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Inactivation of *Salmonella* Enteritidis and *Enterococcus faecium* NRRL B-2354 in corn flour by radio frequency heating with subsequent freezing

Samet Ozturk[a], Shuxiang Liu[b], Jie Xu[b], Juming Tang[b], Jinru Chen[c], Rakesh K. Singh[a], Fanbin Kong[a,*]

[a] Department of Food Science and Technology, The University of Georgia, Athens, GA, USA
[b] Department of Biological Systems Engineering, Washington State University, Pullman, WA, USA
[c] Department of Food Science and Technology, The University of Georgia, Griffin, GA, USA

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ABSTRACT

This study aimed to evaluate the feasibility of using *Enterococcus faecium* NRRL B-2354 (*E. faecium*) as a surrogate of *Salmonella enterica* Enteritidis PT30 (*S.* Enteritidis PT30), validate radio frequency (RF) heating in pasteurizing corn flour, and study the effect of subsequent freezing treatment after RF heating in enhancing the microbial inactivation effect. Corn flour with water activity of 0.45 ± 0.05 at 25 °C was homogeneously inoculated with *S.* Enteritidis PT30 and *E. faecium* separately at 8.50 ± 0.23 log CFU/g. Thermal resistance parameters in corn flour were determined in a water bath at 75, 80, and 85 °C and RF heating was used to pasteurize 3.18 kg corn flour with subsequent freezing storage at −20 °C. Results showed that the thermal resistance of *E. faecium* was higher than *S.* Enteritidis PT30. Samples heated to 85 °C, held for 10 min after heating in a RF system, and then stored at −20 °C for 48 h, resulted in the reduction of *S.* Enteritidis PT30 by 6.59 ± 0.21 log and *E. faecium* by 4.79 ± 0.17 log. *E. faecium* could be used as its surrogate for validation studies in the packaged corn flour. Results also confirmed that RF heating combined with freezing storage treatment could significantly reduce the survival of both microorganisms in corn flour.

1. Introduction

Low-moisture foods (water activity a_w < 0.6), including various dehydrated food powders such as spices, vegetable powder, and wheat flour, have been historically considered as safe due to limited growth of either vegetative or spore-forming bacteria (Blessington, Theofel, & Harris, 2013; Farakos & Frank, 2014). However, a number of foodborne outbreaks were linked to low-moisture foods in recent years (Beuchat et al., 2013; Podolak, Enache, Stone, Black, & Elliott, 2010), which attracted great attention of the scientific community and the food industry to the safety of dry foods and ingredients. Heat resistant pathogens such as *Salmonella* has been shown to increase with the decrease of a_w in foods (Podolak et al., 2010). Therefore, it is critical to develop an effective pasteurization process with reliable performances for eliminating foodborne pathogens in foods with low a_w. Several decontamination methods such as steam treatment, fumigation with ethylene oxide, and irradiation have been developed to reduce the microbial load of low-moisture foods (Lee et al., 2006). However, steam treatment involves use of high temperature and longtime heating due to the low thermal conductivity of the low-moisture food leading to significant loss of nutritional quality (Song et al., 2014; Toofanian & Stegeman, 1988; Waje, Kim, Kim, Todoriki, & Kwon, 2008). Ethylene oxide is regarded as a carcinogen, while irradiated foods are not readily accepted by consumers due to safety concerns (Farkas, 2006; Schweiggert, Carle, & Schieber, 2007; Waje et al., 2008). It is therefore preferred to develop new pasteurization technologies aiming to produce high quality and safe low-moisture foods. Radio frequency (RF) heating is dielectric heating with a frequency range of 3 kHz–300 MHz, which generates heat inside foods through ionic conduction and dipole rotation, providing fast and volumetric heating throughout the foods (Marra, Zhang, & Lyng, 2009). The effect of RF heating on inactivation of pathogenic bacteria has been studied for various foods including shell eggs (Geveke, Bigley, & Brunkhorst, 2017), meat lasagna (Wang, Luechapattanaporn, Wang, & Tang, 2012), almonds (Gao, Tang, Johnson, & Wang, 2012; Gao, Tang, Villa-Rojas, Wang, & Wang, 2011; Gao, Tang, Wang, Powers, & Wang, 2010), wheat flour (Villa-Rojas, Zhu, Marks, & Tang, 2017), shell almond (Li, Kou, Cheng, Zheng, & Wang, 2017), and spices (Kim, Sagong, Choi, Ryu, & Kang, 2012). Our previous studies indicated that RF heating could be a promising technology for pasteurization of food powders (Liu et al., 2018; Ozturk

* Corresponding author.
E-mail address: fkong@uga.edu (F. Kong).

