Effect of different temperatures on the growth and survival of toxigenic and non toxigenic *Vibrio parahaemolyticus* in tissues of Manila clam (*Ruditapes philippinarum*).

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Introduction

Vibrio parahaemolyticus is a human pathogen widely distributed in marine environments with outbreaks associated with consumption of shellfish (Daniels *et al.,* 2000).

Growth behaviour of toxigenic and non toxigenic strains of *V. parahaemolyticus* inside bivalves has not yet been compared. Effective post-harvest treatments to eliminate toxigenic vibrios contamination is an important step to reduce risk of infection associated with seafood consumption. No information is available on the growth and survival of *V. parahaemolyticus* in the Manila clam (*Ruditapes philippinarum*) stored at different temperatures.

The objective of the present work was to model the growth and survival of toxigenic and non toxigenic *V. parahaemolyticus* levels in *Ruditapes philippinarum*, stored at three different temperatures. Temperatures normally produced in the Mediterranean summer (28 ºC), winter (15 ºC) and cooled temperatures (4 ºC) in order to assess the levels of the two types of *V. parahaemolyticus* and identify if there were any differences in bacterial kinetics when compared.

Materials and methods

Live clams came from the delta of the Ebro River and reached the laboratory within the hour. On arrival clams were placed inside a raceway system for depuration. When clams presented undetectable levels of sucrose non-fermenting vibrios, they were placed in 10 L containers with sterile seawater and exposed to either non toxigenic or toxigenic *V. parahaemolyticus* at a concentration of 6.72 ± 0.32 log CFU/ml or 6.16 ± 0.48 log CFU/ml, respectively during 24 h based on previous experiments (Lopez-Joven *et al.* (2009) for 3th International Conference on Vibrio, Rio de Janeiro, Brazil). Isolates were collected from Spain in summer 2006 (Roque et *al.,* 2009) and prepared to a density of 10^4 - 10^5 CFU ml^{-1} in the exposure tank water.

Exposed clams were placed in moist plastic containers and stored at different temperatures (4, 15 and 28 ºC). Three containers were used for each storing temperature: a control container and a container for each type of *V. parahaemolyticus*. Populations of *V. parahaemolyticus* in clams were estimated every 24 h during storage for 96 h. At each time, 3-5 clams were taken out from each container for analysis. Study was repeated. The weight (g) and length (mm) of clams (mean ± standard deviation) was 2.03 ± 0.76 g and 32.80 ± 5.02 mm. Clams were individually homogenized in 10 ml of sterile 2.5 % NaCl solution (SSS). Decimal dilutions were made of the homogenates in SSS and inoculated on Chromagar vibrio. Colonies formed

were counted to calculate the load of *V. parahaemolyticus* (CFU/g). Populations of *V. parahaemolyticus* were expressed as the mean density of all determinations for each temperature and time. Differences between non toxigenic and toxigenic *V. parahaemolyticus* at the same temperature and time were analyzed with one-way analysis of variance (ANOVA) followed by Duncan post-hoc test. Significant differences between means of treatments were established at level of $P = 0.05$. Prior to analysis, quantitative variables were log-transformed to improve homoscedasticity and linearity.

Results and discussion

The populations of non toxigenic or toxigenic *V. parahaemolyticus* in clams increased rapidly from non-detectable to 5.26 ± 0.27 log CFU/g or 4.12 ± 1.43 log CFU/g after 24 h of exposure to seawater containing non toxigenic *V. parahaemolyticus* (6.72 ± 0.32 log CFU/ml) or toxigenic *V. parahaemolyticus* (6.16 ± 0.48 log CFU/ml), respectively, at room temperature. These values were established as initial levels of storage (time 0).

