Dry inoculation methods for nonfat milk powder

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ABSTRACT

Carriers with inoculated microorganisms are often used to validate low-moisture food safety interventions. In this study, we evaluated dry inoculation methods using silicon dioxide (SiO₂) and a small portion of nonfat milk powder (NFMP) as dry carriers for NFMP. Silicon dioxide was characterized by vapor sorption analysis. One milliliter of inoculum of a 5-strain *Salmonella* cocktail (serovars Agona, Reading, Tennessee, Montevideo, and Mbandaka) or *Enterococcus faecium* NRRL B-2354 was inoculated onto 1 g of SiO₂ or 10 g of NFMP as carriers. Both inoculated carriers were air-dried for 72 h [22°C, relative humidity (RH) ~30%], equilibrated to water activity (a_w) 0.25 ± 0.02 (24 h at 22°C, RH 25%), and mixed with preconditioned NFMP ($a_w = 0.25 \pm 0.02$) to reach an inoculation level of 8.2 ± 0.2 log cfu/g. Inoculated NFMP was stored at 22°C, RH 25%, and its bacterial populations were monitored for 30 d. Both sets in equilibrated NFMP were subjected to isothermal treatments in closed aluminum cells at 85, 90, and 95°C. Silicon dioxide maintained moisture content ($0.29 \pm 0.03\%$, dry basis) at different water activities. The NFMP inoculated with both carriers exhibited stable bacterial populations over 30 d at 22°C. Strains in NFMP inoculated with SiO₂ showed equal or higher D-values but equal z-values compared with those inoculated with a small portion of NFMP. *Enterococcus faecium* exhibited comparable thermal resistance to *Salmonella* under all tested conditions. This study supports *E. faecium* as a *Salmonella* surrogate in thermal processing of NFMP and the use of SiO₂ to inoculate NFMP.

Key words: nonfat milk powder, *Salmonella* and *Enterococcus faecium*, dry inoculation, heat resistance, silicon dioxide

INTRODUCTION

Low-moisture foods (LMF) are foods with water activity (a_w) less than 0.65 (Bari et al., 2009). In microbial studies of LMF, the wet inoculation method applies liquid bacterial inoculum directly to samples. For example, nut kernels and black peppercorn were inoculated by dipping them in bacterial culture and then air-drying the kernels on filter paper under ambient conditions (Kim et al., 2012; ABC, 2014). This procedure may not be applicable to powdered foods and herbs, as sticking and agglomeration may occur (Wallack and King, 1988) and prevent uniform inoculation (Tamminga et al., 1977; Doyle et al., 1985; Nazarowec-White and Farber, 1997). Inoculating dry ingredients such as herbs and spices with a wet inoculum may also expose bacteria to water-soluble antimicrobial compounds (Shelef, 1984), which were observed to weaken bacterial cells before performing microbial studies (Hildebrandt et al., 2017).

Consequently, dry carriers, such as talc powder and sand, have been used to transmit bacteria into food matrices (Fustier et al., 1998; Enache et al., 2015). An ideal carrier should distribute bacterial cells evenly without interfering with LMF. A high inoculation level of bacteria in LMF is preferred in subsequent thermal inactivation studies. However, talc powder, which may contain asbestos, was classified as a human carcinogen (American Cancer Society, 2016). Sand is a mixture of minerals with the main compound silicon dioxide (SiO₂), which is a pure, nontoxic mineral that can be used as a potential carrier.

Low-moisture food matrices can also be used as the dry carrier. Specifically, a small portion of the same sample can be inoculated with the bacterial inoculum, conditioned to original a_w as a dry carrier, and then mixed into a large quantity of LMF (Villa-Rojas et al., 2017). Using the same food samples as carriers eliminates potential problems of introducing external materials. However, appropriate handling is needed to bring inoculated carrier back to original a_w and form. For example, dry basil inoculated with liquid inoculum cakes together and needed to be gently massaged and separated.