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et al., 2018; Ozturk, Kong, Singh, Kuzy, & Li, 2017). Specifically, we have studied dielectric properties of various food powders, RF heating rates, and explore different ways to improve heating uniformity using corn flour as a model food (Ozturk et al., 2018, 2017 and; Ozturk, Kong, Trabelsi, & Singh, 2016). In order to use RF heating in food pasteurization, in-plant validation studies are critical to assure the proficiency of the process design and operational parameters to meet safety requirement. However, pathogens are usually not allowed to be directly used for process validation in a production environment due to strict safety considerations. Thus, Busta (2003) suggested the use of non-pathogenic surrogate microorganisms with equal or slightly higher thermal resistance as an alternative approach to conduct microbial validation. *Enterococcus faecium* NRRL B-2354 has been reported as a *Salmonella* surrogate for various low-moisture foods including almond (California, 2007), wheat flour (Liu et al., 2018; Tiwari, Wang, Tang, & Birla, 2011), and various spices (Rachon, Penaloza, & Gibbs, 2016). The feasibility of using *E. faecium* as a surrogate for validation of microbial inactivation has been evaluated in different pasteurization techniques including extrusion (Bianchini et al., 2012), moist-air convection heating (Jeong, Marks, & Ryser, 2011), and infrared pasteurization (Bingol et al., 2011). Additionally, recent studies have been reported on RF inactivation kinetics of pathogen and its surrogate in food powders including wheat flour (Liu et al., 2018), whole black peppercorn and ground black pepper (Wei et al., 2018) and cumin seeds (Chen, Wei, Irmak, Chaves, & Subbiah, 2019), however, not particularly in corn flour. Post-heating treatment could be used to enhance the inactivation of the microbial after pasteurization treatment. For example, a previous study showed that storing sample at freezing temperature following decontamination by the pulsed electric field in green tea infusions successfully reduced the survival of pathogenic microorganisms (Zhao, Yang, & Wang, 2009). It is thus of interest to evaluate the effect of subsequent freezing treatment after RF heating on pasteurization of food powder.

The objectives of this study were to (1) evaluate the potential of *E. faecium* as a surrogate of *Salmonella* to validate RF heating pasteurization of *Salmonella* using corn flour as a model food, and (2) determine the effect of freezing storage (−20 °C for 96 h) on enhancing the microbial inactivation following RF treatment.

2. Material and methods

2.1. Bacterial inoculation of corn flour

Corn flour was purchased from Georgia Spice Company (Atlanta, GA USA). *Salmonella* Enteritidis PT30 (*S*. Enteritidis PT30) and *Enterococcus faecium* NRRL B-2354 (*E. faecium*) were obtained from Dr. Mark Harrison's laboratory at the University of Georgia. Both bacterial strains were cultured in 9 ml of tryptic soy broth (TSB) supplemented with 0.6% (w/v) yeast extract at 37 °C for 24 h and then 1 ml evenly spread on a plate (150 × 15 mm) of tryptic soy agar (TSA). The bacterial lawn on TSA was harvested into 20 ml of sterile 0.1% peptone water and centrifuged for 30 min at 2600 *g*. Then, the supernatant was discarded and the pellet was re-suspended in 3 ml 0.1% peptone water as described by (Liu et al., 2018). One ml of the concentrated pellet of *S*. Enteritidis PT30 or *E*. faecium was mixed into 10 g corn flour in a sterile stomacher bag until a uniform mixture was obtained. After mixing, inoculated corn flour samples (10 g) were used to further inoculate 90 g sample, which was mixed in a stomacher (Seward Stomacher, 400 Lab System, Norfolk, United Kingdom) at 260 rpm for 5 min. Then, 10 samples (1 g each) were randomly selected and enumerated on tryptic soy agar (TSA) plates as described to confirm the uniformity of inoculum distribution. To avoid the effect of a_w on thermal resistances of *S*. Enteritidis PT30 and *E. faecium* in corn flour, inoculated samples were placed in sterile trays and then put into a Hotpack 435315 humidity chamber (SP Industries, Inc., Warminster, PA, USA) for four days to obtain target water activity $a_{w,25°C} = 0.45$.

2.2. Isothermal treatment

Thermal resistances of *S*. Enteritidis PT30 and *E. faecium* in corn flour with $a_{w,25°C} = 0.45 \pm 0.05$ were determined at 3 different temperatures (75, 80, and 85 °C) using aluminum thermal-death-time (TDT) cells purchased from Washington State University, Pullman, WA (Chung, Birla, & Tang, 2008; Villa-Rojas et al., 2013). The cells were fully filled with inoculated corn flour with 0.85 g weight and 4 mm thickness and subjected to isothermal heat treatment in a water bath (Model: SWB-10L-2, Saratoga, CA, USA) maintained at 75, 80, and 85 °C, respectively. To obtain thermal death curves, each isothermal treatment was performed at the same time intervals starting after the come-up times (CUT) ended. The CUT was defined as the time of the corn flour samples to reach target temperature (75, 80, and 85 °C) in the water bath, which was determined by using cells filled with non-inoculated corn flour sample with a T-type thermocouple inserted in the center of the cell to monitor temperature changes. Isothermal treated cells were removed from the water bath and immersed immediately in an ice-water bath for 90 s to stop thermal inactivation in the sample. Triplicates of each set of conditions were performed.

2.3. Enumeration of microbial survival

Thermally treated corn flour in TDT cells was transferred into sterile stomacher bags and diluted 1:10 with 0.1% peptone water. The samples were firstly mixed by hand and then homogenized with a stomacher for 2 min at 260 rpm (Harris, Uesugi, Abd, & McCarthy, 2012). The proper tenfold serial dilutions were spread-plated in duplicate onto modified mTSA for *S*. Enteritidis PT30 or eTSA for *E. faecium* plates to enumerate both *S*. Enteritidis PT30 and E. *faecium* survivors in corn flour, respectively (mTSA: TSA agar, Yeast Extract, 0.05% Ammonium Iron (III) Citrate, and 0.03% Sodium Thiosulfate Pentahydrate ($5H_2O$), and eTSA; TSA agar, Yeast Extract, 0.05% Ammonium Iron (III) Citrate and Esculin Hydrate). The plates were incubated aerobically at 37 °C for 24–48 h, then the colonies were enumerated, and the populations were converted to log CFU/g. Log reductions were calculated by subtracting the survivor counts from the initial population before thermal treatment.

