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Thermal inactivation of *Cronobacter sakazakii* ATCC 29544 in powdered infant formula milk using thermostatic radio frequency

Yue Zhang^a, Yongkang Xie^a, Juming Tang^b, Shaojin Wang^{b,c}, Lu Wang^{a,d}, Guangfei Zhu^a, Xingyi Li^a, Yanhong Liu^{a,*}

^a College of Engineering, China Agricultural University, Beijing, 100083, China

^b Department of Biological Systems Engineering, Washington State University, 213 L.J. Smith Hall, Pullman, WA, 99164-6120, USA

^c Northwest A&F University, College of Mechanical and Electronic Engineering, Yangling, Shaanxi, 712100, China

^d University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

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ABSTRACT

A 27.12 MHz thermostatic radio frequency (RF) system was employed to inactivate *Cronobacter sakazakii* in powdered infant formula milk. A proportional-integral-derivative controller achieved constant material temperature during the holding period. Hot air and similar dielectric material assistance were used to improve RF heating uniformity. The results showed that the microbial thermal resistance was reduced by increasing temperature (55–70 °C) and water activity (0.2–0.4 at 25 °C). Combined RF and hot air pasteurization (RF-HA) was more effective than single RF and material assistance due to better temperature uniformity. $D_{70^\circ\text{C}}$ -value (23.3 min) of *Cronobacter sakazakii* at water activity of 0.2 from RF-HA was not significantly ($P > 0.05$) higher than that under hot water treatment (20.8 min), indicating that thermal effect led to microbial inactivation. Flow cytometry and transmission electron microscopy further confirmed that RF had no additional damage to cell membrane. The findings contribute to better understanding of RF pasteurization mechanism.

1. Introduction

Powdered infant formula milk, the only resource of food for infants under 6 months of age who cannot be breastfed, provides the best possible alternative to breast milk (Drapala, Mulvihill, & O'Mahony, 2018). It contains multiple nutritional ingredients such as milk proteins, lactose, vegetable oils, minerals, vitamins, and prebiotics (Nguyen & Fromberg, 2020; Portela et al., 2019). Despite a series of standard thermal treatments for raw milk like sterilization, concentration and spray drying, microbial contamination is still widespread at manufacturing facilities and production environments (Markoska, Huppertz, Grewal, & Vasiljevic, 2019; Pei et al., 2019). *Cronobacter sakazakii* (*C. sakazakii*), an opportunistic pathogen causing severe foodborne diseases, such as meningitis, bacteremia, and necrotizing enterocolitis with mortality rates of up to 80%, is one of the main microorganisms related to PIFM contamination (Akineden, Wittwer, Geister, Plötz, & Usleber, 2020; Chen et al., 2018). This is because *C. sakazakii* can tolerate extreme osmotic and desiccation stresses in PIFM, and the special biofilms enhance resistance of *C. sakazakii* to disinfectants (Henry & Fouladkhah, 2019; Hu et al., 2018). During preparation before feeding, the presence of viable pathogens in PIFM poses

a considerable risk to neonates when conditions are appropriate for multiplication (Pei et al., 2019). Numerous outbreaks and recalls associated with *C. sakazakii* have been reported in multiple countries, such as France, Belgium, Australia, Brazil, India, Spain, and the United States, causing huge economic losses and raising public concerns about the safety of PIFM (Henry & Fouladkhah, 2019). Therefore, it is imperative to develop an efficient pasteurization method for packaged PIFM to eliminate the risk of microbial contamination.

Conventional heating has been used to pasteurize most low-moisture foods, but both the sensory attributes and nutrients deteriorate due to the long processing time resulting from poor thermal conductivity (Ahmad et al., 2019). Emerging thermal treatments such as ohmic and microwave exhibited promising applications for pasteurizing infant formula products (Pires et al., 2020; Portela et al., 2019). Likewise, numerous non-thermal technologies are under development to pasteurize PIFM, such as high pressure processing (Wazed, Ismail, & Farid, 2020), intense pulsed light (Chen et al., 2018), gamma irradiation (Chen, Zhu, Qin, Qiu, & Zhao, 2018), pulsed electric field (Henry & Fouladkhah, 2019), ultrasounds (Chen et al., 2018), and cold plasma treatment (Oh et al., 2015). Among them, ohmic heating, microwave, high pressure processing, pulsed electric field, and ultrasounds are

* Corresponding author. College of Engineering, China Agricultural University, No. 17 Qinghua Donglu, HaiDian District, Beijing, 100083, China.

E-mail address: liuyanhong@cau.edu.cn (Y. Liu).

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commonly used in liquid dairy products (Lin, Subbiah, Chen, Verma, & Liu, 2020). Irradiation is effective, fast and feasible for pasteurizing a variety of packaged foods, but is banned from PIFM. Intense pulsed light and cold plasma cannot be applied to industrial production due to limited penetration depth. Thus, a novel treatment with fast volumetric heating and a large penetration depth is in great need.

Radio frequency (RF), a physical treatment of exposing material to an electromagnetic field with a frequency range of 3 kHz–300 MHz, is an effective method for inactivating pathogens in food (Kou, Li, Hou, Zhang, & Wang, 2018). However, most studies failed to achieve a constant material temperature, and the samples were continuously heated during pasteurization due to the dielectric heating mechanism (Chen, Wei, Irmak, Chaves, & Subbiah, 2019; Jeong, Baik, & Kang, 2017). Other studies used RF to rapidly heat a material to a target temperature, followed by conventional thermal treatments during holding period, such as hot air (Jiao, Zhong, & Deng, 2016; Lin et al., 2020; Zheng, Zhang, & Wang, 2017), hot water (Yang et al., 2019), or natural heat preservation (Liu, Ozturk, et al., 2018; Ozturk et al., 2019; Xu, Liu, & Tang et al., 2018). No systematic study has been reported on RF pasteurization kinetics of *C. sakazakii*. A major technical challenge for such a study is how to maintain constant material temperature during holding period using RF energy.

