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Citation	Xi, D., Liu, C., & Su, Y. C. (2014). Impacts of Lactobacillus plantarum in Depuration for Reducing Vibrio parahaemolyticus in Pacific Oysters (Crassostrea gigas). Journal of Aquatic Food Product Technology, 23(2), 165-174. doi:10.1080/10498850.2012.701709
DOI	10.1080/10498850.2012.701709
Publisher	Taylor & Francis
Version	Accepted Manuscript
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

Journal of Aquatic Food Product Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/wafp20

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Accepted author version posted online: 31 Jan 2013.

To cite this article: Dunyu Xi , Chengchu Liu & Yi-Cheng Su (2013): Impacts of Lactobacillus plantarum in Depuration for Reducing Vibrio parahaemolyticus in Pacific Oysters (Crassostrea gigas), Journal of Aquatic Food Product Technology, DOI:10.1080/10498850.2012.701709

To link to this article: http://dx.doi.org/10.1080/10498850.2012.701709

Impacts of Lactobacillus plantarum in Depuration for Reducing Vibrio parahaemolyticus in Pacific Oysters (Crassostrea gigas)

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Running head: *Lactobacillus* for reducing *Vibrio parahaemolyticus* in oysters

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Acknowledgements

This study was supported by Oregon State University Agricultural Research Foundation (Project: ARF5031), the National Natural Science Foundation of China (31071557), and the Innovation Program of Shanghai Municipal Education Commission (09ZZ166).

Keywords: *Vibrio parahaemolyticus*, lactic acid bacteria, oysters, depuration, seafood safety.

Abstract

This study investigated potential application of lactic acid bacteria (LAB) in depuration for reducing *Vibrio parahaemolyticus* in oysters. *Lactobacillus plantarum* ATCC 8014, which exhibited strong bactericidal effects against *V. parahaemolyticus* in vitro, was added to artificial seawater for depuration of Pacific oysters (*Crassostrea gigas*) inoculated with *V. parahaemolyticus* BE 98-2029 (O3:K6) to levels of about 10^4 MPN/g at 15±1 and 10±1ºC. Application of *L. plantarum* ATCC 8014 treatment (10^7 CFU/mL) in oyster depuration did not enhance reductions of *V. parahaemolyticus* in oysters depurated at 15±1ºC but significantly decreased ($p<0.05$) levels of *V. parahaemolyticus* in oysters depurated at 10±1°C after 5 days

(3.40 log reductions) when compared with controls (2.75 log reductions). It is not clear if a competitive exclusion by LABs to compete with *V. parahaemolyticus* binding sites in oyster tissues plays a role in the reduction of *V. parahaemolyticus* in the oysters. Further studies utilizing different types of LABs in oyster depuration might provide additional knowledge for application of LAB in depuration for decontaminating *V. parahaemolyticus* in oysters.

Keywords: *Vibrio parahaemolyticus*, lactic acid bacteria, oysters, depuration, seafood safety.

Introduction

Probiotics are "live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host" (FAO/WHO, 2006). Among them, lactic acid bacteria (LAB) have been widely studied for their beneficial effects in humans, including the antibacterial ability against gastrointestinal and urovaginal pathogenic bacteria (Servin, 2004). In addition, antibacterial activity of LAB has also been studied to develop bio-preservatives for application in food products. The antibacterial compounds produced by LAB include organic acids, diacetyl, low molecular weight compounds, and bacteriocins such as nisin (Ouwehand and Besterlund, 2004). Nisin is a small peptide produced by certain strains of *Lactococcus lactis* subsp. *Lactis*. It has been applied as a bio-preservatives in over 48 countries in dairy products (Vandenbergh, 1993) and is considered as "Generally Recognized as Safe" (GRAS) by the United States Food and Drug Administration (FDA, 1995).

In addition to application in foods, utilization of probiotics in aquaculture have shown that certain LAB could enhance the survival of fish larvae exposed to *Vibrio* pathogens by feeding

with rotifers (Gatesoupe, 1994) or commercial dry feed (Gildberg et al., 1997). The survival and growth of oyster larvae increased when they were fed with algae mixed with *Alteromonas* spp. strain CA2 as extra nutritional supply (Douillet and Langdon, 1993). A later experiment defined that the optimal feeding concentration of probiotics was 10^5 cells/mL (Douillet and Langdon, 1994). However, no study has been conducted to determine the potential application of probiotics, especially LAB, for reducing human pathogens like *V. parahaemolyticus* in raw oysters upon harvest.

