Therefore, higher sensitivity of the pathogen to the gastric fluid in winter harvested oysters may also impact the low number of outbreaks.

KEY WORDS: *Vibrio vulnificus*, cell membrane, fatty acid profile, survival, simulated gastrointestinal fluid.

INTRODUCTION. Vibrios are Gram-negative, rod-shaped, non-sporoforming, halophilic, facultatively anaerobic, and motile bacteria (straight or curved) associated with seawater and marine species. Three *Vibrio* species, namely, *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*, are well-documented human pathogens. *V. vulnificus* has an optimal growth temperature of 37 °C the growth does not occur below 13 °C. It is generally believed that the bacterium can survive low temperatures for prolonged periods of time by entering a Viable But Non-Culturable (VBNC) state, where cells metabolize but do not divide [1]. Pre-starvation of *V. vulnificus* in artificial seawater at room temperature for 1 to 2 hours did not prevent entry into VBNC state after temperature downshift to 5 °C, however, if cells were starved at room temperature for ca. 4 hours, they did not enter VBNC state and maintained culturable cell density of at least 5 log₁₀ cfu/ml within 14 days [7].

Vibrio vulnificus emerged as a foodborne pathogen in the late 1980's, primarily associated with seawater and raw oyster consumption. In humans, *V. vulnificus* is highly invasive, capable of causing primary septicemia with mortality rates of approximately 60 %. The majority (~95 %) of all foodborne *V. vulnificus* infections occur in individuals who are immunocompromised, have diabetes, or have high serum iron levels due to

chronic liver damage, such as cirrhosis or hepatitis [9]. Another route of *V. vulnificus* infection includes upper or lower extremity wound contact with contaminated seafoods or seawater. Symptoms of this type of infection usually include localized pain, edema, erythema, and ultimately limb necrosis [6]. J. D. Oliver and R. Bockian [8] showed that *V. vulnificus* viable but not culturable cells retained their virulence against mice by confirming their death after injection of inoculated refrigerated (5 °C, 7 days storage) artificial seawater containing <0.04 CFU of culturable cells but >10⁵ cells in VBNC state. Despite this evidence, questions remain about the ability of cold-starved culturable *V. vulnificus* to cause foodborne infections.

Cellular fatty acid profile of bacterial species fluctuates under different physiological conditions [12]. For example, the ability of bacteria to multiply under suboptimal conditions (low pH, in the presence of toxic chemicals, refrigeration or elevated temperatures) requires membrane fluidity modification, a phenomenon known as homeoviscous adaptation. It has been shown that membrane fluidity can be altered through membrane fatty acid profile modification, with shorter chain or unsaturated fatty acids making the cell membrane more fluid [11]. Studies by K. Linder and J. D. Oliver [5] examined changes in membrane fatty acids in viable but nonculturable state of *V. vulnificus*, however, questions about cold stored

starved but culturable *V. vulnificus* remains unanswered.

The first objective of this study was to investigate *V. vulnificus* fatty acid profile changes after the storage of pre-starved pathogen at different salinities and temperatures using gas chromatography of fatty acid methyl esters (GC-FAME) (MIDI, Inc, Newark, DE). The hypothesis was that a decrease in seawater temperature will cause an increase in short-chain and unsaturated fatty acids present in the cell envelope of *V. vulnificus*.

The second objective was to investigate the ability of starved culturable *V. vulnificus* cells to survive human simulated gastric fluid (pH 4.1) and therefore to maintain virulence against humans.

METHODS OF RESEARCH. *V. vulnificus* strain ATCC 27562 (Lyfocults, Quality Technologies, LLC, Newbury Park, CA, USA) was maintained at room temperature on Trypticase Soy Agar-2 % NaCl (TSA-2, BD Diagnostic Systems, Sparks, MD, USA) slants with 2-week re-streaking period to a fresh TSA-2 slant. The strain was of opaque phenotype. For experimentation, the culture was streaked on TSA-2 and grown for 12 hours at 35 °C. Single colony of the bacterial culture was transferred to 60 ml of Trypticase Soy Broth supplemented with 2 % NaCl (TSB-2, BD Diagnostic Systems, Sparks, MD, USA) and grown in an environmental shaker (PsychroTherm, New Brunswick Scientific Co, Inc, Edison, NJ, USA) for 18 hours at 25 °C under agitation (150 rpm) yielding a turbid broth, which served as a working culture for further studies.

