Temperature effects on the growth and survival of *tdh* positive *Vibrio parahaemolyticus* in tissues of postharvest Manila clam (*Ruditapes philippinarum*)

Carmen Lopez-Joven [a,b] *, Ignacio de Blas [c] and Ana Roque [a]

[a] IRTA-SCR, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Spain

[b] Preventive Veterinary Medicine Institute, Austral University of Chile, Edificio Federico Saelzer 5º Piso, Campus Isla Teja s/n, Valdivia, Chile

[c] Laboratory of Fish Diseases, Faculty of Veterinary Sciences, Instituto Agroalimentario de Aragón (IA2) (Universidad de Zaragoza-CITA), c/ Miguel Servet 177, 50013 Zaragoza, Spain

* Corresponding author: Tel.: +56 6 3244 4359. E-mail address: carmen.lopez@uach.cl (C. Lopez-Joven).

ABSTRACT

Bivalve storage at inadequate temperatures contains greater densities of *Vibrio parahaemolyticus*. The objective of the present study was to determine if there were any differences in growth and survival of potentially pathogenic *V. parahaemolyticus* (*tdh* positive) in relation to nonpathogenic *V. parahaemolyticus* (*tdh-trh* negative) levels in Manila clams (*Ruditapes philippinarum)* when exposed to different postharvest temperatures. Clams were depurated then exposed to known doses of both potential pathogenic and nonpathogenic *V. parahaemolyticus* for 24 h. Clams were then kept at the following temperatures corresponding to the Mediterranean summer (28ºC), winter (15ºC) and refrigeration (4ºC) for 96 h. *Vibrio parahaemolyticus* densities were determined at 0, 24, 48, 72 and 96 h of postharvest storage. Both isolates of *V. parahaemolyticus* multiplied rapidly in live clams held at 28ºC, (increase of 3 logs CFU/g at 72 h). Nonpathogenic *V. parahaemolyticus* in clams stored at 4ºC and 15ºC showed 1 log CFU/g and 2 log CFU/g decrease at 96 h, respectively, while no significant differences were detected for pathogenic *V. parahaemolyticus* at these temperatures after 96 h.

Keywords: *Vibrio parahaemolyticus*; *Ruditapes philippinarum*; Postharvest temperature; Food Safety

1. Introduction

Vibrio parahaemolyticus is a common cause of shellfish-related gastroenteritis (Daniels et al., 2000; Lozano-León et al., 2003). Due to their filter-feeding activity, bivalve molluscs can concentrate bacteria in their tissues. Environmental temperatures, time/temperature abuse and mishandling in postharvest molluscs may easily induce rapid multiplication of *V. parahaemolyticus* (Beuchat, 1982; DePaola et al., 2000). Hemolysins, namely the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH), encoded by *tdh* and *trh* genes respectively, are important virulence factors as they contribute to precipitate the disease (Honda and Iida, 1993). In addition, hemolytic activity based on TDH-producing *V. parahaemolyticus* is present in almost all *V. parahaemolyticus* strains isolated from clinical specimens, whereas it is present only in 1 to 3% of the isolates of environmental origin (Miyamoto et al., 1969). The population dynamics of a number of pathogenic bacteria in molluscs has been modelled (DePaola et al., 2009; Yoon et al., 2008), but not using live clams. Knowing the survival of pathogenic populations of *V. parahaemolyticus* in relation to the total population of *V. parahaemolyticus* during postharvest-process in live molluscs will contribute to prevent outbreaks and to improve the monitoring of harvest sites. Therefore, the objective of the present study was to model the growth and survival of one potentially pathogenic isolate (*tdh* gene positive) and one isolate classified as nonpathogenic (containing neither *tdh* nor *trh* genes), in *Ruditapes philippinarum*, stored at three different temperatures corresponding to the Mediterranean summer (28ºC), winter (15ºC) and refrigeration (4ºC) following artificial contamination with these two isolates of *V. parahaemolyticus* and to determine whether there were any differences in bacterial kinetics survival.

