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Radiofrequency pasteurization against *Salmonella* and *Listeria monocytogenes* in cocoa powder

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ABSTRACT

In this study, thermal processes were developed for cocoa powder against *Salmonella* and *Listeria monocytogenes* in a pilot Radiofrequency (RF) unit and validated using their respective surrogates *Enterococcus faecium* NRRL B-2354 and *Listeria innocua*. For 1.0 kg cocoa powder samples, RF heating to 90 °C provided 1.74–1.84 log reduction of *E. faecium*. A 48 min holding post-RF heating provided an additional 3.65 and 2.24 $\log_{10}$ CFU/g reduction of *E. faecium* for insulated and non-insulated samples, respectively. Compared to *E. faecium*, *L. innocua* was less resistant to RF heating in a_w 0.45 cocoa powder; RF heating to 75 °C coupled with 24 min insulated holding resulted in an additional 5.64 $\log_{10}$ CFU/g reduction. These data suggested that *E. faecium* was a conservative surrogate strain for controlling *Salmonella* and *L. monocytogenes* during RF processing of cocoa powder. To achieve maximal microbial reduction and improve energy efficiency, an insulated holding following RF heating is highly recommended. According to the predictive line obtained from the Bigelow model, a 7.6 min RF heating plus 48 min insulated holding attained a 5-log reduction of *E. faecium* without a negative impact on the color of cocoa powder.

1. Introduction

Cocoa powder and other low-moisture foods are generally regarded as microbiologically safe as low water activity (a_w) environment does not support the growth of foodborne pathogens. However, foodborne pathogens including *Listeria monocytogenes* and *Salmonella* can survive in cocoa powder containing low-moisture foods for long durations. For example, *L. monocytogenes* was able to survive in chocolate-peanut spread (a_w 0.33 and 0.65) over 6 months at 20 °C (Kenney & Beuchat, 2004). *Salmonella* survived in chocolate filling for up to 6 months (Beuchat & Mann, 2015) and in chocolate for up to one year (Tamminga, Beumer, Kampelmacher, & Van Leusden, 1976). In unsweetened cocoa powder, *Salmonella* (Tsai, Ballom, et al., 2019) and *L. monocytogenes* (Tsai, Taylor, et al., 2019) remained detectable after 48-week of storage at 20 °C. Furthermore, upon adaptation to a low a_w food environment, *Salmonella* and *L. monocytogenes* exhibit enhanced heat resistance in low a_w foods such as cocoa powder (Tsai, Ballom, et al., 2019; Tsai, Taylor, et al., 2019), wheat flour (Smith, Hildebrandt, Casulli, Dolan, & Marks, 2016; Taylor, Tsai, Rasco, Tang, & Zhu, 2018), almond meals (Limcharoenchat, James, & Marks, 2019; Villa-Rojas et al., 2013; Zhu, Song, Shen, & Tang, 2020), milk powders (Ballom, Tsai, Taylor, Tang, & Zhu, 2020; Wei, Lau, Chaves, et al., 2020) and others (Rachon, Penaloza, & Gibbs, 2016), posing vexing challenges to the safety of low-moisture foods. Chocolate products have been implicated in outbreaks of salmonellosis (D'Aoust et al., 1975; da Silva do Nascimento et al., 2010; Werber et al., 2005); *L. monocytogenes* was recently associated with a recall of cocoa containing bars (Consumer Affairs, 2020). These underscore a need for the development of effective process controls against *Salmonella* and *L. monocytogenes* in cocoa powder products.

Radiofrequency (RF) pasteurization is a volumetric heating technology that uses electromagnetic energy at frequencies between 1 and 300 MHz to generate heat within foods (Jiao, Tang, & Wang, 2014; Jiao, Tang, Wang, & Koral, 2018; Zhao, Flugstad, Kolbe, Park, & Wells, 2000). Correspondingly, RF heating allows more rapid heating of bulk amounts of food, with deeper penetration and greater uniformity of heating when compared to slow conventional heating due to low thermal conductivity

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in low-moisture foods (Jiao et al., 2018; Zhao et al., 2000). RF heating has been tested for pasteurization of non-fat dry milk (Michael et al., 2014), wheat flour (Liu et al., 2018), red and/or black peppercorn (Hu, Zhao, Hayouka, Wang, & Jiao, 2018; Jeong & Kang, 2014; Wei et al., 2018), corn powder (Ozturk et al., 2019), roasted grain powder (Jeong, Kim, Park, & Kang, 2020), egg white powder (Wei, Lau, Reddy, & Subbiah, 2020), and cumin seeds or cumin powder (Chen, Wei, Irmak, Chaves, & Subbiah, 2019; Ozturk, Kong, & Singh, 2020) to control *Salmonella*. However, the efficacy of RF pasteurization has not been assessed for cocoa powder, nor *L. monocytogenes*.