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Low-moisture foods, such as spray-dried milk powder, do not support the growth of bacterial pathogens (Leistner and Rödel, 1976). However, significant food safety risks may occur when contamination takes place in different processing and handling operations (Podolak et al., 2010). Outbreaks involving nonfat milk powder (NFMP) have been traced back to a factory spray-dryer (Rowe et al., 1987) and dry powder handling machinery (Collins et al., 1968; Kandhai et al., 2004). From these outbreaks, it was determined that cross-contamination of NFMP resulted from contaminated insulation in a spray-dryer and the packaging bottles for the finished products. It is desirable to evaluate the thermal inactivation kinetics of microorganisms in milk powder contaminated after drying with dehydrated microorganisms.

The overall objectives of our study were to evaluate 2 inoculation methods for NFMP, one using SiO₂ and the other using a small portion of NFMP as carriers. A 5-strain *Salmonella* cocktail and potential surrogate *Enterococcus faecium* NRRL B-2354 (ABC, 2014) were tested to estimate the effects of carriers on their thermal resistances and further evaluate if *E. faecium* is a valid *Salmonella* surrogate in thermal processing of NFMP. The specific objectives were to (1) evaluate SiO₂ as the dry carrier, (2) study thermal inactivation kinetics of *Salmonella* cocktail and *E. faecium* at 85, 90, and 95°C with $a_w = 0.25 \pm 0.02$ with 2 inoculation methods, and (3) compare thermal resistance parameters between the 2 sets of strains and those of the same strain set but inoculated by 2 carriers.

MATERIALS AND METHODS

Materials and Strains

Spray-dried NFMP was provided by Michigan Milk Producers Association (Novi, MI). It contained <1.5% fat, <5% moisture, and 33 to 38% (dry basis) total protein without any additives (provided by Michigan Milk Producers Association). The original a_w of NFMP was 0.15 ± 0.01 , measured at 25°C by Aqua Pre (Meter Group Inc., Pullman, WA). Silicon dioxide (99.99%, granulate 0.2–0.7 mm) was purchased from Umicore (Brussels, Belgium) and its initial a_w measured at 25°C was 0.25 to 0.32.

Five *Salmonella* strains and 1 *E. faecium* strain were used in this study. *Salmonella* Agona 447967, *Salmonella* Montevideo 488275, and *Salmonella* Mbandaka 698538 were acquired from the Food and Drug Administration (FDA) Arkansas Regional laboratory (Jefferson, AR). *Salmonella* Reading (ATCC 6967) was from the American Type Culture Collection (Manassas, VA). *Salmonella* Tennessee was from Larry Beuchat at the

University of Georgia (Athens). *Enterococcus faecium* (NRRL B-2354) was acquired from the USDA Agricultural Research Service (Peoria, IL). The cultures were kept at –80°C in tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 20% (vol/vol) glycerol (Becton Dickinson).

Determination of a_w , Moisture Content, and Water Sorption Isotherm Generation

The a_w of samples was determined at 25°C with an Aqua Pre water activity meter. Moisture contents of equilibrated samples were obtained by heating samples of 3 to 5 g at 80°C for 10 h in a vacuum oven under 10 kPa of absolute pressure (AOAC International, 2005).

To explore interactions between water and SiO₂ particles, water sorption isotherms of SiO₂ at 20 and 60°C were obtained with a vapor sorption analyzer (VSA; Decagon Devices Inc., Pullman, WA) following the published method (Yu et al., 2008). Briefly, the moisture content of SiO₂ was obtained by placing samples of 3 to 5 g in a vacuum oven with internal pressure of 10 kPa at 80°C for 10 h. A small amount of SiO₂ (2–3 g) in a metallic cup was placed in the VSA, and initial moisture content of the sample was input into the program. The VSA was then programmed to change a_w from 0.1 to 0.9 (at 0.1-unit intervals) and back to 0.1 at 20 or 60°C. Changes in sample weights were recorded after each time the a_w reached equilibrium with the adjusted relative humidity of the chamber. The moisture content (% dry basis) was calculated from the weight data at each equilibration step. All data points are the average of at least 2 independent samples.