2.4. Inactivation kinetics

The first order kinetics (D-value) Eq. (1), and the Weibull model Eq. (2) were used to express inactivation kinetics (Peleg, 2006)

$$log\frac{N}{N_0} = -\frac{t}{D} \tag{1}$$

$$log\frac{N}{N_0} = -\left(\frac{t}{\delta}\right)^{\alpha} \tag{2}$$

where N_0 is the initial concentration of microorganism (CFU/g), and N is the population of survival (CFU/g) at time t, the isothermal treatment time (min) after CUT; D-value is the time to reduce the microbial population by 10-fold at the inactivation temperature (°C); δ indicates the overall steepness of the survival curve; α is the survival curve factor which indicates whether it is linear (α = 1) or non-linear (α ≠ 1) with a decreasing (α < 1) or increasing (α > 1) inactivation rate with time.

The microbial survival data obtained from isothermal treatment were used to fit the two models and estimate the model parameters. Root mean square error (RMSE) was used to evaluate the performance of the model.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left[log\frac{N}{No}_{data,i} - log\frac{N}{No}_{model,i}\right]^2}{n-p}} \tag{3}$$

where $\log\frac{N}{No}_{data,i}$ is measured log CFU/g reduction, and $log\frac{N}{No}_{model,i}$ is predicted log CFU/g reduction from the model, n is the total number of

observations, and p is the number of model parameters. To estimate the fitness of the model and provide RSME directly, IPMP (Integrated pathogen modeling program) was used (Huang, 2014). ANOVA in Minitab 14 (Minitab Inc., State College, PA) was applied to evaluate differences between measured and predicted D-values among samples. To obtain the z-value, the log of D-values was plotted against temperature, in which the slope refers to the $-1/z$, where z is the change in temperature to alter the D-value by one log-cycle (Gaillard, Leguerinel, & Mafart, 1998).

2.5. Radio frequency heating of inoculated corn flour

A 27.12-MHz, 6-kW pilot scale RF system (COMBI 6-S, Strayfield International, Wokingham, UK) was used in this study. The RF system had two parallel electrodes, and the top electrode position can be adjusted to change the gap between them. A rectangular Polyetherimide (PEI) container ($70 \times 240 \times 300\ mm^3$) was used to hold 3.18 kg corn flour at a fixed 150 mm electrode gap to obtain a better heating rate and uniformity based on our previous study (Samet Ozturk, Fanbin Kong, Rakesh K. Singh, Jesse Daniel Kuzy, & Changying Li, 2017). Two 35 mm-height PEI blocks were used as spacers to hold PEI sample container in the symmetrical center of both the bottom and top electrodes. Additionally, the PEI container surface was covered by a lab-made foam case acting as an insulator to enhance electromagnetic strength around the container, and to prevent significant heat loss while maintaining more uniform heating. To obtain temperature distribution data and determine cold spot area in the middle layer of PEI container, a cheese cloth was used to divide the PEI container into two layers with equal height as described by (Ozturk et al., 2017). The detailed information about heating uniformity assessment was described by (Ozturk et al., 2017) and (Liu et al., 2018). PEI container with uninoculated corn flour (3.18 kg) was put in between the upper and lower electrodes and heated by the RF heating system to reach target temperatures (Fig. 1). The change in temperature during the RF heating was recorded using a fiber optic temperature sensor with an accuracy of ± 1 °C (Fiso Tech. Inc., Quebec, Canada) connected to a data logger. After heating, the RF system was turned off, the PEI container was removed immediately, and the thermal images of middle layers were taken using an infrared camera (FLIR T440, FLIR Systems, Inc., North Billerica, MA, USA) as an indicator of temperature distribution and heating uniformity.

To evaluate the inactivation efficiency of RF heating on both S. Enteritidis PT30 and *E. faecium* in corn flour, inoculated corn flour (5 g) was packed in a sterile plastic bag ($50 \times 50 \times 1\ mm^3$). The bag was then sealed and placed in the cold spot area in the PEI container (Fig. 2), which was in the geometric center of the PEI container as determined by the infrared camera. A separate study was conducted to determine the effect of the plastic bag on heating rates of the samples in the PEI container, in which non-inoculated corn flour sample was loaded in the plastic bag with a fiber optic sensor on the surface of the bag to monitor temperature, as described by Liu et al. (2018). A comparative trial was conducted to record the temperature history during the RF heating process in the corn flour sample of the PEI container without plastic bag inserted. The temperature history in the geometric center of the PEI container with/without plastic bag was compared to reveal any significant impact of the bag on sample temperature.

The PEI container filled with uninoculated corn flour (3.18 kg) with inoculated sealed bag inserted (Fig. 2) was subjected to RF heating, until the geometric center to reach temperatures of 75, 80, and 85 °C, respectively. The RF system was then turned off, and the sealed inoculated sample package was immediately removed from the container and inserted in an ice-bath for 90 s to stop further thermal inactivation process in the sample. The samples were serially diluted and plated for enumerating surviving bacterial population as described above. Additional trials were conducted in which the samples after reaching target temperatures were held in the RF system for 10 min, and the microbial survival was determined to study the effect of holding on pasteurization effect.