The Preventive Controls for Human Food (PCHF) Rule under the Food Safety Modernization Act (FSMA) requires the food industry to ascertain the actual efficacy of intervention against the target foodborne pathogens via validation studies (FDA, 2018). However, it is both highly risky and exorbitantly expensive to validate a process by introducing a target pathogen to a food processing facility. *Enterococcus faecium* NRRL B-2354 is recognized as a suitable surrogate for *Salmonella* during RF pasteurization of wheat flour (Liu et al., 2018), corn flour (Ozturk et al., 2019), black peppercorn (Wei et al., 2018), cumin seeds or powder (Chen et al., 2019; Ozturk et al., 2020) and others. *L. innocua*, which is closely related to *L. monocytogenes* phylogenetically, is considered as an ideal surrogate of *L. monocytogenes* for thermal treatments (Fairchild & Foegeding, 1993; Tobin, Lele, Cutter, Anantheswaran, & LaBorde, 2020). The objectives of this study were to 1) verify the suitability of *E. faecium* and *L. innocua* as a surrogate strain for *Salmonella* and *L. monocytogenes* in thermal processing of cocoa powder; 2) establish an RF pasteurization methodology for cocoa powder process in respect to both *Salmonella* and *L. monocytogenes* using verified surrogates in a pilot RF unit; 3) examine the contribution of insulated holding to microbial lethality following RF heating; and 4) evaluate Bigelow model for microbial survival prediction in cocoa powder subjected to RF pasteurization.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Salmonella Enteritidis PT30, involved in almond outbreaks, was obtained from Dr. Linda J. Harris (University of California, Davis). *Salmonella* Tennessee K4643 and *Salmonella* Agona 447967, implicated in peanut butter and cereal product outbreaks, respectively, were kindly gifted by Dr. Nathan M. Anderson (USFDA, Greater Chicago, Illinois). The *L. monocytogenes* strains (NRRL B-57618, NRRL B-33053, and NRRL B-33466), *E. faecium* NRRL B-2354, and *L. innocua* (NRRL B-33197) were obtained from the culture collection of the National Center for Agricultural Utilization Research (NRRL), USDA Agricultural Research Service (Peoria, IL). *L. innocua* (TVS470 and TVS 471) were kindly gifted by Dr. Trevor V. Suslow (University of California, Davis). Strains were stored at −80 °C in trypticase soy broth that contained 0.6% yeast extract (TSBYE, Becton, Dickinson and Company, Sparks, MD) supplemented with 20% (v/v) glycerol. Each strain was activated twice by consecutive culturing, then plating on 100 × 15 mm tryptic soy agar with 0.6% yeast extract (TSAYE) and incubated at 35 ± 2 °C for 24 h to form a bacterial lawn. Bacterial lawns collected from TSAYE plates were washed twice using sterile phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 8000×*g*, 4 °C for 15 min (Centrifuge 5810 R, Eppendorf North America, Hauppage, NY). Thereafter, the resulting pellets were resuspended in sterile PBS to achieve 10^{10-11} CFU/mL. Three strain cultures were combined in equal volumes to obtain the 3-strain cocktail.

2.2. Cocoa powder inoculation and equilibration

Natural unsweetened 100% cocoa powder (The Hershey Company, USA), purchased from a local grocery store, contains 57.36 ± 0.68% carbohydrate, 25.91 ± 0.09% protein, 6.50 ± 0.48% fat, 6.22 ± 0.03% ash and 4.00 ± 0.62% moisture (Tsai, Taylor, et al., 2019). The receiving cocoa powder was free of *Salmonella* and *L. monocytogenes* and had a background microflora of 2.92 ± 0.03 Log_{10} CFU/g by total plate count enumeration. Inoculation of cocoa powder and equilibration to low a_w were conducted as described previously (Tsai, Ballom, et al., 2019; Tsai, Taylor, et al., 2019). Briefly, 40 g of cocoa powder was inoculated with 400 μL of 3-strain *Salmonella, L. monocytogenes* or *L. innocua* cocktail, or *E. faecium* NRRL B-2354 strain to achieve ~10^{8-9} CFU/g cocoa powder. The inoculated samples were thoroughly mixed and divided equally into two 150 mm Petri dishes in an even layer, placed in a a_w-equilibration chamber (custom designed at Michigan State University) set at the target a_w of 0.45 ± 0.02, and equilibrated for 4-days at room temperature (~22 °C) before use. Water activity was measured using a water activity meter (Aqualab Series 3, Decagon Devices, Inc., Pullman, WA). For each inoculated batch, three sub-samples, 1 g each, from the inoculated cocoa powder were enumerated immediately following inoculation and again after the subsequent equilibration to verify the uniformity of inoculum distribution and initial inoculation level.

2.3. Isothermal inactivation and enumeration of survival bacteria

Following equilibration, the inoculated and a_w equilibrated cocoa powder was loaded into thermal death time (TDT) aluminum test cells designed by Washington State University (Chung, Birla, & Tang, 2008). The TDT cells loaded with samples were subjected to thermal treatment using an ethylene glycol bath (Isotemp® 125, Fischer Scientific, Pittsburgh, PA) preset at a temperature between 70 and 80 °C for *L. innocua*, 75 °C for *L. monocytogenes*, and 90 °C for *Salmonella* and *E. faecium*, respectively. The come-up time (CUT), the time necessary for the sample to reach a target temperature, for the TDT cell was determined by employing a type T thermocouple placed at the geometric center of the TDT cells. For each heat treatment, triplicate TDT cells were sampled at CUT and four additional timepoints for microbial survival analysis. TDT cells removed at each sampling time were immediately cooled in an ice bath for 120 s. Each thermal inactivation was conducted three times independently; there were triplicate samples for each temperature and time combination within an independent repeat.

Following the thermal treatments, the content of each TDT cell was transferred to a Whirl-Pak® bag (Nasco, Ft. Atkinson, WI), diluted with PBS at 1:10, and stomached for 2 min. The resulting bacterial suspension was serial diluted, and appropriate dilutions were plated in duplicates on TSAYE plates, and then incubated at 35 ± 2 °C for 48 h.

