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Enhancing the antibacterial activity of thyme oil against *Salmonella* on eggshell by plasma-assisted process

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ABSTRACT

Salmonella is the main source of microbial contamination on eggs. In the present study, the eggshells infected with *Salmonella* were employed as the test subjects. Thyme oil and cold nitrogen plasma (CNP) were introduced to treat *Salmonella* on eggshells. Both thyme oil and CNP showed low antimicrobial activities against microorganisms when they individually acted to infected eggs for short time. On the contrast, the loss of bacterial viability was obviously enhanced by combined treatment. The subsequent experiments demonstrated that thyme oil concentration and CNP treatment time were positively correlated with antibacterial effects. By CNP assist, thyme oil can effectively inhibit the multiplication of *Salmonella* on eggs under low concentrations. Among various combination groups, 0.5 mg/mL thyme oil and 400 W CNP exhibited the best synergistic antibacterial activity when they were applied for 20 min and 1 min, respectively. The populations of *Salmonella enteritidis* and *Salmonella typhimurium* were reduced below 10 CFU/egg. More importantly, the treatment in combination limited the growth of bacteria on eggs below undetected level during 14 days of storage at different temperatures (4, 12 and 25 °C).

1. Introduction

Egg-associated salmonellosis is an important public health problem in the world. Generally, colonization of *Salmonella* in the reproductive system of laying hens, chicken feces containing *Salmonella* and contaminated environments, such as a nest or hatchery can result in contamination of eggs with *Salmonella* (Park et al., 2015). Sporadic outbreaks of salmonellosis around the world have been potentially associated with direct consumption of infected eggs. Besides, the research data has shown that cross-contamination events linked to eggs have also resulted in outbreaks of salmonellosis (Little et al., 2008). Kitchen surfaces and utensils may become contaminated by the infected eggshells and serve as infection mediums to other foodstuffs (Humphrey, Martin, & Whitehead, 1994).

Actually, eggshell, as the first barrier that microorganisms encounter, is the primary reservoir of *Salmonella* in eggs and leads to the cross-contamination (Muñoz, Domínguez-Gasca, Jimenez-López, & Rodríguez-Navarro, 2015). In consideration of its serious threat to consumers, control measures should be introduced to eliminate *Salmonella* or delay its growth on the outside of a shell egg. In the United States, commercially available eggs must be washed using sanitizers and must be stored at refrigeration temperature (USDA, 2011). The FDA recommends that eggs be pasteurized prior to use in food that are not cooked for 15 s at 155 °C (Mahmoud et al., 2015). Unfortunately, the treatment may induce the presence of pasteurization-resistant *Salmonella*, which can survive heating process (Gurtler et al., 2015). Hence, some new preservation techniques were developed to reduce *Salmonella* levels on raw eggs.

Nonthermal plasma-based sterilization approaches have displayed promising outcomes in preserving food whilst limiting the impact of processing on food quality (Ziuzina, Patil, Cullen, Keener, & Bourke, 2014). Recently, cold plasma technologies are widely applied because of their short processing times, lack of residue formation, and absence of toxicity (Zhang, Oh, Cisneros-Zevallos, & Akbulut, 2013). Although the plasma can avoid the defects of traditional sterilization technologies, it cannot achieve high potency in the inactivation of bacteria when applied on eggshell surfaces. The high roughness on the surface of eggs makes the bacteria easy to stack and affects the interactions between plasma

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and samples, resulting in reduced bactericidal efficiency (Fernández, Noriega, & Thompson, 2013; Noriega, Shama, Laca, Díaz, & Kong, 2011). Besides, longer exposure times might increase the internal temperature of eggs. Consequently, lethal efficacy could only be achieved through intelligent combination of strategies acting synergistically (Espina, Monfort, Álvarez, García-Gonzalo, & Pagán, 2014).

Essential oils (EOs) have captured special interest because of the increasing popular concern about the potential impact of traditional disinfectants on health (Cui, Zhou, & Lin, 2016). One of EOs, thyme oil exhibits excellent inactivation effect against *Salmonella* (Eriotou, Anastasiou, Kokosi, & Samaras, 2011; Mazzarrino et al., 2015). Nevertheless, the application of EOs in food preservation is restricted because they might change the original flavor of food products. As food additives, only a small amount of EOs is accepted, which attenuates their antibacterial effectiveness. Some literature reported that biological activities of essential oils at low concentrations were enhanced by plasma (Matan, Nisoa, Matan, & Aewsiri, 2014; Matan, Puangjinda, Phothisuwan, & Nisoa, 2015). Inspired by previous studies, the synergetic antimicrobial technology was developed in the present work. Thyme oil was introduced to play the role of antibacterial agent by plasma assist to inactivate *Salmonella* on eggshells. Their synergetic antibacterial effects at different storage temperatures were evaluated as well.

