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A gaseous acetic acid treatment to disinfect fenugreek seeds and black pepper inoculated with pathogenic and spoilage bacteria

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ABSTRACT

Contamination of spices by pathogenic and/or spoilage bacteria can be deleterious to consumer's health and cause deterioration of foods, and inactivation of such bacteria is necessary for the food industry. The present study examined the effect of gaseous acetic acid treatment in reducing *Escherichia coli* O157:H7, *Salmonella* Enteritidis and *Bacillus subtilis* populations inoculated on fenugreek seeds and black pepper. Treatment with gaseous acetic acid at 0.3 mmol/L, 0.6 mmol/L and 4.7 mmol/L for 1–3 h significantly reduced the populations of *E. coli* O157:H7 and *Salmonella* Enteritidis on black pepper and fenugreek seeds at 55 °C ($p < 0.05$). The gas treatments at 4.7 mmol/L were more effective in inactivating the pathogens than the treatment at 0.3 mmol/L. An approximately 5.0 log reduction was obtained after 3 h of treatment with 4.7 mmol/L acetic acid. No significant reductions in the population of *B. subtilis* spores inoculated on fenugreek seeds and black pepper were obtained after the gas treatments at 0.3 mmol/L or 0.6 mmol/L ($p > 0.05$). However, the gas treatment at 4.7 mmol/L significantly reduced *B. subtilis* spores ($p < 0.05$), and 4.0 log CFU/g and 3.5 log CFU/g reductions on fenugreek seeds and black pepper, respectively, were obtained after 3 h of treatment.

1. Introduction

Spices have been used as flavoring agents in foods all over the world. The spices can become contaminated with bacteria because of poor producing, handling and processing conditions during the fertilizing, planting, harvesting and transportation processes (Ristori et al., 2007; Emer et al., 2008). The uses of spices with high contamination levels during food preparation might cause serious damage to human health (Rico et al., 2010; Doren et al., 2013). The majority of spice-related outbreaks have been associated with *Salmonella* spp. In 2009, a large outbreak caused by intake of black and red pepper contaminated with *Salmonella* Montevideo occurred in the USA, and 272 cases were identified from July 1st 2009 to April 14th 2010 (Gieraltowski et al., 2013). A large outbreak caused by consumption of spice mixtures containing paprika powders contaminated with *Salmonella* spp. occurred in Germany, and 1000 cases were reported from April to September 1993 (Lehmacher et al., 1995). In fact, some surveillance studies indicated that *Salmonella* spp. was present in the spice, and Hara-Kudo et al. (2006) reported that 1.7% of red pepper (1 of 59 samples) and 2.4% of black pepper (1 of 42 samples) were contaminated with *Salmonella*. Moreira et al. (2009) reported that 18.2% (12 of 66 samples) of black peppers were positive for *Salmonella*. Once the spices are contaminated with *Salmonella*, the pathogens can survive for a long time in appropriate conditions (Zweifel and Stephan, 2012; Keller et al., 2013), and the high risk of foodborne illness would remain unless the pathogens were artificially inactivated. Enterohemorrhagic *Escherichia coli* is the other important pathogen that causes foodborne illness. In 2011, a severe outbreak of Shiga-toxin-producing *E. coli* O104:H4 occurred in European countries, and fenugreek seeds were concluded as the source of the pathogen. More than 4000 cases and 50 deaths were reported during the outbreak (World Health Organization, 2011).

The spices are currently disinfected by using fumigation, such as ethylene oxide, propylene oxide or gamma irradiation (Torlak et al., 2013). However, these techniques have limitations. The fumigation with ethylene oxide and propylene oxide is not approved in EU countries due to residual toxic compounds (Schweiggert et al., 2007). Although gamma irradiation is an effective technology to kill pathogenic and spoilage bacteria without heating or deterioration in quality, the technique is not approved for spice disinfection in some countries (Furuta, 2004). Therefore, development of an

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alternative method to disinfect spices will be beneficial for food industries.

The present study focused on a gaseous acetic acid treatment as an alternative method to disinfect spices. Acetic acid treatment is relatively cheap and does not require a special facility, unlike gamma irradiation. Sholberg and Guance (1995) studied the effect of gaseous acetic acid on bacteria for several types of fruits. Nei et al. (2011) reported the effectiveness of the gaseous acetic acid to inactivate *E. coli* O157:H7 and *Salmonella* inoculated on alfalfa seeds and radish seeds. Weissinger et al. (2001) also reported decreases in *Salmonella* population on mung bean seeds (*Vigna radiata*) by gaseous acetic acid without affecting germination percentage. Nei et al. (2014) tested the gaseous acetic acid treatment for seeds by using a commercial scale device. However, there has not been any report applying the gaseous acetic acid to inactivate pathogenic bacteria on spices, and no data on inactivation of bacterial spores using gaseous acetic acid have been available until now.

The objective of the present study was to evaluate the ability of gaseous acetic acid to inactivate *E. coli* O157:H7, *Salmonella* and *Bacillus subtilis* spores inoculated on black pepper and fenugreek seeds.