2.4. RF pasteurization of inoculated cocoa powder

2.4.1. RF sample preparation and temperature measurements

RF heating was performed as described previously (Liu et al., 2018) using a 27.12-MHz, 6-kW pilot-scale RF unit (COMBI 6-S, Strayfield International, Wokingham, UK). A rectangular container made from polyetherimide (PEI), which had inner dimensions of 25 cm × 15 cm × 7 cm and with double lids, was used to enclose 1.0 kg of cocoa powder. The container was placed between two parallel electrodes, 11.5 cm apart, in the RF unit to obtain consistent heating (Fig. 1A). The top lid of the PEI container was overlaid above the bottom lid to minimize heat loss during the process. On the front side of the PEI container, three evenly spaced holes were predrilled at an even distance to allow three fiber-optic sensors (30-mm-long polyimide tubing, Fiso Technologies Inc., Quebec, Canada) to be placed in the different layers of cocoa powder sample. One sensor was located at the geometric center of the PEI container, where the cold spot of RF heating is located (Liu et al., 2018; Tiwari, Wanga, Tang, & Birla, 2011) (Fig. 1B). The temperature profile during RF processing was recorded using three fiber optic sensors that were connected to a multi-channel temperature-time data recorder (Opsens Inc, TempSens, Quebec, Canada) (Jiao, Shi, Tang, Li, & Wang, 2015).

Inoculated cocoa powder samples were prepared and equilibrated as described earlier. Following adaptation to a_w 0.45 ± 0.02, 1-g aliquots of

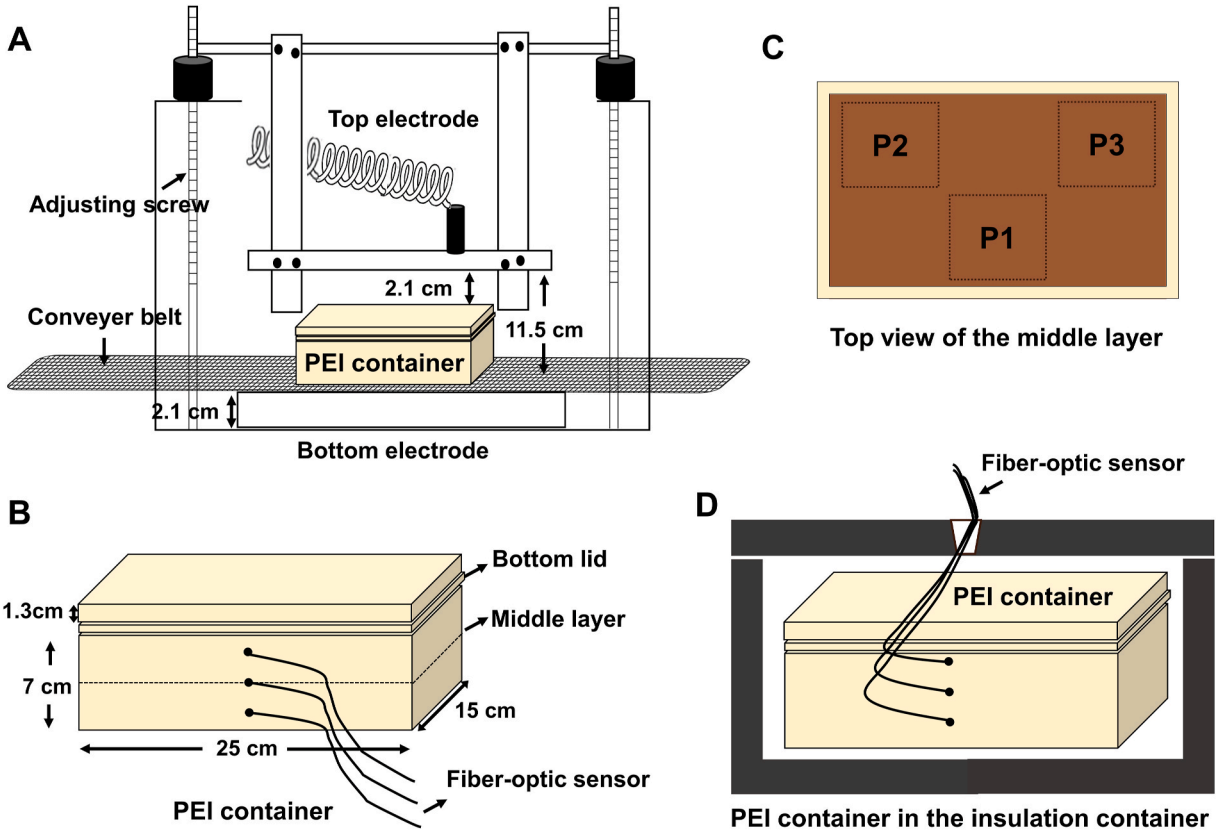


Fig. 1. Schematic diagram of 27.12 MHz, 6 kW radiofrequency heating system with a polyetherimide (PEI) container loaded with inoculated cocoa powder placed in an insulation container. A. Radiofrequency heating cavity with PEI container; the electrode gap between two parallel electrodes set at 11.5 cm; B. PEI container with three fiber-optic sensors; C. Top view of the middle layer showing three locations for inoculated packs, P1–P3 represents three sealed inoculated cocoa powder packs loaded to the master cocoa powder matrix for RF process; D. The PEI container in the insulated container during holding.

cocoa powder inoculated with *L. innocua* or *E. faecium* were sealed in a polyethylene bag. The PEI container was first half filled with uninoculated a_w 0.45 cocoa powder, three inoculated cocoa powder packs were placed on the half-filled PEI container (the middle layer, Fig. 1C), and then the container was filled with more uninoculated cocoa powder till full (~1.0 kg). The surface of the loaded container was flattened using a straight edge before closing the container.

The container loaded with cocoa powder was heated by RF from ~22 °C till the temperature of the sensor at the geometric center reached 75 °C or 90 °C for *L. innocua*- or *E. faecium*-inoculated samples. For the non-insulated RF process, the PEI box was cooled down in the RF oven after the RF system was turned off. For the insulated RF pasteurization, the PEI box was immediately transferred from the RF cavity to the insulated container once the temperature reached 75 °C or 90 °C, then held in the insulated container (Fig. 1D) for up to 24 min with *L. innocua* or up to 48 min with *E. faecium* inoculated cocoa samples.

