1 **Temperature effects on the growth and survival of *tdh* positive *Vibrio***

2 ***parahaemolyticus* in tissues of postharvest Manila clam (*Ruditapes philippinarum*)**

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16 **ABSTRACT**

17 Bivalve storage at inadequate temperatures contains greater densities of *Vibrio*

18 *parahaemolyticus*. The objective of the present study was to determine if there were any

19 differences in growth and survival of potentially pathogenic *V. parahaemolyticus* (*tdh*

20 positive) in relation to nonpathogenic *V. parahaemolyticus* (*tdh-trh* negative) levels in

21 Manila clams (*Ruditapes philippinarum)* when exposed to different postharvest

22 temperatures. Clams were depurated then exposed to known doses of both potential

23 pathogenic and nonpathogenic *V. parahaemolyticus* for 24 h. Clams were then kept at

24 the following temperatures corresponding to the Mediterranean summer (28ºC), winter

25 (15ºC) and refrigeration (4ºC) for 96 h. *Vibrio parahaemolyticus* densities were

26 determined at 0, 24, 48, 72 and 96 h of postharvest storage. Both isolates of *V.*

27 *parahaemolyticus* multiplied rapidly in live clams held at 28ºC, (increase of 3 logs

28 CFU/g at 72 h). Nonpathogenic *V. parahaemolyticus* in clams stored at 4ºC and 15ºC

29 showed 1 log CFU/g and 2 log CFU/g decrease at 96 h, respectively, while no

30 significant differences were detected for pathogenic *V. parahaemolyticus* at these

31 temperatures after 96 h.

32

33 *Keywords*: *Vibrio parahaemolyticus*; *Ruditapes philippinarum*; Postharvest temperature;

34 Food Safety

36 **1. Introduction**

37

38 *Vibrio parahaemolyticus* is a common cause of shellfish-related gastroenteritis (Daniels

39 et al., 2000; Lozano-León et al., 2003). Due to their filter-feeding activity, bivalve

40 molluscs can concentrate bacteria in their tissues. Environmental temperatures,

41 time/temperature abuse and mishandling in postharvest molluscs may easily induce

42 rapid multiplication of *V. parahaemolyticus* (Beuchat, 1982; DePaola et al., 2000).

43 Hemolysins, namely the thermostable direct hemolysin (TDH) and the TDH-related

44 hemolysin (TRH), encoded by *tdh* and *trh* genes respectively, are important virulence

45 factors as they contribute to precipitate the disease (Honda and Iida, 1993). In addition,

46 hemolytic activity based on TDH-producing *V. parahaemolyticus* is present in almost

47 all *V. parahaemolyticus* strains isolated from clinical specimens, whereas it is present

48 only in 1 to 3% of the isolates of environmental origin (Miyamoto et al., 1969). The

49 population dynamics of a number of pathogenic bacteria in molluscs has been modelled

50 (DePaola et al., 2009; Yoon et al., 2008), but not using live clams. Knowing the survival

51 of pathogenic populations of *V. parahaemolyticus* in relation to the total population of

52 *V. parahaemolyticus* during postharvest-process in live molluscs will contribute to

53 prevent outbreaks and to improve the monitoring of harvest sites. Therefore, the

54 objective of the present study was to model the growth and survival of one potentially

55 pathogenic isolate (*tdh* gene positive) and one isolate classified as nonpathogenic

56 (containing neither *tdh* nor *trh* genes), in *Ruditapes philippinarum*, stored at three

57 different temperatures corresponding to the Mediterranean summer (28ºC), winter

58 (15ºC) and refrigeration (4ºC) following artificial contamination with these two isolates

59 of *V. parahaemolyticus* and to determine whether there were any differences in bacterial

60 kinetics survival.

3

61 Results from the study can contribute to the production of data for quantitative risk

62 analysis and harmonize an *in vivo* model for further research.

63

64 **2. Material and methods**

65

66 Experiment assessed two factors: temperature and vibrio. The temperature was divided

67 into three categories (4, 15 and 28ºC), vibrio was divided into *tdh* positive isolate

68 (potentially pathogenic *V. parahaemolyticus*) or *tdh-trh* negative isolate (potentially

69 nonpathogenic *V. parahaemolyticus*), and a control group without *Vibrio* (non-

70 inoculated clam).