Non-uniform heating is a major issue associated with RF heating, so it is necessary to develop effective methods to improve RF heating uniformity to ensure the effectiveness of RF pasteurization. Hot air and similar dielectric materials assistance are common methods used for getting better RF heating uniformity (Ling, Cheng, & Wang, 2019), and these methods may result in different heating uniformity affecting the results of RF pasteurization. Water activity (a_w) has been demonstrated to be an important factor affecting microbial resistance in addition to temperature (Liu, Tang, et al., 2018; Xu et al., 2019). To select the appropriate pasteurization treatment parameters, it is important to systematically study the impact of different factors on the inactivation kinetics of *C. sakazakii* in PIFM.

It has been demonstrated that RF treatment can achieve desired lethal effect of *C. sakazakii* in PIFM in a shorter time than conventional thermal treatment due to the much shorter come-up time (CUT) (Lin et al., 2020). However, the non-thermal effects of RF energy on microorganism inactivation are still controversial. Several published studies considered thermal effects of RF energy as the main cause of inactivating microorganisms in food (Kou et al., 2018; Ling et al., 2019; Liu, Ozturk, et al., 2018; Xu et al., 2018), but there is a lack of information on whether this conclusion applies to the inactivation of *C. sakazakii* in PIFM. The hot water bath pasteurization with only thermal effects could be compared with RF treatment to verify the mechanism of microbial inactivation in PIFM.

Therefore, the objectives of this study were to: (1) develop effective methods to hold the temperature of the PIFM samples based on improved proportional-integral-derivative (PID) temperature control; (2) obtain and compare the thermal inactivation kinetic parameters of *C. sakazakii* under different pasteurization parameters; and (3) study the thermal death mechanism of *C. sakazakii* under RF and hot water (HW) treatments.

2. Materials and methods

2.1. Materials

The PIFM (for newborns, 1 kg/can) was purchased from Beingmate E-commerce Co., Ltd. (Ningbo, China). The protein, fat, and carbohydrate contents were 12.3%, 26.8%, and 55.6%, respectively. The average initial moisture content was $1.99\% \pm 0.01\%$ on wet basis, as determined by a vacuum oven (DZF-6050, Jinghong Instrument Co., Ltd., Shanghai, China) at $60 \pm 5^\circ\text{C}$ and 40–53 kPa for about 5 h until a constant weight according to the method of the National Standards of People's Republic of China (2016a). The initial a_w of the samples was

0.18 ± 0.07 , measured at 25°C using an AquaLab a_w meter (4TEV, Decagon Devices Inc., Pullman, WA, USA). The PIFM samples were sealed in airtight packaging containers and stored at room temperature until use.

2.2. Bacterial strain and cultivation

C. sakazakii (ATCC 29544) was obtained from Huankai Microbial Sci. & Tech. Co., Ltd. (Guangdong, China). A frozen stock culture was added to 500 μL of brain heart infusion (BHI; Beijing Land Bridge, Beijing, China) to obtain the activated cell suspension. Then, the bacteria were streaked onto plate count agar (PCA; Beijing Aoboxing, Beijing, China) slant medium, and incubated at 37°C for 48 h. A loopful was inoculated in 50 mL of tryptic soy broth (TSB; Beijing Aoboxing) and incubated at 37°C for 18 h. Finally, 2 mL of bacterial suspension was transferred to 50 mL TSB and cultivated at 170 rpm/min and 37°C for 6 h to achieve the mid-log phase of *C. sakazakii* (according to an $\text{OD}_{600\text{ nm}}$ growth curve, data shown in Fig. S1). The bacteria were transferred to 50 mL centrifuge tubes and centrifuged at 4°C and $4000 \times g$ for 15 min. The supernatant was discarded and the pellet was washed three times with sterile 0.1% peptone water and re-suspended in 10 mL sterile deionized water. The cell population was approximately 10^9 CFU/mL, which was refrigerated at 4°C for no more than 24 h before inoculating the PIFM samples.

2.3. Sample preparation

2.3.1. Vacuum freeze-dried inoculated and uninoculated PIFM

Freeze-drying has been successfully used to retain the vitality of bacterial surrogates for inoculation studies to validate RF treatments of low-moisture foods. In particular, skim milk was found to be an effective protectant for bacteria cells to avoid cold-injury (Xu, Liu, & Song et al., 2018). Thus, in this study, freeze-drying was used to inoculate *C. sakazakii* into PIFM according to a method described previously with some modifications (Xu et al., 2018). PIFM (60 g) was dissolved in 280 mL sterile deionized water, added to 60 mL of bacterial suspension, and mixed well to obtain the inoculated PIFM solution. The solution was dispensed into four sterile trays (100 mL/tray) with an inner diameter of 180 mm and sealed with sterile gauze. After the cold trap temperature of the freeze dryer (LGJ-10E, Four-ring Science Instrument Plant Beijing Co., Ltd., Beijing, China) decreased to -60°C , the trays were placed into the cold trap and pre-frozen for 2 h to quickly reduce the temperature of the material to -30°C . Then, the trays were vacuum dried for 24 h with temperatures ranging from -30°C to 20°C . The inoculated freeze-dried samples were ground with a mortar and pestle, sealed in a sterile plastic bag, and stored at 4°C for further experiments.

For the uninoculated samples, 60 g PIFM was dissolved in 340 mL sterile deionized water to obtain the uninoculated PIFM solution. The uninoculated freeze-dried PIFM was obtained after vacuum freeze drying.