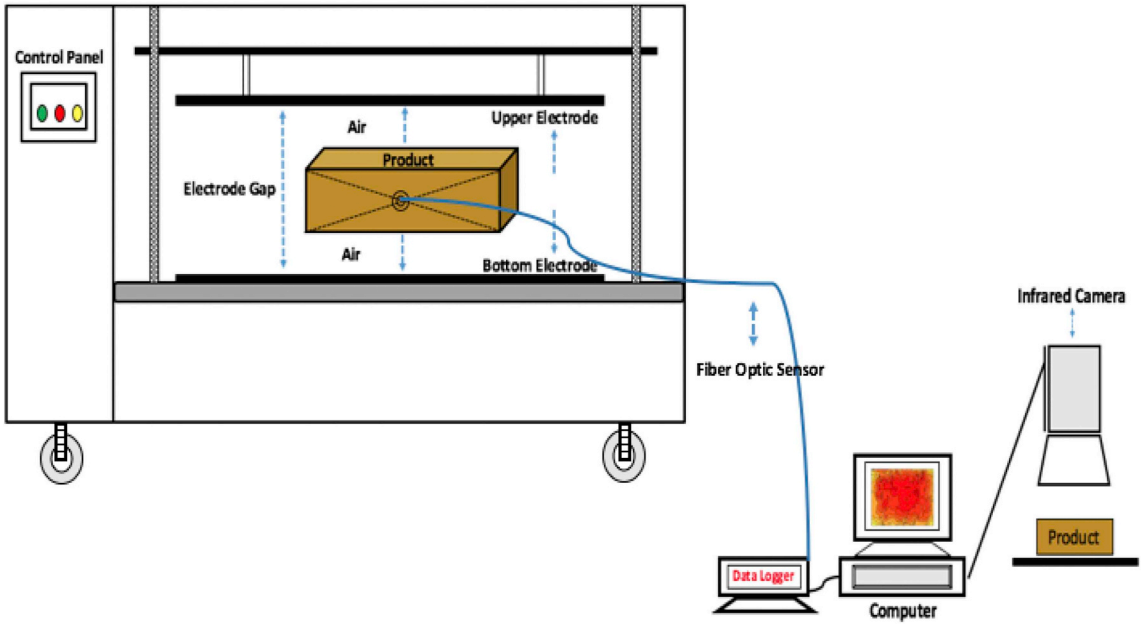

Fig. 1. Schematic diagram of corn flour filled PEI rectangular container placed in the middle of two parallel electrodes in a 6 kW 27.12 MHz radio frequency heating (Ozturk et al., 2017).

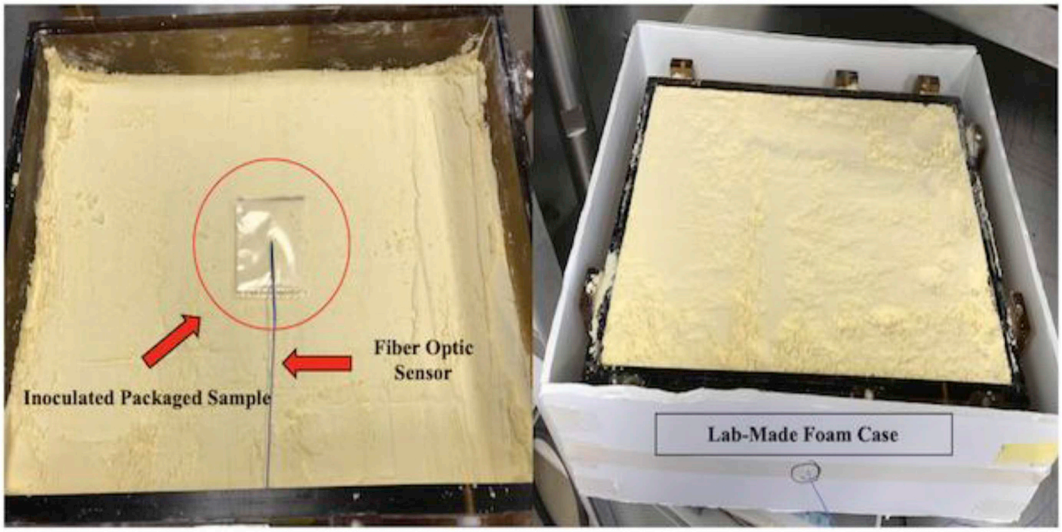

Fig. 2. Left: Sterile plastic bag (50 × 50 × 1 mm^3) with 5 g inoculated (*S.* PT30 and *E. faecium*) corn flour sample in the geometric center of the PEI container; right: PEI container filled with 3.18 kg un-inoculated corn flour covered by lab-made foam case.

2.6. Combination of RF heating and post-freezing treatment on microbial survival

The RF treated inoculated sample packages (with or without 10 min holding) after cooling down to room temperature were stored in a freezer (−20 °C) for up to 96 h. Samples were taken in a 24 h interval to determine the effect of freezing on the survival populations of S. Enteritidis PT30 and *E. faecium*. The stored samples were taken out from the freezer and warmed up to room temperature for enumeration of the survival. Then one-gram sample from each treatment combination was collected randomly, tenfold serially diluted and plated as described previously to enumerate the survivors.