71 Adult clams (*R. philippinarum)* were collected from the Ebro Delta. Clams used in the

72 study were all alive, opened and closed their valves and presented intact shells with

73 normal coloration. Clams were transported to the laboratory in cool boxes containing

74 ice plates. Transport lasted less than 1 h. Upon arrival, clams were placed in a

75 recirculation system flowing sterile seawater in order to depurate them for 12 days

76 (Lopez-Joven et al., 2011a). Five *R. philippinarum* per tank were sampled to confirm

77 that clams had undetectable levels of sucrose nonfermenting vibrios, including *V.*

78 *parahaemolyticus*. *Vibrio parahaemolyticus* used in the study were isolated from the

79 bivalves cultured in the Ebro Delta (Roque et al., 2009), and assumed to be fully

80 adapted to the bivalves and their environment, reason why they were preferred to type

81 strains. Potentially pathogenic isolate, *V. parahaemolyticus* i678 belongs to the serotype

82 O2:K28 containing *tdh* gene (*tdh* positive *Vp),* was isolated from a clam. Nonpathogenic

83 isolate used, *V. parahaemolyticus* i747 (*tdh-trh* negative *Vp),* was isolated from a

84 mussel. Isolates were characterized with API 20E (BioMérieux, Marcy-l'Etoile, France)

85 giving a profile of *V. parahaemolyticus* with 99.5% and 99.9% similarity, respectively.

4

86 Biotype was known to correspond to a non-pandemic strain. Inocula were prepared by

87 resuscitating a cryopreserved isolate in 50 mL of tryptic soy broth (TSB) (Scharlau

88 Chemie S.A., Barcelona, Spain) with 2.0% NaCl at 28ºC for 18-20 h in a rotating shaker

89 (Excella E24 Incubator skaker series. New Brunswick Scientific. Edison, New Jersey,

90 USA). Bacteria were collected by centrifugation at 2000 g, 4ºC, 10 min. Supernatant

91 was discarded and the pellet was suspended in sterile saline solution 2.5% NaCl (SSS).

92 Bacterial solution was added to 10 L containers with sterile seawater containing the

93 depurated clams. Challenge densities were 6.16 ± 0.48 log CFU per clam (*tdh* positive

94 *Vp*, n = 97 clams) or 6.72 ± 0.32 log CFU per clam (*tdh-trh* negative *Vp*, n = 101

95 clams), along with phytoplankton (10^5 cells/mL), axenic *Isochrysis galbana* variety

96 Tahiti (Pérez Camacho et al., 2002) to stimulate bacterial uptake by the clams during 24

97 h (Lopez-Joven et al., 2011b). A control container without *Vibrio* inocula (n = 138

98 clams) was also fed axenic algae.

99 At the end of the 24 h challenge, each batch of clams exposed to *V. parahaemolyticus*

100 and control group, was divided in three equal lots which were placed in different moist

101 plastic containers, and one container from each isolate of *Vibrio* and one for unexposed

102 clams was then stored at each of the temperatures (4, 15 and 28ºC) during 96 h.

103 Nine experimental groups in a full-factorial experiment were investigated using a total

104 of 336 clams.

105 At this point (time 0 h) samples (n = 11 clams exposed to *tdh* positive *Vp*; n = 13 clams

106 exposed to *tdh-trh* negative *Vp*; n = 16 clams unexposed) from each batch were

107 analysed. Concentrations of *V. parahaemolyticus* in clams were estimated every 24 h

108 during storage for 96 h. At each time, 3-5 clams were sampled from each container.

109 Each clam was measured (Digital caliper (0-150 mm) LLG, Mitutoyo CD-15, Tokyo,

110 Japan) and its flesh was weighed. Clams were homogeneous with no significant

5

111 differences among experimental groups. The average length (mm) and weight (g) of

112 clams used in the study were 32.80 ± 5.14 mm and 2.03 ± 0.78 g, respectively.

