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THE ANTIBACTERIAL EFFECT OF FLOWER POLLEN ON *ESCHERICHIA COLI* O157:H7 IN GROUND PORK

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ABSTRACT

This study was conducted to confirm the synergistic inactivation effect of flower pollen when applied with heat treatment to *Escherichia coli* O157:H7. The reduction of the number of *E. coli* O157:H7 was monitored during heat treatment at 55, 57.5, 60, 62.5 and 65C. The D-values in the culture were $D_{55} = 10.03 \pm 0.59$, $D_{57.5} = 3.84 \pm 0.05$, $D_{60} = 2.76 \pm 0.19$, $D_{62.5} = 1.31 \pm 0.20$ and $D_{65} = 1.04 \pm 0.01$ min, respectively, which were reduced to $D_{55} = 3.13 \pm 0.12$, $D_{57.5} = 3.02 \pm 0.14$, $D_{60} = 1.80 \pm 0.05$, $D_{62.5} = 1.11 \pm 0.03$ and $D_{65} = 0.79 \pm 0.01$ min with the addition of pollen (80 mg/mL). The z-value without addition of pollen was 10.24C, which increased to 15.11C with addition of pollen. The D-values in ground pork were $D_{55} = 5.59 \pm 0.11$, $D_{57.5} = 2.58 \pm 0.05$, $D_{60} = 2.07 \pm 0.19$, $D_{62.5} = 1.30 \pm 0.03$ and $D_{65} = 0.87 \pm 0.02$ min. When treated with the addition of pollen (150 mg/mL), the D-values decreased to $D_{55} = 1.87 \pm 0.05$, $D_{57.5} = 1.56 \pm 0.02$, $D_{60} = 1.11 \pm 0.01$, $D_{62.5} = 0.73 \pm 0.01$ and $D_{65} = 0.50 \pm 0.00$ min. The z-value increased from 13.05C to 16.86C.

PRACTICAL APPLICATIONS

To the best of our knowledge, this is the first study applying flower pollen as a natural antimicrobial additive to improve the effect of heat treatment at the time of the sterilization of a meat product. Flower pollen might be provided by cultivating plant resources. It implies that the cost of flower pollen could be dramatically reduced and the cost reduction with suitable degree of antimicrobial activity gives a great benefit to the users in the processed food industries.

INTRODUCTION

Pollen is the fine particulate form of germ cells which is present in the stamens of a flower (Basim *et al.* 2006). Since old times, pollen has been used as a material in drugs because of its high level of flavonoids (Kolesnikov and Gins 1999). In addition, bee pollen with the aggregated form caused by the salivary substances of honeybees has been used as traditional medicine and health supplements (Chung *et al.* 1984; Aliyazicioglu *et al.* 2005). Recent research indicated that pollen has antimicrobial effect against the pathogenic microbials in plants and foods (Basim *et al.* 2006; Erkmen and Özcan 2008) and the antimicrobial effects of the flavonoid derived from plants along with pollen has also been reported (Figueiredo *et al.* 2008; Krishnal *et al.* 2014).

Pathogenic and spoilage microorganisms are important factors affecting food processing, storage and distribution and sales, as they cause foodborne spoilage and food diseases (Choi *et al.* 2005; Kim *et al.* 2006; Manguiat and Fang 2013). In order to prevent contamination by pathogenic microorganisms, synthetic preservatives are mainly used; however, because of their negative effects, such as the accumulation in the body, chronic toxicity and induction of mutation in the human body, study on substitutes for synthetic preservatives using natural antimicrobial substances

is being actively conducted (Chung and Jung 1992; Oh *et al.* 1999; Shim *et al.* 2004; Woo *et al.* 2004).

Meat products are perishable foods, susceptible to physicochemical changes during the storage period. In particular, the microbial contamination facilitates the spoilage of meat products, shortening their shelf life (Lee *et al.* 2004). Among the microorganisms which can contaminate meat during slaughtering and post-slaughtering process, *Escherichia coli* O157:H7 causes a hemorrhagic form of colitis, bloody diarrhea and hemolytic uremic syndrome by producing toxins. It has been suggested that the microbiological safety of meat products must be secured, as they are a significant threatening element to both the food industry and consumers (Stopforth *et al.* 2007).