3. Results and discussion

3.1. D- and z-values of S. Enteritidis PT30 and E. faecium in corn flour

Fig. 3 shows the inactivation kinetics of both microorganisms in corn flour at $a_{w,25°C}$ = 0.45 ± 0.05. Survival data of both *S.* Enteritidis PT30 and *E. faecium* show a good fit to the two models with similar RMSE values (Table 1). The log-linear model was applied to describe thermal resistances and plot z-values in this study (Fig. 4). The D-values for *S.* Enteritidis PT30 at 75, 80, and 85 °C were 14.6 ± 0.51, 6.11 ± 0.45, and 2.03 ± 0.27 min, respectively. As inactivation temperature increased, D-value of *S.* Enteritidis PT30 in corn flour decreased proportionally. Obtained D-values were in a good agreement with values reported in the literature. For instance Smith, Hildebrant, Casulli, Dolan and Marks (2016) and Syamaladevi (2016) reported the$D_{80°C}$ for the same *Salmonella* serotype in wheat flour with the same levels of a_w as 6.9 ± 0.7 and 5.51 ± 0.22 min, respectively. Moreover, the obtained D-values for *S.* Enteritidis PT30 in corn flour were also in good agreement with organic wheat flour, which are 17.65 ± 1.58, 7.17 ± 0.35, and 2.92 ± 0.35 min at 75, 80 and 85 °C, respectively (Liu et al., 2018). The slight difference in the D-values might be due to differences in a food matrix, inoculation method, moisture content, and isothermal treatment method. In this study, *E. faecium* shows significantly ($p < 0.05$) higher heat resistance at applied temperatures with an equivalent z-value as for *S.* Enteritidis PT30. For example, $D_{80°C}$ values of *S.* Enteritidis PT30 and *E. faecium* in corn flour (a_w = 0.45) were 6.11 ± 0.59 min and 10.32 ± 0.82 min, respectively, while the z-values of *S.* Enteritidis PT30 and *E. faecium* were very close, which are 11.8 °C and 11.7 °C, respectively. Several studies reported that *E. faecium* NRRL B-2354 is a promising surrogate for pathogenic bacteria and can be used to validate thermal processing of various foods including high moisture food (dairy products, juices and meat) and low-moisture foods (almonds, walnuts, peanut butter,

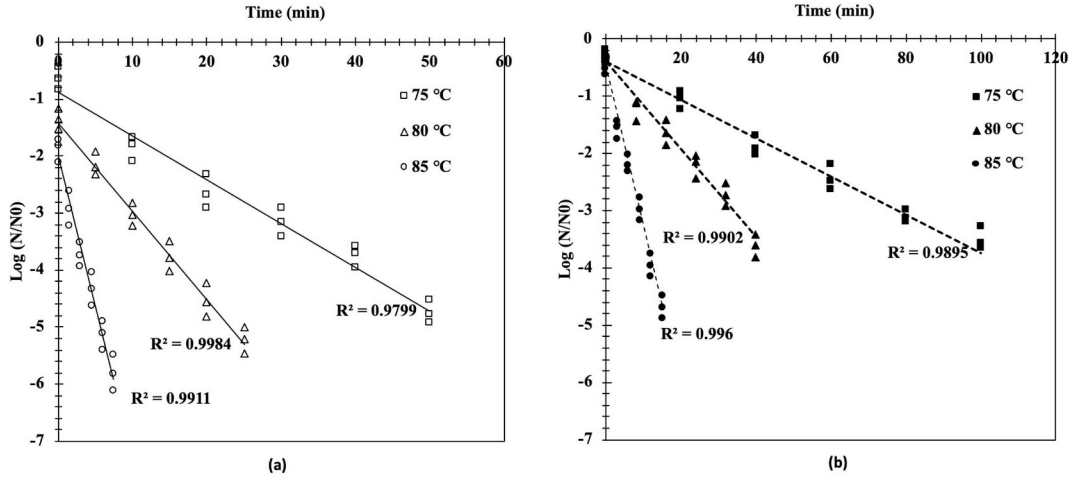

Fig. 3. Inactivation kinetic curves of *S.* PT30 (a) and *E. faecium* NRRL B-2354 (b) in corn flour (a_w 0.45 ± 0.05) at 75, 80 and 85 °C.

Table 1
Parameter estimates for the primary models, as well as the root mean square error (RMSE)

	Temperature (°C)	Linear Model		Weibull Model		
		D-value (min)	RMSE (log CFU/g)	δ(min)	α	RMSE (log CFU/g)
S. PT30	75	14.18 ± 1.24	0.53	15.22 ± 3.42	1.42 ± 0.14	0.62
	80	6.11 ± 0.59	0.24	7.13 ± 0.64	0.72 ± 0.09	0.12
	85	2.02 ± 0.31	0.19	1.67 ± 0.46	0.66 ± 0.04	0.19
E. faecium	75	22.41 ± 1.43	0.42	24.67 ± 4.35	1.02 ± 0.26	0.42
	80	10.32 ± 0.82	0.33	9.12 ± 0.87	0.84 ± 0.16	0.11
	85	3.17 ± 0.26	0.12	2.24 ± 0.52	0.92 ± 0.08	0.17

* The root mean square error (RMSE) was determined by IPMP software using triplicate experimental data.
* Values are means ± standard errors. Parameters were estimated separately for each data set. Smaller RMSE values indicate a better fitness of the model.

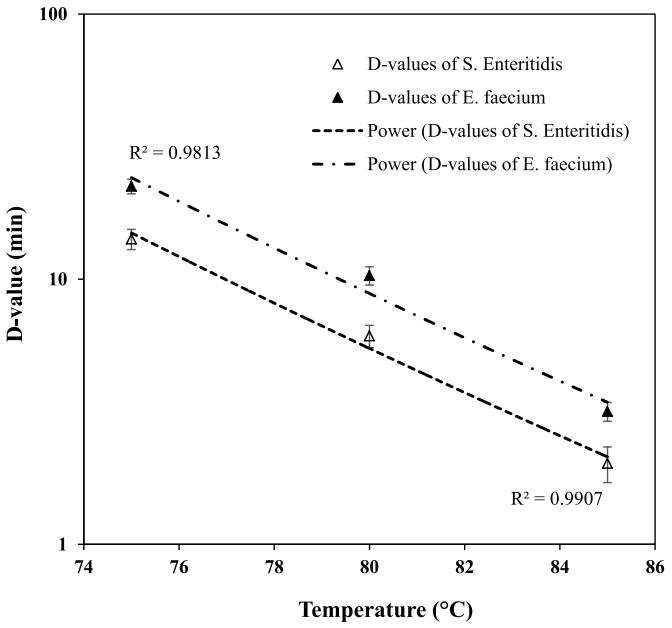

Fig. 4. D-values of *S.* Enteritidis and *E. faecium* at $a_{w,25°C}$ = 0.45 ± 0.05 and their power trend lines (round dot line for *S.* Enteritidis, dashed line for *E. faecium*). Experiments were done in triplicate.

