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Application of freeze-dried *Enterococcus faecium* NRRL B-2354 in radio-frequency pasteurization of wheat flour

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ABSTRACT

This study aimed to assess the applicability of freeze-dried *E. faecium* NRRL B-2354 (FDE) to evaluate the inactivation efficiency of radio-frequency (RF) pasteurization on wheat flour. FDE was prepared by adding equal volume of 10% skim milk solution as a protectant. Microstructure of FDE was evaluated by scanning and transmission electron microscopy. Thermal resistance parameters of FDE in wheat flour were determined at 75 °C, 80 °C, and 85 °C. A 6 kW, 27.12 MHz RF heating system was used to rapidly pasteurize 3.0 kg wheat flour at 75 °C, 80 °C, and 85 °C, respectively. Population reduction of FDE in the geometric center of flour samples were compared with survivor counts and predicted model. Flour samples were equilibrated to water activity 0.45 measured at 25 °C. The results showed that FDE protected by skim milk were well maintained with repeatable D-values $D_{75°C}$ = 14.27 ± 0.85 min, $D_{80°C}$ = 5.92 ± 0.39 min, and $D_{85°C}$ = 2.79 ± 0.15 min, and z-value of 13.1 °C. RF treatment at 75 °C, 80 °C, and 85 °C for 10 min achieved 1.0-, 2.5-, and 4.9-log reduction of FDE, respectively. In RF pasteurization process, the dynamic population reduction of FDE can be predicted by a model based on F-value. RF treatment provides an effective and rapid approach to inactivate target microorganism in flour samples.

1. Introduction

Low-moisture foods (LMFs), such as spices, milk powder, and flour were generally considered safe because of their water activities are lower than 0.6 (Lang et al., 2016). However, many outbreaks of salmonellosis in recent years have been associated with LMFs, such as peanut butter (CDC, 2007), pistachios (FDA, 2016a), raw almonds (Scott et al., 2009) and milk powder (Carrasco, Morales-Rueda, & García-Gimeno, 2012). Those outbreaks underscore the necessity to develop effective treatments to control *Salmonella* in LMFs, and those treatments need to be validated for food companies to be in compliance with regulations. *Salmonella* is a virulent bacteria, and there are limitations for its direct use in the industrial food processing plants for validation studies (Niebuhr, Laury, Acuff, & Dickson, 2008). Consequently, various non-virulent microorganisms are used as surrogates for in-plant process validation (Enache et al., 2015). *Enterococcus faecium* NRRL B-2354 (*E. faecium*), a nonpathogenic microorganism with heat tolerance, was identified and has been widely used as a surrogate for *Salmonella enteritidis* PT30 (*S. enteritidis* PT30) in thermal process validation for food products, such as almonds (Bingol et al., 2011).

In general, validation studies are conducted using liquid cultures of surrogate microorganisms that are directly inoculated into food matrices prior to process validation. The introduction of liquid inoculum into LMFs will change moisture contents (Aguilera, del Valle, & Karel, 1995; Chuy & Labuza, 1994; Kimber, Kaur, Wang, Danyluk, & Harris, 2012; Palipane & Driscoll, 1993). Extended drying/equilibration time after inoculation with liquid inoculum will alter the physical properties of the treated foods. In this case, use of dried bacterial inoculum may provide a potential solution to this challenge because dried inoculum allows better uniformity and minimizes undesirable impacts on physical-chemical properties of food matrices (Hoffmans & Fung, 1992). Freeze-dried bacteria are easy to prepare in large volumes, have powdered consistency, contain extremely low initial water activity (a_w), long-term viability, fast equilibration ability with its surroundings, and offer reduced variability in the inoculum used for process validation (Syamaladevi, Tang, & Zhong, 2016). Characteristics of freeze-dried bacteria, such as viability, virulence, immunological and biochemical properties have also been extensively investigated for the purpose of cultural preservation and transportation (NACMCF, 2010; Swift, 1937). Scarce information has been reported on the application of freeze-dried

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bacteria as a dry-based inoculum for validation of thermal treatment in food processing.

Radio-frequency (RF) heating has been regarded as a promising alternative to conventional heating (Hou, Johnson, & Wang, 2016; Y.; Jiao, Tang, & Wang, 2014; Ozturk, Kong, Trabelsi, & Singh, 2016; Piyasena, Dussault, Koutchma, Ramaswamy, & Awuah, 2003), and has wide applications in food processing, such as baking, thawing, cooking, drying, pest control and pasteurization/sterilization (Wang, Monzon, Johnson, Mitcham, & Tang, 2007b, 2007a; Laycock, Piyasena, & Mittal, 2003; McKenna, Lyng, Brunton, & Shirsat, 2006; Nagaraj, Singh, Hung, & Mohan, 2015; Ponne, Balk, Hancioglu, & Gorris, 1996; Rincon, Singh, & Stelzleni, 2015; Uyar et al., 2015). RF heating generates a more uniformed and faster heating rate in comparison with conventional heating and microwave heating for LMFs (Alfaifi et al., 2013; Casals et al., 2010; Z.; Huang, Marra, Subbiah, & Wang, 2017; S.; Jiao, Deng, Zhong, Wang, & Zhao, 2015; Y.; Jiao, Shi, Tang, Li, & Wang, 2015; Marra, Zhang, & Lyng, 2009). It has been documented that at least 4-log reduction of target pathogens can be reached through RF treatment in wheat flour (Villa-Rojas, Zhu, Marks, & Tang, 2017a), wheat and corn seeds (S. Jiao, Zhong, & Deng, 2016), and black and red pepper spices (Kim, Sagong, Choi, Ryu, & Kang, 2012).

