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Sequential effect of phages and cold nitrogen plasma against *Escherichia coli* O157:H7 biofilms on different vegetables

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ABSTRACT

Escherichia coli O157:H7 (*E. coli* O157:H7) is one of the most common pathogens in fresh vegetables and fruits, and most of the diseases produced by *E. coli* O157:H7 are associated with biofilms. Cold nitrogen plasma (CNP) is a cold sterilization technique which has no residue. However to completely eliminate the biofilm on the surface of vegetables the processing power and time of CNP have to be enhanced, which will impact on the quality of fruits and vegetables. Thus the sequential treatment of CNP and phage techniques was engineered in this study. Compared to treatment performed separately, sequential treatment not only had more mild treatment conditions as 400 W CNP treatment for 2 min and 5% phage treatment for 30 min, but also exhibited more remarkable effect on eradicating *E. coli* O157:H7 biofilms *in vitro* and on vegetables. The population of *E. coli* O157:H7 was approximately reduced by 2 log CFU/cm^2 after individual treatment of 5% phages for 30 min or 500 W CNP for 3 min. While the sequential treatment of CNP (400 W, 2 min) and phages (5%, 30 min) reduced the *E. coli* O157:H7 viable count in biofilm by 5.71 log CFU/cm^2. Therefore, the sequential treatment holds a great promise to improve the current treatment systems of bacterial contamination on different vegetable surfaces.

1. Introduction

In recent years, as public awareness about the malicious negative impacts of the processed food on health enhances gradually, the demand for healthy foods such as fresh vegetables and fruits also increases significantly around the world (O'Beirne et al., 2014; Sagdic et al., 2013). Salad containing different kinds of ready-to-eat vegetables and fruits is a common and popular health foodstuff with good flavor and low calories (Zhao et al., 2014). Furthermore, these ready-to-eat vegetables and fruits in the salad do not need to be heated. Nevertheless, vegetables and fruits are easily contaminated with pathogenic microorganisms from fecal material of animals and manure compost during the process of irrigation, harvesting and transport. Thus with the increase in demand for ready-to-eat vegetable salad, various food borne diseases have occurred in recent years (Cui et al., 2015; Velázquez et al., 2009).

E. coli O157:H7 infection is one of the foodborne diseases that can result in diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Bang et al., 2014). Epidemiological evidences demonstrate that its outbreaks are often associated with the consumption of contaminated vegetables such as cucumber, carrot and lettuce (Poimenidou et al., 2016; Sagdic et al., 2013). There had been 18 food poisoning outbreaks due to *E. coli* O157:H7 contamination of ready-to-eat vegetables in the US between 1998 and 2006 (O'Beirne et al., 2014). *E. coli* O157:H7 forms biofilms on the surface (Macarisin et al., 2014; Torres et al., 2005), stomata, epidermal layer, stratum corneum and vein of the vegetables, which creates the best conditions for bacterial proliferation and aggregation (Seo and Frank, 1999). Once the biofilms are formed, *E. coli* O157:H7 in biofilms are more resistant to antibacterial agent when are compared to planktonic bacteria (Uhlich et al., 2006). Hence, the development of effective approaches for elimination of *E. coli* O157:H7 biofilm on vegetables is urgent for controlling outbreaks of food borne diseases.

Traditional sterilization techniques such as radiation sterilization, microwave sterilization and high pressure sterilization are subjected in some countries as strict regulatory requirements. Excessive use of these sterilization methods not only spends a lot of manpower and resources, but also leads to loss of nutritional value and food organoleptic properties of food, which will reduce the acceptance by consumers (Gutierrez et al., 2008). Hence it is necessary to explore and apply alternative sterilization methods for fresh agricultural products (Snehal et al., 2013). Among them, cold nitrogen plasma (CNP) has been widely investigated as an alternative sterilization technology in biomedical and healthcare sectors recently (Cui et al., 2016a, 2016b, 2016c, 2016d; Ziuzina et al., 2014).

Plasma is an ionized gas, which contains approximately an equal

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amount of positively and negatively charged species including radicals, electrons, ions, excited molecules and UV photons. These reactive species are widely considered to own microbial-inactivation effects (Kim et al., 2014). Some previous studies indicate that non-thermal plasma, such as CNP can be operated at room temperature, has an excellent antibacterial activity against a large number of microorganisms (Dasan et al., 2016; Korachi et al., 2009; Ma et al., 2015; Sun et al., 2012). Compared to traditional sterilization technologies like thermal treatment in food processing, in addition to its remarkable antibacterial activity, CNP technique not only has no residue but also maintains the sensory attributes of the treated foods (Jayasena et al., 2015).

Although CNP can avoid the weakness of traditional sterilization technologies, it cannot achieve high efficiency in inactivating bacteria when applied on vegetable surfaces. The high roughness and porous structure on the surface of fresh vegetables make the bacteria easy to accumulate and affect the interactions between cold plasma and samples, resulting in reduced bactericidal efficiency (Fernandez et al., 2013). In order to overcome these problems, a safe antibacterial agent was employed in association with CNP in this study to improve the antibacterial properties of CNP. The synergetic antibacterial efficacy of CNP technique and clove oil against *E. coli* O157:H7 biofilms on lettuce had been verified by our research group in a previous study (Cui et al., 2016c). However essential oils are volatile and unstable, which are not conducive to long-term sterilization. Therefore, some alternatives are being sought to be used in combination with CNP.