The previous studies showed that *L. monocytogenes* is less resistant during the isothermal treatment of a_w 0.45 cocoa powder compared to *Salmonella.* D-values of *Salmonella* at 70–80 °C were 3.9–5.0 times those of *L. monocytogenes* (Tsai, Ballom, et al., 2019; Tsai, Taylor, et al., 2019). Thus, the *L. innocua* inoculated cocoa powder samples were RF heated to 75 °C while the *E. faecium* inoculated samples were heated to 90 °C.

2.4.2. Sampling and survival determination of RF processed samples

The bacterial survival in each RF process was determined by designating four to five data points that include initial population levels, CUT, and during 3–4 holding times. The CUT was the time point immediately after the desired temperature was achieved using RF heating, i.e., the time needed for the fiber optic probe at the central temperature reached the target temperature. Triplicate inoculated cocoa powder packs were removed at each sampling time and immediately cooled in an ice bath for 120 s to halt any inactivation that may occur due to the residual heat. The survivors of inoculated packs at each sampling point were enumerated as described in the isothermal treatment. For each non-insulated and insulated RF process, each experiment was independently repeated three times.

2.5. D- and z-value analyses in isothermal inactivation

The first-order kinetic/log-linear model was applied for isothermal inactivation of bacteria using TDT cells (Peleg, 2006), and D-value was obtained:

$$\text{Log}\ (N_t/N_0) = -t/D \quad (1)$$

where N_0 is the bacterial population at the CUT, N_t is the bacterial population at time (t), t is the time of the isothermal treatment (min) after the CUT. D is D-value, the time in min at a specific temperature required to reduce the bacterial population by 1- log, which is estimated from the thermal inactivation curve using log-linear regression analysis.

The z-values in °C were determined from the regression of log D-value versus temperature and were calculated as $z = \text{slope}^{-1}$ for the linear trend lines. It was obtained from

$$Z = -\frac{T - T_{ref}}{Log\ D_T - Log\ D_{Tref}} \quad (2)$$

where T is initial temperature, and T_{ref} is reference temperature. D_t = D-value at the initial temperature, D_{Tref} = D-value at the reference temperature, which is D_{90}- and D_{75}-values for *E. faecium* and *L. innocua*, respectively. The z-value of *L. innocua* was generated from isothermal heating using TDT cells in this study. The z-values of *Salmonella, E.*

faecium, and *L. monocytogenes* were published in our previous works using the same model prediction (Tsai, Ballom, et al., 2019; Tsai, Taylor, et al., 2019).

2.6. Model prediction of survivors during RF heating

Bigelow model (Eq. (3)) was used to predict bacterial reduction during RF pasteurization process.

$$\text{Log}\ \frac{N_0}{N_t} = \frac{1}{D_{Tref}} \int_0^t 10^{\frac{T(t)-Tref}{Z}} dt \quad (3)$$

where N_t and N_0 are the survival populations of the target microorganism at time t and 0. T(t) is the measured real-time temperature. T_{ref} is the reference temperature, which is 90 °C and 75 °C for *E. faecium* and *L. innocua*, respectively. The values of D_{Tref} and z were thermal resistance parameters of bacteria in a_w 0.45 cocoa powder generated from the isothermal inactivation. This model has been used for conservative prediction of bacterial reductions during RF pasteurization of other La_wF (Liu et al., 2018; Ozturk, Kong, Singh, Kuzy, & Li, 2017).

2.7. Statistical analyses

Data were analyzed by the generalized linear model one-way analysis of variance (ANOVA) (SAS, Cary, NC). The mean difference between the D-values of the target pathogen and its presumable surrogate was discerned using the paired *t*-test. *P*-values of less than or equal to 0.05 were considered significant. Each thermal inactivation test was repeated three times independently. Microbial thermal inactivation data were presented as mean values ± standard error mean (SEM) averaged from three independent experiments with 3 replicates per treatment within each independent test, n = 9. Mean difference of color measurement L*, a*, and b* values among different heat treatment times were discerned by the LSD multiple-comparison test.