Even though many studies have reported on the usage of dry-based inoculum for thermal challenge studies (Blessington, Theofel, & Harris, 2013; Enache et al., 2015), the potential of freeze-dried bacteria as a target microorganism for validation studies, especially in RF pasteurization, has not been reported. The goal of this study was to evaluate the application of FDE as a surrogate for *Salmonella* in RF pasteurization of wheat flour at 75 °C, 80 °C, and 85 °C. The specific objectives of this study were to: i) analyze microstructure of FDE; ii) determine D- and z-values of FDE in wheat flour through isothermal inactivation study at water activity of 0.45; iii) investigate inactivation efficiency of RF pasteurization in wheat flour using FDE; and iv) predict inactivation kinetics of FDE during RF pasteurization with F-value calculated from yielded D- and z-values.

2. Materials and methods

2.1. Preparation of freeze-dried E. faecium

E. faecium NRRL B-2354 (ATCC® 8459™) was acquired from Dr. Linda Harris at University of California, Davis and stored in 20% glycerol at −80 °C until use. Skim milk protected *E. faecium* was prepared by a method described previously with minor modifications (Syamaladevi et al., 2016; Xu et al., 2017). Briefly, *E. faecium* was grown in trypticase soy broth (TSB, Difco, Detroit, MI, USA) at 37 °C for 24 h with shaking at 200 rpm. Broth culture was centrifuged and washed three times with sterile double-deionized water (dd H_2O). Washed culture was centrifuged, and the supernatant was discarded to obtain bacterial pellet. Subsequently, bacterial pellet was resuspended with dd H_2O and equal volume of 10% skim milk solution (Difco, Detroit, MI, USA). Resuspended bacteria mixed with skim milk was separated into clear Amber glass bottle and freeze-dried for 48 h at −90 °C (FreeZone plus 4.5 L cascade freeze dry system, Labconco Corporation, Kansas City, MO, USA). After freeze-drying, bacteria were vacuum-sealed with a vacuum packaging machine (Ultravac 150, Koch Equipment, Kansas City, MO, USA) in 4 oz Whirl-Pak bags (Nasco, Modesto, CA, USA) and stored at −20 °C until further use.

2.2. Electron microscopy

The microstructure of FDE cells was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *E. faecium* in liquid and freeze-dried forms without the protection of skim milk were used as controls for comparison.

For SEM, FDE were thinly spread onto double coated carbon conductive tabs (Ted Pella Inc., Redding, CA, USA) and gold coated in a vacuum-evaporator (Technics Hummer V Sputter Coater, Technics, San Jose, CA, USA) to a thickness of 6 nm. An environmental field emission gun scanning electron microscope (Quanta 200F, FEI company, Hillsboro, OR, USA) was used to examined the samples and the images were captured by a digital camera (Quartz Imaging Corporation, Vancouver, British Columbia, Canada).

For TEM, FDE were fixed into 2% (v/v) paraformaldehyde/2% (v/v) glutaraldehyde/0.1M phosphate buffer at 4 °C and rinsed by the same buffer. Samples were then transferred into 1% (w/v) osmium tetroxide at 4 °C overnight. Dehydration of samples was performed in a graded ethanol series and propylene oxide for 10 min. Dehydrated bio-samples were polymerized in capsules of 100% Spurr's epoxy resin at 60 °C overnight. Resin embedded samples were ultrathin sectioned (70 nm thickness) by a Reichert Om-U2 ultramicrotome (Leica, Bensheim, Germany) and stained with 2% aqueous uranylacetate and 0.1% lead citrate. The sections of FDE was examined by a transmission electron microscope (model EM 300, Phillips, Eindhoven, Netherlands).

2.3. Preparation of inoculated wheat flour

Soft wheat organic flour was purchased from Eden Foods (Clinton, MI, USA) and conditioned in a Hotpack 435315 humidity chamber (SP Industries, Inc., Warminster, PA, USA) with a humidity control system. Since water activity (a_w) has been shown to be an important factor to influence thermal resistance (as reflected by D-value) of microorganisms (Syamaladevi et al., 2016), one a_w measured at room temperature ($a_{w,\ 25°C}$ = 0.45) was chosen in this study to reduce complexity. The humidity chamber was set to relative humidity 45% at room temperature (25 °C). Flour samples were equilibrated for approximately 4–5 days to reach $a_{w,\ 25°C}$ = 0.45 measured by a water activity meter (Model 3TE, Decagon Devices, Pullman, WA, USA). FDE (10 ± 0.10 mg) was added with 25.0 ± 0.10 g wheat flour (~10^9 CFU/g) and mixed manually for at least 3 min to prepare inoculated wheat flour.