Bacteriophages (phage) are ubiquitous viruses, which can be found wherever bacteria exist. Bacteriophages rely on “preying” bacteria for a living. Hence, bacteriophages have a great potential for use as biocontrol agent in foods. When sufficient time is provided, the phages are able to penetrate the biofilm polymer matrix and gain access to kill the embedded pathogen (Fig. 5). In contrast, oxidizing agents, whether introduced as a chemical treatment or generated through irradiation methods, possess very limited penetration potential. A large number of studies have indicated that phages can be used successfully to reduce food borne pathogens in foods (Bigwood et al., 2008; Leverentz et al., 2003). However, the specificity of phage limits its application for use as the antibacterial agent (Edwards et al., 2016).

In this study, sequential treatment of CNP and phage techniques was performed to achieve a desired sequential antibacterial effect. As a proof of concept, the sequential antibacterial activity of CNP and phages against *E. coli* O157:H7 biofilms on fresh vegetables was also evaluated along with a sensory evaluation.

2. Materials and methods

2.1. Materials and culture

E. coli O157:H7 phage was isolated from domestic sewage from Jiangsu University, Zhenjiang, Jiangsu province, China. A volume of 100 mL of filtered (0.22 mm pore size, Millipore, Cork, Ireland) sewage was mixed into an equal volume of log-phase *E. coli* EHEC O157:H7 CICC 21530. Then, the mixture was added to an agar overlay plate and was incubated overnight at 37 °C. Finally, a single plaque was propagated on the host for three times to ensure virulence and purity (Basiak et al., 2017). After this, isolated *E. coli* O157:H7 phage was further cultivated to achieve a concentration of 10^{11} PFU/mL and stored in SM buffer (0.1 M NaCl, 8 mM $MgSO_4{\cdot}7H_2O$, 0.01% gelatine, 50 mM Tris-HCl in MilliQ pH 7.5) at 4 °C. The tested strain (*E. coli* EHEC O157:H7 CICC 21530) was provided by China Center of Industrial Culture Collection (CICC) and stored with liquid paraffin wax at 4 °C. The stand-by *E. coli* O157:H7 was cultured at 37 °C on nutrient broth (NB) medium. The vegetables chosen in this study were purchased from a local supermarket.

2.2. Antibacterial and antibiofilm activity of E. coli O157:H7 phage

2.2.1. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC and MBC were determined using the broth micro dilution method (Cui et al., 2015). Serial twofold dilutions of *E. coli* O157:H7 phages (from 10^9 PFU/mL to 1.5×10^{10} PFU/mL) in NB medium (peptone, beef extract, sodium chloride) were prepared in tubes. A diluted bacterial suspension of *E. coli* O157:H7 ($10^{5–6}$ CFU/mL) was added to each tube. These tubes were then incubated in concussion incubator (150 rpm) at 37 °C and for 48 h. Cell suspensions from the tube showing no growth were cultured on Nutrient Agar (NA, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The MIC was defined as the lowest concentration of phages that inhibit visible growth of *E. coli* O157:H7. The MBC was regarded as the lowest concentration of phages required to kill all of *E. coli* O157:H7 on the NA after incubation at 37 °C for 48 h. All the experiments were performed in triplicates.

2.2.2. Time-kill analysis of E. coli O157:H7 phages

Time-kill analysis was carried out using the plate colony counting method as described in a previous study (Kwiecin'Ski et al., 2009). *E. coli* O157:H7 phages was diluted into phosphate buffer solution (PBS, 0.03 M, pH 7.2) containing cell suspensions (10^5–10^6 CFU/mL) to obtain concentrations of 0.5 MIC, 1 MIC and 2 MIC. The samples without *E. coli* O157:H7 phages were used as control groups. Then, all the samples were cultured at 37 °C. The number of residual bacteria was measured at 0, 0.5, 1, 2, 4 and 8 h.

2.2.3. Microscopic analysis of the integrity of the cell membrane

The internal morphological changes of *E. coli* O157:H7 treated with phages were observed using a transmission electron microscope (Model: JEM-2100, JEOL, Tokyo, Japan). The bacterial samples treated with 5% phages and the bacterial samples without treatment were separately dyed with 3% (0.3×10^{10} PFU/mL) phosphotungstic acid and dried, followed by microscopic examination (Cui et al., 2016a).

2.2.4. Determination of minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC)

The antibiofilm activity of *E. coli* O157:H7 phages were measured using the MBIC and MBEC assays (Sabaeifard et al., 2014; Wu et al., 2013). MBIC was defined as the lowest concentration of *E. coli* O157:H7 phages that inhibit visible growth of biofilms in the 96-well plate (Corning Incorporated, New York, USA). The wells were filled with tryptone soy broth (TSB) medium containing *E. coli* O157:H7 ($10^{5–6}$ CFU/mL) and *E. coli* O157:H7 phages (from 1% to 15%, vol/vol). After incubation at 37 °C for 48 h, the plate was washed by PBS for three times. A volume of 0.05% (v/v) 3-(4, 5-dimethyl-2-thiazolyl)–2, 5-diphenyl-2-Htetrazolium bromide (MTT) (MTT Cell Proliferation and Cytotoxicity Assay Kit, Beyotime Biotechnology, Shanghai, China) in PBS (pH 7.2) was then added to each well on the plate. After incubation at 37 °C for 4 h, MTT solution was replaced by formazan dissolving solution. The formed formazan crystals were dissolved and the absorbance was measured at 550 nm. The MBEC indicated the lowest concentration of the antimicrobial agent leading to clear biofilm-containing wells. *E. coli* O157:H7 phages (1% to 15%, vol/vol) in PBS were then added into the wells containing the formed biofilms. The formed biofilms in sterile PBS (pH 7.2) without *E. coli* O157:H7 phages were used as control groups. The absorbance was measured at 550 nm for all the trials spectrophotometrically. All the experiments were performed in triplicates.

2.3. Antibiofilm activity of CNP (cold nitrogen plasma)

2.3.1. Operation conditions of CNP

Firstly, sterile stainless steel coupons (2×2 cm^2) were immersed in