E. coli O157:H7 has thermal resistance at various temperatures and environments. Studies on the heat treatment of foods such as ground beef, turkey, lamb, pork and poultry have been reported (Oteiza *et al.* 2003). Thermal processing is the most popular process to inactivate food pathogens in meat products. Compared with other food processing technologies including nonthermal processing technologies, such as hydrostatic pressure, the thermal processing maximally inactivates the pathogenic microorganisms in foods. Generally, the inhibition of microorganisms during thermal processing is described by a single linear regression model including temperature and heating time (Asselt and Zwietering 2005).

The hurdle technologies without using synthetic chemical preservatives are highly demanded from the market (Wordon *et al.* 2012). As environmental friendly hurdle technologies, controlling temperature (high or low), water activity, acidity, redox potential, natural preservatives, competitive microflora, etc., have been widely used and studied (Chawla *et al.* 2006). Multitarget hurdle technology has also been used, demonstrating synergistic effects by interfering with the homeostasis of a microorganism through action on a different target (e.g., cell membrane, DNA, enzyme systems, pH, water activity and redox potential) at the time of utilizing the hurdles within the microbial cells (Leistner 2000).

Therefore, in the present study, the authors investigated the effects of heat treatment at different temperatures with addition of pollen, having various functionality and antibiosis to pork, on the extinction of *E. coli* O157:H7, a pathogenic microorganism in food, through the calculation of D- and z-values. The synergistic effects of heat and pollen were examined. The biological and antibacterial activities of bee pollen have continuously been reported, but data on the antibacterial effect when applied to food were insufficient. To the best of our knowledge, this is the first report of the experimental utilization of flower pollen in combination with heat treatment for inactivating a pathogenic bacterium.

MATERIALS AND METHODS

Bacterial Culture

E. coli O157:H7 (ATCC 43894) used in this study was obtained from the stock culture collection of the Korean Culture Center of Microorganisms (Seoul, Korea). The freeze-dried bacteria were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) and the overnight culture which was incubated at 37C for 24 h was streaking on tryptic soy agar (TSA; Difco Laboratories). A single colony from the TSA plate was inoculated into a test tube containing 10 mL of TSB. A loopful of bacterial culture in TSB was transferred into 10 mL fresh TSB every day to maintain the viability of culture prior to experimental use.

Preparation of Pollen Extraction and Ground Pork

Graminex-NAX 7% paste purchased from Graminex (Saginaw, MI) was used, which comprised of rye grass flower pollen extract. The pollen paste was processed with harvested raw pollen. Subsequently, the pollen particles were extracted using a super-critical CO_2 for removing the exine which may contain allergenic compositions. The pollen paste was dissolved in 50% ethanol containing 20% Tween 20 and the filtrate was used in experiments after filtering with a 0.2 μL nylon membrane syringe filter. Fresh ground pork was kept in a freezer at −18C after purchasing from a local market and was used in experiments after sterilizing at 121C for 15 min in an autoclave. Meat samples were defrosted at 5C before experiments.

Thermal Inactivation of *E. coli* O157:H7

Aliquots of 1 mL of the culture solution of *E. coli* O157:H7 grown up to 10^7~10^8 cfu/mL and 1 mL of *E. coli* O157:H7 culture solution which was mixed with pollen (80 mg/mL) were put into sterilized glass test tubes then treated by heating in water baths at 55, 57.5, 60, 62.5 and 65C, respectively. After the serial dilution of the sample with 0.1% peptone water plated on TSA, the plates were incubated at 37C for 24 h to determine survivors.

The ground sterile pork was weighed into 10 g samples and was put into sterilized bags. Each sample was inoculated with 1 mL of *E. coli* O157:H7 culture grown up to 10^7~10^8 cfu/mL. For combination treatments, each sample was mixed with pollen (150 mg/mL). Both samples were kept under refrigeration for 1 h for bacteria attachment to the ground pork, consequently the addition of 90 mL of 0.1% peptone water was added in the sample and stomached for 2 min at the maximum speed with a sample mixer (WH4000, 3M, Maplewood, MN). The homogenized

samples with inoculated bacteria were put into sterile glass test tubes then maintained in the water bath at 55, 57.5, 60, 62.5 and 65C, respectively. After heat treatment, 100 μL aliquots of the samples were spread on TSA plates then incubated at 37C for 24 h.