2.4. Isothermal inactivation of FDE

Decimal reduction times (D-values) of FDE in wheat flour were obtained using the thermal death time (TDT) method (Chung, Birla, & Tang, 2008). 0.7 g inoculated wheat flour samples were added into TDT test cells (1.8 cm inner diameter, 0.4 cm inner height) and sealed. Subsequently, test cells were submerged in an oil bath (Isotemp 110, Fisher Scientific, Pittsburgh, PA, USA) heated at 75 °C, 80 °C, or 85 °C, respectively. Test cells were removed at the come-up time and four more predetermined time intervals. After treatment, the test cells were cooled immediately in an ice-water bath for 30 s. The come-up time (CUT, the time to reach within 0.5 °C of the set temperatures in the center of the test cells) was determined using a modified TDT cell with a calibrated T-type thermocouple installed in the center. The CUT of treated sample to reach target temperature was 140 s measured by attaching T-type thermocouple to a thermometer (Digiense DuaLogR 99100-50, Cole-Parmer Instruments Co., Vernon Hills, IL, USA). CUT was also regarded as the starting point of thermal death curves (Villa-Rojas, 2015; Villa-Rojas et al., 2017b).

To determine the surviving numbers of FDE, treated samples were scraped into 6.3 mL of 0.1% (w/v) peptone water and processed in a stomacher at 230 rpm (Stomacher 400 circulator, Seward, West Sussex, UK) for 3 min to constitute a ten-fold dilution. The samples were then serially diluted and inoculated on trypticase soy agar (TSA, Difco, BD) followed by incubation at 37 °C for 24 h for enumeration of survivors. Three independent replicate experiments were conducted and the mean number of CFU/g was calculated from the three replicates. An untreated sample was used as a control. Viable counts (CFU/g) of untreated samples were determined by randomly selecting three 1 g portions of inoculated samples and stomaching in 9 mL of 0.1% (w/v) peptone water followed with serial dilution and enumeration as

previously described.

2.5. Radio-frequency pasteurization of FDE in wheat flour

A pilot-scale 6-kW, 27.12-MHz radio-frequency (RF) system (COMBI 6-S, Strayfield Fastran, UK) was used as the source of RF energy in our study. Details about the parameter settings and experimental procedure were previously described by Ozturk et al. (Ozturk, Kong, Singh, Kuzy, & Li, 2017).

Two types of sample bags were prepared by placing 5 ± 0.05 g of flour sample into a sterilized polyethylene bag (7.6 (L) × 7.6 (W) × 0.1 (H) cm^3). Type I bag was filled with an uninoculated flour sample and sealed, leaving a small opening for temperature measurement, while Type II bag was filled with same amount of inoculated flour sample and sealed completely. A rectangular polyetherimide (PEI) container (inner dimension: 30 (L) × 24 (W) × 7 (H) cm^3) was half filled with uninoculated wheat flour loaded with a sample bag in the geometric center (predetermined as the cold spot) (Ozturk et al., 2017), and then filled with more uninoculated flour to reach a total weight of 3.0 ± 0.2 kg.

Type I bag was loaded to determine the heating time (HT) for wheat flour samples in the cold spot of the container during RF treatment. A pre-calibrated fiber optic sensor (Fiso Tech. Inc., Quebec, Canada) connected with a data logger (UM14, Universal Multichannel Instrument, Fiso Tech. Inc., Canada) was passed through a hole of the geometric center of PEI container, penetrated the sample bag, and was in contact with the sample center. The whole container was then treated with RF at 75 °C, 80 °C, or 85 °C, separately. The time to reach within 0.5 °C of the set temperature in the cold spot was regarded as CUT.

Type II bag was loaded in the same way for RF treatment and subsequently sampled at six time points: T_0 (0 min), T_1 (5 min), T_2 (10 min), T_3 (HT), T_4 (HT + 5 min), and T_5 (HT + 10min) at each temperature. During RF treatment, a fiber optic sensor was inserted into a location slightly beneath the sample bag to record temperature change. When the temperature reached the set level, RF system was turned off and the sample bag was kept inside the RF system for additional 5 min or 10 min. Heat treatment was stopped by immediately cooling the treated sample bag into an ice-water bath for 30 s. After treatment, the container was emptied, cooled, refilled with new wheat flour ($a_{w,\ 25°C}$ = 0.45), and then placed back into the RF cavity for another treatment. Enumeration was performed by randomly sampling 1 g of treated inoculated flour and stomaching with 9 mL of 0.1% (w/v) peptone water, followed by plating 10-fold serial dilutions as previously described. Untreated Type II bags placed at room temperature were used as controls (T_0). At each time interval point, three biological replicates (batches inculcated with independently grown FDE) and two technical replicates (samples from the same batch) of each temperature were used in the experiment. This part of the study was performed at the Department of Food Science and Technology, University of Georgia.

2.6. Model prediction of microbial reduction in isothermal inactivation study

The first-order kinetics (Peleg, 2006) was used to model isothermal inactivation data:

$$Log\frac{N}{N_0} = -\frac{t}{D_T} \tag{1}$$

Where N and N_0 (CFU/g) are the population at time t and 0 (CFU/g), respectively, t is the isothermal heating time (min), and D_T (min) is the time required to reduce 90% of bacteria population at temperature T (°C).

