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Short communication

Effect of lysozyme addition on the activity of phages against *Vibrio parahaemolyticus*

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ABSTRACT

Phage therapy gained increased attention as an alternative to antibiotics in order to control bacterial diseases and prevent the spreading of multidrug-resistant bacteria in aquaculture. In this study, the addition of a lytic enzyme (lysozyme) during phage therapy was evaluated for the first time to control *Vibrio* infection. Three phages of *Vibrio parahaemolyticus* (VP-1, VP-2 and VP-3) were used. The results indicate that the combination of lysozyme and phage showed better activity in comparison to the activity of the phage alone. VP-1 and VP-2 phages in the presence of lysozyme were more effective in reducing bacterial concentration (reductions of 4.0 log) than the VP-3 phage (which was the most efficient against *Vibrio*). The application of phages along with lysozyme can be a very useful strategy to eliminate or reduce fish pathogenic bacteria in aquaculture, namely when less effective phages are available.

1. Introduction

It is estimated that the world population obtains at least 20% of its animal protein intake from aquaculture production (FAO, 2012). However, microbial disease outbreaks are one of the major constraints for the development of the aquaculture sector resulting in huge economic losses (Almeida et al., 2009). The typical fish farming diseases are caused by main biological agents but bacterial diseases are a major problem in the expanding aquaculture industry (Almeida et al., 2009; Wahli et al., 2002).

Vibrio species cause vibriosis, a common disease in marine and freshwater fish worldwide, both in natural and commercial production systems (Almeida et al., 2009; Isnansetyo et al., 2009; Toranzo et al., 1991). Vibriosis is caused by bacteria of the genera *Vibrio* (*V. anguillarum*, *V. vulnificus*, *V. parahaemolyticus*, *V. alfinolyticus*) (Almeida et al., 2009; Hanna et al., 1992; Sung et al., 1999; Toranzo et al., 1991). *V. parahaemolyticus* is a halophilic Gram-negative bacterium known to be an important human pathogenic bacterium (Su and Liu, 2007). This bacterium is widely distributed in the marine environments, frequently isolated from a variety of seafood (Daniels et al., 2000; Su and Liu, 2007; Wong et al., 2000). It is frequently associated with the development of acute gastroenteritis in human by consumption of raw or undercooked contaminated seafood, particularly shellfish (Kaysner and De Paola, 2000).

Although the administration of antibiotics in aquaculture has been widely used to control vibriosis, this strategy has a serious negative impact on the environment, increasing the problem of bacterial resistance (Alcaide et al., 2005; Le et al., 2005; Sarter et al., 2007). To reduce the risk of development and dissemination of microbial resistance and to control fish diseases in aquaculture, alternative strategies must be developed. Phage therapy gained increased attention as a possible alternative to antibiotics in aquaculture (Higuera et al., 2013; Inal, 2003; Karunasagar et al., 2007; Martínez-Díaz and Hipólito-Morales, 2013; Nakai and Park, 2002; Park and Nakai, 2003; Shivu et al., 2007; Silva et al., in press; Silva-Aciares et al., 2013; Skurnik and Strauch, 2006; Verner-Jeffreys et al., 2007).

Although the use of phage lytic enzymes (endolysins) has now been described to reduce the number of an extensive range of bacteria (Díez-Martínez et al., 2013; Drulis-Kawa et al., 2012), there is no report assessing the effect of phage therapy in the presence of lytic enzymes. Lytic enzymes are encoded by the phage genome to facilitate the infection or to destroy the bacterial cell wall. The lytic enzymes used to infect the bacterial cell are components of the virion tail, which are able to locally digest the cell wall from the outside to facilitate the injection of the phage genome into the host cell (Hogg, 2005). These kinds of lytic enzymes are widespread in virions, infecting Gram-positive or Gram-negative bacteria (Hogg, 2005). The lytic endolysins used to weaken the cell wall are synthesized in bacterial cells during phage multiplication, by the end of the lytic cycle, acting on the cell wall from inside the cell and facilitating the release of the virions (Umasuthan et al., 2013).