2. Materials and methods

2.1. Bacterial strains and culture

The thyme oil in our study was bought from J.E International (Caussols, France). The strains used in this study were provided by China Center of Industrial Culture Collection (Beijing, China), including *Salmonella enterica* subsp. *enterica serovar Enteritidis* CICC 21482 (*S. enteritidis*) and *Salmonella typhimurium* CICC 22956 (*S. typhimurium*). These strains were shaking cultured in nutrient broth (NB) at 37 °C for 24 h.

2.2. The antibacterial activity of thyme oil in vitro

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

Thyme oil was added into tubes containing NB to prepare the mixtures with different essential oil concentrations (2.0 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL). Subsequently, the mixtures were inoculated with fresh bacterial suspensions to maintain initial bacterial concentration of 10^5–10^6 CFU/mL. The mixtures were then incubated under agitation at 37 °C. The MIC was defined as the lowest concentration of thyme oil capable of inhibiting bacterial growth visually. The MBC was determined as the lowest concentration at which no growth occurred on nutrient agar (NA) plates after spot inoculation and incubation at 37 °C (Low, Martin, Hill, & Kenward, 2011).

2.2.2. Time-kill analysis of thyme oil

The antimicrobial activity of thyme oil was assessed by the plate colony-counting method. First, thyme oil was added to cells suspensions (approximately 10^{5-6} CFU/mL) to obtain the mixtures. Then, the mixtures were incubated at 37 °C and 150 rpm. Bacterial suspensions added with an equivalent amount of phosphate buffer solution (PBS; pH 7.2) were used as controls. Finally, the numbers of residual bacteria were observed at 0, 0.5, 1, 2, 4 and 8 h (Cui, Zhao, & Lin, 2015).

2.2.3. The research of bactericidal mechanism

Following thyme oil treatment, the *S. enteritidis* and *S. typhimurium* suspensions were centrifuged for 10 min at 5717 g. The absorption of supernatant was determined by a microplate reader (Infinite 200 PRO, Tecan Austria GmbH Untersbergstr, Grödig, Austria) at 260 nm in order to analyze the loss of 260-nm absorbing materials (Lee, Kim, Lim, & Ahn, 2014), and the pellets were harvested to measure cellular DNA, ATP concentrations and total protein contents. The cellular DNA was extracted with TIANamp Bacteria Genomic DNA Kit (TIANGEN Biotech Co., Ltd, Beijing, China) and the DNA quantification was conducted by Nanodrop 2000 (Thermo Scientific, USA). Cellular ATP concentrations were measured with a rapid method in accordance with a previous work (Cui, Zhang, et al.., 2015), and the output values were recorded in relative light units (RLU). Quantification of intracellular proteins was determined using the France Trace Protein Detection Kit (Jiancheng Bioengineering Institute, Nanjing, China).

2.3. Preparation and inoculation of eggs

Fresh eggs were bought from local market. Eggs were washed with sterile distilled water, and then sanitized by dipping them in 100 ppm sodium hypochlorite solution for 30 min as described by Shenga, Singh, and Yadav (2010). After aseptically drying, sanitized shell eggs were immersed in *Salmonella* suspensions which contained 10^8–10^9 CFU/mL for 30 min. Subsequently, they were air-dried for approximately 1 h before thyme oil and plasma treatment. This method provided microbial concentrations on eggshells at the level ranging between 6 and 7 Log CFU/egg (Mahmoud et al., 2015).

2.4. Plasma sources and plasma treatment on eggshell surfaces

In this study, a commercial cold nitrogen plasma (CNP) generating device (APLM-SP-YB-D1KW-3232328-2-5, ATV Electronic Technology Co., Ltd, Suzhou, China) was introduced. As shown in Fig. 1, an encapsulated plasma source was installed in a rectangular, parallelepiped plastic container. Nitrogen gas was used to generate plasma at a fixed flow rate of 100 standard cubic centimeters per minute (sccm). The carrier gas could uniformly pass through the anode electrode made of anodized aluminum, and the discharge was generated between the anode and the ground electrode. Besides, the intensity of plasma can be regulated by varying the frequency or electric current. The operation power levels of 300, 400, 500 and 600 W had been used for plasma generation in the present work. The samples were placed on the shelf of the container and they were treated with the CNP for pre-set time.

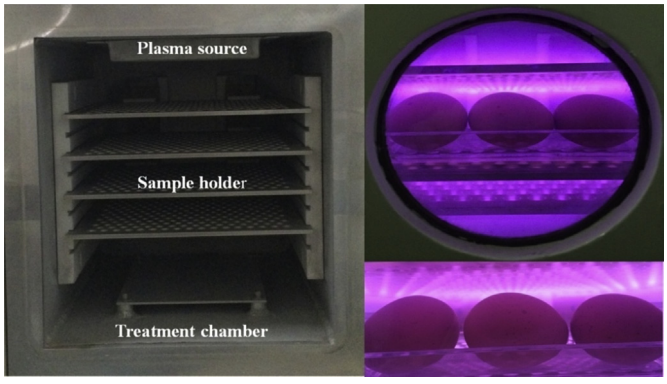

Fig. 1. Schematic diagram of cold nitrogen plasma treatment on eggs.