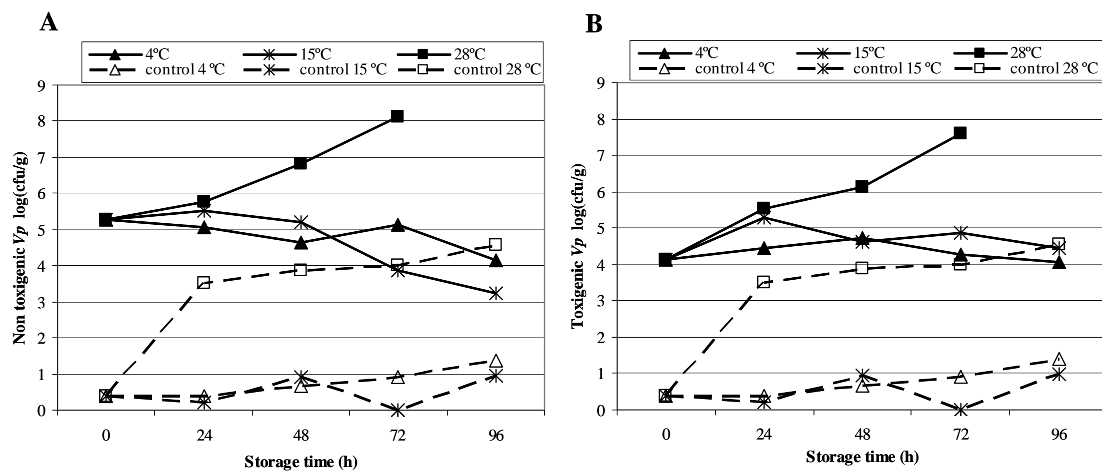

Fig.1. Changes in populations of non toxigenic *V. parahaemolyticus* (Fig. 1A) and toxigenic *V. parahaemolyticus* (Fig. 1B).

Population of non toxigenic *V. parahaemolyticus* increased from 5.26 to 8.11 log CFU/g in clams (Fig. 1A), and population of toxigenic *V. parahaemolyticus* increased from 4.12 to 7.61 log CFU/g (Fig 1B), stored at 28 ºC for 96 h. Duncan post-hoc indicated significant differences ($P < 0{,}001$) in growth and survival of non toxigenic *V. parahaemolyticus* at 48 and 72 h. Populations of toxigenic *V. parahaemolyticus* showed significant differences ($P < 0{,}001$) from 24 h. The study also showed that remaining *V. parahaemolyticus* in control group was able to multiply in clams at 28 ºC. And there were significant differences ($P < 0{,}001$) from 24 h with an increase from 3.50 to 4.00 log CFU/g. At 4 and 15 ºC, significant reductions from 5.26 to 4.15 log CFU/g and from 5.26 to 3.22 log CFU/g were detected at 4 ºC and at 15 ºC ($P < 0{,}001$) of non toxigenic *V. parahaemolyticus* (Fig. 1A). Duncan test indicated that reductions were significant after 96 and 72 h, respectively. However, results of toxigenic *V. parahaemolyticus* remained fairly constant and did not show significant differences in clams stored at 4 ºC ($P = 0{,}350$) and 15 ºC ($P = 0{,}194$), after 96 h. Similarly, population of *V. parahaemolyticus* in control group of clams did not show significative reductions along time, in clams stored at 4 ºC ($P = 0{,}184$) and 15 ° C ($P = 0{,}158$).

Generation times of *V. parahaemolyticus* are very fast (Natarajan et *al.* 1980), and in raw seafood could be a potential public health hazard if the storage temperature is inadequately controlled (Beuchat 1982). Cook and Ruple (1989) noted a rise in *Vibrio* levels in postharvest shellstock oysters stored at 22 and 30 ºC), but they reported no increases at 10 ºC during a 5-day period. Gooch *et al.* (2002) also observed that *V. parahaemolyticus* multiplied rapidly in live oysters held at 26 ºC after harvest and decreased during refrigeration storage at 3 ºC. Shen *et al.* (2009) reported that populations of *V. parahaemolyticus* increased in oysters when the oysters were stored at 15 ºC for 60 h, but remained fairly constant in oysters stored at 10 ºC and decreased gradually in oysters stored at 0 and 5 ºC, after 96 h. Yoon *et al.* (2008) inoculated both pathogenic and nonpathogenic *V. parahaemolyticus* strains into oyster slurry and observed both pathogenic and nonpathogenic *V. parahaemolyticus* decreased at 10 ºC and 15 ºC in oyster slurry, and then increased at 20 ºC and pathogenic *V. parahaemolyticus* decreased more rapidly than nonpathogenic *V. parahaemolyticus* at both temperatures. Present results showed highest densities for both types of vibrios at 28 ºC, but in contrast to the results of Yoon et al. (2008), only non toxigenic *V. parahaemolyticus* decreased after 96 h of storage at 4 and 15 ºC in clams while levels of toxigenic *V. parahaemolyticus* were maintained constant during time at 4 and 15 ºC. Results presented here showed that toxigenic *Vibrio parahemolyticus* might survive better than non toxigenic *Vibrio parahemolyticus* in Manila clam.

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