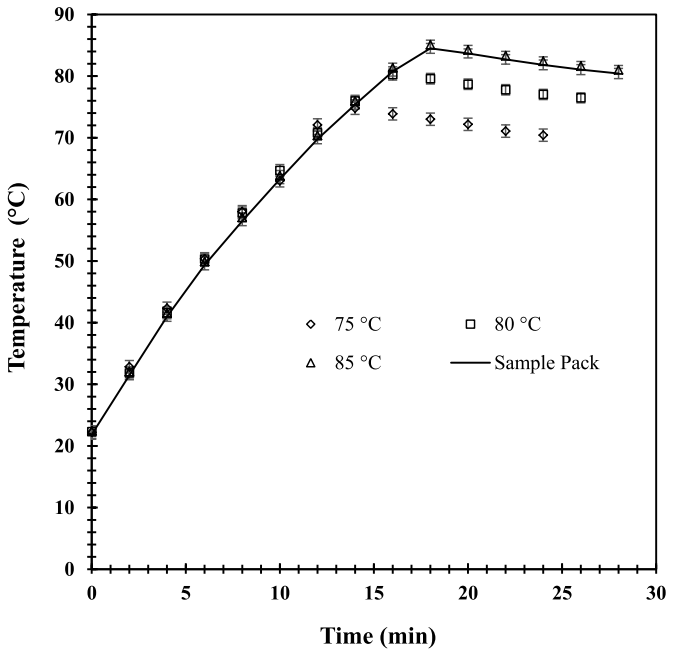

Fig. 5. A typical measured temperature–time curve of corn flour in a PEI container subjected to RF heating at 15 cm electrode gaps with 10 min holding. Empty diamond, square, and triangle markers indicate temperatures in the geometric center of the sample while continuous line represents the temperature on the surface of the inoculated plastic bag.

extruded products) (Annous & Kozempel, 1998; Bianchini et al., 2012; Blessington et al., 2013; Ma, Kornacki, Zhang, Lin, & Doyle, 2007; Piyasena, Dussault, Koutchma, Ramaswamy, & Awuah, 2003). It has been used as a surrogate of *Salmonella* to validate process conditions for achieving 4 to 5-log reduction of *Salmonella* in almonds (Bingol et al., 2011; Kopit, Kim, Siezen, Harris, & Marco, 2014). The present study indicates that *E. faecium* is a conservative, but a suitable surrogate for *S.* Enteritidis PT30 in corn flour in the tested conditions with higher heat resistance.

3.2. Temperature profile during RF heating and uniformity

In this study, corn flour (3.18 kg) held in a PEI container was subjected to RF heating until reaching target temperatures in the geometric center of the sample. Fig. 5 shows the typical temperature-time profiles in the samples. As can be seen, the temperature history on the surface of packed corn flour was close to the central temperature of PEI container without sample package in the geometric center. The determined temperature difference was only 1.0 ± 0.4 °C, which indicates inserting the inoculated plastic bag did not significantly impact the temperature. Thus, the microbial survival inside the bag can be used to indicate the pasteurization effect of RF heating on the samples in the PEI container. Similar results were reported in wheat flour heated by the same RF system (Liu et al., 2018; Xu et al., 2018). Our previous study showed that samples heated in the PEI container surrounded by foam sheet had achieved better heating uniformity in the middle layer with a higher average temperature (Ozturk et al., 2017). As Fig. 6 presents, the corner and edge heating were observed in the middle layers, and the cold spot area was located in the center. After samples were heated in the RF system till 75, 80, 85 °C, the average temperature of the middle layer, in which cold spot area was located, was determined as 72.2 ± 2.4, 78.4 ± 2.9, and 81.9 ± 3.2 °C, respectively. Recent studies also reported similar heating distribution patterns for RF heated coffee bean (Pan, Jiao, Gautz, Tu, & Wang, 2012), rice (Zhou, Ling, Zheng, Zhang, & Wang, 2015), wheat flour (Tiwari et al., 2011) and wheat germ (Ling, Lyng, & Wang, 2018; Ling, Ouyang, & Wang, 2019). Moreover, holding the packaged sample in PEI container for 10 min after RF system was turned off resulted in a decrease in the temperature of the sample around 4.1 ± 1.2 °C (Fig. 5) due to heat conduction throughout the PEI container and heat loss to the air.