Plotting logarithmic D-values of FDE ($D_{75°C}$, $D_{80°C}$, and $D_{85°C}$) against temperature revealed a linear thermal death time (TDT) curve. The z-value measured in °C was the reciprocal of the slope of the TDT curve for FDE. Z-value (°C) were determined by using Equation (2), which is the temperature change (°C) necessary to obtain a change in D (min) by 1-log cycle (Holdsworth, Simpson, & Barbosa-Cánovas, 2008):

$$z = \frac{T - Tr}{log\ Dr - log\ D} \tag{2}$$

where T is the temperature (°C) and D is the decimal reduction time (min) at temperature T (°C).

2.7. Model prediction of microbial reduction in RF pasteurization

The logarithmic reduction in time required to kill the same number of microorganisms as temperature is increased was expressed by calculating lethal rate. The lethal rate is a dimensionless number and can be calculated using Equation (3) (Murphy, Duncan, Johnson, & Davis, 2001; Murphy, Duncan, Johnson, Davis, & Smith, 2002):

$$L = 10^{\frac{T - T_r}{z}} \tag{3}$$

where L is the lethal rate, T is the temperature (°C) at which the lethal rate is calculated, T_r is the reference temperature (°C) at which the equivalent lethal effect is compared, z is the z-value of FDE.

Plotting lethal rates against process time can be used to calculate the F-value of a thermal process. The F-value is defined as the time or equivalent time (min) taken to reduce initial microbial numbers at a specified temperature (°C), by a value, normally a multiple of the D-value for the target microorganism. Equation (4) is commonly used to calculate F-value (Holdsworth et al., 2008; Mullan, 2007):

$$F = \int_0^t L(t)\mathrm{d}t = \int_0^t 10^{\frac{T(t) - T_r}{z}} dt \tag{4}$$

The target microorganism is FDE, T(t) is the temperature history at the cols spot, which has been recorded by the fiber optic sensor. T_r is the reference temperature at 75 °C, 80 °C, or 85 °C. The microbial reduction of FDE can be calculated by Equation (5):

$$R = \frac{F_T}{D_T} \tag{5}$$

where R is the log reduction (CFU/g) of FDE, F_T and D_T refer to F-value (min) and D-value (min) of FDE at temperature T (°C). In this study, F_T is accumulated lethality rate changed with real-time temperature, while D_T is the reference D-value determined from isothermal inactivation study ($D_{75°C}$, $D_{80°C}$, $D_{85°C}$).

2.8. Statistical analysis

The mean and standard deviation of D-values of FDE were calculated using linear survival models through USDA integrated predictive modeling program tools (L. Huang, 2014). The goodness of fit was evaluated using the R^2 coefficient. The significant difference analysis was determined using student's t-test in IBM SPSS statistics 22.

3. Results

3.1. Microstructure of E. faecium

Fig. 1 provides images of FDE right after freeze-drying (Fig. 1, A) and vacuumed-packaging before storage (Fig. 1, B). After freeze-drying, FDE formed powdered residuals in a light-yellow color with extremely low a_w (< 0.02) and moisture content. The glass container of SM-FED was vacuum sealed and stored at −20 °C to protect freeze-dried bacteria from high moisture and temperature environments (Wagman & Weneck, 1963).

The SEM and TEM images were evaluated to assess whether the freeze-drying process affected the microstructure of *E. faecium*. In Fig. 2, *E. faecium* cells were completely embedded with skim milk and difficult to discern from background materials. Freeze-dried skim milk

Fig. 1. Physical appearance of skim milk-protected freeze-dried *E. faecium* (A) after freeze-drying (B) vacuum-packed for storage. The residual mass of freeze-dried bactiera was obtained from 6 mL bacterial suspension mixed with an equal volume of 10% skim milk solution.

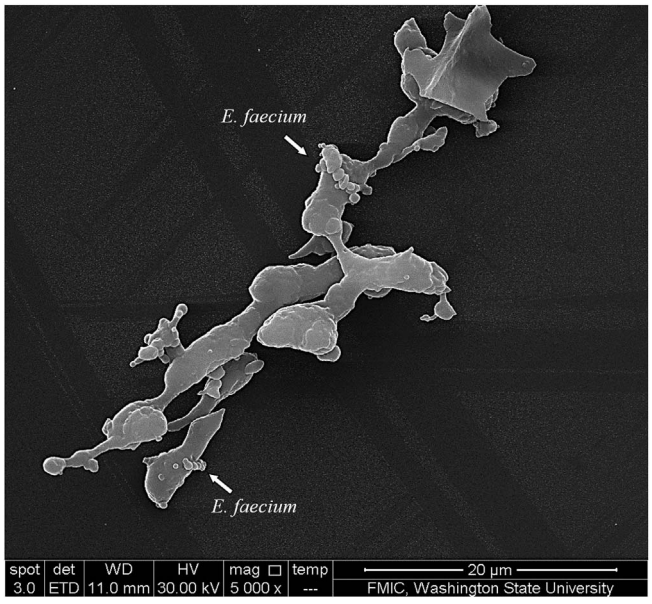

Fig. 2. Scanning electron microscopy of skim milk-protected freeze-dried *E. faecium*. Magnification × 5,000, scale bar = 20 µm.