Thermal D- and z-Value

D-value is the time required to reduce the number of microorganisms to a tenth by heating at a specified temperature. The D-value can be obtained from the negative slope of the survival curve of the microorganism and estimated by Eqs. (1) and (2). z-value is the temperature that decreases the D-value to a tenth and was obtained from the Eq. (3).

$$\log\frac{N}{N_0} = -\frac{kt}{2.303} \quad (1)$$

$$\mathrm{D} = \frac{2.303}{k} \quad (2)$$

$$\log\frac{D_{T_2}}{D_{T_1}} = \frac{1}{z}(T_1 - T_2) \quad (3)$$

Statistical Analysis

All determinations were carried out in two independent experiment blocks, and for each experimental block, three measurements were conducted. Results are shown as the arithmetic mean values ± standard deviation. The heat resistance data were analyzed by regression analysis and analysis of variance (ANOVA) to determine if there were any statistically significant differences among the treatments and pollen using the SAS program (version 9.2; SAS Institute, Inc., Cary, NC: PROC REG, PROC ANOVA). The coefficient of multiple determination (square of the correlation coefficient) was used to estimate the proportion of variability of the response of the regression of D- and z-value fitting (Juneja *et al.* 2009).

RESULTS

Thermal Inactivation of *E. coli* O157:H7 with Pollen in TSB

The reduction in the number of bacteria because of the heat treatment and the addition of pollen was evaluated by calculating the D- and z-values from the survival curve, and the D- and z- values were used to compare the rate of inactivation of *E. coli* O157:H7. The study of Juneja *et al.* (1997), who determined the D- and z-values of *E. coli* O157:H7 in beef and chicken, was referenced for the setting of experimental temperature intervals. When applying heat treatment to *E. coli* O157:H7, the reduction rate of the bacteria decreased as the heat treatment time and temperature increased (Fig. 1a). The D-values obtained were $D_{55} = 10.03 \pm 0.59$, $D_{57.5} = 3.84 \pm 0.05$, $D_{60} = 2.76 \pm 0.19$, $D_{62.5} = 1.31 \pm 0.20$ and $D_{65} = 1.04 \pm 0.01$ min, respectively, and log-linear reduction in the number of bacteria was observed (Table 1). When applying heat to the *E. coli* O157:H7 with the addition of pollen (80 mg/mL), the higher the temperature, the faster the number of bacteria was reduced. The addition of pollen showed higher effect on the inactivation of *E. coli* O157:H7 than heat treatment alone (Fig. 1b). The number of bacteria depending on the heating time demonstrated log-linear reduction, with D-values of $D_{55} = 3.13 \pm 0.12$, $D_{57.5} = 3.02 \pm 0.14$, $D_{60} = 1.80 \pm 0.05$, $D_{62.5} = 1.11 \pm 0.03$ and $D_{65} = 0.79 \pm 0.01$ min, respectively (Table 1). These showed lower values in comparison with heat treatment alone. The results in this study were similar to the research of Kim *et al.* (2013) about propolis which main antibacterial components

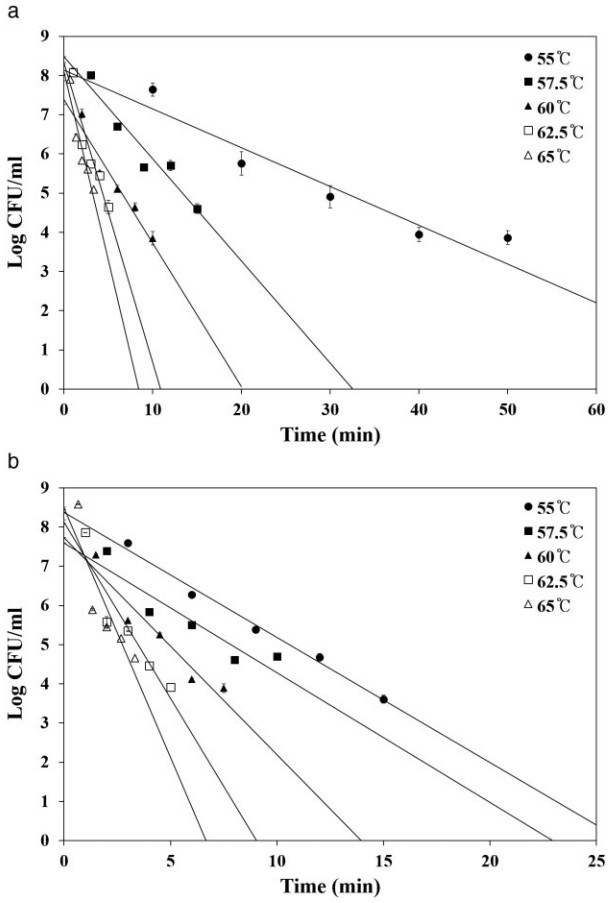

FIG. 1. THERMAL DEATH TIME CURVES OF *ESCHERICHIA COLI* O157:H7. (A) HEAT TREATMENT ALONE AND (B) HEAT TREATMENT WITH POLLEN.