V. parahaemolyticus is a human pathogen occurring naturally in the marine environments and commonly found in molluscan shellfish, particularly oysters. Clinical strains of *V. parahaemolyticus* are differentiated from environmental strains by their ability to produce a thermostable direct hemolysin (TDH) or a TDH-related hemolysin (TRH) (DePaola et al., 2003). Consumption of raw or undercooked shellfish contaminated with *V. parahaemolyticus* can result in food-borne illnesses including gastroenteritis, wound infection, and septicemia (Butt et al., 2004). It is reported that more than six hundred thousand tons of Pacific oysters (*Crassostrea gigas*) were produced worldwide every year between 2000 and 2008 (FAO, 2008). Numerous outbreaks of *V. parahaemolyticus* infections resulting from consumption of raw oysters in the U.S. were documented over the past ten years (CDC, 2005; McLaughlin et al., 2005; CDC, 2006).

Several post-harvest treatments, such as high pressure processing (Ma and Su, 2011), irradiation (Mahmoud and Burrage, 2009), low temperature pasteurization (Andrews et al., 2000), and flash freezing with frozen storage (Liu et al., 2009), have been developed for reducing *V.*

parahaemolyticus in oysters upon harvest. However, these processes require either a significant amount of investment in equipment or operation costs. The processes, except irradiation, also kill oysters during the treatments. There is a need to develop an economic post-harvest process for reducing *V. parahaemolyticus* contamination in oysters without adverse effects to oysters.

Depuration is a process to allow shellfish to purge contaminants in clean seawater either in a natural setting or in land-based facilities (Richards, 1988). Although the process has a long history as a post-harvest treatment of shellfish, it is ineffective in reducing *Vibrio* contamination in oysters at ambient temperature (Vasconcelos and Lee, 1972; Eyles and Davey, 1984). This study was conducted to investigate the effect of LAB on reducing *V. parahaemolyticus* contamination in raw oysters during post-harvest depuration.

Materials and methods

Vibrio parahaemolyticus

Five clinical strains of *V. parahaemolyticus* [10290 (O4:K12, *tdh*$^+$ and *trh*$^+$), 10292 (O6:K18, *tdh*$^+$ and *trh*$^+$), 10293 (O1:K56, *tdh*$^+$ and *trh*$^+$), BE 98-2029 (O3:K6, *tdh*$^+$), and 027-1c1 (O5:K15, *tdh*$^+$ and *trh*$^+$)] obtained from the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA, USA) were used in this study. A cocktail of the five strains was used in the inhibition tests, while strain BE98-2029 was used in oyster challenge experiments. Each strain was individually enriched according to the methods of Ma and Su (2011) to prepare a cell suspension of approximately 10^8 CFU/mL.

Lactic acid bacteria

Three strains of LAB (*Lactobacillus plantarum* ATCC 8014, *Lactobacillus acidophilus* ATCC 314, and *Lactococcus lactis* subsp. *lactis* ATCC 11454) were individually grown in de Man, Rogosa and Sharpen (MRS) broth (Acumedia Manufacturers, Inc., Lansing, MI, USA) at 37°C for 18-24 h. The enriched cultures were streaked onto MRS agar and incubated at 37°C for 72 h. A single colony from each MRS agar plate was transferred to 9 mL MRS broth and incubated at 37°C for 24 h. Cells were harvested by centrifugation at 3000 × g at 5±1°C for 20 min and re-suspended in equal amounts of phosphate buffered saline (PBS).

Preparation of lactic acid bacteria culture broth supernatant

Cell-free supernatant (CFS) was prepared by filtering supernatant of enriched MRS broth after centrifugation (3000 × g) at 5±1°C for 20 min through a 0.2 μm sterile polyethersulfone syringe filter (VWR International, Radnor, PA, USA) and kept at 4°C until usage.