generated fine flaky particles and formed a protective layer for bacteria. TEM examination revealed that liquid *E. faecium* cells were of a spherical shape and organized in pairs (Fig. 3, A1-2). Ribosomes were distributed evenly in the cytoplasmic region. Outer and inner cell membranes were clearly visible and tightly attached to each other. After freeze-drying, cellular integrity was maintained with intact ribosomes (Fig. 3, B1-2). The FDE cells showed few differences in the cytoplasmic region and were covered by a protective layer compared with cells in other formulations (Fig. 3, C1-2, arrows). These electrical-dense materials observed around the outer membrane could potentially serve as protective layers. The particles in the background of the TEM images (Fig. 3, C1-2) were likely caused by the presence of skim milk. At least five images have captured for each sample type at each magnification and only the representative images showed the majority microstructure of cells were illustrated.

3.2. Thermal resistance of FDE in wheat flour

Thermal inactivation curves of FDE in wheat flour ($a_{w,\ 25°C}$ = 0.45) at 75 °C, 80 °C, and 85 °C are shown in Fig. 4. The survival curves of FDE fit well into the first-order kinetic model at all temperature levels (R^2 = 0.92–0.95). Inactivation curves with sharper slopes (higher inactivation rates) were observed as temperature was increased. Average D-values of FDE at 75 °C, 80 °C, and 85 °C in wheat flour based on three independent tests are summarized in Fig. 4.

A z-value of 13.1 °C was obtained by plotting the log-linear regression of D-values versus temperatures (R^2 = 0.99). It is important to note that the $D_{80°C}$-value of FDE obtained in this study (5.92 ± 0.39 min) was significantly higher ($P < 0.05$) than the $D_{80°C}$-value of *S. enteritidis* PT30 (4.25 ± 0.45 min) in wheat flour under the same treatment condition (Villa-Rojas, 2015). This result indicates that FDE is an appropriate surrogate for *S. enteritidis* PT30 for food processing validation studies (Li, Kou, Cheng, Zheng, & Wang, 2017).

3.3. Inactivation kinetics of FDE in RF treatment

In this study, we assumed that no significant migration of moisture occurred in the whea flour sample during RF treatments, and a_w and temperature were similar with those in isothermal inactivation (Liu et al., 2017). Thus, the D- and z-values of FDE determined in the isothermal inactivation study (Fig. 4) can be applied in the calculation of the lethal rate of FDE during RF treatment at given temperatures.

Fig. 5 shows the temperature-time histories at the cold spot of the treated container when placed at the geometric center of a 15 cm electrode gap in the RF system. The time for the cold spot to reach 75 °C, 80 °C, and 85 °C were 13.7 min, 14.3 min, 16.1 min with corresponding average heating rates of 5.47, 5.59, and 5.28 °C·min^{-1},

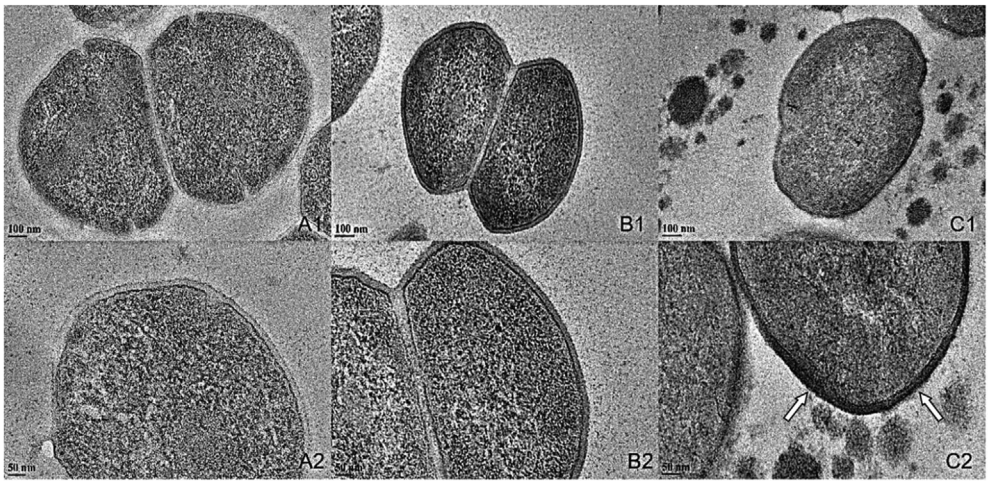

Fig. 3. Ultra-thin sections of *E. faecium* cells with different formulations: A1, A2: liquid, B1, B2: freeze-dried without skim milk, C1, C2: freeze-dried protected with skim milk. 1: magnification × 15,000, scale bar = 100 nm; 2: magnification × 275,000, scale bar = 50 nm.