TABLE 1. D- AND Z-VALUES OF *ESCHERICHIA COLI* O157:H7 BY HEAT AND POLLEN TREATMENT OF POLLEN IN CULTURE

	Heat alone		Heat + Pollen	
Temperature (C)	Mean D-values ± SD†	(R^2)‡	Mean D-values ± SD†	(R^2)‡
55	10.03 ± 0.59	(0.98)	3.13 ± 0.12	(0.99)
57.5	3.84 ± 0.05	(0.99)	3.02 ± 0.14	(0.98)
60	2.76 ± 0.19	(0.99)	1.80 ± 0.05	(0.99)
62.5	1.31 ± 0.20	(0.99)	1.11 ± 0.03	(0.98)
65	1.04 ± 0.01	(0.99)	0.79 ± 0.01	(0.96)
z-value	10.25C	(0.99)	15.12C	(0.99)

† D-values (in minutes) were obtained by linear regression and are the results of two replicate experiments, each performed in duplicate.
‡ Correlation coefficient.

are flavonoids. The rapid inactivation than heat treatment alone of *E. coli* O157:H7 in ground pork was observed by propolis; consequently, the addition of propolis caused the reduction of D-values.

In addition, Basim *et al.* (2006) researched the antibacterial effects of pollen and propolis extracts against plant pathogenic bacteria, and Pascoal *et al.* (2014) reported that pollen extract inhibited the growth of pathogenic bacteria in foods at 37C.

In the present study, the z-value of *E. coli* O157:H7 was 10.24C when heat treated alone, but increased to 15.11C when heat treated with the addition of pollen (Fig. 2). These results indicated that the inactivation effect of pollen with heat treatment to the *E. coli* O157:H7 was not sensible by increasing the temperature. It implies that the heat resistance of *E. coli* O157:H7 might be increased by adding pollen. Similarly, Kim *et al.* (2013) observed that the z-value of the experimental group of *E. coli* O157:H7 with propolis added increase from 6.4C to 10.3C. In addition, in the study of Blackburn *et al.* (1997), the z-value increased from 4.6–5.1C at 0.5% w/w NaCl to 5.8–7.0C at 8.5% w/w NaCl when the combined treatment of heat and NaCl was applied to

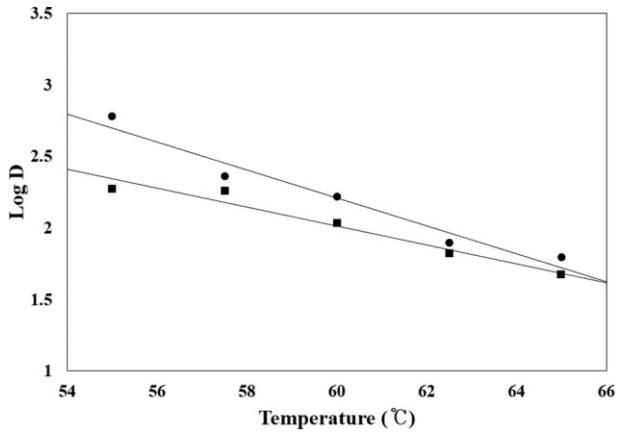

FIG. 2. LOG D-VALUE REDUCTION OF *ESCHERICHIA COLI* O157:H7 BY HEAT TREATMENT (•) AND HEAT TREATMENT WITH POLLEN (■)

Salmonella enteritidis and *E. coli* O157:H7. The D-values and z-values of *E. coli* O157:H7 obtained herein, along with the goodness of fit (R^2) of the models, are shown in Table 1.