The pH of CFS was determined by a pH meter (Symphony Meters, Beverly, MA, USA). Titratable acidity (TA) was determined by titration in triplicate with 0.1 N NaOH standardized with potassium acid phthalate (KPH) to reach an end point of pH 8.20±0.02. TA was reported as equivalent to lactic acid according to the equation (Sadler and Murphy, 2003): acid (w/v) %=($N \times V_1 \times EqWt \times 100$)/($V_2 \times 1000$), where N=normality of titrant (mEq/mL); V_1=volume of titrant (mL); V_2=volume of sample (mL); EqWt=Equivalent weight of lactic acid (90.08 mg/ mEq); and 1000=factor converting gram to milligram (mg/g).

Inhibitory effect of lactic acid bacteria culture broth supernatant on growth of *V. parahaemolyticus*

Effects of LAB culture supernatant on growth of *V. parahaemolyticus* was evaluated using the well diffusion method (Tagg et al., 1976; Lash et al., 2002). One milliliter of the five-strain cocktail cell suspension of *V. parahaemolyticus* was mixed with 100 mL of sterile TSA-salt tempered to 45ºC to prepare *V. parahaemolyticus* in the medium at a level of approximately 10^5 CFU/mL. The mixture was poured onto petri dishes (25 mL) and allowed to solidify at ambient temperature. Wells (0.9 cm in diameter) were created on the TSA-salt plates using a sterile cork borer. An aliquot (200 μL) of CFS or cell suspension (10^8 CFU/mL) from each LAB was added to individual wells. The plates were incubated at 37°C for 20 h and observed for clear zones. The inhibitory effect (%) was calculated as: [(diameter of inhibition zone-diameter of well)/diameter of well] ×100.

To determine the major parameters in CFS contributing to the antibacterial effects against *V. parahaemolyticus*, CFS prepared from 24 h growth of *L. plantarum* ATCC 8014 was adjusted to pH 6.5 by adding 2 N NaOH to neutralize lactic acid and other organic acids or treated with 0.1 mL/mL catalase (MP Biomedicals, LLC, Solon, OH, USA) and 2 mg/mL pepsin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h to eliminate hydrogen peroxide and protein compounds. The neutralized or enzyme-digested CFS was tested for the inhibitory effects on growth of *V. parahaemolyticus* as described previously. In addition, lactic acid solutions (Sigma-Aldrich) at levels of 0.5, 1.0, and 2.0 % (w/v) were also tested for their effects on growth of *V.*

parahaemolyticus. MRS broth and MRS broth treated with catalase (0.1 mL/mL) or pepsin (2 mg/mL) were used as controls. All samples were tested in triplicate.

Oyster preparation

Raw Pacific oysters (*Crassostrea gigas*) were obtained from oyster farms in Oregon and Washington and delivered in a cooler on the day of harvest. Oysters were washed under tap water to remove mud on shells and acclimated in a high-density polyethylene (HDPE) tank (18 by 12 by 12 in; Nalgene, Rochester, NY, USA) containing 20 L artificial seawater (ASW) at ambient temperature (23±1ºC) for 2-4 h upon delivery to the laboratory. The ASW (salinity: 30 ppt) was prepared by dissolving Instant Ocean Salts (Aquatic Eco-System Inc, Apopka, FL, USA) in deionized water according to the manufacturer's instructions. Oysters were analyzed for LAB and *V. parahaemolyticus* before being inoculated with *L. plantarum* or *V. parahaemolyticus*.

Accumulation of *L. plantarum* or *V. parahaemolyticus* in oysters

For accumulation of *L. plantarum* in oysters, about 30 oysters were placed in the HDPE tank of 10 L fresh ASW containing *L. plantarum* ATCC 8014 at a level of approximate 10^6 CFU/mL, with water being circulated at a rate of approximately 12 L/h at 23±1ºC for 20 h. Similarly, accumulation of *V. parahaemolyticus* in oysters was conducted by holding about 80 oysters in the HDPE tank of 20 L fresh ASW containing *V. parahaemolyticus* BE 98-2029 at a level of approximately 10^4 CFU/mL.

Oyster depuration

Twenty-five oysters exposed to ASW containing *L. plantarum* ATCC 8014 were depurated in 60 L of ASW in a laboratory-scale re-circulating (25 L/min) system at 20±1ºC for four days. Populations of *L. plantarum* in oysters and ASW were tested every day to determine the ability of *L. plantarum* to remain colonized in oysters during the depuration process.