Artificial seawater (12, 20, and 35 ppt) was freshly prepared for each replication by dissolving 12, 20, and 35 g of seasalt (CrystalSea MarineMix, Bioassay Laboratory formula, Marine Enterprise International, Baltimore, MD, USA) in 1 L of distilled water followed by filter-sterilization (0.45 µm cellulose acetate, Corning Costar, Corning, NY, USA) and its pH was measured using an Accumet AR-60 pH meter (ThermoFisher Scientific, USA).

Simulated gastric fluid was freshly prepared for each replication by dissolving 20 mg pepsin (from porcine stomach, 424 units/mg solid, Sigma), 0.7 g mucin (type II: crude, from porcine gastric mucosa, Sigma), and 1.7 g NaCl in 200 ml sterile distilled water. Sterile 1.0 N hydrochloric acid was used to adjust pH to 4.1.

Three 6-ml aliquots of a working broth culture of *V. vulnificus* strains ATCC 27562 were transferred to 15-ml tubes and centrifuged (Centrifuge Model 228, ThermoFisher Scientific) for seven minutes at 1.380 x g. Cell pellets were re-suspended in 6 ml of sterile seawater (12, 20, or 35 ppt), and centrifuged again under the same conditions.

This procedure was repeated to obtain double-washed cells. Three 2-ml aliquots from double-washed cell suspensions were transferred to three 18-ml quantities of corresponding sterile seawater (12, 20, or 35 ppt) to achieve a 1:10 dilution. These samples were held at 25 °C for 2 hours and then incubated at 4, 10, and 25 °C for 7 days.

Vibrio vulnificus survival in simulated gastric fluid after exposure to seawater under starvation was assessed on day 0 (after 2-hours adaptation in 20 ppt seawater at 25 °C), and on day 7 for samples stored at 4, 10, or 25 °C. Refrigerated samples were immediately returned to cold storage after aliquot transfer. *Vibrio vulnificus* counts in stored samples were determined before exposure to simulated gastric fluid to estimate cell population in simulated gastric fluid at time point "0 min". To assess survival of the bacterium in simulated gastric fluid, 1 ml aliquots of inoculated stored seawater samples were transferred to 9 ml simulated gastric fluid equilibrated to 37 °C in a water bath. These were vortexed for 10 seconds and immediately returned to the 37 °C water bath. One ml aliquots from each treatment were transferred after 3, 6, 9, 12, and 15 minutes to 9 ml PBS blanks, vortexed, and further serially diluted as needed in 9 ml PBS. One tenth milliliter of each dilution was then plated in duplicate on TSA-2 (48 hours, 37 °C) to evaluate vibrio survival at each time point. Counts were expressed as log₁₀ cfu/ml of simulated gastric fluid. Addition of 1 ml of 20 ppt seawater to 9 ml simulated gastric fluid did not result in simulated gastric fluid pH change. Addition of 1 ml of simulated gastric fluid (pH 4.1) to 9 ml of phosphate-buffered saline (PBS, pH 7.4) did not result in PBS pH change.

As for fatty acid profile analysis, seven 6-ml aliquots of the working broth culture were transferred to 15-ml tubes and centrifuged for seven minutes at 1.380 x g (Centrifuge model 228). Cell pellets were re-suspended in 6 ml of sterile seawater (12, 20, or 35 ppt) and centrifuged again under the same conditions. Double-washed cells were finally re-suspended in 6 ml of sterile seawater (~10⁹ cfu/ml). One sample was analyzed for fatty acid profile after a 2-hour room temperature cell adaptation period (day 0), while three 2-sample sets were immediately transferred to 4, 10, and 25 °C incubators. One sample from each incubator was withdrawn on day 7 and day 14, and analyzed for change in fatty acid profile. In order to assess bacterial fatty acid profile, each analyzed sample was centrifuged for 7 minutes at 2.700 x g to obtain a cell pellet (biomass ca. 40 mg). Procedures for cell lysis and fatty acid saponification, methylation, extraction, and alkali wash were performed as described previously [12].