Thermal Inactivation of *E. coli* O157:H7 in Ground Pork with Pollen

The effect of thermal treatment with addition of pollen on the ground pork inoculated with *E. coli* O157:H7 was investigated (Fig. 3). Log-linear reduction bacteria was observed and D-values obtained at each temperature were $D_{55} = 5.27 \pm 0.01$, $D_{57.5} = 2.71 \pm 0.01$, $D_{60} = 2.05 \pm 0.01$, $D_{62.5} = 1.33 \pm 0.00$ and $D_{65} = 0.86 \pm 0.02$ min (Table 2). When heat treatment was applied to the ground pork inoculated with *E. coli* O157:H7, the reduction of the number of bacteria occurred faster than when heat treatment was applied to *E. coli* O157:H7 alone.

When inoculating the ground pork with *E. coli* O157:H7 together with pollen (150 mg/mL), the number of bacteria also decreased in a log-linear fashion. When the pollen was added, the reduction of bacteria occurred more rapidly. Antibacterial effects of pollen were only observed when the concentration of 150 mg/mL was added, not when 80 mg/mL was inoculated to the 10 g sample of ground pork (data not shown). The D-values of *E. coli* O157:H7 in ground pork with the addition of pollen at varied temperature were $D_{55} = 1.93 \pm 0.03$, $D_{57.5} = 1.66 \pm 0.13$, $D_{60} = 1.13 \pm 0.02$, $D_{62.5} = 0.72 \pm 0.00$ and $D_{65} = 0.49 \pm 0.01$ min, respectively (Table 2), and the changes in the number of bacteria are shown in Fig. 3. The z-value in ground pork was 13.25C, which was raised to 16.02C by the addition of pollen (Fig. 4). It was considered that when applying the heat treatment after adding the *E. coli* O157:H7 and pollen to the ground pork, the heat resistance increased similarly to the case of heat applied to the *E. coli* O157:H7 culture alone with pollen added (Fig. 4, Table 2).

DISCUSSIONS

In this study, the authors identified the synergistic effect of pollen on the thermal inactivation of *E. coli* O157:H7. The

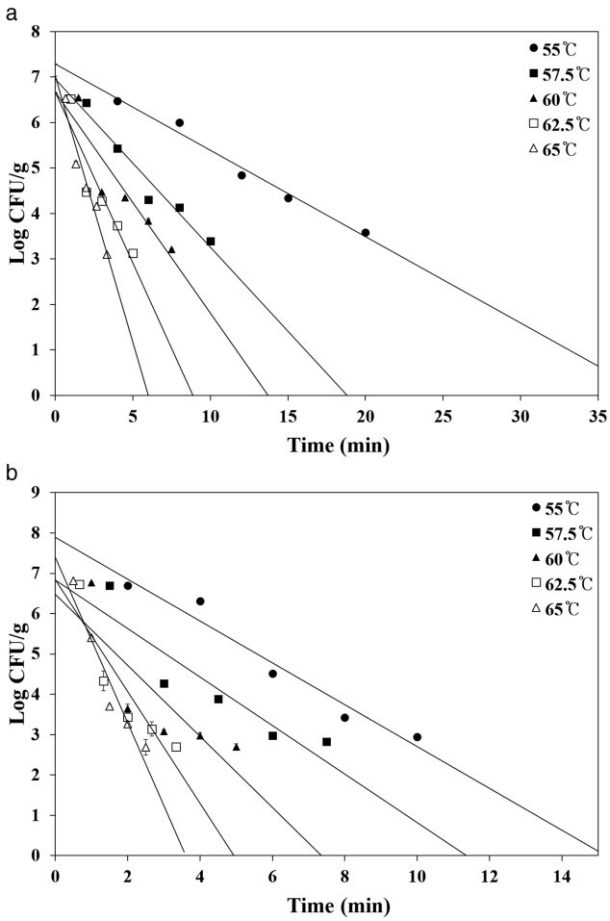

FIG. 3. THERMAL DEATH TIME CURVES OF *ESCHERICHIA COLI* O157:H7. (A) HEAT TREATMENT ALONE AND (B) HEAT TREATMENT WITH POLLEN IN GROUND PORK.