To determine the effects of application of LAB in depuration on reducing *V. parahaemolyticus* in oysters, about 70 oysters contaminated with *V. parahaemolyticus* BE 98-2029 were depurated in the re-circulating (25 L/min) system equipped with a 15-W Gamma UV sterilizer (Current-USA Inc., Vista, CA, USA) and a temperature regulator (Delta Star, Aqua Logic, Inc., San Diego, CA, USA) capable of regulating water temperature between 10 and 15°C. Cells of *L. plantarum* ATCC 8014 were added to the ASW to reach a level of 10^7 CFU/mL. Depuration was conducted at 15±1 and 10±1ºC for the first 24 h without turning on the UV sterilizer followed by four days of process with UV light to inactivate cells of *V. parahaemolyticus* released from oysters into ASW and prevent the ASW from becoming a source of re-contamination during the process.

Oysters inoculated with *V. parahaemolyticus* and depurated in UV-sterilized ASW without addition of *L. plantarum* to ASW were used as a control. Survival of oysters during depuration was observed daily by knocking each oyster on its shell. Oysters which opened shells upon knocking were considered dead. The mortality of oysters was expressed as total number of dead oysters divided by total number of oysters used in the study.

Microbiological tests

Sample preparation

Five oysters were individually analyzed at each time of tests. Each oyster was shucked with a sterile shucking knife in a sterile stainless steel tray and blended with equal volume of sterile PBS at low speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, CT, USA) to prepare a 1:2 dilution sample suspension. Twenty-five grams of the sample suspension were then mixed with 100 mL sterile PBS to make a final 1:10 dilution. Additional 10-fold dilutions of each sample suspension were prepared with sterile PBS. One ASW sample was collected every 24 h during oyster depuration at 20±1ºC and analyzed for LAB.

Detection of lactic acid bacteria

LAB populations in oysters and ASW were determined by the pour plate method using MRS agar with incubation at 37°C for 72 h. Results were reported as the mean value of five oysters plus standard deviation.

Detection of V. parahaemolyticus

Populations of *V. parahaemolyticus* in oysters were determined by the three-tube most probable number (MPN) method according to the U.S. Food and Drug Administration's Bacteriological Analytical Manual (FDA, 2004). Briefly, all the sample suspensions were individually enriched

in sterile alkaline peptone water (APW) and incubated 16-18 h at 37ºC. One loop (1 µL) of enriched APW from a turbid tube was streaked onto individual thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates and incubated at 37ºC for 18-24 h. Formation of round and green colonies on TCBS agar plates was considered positive for *V. parahaemolyticus*. Results were reported as the mean value (MPN/g) from five oysters plus standard deviation.

Statistical analysis

Results of microbiological tests were transferred to log values for statistical analysis. Bacterial populations in oysters at different treatment times were analyzed by t-Test: Paired Two Samples for Means (Excel, Microsoft, Redmond, WA, USA). Significant differences between means of treatments were established at $p<0.05$.

Results

Inhibitory effects of cell-free supernatant on growth of *Vibrio parahaemolyticus*

The pH and titratable acidity of the cell-free supernatant of LAB after 12 and 24 h enrichment are reported in Table 1. CFS of all three strains had lower pH and higher TA after 24 h enrichment than those in CFS collected after 12 h enrichment. The CFS obtained from 24 h of growth of *Lactobacillus plantarum* ATCC 8014 had the lowest pH (4.19) and highest TA (2.16 %). In contrast to CFS, none of the cell suspensions prepared from growth of three LAB after 24 h of enrichment inhibited growth of *V. parahaemolyticus* in vitro (Data not shown). To

investigate the major parameters contributing to the antibacterial activity of CFS from 24 h growth of *L. plantarum* ATCC 8014, the CFS was treated with NaOH to neutralize organic acids or with catalase and pepsin to eliminate hydrogen peroxide and proteinaceous substances. When the CFS was adjusted to pH 6.5, it totally lost the antibacterial activity against growth of *V. parahaemolyticus* (Table 2). The inhibitory effect of the CFS slightly reduced from 66.67% to 55.56% when it was treated with pepsin or catalase (Table 2).

Lactic acid at 0.5, 1.0, and 2.0% (w/v) exhibited inhibitory effects against growth of *V. parahaemolyticus*. Among them, the 2.0% lactic acid exhibited greater inhibitory effect (85.19%) than the 24 h enriched CFS (66.67%) (Table 2). No inhibitory effect was observed for MRS broth either before or after enzyme (catalase or pepsin) treatments on growth of *V. parahaemolyticus*.