2.3.2. Stability test of freeze-dried inoculum

A *C. sakazakii* survival stability test was performed during a 11-day storage at 4°C of the inoculated freeze-dried PIFM. Duplicate samples were randomly removed for enumeration every 24 h until the stable *C. sakazakii* survival period was determined.

2.3.3. Equilibration of samples with different target water activities

The inoculated and uninoculated freeze-dried samples as well as the commercial PIFM were placed in sterile petri dishes and loaded into a humidity chamber (CTHI-150B, STIK Instrument Equipment Ltd., Shanghai, China) at 25°C and 20% relative humidity. The a_w of the samples was measured at 25°C every 2 h, and the dishes were removed when they reached $0.2 (\pm 0.02)$. When the relative humidity was set to 30% and 40%, respectively, samples with a_w values of $0.3 (\pm 0.02)$ and

0.4 (± 0.02) were acquired.

2.4. HW treatment

Aluminum TDT cells (with inner diameter of 1.8 cm and inner height of 0.4 cm) described in Chung, Birla, and Tang (2008) were fully filled with 0.2 g of inoculated freeze-dried sample with an a_w value of 0.2, closed, and immersed in a preheated hot-water bath (60, 65, and 70 °C). The sample core temperatures were monitored with thermocouples and a self-made data logger. Once the target temperatures, namely 60, 65, and 70 °C, were reached after CUT, the samples were held for five predetermined time intervals (0–40 min, depends on temperature). After holding, the TDT cells were immediately removed and placed in an ice-water bath for at least 30 s, and the survivors were enumerated.

2.5. RF treatment

2.5.1. Hot air-assisted RF heating system

These experiments were conducted in a 6 kW, 27.12 MHz pilot-scale RF system (COMBI 6-S, Strayfield International, Wokingham, UK) together with a 6.3 kW auxiliary hot air heating device (HBO-DR-3380-ZF-802, Zhangjin Hot Blower Crop., Ltd., Shanghai, China). The schematic diagram was previously provided in detail by Wang, Tiwari, Jiao, Johnson, and Tang (2010). The none-load and rated-load voltage of the RF top electrode is about 6.2 kV and 6.8 kV, respectively, according to the equipment manual. The gap of the electrode plates was adjustable within 80–200 mm by moving the top electrode (830 × 400 mm); thus, the heating rate could be regulated. Forced hot air, provided through an air distribution box, was blown upwards into the RF cavity through air holes on the bottom electrode. Hot air circulated in the empty RF cavity for 1 h before the treatment to achieve stable heating conditions.

2.5.2. Temperature measurements and control

Five cylindrical petri dishes (35 mm diameter and 12 mm high) made of polystyrene and numbered No. 1 to No. 5 from top to bottom were placed vertically in the middle of the electrodes (Fig. 1a). The core temperatures of the five samples were monitored using fiber optic sensors (UM18, FISO Technologies Inc., Quebec, Canada). Considering that the microbial contamination may be spread by inserting sensors into inoculated PIFM, the temperature of the inoculated sample was not directly measured. According to Wang, Zhang, Gao, Tang, and Wang (2014), when the samples were located in the middle of the two electrodes, the temperature was symmetrically distributed along the center dish which was right in the middle of the two electrodes. Therefore, the inoculated and uninoculated freeze-dried samples were placed separately in the two petri dishes with the same heating pattern, and the temperature of the uninoculated freeze-dried sample can be used to represent that of the inoculated freeze-dried sample.

In our research, the two petri dishes with the same heating pattern were fully filled with 3 g of uninoculated and inoculated freeze-dried samples, respectively, and the others were filled with 3 g of the commercial PIFM samples. A lab-made five-sided foam case was used to cover the dishes to prevent heat loss during heating. Temperatures at three representative periphery locations in the middle layer of the uninoculated freeze-dried sample were also recorded using fiber optic sensors during RF heating (Fig. 1b). The surface temperatures of the samples were captured immediately by an infrared thermal imaging camera (FOTRIC 600, IRS Systems Inc., Shanghai, China) after heating.

A custom-built PID controller was applied to the RF system to control material temperature during holding using RF energy. The core temperature of the petri dish filled with uninoculated freeze-dried sample (location a) was used as the input value, and the target temperature was the set value. The threshold of the temperature controller was not set; that is, the RF system automatically turned off when the sample temperature exceeded the set point and restarted if below the

set point. The top electrode ran in this manner to achieve a constant and accurate RF pasteurization temperature.

2.5.3. Determination of the RF treatment parameters

According to our previous study, heating rates in the range of 5–30 °C/min had no significant effect on heat resistance of *C. sakazakii* in PIFM (Wang et al., 2016). Therefore, the heating rates corresponding to the electrode gaps were selected within the above range.

Three methods were used during the RF treatments. One method combined RF and hot air heating (RF-HA); that is, both RF and hot air energy were used to synergistically heat and hold the sample temperature. Based on our preliminary tests, a hot air temperature of 2–3 °C lower than the target temperature and an electrode gap of 123 mm were chosen for better temperature control. The second method was RF holding after hot air-assisted RF heating (RF-HA-RF); that is, combined RF and hot air was used to heat the samples to the target temperature, then the hot air system was turned off, and RF energy was used to hold the sample temperature, in which the hot air temperature and electrode gap were the same as RF-HA. Therefore, compared with RF-HA, this method relies mainly on RF energy during holding. The third method was RF heating with the samples wrapped with PIFM (RF-MP). The petri dishes were placed in the center of large cylindrical containers (90 mm diameter and 12 mm high) made of polystyrene, and similar sample arrangement and temperature measurements are shown in Fig. 1c and d. The test sample was surrounded by 17 g of PIFM with the same density, height, and material status, so that the heating rates of the two were consistent (data not shown). RF energy was used exclusively, and the electrode gap was fixed at 84 mm to obtain a fast heating rate.