Results from the study can contribute to the production of data for quantitative risk analysis and harmonize an *in vivo* model for further research.

2. Material and methods

Experiment assessed two factors: temperature and vibrio. The temperature was divided into three categories (4, 15 and 28ºC), vibrio was divided into *tdh* positive isolate (potentially pathogenic *V. parahaemolyticus*) or *tdh-trh* negative isolate (potentially nonpathogenic *V. parahaemolyticus*), and a control group without *Vibrio* (non-inoculated clam).

Adult clams (*R. philippinarum)* were collected from the Ebro Delta. Clams used in the study were all alive, opened and closed their valves and presented intact shells with normal coloration. Clams were transported to the laboratory in cool boxes containing ice plates. Transport lasted less than 1 h. Upon arrival, clams were placed in a recirculation system flowing sterile seawater in order to depurate them for 12 days (Lopez-Joven et al., 2011a). Five *R. philippinarum* per tank were sampled to confirm that clams had undetectable levels of sucrose nonfermenting vibrios, including *V. parahaemolyticus*. *Vibrio parahaemolyticus* used in the study were isolated from the bivalves cultured in the Ebro Delta (Roque et al., 2009), and assumed to be fully adapted to the bivalves and their environment, reason why they were preferred to type strains. Potentially pathogenic isolate, *V. parahaemolyticus* i678 belongs to the serotype O2:K28 containing *tdh* gene (*tdh* positive *Vp),* was isolated from a clam. Nonpathogenic isolate used, *V. parahaemolyticus* i747 (*tdh-trh* negative *Vp),* was isolated from a mussel. Isolates were characterized with API 20E (BioMérieux, Marcy-l'Etoile, France) giving a profile of *V. parahaemolyticus* with 99.5% and 99.9% similarity, respectively.

Biotype was known to correspond to a non-pandemic strain. Inocula were prepared by resuscitating a cryopreserved isolate in 50 mL of tryptic soy broth (TSB) (Scharlau Chemie S.A., Barcelona, Spain) with 2.0% NaCl at 28ºC for 18-20 h in a rotating shaker (Excella E24 Incubator skaker series. New Brunswick Scientific. Edison, New Jersey, USA). Bacteria were collected by centrifugation at 2000 g, 4ºC, 10 min. Supernatant was discarded and the pellet was suspended in sterile saline solution 2.5% NaCl (SSS). Bacterial solution was added to 10 L containers with sterile seawater containing the depurated clams. Challenge densities were 6.16 ± 0.48 log CFU per clam (*tdh* positive *Vp*, n = 97 clams) or 6.72 ± 0.32 log CFU per clam (*tdh-trh* negative *Vp*, n = 101 clams), along with phytoplankton (10^5 cells/mL), axenic *Isochrysis galbana* variety Tahiti (Pérez Camacho et al., 2002) to stimulate bacterial uptake by the clams during 24 h (Lopez-Joven et al., 2011b). A control container without *Vibrio* inocula (n = 138 clams) was also fed axenic algae.

At the end of the 24 h challenge, each batch of clams exposed to *V. parahaemolyticus* and control group, was divided in three equal lots which were placed in different moist plastic containers, and one container from each isolate of *Vibrio* and one for unexposed clams was then stored at each of the temperatures (4, 15 and 28ºC) during 96 h. Nine experimental groups in a full-factorial experiment were investigated using a total of 336 clams.