Preparation of Aqueous Inoculum

A modified lawn-harvest method (Hildebrandt et al., 2016) was used to prepare the aqueous inoculum. Briefly, the differential media m-TSAYE [tryptic soy agar supplemented with 0.6% (wt/vol) yeast extract, 0.03% sodium thiosulfate, and 0.05% ammonium ferric citrate] and e-TSAYE [tryptic soy agar supplemented with 0.6% (wt/vol) yeast extract, 0.03% esculin thiosulfate, and 0.05% ammonium ferric citrate] were used to isolate *Salmonella* and *E. faecium* colonies, respectively (Becton Dickinson). After 2 successive transfers in 10 mL of TSBYE [tryptic soy broth with 0.6% (wt/vol) yeast extract], 1 mL of bacterial culture in tubes was spread-plated onto 150 × 10 mm TSAYE plates and incubated at $37 \pm 2^\circ\text{C}$ for 24 h. Bacterial lawns were harvested using 5 mL of sterile 0.1% peptone water and collected to constitute the aqueous inoculum. Each plate produced 3 to 4 mL of inoculum. For the 5-strain *Salmonella* cocktail, inocula with high cell con-

centrations ($\sim 11 \log \text{cfu/g}$) of the 5 strains were equally combined and vortexed into 1 homogeneous mixed inoculum. The experimental procedure is schematically presented in Figure 1.

Inoculation of NFMP

Two dry inoculation methods were compared in this study to inoculate NFMP: using SiO_2 and using a small

Figure 1. Experimental flowchart of the study. The ratio of inoculated SiO_2 and final nonfat milk powder (NFMP) was 1:31 (wt/wt). SiO_2 -*Salmonella* and SiO_2 -*E. faecium* (*Enterococcus faecium*) are the bacteria in NFMP inoculated using SiO_2 as a carrier; NFMP-*Salmonella* and NFMP-*E. faecium* are the bacteria in NFMP inoculated using a small portion of NFMP as a carrier.

portion of NFMP. To achieve a target inoculation level of 8.2 ± 0.2 log cfu/g, we inoculated 1 g of SiO₂ or 10 g of NFMP with the *Salmonella* cocktail or *E. faecium*, conditioned back to their original a_w , equilibrated to $a_w = 0.25 \pm 0.02$, and mixed with 30 and 90 g of pre-equilibrated NFMP ($a_w = 0.25$), respectively (Villa Rojas, 2015).

Use of SiO₂ as a Dry Carrier. One milliliter of each bacterial inoculum prepared was pipetted onto 1 g of sterile SiO₂ (previously autoclaved for 45 min and cooled to room temperature) in a sterile cup, and the SiO₂ was mixed with the inoculum. Inoculated SiO₂ was then conditioned to $a_w = 0.25$ in an equilibration chamber at room temperature ($\sim 22^\circ\text{C}$) for 2 to 3 d. One gram of inoculated SiO₂ was then mixed into 30 g of equilibrated NFMP; the mixture was then stomached at 260 rpm for 3 min in a Seward stomacher (Seward, London, UK). Final inoculated NFMP (SiO₂-*Salmonella* and SiO₂-*E. faecium*) was kept at $a_w = 0.25 \pm 0.02$ for a minimum of 48 h at room temperature before heat treatments.

Use of a Small Portion of NFMP as a Dry Carrier. One milliliter of the prepared bacterial inoculum was mixed by hand with 10 g of NFMP in a sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI). Inoculated NFMP was poured onto a sterile tray and stabilized in an equilibration chamber at room temperature 22°C for 2 to 3 d at $a_w = 0.25 \pm 0.02$. Ten grams of dry inoculated NFMP particles was then pulverized into powdered form with a steel cylinder (Figure 1). The powder-form inoculated NFMP was used as a seed inoculum and mixed into 90 g of equilibrated NFMP to produce a total of 100 g of inoculated NFMP (NFMP-*Salmonella* and NFMP-*E. faecium*), which was stomached at 260 rpm for 3 min in a stomacher. Final inoculated NFMP from both carriers were kept at $a_w = 0.25 \pm 0.02$ for a minimum of 48 h at room temperature before heat treatments.

For each inoculation method, the bacterial populations of 10 randomly selected (1 g, inoculated) NFMP samples were enumerated on the previously described differential media to confirm the uniformity of inoculum distribution. The a_w of 0.25 at 25°C was selected because (1) 0.25 ± 0.02 was the lowest a_w that SiO₂ could achieve, and (2) NFMP would lose its flowability and original texture at a_w above 0.25 ± 0.02 .