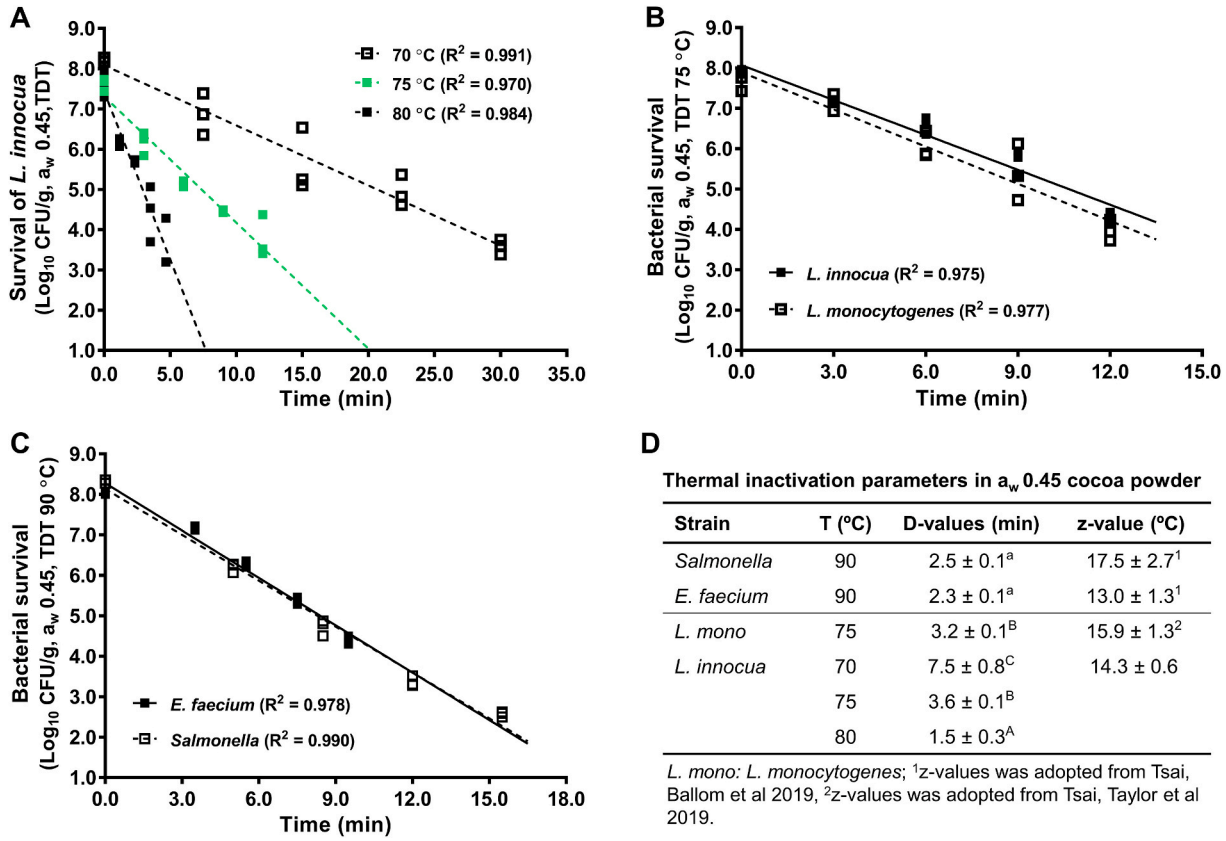
3. Results

3.1. D- and z-values of Salmonella, L. monocytogenes and their surrogates in a_w 0.45 cocoa powder obtained with TDT cells

At each inactivation temperature, the inactivation curves of *L. innocua* in a_w 0.45 cocoa powder showed a log-linear trend (Fig. 2A). The D_{75}-and z-values of *L. innocua* in a_w 0.45 cocoa powder were, 3.6 ± 0.1 min and 14.3 ± 0.6 °C (Fig. 2A and D). The thermal resistance of *L. innocua* was not different ($P > 0.05$) from *L. monocytogenes* in a_w 0.45 cocoa powder at 75 °C. The D_{75}-values for *L. innocua* and *L. monocytogenes* were 3.6 ± 0.1 and 3.2 ± 0.1 min, respectively (Fig. 2B and D). Similarly, the thermal resistance of *E. faecium* did not differ ($P > 0.05$) from that of *Salmonella* with D_{90}-values of 2.3 ± 0.1 and 2.5 ± 0.1 min, respectively (Fig. 2C and D). Data showed that *E. faecium* and *L. innocua* were appropriate surrogate organisms for *Salmonella* and *L. monocytogenes* during thermal processing of a_w 0.45 cocoa powder at the selected temperatures, thus used for subsequent RF pasteurization processing.

3.2. RF pasteurization of E. faecium inoculated cocoa powder

An inoculated cocoa powder sample was first subjected to RF heating



D

Thermal inactivation parameters in a_w 0.45 cocoa powder

Strain	T (°C)	D-values (min)	z-value (°C)
Salmonella	90	2.5 ± 0.1[a]	17.5 ± 2.7[1]
E. faecium	90	2.3 ± 0.1[a]	13.0 ± 1.3[1]
L. mono	75	3.2 ± 0.1[B]	15.9 ± 1.3[2]
L. innocua	70	7.5 ± 0.8[C]	14.3 ± 0.6
	75	3.6 ± 0.1[B]	
	80	1.5 ± 0.3[A]	

L. mono: L. monocytogenes; [1]z-values was adopted from Tsai, Ballom et al 2019, [2]z-values was adopted from Tsai, Taylor et al 2019.

Fig. 2. The representative isothermal inactivation curves and parameters of *Salmonella* and *L. monocytogenes* and their surrogates *E. faecium* and *L. innocua* in a_w 0.45 cocoa powder at the selected temperatures. A. *L. innocua* inactivation curves at 70–80 °C. B. *L. monocytogenes* and *L. innocua* inactivation curves at 75 °C. C. *Salmonella* and *E. faecium* inactivation curves at 90 °C. D. D- and z-values obtained using thermal death time (TDT) cells. [a]mean values between *Salmonella* and *E. faecium* did not differ significantly ($P \leq 0.05$). [A–C]mean values of *Listeria* within a column without a common letter differ significantly ($P \leq 0.05$). Experiments were repeated independently three times. Mean ± SEM, averaged from three independent studies, n = 9. a_w: water activity measured at 22 °C.

to reach the target temperature followed by holding periods. The time required for the center of the 1.0 kg cocoa powder in the PEI container to reach 90 °C (i.e. CUT for 90 °C RF heating) was 7.6 ± 0.2 min. The average heating rate of 9.0 ± 0.3 °C/min was achieved for the 90 °C RF heating of the *E. faecium*-inoculated sample. RF heating during CUT resulted in a 1.74–1.84 log reduction of *E. faecium* in inoculated cocoa powder packs (Table 1).

Typical temperature profiles of the cocoa powder samples subjected to RF heating to 90 °C and holding for 48 min in RF unit or an insulated container after turning off RF are shown in Fig. 3. Transferring the PEI box after RF heating to an insulated chamber slowed down the temperature drop during the holding. By the end of holding (48 min holding in addition to 7.6 min CUT), the temperature of the cocoa powder fell from 90 °C to 61.9 ± 0.6 and 74.2 ± 1.1 °C, respectively, for non-insulated and insulated holding processes (Table 1). Correspondingly, *E. faecium* in cocoa samples showed a significantly greater log reduction in the insulated holding compared to the non-insulated holding samples (Fig. 3 and Table 1). There was 3.98 ± 0.21 and 5.49 ± 0.27 $\log_{10}$ CFU/g reduction of *E. faecium* in non-insulated and insulated samples, respectively (Table 1).