At this point (time 0 h) samples (n = 11 clams exposed to *tdh* positive *Vp*; n = 13 clams exposed to *tdh-trh* negative *Vp*; n = 16 clams unexposed) from each batch were analysed. Concentrations of *V. parahaemolyticus* in clams were estimated every 24 h during storage for 96 h. At each time, 3-5 clams were sampled from each container. Each clam was measured (Digital caliper (0-150 mm) LLG, Mitutoyo CD-15, Tokyo, Japan) and its flesh was weighed. Clams were homogeneous with no significant

differences among experimental groups. The average length (mm) and weight (g) of clams used in the study were 32.80 ± 5.14 mm and 2.03 ± 0.78 g, respectively. Tissue and shell liquor were aseptically removed, weighed and placed into 10 mL of SSS. The resulting mixture was homogenized for 30 s using a digital homogenizer Ultra-Turrax T-25 (IKA-Werke GmbH & Co. KG). Decimal dilutions were made in SSS and volumes of 0.1 mL from each dilution were spread onto Chromagar Vibrio plates and incubated at 37ºC for 18-20 h. The colonies formed (*V. parahaemolyticus* produces mauve-purple colonies onto Chromagar Vibrio) were counted manually to calculate the density of viable cells in the sample (CFU/g).

Experiment was performed twice with an extra replicate for storage at 4ºC in order to verify previous results, taking into account this temperature is used as a critical control point to protect public health at home, restaurant, retail… (Table 1).

Descriptive statistics were performed using mean and standard deviation for quantitative variables, as length and weight, stratified by experiments. Homoscedasticity was tested using Kolmogorov-Smirnov test to check normal distribution, and Levene test for variance homogeneity. Non-homoscedastic variables, as CFU/g, were log-transformed to improve homoscedasticity. Differences between means for log (CFU/g) depending on *tdh*-positive and *tdh-trh* negative *V. parahaemolyticus*, temperature and time were analyzed with one-way analysis of variance (ANOVA) followed by Duncan post-hoc test. A General Linear Model for log (CFU/g) using vibrio strains, temperature and time as fixed factors was carried out to check interactions between factors. Desired alpha error was established at 0.05. Statistical analysis was performed using IBM SPSS 19.0 for Windows software (Armonk, NY, USA).

3. Results and discussion

136

137 At time 0 h, the densities of *V. parahaemolyticus* were not significantly different in all
138 treatments and among repetitions of the same treatment: *tdh*-positive *V.*
139 *parahaemolyticus* (4.12 ± 1.43 log CFU/g) ($P = 0.440$), *tdh-trh* negative *V.*
140 *parahaemolyticus* (5.26 ± 0.27 log CFU/g) ($P = 0.398$), and control group (0.38 ± 0.82
141 log CFU/g) ($P = 0.794$). Bacterial densities are in agreement with previous results
142 (Lopez-Joven et al. 2011b).
143 Results of *tdh* positive *V. parahaemolyticus* fluctuated no significantly in clams stored
144 at 4ºC ($P = 0.357$) and 15ºC ($P = 0.369$) until 96 h (Fig. 1A), whereas significant
145 reductions from 5.26 to 4.13 log CFU/g and from 5.22 to 3.22 log CFU/g were detected
146 ($P < 0.001$) and ($P = 0.004$), for *tdh-trh* negative *V. parahaemolyticus* at 4ºC and 15ºC,
147 respectively (Fig. 1B). Duncan test indicated that reductions were significant after 96 h
148 at 4ºC, and after 72 h at 15ºC. The slight increase for *tdh-trh* negative *V.*
149 *parahaemolyticus* at 72h at 4ºC could be due to immunological factors of some clams.
150 Previous studies showed it is possible to isolate total *V. parahaemolyticus* from bivalves
151 after the end of its shelf-life (between 1 and 2 weeks), when it has been stored at
152 refrigeration temperatures, with no apparent decrease in numbers (Johnson et al., 1973;
153 Muntada-Garriga et al., 1995). On the other hand, other studies showed *V.*
154 *parahaemolyticus* decreased during refrigeration storage (3ºC) from 2.9 log CFU/g to
155 0.8 log CFU/g after 14 days of refrigeration (Gooch et al., 2002). Shen et al. (2009)
156 observed a gradual decrease of *V. parahaemolyticus* in oysters stored at 0 and 5ºC, after
157 96 h. The same study reported populations of *V. parahaemolyticus* remained constant in
158 oysters stored at 10ºC which had been previously observed (Cook and Ruple, 1989), and
159 increased when the oysters were stored at 15ºC for 60 h. Thomson and Thacker (1973),
160 using sterile oyster homogenates inoculated with 10^4 *V. parahaemolyticus* cells, showed