Isothermal Inactivation Study and Storage Stability Test

To obtain thermal death curves for *Salmonella* cocktail and *E. faecium* at 85, 90, and 95°C , moisture-equilibrated inoculated NFMP samples were subjected to isothermal inactivation treatment in aluminum

test cells (18 mm diameter, 4 mm thickness; Chung et al., 2008) as described previously (Liu et al., 2017). Briefly, 0.7-g samples were loaded and sealed in the test cells and immersed in an oil bath (Neslab GP-400, Newington, NH) maintained at 85, 90, or 95°C . The come-up-time (CUT) was determined using a T-type thermocouple located at the center of the test cell loaded with non-inoculated sample; CUT was defined as the time required for the sample center to reach within 0.5°C of the set temperature. Thermal treatment after CUT with uniform time intervals was performed at each temperature. Once removed from the oil bath, the test cells were immediately placed in an ice-water bath for at least 30 s to stop isothermal inactivation.

For the storage test, NFMP inoculated with both strain sets by both carriers were stored at room temperature (22°C) in closed sterile bottles. Five 1-g inoculated NFMP samples were enumerated every week after equilibration for tracking the survivors for up to 30 d.

Recovery and Enumeration

Salmonella cocktail and *E. faecium* survivors were enumerated on plates of differential media. Thermally treated samples were transferred from test cells into sterile stomacher bags, diluted 1:10 with 0.1% peptone water (Becton Dickinson), and homogenized for 3 min at 260 rpm with a Seward Stomacher (Harris et al., 2012). Appropriate serial dilutions of *Salmonella* or *E. faecium* samples were spread-plated in duplicate onto m-TSAYE or e-TSAYE, respectively. The plates were incubated ($37 \pm 2^\circ\text{C}$ for 48 h) and colonies were counted. Log reductions were calculated by subtracting log survivor counts of either microorganism from the log population at time zero, which was measured after the CUT.

Kinetics Analysis

The Mafart-Weibull model was used for describing the thermal resistance because it models both linear and nonlinear trends of inactivation kinetics in simple parameters with good fitness (Albert and Mafart, 2005):

$$\log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^\alpha, \quad [1]$$

where N and N_0 are population counts (cfu/g) at times t and 0, respectively; t is the time of the isothermal treatment (min) after CUT; δ refers to the overall steepness of the survival curve; and α describes the general shape of the curve and whether it is linear ($\alpha = 1$) or nonlinear ($\alpha \neq 1$) with a decreasing ($\alpha < 1$) or

increasing ($\alpha > 1$) inactivation rate with time. When the survivor curve shows a linear trend ($\alpha \approx 1$), equation [1] can also be used as the first-order kinetic model (equation [2]; Gaillard et al., 1998):

$$\log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^1 = -t/D, \quad [2]$$

where D is the time (min) required to reduce the microbial population by 10-fold at a specified temperature ($^{\circ}\text{C}$). Thus, δ -values could be the starting point of a secondary modeling and the z_T -value required to change the D value of target microorganisms by 90% (1 log) in specific LMF could be determined by equation [3] (Gaillard et al., 1998; Leguérinel and Mafart, 2001):

$$z_T = \frac{T_2 - T_1}{\log\left(\frac{D_1}{D_2}\right)}. \quad [3]$$

where D_1 and D_2 are the time (min) required to reduce 1-log of the microbial population at temperature T_1 and T_2 , respectively. When the regressions were used to correlate the log counts of bacteria with time, a pseudo-coefficient of determination was calculated using the equation

$$R^2 = 1 - \frac{\sum_1^n (Y_i - \hat{Y}_i)^2}{\sum_1^n (Y_i - \bar{Y})^2}, \quad [4]$$

where Y_i is the $\log_{10}$ count of bacteria, $\hat{Y}_i$ is the $\log_{10}$ count estimated by equation [1], $\bar{Y}$ is the mean of the $\log_{10}$ count of bacteria, and n is the number of data points of an inactivation curve. The integrated pathogen modeling program (Huang, 2014) was used for generating thermal resistance parameters in equation [1]. Differences between δ -values among samples were evaluated using ANOVA in Minitab 14 (Minitab Inc., State College, PA).