Statistical analysis was done using Statsoft STATISTICA by the Department of System Investigations of SHEI “I. Ya. Horbachevsky Ternopil State Medical University MPH of Ukraine”. A two-factorial design with three replications was utilized to determine the influence of seawater salinity (12, 20, and 35 ppt) and storage day (day 0, day 7, and day 14) on percentage of each fatty acid found at three different storage temperatures (4, 10, and 25 °C). An experimental design of five replications with two factors was used to compare the effects of *V. vulnificus* storage conditions (day 0, 4 °C day 7, 10 °C day 7, and 25 °C day 7) and exposure time (0, 6, 9, 12, and 15 minutes) in simulated gastric fluid, and their combined effect on *V. vulnificus* counts. D-values in simulated gastric fluid were expressed in minutes and reflected the time needed to reduce *V. vulnificus* population by 1 log₁₀ cfu/ml. D-values were calculated for each replication and each storage condition based on 0 to 12 minutes time frame. Fisher’s Significant Difference (LSD) Test was utilized to separate treatment means when differences (p<0.05) occurred among treatments.

RESULTS AND DISCUSSION. Nine major fatty acids were recovered from cellular membranes of *V. vulnificus*; notably, 12:0 3OH, 14:0, 16:1ω9c, 16:0, 18:1ω7c, 18:1ω6c, 18:0, summed feature 2 (16:1 ISO I & 14:0 3OH), and summed feature 3 (15:0 ISO 2OH & 16:1ω7c). Minor fatty acids with abundance percentage of 0 to 0.7 were not reported since they were not recovered consistently due to the low amounts present.

Significance of influence of starvation day and salinity on individual fatty acid percentage is shown in Table. Starvation day, but not salinity, significantly influenced (p<0.05) individual fatty acid percentages for most treatments. The notable exceptions were 18:1ω7c (cis-vaccenic acid) and 18:1ω6c fatty acids, where both starvation day and salinity influenced their percentage. Though not analyzed, it appeared that lower temperatures together with starvation prevented changes in fatty acid profile of *V. vulnificus*.

Results for fatty acid profile analysis for *V. vulnificus* ATCC 27562 are shown in Figure 1. All salinities were combined for a specific starvation day because the influence of salinity was not significant (p>0.05) in most cases. Increased storage time at any temperature caused a decrease (p<0.05) in the amount of C14:0 and summed feature 3 and an increase (p<0.05) in C18:0 (Fig. 1). Storage at 25 °C also caused a significant increase (p<0.05) in C16:0.

As noted by K. Linder and J. D. Oliver [5], a decrease in incubation temperature should cause bacterial cells to increase the percentage of unsaturated fatty acids and short-chain fatty acids in their cell envelopes. At the same time, the percentage of saturated and long-chain fatty acids should decrease in membrane phospholipids in order to maintain sufficient membrane fluidity. Apparently, under starvation with no nutrients available, cells of *V. vulnificus* did not adjust their membrane fluidity as a response to storage temperature during the time frame studied in the present experiment, with a notable exception of a shift from 18:1ω7c to 18:1ω6c fatty acid. The shift involved relocation of a single double bond in 18:1 unsaturated fatty acid from ω7 carbon to ω6 carbon position. This trend was noted for the cells stored at 25 °C where significant amounts of C18:1ω7c fatty acid were found initially (18 to 19 %) followed by transformation into C18:1ω6c during room temperature starvation.

At 4 and 10 °C, transformation of C18:1ω7c to 18:1ω6c was slower, with larger amounts of C18:1ω7c retained on day 7. It appears that *V. vulnificus* cells, while starved and not growing, are trying to “preserve” their original 25 °C-grown state while refrigerated with a notable exception of C18:1ω7c to 18:1ω6c shift (Fig. 1).