Lysins can be classified according to their catalytic activity as lysozymes or muramidases, glucosaminidases and lytic transglycosylases

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(acting on the sugar moiety, glycosidases), N-acetylmuramoyl-L-alanine-amidases (hydrolyzing the amide bond connecting the sugar and peptide constituents of peptidoglycan) and endopeptidases (cleaving the peptide cross-bridge) (Hermoso et al., 2007).

These lytic enzymes, such as lysozymes, are also produced by other eukaryotes and prokaryotes, being involved in non-specific defence mechanisms (Burge et al., 2007). As the isolation and purification of these enzymes are easier than those of phages, they can be externally added in order to facilitate the phage penetration during phage therapy. The aim of the present study was to evaluate the effect of lysozyme from chicken egg white on the activity of three phages of *V. parahaemolyticus* (VP-1, VP-2 and VP-3) in the control of *Vibrio*.

2. Experimental procedures

The bacterial strain *V. parahaemolyticus* was previously isolated in our laboratory from marine water samples of the semi-intensive aquaculture Corte das Freiras (Pereira et al., 2011). Before each assay, one isolated colony was aseptically transferred from cultures in solid Tryptic Soy Agar medium (TSA; Merck, Darmstadt, Germany) to 10 mL of Tryptic Soy Broth medium (TSB; Merck, Darmstadt, Germany) and was grown overnight at 25 °C. An aliquot of this culture (100 μL) was transferred to fresh TSB medium and grown overnight at 25 °C to reach an optical density (O.D. 600) of 0.8 (about 10^9 colony forming units—CFU—per millilitre).

The three phages (VP-1, VP-2 and VP-3) were previously isolated and purified in our laboratory from marine water samples of the Aquaculture Corte das Freiras, using *V. parahaemolyticus* as host (Mateus et al., 2014). The phage suspensions (10^9 plaque forming units (PFU) per millilitre) were maintained in TSB at 4 °C with 2% of chloroform.

Phage therapy was performed with the three phages (VP-1, VP-2 and VP-3) using the bacterium *V. parahaemolyticus* as host. The assays were performed with single-phage suspensions and with a combination of phage with lysozyme (lysozyme from chicken egg white: Sigma-Aldrich: St. Louis, USA).

A diluted overnight culture of *V. parahaemolyticus* (10^5 CFU mL^{-1}) and a phage suspension (10^7 PFU mL^{-1}) were inoculated TSB medium (MOI 100). The lysozyme solution was added to the mixture, which was incubated at 25 °C without agitation (test sample). Four control samples were also included, the bacterial control without lysozyme (BC), the phage control without lysozyme (PC), the bacterial control with lysozyme (BCL) and the phage control with lysozyme (PCL). Aliquots of test samples and of the bacterial and phage controls were collected after 0, 2, 4, 6, 8, 10, 12, 18, 24 h of incubation for host and phage quantification in the test samples, for host quantification in the bacterial control and for phage quantification in the phage control. The bacterial concentration was determined in duplicate, by pour plating, in TSA medium after incubation at 25 °C for 24 h. The phage titre was determined, in duplicate, by the double agar layer method, after of 12–18 h at 25 °C. For the VP-3 phage, assays were performed in the presence of three lysozyme concentrations (3, 10 and 20 mg mL^{-1}) and for the other two phages, VP-1 and VP-2, only one lysozyme concentration was tested (10 mg mL^{-1}). Three independent experiments were performed for each condition.