161 *V. parahaemolyticus* numbers decreased to almost zero after 1 week at 0 and 4ºC. In
162 addition, they observed the cells did not multiply but remained viable for 3 weeks at
163 8ºC, while multiplication occurred at 10 and 12ºC with cell remaining viable for 4
164 weeks or longer.
165 However, hardly any studies separated *tdh*-positive and *tdh-trh* negative *V.*
166 *parahaemolyticus.* Yoon et al. (2008) inoculated both pathogenic and nonpathogenic *V.*
167 *parahaemolyticus* strains into oyster slurry and observed that both *V. parahaemolyticus*
168 decreased at 10ºC and 15ºC. Present results are in contrast with the ones published by
169 Yoon et al. (2008). This discrepancy may be attributable to the matrix used, broth and
170 oyster slurry (Yoon et al., 2008) and live clam (present study). Furthermore, different
171 defense systems in clams and oysters may also have influenced in the differences
172 among studies.
173 In any case, not only present study represents better what happens in a bivalve, it is also
174 probable that bacteria are in better conditions when kept alive inside a live clam. Present
175 results also suggest better adaptation properties in the *tdh*-positive *V. parahaemolyticus*
176 isolate harvested clams.
177 Vibrio levels in *R. philippinarum* control clams (after depuration process and
178 postharvest storage at 4ºC and 15ºC) were either undetectable or were observed in
179 concentrations lower than 1.4 log (CFU/g), which were not statistically significant along
180 time neither at 4ºC ($P = 0.184$) nor at 15ºC ($P = 0.158$). These values do not affect
181 results since the clams were exposed to a huge initial concentration of both isolates of
182 vibrios.
183 Bacterial load was higher at 28ºC than 4 ºC and 15ºC in all treatments. This growth was
184 significantly higher ($P < 0.001$) at 48 and 72 h (Fig. 1). Similar trends were previously
185 reported, where both pathogenic and nonpathogenic *V. parahaemolyticus* strains

186 increased within oysters stored from 20ºC to 30ºC during a 5-day period (Cook and
187 Ruple, 1989; Yoon et al., 2008).
188 At 96 h, only two clams exposed to spiked seawater with *tdh-trh* negative *V.*
189 *parahaemolyticus* were alive, and mean levels was 5.36 log CFU/g.
190 In control group, vibrio levels were more than 3 log CFU/g at 24 h and there were
191 significant differences ($P < 0.001$) from 24 h to 96 h with an increase from 0.38 to 4.56
192 log CFU/g.
193 Even though clams were depurated, probably due to storage temperature, undetectable
194 levels of *V. parahaemolyticus* or viable but non-culturable (VBNC) vibrios began to
195 multiply becoming a potential threat to public health (Feldhusen, 2000; Ramamurthy et
196 al., 2014). Although it would have been preferred to run a simultaneous exposure to the
197 two bacterial strains, this design was not feasible because the method to quantify the
198 bacteria would have been real-time PCR and this technique may not sufficiently
199 sensitive (Nordstrom et al., 2007).
200 Previously described relationships among storage temperature, time and *V.*
201 *parahaemolyticus* load were also analyzed using a General Linear Model. Results
202 showed that this model was significant ($P < 0.001$) and its adjusted determination
203 coefficient (R^2) was 0.811. All factors are significantly associated with vibrio
204 concentration ($P < 0.001$ for almost all individual factors and interactions; except $P =$
205 0.001 for the interaction of temperature and vibrio strain; and $P = 0.023$ for the
206 interaction of temperature, vibrio strain and time).
207 All observed significant differences in this multivariate analysis support the previously
208 described results from bivariate analysis, and confirm the different behavior of control
209 group with higher bacteria loads related with increasing postharvest time and
210 temperature, while *tdh*-positive and *tdh-trh* negative *V. parahaemolyticus* groups

showed similar and constant concentrations at 4ºC and 15ºC along the time and increasing concentrations along the time at 28ºC (at least during the first 72 h).