The predicted *E. faecium* survival lines in both non-insulated and insulated holding along with experimental survival data with respect to RF processing are shown in Fig. 3. For the RF heating coupled with an insulated holding process, more reductions were observed in the experimental data than those obtained from the predicted curve (Table 1 and Fig. 3). The predicted reduction of *E. faecium* for insulated samples was 4.60 ± 0.41 $\log_{10}$ CFU/g reduction compared to 5.49 ± 0.27 $\log_{10}$ CFU/g reduction enumerated after RF heating plus 48 min insulated holding (Table 1). The survival data predicted with the Bigelow model were in good agreement with the experimental survival of *E. faecium* observed in both insulated and non-insulated cocoa powder samples for all holding times with correlation coefficient $R^2 = 0.907$ and RMSE of 0.40 $\log_{10}$ CFU/g.

3.3. RF processing *L. innocua* inoculated cocoa powder followed by insulated holding

For RF treatment of *L. innocua*-inoculated cocoa powder, the time required for the center of 1.0 kg a_w 0.45 cocoa powder to reach the target temperature (i.e. CUT at 75 °C) was 6.2 ± 0.0 min with a heating rate of 8.6 ± 0.1 °C/min. During the insulated holding, the temperature of cocoa powder samples remained relatively stable, and there were 3.5 ± 0.9 °C and 7.7 ± 1.0 °C drop at 6 min and 18 min, respectively (Table 2). Correspondingly, there were 1.15 ± 0.19, 3.42 ± 0.27, 5.05 ± 0.03 $\log_{10}$ CFU/g reduction of *L. innocua* in a_w 0.45 cocoa powder at CUT and CUT plus 6 min and 18 min holding, respectively (Table 2).

The overall predicted *L. innocua* survival curve was similar to that of experimental survival data for RF processing, though smaller reductions were observed in the predicted curve (Fig. 4). The survival data predicted with the Bigelow model were in good agreement with

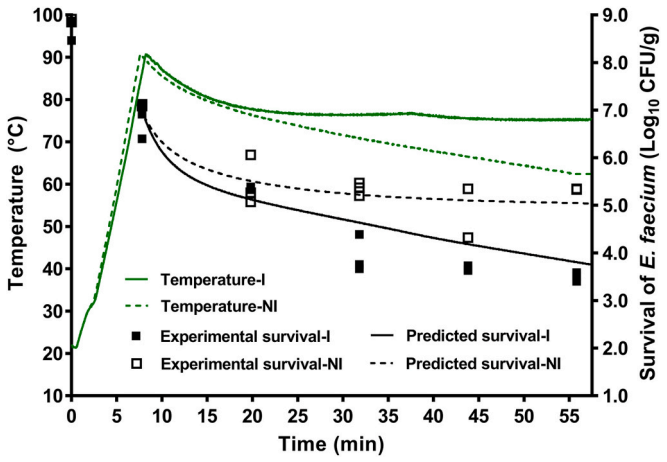


Fig. 3. Temperature profile, experimental and predicted survival of *E. faecium* NRRL-2354 in a_w 0.45 cocoa powder subjected to radiofrequency (RF) heating at 90 °C followed with up to 48 min holding with or without insulation. Temperature–I: Temperature profile during RF process with insulation (green solid line); Temperature-NI: Temperature profile during RF process without insulation (green dashed line). Experimental survival-I: Survival of *E. faecium* analyzed by enumeration during RF processing with insulation (filled black square). Experimental survival-NI: Survival of *E. faecium* analyzed by enumeration during RF processing without insulation (open black square). Predicted survival-I: Predicted survival of *E. faecium* during insulated holding after RF heating (black solid line) using Bigelow model; Predicted survival-NI: Predicted survival of *E. faecium* during non-insulated holding after RF heating (black dashed line) using Bigelow model. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Experimental and predicted reduction of *L. innocua* in cocoa powder during insulated radiofrequency heating process.

Holding time	0 min[a]	6 min	12 min	18 min	24 min
Temperature (°C)	75.2 ± 0.1	71.7 ± 0.9	68.8 ± 1.0	67.5 ± 1.0	67.6 ± 0.0
Experimental	1.15 ± 0.19	3.42 ± 0.27	4.47 ± 0.00	5.05 ± 0.03	5.64 ± 0.11
Predicted	1.15 ± 0.19	2.58 ± 0.11	3.35 ± 0.06	3.91 ± 0.10	4.60 ± 0.00

[a] Time at CUT was set as 0 min. Experimental: Reduction of *L. innocua* analyzed by enumeration, $\log_{10}$ CFU/g. Predicted: Reduction of *L. innocua* predicted using Bigelow model and its D_{75}-value and z-value obtained from isothermal heating, $\log_{10}$ CFU/g. The predicted bacterial reduction at the CUT was assumed to be equal to the experimental result. Mean ± SEM, averaged from three independent studies. *L. innocua* inoculated cocoa powder was equilibrated to a_w 0.45 at 22 °C.

Table 1
Experimental and predicted reduction of *E. faecium* in cocoa powder during radiofrequency heating with insulated or non-insulated holding.