Studies by K. Linder and J. D. Oliver [5] examined changes in membrane fatty acids in viable but nonculturable state of *V. vulnificus*. According to them, C16:1, C16:0, and C18:1 fatty acids predominated on day zero in artificial seawater at 5 °C, comprising 46, 32, and 12 % of total extracted fatty acids, respectively. Only slight

Table – Statistical influence of starvation day (0, 7, and 14) and salinity (12, 20, and 35 ppt) on percentage of individual fatty acids for different treatments (bacterial strain and starvation temperature). Plus (+) denotes significant influence (p<0.05), while minus (-) denotes non-significant influence (p>0.05). Slash character separates the influence of starvation day (left) from salinity (right)

Storage temp, °C	Significance of starvation day/salinity influence on <i>V. vulnificus</i> fatty acid abundance								
	12:0 3OH	14:0	16:1ω9c	16:0	18:1ω7c	18:16c	18:0	sum feature 2	sum feature 3
4	+/-	+/-	-/-	-/-	+/-	+/+	+/-	+/-	+/-
10	+/-	+/-	-/-	-/-	+/+	+/-	+/-	+/-	+/-
25	+/+	+/-	+/-	+/-	+/-	+/-	+/-	-/-	+/-

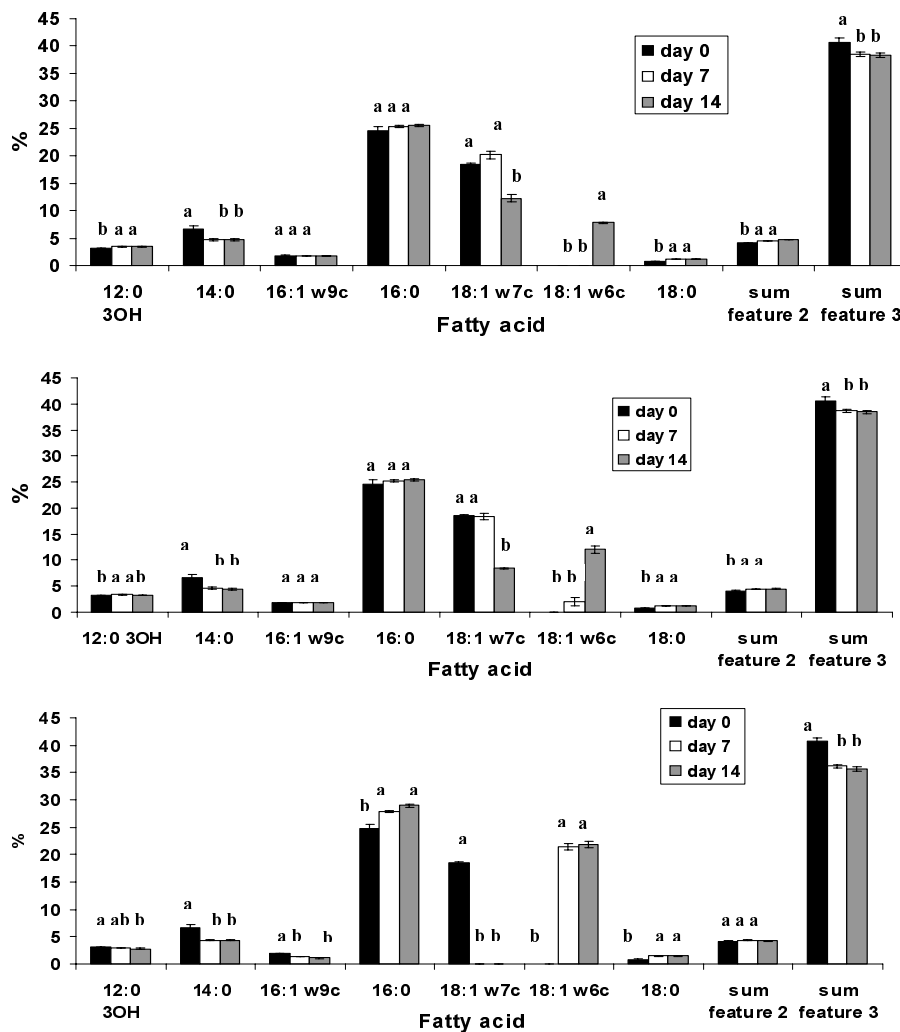


Fig. 1. Fatty acid profile analysis of starved *V. vulnificus* ATCC 27562 in artificial seawater with all salinities combined at 4 °C (top), 10 °C (middle), and 25 °C (bottom). Means with the same letters within the same fatty acid are not significantly different ($p > 0.05$).