2.5.4. RF heating uniformity evaluation

The temperature difference (Dif) between the center and periphery of the middle layer of the treated samples was evaluated using Eq. (1):

$$\text{Dif} = T_a - \frac{T_b + T_c + T_d}{3} \quad (1)$$

where T_a , T_b , T_c , and T_d are the temperatures (°C) measured by the fiber optic sensors at positions a, b, c, and d, respectively.

Heating uniformity of the samples was evaluated using the heating uniformity index (λ), which was calculated with Eq. (2) from the data measured by an infrared camera (Wang, Yue, Tang, & Chen, 2005).

$$\lambda = \frac{\sqrt{\sigma^2 - \sigma_0^2}}{\mu - \mu_0} \quad (2)$$

where σ and σ_0 are final and initial standard deviations of the sample temperatures (°C), and μ and μ_0 are the final and initial average values of the sample temperatures (°C), respectively. A smaller λ value represents better heating uniformity.

2.5.5. RF pasteurization kinetics

The effect of a_w levels of 0.2, 0.3, and 0.4 measured at 25 °C on heat resistance of *C. sakazakii* was determined under the RF-HA treatment. The effect of different RF heating uniformity improvement methods on thermal inactivation kinetics of *C. sakazakii* was explored with a fixed a_w of 0.2. After the core temperature of the sample reached the target points (55, 60, 65, and 70 °C) and was held for the predetermined five time intervals (0–40 min), the RF system was shut down, the five petri dishes were moved out, the inoculated freeze-dried samples were sealed in sterile plastic bags, and transferred to ice water for cooling, ready for enumeration.

2.6. Enumeration method

Each treated sample was poured into a sterile stomacher bag with sterile BHI and mixed well to achieve a 10-fold dilution. Serial dilutions

Fig. 1. Schematic diagram of the arrangement of the inoculated and uninoculated freeze-dried powdered infant formula milk samples in the polystyrene cylindrical petri dishes (a: hot air assistance method; c: material assistance method); and the top view of petri dish No. 2 with four locations (●) in the middle layer of the sample for the temperature measurements (b: hot air assistance method; d: material assistance method). All dimensions are in mm.

(1:10) were performed using sterile 0.1% peptone water, and 1 mL of each was mixed with 20 mL of 46 °C PCA. The plates were incubated at 37 °C for 48 h to obtain the amount of residual microorganisms. The plate colony counts were expressed as CFU/g and log transformed (National Standards of People's Republic of China, 2016b).

2.7. Determination of the D- and z-values

Thermal resistance of microorganisms was estimated by the log-liner model as Eq. (3) (Peleg, 2006).

$$\log \frac{N}{N_0} = -\frac{t}{D} \quad (3)$$

where N_0 is the initial bacteria population (CFU/g); N is the population of survival (CFU/g) at time t ; t is the time of the isothermal treatment (min) after CUT; and D indicates the time (min) required to inactivate 90% of the microbial populations at a target temperature (°C).

The z-value, indicating increase in temperature required to reduce the D-value by 90%, was calculated as absolute value of the inverse of the slope of the log D-value vs. temperature curve. Larger D- and z-values indicate greater heat resistance of *C. sakazakii* (Michael et al., 2014).

The goodness-of-fit of the model was evaluated using the coefficient of determination (R^2) of the predicted and experimental values, and it was calculated using Eq. (4).

$$R^2 = 1 - \frac{\sum_{i=1}^n \left[\log \left(\frac{N}{N_0} \right)_{pre,i} - \log \left(\frac{N}{N_0} \right)_{exp,i} \right]^2}{\sum_{i=1}^n \left[\log \left(\frac{N}{N_0} \right)_{pre,i} - \log \left(\frac{N}{N_0} \right)_{exp,i} \right]^2} \quad (4)$$

where $\log (N/N_0)_{exp,i}$ is the measured reduction of bacteria during treatment; $\log (N/N_0)_{pre,i}$ represents the predicted log reduction using the model; and n is the total number of data. The larger R^2 coefficient corresponds to better effectiveness of fitting.

2.8. Determination of inactivation mechanism

2.8.1. Sample pretreatment

A combination of a_w value of 0.25, temperature of 70 °C, and holding time of 20 min was adopted to compare the effects of the RF-HA and HW treatments on bacterial damage. An unheated inoculated sample was used as control. A 1 g sample was re-suspended in 10 mL sterile phosphate buffered solution (PBS: Hyclone, Logan, UT, USA). Trypsin (7 mg, 1:250; Sigma, St. Louis, MO, USA) was added and incubated at 40 °C for 20 min to digest the protein. The samples were then centrifuged at 6000 × g for 10 min, the lipid (top layer) was removed with a sterile cotton swab, the supernatant was discarded, and the bacterial precipitate was washed twice and re-suspended in PBS. The prepared samples were used for bacterial microstructure and vitality analyses to reveal the *C. sakazakii* inactivation mechanism.

Fig. 2. The *C. sakazakii* survival curve in powdered infant formula milk stored at 4 °C for 11 days. Results of two replicated experiments are shown.

2.8.2. Transmission electron microscopy (TEM) measurement

The bacterial precipitates were fixed with 2.5% (v/v) glutaraldehyde and 0.1M PBS at 4 °C for 2 h and washed by PBS for 20 min. Samples were then transferred into 1% (w/v) osmium tetroxide at 4 °C for 2 h and rinsed by the same buffer for 20 min. Dehydration of samples was performed in a graded acetone series (30, 50, 70, 90, and 100%, v/v). The samples were infiltrated by a series of mixture of Epon 812 solution and acetone (1:3, 1:1, 3:1, and 1:0, v/v) for 12 h, and embedded in capsules of Spurr's epoxy resin at 60 °C for 24 h. Resin embedded samples were ultrathin sectioned (thickness of 60–80 nm) by a ultramicrotome (model EM UC7, Leica, Bensheim, Germany) and stained with 2% aqueous uranylacetate and 0.1% lead citrate. Finally, the sections were examined by a transmission electron microscope (model HT7700, Hitachi, Tokyo, Japan).