Holding time	Non-insulated holding			Insulated holding		
	T (°C)	Log Reduction		T (°C)	Log Reduction	
		Experimental	Predicted		Experimental	Predicted
0 min[a]	90.2 ± 0.0	1.74 ± 0.03	1.74 ± 0.03	90.2 ± 0.0	1.84 ± 0.05	1.84 ± 0.05
12 min	76.0 ± 0.6	3.08 ± 0.26	3.25 ± 0.09	76.9 ± 0.7	3.19 ± 0.16	3.52 ± 0.13
24 min	70.1 ± 0.9	3.21 ± 0.32	3.50 ± 0.12	74.7 ± 1.8	4.46 ± 0.01	3.92 ± 0.22
36 min	65.7 ± 0.9	4.20 ± 0.15	3.61 ± 0.14	74.1 ± 1.4	5.21 ± 0.17	4.27 ± 0.33
48 min	61.9 ± 0.6	3.98 ± 0.21	3.66 ± 0.15	74.2 ± 1.1	5.49 ± 0.27	4.60 ± 0.41

[a] Time at CUT was set as 0 min. Experimental: Reduction of *E. faecium* analyzed by enumeration, $\log_{10}$ CFU/g. Predicted: Reduction of *E. faecium* predicted using Bigelow model and its D_{90}-value and z-value obtained from isothermal heating, $\log_{10}$ CFU/g. The predicted bacterial reduction at the CUT was assumed to be equal to the experimental result. Mean ± SEM, averaged from two independent studies. *E. faecium* inoculated cocoa powder was equilibrated to a_w 0.45 at 22 °C.

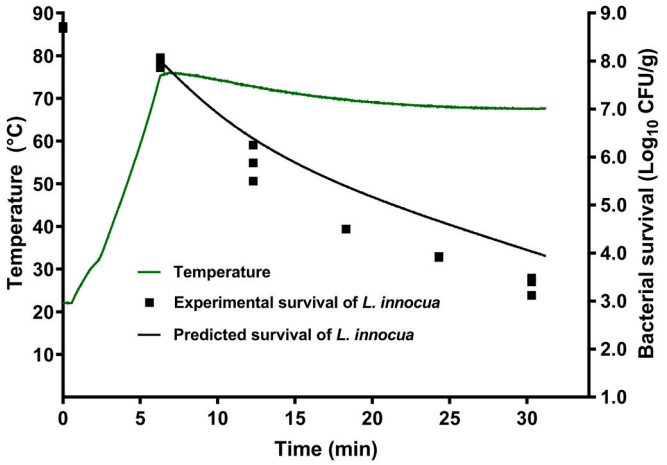


Fig. 4. Temperature profile, experimental and predicted survival of *Listeria* in a_w 0.45 cocoa powder subjected to radiofrequency (RF) heating at 75 °C followed by insulated holding for up to 24 min. Solid green line: temperature profile during RF process. Black-filled square: Experimental survival of *L. innocua*. Black solid line: Predicted survival of *L. innocua* modeled using Bigelow model. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

experimental survivors of *L. innocua* during holding with correlation coefficient $R^2 = 0.961$. Reduction of *L. innocua* per the predicted model was 2.58 ± 0.11 and 3.91 ± 0.10 $\log_{10}$ CFU/g reduction, respectively, 6 min and 18 min after RF heating (Table 2).

3.4. Effect of RF processing on color of cocoa powder

The color of cocoa powder subjected to 90 °C RF heating followed by up to 48 min of insulated heating was analyzed, and no significant change was observed (Table 3, $P > 0.05$).

4. Discussion

4.1. Surrogate strains for Salmonella and L. monocytogenes inactivation in cocoa powder

An appropriate surrogate strain is interpreted as a non-pathogenic strain that has a similar or higher resistance to a particular treatment as its target pathogen (Busta et al., 2003). Ideally, the surrogate strain should have slightly higher resistance than the target pathogen without exhibiting highly excessive resistance (Friedly et al., 2008; Gurtler, Rivera, Zhang, & Geveke, 2010). Based on the isothermal treatment data, for 0.45 a_w cocoa powder, the D_{90}-value of *E. faecium* was not significantly different from that of *Salmonella*, indicating *E. faecium* is a suitable surrogate for thermal inactivation at 90 °C. *E. faecium* was also shown as an appropriate surrogate strain for microbial inactivation against *Salmonella* in other La_wF such as wheat flour (Liu et al., 2018), corn flour (Ozturk et al., 2019), and black peppercorn (Wei et al., 2018). Similarly, the D_{75}-value of *L. innocua* was not significantly different from that of *L. monocytogenes*. This agrees with previous findings (Fairchild & Foegeding, 1993; Tobin et al., 2020) and confirmed that *L. innocua* is an appropriate surrogate strain for *L. monocytogenes* in cocoa powder thermal processing.

Table 3
Color parameters of cocoa powder treated with or without RF.

Treatment	Lightness (L*)	Green/red (a*)	Yellow/blue (b*)
Control	41.31 ± 0.97[a]	13.11 ± 0.23[a]	22.29 ± 0.28[a]
CUT + 24 min	38.49 ± 0.13[a]	13.21 ± 0.09[a]	21.92 ± 0.14[a]
CUT + 48 min	38.63 ± 0.37[a]	13.07 ± 0.07[a]	21.84 ± 0.10[a]

Non-inoculated cocoa powders were subjected to radiofrequency (RF) heating to 90 °C then holding with insulation for 24 min (CUT + 24 min) to 48 min (CUT + 48 min) or without RF heating (Control). Means within a column with no common letter differ significantly at $P < 0.05$. Mean ± SEM, n = 3.