2. Materials and methods

2.1. Test strains

Three strains of *E. coli* O157:H7 (CR-3, MN-28 and MY-29) and two strains of *Salmonella* Enteritidis (SE1 and SE2) isolated from bovine feces were used. The test strains of pathogens were adapted to grow in tryptic soy broth (TSB) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 50 μg/mL of rifampicin, and plating on media containing rifampicin greatly minimizes development of colony interference caused by naturally occurring microorganisms and it facilitates the detection of test pathogens on recovery media (Inatsu et al., 2005). A previous study indicated no differences in growth rates or sensitivity to acids between rifampicin-resistant and wild strains (Inatsu et al., 2003). In addition, the recoveries of the rifampicin-resistant strains injured by acid stress on tryptic soy agar supplemented with rifampicin (TSAR) were higher than other selective medium (Inatsu et al., 2003), and uses of rifampicin-resistant strains and TSAR are appropriate to recover acid-stressed bacteria. A strain of *B. subtilis* (IFO13722) was also used to evaluate the ability of gaseous acetic acid to kill spoilage bacteria.

2.2. Procedure for inoculation of pathogenic and spoilage bacteria

Each strain of *E. coli* O157:H7 and *Salmonella* Enteritidis was cultured at 37 °C in 40 mL of TSB (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) medium supplemented with 50 μg/mL rifampicin (TSBR). Cultures were transferred by loops to TSBR at three successive 24 h intervals immediately before they were used as inocula. Cells of each strain were collected by centrifugation (3000 *g* for 10 min at 4 °C) and resuspended in 40 mL of sterile phosphate-buffered saline (PBS; pH 7.2). Equal volumes of cell suspensions were combined to obtain a bacterial cocktail that contained approximately equal populations of each strain. The final suspension, containing 9.0 log CFU/mL, was maintained at 22 ± 2 °C and was applied to fenugreek seeds and black peppers, produced in India and purchased from seed supplier in Japan, 30 min after preparation. The spores of *B. subtilis* were prepared according to Hamanaka et al. (2011) and Sudehaus et al. (2014) with some modifications. The strain of *B. subtilis* was spread on tryptic soy agar (TSA) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and incubated at 30 °C for ten days. After incubation, the bacterial colonies were harvested and suspended in PBS. The cell suspension was centrifuged (3000 *g* for 10 min) and washed with PBS and then centrifuged and washed twice more. The heat treatment for cell suspension was performed at 70 °C for 30 min to inactivate vegetative cells. The concentration of *B. subtilis* spores was adjusted to 8 log CFU/mL, and the spore suspension was used for inoculation 30 min after preparation. Three hundred grams of the spices were soaked in the bacterial suspension and then mixed gently with a sterile glass rod for 5 min. After the inoculum was decanted, the spices were placed on a sterile perforated tray and dried on a clean bench at room temperature (22 ± 2 °C) for 8 h. After drying, the inoculated spices were mixed well and stored at 4 °C until experimental use. Water contents of fenugreek seeds and black pepper before inoculation were 10.5% w.b. and 11.5% w.b., respectively. After the inoculation and the subsequent drying for 8 h, the water contents of fenugreek seeds and black pepper were 13.1% w.b. and 12.3% w.b., respectively.

2.3. Gaseous acetic acid treatment

A pilot scale gas fumigation device (AG1000-AS, Daisey Machinery Co. Ltd., Saitama, Japan) was constructed. The device is equipped with a heating device, rotating drum, fumigation chamber and gas exhaust, and placed in chemical hood to prevent leakage of the gaseous acetic acid to laboratory. Three hundred grams of each spice were placed in a rotating drum located inside the fumigation chamber. After placing the samples in the drum, the door was closed to completely seal the chamber, and the temperature was increased from room temperature (25 °C) to 55 °C. The required time to reach a temperature of 55 °C was approximately 40 min, and a temperature fluctuation during operation was within 0.5 °C. The specific volume of acetic acid in liquid state was heated to complete vaporization with the heating device located at the top of the equipment, and gaseous acetic acid was introduced to the 30 L of sealed fumigation chamber. The concentration of gaseous acetic acid was maintained at 0.3 mmol/L, 0.6 mmol/L or 4.7 mmol/L during the treatment. After the treatment, the samples were cooled down to room temperature and collected for microbial analysis.

2.4. Microbial analysis

Twenty-five grams of each spice were placed in a stomacher bag, and 225 mL of buffered peptone water (pH 7.2) was added. The seeds were then pummeled for 120 s with a homogenizer (Promedia, SH-001, ELMEX, Tokyo, Japan). Serial decimal dilutions were prepared with buffered peptone water, and the diluted samples were pour plated in quadruplicate on TSA plates supplemented with 50 μg/mL rifampicin (TSAR) to quantify the populations of *E. coli* O157:H7 and *Salmonella*. The rifampicin solution was added to the molten agar before pouring the media into Petri plates. Samples of inoculated enumeration media were incubated at 37 °C for 24–48 h before the presumptive pathogen colonies were counted. The enumeration of *B. subtilis* spores was carried out according to Sagong et al. (2013) with some modifications. Briefly, the homogenized samples of the spices were made by the same method as mentioned above, and the suspensions were heated at 90 °C for 10 min and cooled immediately. Subsequently, the diluted and undiluted suspensions were pour-plated in TSA plates. The plates were incubated at 37 °C for 24 h, and colony formations were counted. The spices were analyzed before the gaseous acetic acid treatment, and no pathogens were detected in uninoculated samples.