RESULTS

The moisture content of SiO_2 granules remained stable at $0.29 \pm 0.03\%$ (dry basis) at all tested a_w levels (0.15–0.85) at both 20 and 60°C (Figure 2). During exposure in the biosafety hood at 22°C and relative humidity $\sim 30\%$, the dynamic a_w of the inoculated 1 g of SiO_2 and 10 g of NFMP (by 1 mL of aqueous inoculum) changed, as shown in Figure 3. The a_w of both samples increased at time zero (immediately after mixing with wet inoculum), then decreased in the biosafety hood. Each line in Figure 3 represents a different condition-

Figure 2. Water sorption isotherms of SiO_2 granules at 20 and 60°C generated by a vapor sorption analyzer. Experiments were performed in 2 replicates. Standard errors were negligible. MC = moisture content.

ing history of microorganisms after inoculation. The highest a_w levels were 0.98 ± 0.01 and 0.56 ± 0.02 for 1 g of SiO_2 and 10 g of NFMP, respectively. The inoculated seed SiO_2 was brought back to original a_w 30 min faster than the inoculated 10 g of NFMP. No significant difference was observed between a_w of NFMP (0.25 ± 0.02) and the lowest a_w of SiO_2 (0.25) after reaching the equilibration.

Approximately 2-log reductions of *Salmonella* and *E. faecium* in carriers were observed after conditioning in a biosafety hood overnight at 22°C , relative humidity

Figure 3. The dynamic change of water activity (a_w) of 1 g of SiO_2 and 10 g of nonfat milk powder (NFMP) during inoculation and equilibration. Inoculated samples were placed in sterile plates, air-dried inside a biosafety hood at 22°C (relative humidity $\sim 30\%$) overnight, and transferred to a relative humidity 25% chamber for equilibration.

Figure 4. Survival of *Salmonella* and *Enterococcus faecium* before and after inoculation and subsequent isothermal (85, 90, and 95°C) inactivation curves in nonfat milk powder (NFMP). Open symbols indicate that samples were inoculated using NFMP as a carrier, and solid symbols indicate that samples were inoculated using SiO₂ as a carrier; SiO₂-*Salmonella* and SiO₂-*E. faecium* (*Enterococcus faecium*) are the bacteria in NFMP inoculated using SiO₂ as a carrier; NFMP-*Salmonella* and NFMP-*E. faecium* are the bacteria in NFMP inoculated using a small portion of NFMP as a carrier.

~30% (Figure 4, left). The NFMP samples inoculated by both carriers exhibited high inoculation levels of 8.2 ± 0.2 log cfu/g. From the separate storage test, no significant loss of bacterial population in NFMP inoculated by both carriers occurred within 30 d of storage at 22°C (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-14478>).

In the isothermal inactivation tests, the CUT of NFMP inoculated by both carriers in the test cells was ~150 s. Thermal inactivation kinetics of *Salmonella* and *E. faecium* in NFMP inoculated by both methods are presented in Figure 4 (right), along with the prediction lines generated from the Mafart-Weibull model ($R^2 =$

0.94–0.99). Thermal resistance parameters from these survivor curves are also reported in Table 1. Linear trends of inactivation kinetics of SiO₂-*Salmonella*, N-*Salmonella*, and SiO₂-*E. faecium* in NFMP are evident, as shown in Figure 4. However, nonlinear inactivation curves trend of NFMP-*E. faecium* in NFMP was indicated by an α value of greater than 2 (Figure 4).

The highest δ -value was estimated from the survivor curves of NFMP-*E. faecium*; NFMP-*Salmonella* showed much lower δ -values ($P < 0.05$) than those of SiO₂-*Salmonella* and SiO₂-*Salmonella* was as resilient as NFMP-*E. faecium* under all tested conditions. The relationship between δ -values, when $\alpha \approx 1$, and tem-

peratures of SiO₂-*Salmonella*, SiO₂-*E. faecium*, and NFMP-*Salmonella* are presented in Figure 5. Estimated z_T-values of these 3 strains were 16.0, 16.4, and 16.5°C, respectively.