2.8.3. Flow cytometry (FCM) analysis

FCM is a sensitive technique used to quantitatively analyze multiple parameters of thousands of individual cells within a heterogeneous population. Use of different fluorescently labeled antibodies or specific probes in combination with FCM enables physiological analysis and enumeration of microbial populations (Longin, Petitgonnet, Guilloux-Benatier, Rousseaux, & Alexandre, 2017). Propidium iodide (PI) can be combined with SYTO9 to simultaneously analyze cell vitality. They are both nucleic acid dyes but with differences in cell membrane penetration and nucleic acid affinity. SYTO9 penetrates both live and dead bacteria and displays strong green fluorescence due to its high permeability, whereas PI only penetrates dead cells with seriously damaged membrane and emits red fluorescence due to its stronger nucleic acid affinity (Yang, Huang, & Yousef, 2017). Injured cells with partially damaged membranes are both green and red, as intracellular PI cannot be sufficient to displace SYTO9 completely (Subires, Yuste, & Capellas, 2014).

In this study, FCM was used to evaluate the ratio of different active bacterial cells in the treated samples according to the modified methods of Gunasekera, Attfield, and Veal (2000) and Li et al. (2016). The membrane repair capacity of *C. sakazakii* cells was monitored by a 7-day refrigerated storage of samples using the method described by Subires et al. (2014). After pasteurization, inoculated sample powders were packaged in sterile air-tight bags, and stored at 4 °C for 7 days to investigate the membrane repair performance of *C. sakazakii*, an indicator for assessing microbiological safety of pasteurized food. Stored

samples were pretreated using the same method as in 2.8.1. Before FCM test, cell density was adjusted to approximately 10^6 – 10^7 cells/mL and 1 mL of the cell suspension was stained with 1.5 μ L SYTO9 and 1.5 μ L PI fluorescent dyes (LIVE/DEAD® BacLight™ bacterial viability kit, Invitrogen, Carlsbad, CA, USA) at room temperature for 15 min, protected from light. The samples were analyzed with a FACS AriaSorp flow cytometer (BD, Franklin Lakes, NJ, USA) equipped with a 15-mW 488-nm argon-ion laser. The SYTO9 fluorescent emission was detected by the FL1 channel (488–520 nm), while PI was detected by the FL2 channel (561–600 nm). Single-stained and unstained cells served as controls to adjust the detectors and compensate for the settings. The data were acquired until approximately 100,000 events were completed.

2.9. Statistic analysis

All data are presented as the mean of three determinations $\pm$ standard deviation and analyzed with SPSS statistical software (version 22.0, SPSS Inc., Chicago, IL, USA). Duncan's multiple comparison test was used to detect differences. A *P*-value < 0.05 was considered significant.

3. Results and discussion

3.1. Stability of *C. sakazakii* survival

The curve of *C. sakazakii* survival during storage at 4 °C is presented in Fig. 2. The inoculated freeze-dried samples were uniformly distributed with standard deviations of the population counts < 0.2 log CFU/g. The number of survivors decreased gradually during adaptation to the dry environment from 0 to 3 day, particularly from 2 to 3 day. The number of survivors was basically stable from 3 to 9 day and began to decline slowly after 9 days. The stationary phase is mainly due to the quick adaption of bacteria to changes in the environment by manipulating their own gene expression (Wu et al., 2020). A similar trend of *E. sakazakii* strains in PIFM during refrigeration temperature (4 °C) has been observed previously (Al-Holy, Lin, Abu-Ghoush, Al-Qadiri, & Rasco, 2009). The stable cell population level may reflect consistent biological characteristics of *C. sakazakii*, which would ensure the reliability of pasteurization experiments; thus, the inoculated freeze-dried sample stored for 3–9 day was selected for subsequent tests, and the cell population was approximately 10^8 CFU/g.

Fig. 3. Temperature-time profiles at the center of five petri dishes when heated to 60 °C during RF treatment. Results of three replicated experiments are shown.

3.2. Temperature distribution and RF heating uniformity evaluation

Temperature-time profiles at the center of five petri dishes when heated to 60 °C during RF treatment are shown in Fig. 3. The heating modes of the symmetrically placed petri dishes No. 2 and No. 4 were basically the same, while the temperatures of dishes No. 1 and No. 5 were lower, and the dish No. 3 had a slightly higher temperature. Therefore, the uninoculated and inoculated freeze-dried samples were placed in dishes No. 2 and No. 4, respectively.

The temperatures of PIFM in petri dish No. 2 measured by the fiber optic sensors and infrared camera after holding for 20 min at 60 °C with the different uniformity improvement methods are listed in Table 1. The core temperature (location a) was always higher than that of the periphery (average of b, c, and d) and upper (μ); that is, the hot spot was in the center of the sample, and the cold spot was located on the edge during holding period among the various methods, because there was heat loss from the edge of the samples to the system and the environment during heating and photographing. Both hot air (RF-HA) and similar dielectric material assistance (RF-MP) greatly improved the RF heating uniformity, and the uniformity of RF-HA was superior to that of RF-MP, followed by RF-HA-RF.

3.3. Thermal inactivation as influenced by temperature and water activity

The CUT and log reduction of *C. sakazakii* heated under different parameter settings during CUT of RF-HA are presented in Table 2. As the a_w increased, the time required for the PIFM to reach a fixed target temperature was shortened, and the heating rate was accelerated. This phenomenon was related to the response of moisture to the RF field.

Table 1

Temperatures and heating uniformities by different uniformity improvement methods at 60 °C with holding time of 20 min.