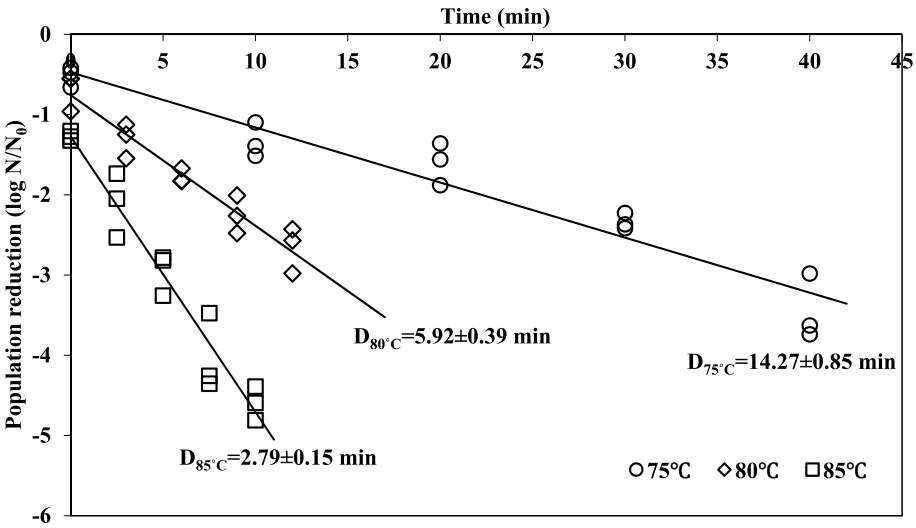

Fig. 4. Inactivation kinetic curves and D-values of skim milk-protected freeze-dried *E. faecium* in wheat flour ($a_{w,\ 25°C}$ = 0.45) at 75 °C, 80 °C and 85 °C.

respectively. During the 10 min holding time (i.e. the time samples were kept in the RF system after reaching CUT), temperatures at the cold spot slightly declined to 3 °C lower than target temperature.

Experimental inactivation kinetics of FDE in wheat flour ($a_{w,\ 25°C}$ = 0.45) during RF treatment at 75 °C, 80 °C, and 85 °C are listed in Table 1. The initial population level of FDE inoculated in wheat flour was 8.74 ± 0.17 log CFU/g. Sample bags treated for 5 min and 10 min were tested to measure the progressive changes of survivors of FDE during heating time. After 10 min heating, 0.37 ± 0.14 log CFU/g of FDE was reduced when compared with the initial population. After the temperature of flour reached CUT, samples were held for 10 min and the survivor counts were determined every 5 min after CUT. The population of FDE in wheat flour decreased as the RF heating time increased, while the degree of decrease varied with the temperature. The population reduction of FDE in wheat flour increased quickly with increasing treatment temperature. Ten min of holding time in RF had achieved 4.93 ± 0.16 log reduction at 85 °C, while only 1.03 ± 0.04 and 2.54 ± 0.11 log reductions were achieved at 75 °C and 80 °C, respectively.

Based on D- and z-values obtained from the isothermal inactivation study, Equation (5) was used to predict microbial reduction of FDE in wheat flour during RF treatment. For example, the predicted curve at 85 °C (black line in Fig. 6) was generated by putting $D_{85°C}$ = 2.79 min, and $F_{85°C}$ = 13.11 min in Equation (5). To get $F_{85°C}$, T_r = 85 °C, z = 13.1 °C and temperature-time history data recorded by the fabric-optic sensor were put in Equation (4) and integrated from time 0–26.1 min.

4. Discussion

Freeze-drying is commonly used for cultural preservation and transportation (Malik, 1978, 1984, 1988). However, the challenging part of freeze-drying technology was that cold-injury may happen to bacterial cells. Skim milk acting as a protective agent of bacteria during freeze-drying and further storage can form protective layers around bacteria cells (Dhewa, Pant, & Mishra, 2014; Lee, Kim, & Park, 2016).