DISCUSSION

SiO₂ as an Inert Carrier in Dry Inoculation Method

One purpose of using a dry carrier for LMF inoculation is to ensure that the dehydration history of microorganisms is repeatable. If microorganisms were dehydrated before inoculating large numbers of samples, mixing of dry inoculum with LMF would be expected to have little effect on bacterial thermal resistance parameters and subsequent application in food safety analyses. Silicon dioxide used in our study did not interact with water molecules at water activities between 0.15 and 0.85. Every batch of microorganisms in SiO₂ can be dehydrated to the same a_w level, regardless of the environmental variables present in individual laboratories. Similarly, sand (Blessington et al., 2013) and glass beads (Hildebrandt et al., 2017) were used in the same way, as they might have similar water sorption isotherms as that of SiO₂.

In the present study, the ratio of inoculated SiO₂ and NFMP was standardized as 1:30 (wt/wt). That is, only 0.023 g of SiO₂ granules were present in every 0.7 g of treated samples, small enough that SiO₂ would not interfere with the heat transfer rate inside aluminum test cells or the water sorption isotherm of NFMP at treatment temperatures. However, caution is still needed, as gravity segregation of inert carrier or target

Figure 5. D-values of SiO₂-*Enterococcus faecium*, SiO₂-*Salmonella*, and *Salmonella* in nonfat milk powder (NFMP-*Salmonella*) in log scale as a function of treatment temperature. Mean ± SEM, n = 3. *D-value is considered as the δ-value from Table 1 when α ≈ 1 (P > 0.05). SiO₂-*Salmonella* and SiO₂-*E. faecium* (*Enterococcus faecium*) are the bacteria in NFMP inoculated using SiO₂ as a carrier; NFMP-*Salmonella* and NFMP-*E. faecium* are the bacteria in NFMP inoculated using a small portion of NFMP as a carrier.

foods may occur depending on their respective densities. Thorough stirring of the mixture may be helpful to ensure bacterial homogeneity before any treatments. Mineral powders of very fine particle size (<200 μm) may perform well in attaching to food particles with less separation, though they can become a problem in laboratories due to their dust-forming property and electrostatic adherence (Bailey, 1984; Halim and Baringer, 2007). Additional studies are needed to identify

Table 1. Parameters estimated from the Mafart-Weibull model and estimated z-values¹ (°C)

Strain ²	Temperature (°C)	Y ₀	δ	α	z-value (°C)
NFMP- <i>Salmonella</i>	85	-0.16 ± 0.07	16.05 ± 1.06 ^{E,c}	1.14 ± 0.05	16.0
	90	-0.04 ± 0.07	10.22 ± 0.56 ^{F,d}	1.36 ± 0.06	
	95	-0.54 ± 0.12	3.80 ± 0.44 ^{G,e}	1.12 ± 0.10	
SiO ₂ - <i>Salmonella</i>	85	-0.30 ± 0.07	59.72 ± 5.14 ^{B,a}	1.02 ± 0.06	16.4
	90	-0.30 ± 0.09	28.13 ± 3.65 ^{D,b}	0.90 ± 0.07	
	95	-0.42 ± 0.09	14.62 ± 1.73 ^{E,c}	0.90 ± 0.06	
NFMP- <i>E. faecium</i>	85	-0.24 ± 0.05	109.60 ± 3.66 ^{A,a}	2.46 ± 0.16*	NA ³
	90	-0.15 ± 0.04	51.59 ± 1.18 ^{C,b}	2.42 ± 0.10*	
	95	-0.30 ± 0.13	28.56 ± 1.48 ^{D,c}	3.09 ± 0.27*	
SiO ₂ - <i>E. faecium</i>	85	-0.51 ± 0.18	68.41 ± 11.67 ^{B,b}	1.16 ± 0.15	16.5
	90	-0.96 ± 0.09	29.71 ± 0.98 ^{D,c}	0.87 ± 0.05	
	95	-0.77 ± 0.15	16.90 ± 2.06 ^{E,d}	1.26 ± 0.11	

^{a-c}Within 1 strain set, D-values were grouped by lowercase letters indicating significant differences (P < 0.05) by Minitab.