Methods	Fiber optic temperature in the middle layer (°C)			Infrared temperature in the upper layer (°C)		
	a	Average of b, c, d	Dif	μ	σ	λ
RF ^b	59.73 ± 0.04 ^{ab}	47.25 ± 3.82 ^b	12.48 ± 3.78 ^a	50.43 ± 0.08 ^b	4.20 ± 0.04 ^a	0.176 ± 0.002 ^a
RF-HA	59.87 ± 0.04 ^a	59.17 ± 0.05 ^a	0.69 ± 0.02 ^b	56.17 ± 1.63 ^a	1.97 ± 0.14 ^b	0.066 ± 0.001 ^c
RF-HA-RF	59.90 ± 0.07 ^a	54.89 ± 1.11 ^a	5.01 ± 1.18 ^b	50.31 ± 0.67 ^b	2.80 ± 0.35 ^b	0.118 ± 0.015 ^b
RF-MP	59.95 ± 0.18 ^a	56.32 ± 0.33 ^a	3.64 ± 0.52 ^b	49.83 ± 1.34 ^b	1.79 ± 0.68 ^b	0.076 ± 0.025 ^c

^a The data shown are the mean values from three independent replicates plus the standard deviations. Different letters in the same column indicate significant difference ($P = 0.05$) between different methods.

^b RF-HA, combined RF and hot air heating; RF-HA-RF, RF holding after hot air assisted RF heating; RF-MP, RF heating with samples wrapped with PIFM; RF, samples heated by RF energy without any uniformity improvement methods.

Similar trends were reported by Jiao et al. (2016) and Zheng, Zhang, Zhou, and Wang (2016). Inactivation of *C. sakazakii* during CUT was positively correlated with target sample temperature and the a_w value, in agreement with the findings of Wang et al. (2016) for *C. sakazakii* in PIFM. Table 2 also illustrates the thermal resistance parameters of *C. sakazakii*, namely D - and z -values, obtained from the holding time at different temperatures and a_w levels. The D -values decreased with increasing temperature and a_w , and an inverse relationship was observed between the a_w levels and the z -values. That is, the higher the temperature and a_w , the poorer the microbial heat resistance, and the shorter the time required to inactivate *C. sakazakii*. Similar results were reported by Villa-Rojas et al. (2013) and Xu et al. (2019) for *Salmonella* Enteritidis PT 30. The enhanced thermal resistance of bacteria in lower a_w material might be attributed to the reduced mobility of available water molecules and the presence of stable protein structures in bacterial cells (Liu, Ozturk, et al., 2018; Syamaladevi et al., 2016; Xu et al., 2019). Freeze-dried *C. sakazakii* culture appears to be weaker than freeze-dried *Enterococcus faecium* which is considered a good surrogate for *Salmonella* Enteritidis PT30 in process validations in food plants (Xu et al., 2018). Overall, the results of the present study on D - and z -values could be used to establish safe thermal processing protocol for pasteurizing PIFM in the dairy industry. In order to improve pasteurization efficiency and minimize effects on product quality, a higher a_w level could be preferred to be chosen for food industrial application.

3.4. Thermal inactivation as influenced by the RF heating uniformity improvement method

The microbial inactivation parameters at a_w value of 0.2 during RF-HA, RF-HA-RF and RF-MP are listed in Table 3. Minimal inactivation of *C. sakazakii* during CUT was detected, which agreed with the finding of Liu, Ozturk, et al. (2018), and no significant difference was observed among the various uniformity improvement methods. RF-HA exhibited the lowest D - and z -values of *C. sakazakii*, while RF-HA-RF resulted in the highest D - and z -values, consistent with the temperature uniformity results shown in Table 1. This observation shows that the lethality the inoculated samples experienced was positively related to the temperature, indicating that the thermal effects might be the main cause of inactivation of *C. sakazakii*. This result proposes the use of combined RF and hot air techniques during the PIFM industrial pasteurization process.

3.5. Comparison of *C. sakazakii* inactivation between the RF-HA and HW treatments

Slightly lower reductions during CUT and higher D - and z -values of *C. sakazakii* were observed in RF-HA compared with HW, but without a significant difference (Table 3), indicating that HW had slightly stronger lethal effects. This phenomenon was caused by the fact that the temperature of the treated sample was tracked at the cold spot during the HW, while the material temperature was tracked at the hot spot

Table 2

The log reduction of *C. sakazakii* during come-up time (CUT) and *D*-, *z*-values of *C. sakazakii* during holding time of combined RF and hot air heating (RF-HA) at different temperatures and water activities at 25 °C.

a_w , 25 °C ± 0.02	Target temperature (°C)	RF-HA treatment				
		CUT (min)	Reduction during CUT (log CFU/g)	<i>D</i> -value (min)	<i>R</i> ²	<i>z</i> -value (°C)
0.2	60	5.85 ± 0.10 ^{cAa}	0.07 ± 0.01 ^{aB}	109.2 ± 18.9 ^{aA}	0.9174	14.90 ± 3.62 ^A
	65	6.52 ± 0.06 ^{bA}	0.11 ± 0.10 ^{aB}	36.4 ± 4.7 ^{bA}	0.9528	
	70	6.96 ± 0.05 ^{aA}	0.16 ± 0.03 ^{aC}	23.3 ± 1.7 ^{bA}	0.9837	
0.3	55	5.61 ± 0.00 ^{cA}	0.07 ± 0.01 ^{cB}	209.1 ± 41.3 ^{aA}	0.8954	14.07 ± 3.11 ^A
	60	5.73 ± 0.06 ^{bcA}	0.12 ± 0.06 ^{cB}	48.7 ± 3.6 ^{bb}	0.9834	
	65	6.32 ± 0.43 ^{abA}	0.22 ± 0.06 ^{bAB}	24.0 ± 3.9 ^{bAB}	0.9262	
0.4	70	6.45 ± 0.47 ^{aA}	0.34 ± 0.01 ^{aB}	17.3 ± 1.5 ^{bAB}	0.9765	13.33 ± 2.57 ^A
	55	4.42 ± 0.08 ^{bb}	0.19 ± 0.00 ^{cA}	133.7 ± 38.2 ^{aA}	0.8600	
	60	4.57 ± 0.21 ^{bb}	0.30 ± 0.04 ^{bA}	33.9 ± 3.9 ^{bb}	0.9620	
	65	5.47 ± 0.20 ^{aB}	0.31 ± 0.03 ^{bA}	13.9 ± 0.7 ^{bb}	0.9922	
	70	5.53 ± 0.26 ^{aB}	0.47 ± 0.04 ^{aA}	10.1 ± 1.8 ^{bb}	0.9111	