4. Conclusions

This research highlighted the different behavior of different biotypes of *V. parahaemolyticus* at three different storage temperatures. Results here suggest that conditions or ability for survival of a potentially pathogenic *V. parahaemolyticus* are better in postharvest Manila clam, as compared to a potentially nonpathogenic *V. parahaemolyticus* at 4ºC and 15ºC. Present study was based on the growth and survival of one single isolate of each potentially pathogenic and nonpathogenic *V. parahaemolyticus*; thus more extensive growth data for various pathogenic strains possessing the *tdh* gene as well as various nonpathogenic strains are needed for the data validation. Furthermore, it is relevant that no differences appear to exist between *tdh* positive *V. parahamolyticus* densities in clams stored at 4ºC and 15ºC, therefore, clams could be stored at either of these temperatures which would facilitate transport conditions after harvesting and depuration.

Acknowledgments

Study financed by Spanish INIA projects RTA 2005 00079 00 00 and RTA 2007 00063 00 00 with Feder funds awarded to AR. CLJ had a doctoral scholarship provided by INIA. We are grateful to the staff from the IRTA-SCR for their help and to the OECD Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems for its sponsorship.

236

237 **References**

238

239 Beuchat, L.R., 1982. *Vibrio parahaemolyticus*: public health significance. Food
240 Technol. 36, 80-83.
241 Cook, D., Ruple, A., 1989. Indicator bacteria and Vibrionaceae multiplication in post-
242 harvest shellstock oysters. J. Food Prot. 52, 343-349.
243 Daniels, N.A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R.M.,
244 Thompson, S., Wilson, S., Bean, N.H., Griffin, P.M., Slutsker, L., 2000. *Vibrio*
245 *parahaemolyticus* infections in the United States, 1973-1998. J. Infect. Dis. 181, 1661-
246 1666.
247 DePaola, A., Kaysner, C.A., Bowers, J., Cook, D.W., 2000. Environmental
248 investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington,
249 Texas, and New York (1997 and 1998). Appl. Environ. Microbiol. 66, 4649-4654.
250 DePaola, A., Jones, J.L., Noe, K.E., Byars, R.H., Bowers, J.C., 2009. Survey of
251 postharvest-processed oysters in the United States for levels of *Vibrio vulnificus* and
252 *Vibrio parahaemolyticus*. J. Food Prot. 72, 2110-2113.
253 Feldhusen, F., 2000. The role of seafood in bacterial foodborne diseases. Microbes
254 Infect. 2, 1651-1660.
255 Gooch, J.A., DePaola, A., Bowers, J., Marshall, D.L., 2002. Growth and survival of
256 *Vibrio parahaemolyticus* in postharvest American oysters. J. Food Prot. 65, 970-974.
257 Honda, T., Iida, T., 1993. The pathogenicity of *Vibrio parahaemolyticus* and the role of
258 the thermostable direct haemolysin and related haemolysins. Rev. Med. Microbiol. 4,
259 106-113.

Johnson Jr., W.G., Salinger, A.C., King, W.C., 1973. Survival of *Vibrio parahaemolyticus* in oyster shellstock at two different storage temperatures. Appl. Microbiol. 26, 122-123.

Lopez-Joven, C., Ruiz-Zarzuela, I., de Blas, I., Furones, M.D., Roque, A., 2011a. Persistence of sucrose fermenting and nonfermenting vibrios in tissues of Manila clam species, *Ruditapes philippinarum*, depurated in seawater at two different temperatures. Food Microbiol. 28, 951-956.