2.1. Statistical analysis

Statistical analysis was performed using SPSS (SPSS 20.0 for Windows, SPSS Inc., USA). The existence of significant differences among the different phage conditions was assessed by one-way analysis of variance (ANOVA). For each situation, the significance of the differences was done by comparing the results obtained in the test samples after correction with the results obtained for the correspondent control samples (difference between the control and the test sample) for the different times of each of the three independent assays. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

The maximum of bacterial inactivation with VP-1 phage without the addition of lysozyme was 2.9 log, achieved after 6 h of incubation. However, the combination of the phage with lysozyme at 10 mg mL^{-1}, reached a maximum of bacterial inactivation of 4 log after 2 h of incubation, being statistically different from the values obtained with the phage without lysozyme (ANOVA, $p < 0.05$). The difference between the two samples was statistically significant until 10 h of incubation (ANOVA, $p < 0.05$) (Fig. 1A).

The bacterial concentration in BC and in BCL was similar during the 24 h of incubation (ANOVA, $p > 0.05$) (Fig. 1A). Phage survival did not decrease during the 24 h of the experiments, neither for the PC nor for PCL (Fig. 1B). When the phage alone and the phage with lysozyme were incubated in the presence of the host, a significant increase of 0.7 log and 1.6 log, respectively, was observed after 24 h of incubation, being the difference between both samples statistically significant (ANOVA, $p < 0.05$) (Fig. 1B).

The maximum of bacterium inactivation with VP-2 phage without lysozyme was 3.6 log after 8 h of incubation. The assays with the combination of the phage and lysozyme at a concentration of 10 mg mL^{-1} were statistically significant from those without lysozyme, with a maximum of bacterial inactivation of 4 log after 4 h of incubation (ANOVA, $p < 0.05$). The difference between the two samples was already observed after 2 h of incubation, with reductions on the bacterial concentration of 3.5 log, when lysozyme was added to the samples, and of 0.8 log without the presence of lysozyme (ANOVA, $p < 0.05$). After 12 h of phage therapy the rate of inactivation was still

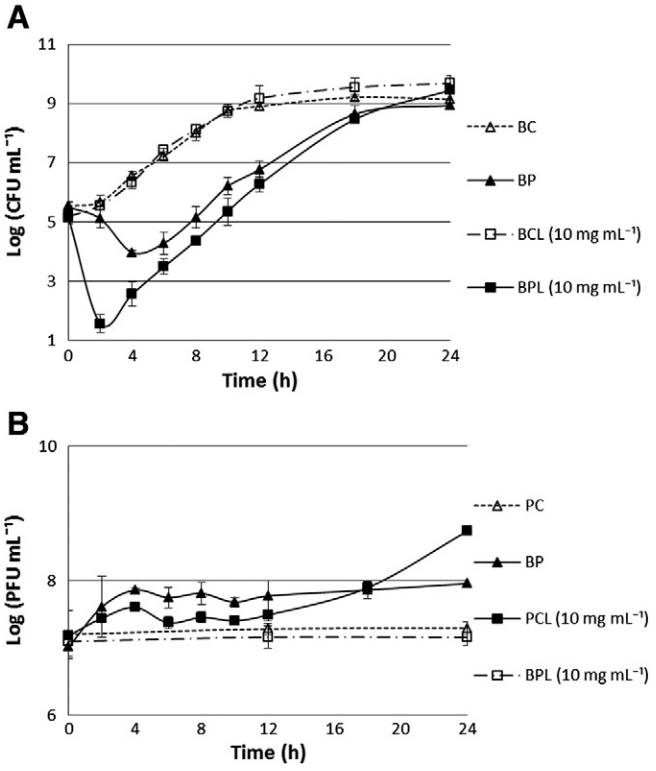

Fig. 1. Inactivation of *V. parahaemolyticus* by VP-1 phage with and without lysozyme (10 mg mL^{-1}). (A) bacterial and (B) phage concentration variation along the 24 hour experiment. BC—bacterial control; BP—bacteria plus phage, BCL—bacterial control with lysozyme, BPL—bacteria plus phage and lysozyme, PC—phage control and PCL—phage control with lysozyme. Values represent the mean of three independent experiments; error bars indicate the standard deviation.