^a Experiments were repeated three times independently. Different lower and upper case letters within a column indicate that means are significantly different at *P* = 0.05 among temperature and water activity, respectively.

Table 3

The log reduction of *C. sakazakii* during come-up time (CUT) and *D*-, *z*-values of *C. sakazakii* during holding time of different heating methods at three temperatures and water activity of 0.2 at 25 °C.

Methods	Target temperature (°C)	a_w , 25 °C = 0.2				
		CUT (min)	Reduction during CUT (log CFU/g)	<i>D</i> -value (min)	<i>R</i> ²	<i>z</i> -value (°C)
RF-HA ^b	60	5.85 ± 0.10 ^{cAa}	0.07 ± 0.01 ^{aA}	109.2 ± 18.9 ^{aA}	0.9174	14.90 ± 3.62 ^A
	65	6.52 ± 0.06 ^{bA}	0.11 ± 0.10 ^{aA}	36.4 ± 4.7 ^{bC}	0.9528	
	70	6.96 ± 0.05 ^{aA}	0.16 ± 0.03 ^{aA}	23.3 ± 1.7 ^{bbC}	0.9837	
RF-HA-RF	60	5.85 ± 0.10 ^{cA}	0.10 ± 0.06 ^{aA}	121.7 ± 18.1 ^{aA}	0.9377	18.09 ± 2.85 ^A
	65	6.52 ± 0.06 ^{bA}	0.12 ± 0.05 ^{aA}	76.6 ± 9.0 ^{bA}	0.9601	
	70	6.96 ± 0.05 ^{aA}	0.13 ± 0.07 ^{aA}	34.0 ± 3.4 ^{cA}	0.9703	
RF-MP	60	1.35 ± 0.08 ^{bb}	0.10 ± 0.07 ^{aA}	114.9 ± 7.4 ^{aA}	0.9876	15.80 ± 1.16 ^A
	65	1.46 ± 0.05 ^{abB}	0.13 ± 0.06 ^{aA}	50.5 ± 6.3 ^{bb}	0.9548	
	70	1.59 ± 0.07 ^{ab}	0.14 ± 0.02 ^{aA}	26.8 ± 3.7 ^{cB}	0.9464	
HW	60	1.46 ± 0.21 ^{aB}	0.11 ± 0.11 ^{aA}	103.6 ± 14.2 ^{aA}	0.9467	14.35 ± 2.22 ^A
	65	1.53 ± 0.07 ^{aB}	0.14 ± 0.04 ^{aA}	37.5 ± 4.5 ^{bC}	0.9584	
	70	1.70 ± 0.14 ^{aB}	0.18 ± 0.01 ^{aA}	20.8 ± 1.5 ^{bC}	0.9848	

^a Experiments were repeated three times independently. Different lower and upper case letters within a column indicate that means are significantly different at *P* = 0.05 among temperature and treatments, respectively.

^b RF-HA, combined RF and hot air heating; RF-HA-RF, RF holding after hot air assisted RF heating; RF-MP, RF heating with samples wrapped with PIFM; HW, hot water treatment.

during the RF-HA treatment; thus, there were more survivors. The changes in the a_w values of the samples after holding for 20 min at 65 °C between the two treatments are compared in Table 4. RF-HA displayed lower a_w values and a greater reduction in a_w than HW, indicating increased thermal resistance of the microorganisms. The possible reason is the different heat transfer mechanisms. Heat was transferred mainly by conduction between the TDT cells and the samples, whereas it was generated within samples by dielectric heating in the RF field, leading to different extents of moisture evaporation and migration inside the petri dishes. It could be further confirmed that the destruction of *C. sakazakii* was mainly due to thermal effects, and similar results were reported for *Escherichia coli* and *Staphylococcus aureus* in apple juice and mashed potatoes (Kou et al., 2018), and for *C. sakazakii* and *Salmonella* species in nonfat dry milk (Michael et al., 2014).

3.6. Microstructure of *C. sakazakii*

The morphological and intracellular variations of *C. sakazakii* cells before and after the HW or RF-HA treatment are presented in Fig. 4. Control cells had a smooth and continuous membrane, dense and uniform cytoplasmic, and stretched nuclear region with low electron density (Fig. 4a), consistent with the findings reported in Wang, Pan, Xie, Yang, and Lin (2010). However, after the HW treatment, the

Table 4

Changes in water activity of different treated powdered infant formula milk samples with water activity of 0.2 at 25 °C after holding for 20 min at 65 °C.

Treatment	a_w , 25 °C		
	Before pasteurization	After pasteurization and cooling	After 12 h equilibrium
HW ^b	0.200 ± 0.0003 ^a	0.171 ± 0.001 ^{cA}	0.182 ± 0.003 ^{bA}
RF-HA	0.200 ± 0.0003 ^{aA}	0.132 ± 0.001 ^{cB}	0.145 ± 0.003 ^{bb}

^a Results of three experiments are shown. Different lower and upper case letters indicate that means are significantly different at *P* = 0.05 among different stages and treatments, respectively.