113 Tissue and shell liquor were aseptically removed, weighed and placed into 10 mL of

114 SSS. The resulting mixture was homogenized for 30 s using a digital homogenizer

115 Ultra-Turrax T-25 (IKA-Werke GmbH & Co. KG). Decimal dilutions were made in

116 SSS and volumes of 0.1 mL from each dilution were spread onto Chromagar Vibrio

117 plates and incubated at 37ºC for 18-20 h. The colonies formed (*V. parahaemolyticus*

118 produces mauve-purple colonies onto Chromagar Vibrio) were counted manually to

119 calculate the density of viable cells in the sample (CFU/g).

120 Experiment was performed twice with an extra replicate for storage at 4ºC in order to

121 verify previous results, taking into account this temperature is used as a critical control

122 point to protect public health at home, restaurant, retail… (Table 1).

123 Descriptive statistics were performed using mean and standard deviation for quantitative

124 variables, as length and weight, stratified by experiments. Homoscedasticity was tested

125 using Kolmogorov-Smirnov test to check normal distribution, and Levene test for

126 variance homogeneity. Non-homoscedastic variables, as CFU/g, were log-transformed

127 to improve homoscedasticity. Differences between means for log (CFU/g) depending on

128 *tdh*-positive and *tdh-trh* negative *V. parahaemolyticus*, temperature and time were

129 analyzed with one-way analysis of variance (ANOVA) followed by Duncan post-hoc

130 test. A General Linear Model for log (CFU/g) using vibrio strains, temperature and time

131 as fixed factors was carried out to check interactions between factors. Desired alpha

132 error was established at 0.05. Statistical analysis was performed using IBM SPSS 19.0

133 for Windows software (Armonk, NY, USA).

134

135 **3. Results and discussion**

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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

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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

8

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

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211 showed similar and constant concentrations at 4ºC and 15ºC along the time and

212 increasing concentrations along the time at 28ºC (at least during the first 72 h).

213

214 **4. Conclusions**

215

216 This research highlighted the different behavior of different biotypes of *V.*

217 *parahaemolyticus* at three different storage temperatures. Results here suggest that

218 conditions or ability for survival of a potentially pathogenic *V. parahaemolyticus* are

219 better in postharvest Manila clam, as compared to a potentially nonpathogenic *V.*

220 *parahaemolyticus* at 4ºC and 15ºC. Present study was based on the growth and survival

221 of one single isolate of each potentially pathogenic and nonpathogenic *V.*

222 *parahaemolyticus*; thus more extensive growth data for various pathogenic strains

223 possessing the *tdh* gene as well as various nonpathogenic strains are needed for the data

224 validation. Furthermore, it is relevant that no differences appear to exist between *tdh*

225 positive *V. parahamolyticus* densities in clams stored at 4ºC and 15ºC, therefore, clams

226 could be stored at either of these temperatures which would facilitate transport

227 conditions after harvesting and depuration.

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236

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238

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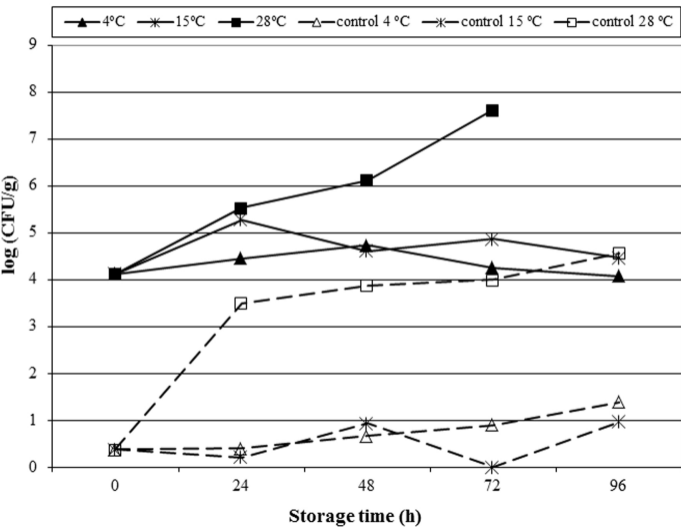
Table 1. Number (n) of *Ruditapes philippinarum* clams analysed at each time point from each batch (exposed to *tdh* positive *Vp; tdh-trh* negative *Vp*; unexposed clams) used in the present study with three different storage temperatures (ºC). Average and standard deviation of *V. parahaemolyticus*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

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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*