L. plantarum attachment to oysters

Changes of LAB populations in oysters when held in re-circulating ASW at 20±1ºC are reported in Table 3. Fresh oysters contained a low level of LAB (1.83 log CFU/g). Exposure of oysters to ASW containing *L. plantarum* ATCC 8014 (6.41 log CFU/mL) for 20 h allowed accumulation of the bacterium in oysters and increased the total LAB in oysters to 4.66 log CFU/g. Populations of *L. plantarum* in oysters decreased slightly but remained moderately colonized in oysters (3.10 log CFU/g) during the process, while the levels of *L. plantarum* in ASW gradually increased to >3.40 log CFU/mL after four days of process.

Effects of *L. plantarum* treatment on reducing *V. parahaemolyticus* in oysters during depuration

The efficacies of *L. plantarum* ATCC 8014 treatment on reducing *V. parahaemolyticus* in oysters during depuration at 15±1 and 10±1ºC are summarized in Table 4. No *V. parahaemolyticus* was detected in fresh oysters before being inoculated with *V. parahaemolyticus*. When oysters were depurated at 15±1ºC, populations of *V. parahaemolyticus* in oysters decreased to <10 MPN/g (>3.06 log reductions) after 4 days of depuration with *L. plantarum* added to ASW at the beginning of the process. At the end of 5 days of depuration, reductions of *V. parahaemolyticus* in oysters increased to >3.42 and >3.30 log MPN/g in oysters with and without the *L. plantarum* treatment, respectively. No significant difference ($p>0.05$) between the treatments was observed.

When the depuration was conducted at 10±1ºC, a greater reduction (3.40 log MPN/g) of *V. parahaemolyticus* in oysters was observed with *L. plantarum* treatment than that (2.75 log MPN/g) in oysters without the treatment after five days of processes, indicating application of LAB in low-temperature depuration could enhance reductions of *V. parahaemolyticus* in oysters (Table 4). All the oysters were able to survive during cold water (10ºC) depuration for five days (Table 5).

Discussion

Studies of the inhibitory effects of cell-free supernatant from *Lactobacillus plantarum* ATCC 8014 on growth of *Vibrio parahaemolyticus* indicate that low pH and high titratable acidity

played the major roles in inhibiting growth of *V. parahaemolyticus*. The CFS of *L. plantarum* ATCC 8014 has been reported to exhibit a wide range of antibacterial spectrum within a narrow pH range (pH 4-5) (Lash et al., 2005). Such a phenomenon was also observed in this study as the CFS lost its inhibitory effect on growth of *V. parahaemolyticus* when the pH of CFS was adjusted from 4.19 to 6.50 (Table 2). The previous study also reported a protein of 122 KDa in the CFS from *L. plantarum* ATCC 8014 to be the major compound exhibiting antibacterial activity. However, such a compound was not observed in this study because the CFS from *L. plantarum* ATCC 8014 still inhibited growth of *V. parahaemolyticus* after being treated with pepsin (Table 2). This observation is consistent with a previous report that *L. plantarum* ATCC 8014 did not produce bacteriocin (Skinner et al., 1999). The possible reasons for contradictory results observed in these studies even using the same strain could be the different types of inhibition tests and different CFS preparation methods. In addition, a number of low molecular mass antimicrobial compounds, including benzoic acid, methylhydantoin, and mevalonolactone, could be produced from growth of *L. plantarum* and be present in CFS. All of those compounds at a level of 10 ppm have been shown to inhibit Gram-negative bacteria when they were applied with 1% lactic acid. However, the inhibitory effect of each of the compounds was much weaker than with that of combined use (Niku-Paavola et al., 1999), which indicates the strong synergic effect of those compounds.

Lactic acid and other organic acids produced by LAB can inhibit growth of a number of Gram-negative bacteria. Many antibacterial compounds of large molecular mass cannot penetrate into the cell due to the function of lipopolysaccharide as the permeability barrier on the outer membranes of Gram-negative bacteria. Lactic acid can act as a permeant and disrupt the outer

membrane of the Gram-negative bacteria so that lactic acid itself or other antibacterial compounds can then enter the cell to exert antibacterial effects (Alakomi et al., 2000). In this study, organic acids produced by *L. plantarum* ATCC 8014 after 24 h enrichment appear to be the major compounds for inhibiting growth of *V. parahaemolyticus* in vitro. However, other compound(s) with antibacterial properties might exist in the CFS. Further analysis is required to determine the structures and percentages of additional antibacterial compounds in CFS.

