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Application of phage therapy during bivalve depuration improves *Escherichia coli* decontamination

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ABSTRACT

The present study investigated the potential application of the bacteriophage (or phage) phT4A, ECA2 and the phage cocktail phT4A/ECA2 to decrease the concentration of *Escherichia coli* during the depuration of natural and artificially contaminated cockles. Depuration in static seawater at multiplicity of infection (MOI) of 1 with single phage suspensions of phT4A and ECA2 was the best condition, as it decreased by ~2.0 log CFU/g the concentration of *E. coli* in artificially contaminated cockles after a 4 h of treatment. When naturally contaminated cockles were treated in static seawater with single phage suspensions and the phage cocktail, similar decreases in the concentration of *E. coli* (~0.7 log CFU/g) were achieved. However, when employing the phage cocktail, a longer treatment time was required to obtain comparable results to those achieved when using single phage suspensions. When naturally contaminated cockles were depurated with phage phT4A in a recirculated seawater system (mimicking industrial depuration conditions), a 0.6 log CFU/g reduction of *E. coli* was achieved after a 2 h of treatment. When the depuration process was performed without phage addition, a 4 h treatment was necessary to obtain a similar decrease. By combining phage therapy and depuration procedures, a reduction in bivalves depuration period can be achieved for, thus decreasing the cost associated with this procedure and even enhance the quality and safety of depurated bivalves destined for human consumption.

1. Introduction

Enteropathogenic strains of *Escherichia coli* are widely distributed in coastal areas and are causative agents of gastroenteritis in humans after consumption of contaminated seafood (Kanayama et al., 2015; Potasman et al., 2002). Cockles (*Cerastoderma edule*) are filter feeder bivalve molluscs that accumulate food particles and small organisms by circulating large volumes of seawater; consequently, microorganisms, including human pathogens, are retained and accumulate in their tissues (Lees, 2000). Moreover, as bivalves are often consumed raw or lightly cooked, they are potential vectors for pathogenic *E. coli*.

Depuration is the preferred option to decontaminate bivalves, being strictly regulated and employed worldwide (WHO, 2010). Briefly, it consists of a flow-through or recirculation system operating with chemically (chlorine, ozone, iodophores or activated oxygen) or physically (UV-C light) disinfected water, for two days, that allow bivalve purification under controlled conditions (Croci et al., 2002; FAO, 2008; Wang et al., 2010). After depuration, bivalves may be destined for human consumption if they present less than 230 most probable number (MPN) *E. coli* per 100 g of flesh and intra-valvular liquid (FIL), as well as no detectable levels of *Salmonella* spp. (FAO, 2008). However, some microorganisms, such as *E. coli* and *Vibrio* spp. are less prone to be eliminated using this process and may persist in bivalves after depuration (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). Additionally, methods commonly employed for seawater disinfection (e.g. chlorine, ozone or UV radiation) may easily drive chemical hazards that negatively affect seafood (Rong et al., 2014). Chlorination and UV may lead to the formation of toxic products, such as monochloramine, and induce specific mutations in the microbial genome, which can ultimately contribute to the selection of resistant genes (Almeida et al., 2014).

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Relaying (relocating bivalves harvested from polluted areas to a cleaner location to allow them to cleanse) is also an approved method to enforce food safety criteria in bivalves, but the regular use of this practice is limited to a few number of countries (FAO, 2008).

Non approved methods have also been evaluated to eliminate human pathogens from bivalves (Rong et al., 2014), namely post-harvest processes, heat/cool pasteurization (Andrews et al., 2000), irradiation (Andrews et al., 2003; Jakabi et al., 2003; Mahmoud and Burrage, 2009; Mahmoud, 2009), high hydrostatic pressure (Ahmadi et al., 2015; Cook, 2003; Kural and Chen, 2008; Ma and Su, 2011; Prapaiwong et al., 2009), ultraviolet (UV) exposure (Phuvasate et al., 2012) and rapid freezing with frozen storage (Liu et al., 2009). Recently, combined post-harvest techniques were also studied, such as high hydrostatic pressure (HHP) combined with moderate heating (Ye et al., 2012) and HHP combined with the use of phages (Ahmadi et al., 2015). However, bivalves die during these processing techniques and these processes can modify their nutritional and organoleptic characteristics (Rong et al., 2014). The development and evaluation of new strategies, with no adverse effects on the bivalves, in order to reduce the concentration of human pathogens in bivalves is essential. One of the most promising approach is the association of phage therapy (application of lytic phages to prevent and/or to treat bacterial infections) to the depuration process. This association will contribute to the improvement of the decontamination efficiency, likely reducing the time required for the depuration, and consequently, the production costs, with additional benefits in bivalve safety and quality.