changes (<2%) were observed in these fatty acids on day 13, while *V. vulnificus* cells lost their culturability from 7 log₁₀ cfu/ml to 3.5 log₁₀ cfu/ml. The present study obtained similar percentages for C16:1, C16:0, and C18:1 fatty acids, which were 47, 24, and 19%, respectively. Fatty acid C16:1 percentage was estimated as a sum of C16:1ω9c, summed feature 2, and summed feature 3. According to [5], *V. vulnificus* fatty acid profile showed a drastic change only at the point of complete non-culturability (5 °C, day 24, 0 log₁₀ cfu/ml). Notably, both C16:0 and C16:1 percentage decreased from 32 and 46% to 11 and 8%, respectively. At the same time, the percentage of fatty acids with chain length of less than 16 carbons increased from 5.4 to 28.9% with the concomitant appearance of long-chain fatty acids (C19:0, C20:0, and C22:1).

Results from the present study are similar to those seen by K. Linder and J. D. Oliver [5]. Fatty acid profiles were similar on day 14 (present study)

versus day 13 [5]. On the other hand, present results did not reveal reductions in C16:0 and C16:1 or appearance of C19:0, C20:0, and C22:1 fatty acids during storage. This is possibly explained by the shorter incubation period used (14 days vs. 24 days) and the lack of nutrients.

The presently used GC-FAME microbial identification system is more sensitive for identification of not only specific fatty acids with defined chain length and number of double bonds, but also for differentiation of the same fatty acid with different double bond location. For example, K. Linder and J. D. Oliver [5] did not observe any significant changes in C18:1 fatty acid during 13 days storage at 5 °C, while the present effort identified a significant relocation of the double bond from ω6c to ω7c position as discussed earlier.

The significance of double bond relocation is difficult to explain. The length of the carbon backbone and number of unsaturated double bonds did not change in octadecenoic acid

(C18:1). These two factors have been shown to be important in influencing membrane fluidity of *Escherichia coli* O157:H7 [12]. Similar shift of C18:1 ω 7c into C18:1 ω 6c was shown by H.-Y. Kahng [2] for *Pseudomonas* sp. strain KK1 capable of growing on naphthalene source. *Pseudomonas* KK1 while growing in the presence of naphthalene had greater C18:1 ω 6c contents at the expense of C18:1 ω 7c. The author concluded that such switch might be a physiological adaptation of

Pseudomonas sp. in response to toxic chemicals and to the utilizable substrate, naphthalene. The same switch was observed in the current study.

The high acidity of gastric fluid is the main barrier infectious bacteria must overcome in order to establish infection in the human gastrointestinal tract [3, 4]. Figure 2 shows inactivation curves of *V. vulnificus*, either untreated or stored at different temperatures, during exposure to simulated gastric fluid.