Raw oysters may contain LAB, and exposure of oysters to artificial seawater containing *L. plantarum* ATCC 8014 allowed the organism to colonize in oysters. These results demonstrated that *L. plantarum* ATCC 8014 could attach to oyster tissues and remain colonized in oysters during the depuration process. Therefore, the organism might compete with *V. parahaemolyticus* for attachment to oyster tissues and prevent the colonization of *V. parahaemolyticus* in oysters. However, the mechanism of colonization of *L. plantarum* ATCC 8014 in oysters remains to be investigated. The sudden increase of LAB populations in ASW after four days of holding oysters was probably because several oysters died after three days in the process (data not shown) and provided nutrients for the multiplication of the bacteria in ASW, so that depuration of oysters at 20ºC for more than 3 days should not be considered for reducing *V. parahaemolyticus* in oysters upon harvest.

Several studies have reported that depuration at ambient temperatures are ineffective in reducing *V. parahaemolyticus* contamination in oysters. Ren and Su (2006) reported that holding laboratory-contaminated Pacific oysters in ASW for 24 h did not yield apparent reductions of *V. parahaemolyticus* or *V. vulnificus* in oysters. A study of depuration of laboratory-contaminated

American oysters (*Crassostrea virginica*) in ASW at 22ºC for 48 h resulted in a limited reduction (1.2 log MPN/g) of *V. parahaemolyticus* in oysters. However, the reduction of *V. parahaemolyticus* in oysters was slightly increased to 2.1 log MPN/g when the depuration was conducted at 15ºC for 48 h (Chae et al., 2009). In this study, we investigated the potential application of LAB in depuration at 10 and 15 ºC for enhancing efficacy in reducing *V. parahaemolyticus* contamination in oysters. Although no significant difference was observed between reductions of *V. parahaemolyticus* in oysters depurated at 15±1ºC (Table 4), addition of *L. plantarum* to ASW for depuration reduced the mortality rate of oysters to 2.9% from 8.8% observed for untreated oysters (Table 5).

Reducing the depuration temperature to 10±1ºC did not enhance the efficacy of depuration in reducing *V. parahaemolyticus* in oysters when compared with reductions observed at 15ºC (Table 4). However, the reduction of *V. parahaemolyticus* in oysters treated with *L. plantarum* after 5 days of depuration at 10ºC (3.40 log MPN/g) was significantly greater than that (2.75 log MPN/g) observed in controls (Table 4). The mechanism for *V. parahaemolyticus* reduction in oysters by the LAB depuration process is not clear. A hypothesis is that *L. plantarum* competes with *V. parahaemolyticus* for attachment sites on tissues in the digestive tract, resulting in a competitive exclusion effect for the attachment of *V. parahaemolyticus* cells to oyster tissues. However, such a phenomenon remains to be investigated. In addition, the feasibility of application of LAB in depuration for reducing *Vibrio* spp. in oysters needs to be further studied to identify the optimal depuration condition to improve the efficacy of the process.

In conclusion, application of *L. plantarum* in depuration resulted in greater than 3.0 log MPN/g reductions of *V. parahaemolyticus* in raw Pacific oysters after five days of process at 10±1ºC with no mortality. It is not clear if a competitive exclusion by LABs to compete with *V. parahaemolyticus* binding sites in oyster tissues plays a role in the reduction of *V. parahaemolyticus* in the oysters. Further studies utilizing different types of LABs in oyster depuration might provide additional knowledge for application of LAB in depuration for decontaminating *V. parahaemolyticus* in oysters.

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Table 1. Titratable acidity (TA) and pH of cell-free supernatant (CFS) from growth of three lactic acid bacteria at 37ºC.

Bacteria	pH	TA[†] (%)	Growth Time (h)
L. acidophilus ATCC 314	5.29±0.01[*]	0.689±0.010	12
	5.00±0.03	0.978±0.021	24
L. lactis subsp. *lactis* ATCC 11454	5.08±0.03	0.672±0.019	12
	4.79±0.02	0.838±0.016	24
L. plantarum ATCC 8014	4.78±0.01	1.137±0.013	12
	4.19±0.02	2.164±0.047	24

[*] Data are means of three determinations ± SD.