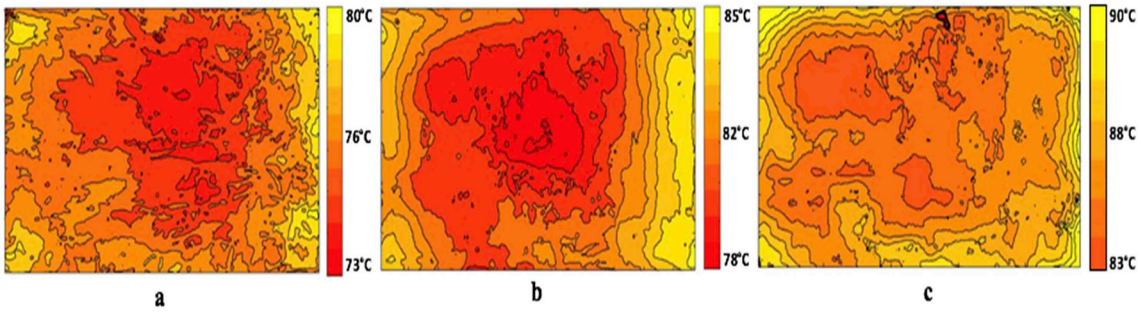

Fig. 6. Temperature distribution in the middle layer of RF heated corn flour. The samples were heated to reach 75 (a), 80 (b), and 85 °C (c).

3.3. Microbial survival after RF heating with or without holding and subsequent freezing

This study was conducted to study how combinations of RF heating, holding and freezing storage enhance the inactivation effect on both *S.* Enteritidis PT30 and *E. faecium* in corn flour. Inactivation effect of RF heating on various foodborne pathogens and their potential surrogates have been reported for different low-moisture foods such as wheat flour and almond kernel (Liu et al., 2018; Villa-Rojas et al., 2013, 2017), black and red peppers (Jeong & Kang, 2014). In this study, the cold spot

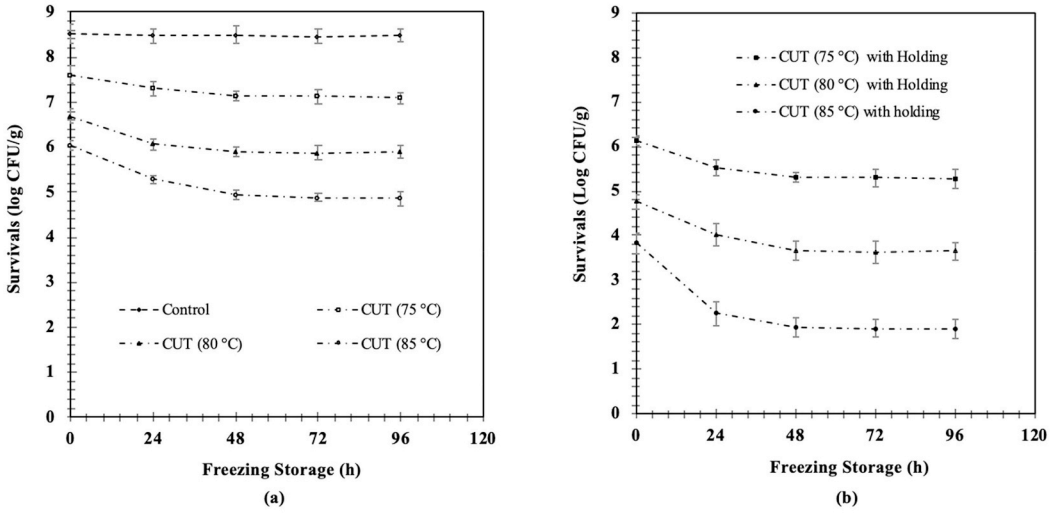

Fig. 7. Effect of freezing storage at −20 °C on survivals of *S.* PT30 in untreated and RF treated corn flour. The graph (a) indicates samples were frozen stored right after come-up-time (CUT) to reach target temperatures (75, 80 and 85 °C), and the graph (b) indicates samples were held for 10 min in the RF system before freezing storage.

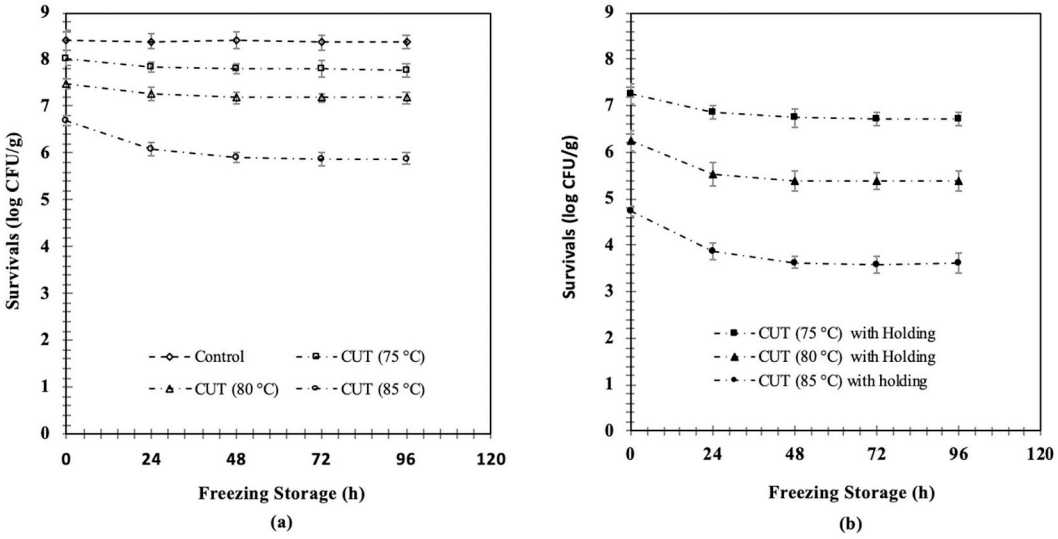

Fig. 8. Effect of freezing storage at −20 °C on survivals of *E. faecium* in untreated and RF treated corn flour. The graph (a) indicates samples were frozen stored right after come-up-time (CUT) to reach target temperatures (75, 80 and 85 °C), and the graph (b) indicates samples were held for 10 min in the RF system before freezing storage.