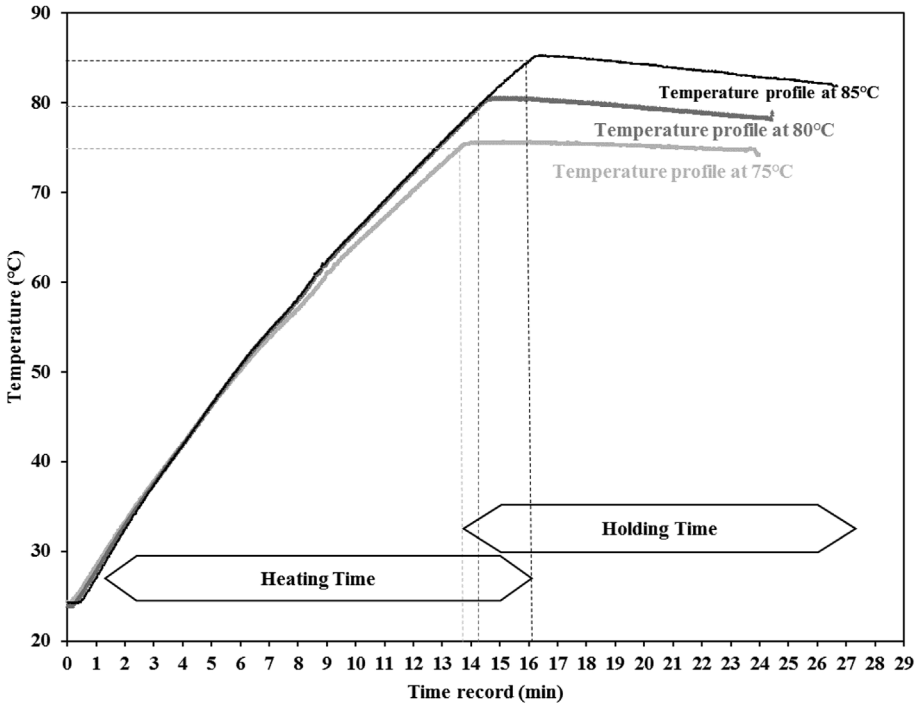

Fig. 5. Temperature-time history of wheat flour at the cold spot during RF treatment (Light grey: 75 °C, Grey: 80 °C, Black: 85 °C). Two stages of RF treatment were named as heating time and holding time and showed separately.

Table 1
Population reduction (log CFU/g) and processing time (min) of skim milk-protected freeze-dried *E. faecium* in wheat flour during RF treatment.

RF process	Population reduction during heating (log CFU/g)			Population reduction during holding (log CFU/g)		
75 °C	0.00 ± 0.00 (0.0)[a]	0.05 ± 0.04 (5.0)	0.37 ± 0.14 (10.0)	0.62 ± 0.06 (13.7)	0.76 ± 0.12 (18.7)	1.03 ± 0.04 (23.7)
80 °C				0.94 ± 0.12 (14.3)	1.65 ± 0.07 (19.3)	2.54 ± 0.11 (24.3)
85 °C				1.33 ± 0.15 (16.1)	4.35 ± 0.03 (21.1)	4.93 ± 0.16 (26.1)

[a] Number in the bracket indicated the processing time in minutes.

Freeze-dried *E. faecium* developed in this study has been previously confirmed to have high stability in survivability and heat tolerance throughout storage (vacuumed packed and stored at −20 °C) (Xu et al., 2017). TEM images indicate that cellular integrity was well maintained after freeze-drying and no significant ultrastructural differences were observed among *E. faecium* cells of different formulations (liquid and freeze-dried with/without skim milk).

In this study, FDE showed higher thermal resistance (5.92 ± 0.39 min) than liquid-form *S. enteritidis* PT30 in wheat flour ($a_{w,\ 25°C}$ = 0.45) under the same treatment condition. $D_{80°C}$-value of *S. enteritidis* PT30 in the same flour product was 4.3 ± 0.2 min (Tadapaneni, Syamaladevi, Villa-Rojas, & Tang, 2017). Lower D-values of *S. enteritidis* PT30 at 75 °C and 85 °C were also observed in an all-purpose wheat flour ($D_{75°C}$ = 12.7 ± 1.3 min, $D_{85°C}$ = 2.3 ± 0.1 min) (Villa-Rojas, 2015). *S. enteritidis* PT30 in all-purpose wheat flour showed a z-value of 13.5 °C (Villa-Rojas, 2015), which was statistically the same ($P < 0.05$) with that of FDE (13.1 °C) in this study. Previous studies reported the use of liquid *E. faecium* NRRL B-2354 as a surrogate (Bianchini et al., 2014; Rachon, Peñaloza, & Gibbs, 2016). Compared with liquid *E. faecium*, FDE used in this study showed lower thermal resistance under the same treatment conditions (Liu et al., 2017). The decreased thermal resistance of FDE might have been caused by partical injury of bacteria cells during freeze-drying and subsequent storage (Peiren, Hellemans, & De Vos, 2016; Ray, Jezeski, & Busta, 1971). The $D_{85°C}$-value (4.1 ± 0.2 min) of liquid-formulation *E. faecium* in the same flour product was observed as twice as that of *S. enteritidis* PT30 (2.3 ± 0.1 min) (Liu et al., 2017). The liquid-form *E. faecium* was too tough to be eliminated than *Salmonella* which may result in overestimated process time. In this case, a non-pathogenic microorganism with a similar or a slightly higher thermal resistance compared to the target microorganism meets a better criteria for surrogate selection and is more practical for industrial applications in terms of safety considerations and energy consumption (Sinclair, Rose, Hashsham, Gerba, & Haas, 2012).