Lopez-Joven, C., de Blas, I., Ruiz-Zarzuela, I., Furones, M.D., Roque, A., 2011b. Experimental uptake and retention of pathogenic and nonpathogenic *Vibrio parahaemolyticus* in two species of clams: *Ruditapes decussatus* and *Ruditapes philippinarum*. J. Appl. Microbiol. 111, 197-208.

Lozano-León, A., Torres, J., Osorio, C.R., Martínez-Urtaza, J., 2003. Identification of *tdh*-positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain. FEMS Microbiol. Lett. 226, 281-284.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K., Yamai, S., 1969. *In vitro* hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J. Bacteriol. 100, 1147-1149.

Muntada-Garriga, J.M., Rodriguez-Jerez, J.J., Lopez-Sabater, E.I., Mora-Ventura, M.T., 1995. Effect of chill and freezing temperatures on survival of *Vibrio parahaemolyticus* inoculated in homogenates of oyster meat. Lett. Appl. Microbiol. 20, 225-227.

Nordstrom, J.L., Vickery, M.C.L., Blackstone, G.M., Murray, S.L. and DePaola, A., 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl. Environ. Microbiol. 73, 5840-5847.

Pérez Camacho, A., Delgado, M., Albentosa, M., 2002. Influencia del tamaño y la concentración de alimento sobre las tasas de aclaramiento e ingestión de la almeja *Ruditapes decussatus* (Linnaeus, 1758). Bol. Inst. Esp. Oceanogr. 18, 315-320.

Ramamurthy, T., Ghosh, A., Pazhani, G.P., Shinoda, S., 2014. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Front. Public Health. 2, 1-9.

Roque, A., Lopez-Joven, C., Lacuesta, B., Elandaloussi, L., Wagley, S., Furones, M.D., Ruiz-Zarzuela, I., de Blas, I., Rangdale, R., Gomez-Gil, B., 2009. Detection and identification of *tdh*- and *trh*-positive *Vibrio parahaemolyticus* strains from four species of cultured bivalve molluscs on the Spanish Mediterranean Coast. Appl. Environ. Microbiol. 75, 7574-7577.

Shen, X., Cai, Y., Liu, C., Liu, W., Hui, Y., Su, Y.C., 2009. Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (*Crassostrea plicatula*). Int. J. Food Microbiol. 136, 129-132.

Thomson, W.K., Thacker, C.L., 1973. Effect of temperature in *Vibrio parahaemolyticus* in oysters at refrigerator and deep freeze temperature. Can. Inst. Food Sci. Technol. J. 6, 156-158.

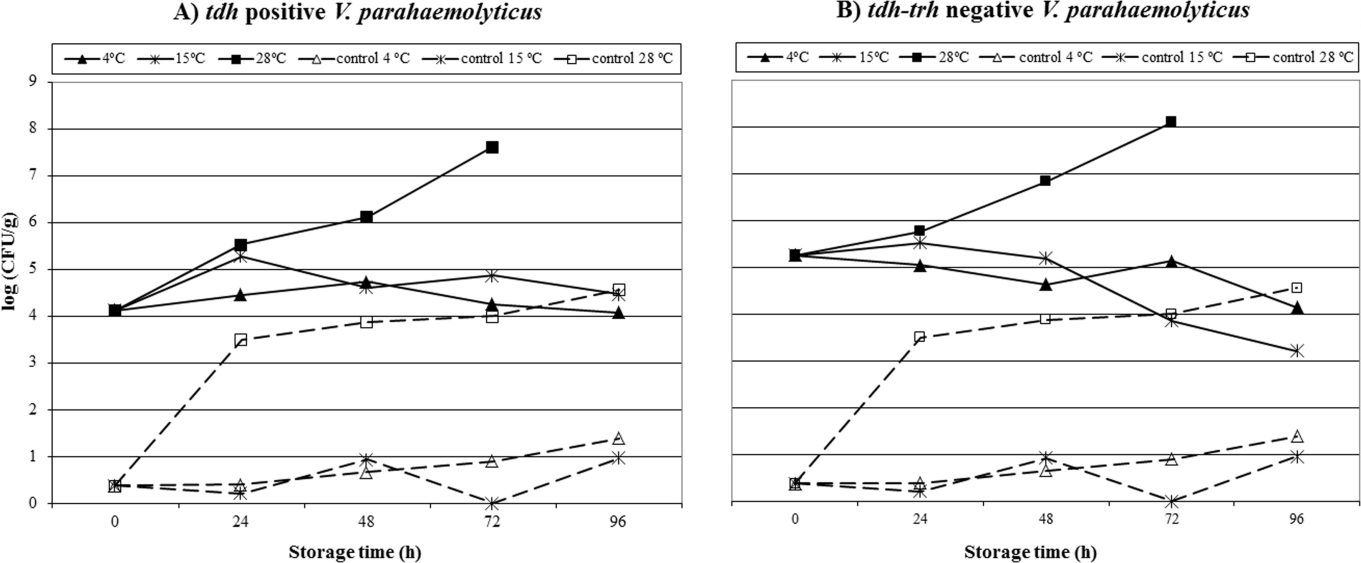
Yoon, K.S., Min, K.J., Jung, Y.J., Kwon, K.Y., Lee, J.K., Oh, S.W., 2008. A model of the effect of temperature on the growth of pathogenic and nonpathogenic *Vibrio parahaemolyticus* isolated from oysters in Korea. Food Microbiol. 25, 635-641.