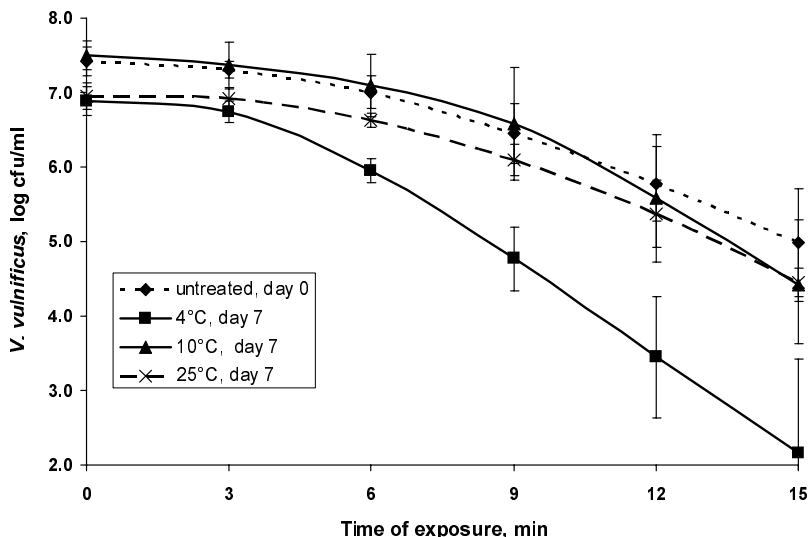


Fig. 2. Survival of *V. vulnificus* ATCC 27562 in Simulated Gastric Fluid (SGF) after storage in sterile 20 ppt seawater for 7 days at 4, 10, and 25 °C.

There was a significant influence ($p < 0.05$) of *V. vulnificus* storage condition and exposure time to simulated gastric fluid, as well as their combined effect, on *V. vulnificus* counts. There also was a significant treatment difference ($p < 0.05$) between D-values of *V. vulnificus* inactivation by simulated gastric fluid (Fig. 2). D-value for untreated *V. vulnificus* was 7.8 ± 2.4 minutes, while the D-value for 7-day, 25 °C stored *V. vulnificus* was 9.1 ± 3.9 minutes.

J. Koo et al. [3] found D-values for three non-starved *V. vulnificus* strains ranged from 2.8 to 4.1 minutes at pH 4.0 in the same simulated gastric fluid composition. It appeared that simulated gastric fluid had better bactericidal ability compared to acidified nutrient broth at the same pH level. For example, J. Koo et al. [4] showed D-values ranged from 26.7 to 51.8 minutes for three strains of *V. vulnificus* in acidified TSB-2 % NaCl with hydrochloric acid at pH 4.0.

Interestingly, as the storage temperature decreased, *V. vulnificus* became more susceptible to the bactericidal effect of low pH, with D-values of 3.7 ± 0.89 minutes and 6.6 ± 1.99 minutes for *V. vulnificus* stored at 4 and 10 °C, respectively (Fig. 3).

It appears that low temperature adaptation may offer less resistance to low pH that might be experienced by the bacterium during gastric transit. Based on the obtained data, *V. vulnificus* might be more susceptible to lethal effects of gastric fluid after starvation storage at refrigerated temperatures. This may provide additional explanation why the majority of *V. vulnificus* cases (89 %) associated with raw oyster consumption occur when seawater harvesting temperatures exceed 22 °C [10].

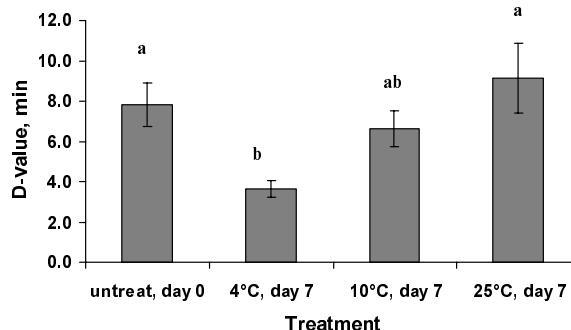


Fig. 3. Inactivation D-values of starved *V. vulnificus* stored under different conditions after exposure to simulated gastric fluid. Means with the same letter are not significantly different ($p > 0.05$).

CONCLUSIONS. Under starvation conditions, cells of *V. vulnificus* did not adjust their membrane fluidity to storage temperature within the investigated time frame. However, a significant switch ($p < 0.05$) from C18:1 ω 7c into C18:1 ω 6c by double bond relocation was observed. The relocation occurred faster at ambient temperatures compared to refrigerated temperatures.

It is generally known that majority of *V. vulnificus* associated infections occur during summer hot months. *Vibrio vulnificus* ATCC 27562 was significantly less resistant to simulated gastric fluid (pH 4.0) after 7-day storage at 4 °C compared to the control, with D-values of 3.7 and 7.8 minutes, respectively. Therefore, higher sensitivity of the pathogen to the gastric fluid in winter harvested oysters may also impact the low number of outbreaks.