4.2. The inoculated packs and their placements during RF heating

In this study, a bulk cocoa powder loaded with small size inoculated packs was subjected for RF heating. The temperature of bulk dry foods under RF heating was reported to be the highest in the middle layer when compared to the top and bottom layers (Ozturk et al., 2017; Tiwari et al., 2011; Xu, Yang, Jin, Barnett, & Tang, 2020), but the microbial reduction in the middle layer was not different from the top/bottom layers during RF pasteurization of wheat flour (Xu et al., 2020). Correspondingly, in our study, three inoculated packs were embedded into the middle layer of bulk cocoa powder. These inoculated packs were transparent to RF waves and did not influence the temperature profile/distribution during RF heating, while it facilitated the inoculated sample preparation and sampling for microbial enumeration. The same approach was used for RF pasteurization of black peppercorn (Wei et al., 2018), corn flour (Ozturk et al., 2019), egg white powder (Wei, Lau, Reddy, & Subbiah, 2020), and wheat flour (Liu et al., 2018).

4.3. RF heating microbial inactivation

The microbial killing pattern of bulk low-moisture foods during RF processing is closely related to heating uniformity and temperature distribution of RF heating, and subsequently, the holding condition. The fast heating rate of RF pasteurization is associated with shorter processing times but resulted in less uniformity in terms of temperature and microbial inactivation (Ozturk et al., 2017; Xu et al., 2020). To balance the heating uniformity and throughput, the electrode gap of 11.5 cm corresponding to a median heating rate was used in this study. The uniformity of RF heating was also influenced by food composition/density and the dielectric properties of material surrounding low-moisture foods (Jiao et al., 2014; Ozturk et al., 2017). The uniformity of RF heating improved as the bulk density of dry food increased but decreased as the moisture content of processed foods increased (Ozturk et al., 2017). Thus, for each RF treatment, a constant weight of cocoa powder was loaded into the PEI boxes, and the inoculated and surrounding bulk cocoa powder was equilibrated to the target a_w before RF processing.

RF heating to reach 75 °C and 90 °C for *E. faecium* and *L. innocua* inoculated sample, respectively, caused a greater microbial inactivation (1.7–1.8 and 1.2 $\log_{10}$ CFU/g reduction for *E. faecium* and *L. innocua*) than those observed during post-RF holding. This phenomenon was also observed in RF pasteurization of wheat flour (Liu et al., 2018; Xu et al., 2020). The holding time post RF heating contributed significantly to microbial lethality of RF processed cocoa powder especially for the insulated holding process. A 48 min of natural cooling (holding without insulation) or insulated holding after RF heating to 90 °C caused an additional 2.24 and 3.65 $\log_{10}$ CFU/g reduction of *E. faecium,* respectively. Similarly, 24 min insulated holding post RF heating to 75 °C resulted in an additional 4.49 $\log_{10}$ CFU/g reduction of *L. innocua*. In agreement, 1-h holding in a hot-air oven following 80 °C RF heating engendered 2.8–3.9 more $\log_{10}$ CFU/g reductions of *E. faecium* in egg white powder (Wei, Lau, Reddy, & Subbiah, 2020). Data suggested that reducing heat loss of RF treated samples during holding had a major impact on microbial lethality of RF processed samples. In the industrial processes, products after RF treatments are commonly cooled down naturally. To improve energy efficiency and to achieve the maximal microbial reduction, during industry RF processing of low-moisture foods include an insulated tunnel, a thermal blanket, or hot air convection system following by RF heating is highly recommended.

Survival of *E. faecium* and *L. innocua* in cocoa powder during RF heating plus insulated holding were in good agreement with the

predicted survival of *E. faecium* and *L. innocua* from the Bigelow model with high correlation coefficient values. For both surrogate strains, the experimental survival enumerated during insulated holding processes was smaller than those predicted survival lines from the Bigelow model, which might be due to a slight temperature difference between the center spot where the temperature sensor is located and the actual temperature of inoculated packs, or the difference in D- and z-values obtained from TDT cells and actual D- and z-values during RF processing. In support of our finding, the reduction of *E. faecium* in wheat flour subjected to 80 °C RF heating followed by natural cooling was larger than that from the Bigelow model prediction (Liu et al., 2018).

4.4. RF processing on color of cocoa powder

RF pasteurization at 90 °C and followed by up to 48 min of insulated holding, which is predicted to have a 5-log reduction of *E. faecium*, had no significant impact on cocoa powder color. This indicated that RF is a viable microbial inactivation method for bulk cocoa powder pasteurization. Similarly, RF heating had no effects on the color parameters of corn flour (Ozturk et al., 2017), powdered red/black pepper (Jeong & Kang, 2014), and roasted grain powder (Jeong et al., 2020). However, RF processing at 75–90 °C caused a significant change in the color parameter in low-heat processed nonfat dry milk (Chen et al., 2013).

5. Conclusion

E. faecium and *L. innocua* were appropriate surrogate strains for controlling *Salmonella* and *L. monocytogenes*, respectively, during RF processing of cocoa powder. Continuous RF-assisted thermal processing provides a viable pasteurization strategy for cocoa powder. Insulated holding post RF heating improved both energy efficiency and efficacy of RF microbial inactivation. A 7.6 min RF heating followed by 48 min insulated holding results in a predicted 4.6-log reduction of *E. faecium*. For *L. innocua* cocoa samples, a 24 min insulated holding post RF heating to 75 °C resulted in a predicted 4.6 log reduction. Bigelow model provides a good prediction to the experimental microbial reduction in cocoa powder processed by pilot-scale RF pasteurization. Data of this study provide practical information to the food industry for microbial validation of cocoa powder as well as other dry foods. The inoculated pack method used in this pilot-scale RF validation provides a convenient and applicable method for commercial bulk RF processing of low-moisture foods.

CRediT authorship contribution statement

Kenneth Ballom: Investigation, Writing – review & editing. **Nitin Dhowlaghar:** Investigation, Formal analysis, Writing – review & editing. **Hsieh-Chin Tsai:** Investigation. **Ren Yang:** Writing – review & editing. **Juming Tang:** Writing – review & editing. **Mei-Jun Zhu:** Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors have no known conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111490.

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