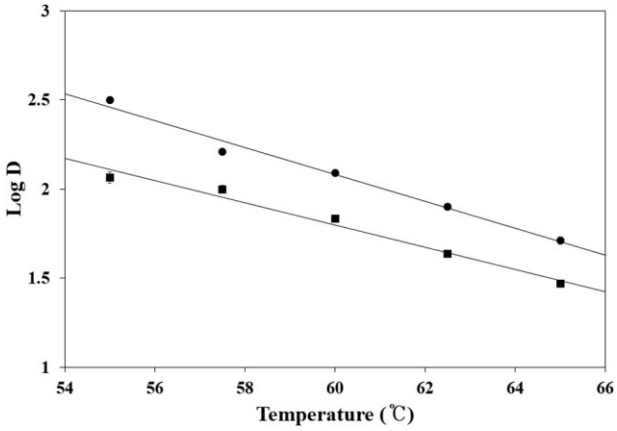

FIG. 4. LOG D-VALUE REDUCTION OF *ESCHERICHIA COLI* O157:H7 IN GROUND PORK BY HEAT TREATMENT (•) AND HEAT TREATMENT WITH POLLEN (■)

outer membrane of Gram-negative bacteria such as *E. coli* O157:H7 consists of low molecular weight hydrophilic substances and forms a permeability barrier to hydrophobic compounds, having diffusion channels for the hydrophobic compounds (Costerton *et al.* 1974; Nikaido 1976). Grau (1978) directly studied the changes of permeability in the cell membrane using lactose, proline and alpha-methylglucoside independently transported.

The major polyphenols of pollen are the flavonoids, including phenolic acids, esters, phenolic aldehydes and ketones or the like, which inhibit the biosynthesis of nucleic acid by inhibiting the activity of DNA gyrase in bacteria, causing changes in the fluidity of the cell membrane and subsequent destruction of cells by eluting the intracellular components (He *et al.* 2014). Thus, our study showed that the cell destruction of *E. coli* O157:H7 by heat and the flavonoids of pollen increased in comparison with that incurred by the heat treatment alone.

Previous studies demonstrated that the z-value was increased because of the increase of heat resistance of the microorganisms when a combined effect occurred. Similar to the results obtained in this study, Juneja *et al.* (2009) reported that when tea leaf and apple skin powders were added for antibacterial effects in sous-vide cooked ground beef, the z-value of *E. coli* O157:H7 was increased from 4.23C to 5.06C in comparison with the control group. In addition, Steenstrup and Floros (2006) reported that the

TABLE 2. D- AND Z-VALUES OF *ESCHERICHIA COLI* O157:H7 BY HEAT AND POLLEN TREATMENT IN GROUND PORK

	Heat alone		Heat + Pollen	
Temperature (C)	Mean D-values ± SD†	(R^2)‡	Mean D-values ± SD†	(R^2)‡
55	5.27 ± 0.01	(0.99)	1.93 ± 0.03	(0.99)
57.5	2.71 ± 0.01	(0.99)	1.66 ± 0.02	(0.95)
60	2.05 ± 0.01	(0.97)	1.13 ± 0.02	(0.90)
62.5	1.33 ± 0.00	(0.97)	0.72 ± 0.00	(0.95)
65	0.86 ± 0.02	(0.99)	0.49 ± 0.01	(0.98)
z-value	13.25C	(0.99)	16.03C	(0.99)

† D-values (in minutes) were obtained by linear regression and are the results of two replicate experiments, each performed in duplicate.
‡ Correlation coefficient.

addition of malic acid, potassium sorbate and sodium benzoate to cider caused an increase in the z-value of the *E. coli* O157:H7 from 6.3C to 26.5C in comparison with the control group. In this study, the z-value in the ground pork increased from 13.25C in the control to 16.03C when pollen was added. Because less influence on microorganisms is observed due to the increase of temperature at high z-values, the increase of z-value significantly influences processing (Steenstrup and Floros 2006). For safe processing, when additives such as pollen which increase the z-values are added, concern should be given to increases in the temperature of heat treatment. In other words, if the z-value is high, sterilization at low temperature is more advantageous.

CONCLUSION

When applying the combined treatment of both pollen and heat to ground pork, it showed synergistic effects for inactivation. The flavonoids in the pollen caused the cell destruction of *E. coli* O157:H7 and reduced the heat resistance, increasing the effect of heat treatment. The reduction of D-value and increase of z-value were confirmed by fitting these results using the linear model. The reduction of D-values by adding pollen might give a great advantage for low temperature heat treatment, but because the z-value increased by adding pollen, there is a need to pay more attention to controlling the heating temperature. Thus, our study clearly showed that the addition of flower pollen to ground pork may be applicable for sterilization to protect against foodborne pathogens.

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