The RF heating was designed and conducted based on the following facts: i) the cold spot of the treated flour sample was located in the geometric center of the container (Tiwari, Wang, Tang, & Birla, 2011); ii) the introduction of a small sample bag in the cold spot will not significantly influence the temperature distribution of the flour sample during RF treatment (Liu et al., 2017); and iii) the small amount of flour in the sample bag were cooled very rapidly after treatment with no appreciable microbial destruction during cooling. RF treatment allows rapid heating of bulk flour samples, and RF heating has been regarded as an effective thermal treatment for controlling *Salmonella* in low-moisture foods. It has been reported that 40 s RF heating could generate 3.38 log CFU/g reduction of *S. typhimurium* in red peppers (Kim et al., 2012), and 3.7-, 6.0-, and 5.6-log reductions of *S. enteritidis*, *S. typhimurium*, and *S. senftenberg*, respectively in surface-inoculated raw shelled almonds (Jeong, Baik, & Kang, 2017). In this study, RF heating at 85 °C achieved nearly 5-log reduction of FDE within 27 min in 3.0 kg

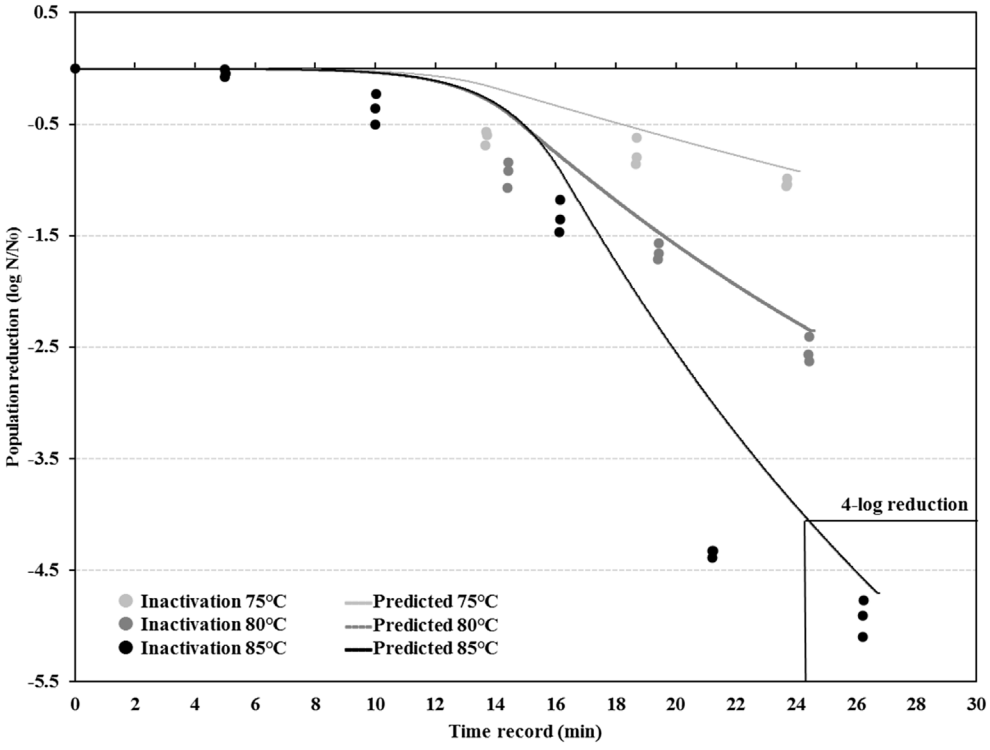

Fig. 6. Inactivation kinetics of skim milk-protected freeze-dried *E. faecium* in RF-pasteurized wheat flour at different temperatures. Discrete data points represented population reduction of FDE during RF treatment from experimental data. Solid lines showed population reduction of FDE predicted by a model (Light grey: 75 °C, Grey: 80 °C, Black: 85 °C).

wheat flour. Validation processes often aim for a 3–5 log reduction, and as a margin of safety, the target is to obtain additional log reductions beyond the expected level of contamination (Bianchini et al., 2014). To comply with the USDA pasteurization standard of at least 4-log reduction of a surrogate (FDA, 2016b), 24 min RF treatment at 85 °C will generate enough lethality of bulk flour samples with $a_{w,\ 25°C}$ of 0.45.

The progressive log reduction of FDE in the geometric center of wheat flour can be predicted based on D- and z-values of FDE and temperature history from RF heating (at different temperatures with holding time up to 10 min). Compared with experimental data, dynamic population reduction of FDE in wheat flour can be well predicted by the equation. This model has also be used to predict population reduction of liquid *E. faecium* in wheat flour during RF treatment at 80 °C (Liu et al., 2017). In our study, the observed log reductions collected from experimental data were higher than the predicted model, and similar results were also observed (Liu et al., 2017). This may be caused by the unavoidable delay in taking the sample bag out of the container after each sampling time point. Moreover, experimental data collected from sealed sample bag located in the cold spot of the container, representing the least heated area, provided conservative results with greater log reductions of the target microorganism than predicted. Greater lethality in sample bags at other locations would be achieved under the same process since they had a higher temperature than the cold spot. In the real practice, more lethality of the target microorganism could be generated if a gradual cooling process is taken into consideration.

This study concludes that freeze-drying process did not cause visible changes in the microstructure of *E. faecium*. Freeze-dried *E. faecium* protected with skim milk proved to be an appropriate surrogate of *Salmonella* in LMFs, such as wheat flour. RF pasteurization provided rapid heating to inactivate pathogens in LMFs, and the RF process was validated by using freeze-dried *E. faecium* as a new surrogate. The inoculated sample bags used in this study could avoid using large inoculated population of surrogate, and eliminate the possibility of cross-contamination caused flour aerosols in commercial operation.

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