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Phage inactivation of *Salmonella enterica* in cockles during depuration

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Introduction

Salmonella enterica subsp *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the most frequent causative agent of human gastroenteritis, after consumption of contaminated seafood.

Depuration is a useful method to eliminate microorganisms from bivalves when occur under conditions that maximize the natural filtering activity, which results in expulsion of intestinal contents. However, some pathogenic microorganisms are resistant to this process [1,2,3].

To reduce the risk of the development and transmission of infections caused by microbial pathogens, including multidrug-resistant bacteria, other technologies associated with depuration, such as phage therapy must be developed.

This study investigated the potential application of the bacteriophage SE-5 during depuration to reduce *S.* Typhimurium in cockles (*Cerastoderma edule*) at different multiplicity of infection (MOI).

Material and Methods

Bacteria and growth conditions

S. Typhimurium (ATCC 13311) grew at 37 ºC, pH 7.3, in tryptic soy broth.

Bacteriophages

SE-5 phage was isolated from sewage water, according to the procedure described by Pereira et al, 2011 [4].

Bacterial inactivation by phages

Phage therapy was performed using SE-5 (90µL of 10^9 PFU mL^{-1}) phage and bacterium *S.* Typhimurium (2.5 µL of 10^9 CFU mL^{-1}) as host, at a MOI of 100.

Bacteria and phage were inoculated in TSB and incubated at 25 C.

Two control samples were included, the bacterial control and the phage control, respectively, without phages and without bacteria.

For host quantification, aliquots were serially diluted, plated by pour plating method in duplicate and incubated at 37 C for 24 hours.

The phage titre was determined by the double-layer method and incubated at 37 C for 6 hours (Figure 1).

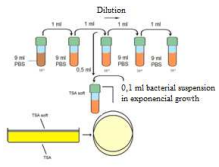

Figure 1 : Soft agar overlay technique

Accumulation of *S.* Typhimurium in cockles

Fresh bacterial culture was prepared (~10^9 colony-forming units (CFU / mL) and poured into a set of three aquaria with 0.6 L of natural seawater.

The final concentration of *S.* Typhimurium in the four infected groups was 10^5, 10^6, 10^7 and 10^8 CFU/mL, respectively. Negative control was included (without *S.* Typhimurium)

Cockles depuration with phages

Challenged cockles with 10^6 colony-forming units (CFU/mL) of *S.* Typhimurium in the seawater and each infected group was treated with four different MOI values: 100, 10, 1 and 0.1.

For each infected group (10^5, 10^6, 10^7 and 10^8 CFU/mL), cockles (n = 30) infected with *S.* Typhimurium were divided into four treatment groups (n = 30 each):

- Control bacteria (BC) : fish group infected with the bacterium (10^6 $CFUmL^{-1}$) but not treated with the phage.
- Control phage: fish group not infected with the bacterium but added of phages
- Control bivalve (SB): Bivalve group without phage and without bacteria
- Phage treatment with an MOI of 0.1
- Phage treatment with an MOI of 1
- Phage treatment with an MOI of 10
- Phage treatment with an MOI of 100

Infected cockles were depurated in non-recirculating seawater at 16 ℃ for 12 h.

Bacterial and phage concentration was determined as described before.

Results and Discussion

Phage isolation and enrichment

SE-5 formed clear plaques on the host strain with a diameter of 2-5 mm (Figure 2).

Figure 2 : Phage plaques of the SE-5 phage.

Bacterial inactivation by phages

The maximum of bacterium inactivation by the SE-5 phage was 2.5 log, achieved after 12 h of applying phage therapy. However, only after 4 h of treatment, the rate of inactivation was already significant (1.9 log) (Figure 3).

The phage control remained constant along the experiment. However, when phages incubated in the presence of its host, the concentration increased significantly (0.35 log) after 2 h of phage addition, remaining relatively constant after and until the end of experiment.

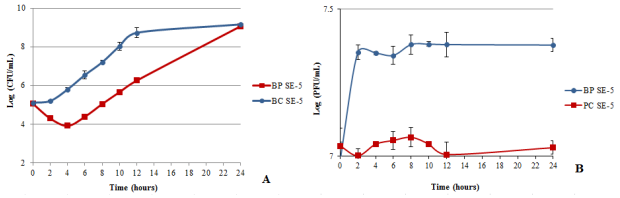

Figure 3. Inactivation of *S.* Typhimurium by the SE-5 phage at a MOI of 100 during the 24 h. **A.** Bacterial concentration: BC – Bacteria control; BP – Bacteria plus phage. **B.** Phage concentration: PC – phage control; BP – Bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation

Accumulation of *S.* Typhimurium in cockles

Live cockles were assessed after 36 h of pathogen accumulation at 16 ℃.

Accumulation of *S.* Typhimurium in cockles increased rapidly during the first 6 h, and then remained constant.

This suggests that *S.* Typhimurium could colonize the cockles up to a certain level after accumulation for 12 h. Therefore, phage treatments were performed after 12 h of bacterial addition to the sea water.

Cockles depuration with phages

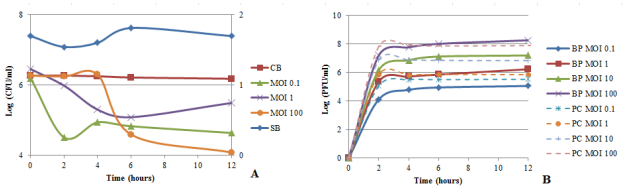

Figure 4. Inactivation of *S.* Typhimurium by the SE-5 phage in cockles during the 12 h. **A.** Bacterial concentration: SB- Group without phage and without bacteria BC – Bacteria control; MOI 0.1, 1, 10, 100 – Group infected with bacteria and treated with phage with an MOI of 0.1, 1, 10 and 100, respectively. **B.** Phage concentration: PC – phage control; BP MOI 0.1, 1, 10, 100 – Group infected with bacteria and treated with phage with an MOI of 0.1, 1, 10 and 100, respectively. Values represent the mean of three experiments; error bars represent the standard deviation.

Depuration with phages at MOI 0.1 was the best condition to inactivate *S.* Typhimurium in cockles, the concentration was reduced by 1.7 log CFU/g after 2 h of depuration. Reduction for the other MOI values (MOI= 1; MOI = 10; MOI = 100), was 1.13, 1.21 and 2.10 log CFU/g after 6 h, 12 and 12 h of treatment, respectively (Figure 4).

Accumulation of SE-5 phages in cockles increased rapidly during the first 2 h, and then remained constant along the experiment (Figure 4).

Conclusions

To our knowledge, this is the first report of a bivalve depuration using bacteriophage in the cockle treatment process to inactivate S. Typhimurium .

The data of this study indicate clearly that the application of the bacteriophages could reduce significantly the population of *S.* Typhimurium in contaminated the cockles alive during the depuration process. Therefore, the application of bacteriophage was effectively proven to be useful for shellfish depuration.

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