^{A-G}Uppercase letters indicate significant differences within a column (P < 0.05).

¹z-value is estimated only if α shows no significant difference from 1 (P > 0.05). All the z-values are equivalent (P > 0.05). δ-values were considered as D-values and grouped from largest to smallest in all tests.

²SiO₂-*Salmonella* and SiO₂-*E. faecium* (*Enterococcus faecium*) are the bacteria in NFMP inoculated using SiO₂ as a carrier; NFMP-*Salmonella* and NFMP-*E. faecium* are the bacteria in NFMP inoculated using a small portion of NFMP as a carrier.

³NA = not available.

*α is significantly different from 1.0 (P < 0.05).

alternative dry carriers and their applicable food matrices.

Effect of Dry Carriers on Bacterial Survivability and Thermal Resistance

To achieve the same inoculation level of 8.2 ± 0.2 log cfu/g before storage test and isothermal inactivation studies, the ratio of the amounts of the starting aqueous inoculum and final inoculated NFMP samples prepared by the 2 carriers was different: 1 mL to 30 g of NFMP for SiO₂-microorganisms and 1 mL to 100 g of NFMP for NFMP-microorganisms. These 2 ratios were determined from a preliminary test, from which bacterial survival rates were observed to be different. Both strain sets in 10 g of SiO₂ retained lower bacterial populations, and the population loss in the SiO₂ drying process lead to higher microbial thermal resistance.

In the storage tests, microorganisms prepared by both inoculation methods maintained strong survivability during 30 d following equilibration. Stable survivability of 2 bacterial sets was also observed from previous studies in nut kernels (Blessington et al., 2013) and wheat flour (Villa Rojas, 2015; Xu et al., 2018). The suggested time of using *E. faecium*-inoculated almonds was 30 d from the date of inoculation (ABC, 2014).

In NFMP, SiO₂-*Salmonella* displayed much stronger heat resistance than NFMP-*Salmonella* ($P < 0.05$). This difference in thermal inactivation might have been the result of the different conditioning histories of microorganisms in the 2 carriers (e.g., affected by nutrient content, drying rate, inoculum size). The different rate of drying and the different environment during drying is relevant to the survivors before thermal treatments and, hence, their thermal resistances. The presence of compatible solutes (including disaccharides, such as lactose) and the presence of dead cells during drying can substantially alter desiccation tolerance and heat resistance of desiccated cells by altering the composition of cytoplasmic solutes (Doyle et al., 1985). The differential effect of the 2 drying methods on heat resistance of *Salmonella* and *E. faecium* further substantiate that organism-dependent factors (e.g., accumulation or synthesis of compatible solutes) rather than process-driven factors account for the difference (Podolak et al., 2010; Gruzdev et al., 2011).

Such large differences of thermal inactivation kinetics of microorganisms prepared by 2 methods would have a major effect on process validation. In our study, the aqueous inoculation method resulted in lower thermal resistance of the target pathogen (Table 1); however, many researchers have used this method to estimate thermal resistance of *Salmonella* in LMF and applied the results in the design of thermal processes (Bari et

al., 2009; Jeong et al., 2009; Liu et al., 2017). These treatments might be sufficient to control pathogens in LMF only if the aqueous inoculation method reflects the natural contamination. Full investigation of possible contamination routes is needed to assess the efficiency of current process validation procedure.

Thermal inactivation kinetics of SiO₂- and NFMP-*E. faecium* in NFMP were different, as described by α values. The population of NFMP-*E. faecium* was stable at the beginning of heat treatment and declined rapidly at a certain point (e.g., inactivation rate increased suddenly at 95°C, 50 min). Chemical reactions and quality degradation may occur in NFMP during thermal treatments and thus influence inactivation rates of inherent microorganisms. The NFMP inoculated by NFMP-microorganisms showed color and flavor changes at the end of the isothermal treatments (data not shown).