area in the middle layer of the PEI container was used as the least heating zone to validate the RF heating process where packaged inoculated corn flour was loaded. RF heating during CUT to reach 75, 80, and 85 °C resulted in 0.91 ± 0.07, 1.84 ± 0.13, and 2.49 ± 0.11 log CFU/g reductions in *S*. Enteritidis PT30 population, and 0.39 ± 0.09, 0.94 ± 0.05, and 1.72 ± 0.18 log CFU/g reduction in *E. faecium* population, respectively. Additionally, holding samples for 10 min after CUT, which are 14, 16, and 18 min, for targeted temperature 75, 80, and 85 °C, respectively, increased the reduction of both microorganisms in the corn flour (Figs. 7–8). As shown in Figs. 7–8, holding inoculated sample bag in the PEI container after reaching 80 °C resulted in additional 1.92 ± 0.17 and 1.24 ± 0.09 log CFU/g reductions for *S*. Enteritidis PT30 and *E. faecium*, respectively. The enhancing effect of holding on the inactivation of both *S*. Enteritidis PT30 and *E. faecium* in corn flour after RF heating shows a similar trend with wheat flour (Liu et al., 2018; Xu et al., 2018) and shell almond (California, 2007).

As the bactericidal action of RF heating with 10 min holding for 80 °C resulting in 3.76 ± 0.24 log CFU/g reduction of *S*. Enteritidis PT30 in corn flour was not enough to achieve 5 log (5D) reduction to be considered a reliable microbial validation of RF heating in corn flour. Therefore, to enhance the microbial inactivation of *S*. Enteritidis PT30 in corn flour, it is necessary to consider the hurdle effect of RF heating with other technologies. In this study, freezing treatment at −20 °C for 96 h was applied following RF heating. The reason to apply freezing storage treatment to RF heated samples was to inactivate sub-lethally injured microorganisms in corn flour. Mattick (2016) reported that the inactivation of bacteria in foods with high-a_w (close to 1) is based on protein denaturation, which requires sufficient water to hydrate protein. On the other hand, microbial inactivation in foods with low-a_w (less than 0.6) is mostly associated with injured cell membrane through lipid fluidization because sufficient water is not presented for protein denaturation in low-moisture foods (Rychlik & Barrow, 2005). Additionally, Russell (2002) reported that for healthy cells, lower temperatures promotes redeeming changes in fatty acid composition which increases the fluidity of cell membrane to acclimate freezing temperature conditions. However, RF heating can discompose the membrane stability, and as an effect, lipid change may not be effective in acclimating damaged bacterial cell to growth at cold temperatures. Bacteria in low moisture food as subjected to RF heating might be sublethally injured especially in the cold spot areas (Fig. 6). When environmental conditions such as water, temperature or nutrient permit, microorganisms with sublethally injured cell membrane can recover and become metabolically active. Therefore, RF heating with or without holding combined with freezing storage treatment may enhance the inactivation level of *S*. Enteritidis PT30 and *E. faecium* in corn flour through inhibition of repair process and recovery of sublethally injured microorganisms.

The effect of various treatment combinations on survival is presented in Figs. 7–8 for both microorganisms. As represented in Figs. 7–8, freezing storage treatment (−20 °C) for 24 h caused reduced survival of *S*. Enteritidis PT30 and *E. faecium* populations in RF heated sample with or without holding; however, no further decrease in survival was observed with longer than 48 h holding in freezing temperature. Therefore, extended cold storage time beyond the 24 h is not necessary. In addition, no significant change ($p < 0.05$) in the initial load of both un-treated *S*. Enteritidis PT30 and *E. faecium* (approximately 8.5 ± 0.23 log CFU/g) was observed in corn flour during freezing storage treatment (See control in Figs. 7–8). The results evidenced that freezing storage treatment following RF heating with holding significantly enhanced the inactivation efficiency of RF heating. The combination of RF heating at 85 °C with holding and freezing storage treatment for 48 h resulted in a 6.59 log CFU/g reduction in *S*. Enteritidis PT30 population and 4.79 log CFU/g reduction in *E. faecium* population in corn flour. The effect of freezing storage treatment on the survival of microorganisms has also been reported for green tea infusions as treated by the pulsed electric field (Zhao et al., 2009). We speculated that RF heating can generate sublethally injured microorganisms which were not able to recover or repair themselves at freezing temperature, while un-treated healthy cells could survive during cold storage at −20 °C for 96 h. Thus, RF treatments combined with freezing storage treatment successfully enhanced microbial inactivation in a synergistic manner, possibly through inhibition of repair process and recovery of sublethally injured microorganisms. Additional studies are needed to further explore optimum storage time for industrial applications and to understand the survival mechanism of sublethally injured cells in the course of RF heating and freezing storage.

4. Conclusions

This study indicates that *E. faecium* can be used as a surrogate of *Salmonella* to validate RF heating for pasteurization of corn flour due to its higher thermal resistances than *S*. Enteritidis PT30. A combination of RF heating with holding and subsequent freezing storage treatment enhanced the inactivation level of pathogenic microorganisms in corn flour. The results of this study can be used to develop RF heating in combination with freezing as an alternative approach for pasteurization of other low-moisture foods. However, further studies are needed to explore optimum conditions, including holding and freezing time, in order to achieve safety requirements while maintaining food quality in low-moisture foods.

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