Table 1. Number (n) of *Ruditapes philippinarum* clams analysed at each time point from each batch (exposed to *tdh* positive *Vp; tdh-trh* negative *Vp*; unexposed clams) used in the present study with three different storage temperatures (ºC). Average and standard deviation of *V. parahaemolyticus* log levels (expressed as mean ± standard deviation).

	A (*tdh* positive *Vp*)						**B (*tdh-trh* negative *Vp*)**						**Control Group (unexposed clams)**					
	n	Log CFU/g					n	Log CFU/g					n	Log CFU/g				
0 h	11	4.12 ± 1.43					13	5.26 ± 0.27					16	0.38 ± 0.82				
		4ºC		**15ºC**		**28ºC**		**4ºC**		**15ºC**		**28ºC**		**4ºC**		**15ºC**		**28ºC**
	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g	n	Log CFU/g
24 h	11	4.46 ± 0.60	6	5.28 ± 0.43	6	5.53 ± 0.52	11	5.05 ± 0.57	6	5.52 ± 0.43	6	5.77 ± 0.44	14	0.40 ± 1.06	9	0.22 ± 0.65	9	3.50 ± 1.59
48 h	11	4.73 ± 0.63	6	4.61 ± 0.83	6	6.12 ± 0.79	11	4.64 ± 0.84	6	5.19 ± 1.19	6	6.84 ± 0.75	14	0.67 ± 1.14	9	0.93 ± 1.40	9	3.88 ± 0.80
72 h	11	4.26 ± 0.58	6	4.87 ± 0.53	6	7.61 ± 0.90	11	5.14 ± 0.46	6	3.86 ± 2.12	6	8.11 ± 0.69	14	0.90 ± 1.38	9	0.00 ± 0.00	9	4.00 ± 2.19
96 h	11	4.08 ± 0.57	6	4.46 ± 0.32	0		11	4.15 ± 0.64	6	3.22 ± 0.68	2	5.37 ± 0.95	14	1.39 ± 1.72	9	0.97 ± 1.45	3	4.56 ± 1.62

Figure captions

Figure 1. Growth of *tdh* positive (A), and *tdh-trh* negative (B) *V. parahaemolyticus* in clams during 96 h storage study at refrigeration temperatures (4ºC), Mediterranean winter temperatures (15ºC), and Mediterranean summer temperatures (28ºC).


A) tdh positive V. parahaemolyticus
B) tdh-trh negative V. parahaemolyticus
4°C
15°C
28°C
control 4 °C
control 15 °C
control 28 °C
log (CFU/g)
Storage time (h)
0
24
48
72
96