^b RF-HA, combined RF and hot air heating; HW, hot water treatment.

cytoplasm lost considerable electron density and the ribonucleoprotein particles aggregated. The membranes were perturbed, roughed and deformed, and pronounced bulging and invagination of membrane appeared. The separation of the cytoplasm and the outer membranes occurred, and even the cell membranes were significantly ruptured (Fig. 4b). These phenomena were also found in RF treated *C. sakazakii* cells (Fig. 4c). In addition, there was no special damage in RF treated *C.*

Fig. 4. Representative transmission electron micrographs of control *C. sakazakii* cells and those treated by hot water heating (HW) or combined RF and hot air heating (RF-HA) in magnification of $49,000 \times$. Scale bars correspond to 200 nm. A: nuclear region; B: aggregation of ribonucleoprotein particles; C: membrane perturbations; D: separation of the cytoplasm and the outer membranes; and E: cell membranes rupture.

sakazakii samples compared with those of HW treated ones. We can deduced that the two treatments might destroy the membranes in a way of local pore formation, leading to leakage of the cytoplasm and ultimately disintegrating the entire cells (Liu, Wang, Cao, Jiang, & Hou, 2019). This finding matched with the statement that the microbial inactivation mechanism in low-moisture foods is likely due to injured cell membrane, different from protein denaturation in foods with high a_w (Ling et al., 2019). Since the treated samples had cells in various states, the degree of membrane damage needed to be quantified.

3.7. *C. sakazakii* vitality

The degree of bacterial cell membrane damage was quantified by FCM, and the ratio of different active bacterial cells was obtained. The proportions of live, injured, and dead cells in the control (inoculated PIFM without heat treatment), HW, and RF-HA treatments are presented in Fig. 5. The Q1 (SYTO9-/PI+), Q2 (SYTO9+/PI+), and Q4 (SYTO9+/PI-) gates represented dead, injured, and live bacterial cells,

respectively. Before the heat treatment, the live cells in the inoculated sample maintained a relatively high percentage (63%) and the proportions of dead and injured cells were 4.3% and 13.6%, respectively (Fig. 5a), suggesting that the cell membranes were partially injured during the vacuum freeze drying inoculation process. Throughout the heat treatments, the percentages of injured and dead cells increased and that of healthy cells decreased. After the HW treatment, the ratios of dead and injured cells increased to 25.0% and 36.9% (Fig. 5b), respectively, whereas the percentages were 14.6% and 26.5% after the RF-HA treatment (Fig. 5c), coincident with the thermal inactivation results presented above, demonstrating that cell membranes could not be additionally damaged by RF. A possible influencing factor might be the electric field intensity (Kou et al., 2018). According to dielectric rupture theory, a critical electric field density of 13.33 kV/cm is required to induce cells with a typical radius of 0.5 μm to form pores (Jeyamkondan, Jayas, & Holley, 1999), while the electric field intensity of the 27.12 MHz RF system was 0.3 kV/cm or so by computer simulation (Huang, Zhu, Yan, & Wang, 2015). Therefore, the electric field

Fig. 5. Representative flow cytometry cell vitality and membrane integrity assessments of *C. sakazakii* in inoculated freeze-dried powdered infant formula milk after the hot water (HW) and combined RF and hot air heating (RF-HA) treatments. Q1: dead cells, Q2: injured cells, Q3: negative, Q4: live cells. (a) Control, day 1, (b) HW, day 1, (c) RF-HA, day 1, (d) Control, day 7, (e) HW, day 7, and (f) RF-HA, day 7.

intensity used in this study may be too low to break *C. sakazakii* cell. Moreover, there was no obvious change in membrane integrity after 7-day refrigerated storage (Fig. 5d–f), suggesting that sublethally injured *C. sakazakii* cells in both the RF-HA and HW-treated samples did not undergo membrane repair during exposure to refrigeration. The fact that the injured state cannot be restored to the normal state with metabolic activity indicated both RF-HA and HW can pasteurize PIFM thoroughly so as to ensure safe shelf life, and will not constitute potential risks to the food processing (Wu et al., 2020).

4. Conclusions

In this study, a PID temperature controller without a set threshold was applied to the RF pasteurization process to achieve a constant material temperature during holding time. Temperature, a_w level, and RF heating uniformity improvement method had major effects on the thermal inactivation characteristics of *C. sakazakii*, which was inoculated in PIFM by vacuum freeze drying. The heat resistance of *C. sakazakii* decreased with increasing temperature and a_w value during the RF-HA treatment, which achieved a better *C. sakazakii* inactivation effect than RF-HA-RF and RF-MP due to improved temperature uniformity. The non-thermal effects were not found in the 27.12 MHz RF system for *C. sakazakii* inactivation after comparing the thermal resistance of *C. sakazakii* under the RF and HW treatments. FCM and TEM analyses further confirmed that the RF treatment did not have a special cell membrane damage effect. Although also primarily attributing to thermal effect for pasteurization, RF heating can provide an outstandingly higher heating rate compared to traditional treatment, so it has great potential to be used for the production of PIFM to achieve higher pasteurization efficiency. Future research should explore quality changes to evaluate the edible safety of pasteurized PIFM.

CRediT authorship contribution statement

Yue Zhang: Investigation, Data curation, Writing - original draft. **Yongkang Xie:** Investigation. **Juming Tang:** Writing - review & editing. **Shaojin Wang:** Writing - review & editing. **Lu Wang:** Methodology. **Guangfei Zhu:** Investigation. **Xingyi Li:** Software. **Yanhong Liu:** Funding acquisition, Methodology, Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

The authors state that they have no conflict of interests or personal relationships that could influence the work reported in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2020.107270>.

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