The use of lytic phages to reduce food-borne pathogens has emerged as a promising tool for food safety (Ahmadi et al., 2015; Atterbury et al., 2007; Denes and Wiedmann, 2014; Endersen et al., 2014; Goodridge and Bisha, 2011; Jun et al., 2014; Raya et al., 2006; Rong et al., 2014; Sillankorva et al., 2012). These viruses are target-specific, self-replicating, rapid bactericides and do not modify normal food properties. Some studies have already been conducted to eliminate human pathogens from raw bivalves using phages (Ahmadi et al., 2015; Jun et al., 2014; Pelon et al., 2005). However, to date, only two studies have reported the simultaneous use of depuration and phage therapy to eliminate *Vibrio parahaemolyticus* in bivalves (Jun et al., 2014; Rong et al., 2014). Rong et al. (2014) reported the effectiveness (decrease of approximately 1.0 log CFU/g relatively to the bacterial control) of phage application to reduce the population of *V. parahaemolyticus* in the artificially contaminated oysters during depuration in static systems. Jun et al. (2014) also reported the decrease of *V. parahaemolyticus* (by approximately 5.8 log CFU/g) after 72 h of application of phage pVp-1 in artificially contaminated oysters in static systems. Besides adding phages to seawater to control bacteria, these authors have also tested the addition of phages to the surface of oysters previously contaminated with the bacteria. The results reported by Jun et al. (2014) show a decrease of approximately 5.9 log CFU/after 12 h of phage application to the oysters surface. However, the study of Rong et al. (2014) and Jun et al. (2014) have not replicated industrial depuration procedures, neither tested the suitability of this approach on naturally contaminated bivalves.

The aim of the present study was to evaluate, for the first time, the efficiency of two new phages of *E. coli* (phT4A and ECA2), individually or combined in a cocktail, to control *E. coli* in natural and artificially contaminated cockles in static water and during depuration mimicking industrial procedures currently employed.

2. Material and methods

2.1. Bacterial strains

E. coli (ATCC 13706), a microbiological indicator of shellfish depuration efficiency, was used to contaminate cockles. Fresh plate bacterial cultures were maintained in solid Tryptic Soy Agar (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth (TSB; Liofilchem, Italy) and was grown overnight at 37 °C. An aliquot of this culture (100 μL) was aseptically transferred to 10 mL of fresh TSB and grown overnight at 37 °C to reach an optical density (O.D600 = 0.8), corresponding to about 10^9 cells per mL, according to a growth curve previously determined.

2.2. Phage isolation and purification

Two phages were isolated from sewage network of Aveiro (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro), collected at different times. Sewage water was filtered through 0.45 μm pore size polycarbonate membranes (Millipore, Bedford, MA, USA). The filtrate was added to double-concentrated TSB medium with 1 mL of fresh culture of the host, *E. coli* (ATCC 13706). The mixtures were incubated at 37 °C for 18 h at 80 rpm, and then filtered through a 0.2 μm membrane (Millipore Bedford, MA, USA). The phage concentration of the supernatants was determined by the double-layer agar method using TSA as culture medium (Adams, 1959). Plates were incubated at 37 °C and examined for the presence of lytic plaques after 12 h. One single plaque was removed from the agar and incubated in SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 20 mM Tris–HCl, 2% (w/v) gelatin, pH 7.5) at 37 °C during 6 h. The sample was centrifuged and the supernatant was used as a phage source for a second isolation procedure. Three successive single-plaque isolation cycles were performed to obtain pure phage stocks. All lysates were centrifuged at 10.000 *g* for 10 min at 4 °C, to remove intact bacteria or bacterial debris. The phage stock was stored at 4 °C. The phages produced on *E. coli* were designated as phT4A and ECA2.

The phage suspension titre was determined by the double-layer agar method using TSA as culture medium (Adams, 1959). Successive dilutions of the phage suspension in phosphate buffered saline solution [137 mM NaCl (Sigma), 2.7 mM KCl (Sigma), 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.76 mM KH_2PO_4 (Sigma), pH 7.4] were performed. Five hundred microliters of each dilution and 100 μL of the respective bacterial host culture were added to 3 mL of TSB 0.6% top agar layer and plated over a TSA plate. The plates were incubated at 37 °C for 8 h and the number of lysis plaques was counted. The results were expressed as plaque forming units (PFU)/mL.

2.3. Collection of cockle samples

Cockles were selected as biological models to test the efficacy of phage therapy against *E. coli* infections and were purchased from Falcamar Lda. (Vila do Conde, Portugal), a bivalve wholesaler, after being depurated according to industrial processing protocols (48 h at 16 ± 1 °C, in seawater irradiated with UV-C). These specimens were later artificially contaminated with *E. coli* in the laboratory (see below). Naturally contaminated cockles were collected by hand in Mira Channel (Ria de Aveiro, Portugal; 40º36′30″N, 8º44′52″W), a bivalve production area ranked as B (230–4600 MPN *E. coli* per 100 g of FIL) (Despacho n.º 15264, 2013). Mira channel is a recreational area subjected to anthropogenic contamination (Pereira et al., 2015). Cockles were collected in June, July, September and October 2015 and were transported to the laboratory in a container with seawater at 16 ± 1 °C, under an oxygen saturated atmosphere. Experiments were performed no more than 30 min post-collection.

2.4. Phage application during cockles depuration in static seawater

2.4.1. Accumulation of E. coli in cockles

Non contaminated cockles were maintained in independent