[†] Expressed as equivalent weight of lactic acid.

Table 2. Inhibitory effects of cell-free supernatant (CFS) from growth of *Lactobacillus plantarum* ATCC 8014, and lactic acid on growth of a mixture of five *Vibrio parahaemolyticus* strains (10^5 log CFU/mL) in well diffusion tests.

Sample	Treatments	Inhibitory effect (%)*
L. plantarum ATCC 8014	Control (pH 4.19)	66.67±0.00†
	Neutralization (pH 6.50)	N‡
	Catalase (0.1 mL/mL)	55.56±0.00
	Pepsin (2 mg/mL)	55.56±0.00
Lactic acid	0.5 % (w/v)	33.33±0.00
	1.0 % (w/v)	55.56±0.00
	2.0 % (w/v)	85.19±6.42
MRS broth	Control (pH 6.50)	N

Catalase (0.1 mL/mL)	N
Pepsin (2 mg/mL)	N

*Calculated as: [(diameter of inhibition zone - diameter of well) / diameter of well] ×100.

† Data are means of three determinations ± SD.

‡ No inhibitory effect.

Table 3. Changes of lactic acid bacteria populations in oysters and artificial seawater (ASW) during depuration at 20±1ºC.

Time (d)	Oysters (log CFU/g)	ASW (log CFU/mL)
0[*]	4.66±0.18 A[†]	ND[‡]
1	4.00±0.60 B	1.78
2	3.67±0.24 B	1.58
3	3.46±0.68 B	1.74
4	3.10±0.47 B	>3.40

[*] After 20 h of inoculation of oysters (initial lactic acid bacteria populations: 1.83±0.44 log CFU/g) with *Lactobacillus plantarum* ATCC 8014 (6.41 log CFU/mL) at 23±1ºC.

[†] Data are means of five determinations ± SD. Same letter in each column indicates the means are not significantly different ($p>0.05$).

[‡] Not detected using MRS agar.

Table 4. Reductions of *Vibrio parahaemolyticus* BE 98-2029 in laboratory inoculated oysters during depuration at 15±1 and 10±1ºC.

	V. parahaemolyticus populations (Log MPN/g) in oysters			
Time (d)	LAB Treatment* (15±1ºC)	Control (15±1ºC)	LAB Treatment* (10±1ºC)	Control (10±1ºC)
0	3.91±0.45 A†	3.91±0.45 A	4.68±0.24 A	4.68±0.24 A
1	3.00±0.37 B (0.91)‡	2.76±0.44 B (1.15)	3.44±0.22 B (1.24)	3.61±0.64 B (1.07)
2	1.85±0.33 C (2.06)	2.07±0.32 C (1.84)	2.98±0.38 C (1.70)	2.86±0.39 C (1.82)
3	1.78±0.28 C (2.13)	1.42±0.12 D (2.49)	2.12±0.40 D (2.56)	2.48±0.53 CD (2.20)
4	<0.85±0.35 D (>3.06)	<1.17±0.78 DE (>2.74)	2.06±0.46 DE (2.62)	1.82±0.52 D (2.86)
5	<0.49±0.04 E	<0.61±0.20 E	1.28±0.53 E (3.40)	1.93±0.53 D

(>3.42)	(>3.30)	(2.75)

[*] *Lactobacillus plantarum* ATCC 8014 treatment (10^7 CFU/mL).

[†] Data are means of five determinations ± SD. Same letter in each column indicates the means are not significantly different ($p>0.05$).

[‡] Reductions of *V. parahaemolyticus* populations.

Table 5. Mortality of oysters during depuration at 15±1 and 10±1ºC.

Time (d)	Treatment* (%) 15±1ºC	Control (%) 15±1ºC	Treatment (%) 10±1ºC	Control (%) 10±1ºC
1	0 (0/35) †	0 (0/34)	0	0
2	2.9 (1/35)	0 (0/34)	0	0
3	2.9 (1/35)	2.9 (1/34)	0	0
4	2.9 (1/35)	8.8 (3/34)	0	0
5	2.9 (1/35)	8.8 (3/34)	0	0

* *Lactobacillus plantarum* ATCC 8014 treatment (10^7 CFU/mL).

† Total number of dead oysters divided by the total number of oysters.