In terms of SiO₂-*E. faecium*, a linear trend was observed similar to the typical inactivation kinetics reported in wheat flour (Liu et al., 2017), almond kernels (Jeong et al., 2011), and balanced carbohydrate-protein meals (Bianchini et al., 2014). Although the δ -values of NFMP-*E. faecium* were consistently higher than those of SiO₂-*E. faecium*, it is not appropriate to conclude that *E. faecium* was more heat resistant than the other because of different α values. In Figure 4, the predictive lines of SiO₂- and NFMP-*E. faecium* at 85, 90, and 95°C crossed when survivor populations dropped to 4 to 4.2 log cfu/g (see auxiliary arrows in Figure 4). This phenomenon resulted in complexity in modeling and interpretation of thermal inactivation kinetics of microorganisms.

The z values of SiO₂-*Salmonella*, SiO₂-*E. faecium*, and NFMP-*Salmonella* in NFMP at $a_w = 0.25 \pm 0.02$ varied from 16.0 to 16.5°C. These data match previously reported z values of *Salmonella* Enteritidis (16.9°C) and *E. faecium* (16.2°C) in wheat flour at $a_w = 0.30 \pm 0.02$ (Liu et al., 2018) and those of *Salmonella* Enteritidis in wheat flour ($16.7 \pm 0.73^\circ\text{C}$) modeled from D-values at 75 to 85°C at $a_w = 0.31\text{--}0.70$ (Smith et al., 2016). From reported data, z values of both strain sets might be independent of inert carriers (NFMP or SiO₂) and food matrices.

Appropriate preparation procedures of inoculated LMF should mimic the worst-case scenario of contamination in realistic applications and aid in the development of LMF thermal processing. Information has been provided to inoculate specific LMF (GMA, 2009; ABC, 2014), but is not applicable to all LMF. In our study, a dry inoculation method using SiO₂ as the inert carrier alleviated the need for complicated food inoculation procedures (such as equilibration and pulverization) and facilitated evenly distributed bacterial cells in NFMP. In addition, SiO₂ granules provided

a constant low-moisture environment after inoculation, and showed potential for introducing microorganisms into various LMF, especially LMF that could not be restored back to original form after wetting. The NFMP itself served as a carrier as well, though it required an extra pulverization step. From our study, 2 observations of NFMP as a dry carrier were (1) NFMP-*Salmonella* were less thermally resistant than SiO₂-*Salmonella* and (2) NFMP-*E. faecium* demonstrated an accelerated inactivation rate, which is not fully understood yet.

Validation of *E. faecium* as a *Salmonella* Surrogate in Thermal Processing of NFMP

A valid surrogate in thermal processes needs to perform with equal or higher thermal resistance than target pathogens under the same conditions (FDA, 2014). In our study, SiO₂-*Salmonella* yielded unexpectedly higher δ -values than NFMP-*Salmonella* at the tested temperatures. Further research is necessary to explain the significantly higher thermal tolerance when the SiO₂ inoculation method is used. However, within the SiO₂ inoculation method, D-values for *E. faecium* were not significantly different from those for *Salmonella* at the 3 treatment temperatures. When NFMP was used as an inoculum carrier, declines of *Salmonella* were linear, whereas declines of *E. faecium* were nonlinear. In most cases, the decline of *Salmonella* exceeded that of *E. faecium*; therefore, *E. faecium* appears to be a valid *Salmonella* surrogate in thermal processing of NFMP.

CONCLUSIONS

Silicon dioxide granules have a stable moisture content, and hence can apply repeatable dehydration stress to microorganisms before LMF inoculation. *Salmonella* and *E. faecium* survived incorporation into 2 dry carriers and yielded equivalent population concentrations. The SiO₂-*Salmonella* was found to have unexpectedly high thermal resistance, which focuses greater attention on the source of contamination and should aid in the development of intervention treatments. Different inactivation kinetics of SiO₂- and NFMP-*E. faecium* were observed in this study. The NFMP-*E. faecium* yielded equal or higher thermal resistance than SiO₂- and NFMP-*Salmonella* at the test temperatures, indicating that *E. faecium* is a valid *Salmonella* surrogate in thermal processing of NFMP. More information is needed to understand the significantly higher thermal resistance of *Salmonella* observed with the SiO₂ inoculation method and determine if this scenario is reflective of natural routes of contamination before this method can be recommended for use in validating commercial operations.

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