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 ТЕРНОПІЛЬСЬКИЙ ДЕРЖАВНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ ІМЕНІ І. Я. ГОРБАЧЕВСЬКОГО

ВПЛИВ НИЗЬКОТЕМПЕРАТУРНОГО РЕЖИМУ ЗБЕРІГАННЯ ТА СОЛОНОСТІ ВОДИ НА СКЛАД ЖИРНИХ КИСЛОТ ТА ІНАКТИВАЦІЮ БАКТЕРІАЛЬНОГО ПАТОГЕНУ *VIBRIO VULNIFICUS* ШТУЧНИМ ШЛУНКОВИМ СОКОМ

Резюме

За умов голоду в штучній морській воді клітини *Vibrio vulnificus* не змогли саморегулювати плинність клітинних мембран залежно від температури зберігання в межах досліджуваного періоду часу. Проте

спостерігали статистично вагомий ефект заміщення ($p < 0,05$) жирних кислот (C18:1 ω 6 замість C18:1 ω 7), що полягав у переміщенні подвійного зв'язку в жирній кислоті. Ці зміни відбувалися швидше за кімнатної температури навколишнього середовища порівняно зі зниженими температурами. Загальновідомо, що більшість інфекцій, спровокованих *V. Vulnificus*, асоціюється з періодом спекотних літніх місяців. *V. vulnificus* ATCC 27562 був значно менш стійким до дії штучної шлункової рідини (рН 4, 1) після семиденного зберігання при 4 °С порівняно з контролем з D-значеннями інактивації 3,7 і 7,8 хв відповідно. Таким чином, вища чутливість збудника до шлункового соку в зимовий період збирання устриць може також впливати на невелику кількість спалахів вогнищ захворювання.

КЛЮЧОВІ СЛОВА: *Vibrio vulnificus*, клітинна мембрана, жирні кислоти, виживання, шлункова рідина.

А. С. Токарский, Т. Я. Ярошенко, И. П. Кузьмак
ТЕРНОПОЛЬСКИЙ ГОСУДАРСТВЕННЫЙ МЕДИЦИНСКИЙ УНИВЕРСИТЕТ ИМЕНИ И. Я. ГОРБАЧЕВСКОГО

ВЛИЯНИЕ НИЗКОТЕМПЕРАТУРНОГО РЕЖИМА ХРАНЕНИЯ И СОЛЕННОСТИ ВОДЫ НА СОСТАВ ЖИРНЫХ КИСЛОТ И ИНАКТИВАЦИЮ БАКТЕРИАЛЬНОГО ПАТОГЕНА *VIBRIO VULNIFICUS* ШТУЧНЫМ ЖЕЛУДОЧНЫМ СОКОМ

Резюме

В условиях голода в искусственной морской воде клетки *Vibrio vulnificus* не смогли саморегулировать текучесть клеточных мембран в зависимости от температуры хранения в пределах исследуемого периода времени. Тем не менее, наблюдали статистически весомый эффект замещения ($p < 0,05$) жирных кислот (C18:1 ω 6 вместо C18:1 ω 7) путем перемещения двойной связи в жирной кислоте. Эти изменения происходили быстрее при комнатной температуре окружающей среды по сравнению с пониженными температурами. Общеизвестно, что большинство инфекций, спровоцированных *V. Vulnificus*, ассоциируется с периодом жарких летних месяцев. *V. vulnificus* ATCC 27562 был значительно менее устойчив к искусственной желудочной жидкости (рН 4, 1) после семидневного хранения при 4 °С по сравнению с контролем с D-значениями инактивации 3,7 и 7,8 мин соответственно. Таким образом, более высокая чувствительность возбудителя к желудочному соку в зимний период сбора устриц может также влиять на небольшое количество вспышек очагов заболевания.

КЛЮЧЕВЫЕ СЛОВА: *Vibrio vulnificus*, клеточная мембрана, жирные кислоты, выживание, желудочная жидкость.

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